ASPECTS OF THE DIGESTIVE PHYSIOLOGY OF LARVAE OF THE NORTH AFRICAN CATFISH, *Clarias gariepinus* (Burchell 1822), DURING EARLY DEVELOPMENT

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A thesis submitted for Doctor of Philosophy in Aquaculture at the University of Stirling, Institute of Aquaculture

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Declaration

"I hereby declare that this submission is my own work and that, to the best of my knowledge, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the qualification of any other degree or diploma of a University or other institution of higher learning, except where due acknowledgement is made".

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Abstract

Aspects of the digestive physiology of larvae of the North African catfish, *Clarias gariepinus* (Burchell, 1822), during early development

The development of a cost-effective off-the-shelf micro-particulate diet with a nutritionally optimal formulation for larval North African catfish, *Clarias gariepinus* (Burchell, 1822) is one of the most important requirements for the aquaculture sector in Uganda. North African catfish contribute ~50% of Uganda's production of farmed fish, both in terms of tonnage and economic value, however, studies conducted thus far to develop a micro-diet have taken no account of the digestive physiology of the fish itself, but rather have dwelt more on the composition of the diet and its apparent performance in terms of fish growth. Under these conditions, however, large mortalities are experienced during early development. Only a few studies have been conducted concerning the digestive physiology of the larvae or have characterised aspects of the key digestive enzymes produced by this species. Hence a major mismatch has existed between the general approach to creating micro-diets and the level of knowledge of the functional development of the sensory and digestive system in larval fish, including their theoretical digestive capabilities. This study sought to characterise the ontogeny of key digestive enzymes in North African catfish larvae from hatching to 21 days post-hatch (dph) with special emphasis on the period 2-4 dph where live feed is the more accepted diet for the fish larvae. In real-world terms, however, extensive use of live-feed makes farming extremely labour-intensive and it is costly to operate a catfish hatchery providing a consistent product and at the same time generate profit for farmers.

The work described in this thesis was conducted at the University of Stirling's Tropical Aquarium, a facility of the Institute of Aquaculture, which houses a population of North African catfish introduced into the facility over 30 years ago and assumed to be, but not fully verified as, *Clarias gariepinus* Burchell 1822. In order to ensure that the results of the research presented in the thesis could be effectively applied in Uganda, the identity of this species needed to be confirmed. From a morphological and meristic characterisation, in addition to use of a molecular taxonomic classification, the species held at the Institute of Aquaculture and employed for the present study was confirmed to be *Clarias gariepinus,*

(Burchell, 1822) rather than a hybrid with, *Clarias anguillaris* which is a situation that is common in African populations.

Studies were undertaken to determine the point at which when exogenous feeding should be commenced and to establish when fish could be weaned on to an inert formulated diet in order to improve growth and survival during larval rearing. From the growth studies conducted, findings revealed that at 2Dph a mixed diet of (live) *Artemia* and (inert) Coppens diet gave better growth than both Coppens and *Artemia* fed alone, which gave similar growth rates.

The histomorphology of both adult and larval *C. gariepinus* was examined in order to determine the course of development of digestive structures in the larva. In addition, new *in situ* labelling procedures were developed to highlight the localisation of expression of transcripts for key digestive enzymes comprising two alkaline proteolytic enzymes, namely chymotrypsin and trypsin, one acidic proteolytic enzyme, pepsin, and one chitinolytic enzyme, gastric chitinase. Larval development followed a time-course similar to that described by other researchers with the pancreas being discrete in the larval stages but being more dispersed throughout the intestinal region in the adults. The stomach was present by 4 to 5 Dph, supporting a suggestion that alkaline protease digestion dominates early in development, switching to a more acidic digestive mode following development of the stomach.

In situ labelling protocols showed trypsin and chymotrypsin to be localised in the pancreas by as early as 2 Dph, while pepsin and gastric chitinase were localised in the stomach. More specifically, pepsin was localised to oxynticopeptic cells and gastric chitinase to the gastric pits with the former being present by 4Dph and the latter being present by 6Dph.

Quantitative real-time PCR (qRT-PCR) was employed to investigate the timing and level of transcript expression for trypsin, chymotrypsin, pepsin and gastric chitinase. First expression was observed, perhaps unsurprisingly, to occur largely at a time when the tissue representing the site of production was first observable by histological examination and expression increased from this time. Trypsin and chymotrypsin expression were detectable from 1Dph, with clear expression by 2Dph, coinciding with the appearance of the first anlage of the pancreas in histology. Pepsin and gastric chitinase mRNA was detected at 3Dph and was

highly expressed from 4dph onwards. This coincided with the appearance of the first anlage of the stomach in histology.

Important new resources for North African catfish, including the first assembled and annotated transcriptome are now available for this species. These were created from a mixed tissue / stage transcriptome library. Using this transcriptome resource as a basis, an oligo-microarray was constructed, which allowed broad-scale transcriptome analysis to be conducted on samples from early larvae fed using two diet regimes. During development on the standard diet, a number of pathways were observed to be over-represented in terms of transcript expression between 1 Dph and 4 Dph. These included protein digestion and absorption, bile secretion, complement and coagulation cascades, glycolysis, gluconeogenesis, PPAR signalling pathways, and ribosomal pathways. Together these pathways denoted changes in elements of growth, protein production, digestion, the immune system and synaptic transmission *inter alia*.

From the research presented in this thesis, it is clear that the key digestive enzymes studied here are rapidly switched on during the first few days of development and first feeding. Different dietary regimes had important effects on expression of digestive enzymes and knock-on effects for digestion, growth, and immune system development, which may have important consequences for larval health and survival under aquaculture conditions.

The results of this work have the potential to assist the Ugandan feed industry in that they can be used as a baseline to make improved diets for early weaning in larval fish, using increased knowledge of which enzymes are involved in larval digestion and when they are produced by the fish. Clues about the course of development of larval immunity, particularly innate immunity, may help to provide strategies to mitigate the high losses experienced by farmers in early development and may assist in the management of high intensity catfish hatcheries. The findings of the work presented in this thesis can help to better formulate inert feeds and optimise timing of introduction to larval stages to maximise growth and survival. Public transcriptomic resources produced in the course of this work can be used by other researchers as a basis for larger broad-scale transcriptomic investigations using microarray, RNAseq or allied technologies to characterise different states including disease, nutrition, physiology and development which

could be used to *e.g*. improve diets, develop vaccines, or increase production values such as growth characteristics.

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This thesis has benefited enormously from the knowledge, guidance and encouragement of Professor James Bron, thank you so much for your excellent supervision and kindness throughout this long project tenure without your kind gestures this thesis wouldn't be accomplished as you took off time your schedule to give me a firm foundation to do molecular aspects in field of fish nutrition, a subject I barely covered in year one of my BSc degree to allow me understand key concepts including things like FASTA files, Bioedit and later,the use of the instruments including the photomicroscopy equipment and many available resources online to effectively execute this study.

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List of manuscripts in preparation

Nattabi J. K., Taggart J. Ireland, J. and Bron J.E. (Manuscript in preparation). Cloning and expression analysis of four digestive enzymes from (*Clarias gariepinus* Burchell 1822) during early development: Deciphering gastrointestinal functionality.

Nattabi J. K., Taggart J. Ireland, J. and Bron J.E. (Manuscript in preparation). Gene expression of four named digestive enzymes during the larval development of North African catfish (*Clarias gariepinus*, Burchell 1822).

Nattabi J. K., Taggart J. Ireland, J. and Bron J.E. (Manuscript in preparation). Microarray analysis of transcript expression during early larval development of African catfish, *Clarias gariepinus*.

List of conference proceedings

Nattabi J.K April 2015 Microarray analysis of transcript expression during early larval development of African catfish, *Clarias gariepinus* at the Institute of Aquaculture PhD Research conference, - Poster presentation.

Nattabi J.K (2013) Development of digestive capacity in larvae of the African catfish (*Clariasgariepinus*, Burchell 1822), A transcriptomic perspective. Presented a Poster at the Aquaculture conference: "To the Next 40 Years of Sustainable Global Aquaculture" held in Las Palmas de Gran Canarias, Spain Organised by: Elsevier from 3rd to 7thNovember

Nattabi J.K. (2013) Development of digestive capacity in larvae of the North African Catfish (*Clarias gariepinus*, Burchell 1822), A Transcriptomic Perspective (Gave a talk over the lunch time seminar series for Postgraduate students at the Institute of Aquaculture University of Stirling15th May 2013.

Nattabi J.K (2012) Development of digestive capacity in larvae of the African Catfish (*Clarias gariepinus*, Burchell 1822), A Genomic Perspective" at the Institute of Aquaculture PhD Research conference, themed "Innovation towards enhanced Aquatic food Security". Poster presentation24th October 2012.

CHAPTER ONE

1.1 Introduction

1.1.1 The role of fisheries in sustainable development, poverty reduction and food security

1.1.1 Fisheries in sustainable development

Fisheries are an important source of food security. They provide a source of protein for human diets, income, employment and fish food and animal feed components (Belhabib, Sumaila, & Pauly, 2015; Belton & Thilsted, 2014; Fisher *et al*., 2017; Lynch *et al*., 2017; Youn *et al*., 2014). It is currently estimated that 38 million people derive their livelihoods from fish and fish products worldwide. Fish, as a source of high quality protein, can play an important role in improving Africa's food security in the face of the increasing population pressures.

There is a projected need to increase fisheries and aquaculture production by 2030 (World Bank, 2013) in line with the increasing world population and *per capita* income. For Least Developed Countries (LDCs), most of which lie in Africa, climate change and extremes are likely to exacerbate already declining fish stocks due to increased temperature, severe weather, fish movement to cooler waters and reduced fish sizes.

The total world fishery production (capture and aquaculture) is projected to reach 196 Million tonnes by 2025, with most of this increase coming from the developing world (FAO 2016). However, this is yet to be realised as the developing world is still faced with challenges, among which is the production of sufficient affordable feed to sustain aquaculture, as feed costs represent approximately 60% of the costs of production. Fisheries and aquaculture form about 1.3% of the GDP of Africa, worth $24 billion, and will likely increase with the increasing population and much neededrequirement for cheapprotein. Over 1.2 million people are employed in this sector, of which 58% are engaged in fishing / production and the remaining 42% are in the processing sector (Worldbank.org, 2018). Fisheries are considered a communal responsibility in terms of the traditional African approach to managing resources.

Aquaculture is one of the fastest growing food producing sectors in the world, with nearly 50% of global fish consumption being derived from aquaculture. It thus contributes greatly to food and nutrition security (Subasinghe, 2014)). The Food

and Agriculture Organization (U. FAO) of the United Nations (UN) estimated that by 2050, in order to meet the increasing population, aquaculture must increase by 60% to meet demands occurring in the face of stagnation of fisheries and in addition to other agriculture outputs from crops and livestock (FAO, 2014). Fish production is reported to have steadily increased, with food fish increasing at an annual rate of 3.2% which is double the estimated world population growth at 1.6% (FAO, 2014). At a growth rate of 6.2%, aquaculture is the main contributor to this reported increment in world fish production.

1.1.2 Fisheries and food security

Fish and fisheries products have proven to be extremely important sources of animal protein, playing an important role in promoting good health, providing employment and alleviating poverty. They also provide nutritional security to many people all over the world. The trade of fisheries and aquaculture products is an important source of income and employment. By 2003, over 200 million people were reported to be deriving their nutritional security from fish and fisheries products worldwide (Béné*et al*., 2016; Kawarazuka & Béné, 2010;Béné & Heck, 2005).Fisheries and aquaculture have thus continued to play an important role in nutrition, food security and livelihoods (FAO, 2014a).

Food insecurity is currently one of the more visible dimensions of poverty and is the first sign of extreme living conditions (FAO 2016). The contrasting condition, food security, has been defined by FAO as a "condition when people at all times have physical and economic access to sufficient, safe and nutritious food to meet their dietary needs. Fish is critically important to food security and good nutrition and provide more than 4.5 billion people with 15% of their average *per capita* intake of animal protein (Béné *et al*., 2015; Thilsted, Subasinghe, & Karunasagar, 2014).

1.1.3 Fisheries and poverty reduction

Tackling food insecurity and poverty are fundamental to global advancement in theface of increased global population (Béné *et al*., 2016).Fish is an important economic commodity in food security and poverty reduction, especially on the African continent as a source of nutrition. However, sustainability of supply and demand is still inadequately addressed.

Fish has been an important food source back to prehistory and for many people is culturally imprinted in their traditional diets. It is estimated that 17% of globe take in fish as a source of animal protein and also recently because it has been realised that it is a good source of healthy animal protein rich in long chain omega -3 fatty acids, namely eicosapentaenoic acid (EPA) and doxosachexanoic acid (DHA) which are important in brain development especially in young children and in adults lowering heart related conditions (Bogard, 2017; Fisheries, 2014; Thilsted *et al*., 2014). It should be noted, however, that this latter view has been recently challenged (Abdelhamid *et al*., 2018).

The *per capita* fish consumption in Sub-Saharan Africa is predicted to reduce at an annual rate of 1 per cent to 5.6 kilograms during the years 2010–30. However, due to rapid population growth, which was estimated at 2.3 percent annually during that same time frame, total food fish consumption demand, will grow substantially by 30 per cent.On the other hand; projected production increase is only marginal. Capture production was projected to increase from an average of 5,422 thousand tonnes in 2007–09 to 5,472 thousand tonnes in 2030, while aquaculture was projected to increase from 231 thousand tonnes to 464 thousand tonnes during the same period.

FAO's most recent estimates indicate that globally, 842 million people i.e about 12 per cent are unable to meet their nutritional requirements (Brendan *et al* 2017). This implies that one in eight people in the world are likely suffering from chronic hunger. Although the statistics of underweight persons reported by 2016 had increased by about 38 million persons up from 2015, it is only 11% when compared to 15 years ago when the occurrence was as high as 900 million, however, this increase is a signal that the world will not attain the goal of getting rid of hunger by the year 2030 (FAO, IFAD, UNICEF, WFP and WHO. 2017).

It is worthwhile noting that the biggest population with a high prevalence of underweight is found in the developing world (FAO, IFAD, UNICEF, WFP and WHO. 2017).

Aquaculture is now recognised as a viable and profitable enterprise worldwide (Pulvenis, 2008). According to FAO 1988, cited in Edwards &Demaine 1998, aquaculture can be simply defined in terms of "the farming of aquatic organisms such as fish, crustaceans, molluscs and aquatic plants".

The growth of world aquaculture production in the first 15 years in the new millennium gradually slowed down and between 2001-2015 the average annual growth rate was 5.9%, which is significantly lower than the double-digit growth percentage rate seen in the 1980s and the 1990s. At a continent level, African aquaculture exhibited increased growth during 2001-2015 averaging 10.4%, followed by Asia (6%) and Americas (5.7%). In Oceania and Europe aquaculture growth was only 2.9% and 2.5% respectively, over the last 15 years (Zhou, 2017). The total world aquaculture production reached 106 million tonnes (MT) in 2015, with estimated value of US$ 163 billion of which food fish contributed 76.6 million tonnes, worth US$ 157.9 billion tonnes by 2015 (Zhou, 2017). Farmed aquatic plant production by 2015 was 29.4 million tonnes (MT), worth US$ 4.8 billion (FAO 2017). It is worthwhile noting that as a result of overexploitation due to the increase in world population over the last century, aquaculture is increasingly making up the deficit of the much-needed cheap protein source to feed the ever-increasing world population. It is estimated that in order to maintain the current level of *per capita* consumption, global aquaculture production will need to reach 80 million tonnes by 2050 (Subasinghe 2005).

As aquaculture technology has developed in a bid to foster better harvests and faster growth, the rapid growth in the industry has led to a need to move to a more enriched diet leading to replacement of natural foods with commercially prepared diets. In many aquaculture operations today, feed accounts for more than one-half of the variable operating cost (Shipton & Hasan, 2013; Shipton, Hecht, & Shipton, 2013; Tacon & Hasan, 2007). Therefore, knowledge of nutrition and practical feeding of fish is essential to successful aquaculture.

Feeding fish in their aqueous environment involves considerations beyond those required for feeding land animals. These considerations include the nutrient contribution of natural aquatic organisms in pond culture, the effects of feeding and diet composition on dissolved oxygen and other water quality factors, and the loss of nutrients if feed is not consumed immediately (Pouomogne 2007; Rana *et al.,* 2009; Hasan *et al.,* 2013; Hasan & New 2013; Romana-Eguia *et al.,* 2013; Sarder 2013). Fish feeds require processing methods that provide special physical properties to facilitate feeding in water, and variation in feeding behaviour requires special feeding regimens for various species. The effects of diet composition and

feeding practice on the quality of the effluent from the culture system are also an important consideration.

Status of aquaculture in Africa

Aquaculture is growing at an impressive rate in Africa, becoming an increasingly important aspect of the animal food producing sector. Over the last fifteen years, statistics show that Africa is making steady progress, with African aquaculture showing a 3.6% growth up to 2015 with a contribution of 2.3 % to global aquaculture production between 2014 and 2015 as evidenced by the recent statistics as shown in Figure1-1 below.

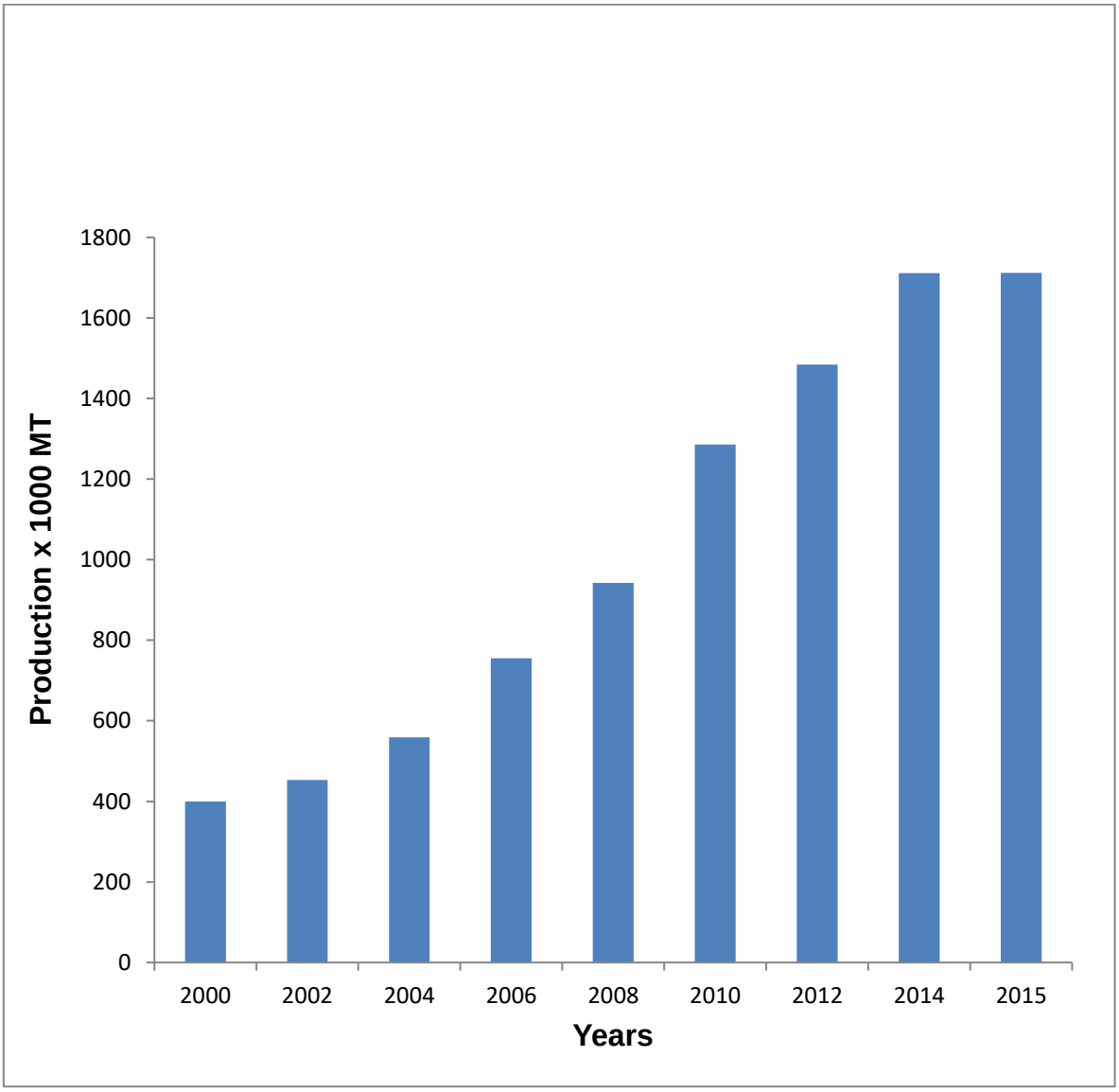


Figure 1-1 Showing aquaculture production in thousands of tonnes from 2000 to 2015 (adapted from Xiao-Wei FAO 2017)

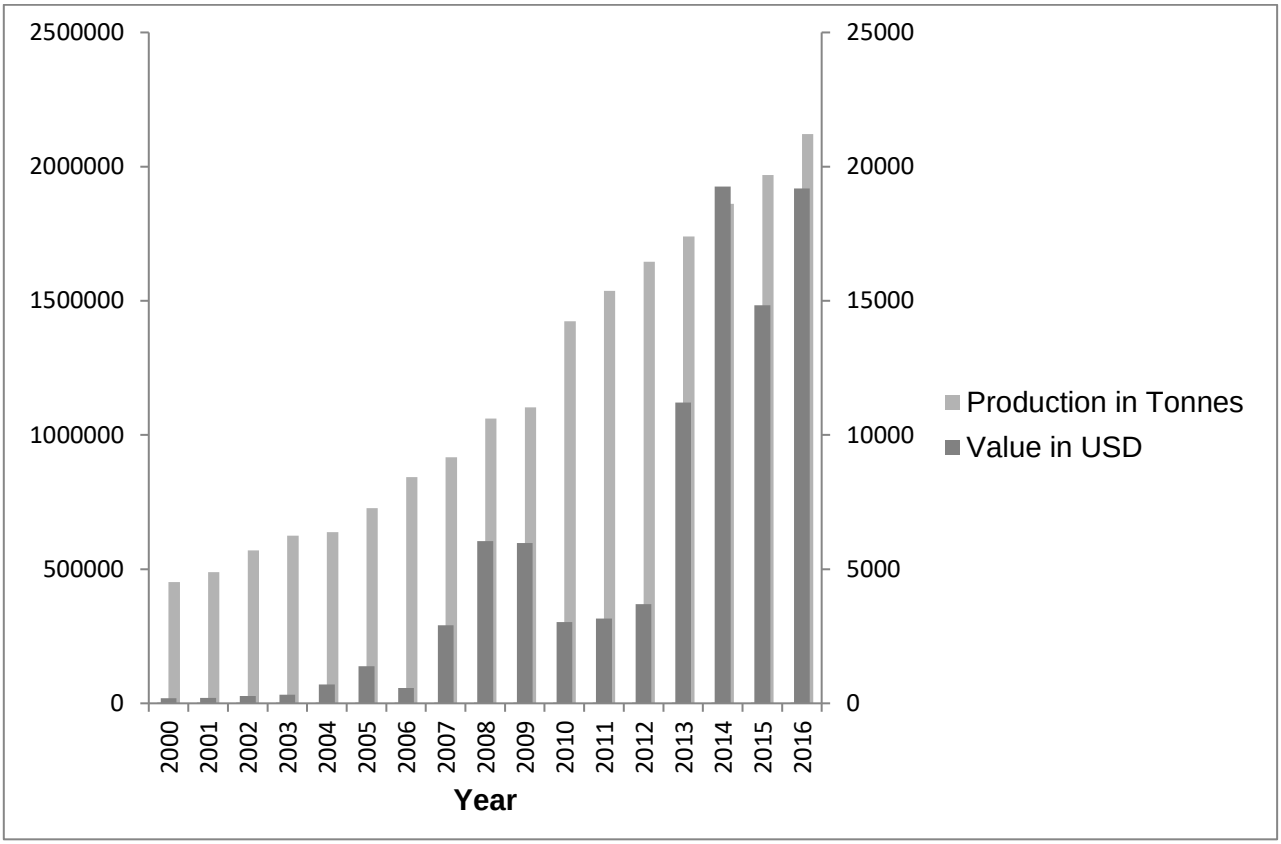


Figure 1-2 Trends in African aquaculture by quantity (Thousands of Tonnes) and value x 1000 USD) 2000-2015. (Source: Data adapted from FAO. 2018. Fishery and Aquaculture Statistics. Global aquaculture production 1950-2016 (FishstatJ). In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated 2018. www.fao.org/fishery/statistics/software/fishstatj/en accessed online 3/5/2018 and replotted by author).

According to FAO 2018, world fisheries Statistics there has been an increased contribution of Africa to global production at the continent level, with African aquaculture exhibiting increased growth during 2001-2016. This can be attributed to in particular to rapid development of inland fisheries based on freshwater fish farming in sub-Saharan Africa, most notably in Nigeria, Uganda, Ghana and Kenya. African aquaculture production is overwhelmingly dominated by finfish culture coupled with strong growth in brackish-water culture in Egypt as seen in terms of percentage value and tonnage of production (Figure, 1-3) below.

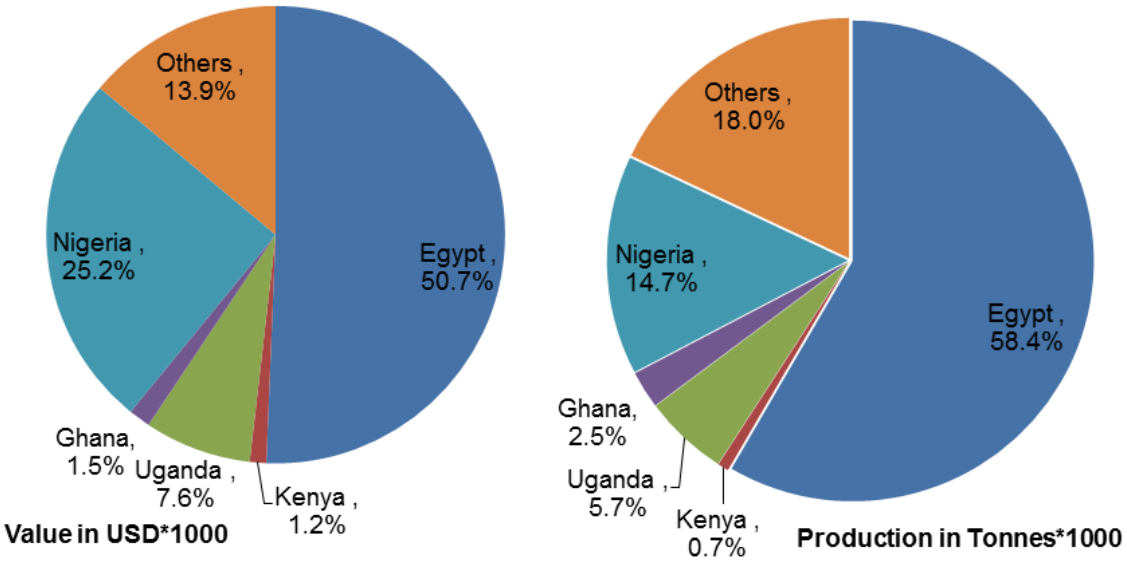


Figure 1-3 showing the top 8 aquaculture producers in Africa by 20156 Data sourced from FAO. 2018. Fishery and Aquaculture Statistics. Global aquaculture production 1950-2016 (FishstatJ). In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated 2018. www.fao.org/fishery/statistics/software/fishstatj/en accessed on 5/5/2018

1.1.2 Contribution of the major aquaculture producing Sub-Saharan Africa (SSA) countries and contribution to total aquaculture production in Africa

African aquaculture has come a long way since it was first introduced over 50 years ago, having followed a challenging trajectory (Machena & Moehl 2001). However, it has experienced ever-increasing growth in the face of the growing young population in SSA coupled with stagnated wild fisheries. Aquaculture production in SSA is projected to reach 1 million tonnes by 2025, an increase of 84% (Satia 2017). The population of SSA by mid-2014 was reported to be 920 million with an annual increase of 2.5 % in 2015 (Satia, 2017).This has implications for the future for SSA and the global economy as the youngest labour-active age group will likely emerge from SSA given a projected increase between 1.5 and 2.5 percent by 2050 (UN -DESA, 2015; Satia, 2011;. Satia, 2017). In the last five years the main drivers of growth are suggested to have been the increased importance of efforts to improve food security, job creation, economic growth and resource use (Satia 2017).

1.1.3.1 Fish farming environments and production in Sub-Saharan Africa (SSA)

They are three aquatic environments including freshwater inland, brackish water and marine (coastal) with most production concentrated inland (Satia 2017) Coastal aquaculture is only practiced in Republic of Madagascar, Mozambique and Republic of Tanzania.

Fish farming production systems and technologies in SSA show great diversity, ranging from extensive aquaculture in ponds, lagoons and coastal areas; semi-intensive farming in ponds and lagoons and intensive culture using tanks, cages and recirculating aquaculture systems (Satia, 2011; Satia, 2017). Of these the currently the most profitable system for tilapia and catfish is cages placed in large public waters with a major advantage of reduced capital investment per unit of fish production due to the lack of requirement for land ownership.

1.1.3.2 Regional aquaculture production in Sub-Saharan Africa SSA 2015

According to Satia (2017) most of the aquaculture production in Sub-Saharan Africa is based on inland waters including ponds, lagoons and cages based in large water bodies. Aquaculture is based on subsistence level, small scale and market driven and on large industrial scales. Aquaculture has also registered a seven-fold increase between 2004-2016 but with no value addition being done to the products being produced. About 98% of aquaculture production from SSA has been derived from Inland waters and from the indigenous species of African Catfishes and Tilapia. Seven countries have been identified namely Nigeria, Uganda, Ghana, Kenya, South Africa, Madagascar and Zambia as the major aquaculture countries in sub-Saharan Africa (SSA contributing about 93% by 2015 (Satia 2017). There have been a number of new emerging countries taking on aquaculture in recent years, notable among them being Ghana.

1.1.3 Status of fisheries and aquaculture in Uganda

Fisheries resources are among the most significant natural resources of Uganda. (Department of Fisheries Resources (DFR), Uganda DFR report 2011, Musoke 2013). With about 20 per cent of its surface area covered by water, Uganda's freshwater resources comprise: open water 46,900 km^2, swamps 7,300 km^2 and rivers 2000 km^2. Uganda's fisheries landscape includes six major lakes namely Victoria, Kyoga, Albert, Edward, George and Wamala plus, over 160 lakes of notable size and a network of rivers including the River Nile, swamps and flood plains as shown in Figure 1- 4 below.

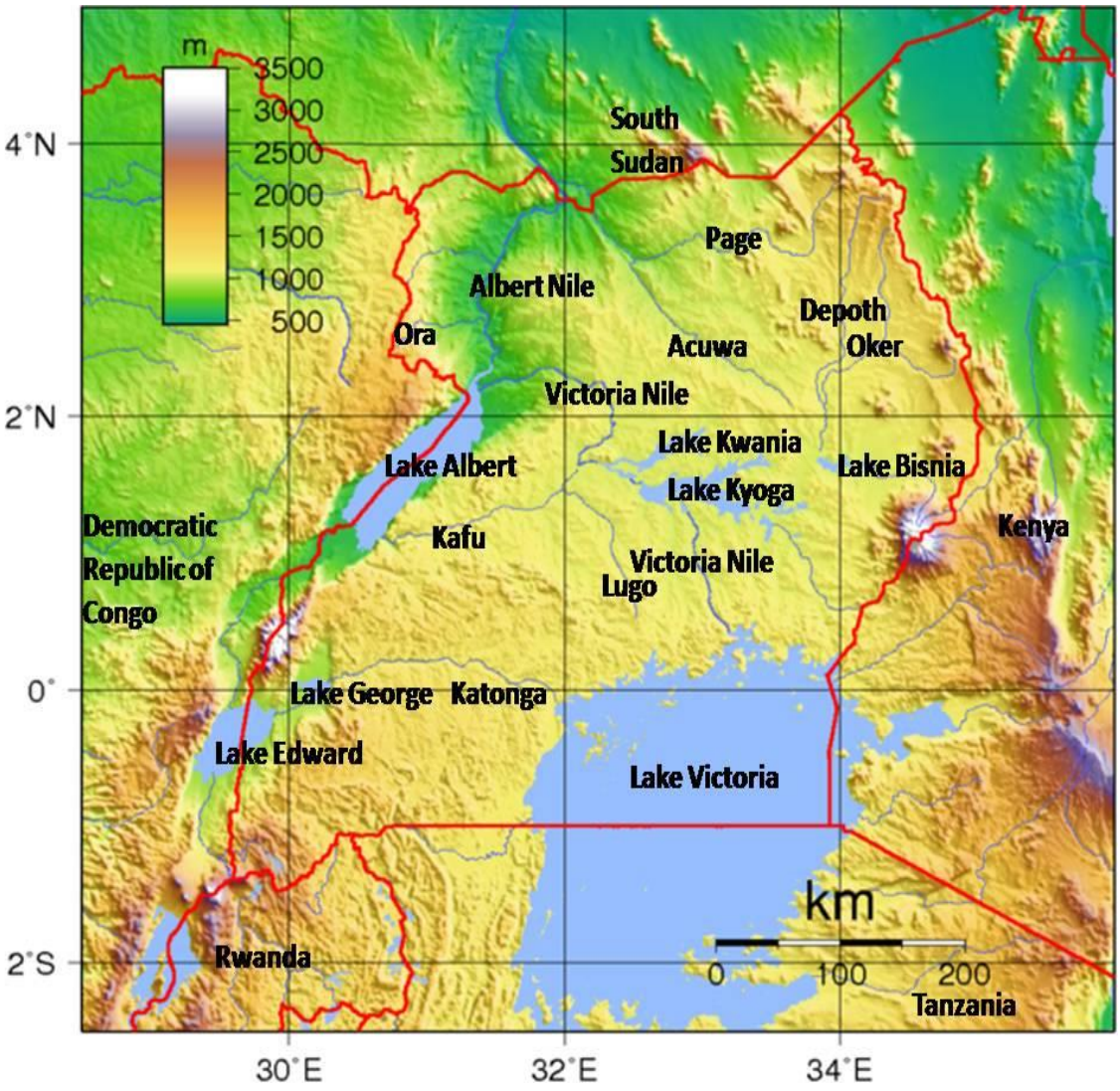


Figure 1-4 Map of Uganda showing the principal freshwater resources of Uganda: Modified from https://geology.com/world/uganda-satellite-image.shtml

1.1.3.3 Fisheries subsector of Uganda

The fisheries of Uganda are very dependent on the abundant water resources available, with natural water bodies covering over 20% as indicated above.Lake Victoria is by far the largest economically most important contributor to the National fisheries. However other lakes around *i.e*. George, Albert and Kyoga along with the Nile River and the minor lakes, swamps and streams also contribute substantially to the annual national catch (FAO 2012 old site).

Since Uganda is well endowed with inland waters it is also rated among the major 17 contributors *i.e* China, India, Bangladesh, Myanmar, Cambodia, Indonesia, Uganda, Nigeria, Tanzania, Russian Federation, Egypt, Congo, Brazil, Philippines,

Thailand, Kenya and Mexico, to the 80% capture fish production as of 2015 (FAO 2017b). Fish remains the leading animal protein provider for rural communities in Uganda, with over 50 percent of households depending on fish as the major source of animal protein for dietary purposes (Owani, Hishamunda & Cai, 2012).

Fisheries resources are important to the populace of Uganda as it is the major source of protein and in addition about 1 million to 1.5 million are employed by this sector and derive an income from the fisheries and aquaculture sector. The fisheries subsector further contributes to national welfare through about 5.3 million people being directly dependent on fisheries as the major source of household income. It also provides food security to the fish eating population and is a major animal protein source for the 34.5 million people of Uganda.The silver fish, Mukene are important in provision of micronutrients (n.b. hidden hunger) to the population. (DFR 2012, Project Red Velvet 2015,DFR 2016). According to FAO,Uganda ranks third in Africa after Nigeria in terms of people engaged in this subsector (Project Red Velvet 2015).

Uganda is also the third largest producer in Africa after Egypt and Nigeria (Shipton *et al*., 2013). Fifty per cent (50%) of the aquaculture production is from reservoirs stocked by Government while the other 50% comes predominantly from smallholder farmers. Currently 40% of the food fish is exported, leaving 330,000 MT for local consumption. Uganda's current population is estimated at 34.7 million, which translates to a *per capita* fish consumption of 8.0 Kg, which is only half the world average of 17 Kg (FAO 2012). This is way below the World Health Organisation's (WHO) recommended *per capita* consumption of 25 Kg. (National Investment Policy for Aquaculture Parks in Uganda report 2012, Agriculture Ministry of Agriculture Animal Industry & Fisheries, (MAAIF) Uganda).

From a global perspective, fish stocks in Ugandan waters, especially of Lake Victoria the largest inland fishery in the world, are declining. Nile perch catches declined by 46 % from 2011- 2015, while tilapia catches were lower by 38% during the same period (NARO_NAFFIRI Uganda National fisheries catchment survey 2017). The survey also estimated that within one year between 2014 and 2015 a 44.5% decline was observed from 269,533 metric tonnes to 149,382. This is mainly due to the use of poor fishing practices since the resource is open access and anyone can fish. However the government of Uganda has identified the

fisheries sub-sector as a key investment area, since it was the second highest foreign earner after coffee until 2014 but has been overtaken by tourism as number one export earner in a country very much dependant on Agriculture (Uganda Bureau of Statistics (UBOS), 2016). In 2016 the Government deployed the National Army together with concerned parties to enforce the laws in order to improve fish stocks. This also concurs with the Ministry of Agriculture Animal Industry and Fisheries (MAAIF) records, which show that fish exports to international markets rose from 4,751 tonnes worth $5,308 (about Shs 17.5 million) in 1991 to 36,616 tonnes valued at $143,618 (about Shs 486 million) in 2005 but have now declined to 17,597 tonnes valued at $134,791 (about Shs 444.8 million). However, since the country is well endowed with water resources, aquaculture is steadily picking up to fill the gap left by the stagnated wild fisheries sub-sector, as seen in Figure 1-5 below.

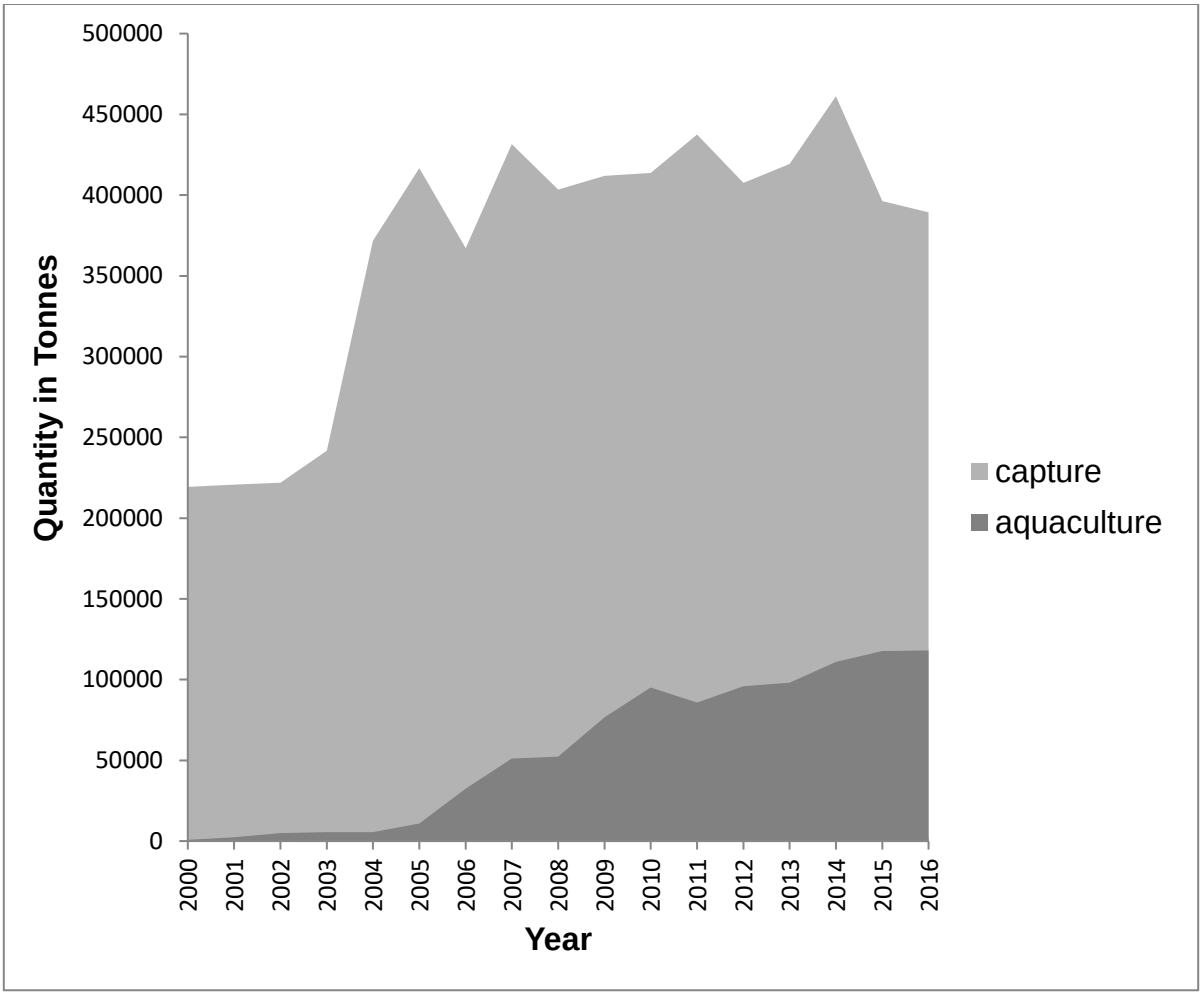


Figure 1-5 Trends in capture fisheries and aquaculture production by quantity for Uganda 2000-2016. Source: Data from FAO 2018. Fishery and Aquaculture Statistics. Global capture production 1950-2016 (FishstatJ). In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated 2018. www.fao.org/fishery/statistics/software/fishstatj/en acessesed 3/5/2018

1.1.3.4 Aquaculture subsector of Uganda

Aquaculture was introduced in Uganda in the 1950s with the main aim of enhancing rural family's nutrition through increasing animal protein content in the diet, to reduce prevailing malnutrition. Statistics indicate that annual harvests increased in the late 1990s as seen on Figure 1-6 below. (Walakira *et al* 2011).

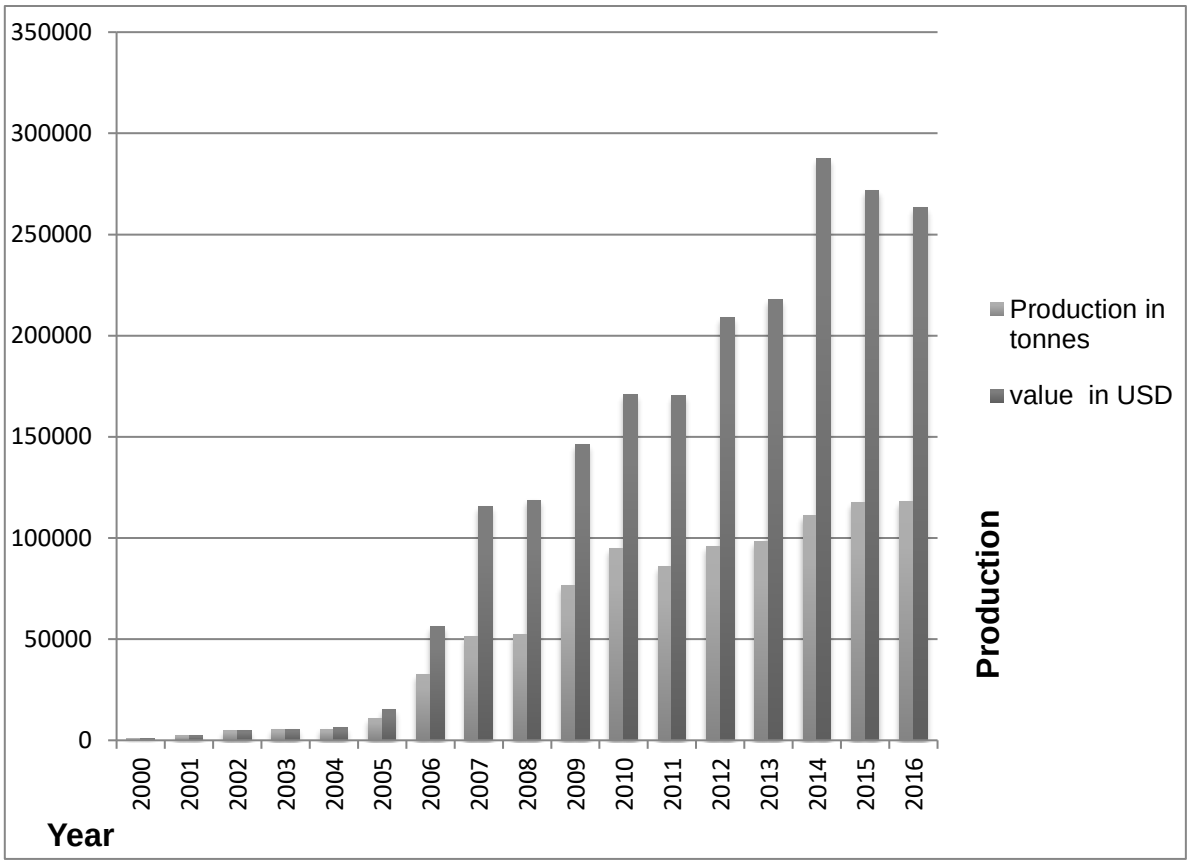


Figure 1-6 Trends of Aquaculture production 2001 -2016 by quantity (tonnes) and Value (USD) to the economy of Uganda. Source FAO. 20178. FAO. 2018. Fishery and Aquaculture Statistics. Global aquaculture production 1950-2016 (FishstatJ). In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated 2018. www.fao.org/fishery/statistics/software/fishstatj/en accessed on 3/5/2018

Aquaculture in Uganda has yet to reach its full potential. According to Department of Fisheries Resources (DFR) Uganda, by 2011 both cage and pond aquaculture was developing all around the country, not only for subsistence but more and more for commercial purposes. As seen from the Figure 1-6 above, there are declining trends in terms of value for aquaculture. This may reflect the fact that farmers are abandoning the venture as quality seed and cheap feed plus a clear marketing outlet may be a problem. Much of aquaculture is practiced on a private basis and synchronising production for the market is still in infancy with just one cooperative group and one value addition company and both are located in the capital where farmers in the rural setting can't easily transport their products when they are ready.

The freshwater aquaculture sector comprises 3 categories of production system, namely pond culture as the most commonly used method followed by cage/pen culture (second most used).and finally use of more recent systems, including tank/raceway culture. The difference in productivity between commercial and subsistence aquaculture is quite substantial, as it varied from around 1 500 kg/ha for smallholder farming to 15 000 kg/ha for commercial farming. By 2005 there were 60% fish farmers at subsistence level (Food and Agriculture Organisation, 2007) today 20 to 30% of them have upgraded their subsistence farms to profitable small scale production units. It is estimated that there are 2 000 such farmers who own nearly 5 000 out of the national total estimated at 20 000 ponds (Food and Agricultural Organisation, 2016).

Small-scale fish farmers have learnt that adopting new technologies is not enough to increase their productivity unless the fish value chain for their products is enhanced at the same time. The Department constructed four regional fish fry centres in Mbale, Gulu, Kajjansi and Bushenyi to boost seed production dealing mainly in catfish production. The cage culture industry emerged in 2006 with over 750 cages owned by groups or individuals (DFR 2011), however, tilapia is the major species raised in cages. Adoption of cage-culture artificial enclosures in natural water bodies such as Lake Victoria (Mukono, Ssese Islands), Lake Nabisojjo (Luwero/Nakaseke) and Lake Banyaruguru have also made a significant contribution to aquaculture production, however, this is not reflected in the aquaculture values, probably becaue records are not being kept or not being released in fullness for fear of government levying taxes (pers. observation). Nearly 2,000 farmers are considered to be emerging commercial aquaculture entrepreneurs while another 3,000 are considered as progressive small-scale fish farmers. Another 21,000 remain in the category of subsistence producers (DFR, 2010/2011). There are currently 10+ medium-sized commercial hatcheries and another 81 small business operated hatcheries, all of which are privately owned.

1.2 Countries making a major contribution of North African catfish to sub--Saharan Africa aquaculture production

North African Catfish has been observed to be grown under aquaculture in SSA in the following nations: Burundi, Central African Republic, Gabon, Ghana, Guinea, Chad, Congo, Benin, Equatorial Guinea, Namibia, Niger, Nigeria, Kenya, Malawi, Mali, Lesotho, Côte d'Ivoire, Swaziland, South Africa, Uganda, Tanzania, United Republic. of Zimbabwe, Sierra Leone, Senegal, Rwanda, Sudan, Zambia, Congo, Burkina Faso and Morocco (FAO 2018). According to Hecht (2013), the major countries contributing to North African catfish production from 2006 to 2011 were Nigeria followed by Uganda and Kenya,and these were still the leading contributors until 2016 (FAO 2018) as seen in Figure 1-7.

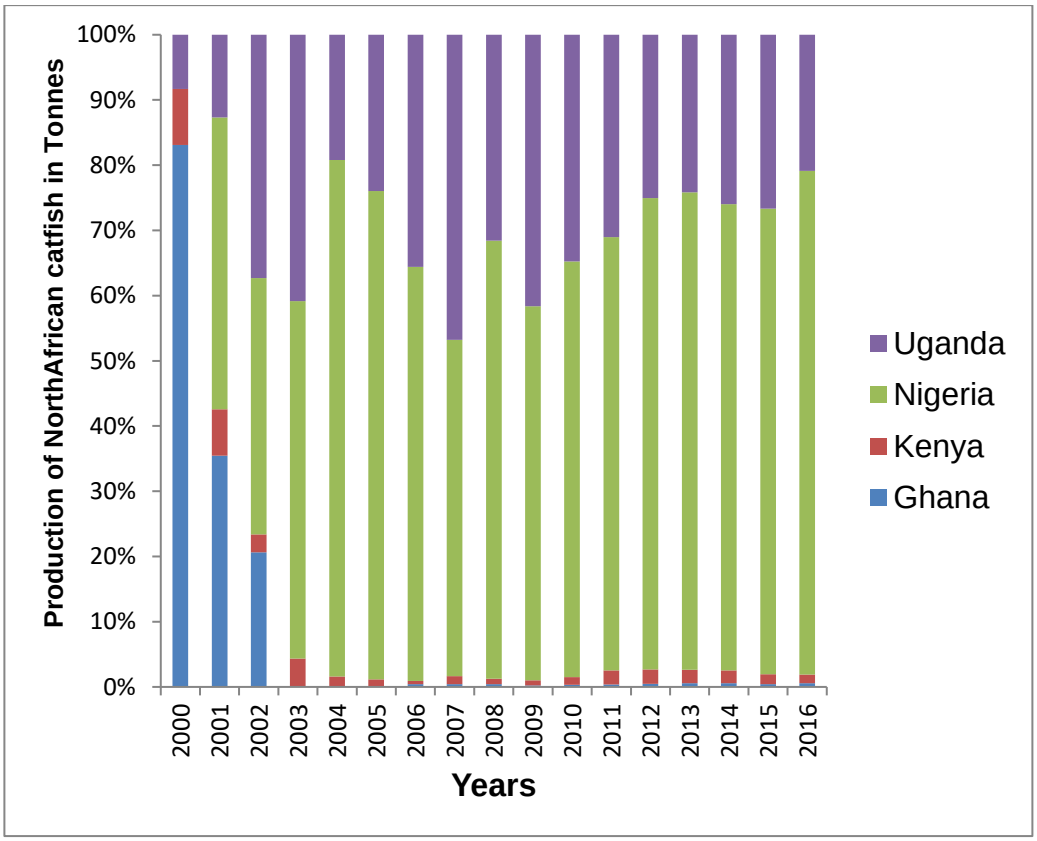


Figure 1-7 Showing the Percentage contribution of the major NorthAfrican Catfish producers in the SSA sourced from FAO. 2018. Fishery and Aquaculture Statistics. Global aquaculture production 1950-2016 (FishstatJ). In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated 2018. www.fao.org/fishery/statistics/software/fishstatj/en accessed 9/5/2018

1.2.1 North African catfish (*Clariasgariepinus*) farming sector in Uganda

1.1.3.5 Main cultured species

Currently the most common species of fish cultured in Uganda are the North African catfish, *Clariasgariepinus (*Burchell 1822), Nile tilapia, *Oreochromis niloticus (*Linnaeus, 1758) and carp (*Cyprinus carpio*) (Linnaeus, 1758) with the North African catfish and Nile Tilapia both popular by 2015 with Nile tilapia being more valuable . (FAO 2017).as shown in Figure 1-8 and 1-9 below.

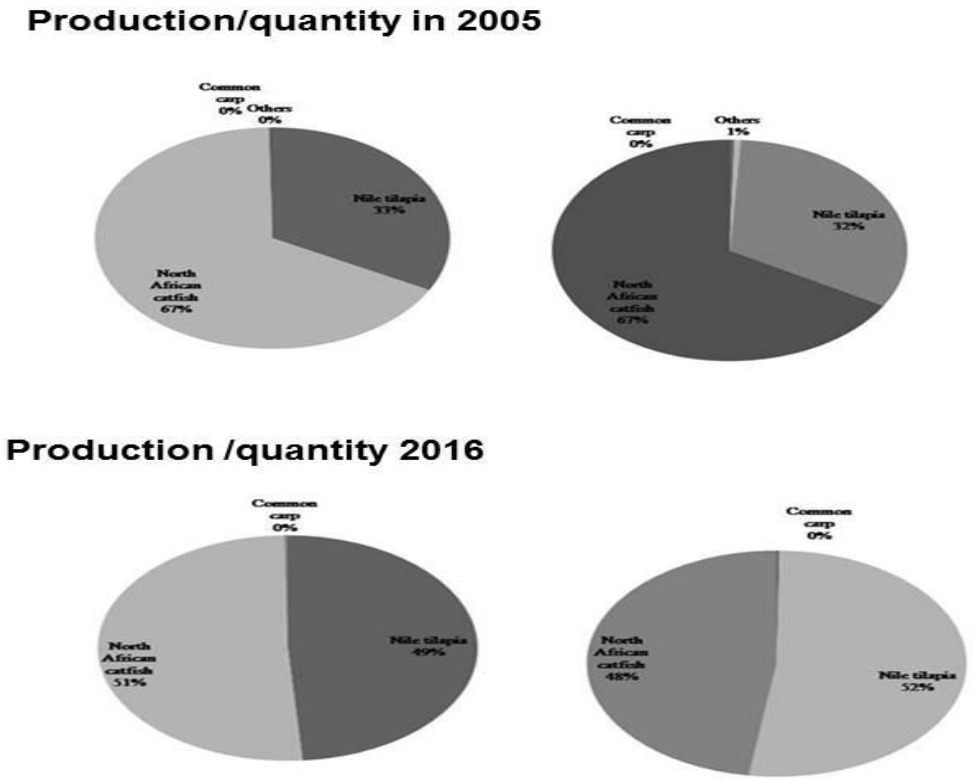


Figure 1-8 showing the contribution of the Three most cultured fish species 10 years ago (2005) and changing trend 10 year later (2016) adopted from Fishery and Aquaculture Statistics. Global aquaculture production 1950-2016 (FishstatJ). In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated 2018. www.fao.org/fishery/statistics/software/fishstatj/en accessed on 4/5/2018

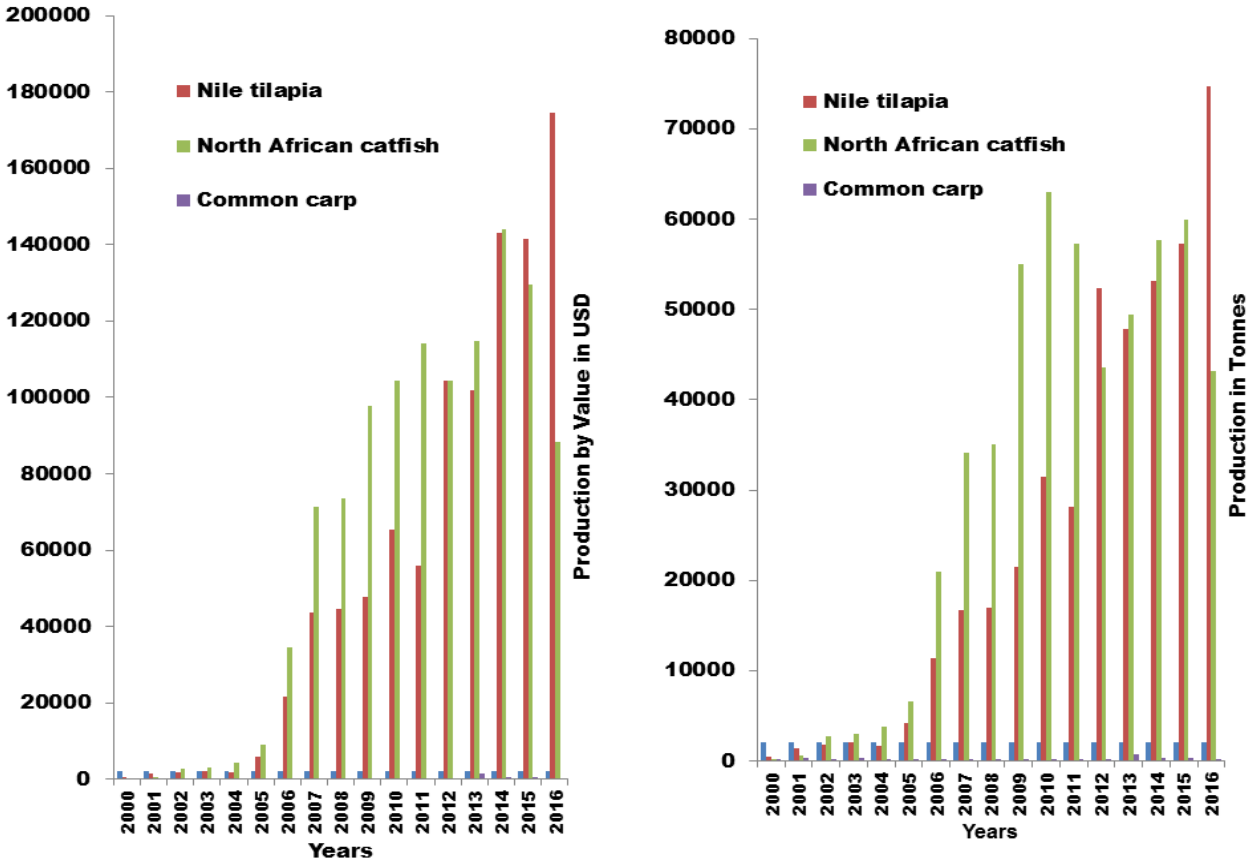


Figure 1-9 The contribution to Ugandan aquaculture by value and quantity by species as FAO. 2018. Fishery and Aquaculture Statistics. Global aquaculture production 1950-2016 (FishstatJ). In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated 2018. www.fao.org/fishery/statistics/software/fishstatj/en accessed on 4/5/2018

This is quite different from the trend depicted 15 years ago as according to FAO 1950-2016. It can be seen from the statistics for species predominance from 2000-2016 that for (*Clarias gariepinus*), North African catfish and (*Oreochromis niloticus*) Nile tilapia, the former has dominated over the last ten years, however, the latter is increasing its presence due to the advent of cage fish farming in Lake Victoria. This has promoted commercial fish farming of Nile tilapia which provides better yields with grow-out farming when compared to North African Catfish, which is a bottom feeder and can't be effectively accommodated in the food web in the open water cages. Most of the North African catfish boom in the past has been attributed to their use in the bait catch fishery for Nile perch, which has since declined.

1.1.3.6 Current status of catfish hatcheries in Uganda.

There are 35 operational hatcheries in Uganda, over 50% concentrated in East and Central Uganda, 11 are located in central Uganda and 8 hatcheries in Eastern Uganda (Mwanja *et al.,* 2015). Of these, three commercial hatcheries are large-scale in nature of which two of were located in central region, while one was found in the East producing between 500,000 and one million catfish seed annually depending on the demand (Mwanja *et al.* 2015).

1.3 General information on Catfish

1.1.4 Overview

Catfish, members of the order Siluriformes, are a highly diverse group of mainly freshwater species. They represent about one third of all freshwater fish species, comprising 34 families, ~437 genera and more than 2700 species (Devaere*et al* 2007,Lundberg & Friel,2003,De Pinna,1998, Teugels, 1996).They are named for the fact that they frequently possess prominent barbels which have a resemblance to cat's whiskers.

Lundberg & Friel (2003) reported that catfish ranged across different sizes as well as behaviour, with the largest recorded being the Mekong giant catfish (*Pangasianodongigas*) in south Asia followed by the Wels catfish (*Silurusglanis)* of Eurasia. Also in existence are the smaller members such as aspredinids and trichomycterids, which are among the smallest vertebrates in the world, however, most catfish are of moderate size of about 5-20cm. Most catfish have been reported to be omnivorous (Nelson 1999) although tiny parasitic species commonly called the *Candiru, Vandellia cirrhosa* also occur. They occur as naked types as well as armour-plated types, neither having scales. Despite their name, not all catfish have prominent barbels; members of the Siluriformes order are defined by features of the skull and swim bladder. Catfish are of considerable commercial importance; many of the larger species are farmed or fished for food. Many of the smaller species, particularly the genus *Corydora*s, are important for the aquarium hobbyists. Catfish are nocturnal. Among the major farmed catfish families are members of the *Ictaluridae*, *Pangasidae*, *Siluridae* and *Clariidae*.

1.1.4.1 Ictaluridae

Ictalurus punctatus (Rafinesque, 1818), also known as the Channel catfish, is a member of the family Ictaluridae and is one of the most important commercially produced freshwater fish species in North America. 90% of the farmed fish production occurs in the Mississippi River valley region (Stickney 2004, Olin 2011). Channel catfish is native to the flowing rivers of North America, including southern Canada and Northern Mexico. Like African catfish, their distant relatives, Channel catfish are opportunistic omnivores. In the USA, channel catfish are raised in ponds, tanks or raceways. As well as food, the fish is also grown for recreational purposes *i.e.* sport fishing. This species was introduced to China in 1984 for domestic consumption.

1.1.4.2 Pangasidae

Pangasius hypophthalmus (Sauvage, 1878), belongs to the family Pangasidae and is another of the cultured catfishes, in particular being widely farmed in the Mekong Delta, Vietnam. (Griffiths, Van Khanh &Trong 2010). It has also been reported to be cultured in Indonesia, Malaysia, Cambodia, Bangladesh, China and Lao People's Democratic Republic. *P. hypophthalmus* is a riverine freshwater species and is omnivorous in feeding mode. Like the African catfish, it is produced by induced artificial breeding in hatcheries using hormones such as human chorionic gonadotrophic hormone (HCG) or pituitary extracts. Vietnam is the world's largest producer of *Pangasius.* The market for this species extends across over 80 countries, however, the EU remains the most significant market contributing 35% by volume and 40% by value (Griffiths, Van Khanh&Trong 2010).

1.1.4.3 Siluridae

The European catfish or Wels *Silurus glanis* (Linnaeus, 1758) is one of the best co-habitant species for use in intensive carp ponds or ponds stocked with a polyculture of various cyprinid species. Its oxygen demand is considerably lower than that of other European carnivorous fish in Europe. Its feeding habits are also advantageous, as the diet is not restricted to live fish, but includes tadpoles, insects and their larvae, worms and dead animals. Wels meat is of high palatability, without bones and scales, its capacity for growth is amongst the

highest of any fish. (Coche & Edwards, 1989). It is cultured in various countries in Europe but its production was < 1500 metric tonnes in 2011 (FAO 2013).

1.1.4.4 Clariidae

The family Clariidae, is a group of specialized, air-breathing catfish (Teugels & Adriaens, 2003; Devaere 2007,). There are 179 species in the genus *Clarias* (Siluroidae, Clariidae) recognized in FishBase (2013). Although more than 100 different species of the Genus *Clarias* have been described in Africa, a recent systematic revision based on morphological, anatomical and biographical studies has been carried out by Teugels (1986a, 1986b). 32 valid species were recognised but it is recognised that the large African species which are of most interest for aquaculture belong to the subgenus *Clarias*.

In earlier systematic studies on the large African catfish species Boulenger (1911) and David (1935) recognized five species of within this subgenus. Both authors used morphological criteria such as form of vomerine teeth, ratio of vomerine to premaxillary teeth band and the number of gillrakers. The five species were; *Clarias anguillaris, Clarias senegalensis Clarias lazera, Clarias mossambicus,* and *Clarias gariepinus.* In 1982 Teugels revised the subgenus *Clarias* and found only two species (*C. gariepinus* and *C. anguillaris*) based on the number of gill rakers on the first branchial arch, it was confirmed that for *C. anguillaris* the number of gill rakers was rather low with a range between 14 to 40 while for *C. gariepinus* was relatively high 20 to 100 (De Graaf & Jansens in FAO 1996).

Although the Siluroidae are not among the model species employed for fundamental genetic and genomic research, the families Clariidae and Ictaluridae represent the best studied catfish families. Also, during the evaluation of strains for aquaculture the two families Ictaluridae and Clariidae have been considered for the longest period among the catfish species. Considerable data concerning the Channel Catfish *Ictarulus punctatus* exist, and the work reported in this thesis exploits these data and the recognised phylogenetic relationship with African catfish, distant albeit (Diogo 2004 and Wong *et al* 2011) Figures 1-10, 1-11.

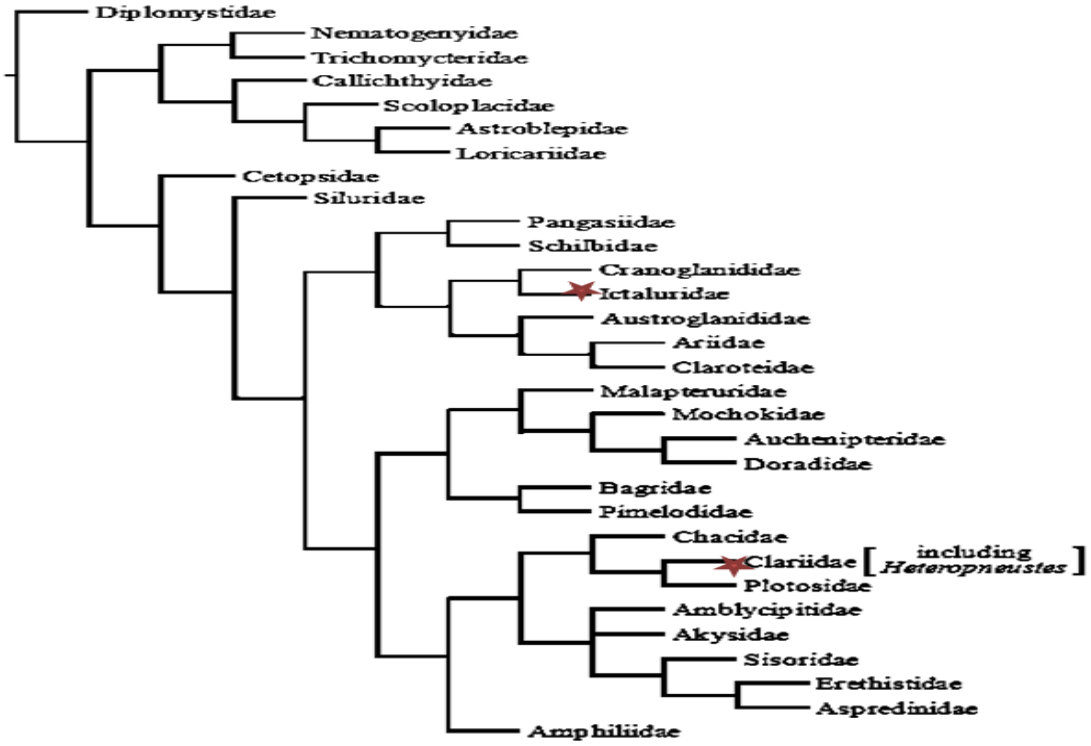


Figure 1-10 The relationships between the major families of siluriforms (adapted from Diogo 2004, Figure 12.9) (species of interest starred)

The phylogenetic relationship of nine key catfish species, two of which are the basis for the current studies *i.e. Ictarulus punctatus* of family Ictaluridae and *Clarias gariepinus* family Clariidae, are shown in Figure 1-11

1.1.5 Description of the genus and species

The North African catfish sometimes referred to as the African catfish genus can be defined as displaying an eel-like shape, as seen in Figure 1-11, having an elongated cylindrical body with dorsal and anal fins being extremely long (nearly reaching or reaching the caudal fin) and with both fins containing only soft fin rays (Van Oijen., 1995 in Witte and Van Densen 1995, Gunder 2004 and Pouomogne 2008).

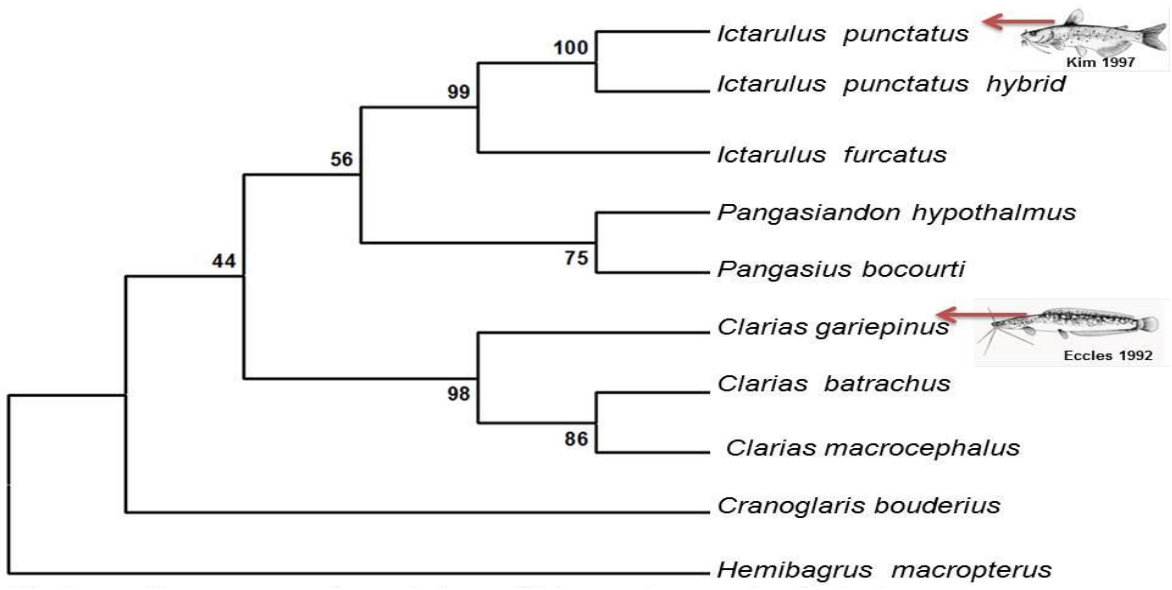


Figure 1-11 Relationships between key catfish species according to DNA barcoding and phylogenetic clustering. Figure adapted from Wong 2011, Figure 1. Species of interest arrowed.

The outer pectoral ray is in the form of a spine and the pelvic fin normally has six soft rays. The head is flattened, highly ossified, the skull bones (above and on the sides) forming a casque and the body is covered with a smooth apparently scale-less skin (scales embedded or partly / completely absent). The skin is generally darkly pigmented on the dorsal and lateral parts of the body and is generally creamy below (Witte &Van Densen 1995). The colour is uniform marbled and changes from greyish olive to blackish according to the substrate. On the other hand fish less than 9 cm have been reported to have a similar colouration except that the dark bands on the head are absent, larger young fish 10-30cm TL are mottled grey dorsally,this marbled colouration may persist in larger individuals. On exposure to light skin the colour generally becomes lighter.

They have four pairs of unbranched barbels, one nasal, one maxilla (longest and most mobile) on the vomer and two mandibulars (inner and outer) on the jaw as seen in Figure 1-12. The major function of the barbels is prey detection (De Graaf & Janssen 1996). Tooth plates are present on the jaws as well as on the vomer.

A supra-branchial or accessory respiratory organ, composed of a paired pear-shaped air-chamber containing two arborescent structures is generally present. These arborescent or cauliflower-like structures located on the second and forth branchial arcs, are supported by cartilage and covered by highly vascularised

tissue which can absorb oxygen from atmospheric air (Van Oijen., 1995; De Graaf and Janssen, 1996, Pouomogne, 2008).The air chamber communicates with the pharynx and with the gill chamber.

The accessory air breathing organ allows the fish to survive for many hours out of the water or for many weeks. The male and females of *C. gariepinus* can be easily recognized as the male has a distinct sexual papilla, located just behind the anus. The sexual papilla is absent in females.

Figure 1-12 North African catfish raised in the Tropical aquarium, Institute of Aquaculture, University of Stirling showing key external features of the Genus.

1.4 Natural range of Occurrence

Clariasgariepinus, which is widely considered to be one of the most important tropical catfish species for aquaculture, has a wide distribution in Africa, from the Nile to West Africa and from Algeria to Southern Africa. They also occur in Israel, Syria and South of Turkey.*Clariasanguillaris* has a more restricted distribution and is found in Mauritania, in most West African basins and in the Nile. In general *C. gariepinus* lives in most river basins sympatrically with *C. anguillaris* (Teugels & Adriaens, 2003).

1.5 Feeding in the wild

C. gariepinus has been described by (De Graaf & Janssen, 1996) De Graaf & Janssen 1996 as a slow moving omnivorous predatory fish which feeds on a variety of foods items from minute zooplankton to fish half of its own length or 10% of its own body weight.

1.6 Aspects of aquaculture for North African catfish *Clarias gariepinus*

North African catfish is one of the major fish species cultured in Africa and is endemic to the African continent. *Clarias gariepinus* is generally considered to be one of the most important tropical catfish species for aquaculture, has an almost Pan-African distribution.*C. gariepinus* has been widely introduced to other parts of the world including the Netherlands, Hungary, much of South-East Asia and East Asia. This species can be cultivated in areas with a tropical climate, areas with access to geothermal waters or with the use of heated recirculating water systems. It is considered to be a very hardy fish, in aquaculture terms, which can be densely stocked in low oxygen waters, making it ideal for culture in areas with a limited water supply. Its air-breathing ability, high fecundity, fast growth rate, resistance to disease and high feed conversion efficiency makes *C. gariepinus* a freshwater species with an extremely wide latitudinal range.

Unfortunately, the features that make *C. gariepinus* an ideal aquaculture species also give it the qualities of an aggressive and successful invasive species. It has a high fecundity, has a plastic phenotype, rapid growth, wide habitat preferences, tolerance to extreme water conditions and the ability to subsist on a wide variety of prey such that it can devastate indigenous fish and aquatic invertebrate populations (Bruton, 1986).

North African catfish in Africa is generally farmed in concrete tanks in the early stages especially in hatcheries and then transferred for onward growth into ponds (Pers. observation 2009 at various fish farms in Uganda). Farming of North African catfish started in early 1950´s and was adopted in the 1970's after field trials which showed that African Catfish is a fast-growing fish which is very robust (Pouomogne,2010). African catfish tolerates a large variety of feedstuffs and is very resistant to changing and sub-optimal water conditions. It can be farmed in high densities reaching production levels of 6-16 MT/ha on an annual basis when raised in monocultures and fed high quality fish feed.

Most of the African Catfish is sold alive into the market. The African catfish are harvested at the age of 6 to 8 months with an average weight of 200-500g.

Fresh African catfish is mildly flavoured and has a tender texture. If heavy and continuous overfeeding occurs, resulting in phytoplankton overgrowth/bloom, this may occasionally lead to an off flavour or “muddy” taste.

Spawning in catfish can be initiated semi-artificially or artificially by hormone injection or injection of ground catfish or tilapia pituitary glands into the ripe female. (Mwanja *et al* 2015).

Spawning takes place in the night following injection where the female is placed with a male in a special net or the stripping of the female and harvesting of the male gonads is done facilitating wet fertilization. The latter is the most commonly employed technique for larval production. The following day both the female and the male are removed. The eggs hatch rapidly and after 3- 4 days the larvae are developed and transferred to the nursing pond where they are grown for 3-4 weeks until they have developed to fingerlings and are transferred to the growing pond (Mwanja *et al* 2015).

In Uganda, the development of *Clarias* culture has been more related to its use as baits for fishing in Lake Victoria. More recently, with the development of balanced extruded feed, there has been a huge diversification of culture environment in most African countries where this catfish is reared, including the use of concrete or fibreglass tanks and water recirculation systems (Pouomogne*,* 2010*;* Hecht 2013*).* In some cases, *C. gariepinus* are used as ‘police-fish’ to control over-breeding in mixed-sex tilapia culture in earthen ponds (Isyagi 2009).

1.7 Early development in larvae of North African catfish

Haylor (1998) described the following life stages in the development of African catfish:

Embryonic stage: Early development takes place inside the spherical membrane of the egg. African catfish eggs are about 1.5mm in diameter and usually green. The egg stage lasts between 18 and 57 hours depending on conditions.The embryo receives its food entirely from yolk for a further 48 to 96 hours after hatching.

Larval stage: following the successful commencement of exogenous feeding, the developing catfish is referred to as a larva. During this period growth is very rapid

and many developments take place. The larva develops a stomach and the digestive system begins to work .The body becomes darker and fins become fully formed .Finally the larva develops and can use special organs in its gill chamber to breath air if necessary. The larval period lasts between 14-42 days depending on conditions. Newly hatched catfish fry, which are only about 6 mm in length, are usually held in indoor trough sand tanks for up to 10 days before being released into outdoor nursery ponds. Initially, catfish fry use their yolk sac as an energy and nutrient source. Once the yolk sac is absorbed (~3–5 days after hatching), fry begin to seek food and need frequent feeding. In hatcheries, fry are fed on finely ground meal or flour-type feeds containing 45–50% protein, which is largely supplied from fish meal.

A number of studies have been done on the development of larval catfish with different authors giving various life stages as shown in Tables 1-1,1- 2. and 1- 3. (Olanyi &Omitigun, Osman *et al* 2008, Fleuren 2008 and Isyagi *et al* 2009 as shown). They have employed various methods including microscopy and photomicrography (Olanyi&Omitigun 2013, histology, (Verreth 1994, Osman *et al* 2008), growth measurements defined in terms of weight and length (Fleuren and Isyagi, 2009). This thesis employs the definitions of Haylor (1998) as shown in Table 1-1., which employed microscopy and morphometric observations.

Table 0-1 Studies of early development in African catfish *Clarias gariepinus* compared within different studies (adapted from Haylor, 1998).

Embryonic stages	**Present study 2014 at 27.5 ±0.5 ˚C**	**Olaniyi &Omitigun 2013 at 28.5± 0.5˚C**	**Osman *et al* 2008 at 24˚C**
Unfertilized oocyte		0 minutes	
Fertilization		0 minutes	
Blastodisc		24minutes	
One cell stage		36 minutes	
Two cell stage		38 minutes	50 minutes
Four cell stage		55 minutes	70 minutes
Eight cell stage		67 minutes	100 minutes
Sixteen cell stage		80 minutes	120 minutes
Thirty two cell stage		83 minutes	135 minutes
Morula		111 minutes	4 hours

Blastula		129 minutes (2 hours 15 minutes)	6 hours
Gastrula		367 minutes (5 hours 45 minutes)	12 hours
Organogenesis		588 minutes (9.8 hours)	20 hours
Fully formed embryo	24 hours		30 hours
Hatching	24 hrs+6 hours	1040 minutes (17 hours)	40 hrs+ 8 hours
At hatching(Day 0)	4.8mm		4.6mm
24hours post hatch(Day 1)	6.8mm	5.5±0.5 mm	5.2mm
48 hours post hatch (Day 2)	7.9mm	6.5±0.5mm	5.4mm
72 hours post hatch (Day 3)	8.6mm	8.7±0.5mm	6.0mm
96 hours post hatch (Day 4)	9.1mm	9.3±0.5mm	6.2mm
120 hours post hatch (Day 5)	10.7mm		7.0mm

Studies on the categorisation of fish size groupings were also conducted by Isyagi*et al* 2009 who came up with the description below based on growth parameters of length and weight as seen in Table1-2 and 1-3.

Table 0-2 Description of the Different Categories of Catfish Sizes*

Category	**Length (cm)**	**Average weight (g)**
Fry	<7	3
Small fingerlings	7 – 10	2-6
Medium fingerlings	10 – 12	6 – 9
Large fingerlings	12 – 15	9 – 20
Extra-large fingerlings	> 15	20

*Source: Isyagi *et al* 2009

Table 0-3 Feed and water quality parameters for the different rearing stages of African catfish*

Growth phase	Age in weeks	Weight (g)	System	Feed	Water quality NH_4,NO_2 pH Temp
Fry larvae	0-2	0.05-0.1	Incubation	Dry diet 0.2-0.3mm	<3,<1,7,28
Fingerling	3-5	0.1-1.0	Advanced fry system	Dry diet 0.3-0.8mm	<4, <2, 7,28
Juvenile	5-8	1.0-8g	Culture	Dry diet 0.8-1.5 mm	<10,<2,7,28
Brood stock	>-1.5 year	>4kg	Brood stock system	Brood stock 9mm	<20<3,7,25

*Source: Willy Fleuren 2008 in Ponzoni RW, Nguyen NH 2008 proceedings of a workshop on the development of genetic improvement program for African catfish, *Clariasgariepinus*

1.1.6 Timing of feeding

1.1.6.1 Feeding larval fish

Larval fish undergo different phases of larval metamorphosis and at a certain phase can be weaned to dry, prepared diets.

The timing of first feeding is very important. If feed is offered too early, the fish digestive system may still be poorly developed and as a result become damaged or blocked, while food not used up will cause deterioration in water quality. On the other hand, if feed is offered too late, fish will begin to hunt for food and prey on other fry. A severe cannibalistic tendency is recognised in African catfish (Haylor & Muir 1998, Nkhonjera 2003).

If fish completely use up their yolk reserves before feed is available to them, they will lose the capacity to forage and die. This stage is sometimes described as the point of no return (PNR). The onset of feeding should therefore fall after the beginning of foraging behaviour but before complete yolk absorption.

The window of opportunity during which feeding can be established begins earlier and becomes smaller as temperature increases. For example at 20ºC the time between the onset of foraging behaviour and PNR is over 40 hours, beginning late on the fourth day after fertilisation while at 34ºC this period is less than 12 hours, beginning early on the second day after fertilisation as seen in Figure 1-13 and 1-14.

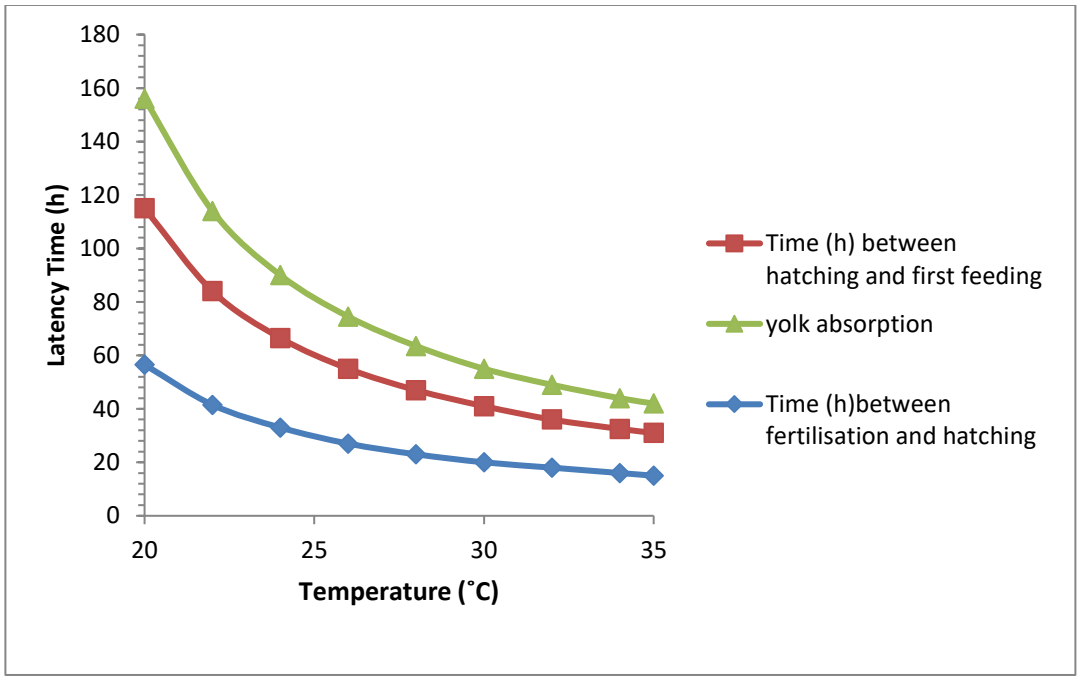


Figure 1-13 Influence of temperature on the early development of African catfish Redrawn from data. Source Haylor and Muir 1998 as Table 7.1 sourced from Haylor & Muir 1994, Haylor & Oyegunwa, 1993. NB The time is to the nearest 30 minutes, days.

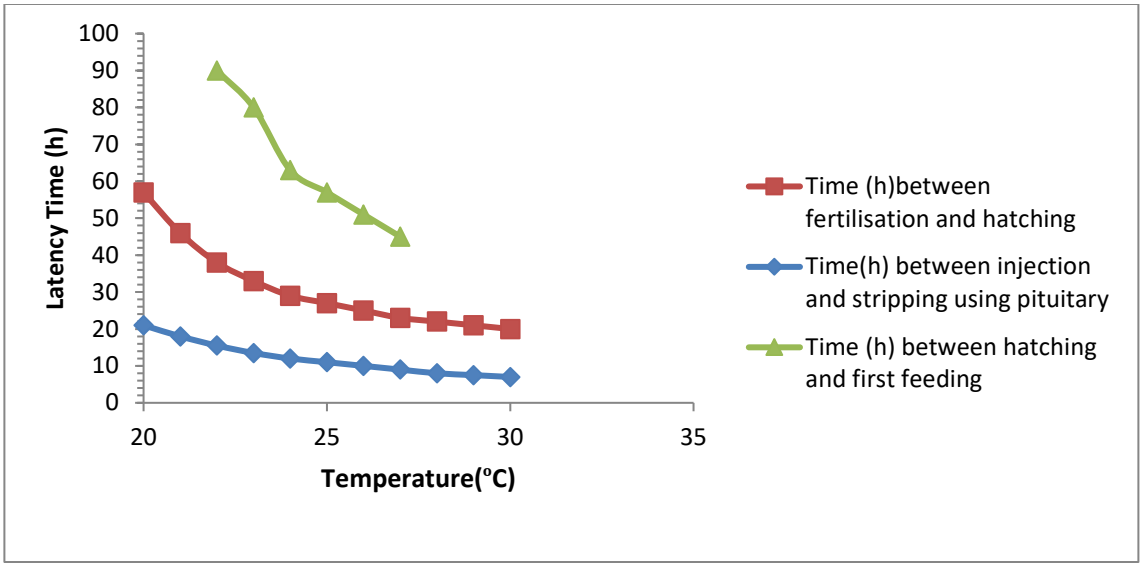


Figure 1-14 Relationship between the time interval between injection and stripping, the time interval between fertilization and hatching and time between hatching and first feeding in relation to water temperature (replotted from data. Data Source: Tendo fish farm Entebbe Uganda 2009, 2010 (Adapted from Hogendoorn and Vismans 1980 in De Graaf and Janssen 1996)

1.1.6.2 Fish hatchery design and operations in Uganda

African catfish hatcheries mainly employ rectangular concrete tanks of mean total depth 0.5 metres. About 20% of the tanks are lined with ceramic tiles, 30% with emulsion paint and 50% operate on rough concrete surface (Mwanja et al., 2015).

Nursing, weaning larvae and raising the fry after hatching the larvae fed on yolk until its exhaustion after 3 to 4 days. Weaning was done immediately on natural feeds, commercial - artemia and artificial dry feeds. However, several farms used them in combination, either artemia and dry feeds (11%), live pond organisms or dry feeds (8%), or Artemia and live pond organisms (11%). About 25% and 31% hatchery operators use dry powdered feed and artemia alone respectively (Mwanja *et al*., 2015).

1.1.6.3 Management of broodstock catfish in Uganda

About 40% of the hatchery operators in Uganda collect the brood fish from other producers, 30% procure brood stock from the Aquaculture Research Development Centre -Kajjansi under NARO -Naffiri, while 30% percent obtain the brood fish from the wild. All hatchery operators condition their broodstock in earthen ponds under natural aeration and artificial feeding.

1.1.6.4 Feed Management for larval *Clarias gariepinus* in Uganda

North African Catfish, *Clariasgariepinus* is commonly spawned and their larvae reared in hatcheries for ten to 14 days, after which they are reared in nursery ponds or in tanks (Hecht 2013). Extensive rearing of larvae, after yolk sac absorption, in ponds is now less often practiced than in the past.

In the retina of the African catfish *Clarias gariepinus*, the pigment epithelium and the tapetum are formed in newly hatched larvae, the cones develop within 2 days, and the rods within 3 days after hatching. Early appearance of the retinal elements enables African catfish larvae to see and feed well even in dim light (Appelbaum & Kamler 2000; Kawamura *et al.,* 2016).

Feed management practices in hatcheries are closely matched with the physiological and endocrinological ontogeny of the fish. For optimal survival and growth, live food (mainly Artemia) is required for the first five days after the start of exogenous feeding, after which the fish can be weaned onto a dry starter feed. Up to a size of 5 g. The species has a high protein demand (>50 percent). Extensive farming of catfish in ponds is largely a subsistence activity and is practiced mainly in polyculture with tilapia that serve as prey fish, accompanied by use of a single-ingredient feed such as maize or wheat bran.

Semi-intensive on-growing of catfish in static and flow-through ponds, as well as under high-density tank culture conditions requires a complete feed. Production levels achieved in these three systems are 15–24 tonnes/ha/cycle, 40 tonnes/ha/cycle and 385 kg/m3/cycle, respectively (Hecht 2013).

1.8 Market for farmed fish in Uganda

The African catfish is a commercially farmed fish with great potential to contribute to reducing the fish gap in Uganda. However, high fish mortalities in catfish hatcheries are a major concern as less than 150,000 catfish fry are produced relative to the estimated 30 million catfish fingerlings that are required annually (Owori-Wadunde & Kityo 2014).

The estimated 20,000 tonnes of the annual production of Uganda's catfish is exported to the regional market. Production of this tonnage requires an estimated 30 million catfish fingerlings. Moreover there exists the need for about 170 million catfish juveniles for the Nile perch fishery as bait.

The trade in African catfish fingerlings and juveniles was estimated to be worth UGX 40 billion (Owori-Wadunde and Kityo 2014) at a unit UGX 200/=per fingerling /juvenile).

The price has slightly increased more recently, with a size f 5cm-6cm costing 300 UGX, 7-8cm costing 400UGX. Broodstock go for 10000-15000 approximately 3USD -5USD and as for consumers, each table-size fish goes for 7000-8000 UGX about 2 USD. However, one factory namely Greenfields offers 6000 UGX (1.5USD) for cultured catfish per Kg. (Pers. Comm. Kiddu, 2017). Farmed African catfish has no structured marketing process, using the same market as catches from the wild market and this in some way affects the prices, as farming fish involves a lot of financial input, unlike wild caught fish. However, in 2009 assistance from USAID-FISH project resulted in the formation of Walimi Fisheries Cooperative Society Wafficos to aid in marketing, provision of farm inputs like feeds *etc*., consultancy services for upcoming farmers and a catfish culture manual. This society has grown from strength to strength, mounting an Aquaculture themed symposium for the last four years and providing access to new knowledge for aquaculture stakeholders.

1.1.7 Farmed Fish Products available on market and Value addition

According to Project Velvet 2015, opportunities exist in Uganda for: Fish processing at lakes other than Lake Victoria; Commercial aquaculture to supplement reduced fish production from traditional sources; Setting up of nurseries and hatcheries for quality seed and fingerlings for stocking of fish farms; and production of high quality feed that meets international standards. While fish processing and value addition is ISO 9001 certified for fisheries for export to the EU as of 2003 (Hempel, 2010), currently the processing and value addition for cultured/farmed fish is its infancy (Pers. observation).

As of 2009 catfish has been sold as whole fresh catfish, whole smoked catfish (gutted), catfish frozen fillets and catfish smoked fillets (USAID -FISH project 2009). However, in 2011 a new product was introduced namely fish sausage from catfish. This an innovation spearheaded by Lovin Kobusigye (http://www.monitor.co.ug/Business/Prosper/Making-a-kill-from-fish-sausages/688616-1885534-mhc5jcz/index.html 2013) formerly working with Wafficos and now an entrepreneur diversifying the products to include minces,

liquid smoked loins/portions, marinades, surimi. and *Alestesbaremose* (agarra) sausages.

The Nile perch was previously the main source of filleted fish on the local market. Because it was cheap, most consumers purchased Nile perch fillet for their children. However, it is mostly the Nile perch that is exported as fillet out of the country and alternatives have to be sought. Catfish fillets are now beginning to find their way into the local market where they offer a good substitute. In addition, catches of the *Bagrus docmac* (Semutundu), a delicacy in Buganda, have declined so much that one hardly finds these fish in the local markets anymore. More people are now buying high quality smoked catfish from farms as an alternative. This African catfish is also a delicacy in the Eastern and Northern parts of the country. Consequently, by 2007/2008, about 33 tonnes of catfish were sold from 20 farms that reported to the United States American people's investment in Development (USAID) under the Investment for Sustainable Harvest FISH Project.

1.9 Confirmation of the Clarias species cultured at the Tropical Aquarium, Institute of Aquaculture, University of Stirling

1.9.1 History of the African catfish species cultured at the University of Stirling, Institute of Aquaculture

The catfish species under investigation was introduced to the Institute of Aquaculture, University of Stirling in 1990/1991.They came from a tilapia farm in the Kafue River basin in Zambia, which drains Zambezi riverine systems. The farm, called Kafue fisheries and owned by Flynn, was located not far from Mazabuka town. The broodstock were originally sourced from wild fish stocks (Kim Jauncey 2010 pers. comm.).as shown on map Fig 1-15 below.

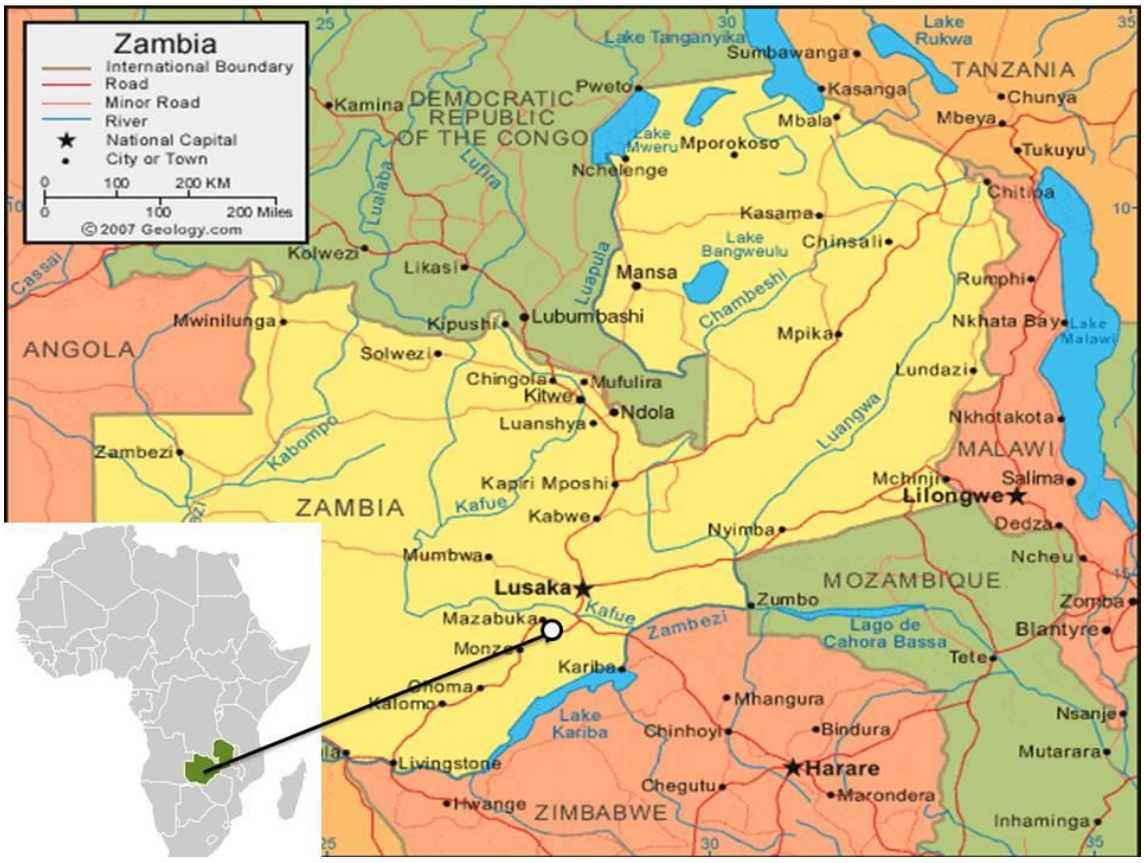


Figure 1-15showing the location of the source of parent stock currently kept for research in the Tropical Aquarium at University of Stirling.
source:https://geology.com/world/zambia-satellite-image.shtml

No studies have so far been undertaken to confirm the exact identity of the species currently used for studies at the Institute of Aquaculture. In this respect it is worthwhile noting that in the wild, populations of *C. gariepinus* and *C. Anguillaris* often co-exist sympatrically, especially in riverine systems (Teugels, 1982) and cursory physical inspection may not be sufficient to discriminate the species. While the genus *Clarias* comprises 32 recognised African species, (Teugels, 1982, 1986) the sub-genus *Clarias*is made up of only two valid species, *Clarias gariepinus* and *Clarias anguillaris*, which cannot be differentiated morphologically except by counting the number of gill rakers on dead fish (Popoola, Fasakin, & Awopetu, 2014;Nwafili & Tianxiang 2007; Popoola *et al.,* 2014; Solomon *et al.,* 2015).

1.10 Contextual framework

From the available information, North African catfish / sharp tooth catfish (*C. gariepinus*) currently contributes an estimated 59% of aquaculture production in Uganda and is also an important aquaculture species in Egypt and Nigeria (FAO 2017). The major constraints to the culture of catfish in Uganda include: lack of sufficient quality and quantity of catfish larvae required by grow-out farmers (FAO 2012); low catfish larval survival rates (25%-35%) partly due to cannibalism; nutritional problems, accompanied by high costs of live feed, *Artemia*, which cost up to $50 / Kg (Bwanika pers comm. 2010). The reported work aims to improve understanding of larval feeding and development during the critical first four days post hatch (Dph) in an effort to help improve early larval survival and growth

1.10.1 Key Objectives

1.10.2 Objectives

The key objectives of the described research were:

1. To confirm the catfish species raised at the Institute of Aquaculture in order to allow application of the knowledge gained to the culture of the species of interest, *Clariasgariepinus,* in Uganda. Applications of the proposed work include better formulation of micro-diets for larvae and improved seed quantity and quality resulting from a better understanding of nutritional physiology.
2. To establish whether the key enzymes pepsin, chymotrypsin, trypsin and gastric chitinase are expressed during early development of *C. gariepinus* larvae
3. To examine the ontogeny of transcript (mRNA) expression for the above enzymes during early development
4. To determine the sites of enzyme expression in developing larvae
5. To contextualise enzyme expression within larger developmental transcriptomic events and examine whether dietary composition / timing affects expression of these enzymes

1.10.3 Research questions

The key research questions asked at the inception of this research were:

1. Are proteolytic enzymes pepsin, chymotrypsin and gastric chitinase expressed in *Clarias gariepinus* larvae?
2. What are the sites of enzymes expression?
3. What is the ontogeny of transcript (mRNA) expression of the above enzymes?
4. Does dietary composition / timing affect the transcript expression of the named enzymes?

2 CHAPTER TWO

2.1 General Materials and Methods

2.1.1 Broodstock source and husbandry of African Catfish, Clarias gariepinus (Burchell, 1822)

North African catfish, *Clarias gariepinus* was raised in the Tropical Aquarium facility located at the Institute of Aquaculture, University of Stirling, Scotland United Kingdom between October 2010 and December 2013.

2.1.1.1 Experimental set up, feeding and brood stock selection

The broodstock fish were raised in a recirculating aquaculture system (RAS) at a stocking density of 20 adults per m^{-3}. The husbandry system comprised 4 centrally drained rectangular tanks with lids (Figure 2-1).

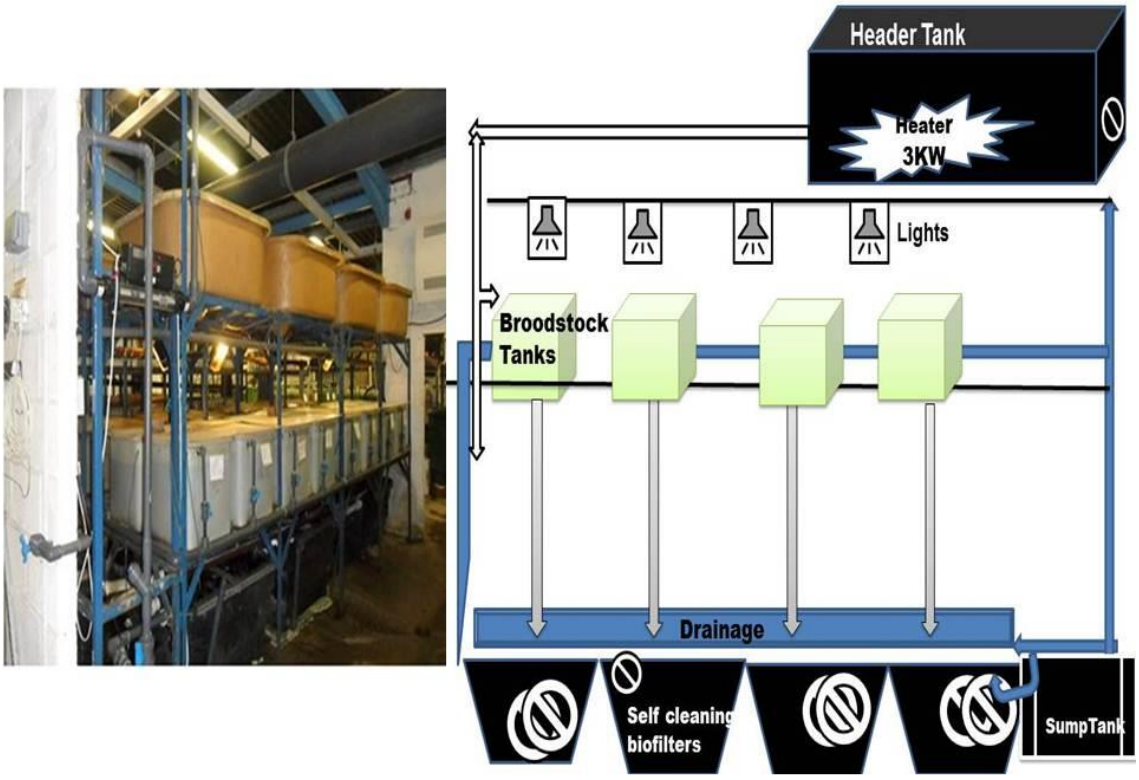


Figure 2-1 Setup of the tanks where broodstock catfish were raised. Left hand photo shows actual setup with middle grey tanks holding fish, bottom black tanks being bio filters and top brown tanks being header tanks. Right hand image comprises diagrammatic overview of system employed

Each unit held a capacity of 280L of water with dimensions of 1m length and 1m width and 0.28m depth. Water was supplied to each tank at a flow rate of

12L min^{-1} from a 350 L header tank. Water was drained through central stand pipes and passed through four biofilter tanks. The water then passed to a sump tank and was pumped up to a 350L header tank containing air stones for aeration and heated with 3KW thermostatically controlled immersion heaters to maintain a temperature of 28.5±1°C throughout the experimental period. About 20% of the water in the system was replaced weekly with fresh water to maintain the water quality and the system subjected to a full exchange every four weeks. A photoperiod of 12 hours and 12 hours of dark was maintained with the light phase extending between 08:00 to 20:00. The setup of the system is shown in fig 2-1

2.1.1.2 Feeding the Broodstock

The fish were fed four times per day by hand to satiety on Skretting Expanded 60, a complete trout diet (Skretting, Preston, UK) during the study period 2010 to 2012 and for the latter part of my study period in 2013 they were switched to a different complete feed for trout known as completed feed for Trout small shaped pellets for trout 150 EWOS Scotland, UK). (See composition in appendix 1).

Two weeks prior to stripping, a pair of fish from the parental stock (male and female) were selected and fed more frequently *i.e.* four times a day unlike the usual twice a day just above maintaince to prepare them for induction for artificial breeding.

Female brood stock of mean size 1000g+/-200g were used for the four cycles of sample collection and males of mean size 1500g+/-500g were selected. A female was considered to be ripe if she had a well distended abdomen.

2.1.1.3 Induced breeding.

Selected female broodstock were anaesthetised using benzocaine (prepared by dissolving 100 g of benzocaine dissolved in 1 L ethanol or 10g benzocaine $100mL^{-1}$ ethanol made the stock solution which was and dosed at 10 mL of the stock solution per litre of water). The female fish was injected with Ovaprim™ (Syndel, USA) at 0.5mL Kg^{-1} using a 2 mL graduated syringe. Fish were injected intramuscularly at an angle of 30-45°underneath the pectoral fins with half of the dose delivered on either side. The fish was then placed in a tank and left to recover, with tight covering to prevent escape.

The injected female was removed from the tank after a latency period of 16 hours, anaesthetised as previously described and the eggs stripped into a dry bowl. A male catfish was then euthanized in accordance with UK Home Office Schedule 1

by terminal anaesthesia and destruction of the brain. Sperm / milt were removed by opening the abdomen using a pair of scissors and a sterile scalpel blade (Solmedia SCL220 No 22, UK) followed by laceration of the sperm ducts. Milt was released into a dry Petri dish. This was then added to the stripped eggs (wet fertilisation). Water was then taken from the culture system where they fish were fertilised and added to the eggs and milt, mixed and poured on meshed netting which was placed into a horizontal rectangular substrate incubator as shown in figure 2-4 below.

2.1.1.4 Water quality parameters

The following water quality parameters were monitored once a week (Table 2-1). pH was measured using a Tropic Marin freshwater (Wartenberg, Germany) pH test kit. Ammonia-Ammonium (NH3/NH4+) was measured using Tropic Marin fresh water ammonia /ammonium test kit (Wartenberg, Germany) and Nitrites / Nitrates were measured using the Tropic Marin freshwater nitrite / nitrate test kit (Wartenberg, Germany). Total water hardness GH was measured using the Tropic Marin GH kit (Wartenberg, Germany) and dissolved oxygen and water temperature were measured using a YSI Environment/Eco sense D0200 Probe.

Table 2-1 showing Water quality parameters measured

Parameters	**Range**	**Acceptable ranges**
pH	7.0-8.0	6.5-8.0[1]
Total Ammonia (mg L^{-1})	< 0.01	<0.05[1]
Nitrite –Nitrates (mg L^{-1})	<0.01	.5-0.7[1,3]
Total water hardness (°dH)	4-5	3-10[1]
Dissolved Oxygen (mg L^{-1})	5.9-8.5	>3.001[1]
Temperature °C	26.5- 28.5	10-32[2]

[1]Md. Zulfikar Ali 2001 (Ali 2001) adapted from (Viveen *et al.,* 1985; Peteri *et al.,* 1992)

[2]Md. Zulfikar Ali 2001 (Ali 2001) adapted from (Hoffman *et al.,* 1991) and (Pers comm with Kim Jauncey 2011)

[3]Baßmann, B., Brenner, M., & W. Palm, H. (2017).

2.1.2 Larval catfish Clarias gariepinus rearing and husbandry protocols

Larvae of African catfish, *Clarias gariepinus* were hatched at the model hatchery facility in May and December 2011, August 2012, July 2013 and November 2013,

with approximately 6,000 - 10,000 larvae hatched per run. These were raised initially in 2011 to 28 days but in the succeeding sampling they were raised only up to 10 days, with fish euthanized at the end of the experimental period. The system was constructed by the researcher based on the description of the system in tables 2-2 and 2-3 and figure 2-2 below.

Table 2-2 showing the description of experimental system .

Description	**Quantity**
Experimental Tanks Number Volume Depth Water flow rate	 32 3.5 litre 10cm 15 mL s^{-1}
Filtration Tank Number Volume	 1 30 litres
Volume of Sump Tank(L)	100 litres
Volume of Header Tank (L)	114 litres

Fish were raised in a RAS system set up in white 5 litre niftlid round bottomed buckets (Solmedia laboratory suppliesP45000, UK) at a stocking density of 100 fish per litre in triplicate sets from a hatchery of volume 3.5 L at one (1) day post hatch (dph). The flow rate was 15mL s^{-1} and hatchery was supplied with UV to sterilise / disinfect the system (Vecton UV water steriliser, Tropical Marine Centre Hertfordshire England, UV 25 UK 150 litres) and heated with a coil 400W and managed semi-automatically with a thermostat for the temperature fluctuations. Photoperiod was maintained at 12hour Light and 12 hour darkness with the light phase extending from 08:00 to 20:00.as shown in figure 2-2 below

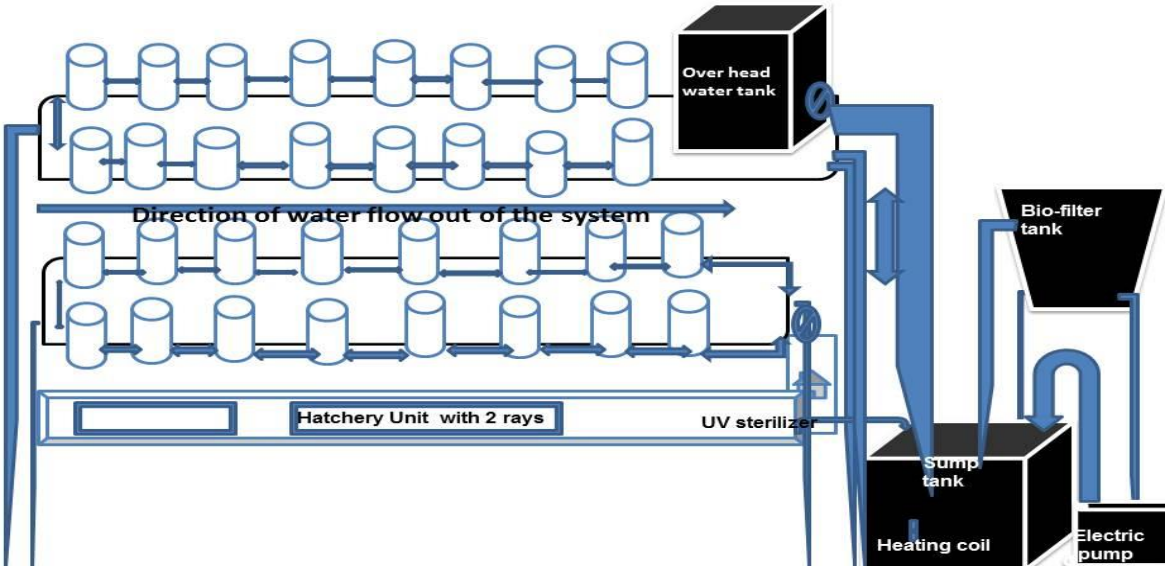


Figure 2-2 shows the setup of the larval catfish rearing system in the Tropical Aquarium

2.1.2.1 Incubation type

The study employed the substrate hatching facility using a mesh net for placing the eggs in rectangular types of incubator and placing it in fibreglass tank volume 92LA rectangular facility of length 1000mm by 100mm width and 100mm height was used .Water was filled up to the 92mm mark and two meshed rectangular nets were placed parallel to the each other to cover the entire incubator. The mesh acted the substrate over which the eggs attached upon fertilized and when they hatched they passed through and settled at the bottom. A 1-layer hatching substrate (40mmx40 mm) was used. The eggs were incubated on the green or grey hatching substrate as usually the practice. After hatching the larvae moved through the spaces (about 2x15 mm) and sheltered in the substrate nipples of the soft rubber like plastic substrate (5mm high). The hatching substrate imitated the gravel substrate naturally occurring in their home rangein the lakes or rivers and provided optimal developmental conditions for the fish larvae. The water in the incubator was heated up to 28ºC as seen in the sample incubator fig 2-3

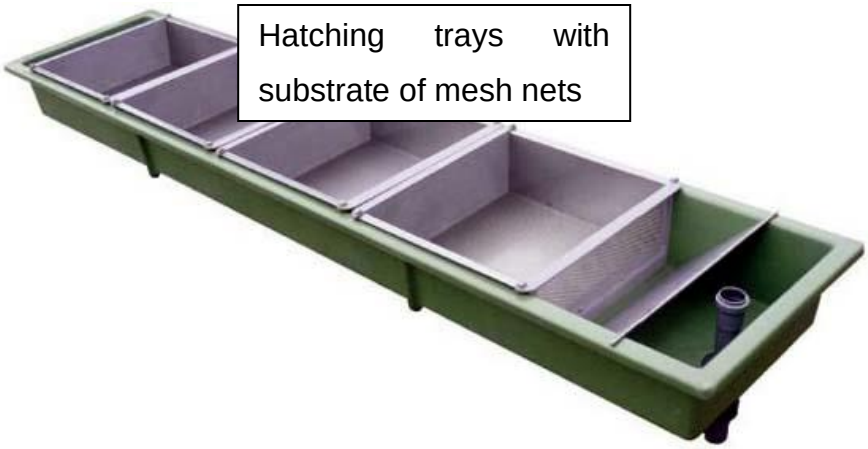


Fig 2-3 showing the typical incubator used in the study sourced from http://www.aquaculture-com.net/breeding.htm accessed 21/07/2018

2.1.2.2 Estimation of percentage fertilisation and hatchability

The percentage fertilisation and hatchability were calculated in accordance to the following: Adebayo and Popoola 2008 and Agbebi *et al* 2013

Relative fecundity =Total number of eggs/Body weight of female.

Fertilisation rate = (Number of fertilized eggs /Total number of eggs counted) X100

Hatching rate= (Number of hatched eggs /Total number of eggs in the batch) X100

Survival (%) = (Number of hatching live up to larval stage/Total number of hatching)X 100

The eggs were weighed in grams upon stripping using the assumption that for small or big eggs 1 gram contains 750 or 700 eggs respectively (Suleiman Isa pers. Com. 2013) with estimates for this study shown below in table 2-3

Table 2-3 showing the estimation of percentage fertilisation and hatchability

Fish wt (g)	Date	Egg weight (g)	Total number of eggs	Fertilized eggs	Hatched eggs
900	9-Dec-2013	66	49,500	44,550	40,950
1300	8-Jul-13	187.8	131,460	111,741	108,141
1500	3-Dec-2012	270	189,000	168,210	185,400
1600	18-Apr-2012	199	139,300	125,370	121,770
Fish wt (g)	**Date**	**Fecundity**	**Fertilization rate %**	**Hatch rate %**	**Survival %**
900	9-Dec-13	55	90	83	73.3
1300	8-Jul-13	101	85	82	83.2
1500	3-Dec-2012	126	89	87	97.2
1600	18-Apr-12	87	90	87	73.9

2.1.3 RNA extraction of the digestive system of adult and larval North African catfish

RNA was extracted using an organic extraction analysis protocol. Thirty mg of adult North African catfish pancreas, stomach, midgut, hindgut, foregut and liver were processed individually while in the case of larvae, whole fish were used since it was difficult to extract the digestive system for them. Tissue was placed in 2 mL screw cap microtubes (Alpha laboratories, UK) containing two 3 mm glass beads (Merck KGaA,USA) and homogenised in 1 mL of Trizol (TRI)Reagent (Sigma Aldrich USA) for 30-45 seconds in a mini bead beater (Thistle Scientific,UK), until the tissue was significantly disrupted. The sample was then incubated at room temperature for 5 minutes.

100 µL 1-bromo-3-chloropropane [BCP] (Sigma Aldrich USA) was added and the tube was shaken vigorously by hand for 15 seconds. It was then left to incubate at room temperature for a further 15 minutes. The sample was then centrifuged (SciQuip 4K15 Sigma) at 20,000 × g for 15 minutes at 4ºC. Then 400µL of the upper aqueous phase was transferred to a nuclease-free 1.5 ml microfuge tube (Axygen fisher Scientific, UK).

200 µL of RNA precipitation solution comprised of 1.2M NaCl and 0.8M Sodium Citrate Sesquihydratewas added followed by an equal volume of 200µLisopropanol (Fluka) to the aqueous phase. The sample was then gently inverted 4-6 times. This was followed by incubation for 10 minutes at room temperature. The sample was then centrifuged at 20,000 × g for 10 minutes at 4°C. A precipitate of RNA formed a gel-like pellet on the side/bottom of the tube. This was followed by a washing phase during which the supernatant was removed and then the pellet was washed with 1 mL of 75% ethanol (Fisher Scientific). This was followed by brief flick-mixing of the sample and the sample was then centrifuged at 2000 × g for 5 minutes at room temperature. The supernatant was carefully removed using a 1 mL (Gilson) pipette followed by re-spinning down the sample and further removal of the supernatant using 20 µL (Gilson) pipette. Finally the RNA pellet was air dried at room temp for 3-5 minutes, until all visible traces of ethanol were evaporated.

The RNA was resuspended in 30µL of RNase free water and then incubated overnight on ice. The sample concentration was then measured using a Nanodrop 2000 spectrophotometer (Thistle Scientific, U.K.). RNA integrity checks were then

performed by heating a 1.0 µg aliquot of RNA for 5 minutes at 75ºC, chilling briefly on ice and running on 1% agarose gel containing ethidium bromide (0.05µg mL^{-1}) along with a 100 bp size marker. Photography of the gel was carried out using a Syngene gel doc system (Syngene, USA).

2.1.3.1 cDNA Synthesis

cDNA was produced using 2µg of RNA with a TaqMan Reverse Transcription kit according to manufacturer's instructions (Applied Bio systems, N808-0234) for a total reaction volume of 20µL.

RNA was diluted to make 2µg $µL^{-1}$ stock in a 10µL volume placed in a 0.2mL nuclease-free tube (Thermo scientific). This was then followed by denaturing at 70° C for 5 minutes on a thermo cycler (Biometra T gradient) followed by placing samples on ice. The next step was the preparation of a master mix comprising of 2µL of 10XRT buffer, 0.8µL of 25XDNTP (100mM) 1.5µL of 10XRandom10XRandom primer, 0.5µL of 20µM Oligo (dT), 1µL Multiscribe Reverse Transcriptase and 4.2µL of Nuclease free water. The 10µL mix was added to the former sample on ice, flick-mixed and briefly centrifuged after thorough mixing. Reactions were placed on the thermal cycler under the following conditions: 25°C for 10min followed by 48°C for 60min then 95°C for 5min followed by a final holding step of 25°C for 2s.

3 CHAPTER THREE

3.1 Confirmation of species under culture at IOA and investigation of the growth performance of larval North African Catfish *Clarias gariepinus* fed on live feed or off-the-shelf micro- diet introduced between during and after the yolk sac period.

3.2 Introduction

Fresh water and marine fish larvae have been documented to exhibit one of the fastest growth rates among vertebrates, with growth rates of 50% of daily body weight recorded (Conceicao, 1997). Feed intake in terms of quality and quantity contributes, to a great extent, to the growth attained in fish (Kaushik & Médale, 1994).

Fish occurring in the tropics display a fastdistinct type of growth, which is not the case for temperate species whose growth patterns are indistinct (Dutta, 1994). Since *Clarias* is a tropical genus, then it might be expected to reveal a distinct growth pattern. Growth is usually measured in terms of length and weight increase over time and in aquaculture it can be measured for production in terms of the specific growth rate: *ln (weight at harvest - weight at stocking) / production period * 100*. A relationship between weight and length can also be used to provide an index of the state of well-being of a fish, termed the condition factor 'K'. Growth can be characterised by various other measures, including, among others, protein retention in the tissues (Gisbert *et al.*, 2008), RNA: DNA ratio, hepatosomatic index, and glycine uptake, which provide different insights into the growth process. The quality and quantity of food plays a significant role in growth regulation,, however, it is also controlled by other exogenous factors including, among others, temperature, oxygen concentration, salinity and photoperiod (Kaushik & Médale, 1994). It is also critical to note that genotypes, endocrine status and physiological condition of the individual carry equal importance as endogenous regulators of growth (Dutta, 1994). However, not much has been done from the physiological point of view in many individual fish species. The research presented in this thesis seeks to reveal how the individual expresses enzymes important to digestion, which in turn affect growth. Downstream chapters 4, 5, and 6 cover gene expression from individual transcripts to broad scale microarray transcriptomics. This work was intended to help in the formulation and timing of diets based on

what occurs in and is required by the individual rather than formulation according to the ingredients that are most readily available.

Modern fish nutrition research covers a broad range of interrelated fields and often requires integration of knowledge coming from advances in chemistry, biochemistry, physiology, microbiology, immunology and molecular biology (Jobling 2016). Notwithstanding new advancements in manufacture of off-the-shelf feeds for larval fish, lack of acceptability / suitability of such feeds for many larval fish result in most species being weaned on live feeds (Conceição *et al.*, 2010). The most commonly used live feeds include *Brachionus spp.* a rotifer and the branchiopod brine shrimp *Artemia sp.* for which well laid-out guidelines exist for culture and enrichment to get the desired quantities and quality. These organisms may, however, be deficient in several key nutrients including HUFA, vitamins, minerals and amino acids (Conceição *et al.*, 2010).

Others including free-living copepods have also been used and have been found to provide superior survival rates, growth rates and quality, however, their production is more labour intensive and no standardised guidelines are readily available. Good overview and control of the supply line of nutrients is important for developing diets for use in larval culture and for the adaptation of rearing conditions that meet larval requirements for the optimal presentation of food organisms and or micro diets. Fish larvae fed micro diets have not, at this point, matched the growth and survival performances demonstrated by larvae fed live feeds such as rotifers and *Artemia (*Kolkovski, 2013).

Many studies have been conducted concerning the morphology and functional capacities for nutrient uptake of larvae of *C. gariepinus*. It has been observed that at the beginning of exogenous diet intake, the larvae of *C. gariepinus* possess an advanced digestive system with a functional pancreas, liver and nutrient absorption capabilities, but lack a functional stomach in the first few days (Verreth *et al.*, 1993). This means they are nominally capable of digestion and deploying enzymes, however, they are thought not to consume live feeds because pepsin activity is not yet in place.

Several studies on nutrition of larval North African catfish, *C. gariepinus* have been conducted, however, there is not a single starter feed identified to date which can be found off-the-shelf for use by fish farmers involved in hatchery operations in

Uganda and elsewhere, and who urgently require rationally formulated alternatives. Live-feed studies performed to date include experiments with the free-living copepod *Cyclops* spp. and *Artemia* in which a mixture of the two produced better growth performances when compared to feeding just one of the them, however, fish fed the *Cyclops* alone yielded a better growth performance than fish larvae fed on *Artemia* at the switch to exogenous feeding (Chepkwemoi *et al.*, 2013).

In parallel studies on the rotifer *Brachionus calyciflorus* and *Artemia* employed as live feed to larval North African catfish. The fish fed on a mixture of *B. calyciflorus* and *Artemia* showed best growth performance (Abaho *et al.*, 2016). In both cases fatty acid profiles of *Cyclops* and rotifers were better than *Artemia* in terms. of DHA, AA, EPA. This may explain why they performed better as feeds, because these nutrients are important in the early stages of development *e.g*. development of the nervous system which is vital in feeding coordination (Abaho *et al.*, 2016; Chepkwemoi *et al.*, 2013).

Other studies have examined the use of various sources of components for a making an acceptable diet for larval North African catfish such as use of *Daphnia*, *Ostracoda*, fish meal, soybean meal, dry enzyme diets (Kemigabo *et al.*, 2016; Mosha, 2015; Ngupula *et al.*, 2014; Olurin *et al.*, 2012; Olurin & Oluwo, 2010). Despite this, better growth performance continues to be experienced using live diets for weaning (Evolubi *et al.*, 2016) followed by an off-the-shelf diet, but it is not possible to produce live diets in sufficient numbers to promote intensive fish farming of North African catfish in all cases.

More recent studies have involved use of dry feed in combination with de-capsulated Artemia, Moina both live feeds (Aruho, *et al.*, 2017; Yakubu *et al.*, 2015) with results indicative of the fact that the dry diet CP 57%, a commercial diet imported in to Uganda, can be used as a starter diet for larval North African catfish, although this would be expensive for the farmers and therefore there remains a need to find an off-the-shelf diet composition that suits the species.

The present researcher set out to look at the digestive physiology of larvae of this species at the transcriptomic level, raising fish under 6 feed treatment regimens and subjecting selected samples from these studies to gene expression analysis, which is described in chapters 5 and, 6 of this thesis.Prior to investigating the growth performance,work was undertaken to confirm the species of the catfish

stock held at the University of Stirling's Tropical Aquarium, a facility of the Institute of Aquaculture (IOA), which houses a population of North African catfish.These catfish were introduced into the facility over 30 years ago and assumed to be, but not fully verified as,*Clarias gariepinus* Burchell 1822. In order to ensure that the results of the research presented in the thesis could be effectively applied in Uganda, the species of the IOA stock was therefore confirmed.Confirmation of the species ensured that the present researcher could work with the same species that she was likely to encounter when back in Uganda as it is currently the species of most significance to the aquaculture sector. Confirmation of species also ensured that the collected data could be interpreted in an appropriate context with respect to the work of other researchers.

This chapter focuses on the confirmation of the species under culture at IOA and the investigation of the growth of North African Catfish larvae relating to the use of micro diets as a sole or partial feed for weaning and early introduction. The aim of the latter was to understand what happens in terms of growth for the first 10 Dph when live (*Artemia*) or dry feed (Coppens, an off-the-shelf microdiet) is introduced solely or in mixtures for the first 4 days of growth. The information provided by the work undertaken in this chapter was then used to design downstream qPCR chapter 5, and microarray studies chapter 6 aimed at helping to interpret the results from a molecular point of view and assisting progress towards weaning them from a live to a dry dietfor the identified species.

3.3 Objectives

3.3.1 Major objective

To confirm the species of the studied *Clarias*species using adult and juvenile specimens and to investigate the effect of introduction of live and dry feeds earlier or later during the yolk sac period and subsequently on the expression level of enzyme transcripts detailed in chapters 4 and 5 below.

3.3.2 Specific objectives

i. To confirm the species using morphometric and meristic characteristics in addition to molecular techniques.

ii. To determine the effects on growth rate of introduction of live diet (*Artemia*) and dry diet (Coppens standard diet) for Catfish larvae during the yolk sac period *i.e*. days 1, 2 and 3 Days post hatch (Dph) on growth and onset of enzyme expression.

3.4 Review of related literature

3.4.1 Confirmation of the species currently kept at the tropical aquarium facilities at Institute of Aquaculture, University of Stirling

3.4.1.1 Introduction

Taxonomy has been defined as the bioscience through which organisms are classified, named and identified (Singh, 2012). It is based on 3 principles *i.e*. looking for similarities and evolutionary resemblance between organisms, termed classification, naming the organisms, termed nomenclature and looking for particular characteristics that are unique to a given taxon, referred to as identification (Padial, Miralles, De la Riva, & Vences, 2010; Singh, 2012).

In the recent past, integrative taxonomists have employed five feature classes to identify, classify and name organisms. These comprise features relating to biochemical, molecular, morphological, behavioural and ecological characters and can be qualitative or quantitative and can be used to explain variation that is discrete or continuous such as meristics and morphometrics (Padial*et al*., 2010).

In most studies on fish taxonomy, morphology is the most commonly employed method to gain knowledge of the identity, name and class of a given species, probably because it is an easier and cheaper means of characterising a given species (Leis, 2015). Meristic characters, primarily fin rays and scales, are used because in evolutionary terms they relate to body segmentation. Other characters that can be counted, such as cephalic pores, are sometimes referred to as meristic. Countable characters vary within and among species, so they are useful in describing or identifying fishes. However, meristic characters can be influenced substantially by environmental factors, especially by temperature during early development, which can make them a less reliable taxonomic trait.

3.4.2 Morphological characterisation of fish

Accurate identification of species should be the initial stage in any biological research work and plays a key role, especially in terms of behavioural, ecological, biodiversity and conservation studies, which may be sensitive to the presence of interspecific hybrids in fish and which require their early detection. In terms of identification in fish and other organisms, morphometric measurements and meristic counts are considered the easiest and most straightforward methods for

taxonomic studies, these usually being termed as morphological systematics studies (Nayman, 1965 in Brraich& Akhter 2015, Doung 2017).

They are two key types of morphological characters used in taxonomy, morphometric and meristic, which are frequently employed to identify stocks of exploited fish species. Morphometric characters are classified into genetically, intermediate and environmentally controlled characters (Johal *et al,.*1994).Ameristic character is a discrete countable trait, such as number of gill rakers or number of dorsal fin spines, while morphometric measurements examine size and shape using a continuous measurable trait such as length or weight. Morphometric and meristic studies provide effective tools for assessing the discreteness of a particular species versus other similar species (or sub-species). Species identification and population discrimination are very important in natural fisheries management and biodiversity conservation (Ibañez *et al,* 2007, Brraich& Akhter 2015). It is necessary to always identify individual specimens to the correct population in order to better understand their biological traits including growth, nutrition, mortality, fecundity and other relationships. Morphometric and meristic analysis tools can be used in differentiating closely related species, which have high similarity across various parameters (Fagbuaro *et al* 2015). It has been noted that not only are these characters important for their taxonomic significance but they can also be employed for understanding reproductive processes and health status of the species under study since condition factors and relative gut length can be derived from these parameters to give an indication of growth and health (Adeoye *et al* 2016, Manimegalai *et al* 2010).

Morphometric characteristics are usually phenotypically plastic and can be influenced by environmental factors such as seasonality (Ikpeme *et al.,* 2016; Fagbuaro *et al.,* 2015). Morphometric features usually employed to identify fish species include body shape, standard length, head length, eye diameter, and mouth gap (Wasala *et al.,* 2007), Dorsal head length (DHL), Interorbital length (IL), Occipital process length (OPL), Occipital process Width (OPW), Premaxillary Tooth plate Width Vomerine Tooth plate Width, Barbel length (BL), Prepectoral Length (Applebaum, Perez, Lazo, & Holt), Prepelvic Length (PpeL), Predorsal Length (PDL), Preanal Length (PAL), Dorsal Fin Base (Gourène& Teugels ; Teugels 1982b,c; Teugels 1982a; Teugels 1984b; Teugels 1984a,1986; Teugels *et al.,* 1990a; Teugels *et al.,* 1990b; Teugels 1992; Teugels 1996; Agnèse *et*

*al.,*1997; Rognon *et al.,* 1998; Agnese & Teugels 2001; Agnese *et al.,* 2001; Jean François Agnèsea 2001; Teugels & Adriaens 2003; Sudarto 2004; Agnese & Teugels 2005; J.-F. Agnese and 2005; Więcaszek B. 2010; Duong *et al.,* 2017a) and meristic counts can include the number of gill rakers present on the first gill arch, number of fin rays (Grabherr*et al*.) and spines (nfs) (Teugels 1986, Ola - Oladimeji *et al* 2016), number of scales (nS), number of barbels (nB), number of vertebrae in vertebral column (VC), Dorsal fin rays (DFR), pectoral fin rays (PFR) (Ola -Oladimeji *et al* 2016). Additional morphological features that are still being used in the separation of *Clarias*species are mainly: the form of the vomerine teeth, the shape of the tooth bands, the ratio of the vomerine to the premaxillary bands, and the number of gillrakers on the first branchial arch.

3.4.3 Morphological studies on *Clarias*

Various studies have been conducted on the morphological characterisation of *Clariasgariepinus*, with the most complete being by Teugels (Teugels 1982a; Teugels 1984b; Teugels *et al*. 1990b). Two species, *C. anguillaris and C. gariepinus* are believed to occur in and around most riverine systems in Africa. These two species belong to the sub-genus *Clarias (Clarias*), which contains the large African species that have been of most interest to aquaculturists. *C. gariepinus*is considered to be the most important catfish for aquaculture (deGraaf and Janssen, 1996). The species recognised by Boulenger (1911) and David (1935) as belonging to the subgenus *Clarias* in Africa were *C. anguillaris, C. gariepinus, C. mossambicus, C. senegalensis* and *C. lazera*. The last two of these species have now been reduced to synonymy with the first two, respectively. Teugels (1982a, b) examined some aspects of the taxonomy of Clarias species in Africa and Teugels (1986) revised the sub-genus *Clarias (Clarias),* among other subgenera, recognizing only two of the five species recognised by Boulenger (1911) and David (1935), *i.e.C.anguillaris* and *C. gariepinus.* In his review of the African species of the genus, Teugels (1986) recognised 6 subgenera: *Clarias* (Clarias) Scoploli, 1777; *Clarias* (*Dinotopteroides*) Teugels, 1982; *Clarias* (*Clarioides*) David, 1935; *Clarias* (*Platycephaloides*) Teugels, 1982; *Clarias (Brevicephaloides*) and *Clarias (AguilloClarias)*Teugels, 1982. Teugels excluded *Clarias (Allabenchelys*) and said that while it was recognised by David (1935), he considered it a junior synonym of the genus *Clariallabes* Boulenger, (1900).

Clarias typically have about 10-20 gill rakers on the first gill arch. except for two species of the subgenus *Clarias*, where the range of gill rakers varies between 16-50 gill rakers in *C. anguillaris* and 24-110 in *C. gariepinus* for specimens of 650 and 600mm TL respectively, according to Teugels (1986). However, the larger specimens ranging 1-1.5 m upon extrapolation of regression lines would result in the number of gillrakers being about 130-180 and 150-300 respectively. (Roberts 1989).

A number of studies have been conducted on the morphological characterisation of *C. gariepinus*, both cultured and wild fish, and have focused on the morphometric variation between populations, on diploidy and triploidy, and on the use of different analytical tools, namely multivariate analysis and Principal Component Analysis (PCA). Studies carried out on *C. gariepinus* morphology using Principal Component Analysis (PCA) (Turan 2005; Więcaszek *et al.,* 2010; Fagbuaro *et al*. 2015; Fagbuaro *et al.,* 2016; Ola-Oladimeji *et al.,* 2016; Normala *et al.,* 2017; Ola-Oladimeji *et al.,* 2017) showed that there was morphologic differentiation largely located in the head of the fish, indicating the importance of characterising a given population and using such knowledge in selection for breeding purposes (Kipper *et al.,* 2013; Ikpeme *et al.,* 2015; Oguntade *et al.,* 2015; Folasade *et al.,* 2016; Ikpeme *et al.,* 2016b; Chinakwe & Solomon 2017). It is important to recognise, however, that the detected differentiation could also be related to environmental conditions including temperature, turbidity, food availability and water depth, which underlines the need to apply genetic techniques to confirm the genomic basis for the detected phenotypic differentiation. This perhaps explains why researchers may have to undertake the extra step of further confirming the identity of species of interest using mitochondrial DNA to allow detection of any small genomic differences (Singh 2012).

More recent studies of *C. gariepinus* have examined not only morphological variations (Popoola *et al*. 2014; Fagbuaro *et al* 2015) but also genetic relatedness (Ikpeme*et al*. 2016b) of wild and cultured fish and differences between triploid and diploid populations (Normala*et al*. 2017). Results from various studies have shown differences in morphometric characters (Compaoré *et al.,* 2015; Fagbuaro *et al.,* 2015; Solomon *et al.,* 2015; Solomon *et al.,* 2016), with wild populations having higher morphometric measurements than cultured *C. gariepinus*. These findings

were similar to those of Turan *et al (*2005) who attributed these differences to either genetic factors or to phenotypic plasticity in response to key aspects of the rearing environment. According to Solomon *et al.* (2015b), differences were also significantly related to sex variation. Results from the various studies showed differences in meristic counts (Ola-Oladimeji *et al*. 2016; Eyo 2017), something which can be attributed to chromosomal aberration causing deformities at embryonic development.

Studies on ploidy discrimination using morphological variables were not efficient, which highlighted the future importance of investigation by use of genetic markers in ploidy determination rather than the current cheapest method of erythrocyte measurement (Normala *et al.,* 2017).

Studies on morphological differentiation among cultured and wild *Clarias macrocephalus* and their hybrids with *C. gariepinus* (Duong *et al*. 2017) indicated that *C. macrocephalus* and *C. gariepinus* and their hybrids could be morphologically differentiated, with similar divergence recognised between cultured and wild fish. Important discriminatory characters included the number of gill rakers and head characters (Wiecaszek & Krzykawski 2010; Duong *et al*. 2017; Thuy-Yen Duong 2017). This reaffirms the outcome of earlier work by other authors who found that the head is important for characterisation (Turan*et al* 2005, Więcaszek*et al* 2010 and Ola- Oladimeji *et al* 2016), however, the hybrids in this case were intermediate. The meristic measurements which are highly heritable and are commonly a measure for classification in fish, conformed to the known number for *C. gariepinus*, being almost double those in *C. marcephalus* of 20. However, an earlier study in *C. gariepinus* indicated that variations could occur, especially for cultured species, with this also demonstrated for other species including *Tilapia zilli (*Ezeafulukwe *et al*., 2015).

3.4.4 Species identification using DNA

Accurate species identification has long been dependent on morphological analyses performed by taxonomists. It is, however, recognised that morphological approaches to species identification have limitations, particularly since close morphological similarities between closely related organisms create challenges in discriminating them from one another. In addition to this, taxonomists believe that only about 1.7 million species of the estimated 4-11 million that live on this planet

have been morphologically identified and catalogued, (Pennsi 2003), with molecular tools therefore representing a key resource for future species identification and recognition. Other challenges relate to the determination of whether similar specimens from different habitats are different species or simply look different due the adaptability of an organism to changes in its environment (Hebert *et al.,* 2003). Morphological identification methods are also often dependent upon sex and life stage of the specimen (Hebert *et al.,* 2003) and this may lead to difficulties in recognition of, for example, juvenile specimens. With the advent of new tools for molecular biology in the 1980s, biologists have since been able to determine even the smallest variations within species and individual strains. Various molecular approaches now play a central role in determining phylogeny and nomenclature (Singh 2012).

3.4.5 Genetic variation of the *Clariidae*

According to Volckaert& Agnese 1996 the genetic characterization of catfishes by means of phenotypic markers, karyotyping and protein and DNA polymorphisms can make a key contribution to the disciplines of systematics, population genetics, quantitative genetics, biochemistry, molecular biology and aquaculture. Although the siluroidae were not among the model species employed for fundamental genetic research, the families; Clariidae and the Ictaluridae represent the best studied catfish families. The family Clariidae found in Africa and Asia are known to have undergone genetic differentiation leading to speciation (Agnese *et al* 2005). Studies in Lake Victoria on the Tanzanian portion have indicated that four genera occur namely C*. gariepinus, C. liocephalus, C. wern*alli and *C. alluandi* (Mwita 2014) Witte & Vandasen 1995). In other earlier studies, still employing morphological characters, biochemical, genetic and electrophoretic polymorphism it was found that wild populations of *C. gariepinus* and *C. anguillaris*were more divergent from each other with distinct populations, namely the Nilo-Sudanian populations and the other including Lake Victoria and southern African populations (Van der Bank *et al.,* 1992; Rognon *et al*., 1998).

More recent studies byYogbabaliat 2014;using the PCR amplification of the cytochrome b gene in *Clarias*and *Heterobranchus*species showed its utility for untangling species relationships. The clustering of genotypes for wild *C. gariepinusand C. galamensis* revealed their close relationship, which could be

attributed to the fact that they were both obtained from the wild. Although there were variations in the phenotype of wild and cultured *Heterobranchus* and *Clarias species*, the clustered groups of *Heterobranchus longifilis, H. isopterus, H. bidorsalis* and *Clarias gariepinus* shows a close relationship in their genotypes which is indicative of their phylogeny. Findings on serum protein pattern in interspecific and intergeneric hybrids of *Heterobranchus longifilis, Clarias gariepinus* and *Clarias anguillaris* using SDS-PAGE (Agnèse *et al.,* 1995; Agnèse *et al.* 1997; Agnese *et al.* 2001; Akinwande *et al.,* 2012; Fagbenro & Adebayo 2012) found a high similarity coefficient between these species, hinting at a very low genetic diversity. The close relationship between the *Heterobranchus* species and the species of the subgenus *Clarias* to which *C. gariepinus* and *C. anguillaris* belong has been emphasized by (Teugels 1986; Teugels *et al.*, 1990; Teugels & Adriaens 2003) in a revisionary study using osteological features.

3.5 Ontogenetic development and important structural and functional changes in the digestive system of fish larvae

Fish larvae undergo a number of critical stages in their life history, during which substantial developments occur in both structure and function of body tissues, organs and systems as growth takes place. Fish larval nutriment commences using an endogenous source, where larvae obtain nourishment from yolk reserves contributed from the maternal fish and switch to exogenous feeding when the digestive system is somewhat fully developed (Hamza *et al.*, 2015; Shan *et al.*, 2016). In the early feeding stage, fish are also introduced to mixed nourishment during the switch from endogenous to exogenous nutriment. The later stage of switching to the mixed feeding, nutriment is vital for fish survival. Therefore, an understanding of the structural and functional changes of the larval digestive system is important in helping to select the right feed in order to increase fish survival during larval rearing. It has been noted that for most aquaculture enterprises, high fish losses usually occur during the early stages, matching the period of initial exogenous feeding. It is therefore important that the timing of food introduction is appropriate because food ingestion in first-feeding larvae determines subsequent fish survival and growth (Hurst *et al.,* 2010; Ma *et al.,* 2012; Peck *et al.,* 2012; Rønnestad *et al.,* 2013a; Lechner *et al.,* 2016). (Hurst *et al.*, 2010; Lechner *et al.*, 2016; Rønnestad *et al.*, 2013).

As with marine fish larvae, freshwater fish larvae are very vulnerable during the first stages of development and have strict requirements for biotic and abiotic conditions that allow them to survive, grow and attain the appropriate size / length. (Hamre *et al.,* 2013).

Feed is one of the highest inputs in terms of costs in the farming of fish and therefore optimising feeds to provide the right nutrition should be cost effective, resulting in healthy, high quality food. Fish larvae show patterns in daily intake rather than continuous food intake throughout the day (Navarro-Guillén *et al.*, 2017). The frequency of feeding plus the quantity fed per meal are thought to be key in affecting the process of digestion, rate of body uptake and ultimately the fish growth performance. Nevertheless, our knowledge of the effect of feeding regimes in fish larvae growth is still scarce and limited to a few species (Navarro-Guillén et al. 2017). It has been observed that feeding patterns in many animals, including fish, occur in cyclic patterns, often involving circadian rhythms (Bloch *et al.,* 2013) which are brought about and controlled by transcriptional-translational feedback loops of a group of circadian clock genes and their derived proteins (Vera *et al.,* 2013; Paredes *et al.,* 2014; Mata-Sotres *et al.,* 2015; Mukherjee & Maitra 2015; Yúfera *et al.,* 2017).

3.5.1.1 Nutrition and early development in larval fish growth

During the different life stages of vertebrates, including fish, different diets are eaten and these result in different functional requirements for the gastrointestinal tract.)

In most fish larvae the need for live feed is essential in the early stages, especially at the switch from the endogenous yolk sac period to the exogenous period as most don't really have a fully developed gastric intestinal tract (GIT). Although live feeds are used as starter feeds for most fish larvae, they are expensive and not as convenient to use as formulated feeds because they need a lot of space when compared formulated feeds. This need for sustenance on live feed has hampered the development of an early life fish rearing diet off-the-shelf, which is able to provide good survival and growth and improve fish farming practices. In farm production, any diet that reduces the reliance on live food production is of technical and economic interest in rearing larval fish (Zhu *et al.*, 2014).

Nutrigenomics, which is the study of gene expression with response to dietary nutrients, is a new approach used to understand the basics of metabolic changes in animals that occur in response to feeding different dietary nutrients (Martin & Król, 2017; Müller & Kersten, 2003; Mutch *et al.*, 2005; Ordovas & Mooser, 2004; Pavlidis *et al.*, 2015). Since cell growth is directly related to the animal's metabolic alterations when fed with different nutrients, the results from these kinds of studies can help in the understanding of animal's response to different feed ingredients when fed. This is an interesting area that must be critically explored (Overturf *et al.,* 2010; Panserat & Kaushik 2010; Overturf *et al.,* 2016; Daniel 2017). When fish larvae commence exogenous feeding, the flow of nutrients formerly supplied only from yolk reserves becomes supplemented through the digestive tract. The majority of marine fish larvae currently targeted for cultivation hatch from pelagic eggs and their digestive system is still developing at the onset of exogenous feeding, as is the case for North African Catfish larvae. A fully developed digestive tract, including gastric digestion, develops during metamorphosis. Although the larval gut is not completely developed at the onset of exogenous feeding, it is sufficient to support larval growth by digesting such prey as is available under natural conditions. The physiological constraints of the gut with respect to digestion of cultivated live prey and particularly formulated starter feeds still remain to be elucidated (Rønnestad, 2002).

Recent studies on control and efficiency of digestive function of fish larvae have concentrated on larval nutrition in terms of polyunsaturated fatty acid metabolism and requirements, however, even within these area quantitative requirements still have to be determined in most European fish larvae (Rathore *et al.,* 2016). Fish larvae of the rest of world have been even more poorly studied and this includes larvae of the North African Catfish, *C. gariepinus*.

3.6 Materials and Methods

3.6.1 Fish samples for Taxonomic analysis (Morphometrics and Meristics)

The material used in study of morphometric and meristics comprised Twenty five, 3 month old juvenile catfish. These were hatched out in August 2012 and kept until December 2012 for taxonomic studies using morphometric and meristics. Sixteen morphometric characters were measured using Vernier callipers, with measurements taken to the nearest 0.01mm and 8 meristic counts were made. These were decided based upon earlier studies by Agnese *et al* (1997) who inferred them from the earlier studies of Teugels (1986) who had identified them as being of diagnostic value in catfish taxonomy and which were modified by Więcaszek*et al* (2010) (Figure 3-1).

The lengths were converted to percent fish standard length (SL) and fish head characters to percent dorsal head length (DHL) as shown in Figure 3-1.

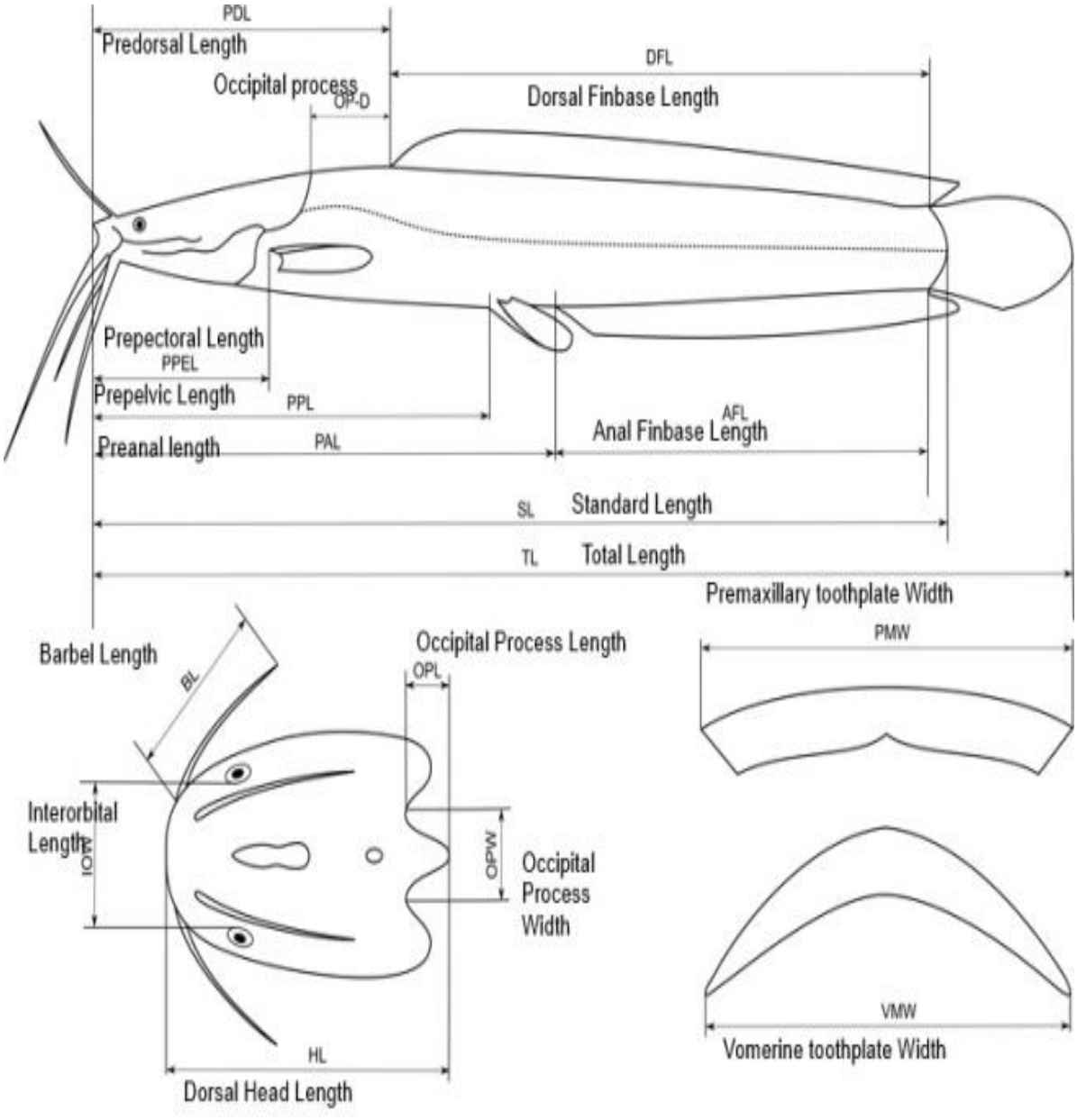


Figure 3-1 showing morphometric characteristics for North African Catfish. Figure adapted from Więcaszek*et al* (2010) as Fig 1. Formerly from Agnèse *et al*. (1997) and Teugels (1998)

Data, in terms of lengths, weight and meristic counts were collected both onsite and in the laboratory and were coded in Excel. Descriptive analysis was done both in Excel and further analysis for statistical differences was done using SPSS (IBM, v21).

3.6.1.1 Fish sample for molecular identification.

One female 3 year old adult catfish was sacrificed using terminal anaesthesia and destruction of the brain, in accordance with the legislation governing the use of animals in research in the United Kingdom (UK) as laid down in the Animals (Scientific Procedures) Act 1986.

The fish was dissected and the heart and liver were removed and placed in 95% Ethanol and transferred to the fridge at 4˚C and kept for further laboratory analysis. In the laboratory, DNA was extracted using the REALPURE Genomic DNA extraction kit (Real pure, USA) and was stored at -20°C in the freezer (Liebherr Comfort QMS2).

3.6.1.2 DNA amplification and sequencing.

Part of the cytochrome b (cytb) gene template suitable for sequencing was obtained using polymerase chain reaction (PCR), using the following universally conserved mtDNAcytb primers: L15267 (5'AATGACTTGAAGAACCACCGT-3') (Briolay*et al*, 1998 modified from Kocher *et al,.* 1989) and H15891 (5'-GTTTGATCCCGTTTCGTGTA-3) (Briolay*et al* 1998 modified from Kocher *et al,.*1989).

PCR was performed in 50µL total volume comprising 25µL MyTAQ^{TM} HS Mix (Bioline), 12µL RNase Free Water (Milli-Q Integral Water Purification System Biocel mil), 1.5 µL of primers (5 picomoles μL^{-1} of L15267 and H15891) and 10µL of mitochondrial, mtDNA Template and run under the following conditions as shown in Table 3-1.

Table 3-1 showing the PCR cycle parameters for mtDNA amplification

Steps	Temperature	Time
1	95°C	2min
2	95°C	30 sec
3	60°C	30 sec
4	72°C	2min
5	Go to Step 2, 33 more times	
6	72°C	2min
7	10°C	20 sec

In addition, a No Template Control (NTC) was run alongside to monitor for potential contamination. The size of PCR product was verified by electrophoresis in a 1.5% agarose gel using a Gene Ruler 100 bp DNA Ladder marker (Thermo Scientific).

The amplified cytochrome b gene was isolated and purified using a QIAquick PCR Purification Kit according to the following steps:

250µL of PBs was diluted in the ratio of 1:5 and added to the amplified cytochrome b product in order to get rid of primer dimers. This was followed by the addition of 2µl of 3M sodium acetate. The mixture was spun down and placed on elute columns and left for 5 minutes to settle. It was then pulsed once and spun at 17800 x g for 1 minute. The mixture was changed to a second set of round bottom of tubes volume 1.5mL. 730µL of Buffer PE ethanol divided into 380µL and 350µL for two washes while swirling the tube. It was then spun for 1 minute at 18000 x g. It was then moved to a new set of dry tubes of volume 1.5mL. It was then spun with the tops open to enable the sample to dry for 1 minute at 17800 x g and placed on the driblock at 65ºCfor 5 minutes to remove any ethanol remaining in the sample. This was followed by elution of the sample by addition of 20µL of heated RNASE free water at a temperature of 65ºC.ad placed on the driblock at 65ºC for 1 minute. It was then spun at 20,000 x g. 30ng was run on a gel to check quality.

The purified PCR products were cycle-sequenced using a Genomelab™ Dye Terminator cycle with Quick start kit and run on an automatic capillary sequencer known as the Beckman Coulter machine CEQ 8000 DNA Sequencer and associated software was used for size determination of the fluorescently labelled PCR products.

3.6.1.3 Sequence analysis.

Sequences obtained were checked and aligned using the DNASTAR Seqman™Sequence Analysis tool for Windows 32 (DNASTAR Inc. 6.1 ©1989-2005). Using NCBI the resultant contigs were blasted using BLASTx and BLASTn and were compared with, known sequences for *Clarias buettikoferi (AF126829), C gariepinus (AF126823), C. ebriensis (AF126822*) and (AF126824*. C anguillaris* (Nwafili & Tianxiang 2007) and AP012010.1| *Clarias sp*. NM-2010 mitochondrial DNA, complete genome, HQ701705, HQ701706, HQ701707 partial and a phylogenetic tree was developed within NCBI.

3.6.2 Feeding of Larvae

The experiments conducted involved 4 trials, of which two were run from 18th April 2012 to 16th May 2012 (28 days) and 3rd December 2012 to 15thDecember 2012 (2 days of seed production+10 days feeding trial). Trials 3 and 4 were performed on 8th July 2013 to 20th July 2013 and 9th December 2013 to 21st December 2013 (2 days of seed production+10 days feeding trial).

During Trials 1 and 2 (Normal/standard feeding regime), larvae were fed from 1 day post hatch (dph) on brine shrimp, *Artemia salina* (Bio Marine USA). Cysts were hatched 18 hours prior to feeding by taking 4g of the brine shrimp eggs and placing them in 40g of sea salt in 2L of water and aerating for 18-24 hours.

In Trial 1, larvae were fed on *Artemia* from 1Dph -4Dph followed by a mixed diet of *Artemia* and trout crumble starter diet (microdiet) in the ratio 1:1 to 7 to 8 Dph,after which larvae were completely switched on to the trout microdiet till 28Dph. It should be noted that trial one was conductedin order to determine the optimal target period for subsequent trials and following this trial, further trials were shortened from 28days to 12 days post hatch (2 days of seed production+10 days feeding trial).

During Trial 2, the larvae were fed on crushed/ground pellets of complete feed 40 for trout expanded (Skretting, Preston UK) from day 1 post hatch for the first experimental treatment and then day 3 post hatch for the second experimental treatment, followed by a mixed diet f comprising of artemia andSkretting Preston diet or six and four days respectively before switching to the dry microdiets completely upto 10Dph. Trial 2 was used to provide samples for the qPCR analysis described in chapter 5.

Trials 3 and 4 involvedthe introduction of exogenous feed on days 1, 2 and 3 Dph respectively, during which period the yolk sac was being reabsorbed. Where T represents a treatment, each experiment involved 6 treatments, each comprising triplicate samples of 350-400 individual fish per tank (estimated, as individual counting would damage larvae). A standard catfish diet, 0.2-0.3mm CATCo Excellent crumble (Coppens, Netherlands) was subsequently employed for some treatments. Fish were fed twice a day at 09:00 and 16:00 to satiation. Excess food was siphoned out on a daily basis, using a toothbrush to clean the clogged mesh net which prevented *Artemia* escaping via the overflow pipe.

For Trial 3: T1 fish were fed on 100% *Artemia* from day 1Dph, T2 were fed on 100% *Artemia* from day 2, T3 were fed on 100% *Artemia* from day 3, T4 were fed on 50% *Artemia* and 50% Coppens diet from day 1, T5 were fed on 50% *Artemia* and 50% Coppens diet from day 2, T6 were fed on 50% *Artemia* and 50% Coppens diet from day 3. The trial structure is summarised in Table 3-2.

Table 3-2 Feeding treatment structure for Trial 3

Larval *Clarias gariepinus* feed ing regime							
Snr	**Tissue**	**Feeding regime**					
		100% Artemia salina naupli			**Catco (Coppens) from the Netherlands**		
		Day 1	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day 3**
1	Eggs						
2	Fertilisation (eggs and milt)						
3	6hr Post fertilisation (PF)						
4	12hr Post fertilisation (PF)						
5	24 hr Post fertilisation (PF)						
6	Day 0± 6 hours						
7	Day 1	**T1_Day 1**	**T2_Day 1**	**T3_DAY 1**	**T4_Day 1**	**T5_Day 1**	**T6_DAY 1**
8	Day 2	**T1_Day 2**	**T2_Day 2**	**T3_DAY 2**	**T4_Day 2**	**T5_Day 2**	**T6_DAY 2**
9	Day 3	**T1_DAY3**	**T2_DAY 3**	**T3_DAY 3**	**T4_DAY3**	**T5_DAY 3**	**T6_DAY 3**
10	Day 4						
11	Day 5						
12	Day 6						
13	Day 7						
14	Day 8						
15	Day 9						
16	Day 10						
	Start feed	Artemia					
	Mixed diet	Catco and artemia			Egg yolk stage		
	Dry diet	Catco and artemia					

For Trial 4: T1 were fed on 100%Coppens diet from day 1Dph, T2 were fed on 100% Coppens diet from day 2, T3 were fed on100% Coppens diet from day 3, T4 were fed on 50% *Artemia* and 50% Coppens diet from day 1, T5 were fed on 50% *Artemia* and 50% Coppens diet from day 2, T6 were fed on 50% *Artemia* and 50% Coppens diet from day 3. The fish were then fed on the dry diet until day 10 as seen in table 3-3 below.

Table 3-3 Feeding treatment structure for Trial 4

Larval Clarias gariepinus							
Snr	**Tissue**	**Feeding regime**					
		100% Artemia salina naupli &Catco (coppens)			**Catco (Coppens) from the Netherlands**		
		Day 1	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day 3**
1	Eggs						
2	Fertilisation (eggs and milt)						
3	6hr Post fertilisation (PF)						
4	12hr Post fertilisation (PF)						
5	24 hr Post fertilisation (PF)						
6	Day 0± 6 hours						
7	Day 1	**T1_Day 1**	**T2_Day 1**	**T3_Day 1**	**T4_Day 1**	**T5_Day 1**	**T6_Day 1**
8	Day 2	**T1_Day 2**	**T2_Day 2**	**T3_Day 2**	**T4_Day 2**	**T5_Day 2**	**T6_Day 2**
9	Day 3	**T1_Day 3**	**T2_Day 3**	**T3_Day3**	**T4_Day3**	**T5_DAY3**	**T6_Day 3**
10	Day 4						
11	Day 5						
12	Day 6						
13	Day 7						
14	Day 8						
15	Day 9						
16	Day 10						
	Start feed	Artemia					
	Mixed diet	Catco and artemia			Egg yolk stage		
	Dry diet	Catco					

Trials 3 and 4 were aimed at selecting the optimal time for the switch to the dry diet as regards to improving growth. While all samples could not be screened by microarray (Chapter 6) due to cost and time constraints, T2 and T5 from Trial 4 were selectedfor microarray analysis following assessment of outcomes (see Results section).

3.6.3 Growth study sample collection

To estimate the rate of growth the larvae were collected at hatching (0 hrs), 6, 12 and 24 hrs post-fertilisation. It is worth noting that hatching took place 24+/-6hrs at the temperature range 26.5-28.5°C over the sampling period, Then after the hatching period, the dead eggs were removed and then samples were collected at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 14, 21, 28 Days post hatch (Dph) for the initial trial (Trial 1) but this was optimised to 0, 1, 2, 3, 4, 6, 8, 10 Dph for the succeeding trials (Trials 2-4) after the realisation that this time period covered the important developmental milestones for this study. For growth estimation 10 fish per tank sampled in triplicate were sampled.

Fish were terminally anaesthetized using benzocaine prepared as a stock solution comprising of 100 grams of benzocaine in 1 litre of ethanol of which 10 mLs of stock solutionlitre^{-1} was used in a water bath. Photographs were taken using an SZ40 dissecting Microscope with an Axiocam camera attached and Axiocam vision (Carl Zeiss, UK) program. The fish length was then measured using ImageJ Fiji version. 5.1.1 software. Weights were taken using a sensitive balance (to 4 decimal places mg) Prior to weighing the fish in the initial small stages 0-1 dph, 5 individuals were weighed after blot drying the water off using blotting tissue (Scott FSC 13545) while for later stages each individual fish was weighed separately. For each replicate 30 fish were weighed.

3.7 Results

3.7.1 Morphometrics and meristics for identification of the catfish species

From Table 3-4 it can be seen that the weight of the fish were between 29 to 200g in terms of weight and total length ranging from 165-300mm placing the size classes into a large size range with some showing faster growth as they came from the same generation before they were used for purposes of identification.

Based on % standard length in the sample as seen in Table 3-4, total length, pre-anal length and dorsal –fin base were observed to be among the more stable variable characteristics together with the most variable character being located on the head region.

Table 3-4 Morphometric characters, converted to percent fish standard length of the studied samples of juvenile *C. gariepinus* (n=25)

Body Character	Range Standard length	Mean ± Std. Error %SL		Std. Deviation	CV
Weight in g	29.40-201.60	102.9520	8.00433	40.02163	0.389
Total length (mm) (TL)	165.0-300.5*	242.276	6.1483	30.7414	0.127
Standard length (mm) (SL)	145.0-275.6	217.452	5.7878	28.9391	0.133
Dorsal head length (mm) (DHL)	37.50-69.50	54.8000	1.50367	7.51835	0.137
Interorbital length (mm) (IL)	12.50-37.00	22.6404	1.08031	5.40154	0.239
Occipital process length (mm) (OPL)	5.55-15.05	10.0900	.41600	2.08001	0.206
Occipital process width (mm) (OPW)	10.90-21.70	16.0444	.57007	2.85033	0.178
Premaxillary Tooth plate width (mm)	10.90-22.10	14.9420	.56942	2.84710	0.191
Vomerine Tooth plate width (mm) (	7.30-14.90	11.4380	.37904	1.89518	0.166
Barbel length (mm) (BL)	38.75-69.80	58.0220	1.48395	7.41974	0.128
Prepectoral length (mm) (	25.3-60.0	44.860	1.6174	8.0870	0.180
Prepelvic length (mm) (PpeL)	53.0-124.0	93.308	3.0188	15.0941	0.162
Predorsal length (mm) (PDL)	46.15-135.00	71.9180	3.16761	15.83806	0.220
Preanal length (mm) PAL)	12.80-138.00*	106.8460	5.21661	26.08305	0.244
Dorsal Fin Base length (mm) (DFB)	85.50-185.90*	137.7220	4.43830	22.19152	0.161
Anal Fin Base length (mm)	63.5-135.5	99.328	3.7760	18.8800	0.190
Distance from dorsal fin to the occipital Process	7.50-16.20*	12.3680	0.53162	2.65808	0.215
Ratio of length to width of occipital process	0.440-880	0.64082	0.025086	0.125431	0.196

In the case of the morphometric characters of the fish head characters of juvenile *C. gariepinus* as % of dorsal head length as seen in table 3-5 there is observed variable characterisation of the head characters as a % of the dorsal head length particularly the length of the occipital process and maxillary barbellbarbel (CV 21.4% and 13.1%)%).

Table 3-5 Table showing the morphometric characters of the fish head characters of juvenile *C. gariepinus* as % of dorsal head length (n=25).

Body Character	%head length Range	Mean± Std. Error %HL		Std. Deviation	CV
BL	69.784__140.000	106.90559	2.804913	14.024565	0.131
OPL	11.190__31.095	18.57256	0.793278	3.966388	0.214
OPW	21.976__36.655	29.31761	0.710064	3.550321	0.121
IO	23.496__69.333	41.77767	2.309829	11.549144	0.276
PMW	4.719__8.444	6.88344	0.189742	.948711	0.138
OP-D	3.333__8.268	5.72317	0.238952	1.194761	0.209
VMW	4.314__6.622	5.26833	0.123002	.615011	0.117

3.7.1.1 The relationship of the morphometric characters with standard length and dorsal head length of studied fish samples

In both tables 3-6 and 3-7 the relationship (R^2) of the morphometric characters to standard length and dorsal head length of the studied fish sample are all significant except PAL; however, none are close to 1.

Table 3-6 showing the relationship between the standard fish length and particular morphometric characters of juvenile *Clarias gariepinus* (Burchell 1822)

Character	Regression y (x)	R^2	R	pP
HL	0.216x +7.838	0.691	0.831	< 0.05*
IO	0.111x -1.594	0.357	0.597	0.002*
OPL	0.042x +0.875	0.348	0.590	0.002*
OPW	0.069x + 0.981	0.495	0.703	< 0.05*
PMW	0.065x +0.734	0.441	0.664	< 0.05*
VMW	0.047x +1.183	0.519	0.720	< 0.05*
BL	0.109x +34.341	0.180	0.425	0.034*
PPEL	0.212x – 1.216	0.575	0.758	< 0.05*
PPL	0.368x +13.348	0.497	0.705	< 0.05*
PDL	0.433x -22.291	0.627	0.792	< 0.05*
PAL	0.258x +50.765	0.082	0.286	0.166

DFL	0.614x +4.2	0.641	0.801	< 0.05*
AFL	0.5x -9.43	0.588	0.767	< 0.05*
OP-D	0.039 x +3.941	0.178	0.422	0.036*

*Correlation coefficient is statistically significant

In the In case of meristic counts only the lateral line counts were significantly related to the standard length of the fish (Table 3-7).

Table 3-7 showing Relationship between standard fish length and the meristic characteristics.

	Regression equation y (x)	**R^2**	**R**	**P**
Soft ray on dorsal fin	0.35x -2.902	0.353	0.594	0.07
Soft ray on anal fin	0.130x+ 22.688	0.101	0.38	0.37
Soft ray on pectoral fin	0.037x-2.62	0.477	0.691	0.027
Soft rays on pelvic fin	0.021x +0.66	0.072	0.268	0.454
Gill rakers on the 1st gill arch	0.304 x -9.889	0.534	0.731	0.016
Lateral line counts	0.008x +44.963	0.001	0.028	0.939*
Vertebrae column	-0.65x +69.106	0.069	0.263	0.464

*Correlation coefficient is statistically significant

3.7.2 Molecular analysis results

3.7.2.1 Sequence contigs for *Clarias gariepinus* cytochrome b (cytb) gene, complete cds; mitochondrial gene for mitochondrial product

The *Clarias gariepinus* cytochrome b (cytb) gene was successfully amplified, sequenced and assembled using the universal primers employed (Figure 3-2.8) and the resultant sequence was blasted in NCBI to create the phylogenetic trees shown in Figure 3-3 below and BLASTn Figure 3-4 below.

>gi|19338919|gb|AF475153.1| *Clariasgariepinus* cytochrome b (cytb) gene, complete cds; mitochondrial gene for mitochondrial product

ATGGCAAGTCTCCGAAAAACACACCCATTATTCAAAATTGTCAACGACGCACTCATCGAC
CTTCCCGCCCCCTCTAATATCTCCGCATGATGAAACTTCGGCTCTCTACTATTATTATGT
CTTGGAGTACAAATCCTCACAGGTCTATTCCTAGCCATACACTACACTTCTGATATCTCA
ACCGCATTCTCATCAGTAGTACACATCTGCCGAGACGTCAACTACGGATGAATTATCCGA
AACCTTCACGCCAACGGAGCATCCTTCTTCTTCATCTGCATCTACCTTCACATTGGCCGC
GGTCTTTACTACGGCTCATACCTATACAAAGAAACATGAAACATTGGCGTTGTACTACTC
CTTTTAGTAATAATAACAGCCTTTGTAGGATACGTACTACCATGAGGACAAATATCCTTC
TGAGGTGCCACAGTAATCACAAACCTCTTATCAGCCGTACCTTACATAGGAGATGCCCTA
GTCCAATGAATCTGAGGAGGTTTCTCCGTAGACAATGCAACACTTACACGATTTTTCGCA
TTCCACTTCCTCCTACCATTCACAATCATCGCAGCTACAATTCTACACGCTCTATTCCTA
CACGAAACAGGATCAAACAACCCAATTGGATTAAACTCCGACGCAGACAAAATCTCATTC
CACCCATATTTCTCCTACAAAGACCTACTAGGCTTTATCATTCTATTAACAGCCCTAGCA
TATCTAAGCCTATTCTCCCCAAACCTTCTAGGCGACCCAGAAAACTTCACCCCCGCTAAT
CCCTTAGTAACTCCACCTCACATTAAACCAGAGTGATACTTCCTATTCGCATACGCCATC
CTCCGATCCATCCCAAATAAACTAGGCGGCGTATTAGCACTATTATTCTCCATCCTAGTA
CTAATAGTAGTACCACTACTACACCTCTCAAAACAACAAGGCCTAACCTTCCGACCTTTA
TCCCAAATCTTATTCTGAACCCTAGTAGCAGATGTAATAATCTTAACATGAATCGGCGGC
ATACCAGTAGAGCACCCCTTCATCATTATCGGACAAATTGCCTCCATCCTCTACTTCTCC
CTATTCCTCATCCTAAACCCACTAGCAGCCTGACTAGAAAATAAACTACTCAACCTAAAT
TGCCCTAGTAGCTTAGCCACTAAAGCGCCGGTCTTGTAATCCGGAGATCGAAGGTTAAAA
TCCTTCCTAGCGCCAGAAAAGAGAGATTTACTCCACAT

Figure 3-4 showing the obtained *Clarias gariepinus* cytochrome b (cytb) contig sequence

3.7.2.2 Phylogenetic trees showing the identified isolate

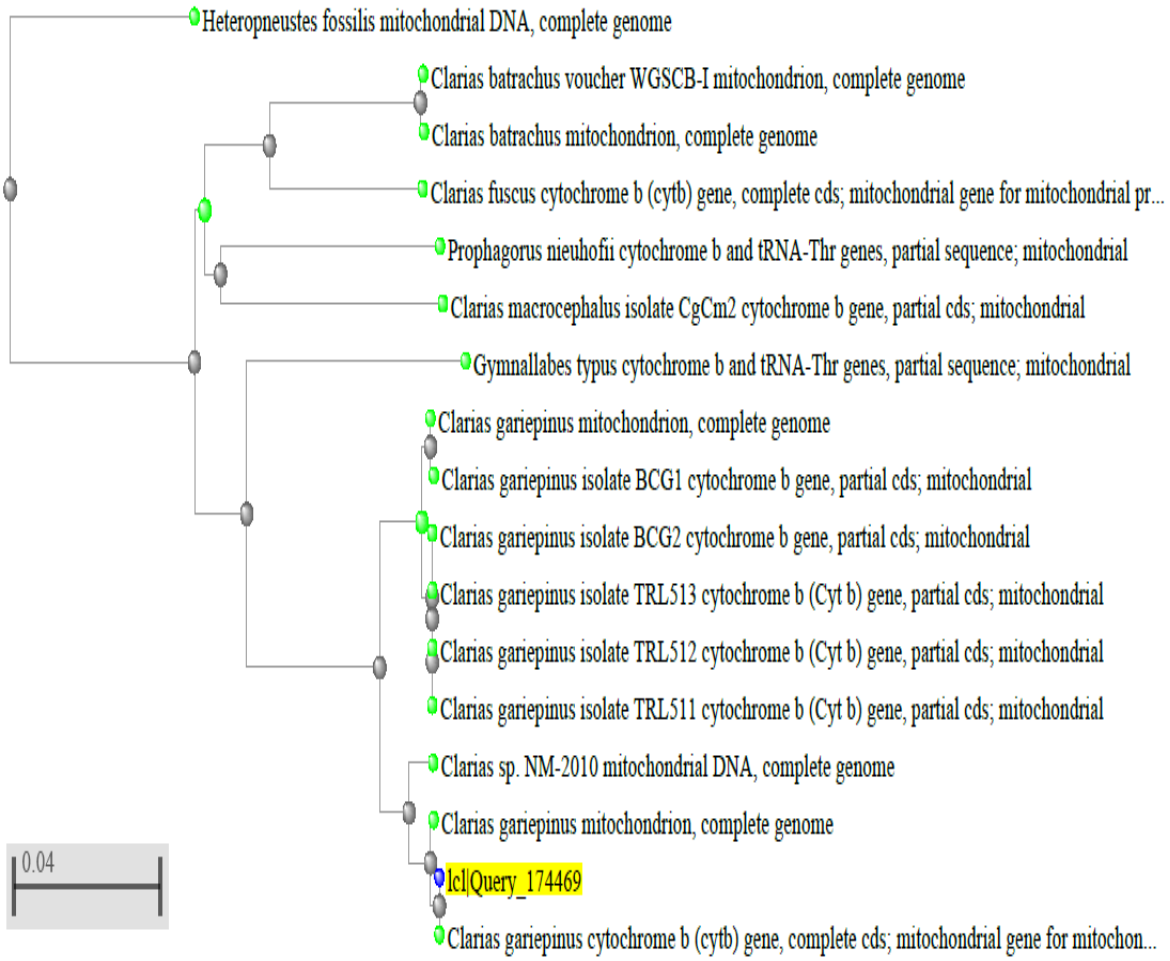
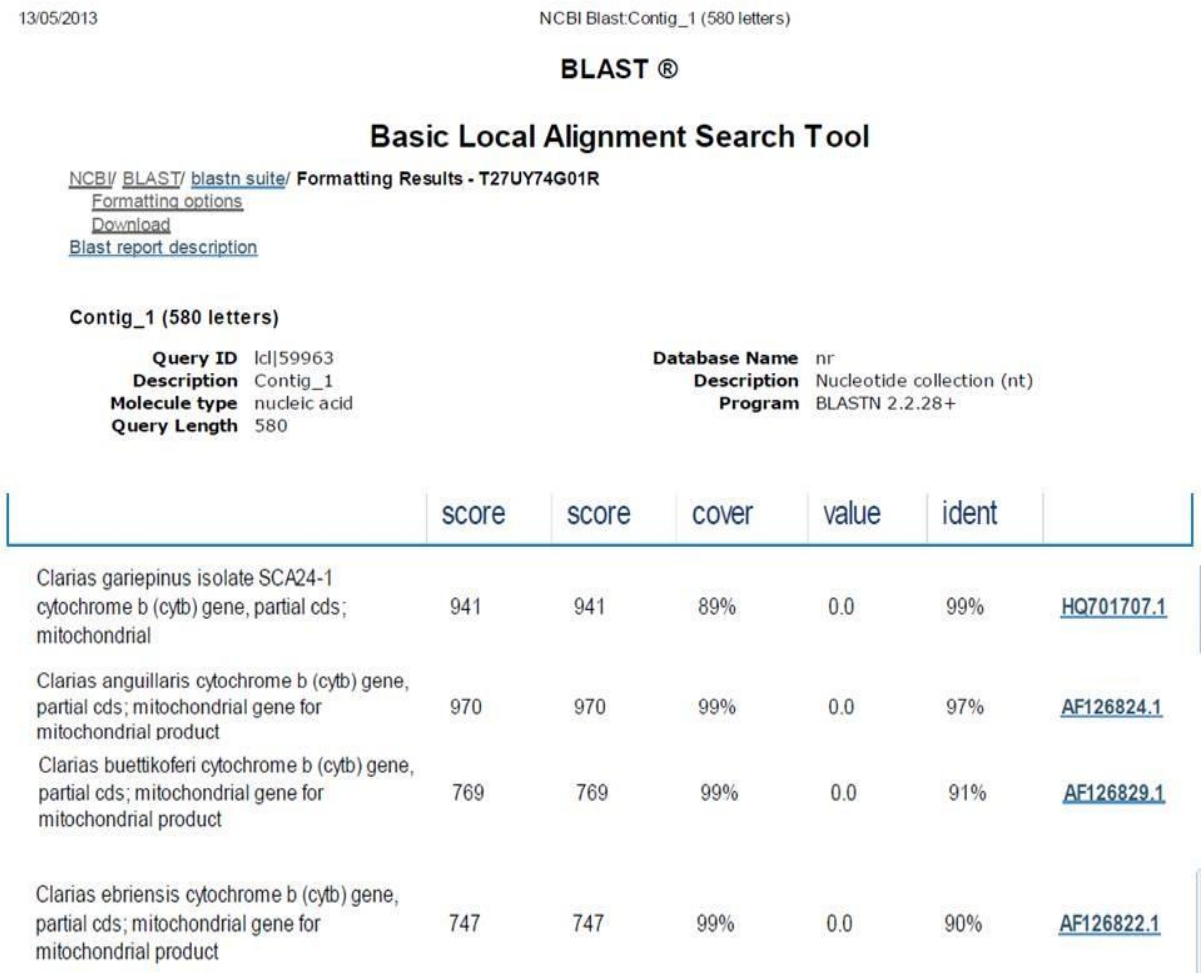


Figure 3-5 Phylogenetic tree showing the submitted isolate (yellow highlighted query 174469) clustering with NCBI sequences from *Clariasgariepinus*.

When the sequenced segment in Figure 3-4 was uploaded into NCBI and a phylogenetic tree constructed (Figure 3-5), clustering indicated a close relationship between the query sequence and those previously verified as *Clarias gariepinus*.

3.7.2.3 BLASTn of submitted sequence contig for similarity

From the molecular analysis using mitochondrial DNA, BLASTn results (Figure 3-6) indicated that the submitted mitochondrial isolate was 99% similar in comparison with *C. gariepinus* (AF126823) 91% *Clarias buettikoferi* (AF126829), 90% *C. ebriensis* (AF126822) and (AF126824. 97% *C anguillaris* (Nwafili&Tianxiang 2007). This suggests that the submitted isolate and the population from which it was obtained comprise *C. gariepinus*.



13/05/2013 NCBI Blast:Contig_1 (580 letters)

BLAST ®

Basic Local Alignment Search Tool

NCBI/ BLAST/ blastn suite/ **Formatting Results - T27UY74G01R**

Formatting options

Download

Blast report description

Contig_1 (580 letters)

Query ID lcl|59963
Description Contig_1
Molecule type nucleic acid
Query Length 580

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.2.28+

	score	score	cover	value	ident	
Clarias gariepinus isolate SCA24-1 cytochrome b (cytb) gene, partial cds; mitochondrial	941	941	89%	0.0	99%	HQ701707.1
Clarias anguillaris cytochrome b (cytb) gene, partial cds; mitochondrial gene for mitochondrial product	970	970	99%	0.0	97%	AF126824.1
Clarias buettikoferi cytochrome b (cytb) gene, partial cds; mitochondrial gene for mitochondrial product	769	769	99%	0.0	91%	AF126829.1
Clarias ebriensis cytochrome b (cytb) gene, partial cds; mitochondrial gene for mitochondrial product	747	747	99%	0.0	90%	AF126822.1

Figure 3-6 showing NCBI BLASTn results of submitted Contig_1 against existing sequences of *Clariasbuettikoferi* (AF126829), *C. gariepinus* (AF126823), *C. ebriensis* (AF126822) and *C. anguillaris (*AF126824.1)

3.7.3 Discussion

The taxonomic approaches employed in the work described in the present chapter, together indicate that the African catfish species maintained in the Institute of Aquaculture's tropical aquarium most closely resembles *Clarias gariepinus* as confirmed by the NCBI BLASTn against other close relatives namely *Clarias buettikoferi*, *C. ebriensis* (AF126822), which places it close to 99% similar to *Clarias gariepinus*. Using morphometrics and meristics showed that the characters on the head are variable and that total length and preanal length are stable characters as found by Turan*et al*. 2005, Więcaszek*et al*. 2010 and Ola-Oladimeji *et al*. 2016 who found that the head is important for characterisation. Morphometric and meristic measurements confirm that the species maintained in

the Institute of Aquaculture's tropical aquarium most closely resembles *Clarias gariepinus.*

3.7.4 Conclusion

From the morphological and meristic characterisation, in addition to the molecular taxonomic classification described in this chapter, the species under study has been identified as the North African catfish, *Clarias gariepinus (*Burchell, 1822). Establishing this is critical since the aim of the present researcher has been to work on a species that is relevant to aquaculture in the researcher's home country, Uganda. It is the intention of the researcher to use the knowledge gained through the research contained in this thesis to provide a solution for the lack of available diet for larval stages, in order to mitigate the high losses incurred in early life.

3.7.5 Growth Results

3.8 Larval feeding experiments

Figures 3-7, 3-8 and 3-9 show images of catfish larvae taken at different stages during development. Images show the gradual reabsorption of the yolk sac at days 0-3 Dph and the development of the stomach and other elements of the digestive canal.

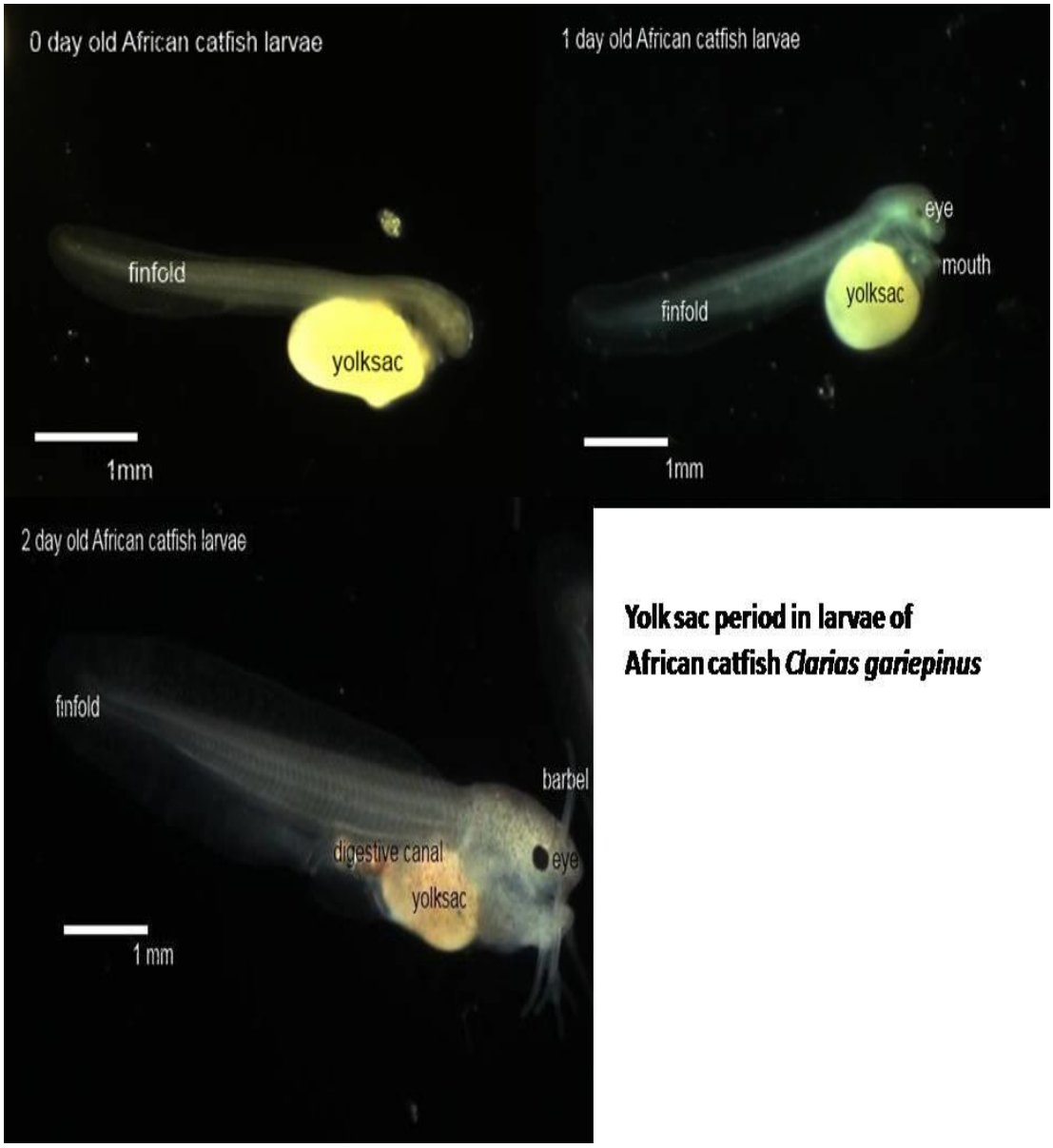


Figure 3-7 showing morphology of larvae of *Clarias gariepinus* at 0 –2days post hatch

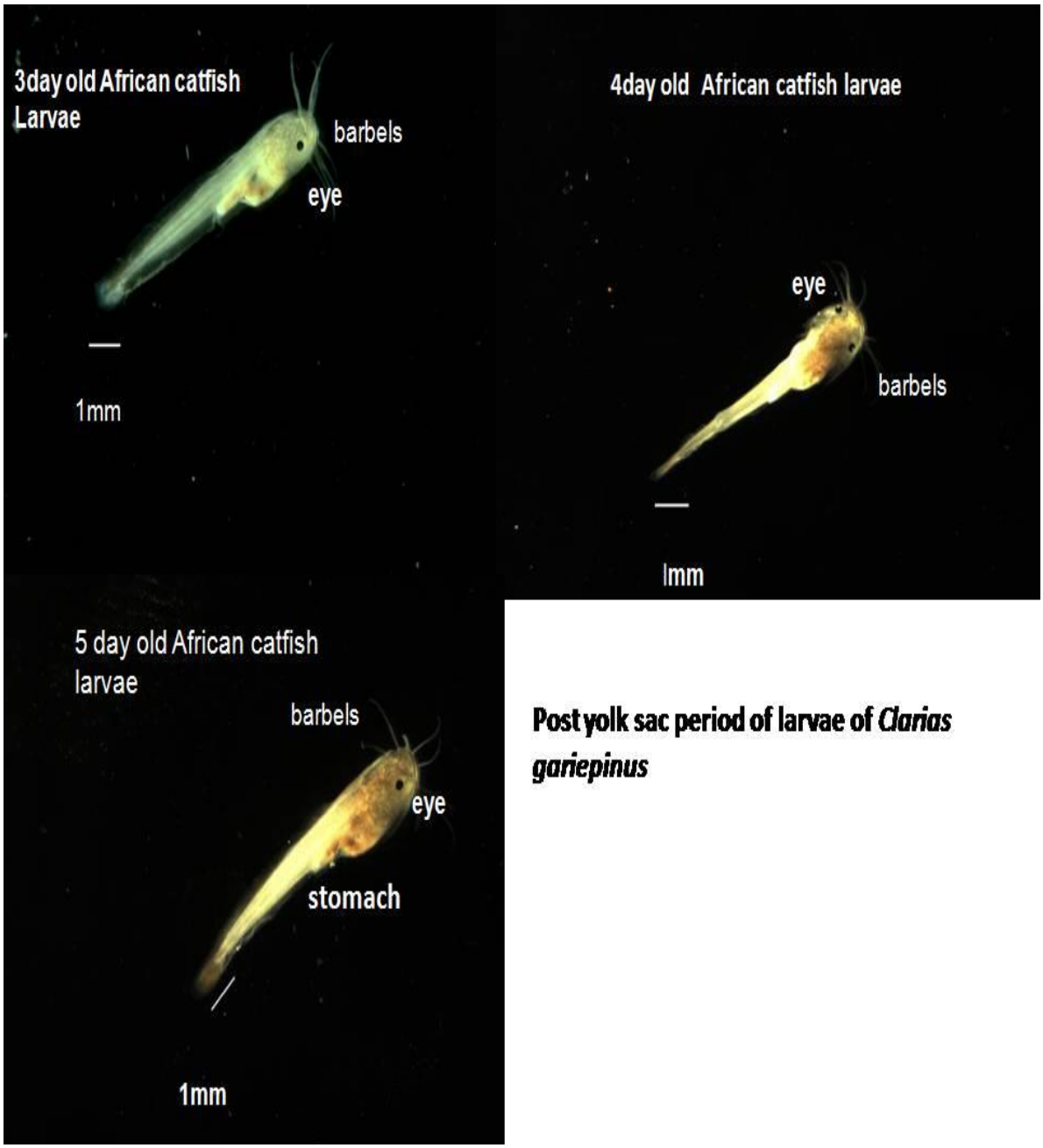


Figure 3-8 showing morphology of larvae of *Clarias gariepinus* at 3–5 days post hatch

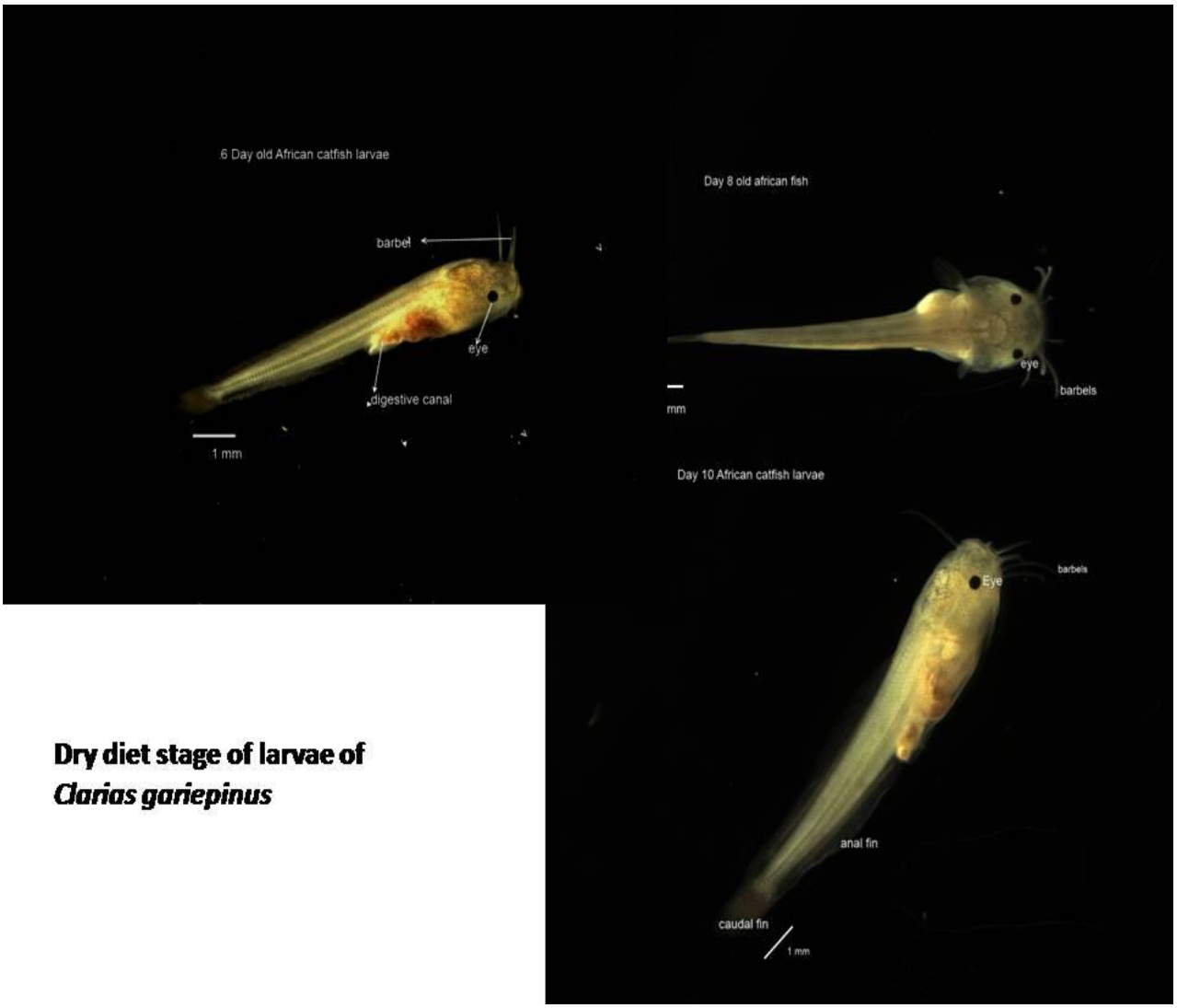


Figure3-9 showing morphology of larvae of *Clarias gariepinus* at 6, 8 and 10 days post hatch

3.8.1 Results for growth of fish larvae in Trial 1

Catfish larvae presented with a standard feeding regime over 28 days exhibited a distinct growth curve attaining about 33mm in the 28days with relatively shallow growth rate observed from 1 Dph till 10 Dph and rapid growth from 10Dph to14Dph. A further growth rate increase is also evident at 21Dph to 28Dph as seen in Figure 3-10.

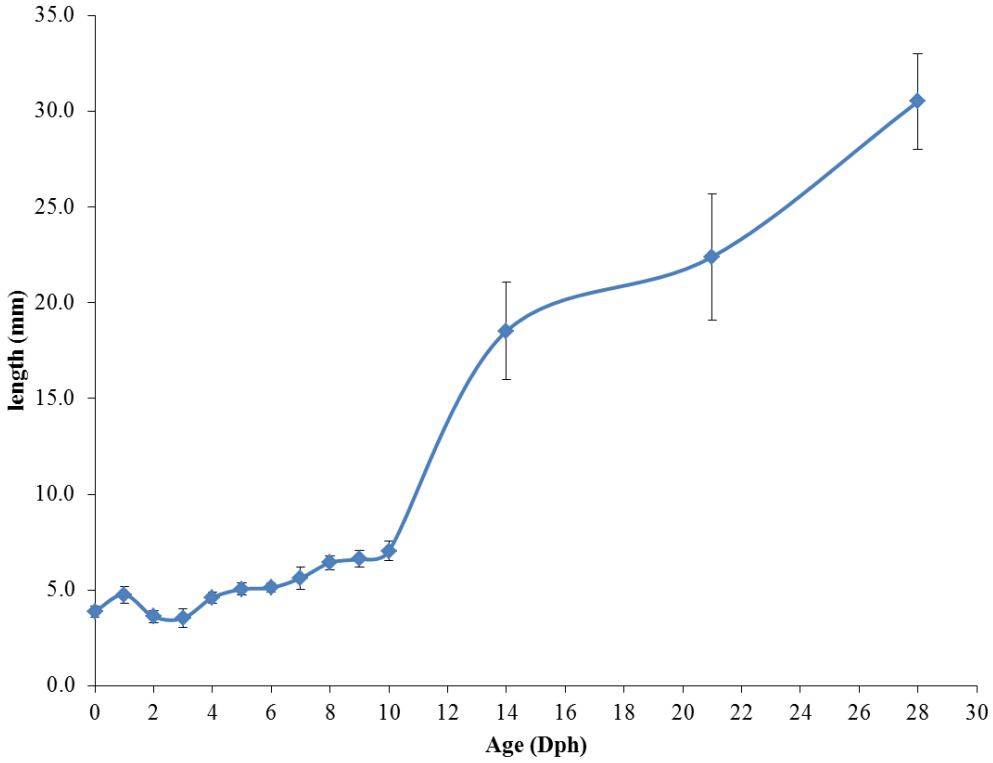


Figure 3-10 Graph showing growth of larvae of African catfish *Clarias gariepinus* in Trial 1 over 28 days.

3.8.2 Results for growth of fish larvae in Trial 2.

Trial 2 examined growth in terms of length when fry were fed on *Artemia* 1,2,3 Days post hatch followed by a mixed diet of *Artemia* and trout crumble starter diet (microdiet) in the ratio 1:1 to 7 to 8 Dph,after which larvae were completely switched on to the trout microdiet till 10 days of culture. Samples collected from this trial were used to run the initial standard PCR run in chapter 5 and histological observations were made for larvae of *Clarias gariepinus* raised upto 10 days post hatch. In addition, the growth pattern was established as displayed in Figure 3-11 below showing a sigmoid curve.

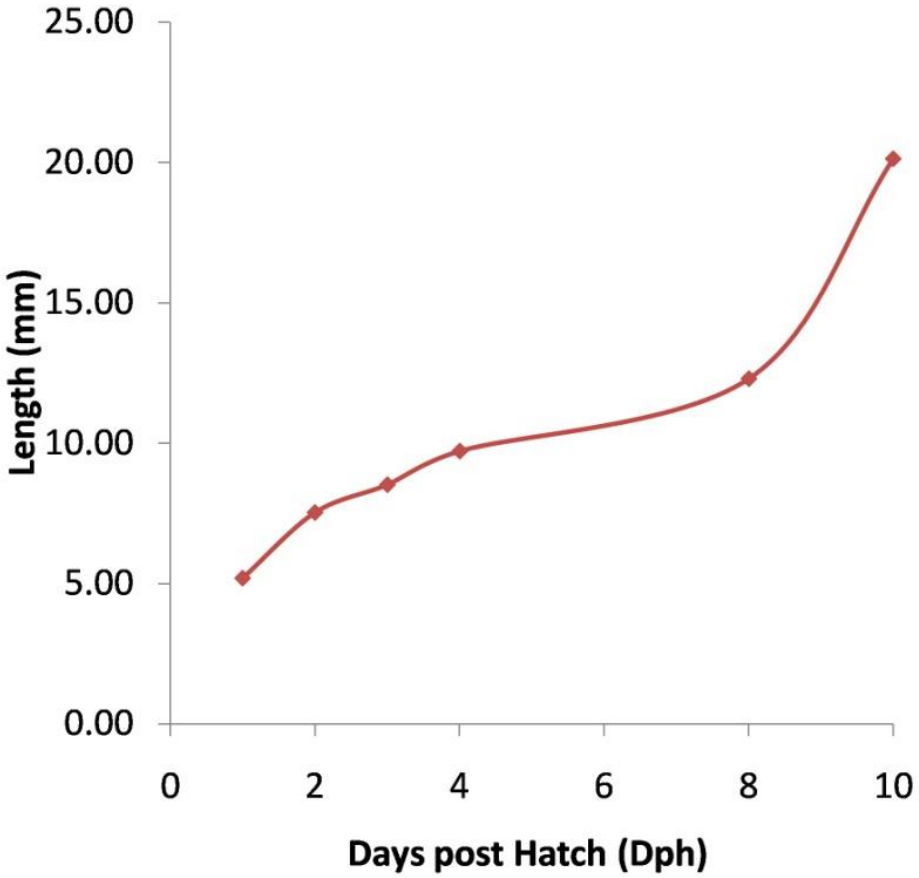


Figure 3-11 showing the growth in *Clarias gariepinus* larvae terms of length raised for 10 days in Trial 2.

3.8.3 Results for growth of fish larvae in Trial 3.

3.8.3.1.1 Growth in terms of weight gain was examined for larvae fed on *Artemia* and *Artemia* +Coppens fed on 1,2,3 Days post hatch over 10 days of culture. In terms of weight, as seen in Figure 3-12 below, weight gain is more prominent when larvae were fed on a mixed diet comprising of *Artemia* and Coppens Catco diet when presented to growing larvae at 2 and 1 Dph and this was closely followed, in terms of performance, by the usual feeding regime of introduction of the mixed diet at 3Dph. However, growth performance was not

observed to be as good when *Artemia* alone was introduced at 1 Dph and 2Dph.

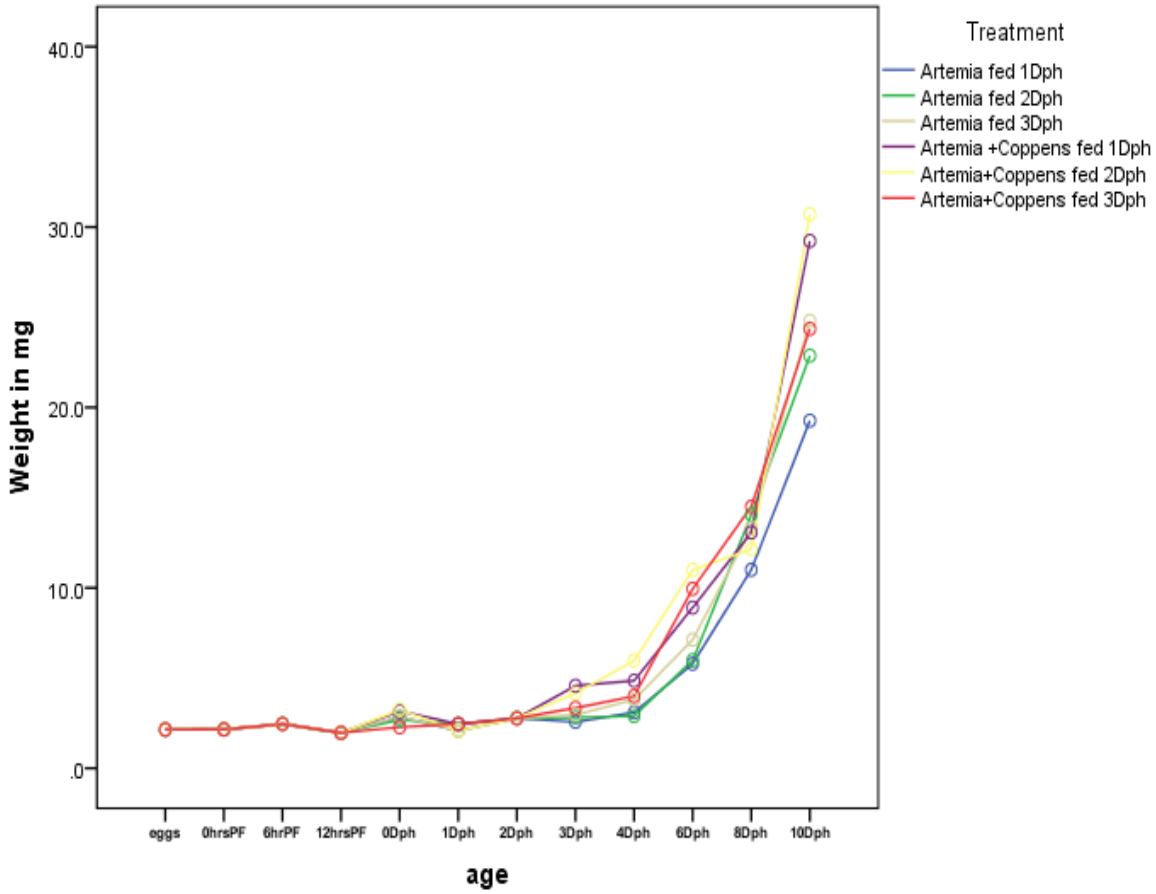


Figure 3-12 Growth in terms of weight gain when fed on *Artemia* and *Artemia* +Coppens fed on 1,2,3 Days post hatch over 10 days of culture. Error bars have been omitted for the sake of clarity.

From statistical analysis, a general linear model (GLM) for repeated measures was performed to test for significant differences in weight gain for the fish when live feed *Artemia* and a mixture of live feed *Artemia* and Coppens (dry diet) was introduced at days 1, 2 and 3Dph. Mauchly's test for sphericity was significant ($p<0.001$) indicating that the assumption for sphericity was not met and therefore a Greenhouse-geisser correction was applied. Results showed that there were significant differences in weight with time / age at which the live feed (*Artemia*)

was introduced and the interaction with tank and treatment also showed the same trend as indicated below:

Age[F (1.377,223.142)=811.200,p<0.001];
Age*Tank [F (2.755, 223.142)=5.799,p<0.001]
Age*treatment [F (6.88,223.142)=5.204,p.>0.001]
Age*Tank*Treatment [F (13.774, 223.142)=1.379,p=0.001]

There was a significant difference in the weight of fish with the age (days), greenhouse-Geisser correction F= (2.764, 447.751) =1767.715, P< 0.05. There is a significant difference in the length of fish on the interaction age and treatment F= 9.889, p <0.05. Effect of age and tank on the length of fish was also significant, p=0.007 with Greenhouse-Geisser correction. Effect of the interaction of age, tank and treatment was not significant on the length of fish, p=0.792.

With respect to length (Figure 3-13), the results in terms of growth performance showed that the fish attained better growth when fed on a mixed diet of *Artemia* and CATCoCoppens diet introduced at 1Dph and 2 Dph unlike for weight they seemed to performed better at 1Dph.As was the case with weight, lowest performance in terms of length was observed in fish larvae fed on live feed *Artemia* at 1Dph.

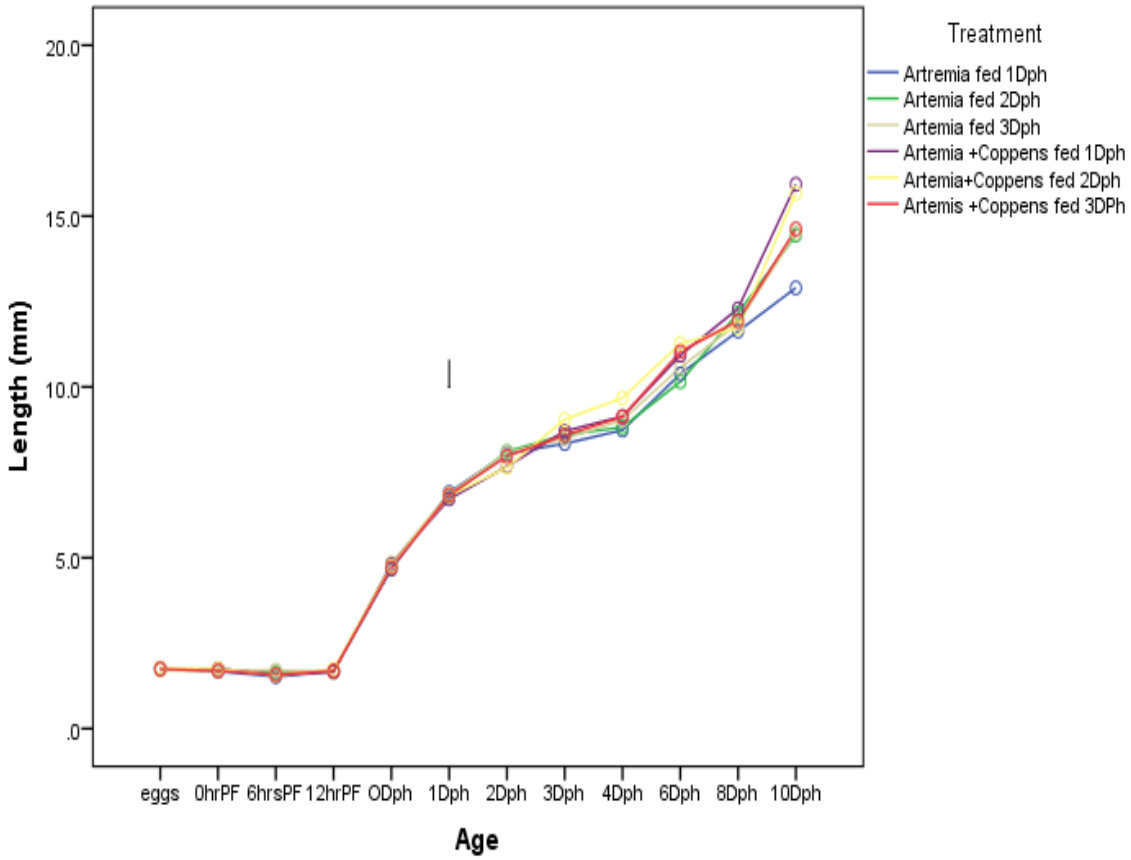


Figure3-13 showing the growth in terms of length gain when fed on *Artemia* and *Artemia* +Coppens fed on 1, 2, 3 Days post hatch over 10days of culture. Error bars have been omitted for the sake of clarity.

From repeated measures GLM (see below) there was a significant difference in the length of fish with the age (days), greenhouse-Geisser correction.There wasalso a significant difference in the length of fish on the interaction age and treatment Age*treatment [F16.692, 540.810=5.204, p< 0.001]. The effect of age and tank on the length of fish was similarly significant, p=0.007 with Greenhouse-Geisser correction. Effect of the interaction of age, tank and treatment was not significant on the length of fish, p>0.001]

Age*Tank [F (6.677,540.810)=5.799,p<0.001]

Age*treatment [F16.692, 540.810=5.204, p< 0.001]

Age*Tank*Treatment [F (13.774, 223.142)=1.379,p>0.001]

Age =[F (3.338,540.810)=10.613,P<0.0001

Trial 3 summary

Larvae of *C. gariepinus* were fed on *Artemia* (live feed) and a mix of *Artemia* and Coppens (Dry feed) were introduced at days 1, 2 and 3 days post hatch (Dph) and growth determined in terms of weight and length up to 10 Days post hatch as shown in Figure 3-5 and 3-6 . Results indicated that in terms of weight, larvae fed on *Artemia* + Coppens at 2 Dph performed better, gaining about 30mg at 10 Dph followed by *Artemia* + Coppens at 3Dph and *Artemia* +Coppens at 1Dph, however, the introduction of *Artemia* alone was outperformed by the mixture as *Artemia*introduction showed a weight gain of only about 18mg at 10Dph. Weaning the larvae at 2Dph in both instances performed better than introduction at 1Dph and 3Dph.

3.8.4 Results for growth of fish larvae in Trial 4

Trial 4 examined growth in terms of in terms of weight gain and length when fed on Coppens (dry diet) and Artemia +Coppens (mixed diet) fed on 1, 2, 3 Days post hatch over 10 days of culture. Results in terms of growth based on weight figure (Figure 3-14)showed that, in terms of growth performance the introduction of a dry diet (Coppens) at 1Dph, 2 Dph and 3 Dph showed differences in the growth in that introduction of Coppens at 1Dph significantly differed from introduction at 2 Dph when fish were raised to 10 Dph. Introduction of Coppens at 3Dph differed from all the other 5 times of introduction of the diet composition, however, growth in fish larval fed on dry diet at 2 Dph and 3Dph differ from those fed on *Artemia* and Coppens from 1 Dph as well as from *Artemia* plus Coppens introduced at 2 Dph. A mixture of Coppens and *Artemia* given at 3 Dph differs from introduction of dry diet on any of the 3 days of start feed.

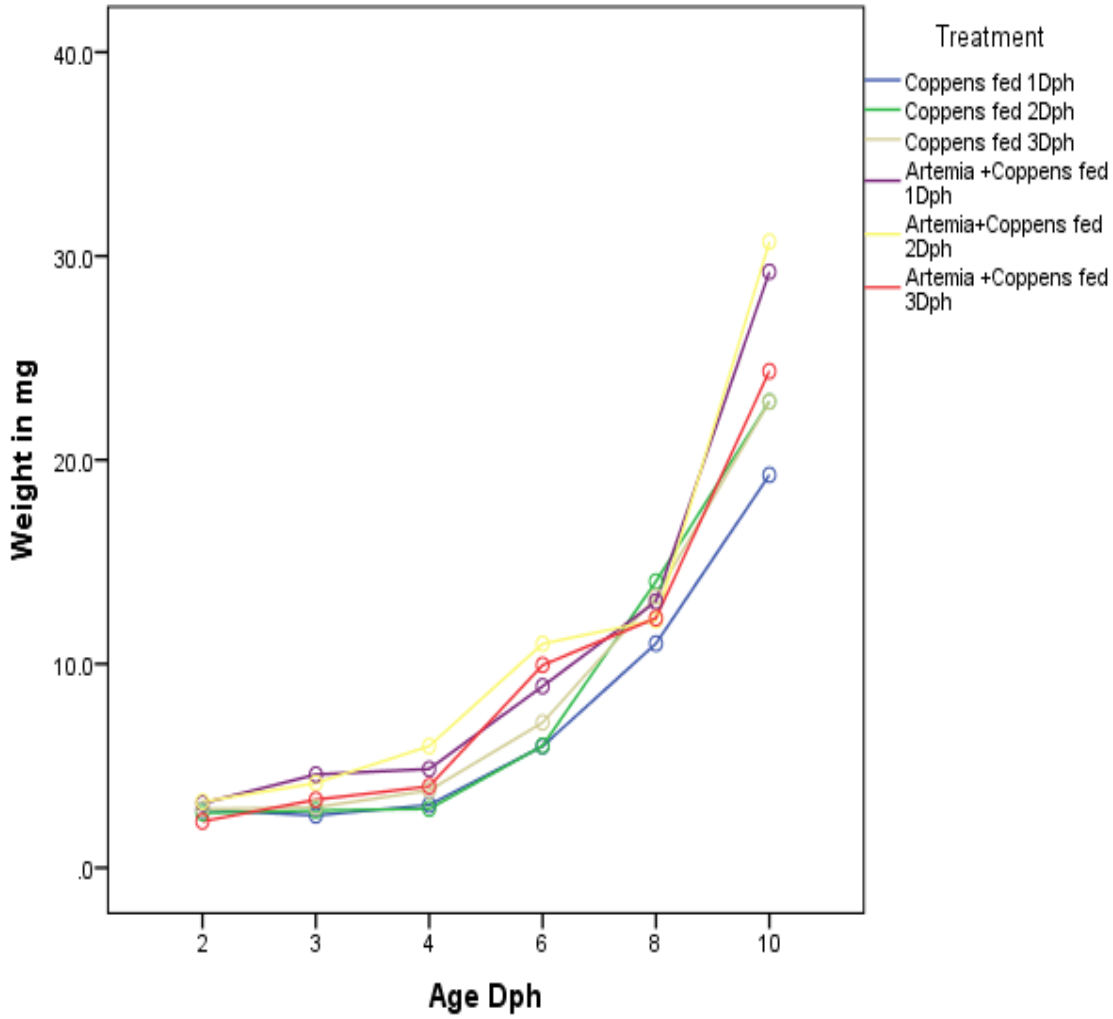


Figure3-14Growth in terms of weight gain when fed on *Artemia* and *Artemia* +Coppens fed on 1, 2, 3 Days post hatch over 10 days of culture. Error bars have been omitted for the sake of clarity

Descriptive statistics showed that Age 1 (mean =5.2;SD=0.330); Age 2 (mean =7.4 32;SD=0.492); Age3 (mean =7.662; SD=0.869).

The General linear model for repeated measures was performed to test for significant differences in weight gain for the fish when dry feed (Coppens) and a mixture of *Artemia* and Dry diet (Coppens) was introduced at days 1, 2 and 3Dph. Mauchly's test for sphericity was above significant ($p<0.001$) and the assumption for sphericity was not met and therefor the Greenhouse-geisser was employed. The analysis indicated that there were significant differences in the weight and with the impact of treatments over time.

Age [F (1.275, 221.815) =536.104,p<0.001]

Age*treatment [F (6.375, 221.815)=3.674.590,p.< 0.001]

Best length at 10 Dph (Figure 3-15) was achieved for fish fed *Artemia* + Coppens from 1 Dph, with those fed mixed diet at 2 and 3Dph reaching similar weights. Fish fed Coppensonly fared more poorly.

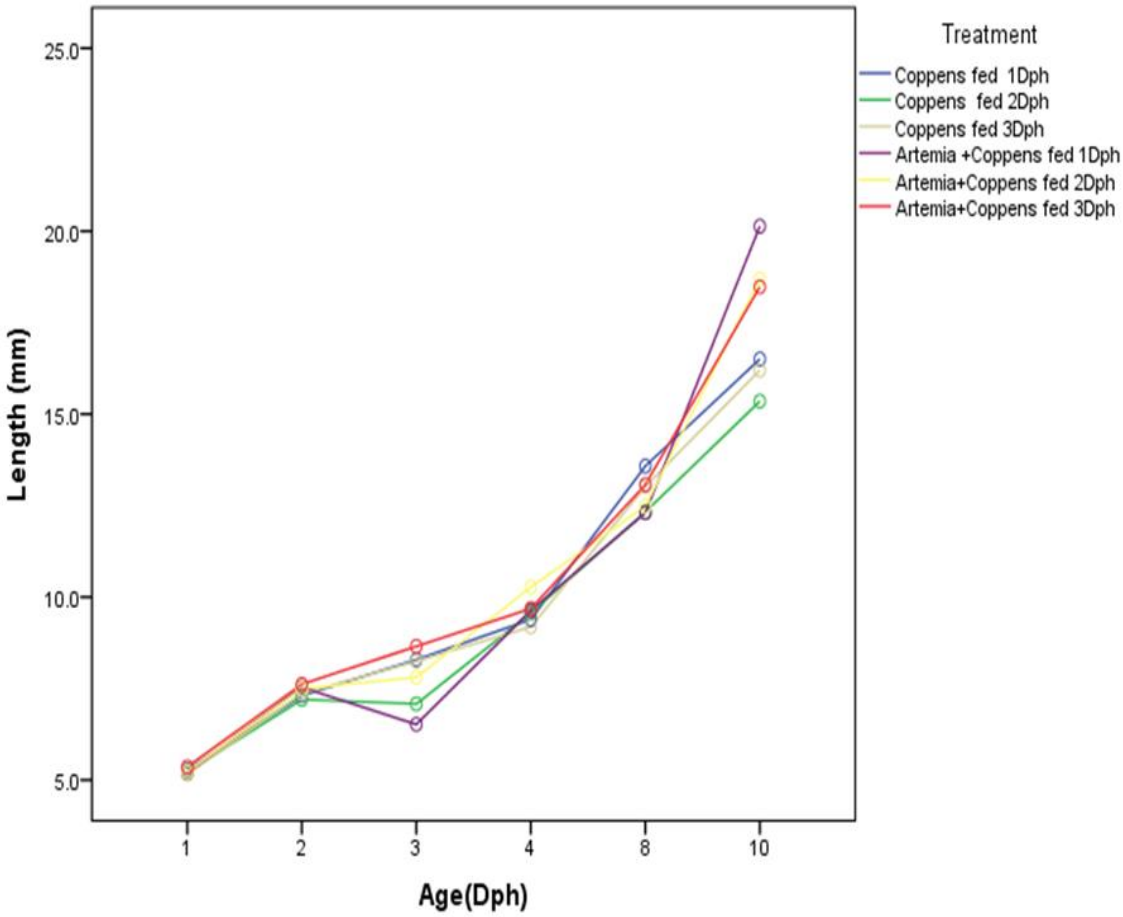


Figure 3-15Growth in terms of length gain when fed on *Artemia* and *Artemia* +Coppens fed on 1, 2 and 3 Days post hatch over 10 days of culture. Error bars have been omitted for the sake of clarity

The General linear model for repeated measures was performed to test for significant differences in length gain for the fish when dry feed (Coppens) and a mixture of *Artemia* and dry feed were fed at days 1, 2, and 3Dph. Mauchly's test for sphericity was significant (p<0.001) and the assumption for sphericity was not met and therefore the Greenhouse-geisser correction was employed. Analysis

indicated that there were significant differences in the length with an interaction of age x treatment:

Age[F (2.055,357.585)=2.914.618,p<0.001];
Age*treatment[F (10.275,221.815)=17.590,p.>0.001]

Trial 4 summary

The General linear model for repeated measures was performed on the data illustrated in Figures 3-14 and 3-15 to test for significant differences in weight gain for fish when live feed *Artemia* and a mixture was introduced at days 1, 2 and 3 Dp. Mauchly's test for sphericity was significant ($p<0.001$) and the assumption for sphericity was not met and therefore the Greenhouse-geisser correction was employed. Analysis showed that there were significant differences in weight according to the time /age at which the live feed (*Artemia*) was introduced and the interaction with tank and treatment also showed the same trend.

3.9 Discussion

Work conducted in this chapter has demonstrated that the stock species at IOA is *Clariasgariepinus* rather than one of the morphologically similar species or hybrids. Findings in terms of growth performance in larvae of *C. gariepinus* from this study show that it is possible to wean larvae at 2Dph with a suitable mixed diet of live and microdiet, here *Artemia* and CATCoCoppens, as seen in results above .This is before the exhaustion of the yolk sac period which usually occurs by 3Dph .This concurs with other studies including one in Uganda by Aruho, *et al.* (2017), where the newly introduced Raanan feed (a product from Israel) was able to improve growth performance when fed to larvae during the switch to exogenous feeding.In that study, however,feed was introduced based on the procedures that farmers currently employ when weaning fish, in that they wait for yolk absorption and then start feeding exogenously.An equally well formulated diet could, from these results, reduce the cost of mass production of catfish fry in hatcheries in Uganda and lessen the burden of preparing live feed cultures. It is also possible that if the dry feed used contained relevant nutritive ingredients that could support first growth of larvae, then successful weaning of catfish larvae exclusively on dry feed alone might be feasible.

3.10 Conclusions

This study has demonstrated that *Clarias gariepinus* are able to uptake of artemia, mixed diet comprising of artemia +Catco Coppens and Catcocoppens.However, the growth performance varies with the best growth performance exhibited when a mixed diet of artemia and CATCocoppens is introduced at 2DPh. This suggests that *Clarias* larvae should be fed exogenously at 2Dph; and a more suitable combination of artemia and comparable microdiet to CATCoCoppens in combination with live Artemia could be used at 2 Dph to feed to larvae of *Clarias gariepinus*.

4 CHAPTER FOUR

Cloning and expression analysis of four key digestive enzymes during early development in larvae of North African catfish, Clarias gariepinus (Burchell 1822): Deciphering gastrointestinal functionality

4.1 Introduction

Understanding the activity and restrictions of dealing with capabilities of the digestive system during early development in fish is an important research area for the growing aquaculture sector (Balon, 1986). Candidate fish species introduced for farming have widely differing nutritional needs during ontogeny (Haylor, 1992; Moguel *et al.*, 2016), however, knowledge of fish nutrition is still in infancy. While nutrition of larval fish for most candidate fish species in the developed world has been extensively studied, knowledge of candidates in the developing world still lags behind, particularly with respect to studies concerning mRNA expression and including the fish species investigated in the work described in this chapter, *Clarias gariepinus* (Burchell 1822). Research investigating enzyme activity or transcript expression during the larval stage of fish can play an important role in discerning the digestive physiology of the fish. A lot of work has been conducted on the digestive physiology of both marine and freshwater fish as it is the best way to establish the nutritional requirements of the fish. This allows researchers to develop suitable feeding protocols, which facilitates intensive production of larvae and onward growth and ultimately helps supply protein for the increasing population worldwide (Galaviz *et al.*, 2011; Galaviz *et al.*, 2015; Gisbert *et al.*, 2009; Koch *et al.*, 2014; Kurokawa *et al.*, 2002; Srichanun *et al.*, 2013; Srivastava *et al.*, 2002; Verreth *et al.*, 1994). However, most of the studied species, including *C. gariepinus* larvae (Verreth *et al.*, 2007) and silversides *Alestes baremoze* (Kasozi *et al.*, 2017) have been researched from the point of view of biochemical and histological / histochemical analysis. A number of studies have attempted to determine the timing for initiation of exogenous feeding and/or gastrointestinal functionality in larval *C. gariepinus* (Onura *et al.*, 2018, Ing & Chew, 2015; Nyang'ate; Segner *et al.*, 1993; Segner & Verreth, 1995; Verreth *et al.*, 1993; Verreth *et al.*, 1987; Verreth *et al.*, 1992), however, the current study employed both histological and molecular techniques to try and characterise development of

digestive capacity in this species. Hence the objective of the study described in this chapter has been to describe the histological and physiological development of the gastrointestinal system in *C. gariepinus* from the time of first-feeding until metamorphosis.

Few species of fish larvae have been studied at the molecular level, especially using i*n situ* expression hybridisation. *In situ* hybridization (ISH) is a technique used to localize nucleic acid sequences in tissue sections or cell culture samples, employing a known mRNA target sequence to provide specificity of labelling and being able to highlight the cellular / tissue / organ site of expression of a given transcript. This method is therefore nominally capable of following the expression of transcripts for digestive enzymes, allowing an estimate of potential GIT functionality, free from interfering signals from live prey enzymes that would be detected in biochemical assays, to be obtained.

For this research work, the focus was on mRNA detection using an ISH protocol based on a tagged nucleotide sequence (i.e. “the probe”) complementary to the sequence of the mRNA to be detected that was applied to the tissue sample.

It is important to note that because larvae are quite small the researcher started by using the adult catfish to allow for processing the right Dig labelled products to be used on the target organs as per literature as the larvae are too small to dissect

4.1.1 Specific objectives

I. To review the histo- morphology of the digestive system of adults, followed by larvae.

II. To determine the molecular events governing the ontogeny of digestive capacity based on localised *insitu* expression hybridization of four enzymes namely trypsin, chymotrypsin, pepsin and gastric chitinase.

4.2 Review of related Literature

4.2.1 Relationship with general physiology organization of the digestive system in fish and fish nutriment.

Studies of physiology are a source of understanding of the physiological mechanisms that allow for survival and conservation of tamed animals and preservation of wild animals respectively in their various environments (Stevens & Hume, 2004). The physiology of the digestive system of teleost fish in terms of function is one of the broad areas that has been studied (Bakke *et al.*, 2010; Wilson & Castro, 2010), Fish exhibit a similar essential plan in terms of gastrointestinal tract to other vertebrates, however, there exists a difference shown in terms of phylogeny and ontogeny, diet and environment.

Fish largely subsist in water, which means that all the activities including feeding, excretion, and gaseous exchange all occur in the same medium. Morphological studies have improved knowledge of the spatial organization and relationship of physiological and biochemical data and the molecular dimension that is quickly being exemplified through molecular techniques directed at the genome, transcriptome, and proteome. Morphological information is also important in understanding fish nutrition in ecology and aquaculture, and during development as well as mechanisms for physiological adaptations to a changing environment (Wilson & Castro, 2010).The ability of any organism to digest a given substance rests predominantly on whether the appropriate enzyme is present or not and then whether the required conditions for operation of that enzyme exist or not.

4.2.2 Functional morphology of the digestive system in fish.

4.2.2.1 General organization of the digestive system in fish.

The digestive system in fish plays a functional role in distribution of nourishment to the body .This process is very critical in the development of fish larval especially at the beginning of development when feed is taken in orally with the need to break it down and use it by the growing larvae since in most species the larval gut is simple (Rønnestad *et al.*, 2007; Rønnestad & Morais, 2008). The digestive system has been described to comprise of the gastro-intestinal tract (GIT) made up of foregut, midgut, & hind gut and its associated accessory organs made up of the liver, bile and pancreatic exocrine gland (Gabaudan & Bruno, 1986; Rønnestad & Morais, 2008; Wilson & Castro, 2010).

4.2.3 Histo-anatomy of the digestive system of larval fish.

In general terms the histo-anatomy of fish at various stages of development from the embryonic stage to post embryonic stage right up to the adult stages show differences within species, however, the basic plan is similar. The wall of the digestive tube is constituted by 3 layers namely the mucosa which includes the epithelium and the sub epithelial connective tissue, the muscularis externa formed by a circular and a longitudinal muscular layer and the serosa (Gabaudan & Bruno, 1986; Rønnestad & Morais, 2008)

4.2.3.1 Histo-anatomy of the digestive system in adult *Clarias gariepinus*

4.2.3.1.1 Introduction

The gross morphology and histology of the digestive system of juvenile and adult *Clarias gariepinus* has been described by the following authors Moawad *et al.*, 2017, Ikpegbu, Year missing ; Ikpegbu, Nlebedum, Nnadozie*, et al.*, 2014; Ikpegbu, Nlebedum, & Ibe, 2014; Ikpegbu *et al.*, 2014 ;; Uys, 1989 as having a wide, sub terminal mouth with a wide gape for taking in large prey and or large volumes of water during filter feeding. The fish possess bands of fine, sharp, curved teeth on the premaxillary and dentary bones which allow it to hold prey to stop its escape. The vomerine and pharyngeal teeth also perform the above function in addition help to disable the prey by crushing it. Some biting may be done, especially by the less sharp teeth on the vomerine band, but large prey is taken in whole. The anterior margins of the five branchial arches possess long gill rakers, and the posterior margins of the third and fourth arches have gill rakers that interdigitate with those on the anterior margin of the next arch (Uys, 1989). The gill rakers form a straining apparatus which allows the fish to filter-feed on mid-water organisms and surface feed substances.

4.2.3.2 Developmental patterns in morphology and histology of the digestive tract of larval fish

The digestive system of larval fish in terms of developmental history has been defined as differing among fish species (Balon, 1981; Flegler-Balon, 1989) This is based on the life history patterns, these being divided into precocial species that develop directly from embryos to juveniles with an intermediate stage as seen in salmonids,andgobids and altricial species which develop from a distinct larval stage with temporal structures that change during metamorphosis eg (*Solea senegalensis, Kaup* 1858) senegalese sole, and, *Clarias gariepinus* Burchell 1822).North African catfish One outstanding feature of altricial species is possession of distinct larval stage that lacks a functional gastric stomach.

In general, the alimentary tract of first-feeding larvae is a simple and a relatively undifferentiated tube with only one sphincter with an intestinal-rectal valve before the anal opening.Some species have a rotated gut at the onset of exogenous feeding while other species have a straight gut .However as ontogeny continues to occur the ontogenetic development of the intestine changes, including among other an increase in length and mucosal surface area, as well as an intensification of the activities of brush border enzymes (Segner *et al.*, 1994).other changes include the increase in area, sometimes with increase in number of rotated intestinal loops and an increase in the epithelial folding. The folding of the intestinal wall appears in some species as a regular pattern while in other the folds appear more irregular. The intestinal tube consists of a simple epithelium surrounded by connective tissue, smooth muscle and enteric nerves. There are different cell types that can be identified in the epithelial layer (Mitra *et al.*, 2015). These include enterocytes (the most numerous of all cell types, responsible for nutrient absorption, single enteroendocrine cells which produce and secrete peptide hormones and mucus producing cells known as goblet cells.

The ultra-structural characteristics of the enterocytes of pre-feeding larvae have been described as being essentially the same as in adult fish species with possession of functional lipid absorption structures, such as well-developed endoplasmatic reticulum (ER) and Golgi apparatus at the time of first-feeding has been noted having functional enzymes from pancreas, enterocytes and able to digest lipids.

Just like other fish species the larvae of North African catfish exhibit exogenous feeding and measure 5.5-6mm and weigh 2.2-2.4mg (Helmut Segner&Verreth, 1995) at 2-3 Dph*Clariasgariepinus* larvae, possess the digestive tube is simple, and no stomach is evident. The Yolk sac stage lasts for 48hours post hatch. However dependant on the prevailing temperatures of the rearing environment at around 5 Dph the initial anlage of stomach develops in the most caudal part of the "oesophagus". This is characterized by the formation of oxyntic glands in the thickened mucosa (Ing & Chew, 2015; Osman *et al.*, 2008; Segner *et al.*, 1994; Segner & Verreth, 1995;Uys, 1989; Verreth *et al.*, 1992) (Stroband& Kroon 1981;Uys 1989; Verreth et al., 1992; Segner&Verreth 1995; Osman et al. 2008; Ing & Chew 2015) and also by the presence of the digestive tract (as seen in Chapter 6 of this thesis By the 10th day peptic activity in the stomach is macroscopically distinct also observed in this study. According to Stroband & Kroon, 1981, the pyloric region of the stomach is histologically fully developed by around 15Dph. After this stage, no important changes seem to take place in the morphology of the digestive tract. Description of the histo-anatomy of the gastro-intestinal tract in fish

4.2.3.2.1 Oesophagus

The oesophagus in fish is comprised of the mucosa made up stratified squamous epithelium, numerous goblet cells in the epithelium; sub epithelial connective tissue generally formed by an inner dense layer and an outer loose layer with many leucocytes. It is also comprised of the muscularis externa made up of two layers of striated muscle, an inner longitudinal-one and an outer circular-one the serosa (Gabaudan & Bruno, 1986).

The oesophagus is vital in relaying food to the stomach as it has the ability to stretch its striated muscles to allow for this since fish have be known to feed intermittently

4.2.3.2.1.1 Description of the morphology of the oesophagus of *Clarias gariepinus*

The oesophagus adult *Clarias gariepinus* is described as a short and capable of expansion thereby allowing for, passage to the highly expansive stomach which of is divided into corpus and pyloric regions (Uys, 1989).

The digestive system in the newly hatched embryo of North African catfish larvae is formed by a monostratified layer of cubical/columnar cells encompassing a

narrow undifferentiated (uniform) lumen, connected dorsally to the yolk sac. Mouth and anus are still closed. At 3 Dph, the first anlage of the liver and the pancreas are localized. At 4 Dph, buccal cavity, oesophagus and intestine were differentiated, but mouth and anus were still closed the buccal cavity is lined with squamous epithelium and the oesophagus appeared as a simple columnar epithelium. The lining of the remainder of the alimentary canal consisted of columnar epithelium. The opening between the intestine and the buccal cavity was not yet established and only opened at 6DPH Thereafter, lining of the oesophagus transformed into stratified epithelium) An intestinal valve is present between the oesophagus and the intestine, dividing the digestive tract into three distinct regions: buccal cavity, oesophagus and intestine. A constriction further divided the intestine into a wide anterior intestine and a narrow posterior intestine. Numerous epithelial folds (columnar epithelium) were observed throughout the intestine At this stage, the mouth and the anus opened (Osman *et al*., 2008).

4.2.3.2.2 Stomach

4.2.3.2.2.1General description

The shape of stomach in fish varies with fish species but can be basically described as generally being straight, Y shaped/ siphon shaped with variation in term of length very much dependant on diet intake and could classified as 0. 2 to 2. 5 times the length of the fish for the carnivores, 0. 6 to 8. 0 times for omnivores and 0. 8 to 15. 0 times for herbivores. However sometimes the fish are stomach less and may be swapped with the intestinal bulb. Stomach-less fish basically don’t not possess acid digestion (Gabaudan& Bruno, 1986) The general structure of the stomach of fish is described as comprising of 3 parts namely the cardiac and fundic which contain gastric glands and pyloric region which has no glands (Gabaudan& Bruno, 1986; Rønnestad & Morais, 2008).

In terms of histology the stomach has been described as having a mucosa, comprised of high columnar, simple epithelium. Histologically and topographically, the start of the stomach is distinct with the sudden change in the epithelium to the columnar mucous cells of the stomach and the appearance of gastric glands. Since this transition can be gradual the term esogaster is sometimes applied to the transition zone between the oesophagus and stomach. (Wilson and Carlson 2010).The luminal surface of the epitheliocytes has short and widely spaced microvilli and it is covered by a mucous coat. Usually there are no goblet cells but

the apical cytoplasm contains mucous granules which are secreted into the gastric lumen by exocytosis. Exocrine glandular cells are located in the connective tissue. They are radially arranged.

The stomach also posesses the *muscularis externa* composed of two layers of smooth muscle, an inner circular-one and an outer longitudinal-one i.e. it is composed of four layers namely mucosa, sub mucosa, muscularis and serosa. (Ghosh & Chakrabarti, 2015).

The stomach plays an important role as it the site of acid digestion of proteins because it has the gastric glands that secrete hydrochloric acid and pepsinogen an active precursor which becomes pepsin in the presence of a low pH (Rønnestad *et al.*, 2007; Rønnestad & Morais, 2008; Rønnestad *et al.*, 2013). The presence of lipases and chitinase has also been reported in the stomach of some species.

4.2.4 Histomorphological, histochemical, and ultrastructural studies of the stomach of the adult North African catfish (*Clarias gariepinus*)

The stomach has been described as being muscular and is capable of breaking down the food by churning it around thereby facilitating the digestive process. The stomach in *Clarias gariepinus* has been described as being J shaped which is distinctively divided into a descending glandular portion known as the corpus and ascending glandular portion known as the pyloric (Moawad *et al.*, 2017;Ikpegbu, Year missing ; Ikpegbu, Nlebedum, Nnadozie*, et al.*, 2014; Ikpegbu, Nlebedum, & Ibe, 2014; Ikpegbu *et al.*, 2014 ;). The stomach is a J-shaped sac divided into three regions namely: cardiac; fundic; and pyloric. In terms of histology, the stomach wall is comprised of four layers known as mucosa; sub mucosa; muscularis externa; and serosa. The mucosa of the three portions has thick longitudinal folds lined with simple high columnar cells containing oval basally located nuclei. These cells contain apically located muco substances. Many gastric pits are present and made by invaginations of the mucosal layer into the underlying lamina propria and continuous with the openings of the gastric glands. Only the cardiac and fundic regions contained mucosal glands. The fundic glands are lined with oxynticopeptic cells which are sites of production of pepsinogen and HCl in preparation for acidic proteolysis the usual mode utilised by adult fish to break down their food .Enteroendocrine cells were distributed in the gastric wall

within the epithelial cells of the gastric mucosa and gland. The lamina propria composed of extensive collagen fibres, many blood vessels, and nerves (Ikpegbu, Year missing ; Ikpegbu, Nlebedum, Nnadozie*, et al.*, 2014). Strands of smooth muscle fibres situated between the lamina propria and the sub mucosa forming lamina Muscularis mucosa. Loose connective tissue was the main component of the tunica sub mucosa. The pyloric portion has the thickest mucosa and the serosa coat of the stomach was formed of loose connective tissue containing blood vessels (Moawad *et al* 2017).

Studies by histological features and histochemical characterization of the stomach were investigated in *Mystus cavasius* (Hamilton1822), *Oreochromis niloticus* (Linnaeus 1758) and *Gudu siachapra* (Hamilton 1822) having different feeding habits. Histologically the stomach of all the three fishes was made up of mucosa, sub mucosa, Muscularis and serosa. The mucosa of superficial epithelium consists of a single layer of compactly arranged columnar epithelial cells. Prominent gastric glands are present in *M. cavasius* whereas in *G. chapra* the gastric glands are totally absent in the gizzard like stomach. However, in O. niloticus tubular gastric glands are present in the glandular epithelium of caeca like stomach (Ghosh & Chakrabarti 2015).

The intestine is simple, relatively short and thin walled. The walls of the terminal portion of the intestine (rectum) are slightly thicker and more muscular than those of the rest of the intestine. The walls of the terminal portion of the intestines are slightly thicker and more muscular than the rest of the intestine (Uys 1989)

4.2.4.1 Development of the histo-morphology of the larval fish stomach

Altricial fish species, including *Clarias gariepinus,* develop a functional stomach during metamorphosis. One of the major functions of the fully functional stomach is the breakdown of large food particles, which is achieved by mechanical grinding in combination with acid denaturation and enzyme breakdown. Together, these actions allow the regulated release of partly digested food (chyme) into the midgut and this helps to maintain an optimal ratio of food to digestive enzyme in the midgut intestine (Rønnestad & Morais, 2008; Rønnestad *et al.*, 2003; Rønnestad *et al.*, 2013).

The stomach develops from an intermediate region between the oesophagus and the intestine. In larval stages that have a rotated gut it can first be recognised as a pouch-like bulb in the foregut with functional oesophageal and pyloric valves before the onset of gastric secretion. This suggests that the presumptive stomach is capable short-term food storage.

In fish larvae the augmentation of gastric HCl production and secretion has been established by histological observations and pH measurements, and by reverse transcription-polymerase chain reaction.

Various fish studies have indicated that fish larvae do not possess HCl-producing cells (gastric glandular acini at first-feeding (Rønnestad *et al.*, 2000; Rønnestad *et al.*, 2003). For example studies employing *in situ* expression hybridisation coupled with RT-PCR in winter flounder (*Pseudopleuronectes americanus).* It is evident that *mRNA* expression of proton pumping genes takes place at 20 days post-hatch and corresponds to the appearance of gastric glands as an indicator for pepsin activity (Douglas *et al.*, 1999; Gawlicka *et al.*, 2001). These studies also indicated that, according to *in situ* hybridization methods, the sites of pepsinogen synthesis and hydrochloric acid secretion were localized in the glandular stomach mucosa and in the case of winter flounder, the specialised cells are of the oxynticopeptic type (Gawlicka *et al.*, 2001). In another study in Red porgy (*Pagrus pagrus*), pepsinogen and proton pump expression were detected from 30 days post-hatching (dph), increasing with development. Proton pump expression was localized in the gastric glands of red porgy larvae as revealed by *in situ*hybridization, showing increasing signal intensity during digestive system development (Darias *et al.*, 2007a).

In another study employing *in situ* hybridization (ISH) the onset of expression of the gastric enzyme pepsinogen was described as well as the complete development of gastric glands in the larval stomach of (*Paralichthys olivaceus),* Japanese flounder (*Verasper variegatus)* spotted halibut, (*Epinephelusseptemfasciatus)* seven-band grouper) and *Serioladumerili)* greater amberjack. Pepsinogen expression was observed to begin in the cardiac region of the larval stomach at 25 days post-hatching (dph) in Japanese flounder, 36 dph in spotted halibut, 38 dph in seven band grouper and 13 dph in greater amberjack. The gastric glands of these species were fully developed in the stomach approximately 5–12 days after the initiation of pepsinogen transcript expression also coinciding with external manifestations of metamorphosis (Wu *et al.*, 2011). This was also seen in Atlantic halibut (*Hipoglossus hipoglosus*) where onset of gastric function was described using anatomical, biochemical, molecular and *in vivo* observations combined with quantitative PCR (qPCR). Similarly in this study, the development of the gastric proton pump (H/K^+) and pepsinogen showed that gastric proteolytic capacity was present during the climax of metamorphosis (Gomes *et al.*, 2014).

The fact that the development of the gastric / proton pump coincides with metamorphosis from juvenile to the adult morphologies suggest that this is a point when the fish could be switched to a micro diet. For the above species, this is too long to sustain live feed for proper growth. Such studies allow for comparison between enzyme activity patterns and expression at the molecular level (Yúfera *et al.*, 2018) and also suggest that time course profiles of transcript expression are genetically programmed and only affected to a minimal extent by diet changes.

4.2.4.2 Intestine

The intestine in fish has been described in terms of ultra-structure and function (Gabaudan & Bruno, 1986; Rønnestad & Morais, 2008; Wilson & Castro, 2010). It is comprised of, 3 parts namely anterior intestine, posterior intestine and hind gut /rectum.

On the basis of ultra-structure and functional criteria, the intestine of larvae of *Clarias gariepinus* can be divided into three regions; anterior intestine, posterior intestine and rectum.

In fish the digestive enzymes are active in the intestinal lumen at a pH close to neutrality and are secreted by either the exocrine pancreas or by the intestinal

mucosa..The intestinal mucosa is responsible for production of proteases including amino peptidase, dipeptidase and tripeptidase, alcaline and acid nucleosidases, poly nucleotidases, lecithinase, lipase and esterase and amylase, maltase and laminase; Gabaudan 1986; Infante&Cahu 1994; Chakrabarti *et al.*1995). On the other hand the exocrine pancreas secretes the following digestive enzymes; proteases including trypsin, chymotrypsin, carboxy peptidase and elastase (all stored and secreted as proenzymes and activated in the intestinal lumen, amylase, lipases and chitinase in some species (Nilsson &Fänge 1969; Yoshinaka et al., 1984; Gabaudan 1986; Infante&Cahu 1994; Chakrabarti *et al*., 1995; Alarcón*et al.,* 1998; Falcón-Hidalgo *et al*., 2011).

4.2.4.2.1 Anterior intestine

The anterior intestine has been described to possess a mucosa which appears as numerous branched folds. The epithelium is composed of a simple layer of high columnar cells, the enterocytes, among which occur goblet cells. The apical plasma membrane forms microvilli which make the brush border thus increasing the exchange surface between the lumen and the enterocytes. The cytoplasm contains lipid vacuoles and lipid particles.

4.2.4.2.1.1Role of the intestine

The intestine performs two major roles namely digestion and absorption. In terms of digestion when exocrine pancreas excretes the following proteases namely trypsin, chymotrypsin, carboxypeptidase and elastase these are stored and secreted as proenzymes and activated in the intestinal lumen plus others including amylase, lipases and chitinases (in some species). Apart from storing the former enzymes in preparation for digestion the intestinal lumen also secretes the following proteases namely aminopeptidase di- and tripeptidase; alcaline and acid nucleosidases; polynucleotidases; lecithinase, lipase and esterase; amylase, maltase, laminarinase.

In its role for absorption, the intestine is involved in uptake of nutriments which are absorbed by the enterocytes and proceed to the circulatory system through blood capillaries located in the sub epithelial connective tissue. Then, they are carried to the liver by the portal vein. Histochemical studies have suggest that amino acids, fatty acids and glucose are mostly absorbed by the anterior intestine while macromolecules (including proteins), water and minerals would be absorbed by the posterior intestine.

4.2.4.2.2 Midgut

The midgut plays a key role in the digestion and absorption of ingested feeds in larval fish. In most species, the main degradation and absorption takes place in the midgut segment and it is often not possible to recognise the feed when the chyme is passed into the hindgut/rectum and the midgut has been described as appearing alkaline throughout development.

4.2.4.2.3 Hindgut/rectum

In fish larvae the hindgut is recognised as discrete chamber from the onset of first-feeding while in adult vertebrates the hindgut is mainly associated with final adjustments in the water and ion composition of the faeces though in larval fish not much is fully understood. Histological studies of the epithelial lining show frequent pinocytotic invaginations and intracellular vesicles (Kurokawa *et al*., 1996).

4.2.4.3 Pyloric caeca

In fish the pyloric caeca occur as finger-like projections of the anterior intestine wall located just past the pyloric sphincter. They have been found to have a similar histology and role comparable to the anterior intestine. They play a role in food retention and increase the intestinal evacuation time.

4.2.4.3.1.1Pyloric caeca in fish larvae

Pyloric caeca develop during the transition from larvae to juveniles and originate in the anterior part of the intestine. The pyloric caeca are blind-ended appendages that serve to increase intestinal absorptive surface area without increasing the length or thickness of the digestive tract itself .The number of caeca varies from a few to >100 and they initially grow proportionally faster in length than the rest of the intestine until they reach a constant ratio to the length of the intestine in juveniles (Rønnestad & Morais, 2008), They often develop a folding of epithelial lining which contributes significantly to increase the surface area. Functionally, pyloric caeca appear to be involved in mixing the chyme with digestive secretions in the region of the pyloric caeca

4.2.4.3.2 Accessory organs associated with the digestive system

Histological examination of the timing of ontogenetic development of the digestive tract and accessory glands is considered to be one of the most accurate method for the evaluation of proper function and, thus, nutritional status of fish larvae (M. A. Galaviz, López, Gasca, *et al*., 2015; Gisbert &Doroshov, 2003; Papadakis *et al*., 2013; Zambonino-Infante *et al*., 2008

4.2.4.3.3 Liver

In terms of structure the liver has been described to comprise of hepatic parenchyma made of polygonal cells called hepatocytes (Gabaudan& Bruno, 1986; Rønnestad & Morais, 2008). Hepatocytes are radially arranged around a central vein in two-cell thick laminae separated by sinusoids. The cytoplasm contains glycogen and lipids. The hepatic artery and the portal vein enter into the liver. The junction of several hepatocytes forms the bile canaliculi which drain the bile to the gall bladder where it is stored. The bile is then delivered to the intestinal lumen by the common bile duct.

The liver plays an important role in bile secretion which acts as an emulsifier for the lipids up taken in the diet in the intestinal lumen thereby allowing for enzymatic activity. Bile is also important in neutralization of powerful acidic intestinal composition coming into the intestine during the digestion process (Wilson & Castro, 2010).

However, the liver has various other roles which are not digestive in nature and include among others, storage of energy (in the form of lipids and glycogen), protein synthesis, site of gluconeogenesis and deamination of aminoacids and detoxification and inactivation of toxic substances.

4.2.4.3.4 Pancreas

4.2.4.3.4.1General description

The pancreas is another of the accessory glands in the digestive systems .The pancreas is both an exocrine and endocrine gland (Rønnestad *et al.*, 2007). As an endocrine gland the pancreas secretes the hormones (glucagon and insulin) and as an exocrine gland, the pancreas secretes digestive enzymes. The exocrine pancreas is usually diffuse and consists of scattered acini in the mesenteries and within the liver surrounding branches of the hepatic portal vein. A pancreatic canal opens into the intestine next to the common bile duct. The pyramidal exocrine cells secrete their products into the lumen of the acini. Their nucleus is basal and spherical; the cytoplasm is basophilic except at the apex where numerous acidophilic granules can be found. These granules are zymogen granules containing the digestive proenzymes. In terms of function as an exocrine gland the pancreas secretes the following proteases namely trypsin, chymotrypsin, carboxypeptidase and elastase all of which are stored and secreted as

proenzymes and activated in the intestinal lumen, amylase, lipases and chitinases in some species (Rønnestad & Morais, 2008).

4.2.5 Classes of digestive enzymes

Enzymes are biological catalysts which are involved in chemical reactions in organismal systems (Kim & Dewapriya2014; Rao, *et al.*1998).Digestiveenzymesin fish can be divided into the broadly into the following classes: proteases, lipases, amylases,cellulases and chitinases (Fish1960; Rust 2003).

Koolman & Roehm (2005) define enzymes as proteins specialised in catalysis of organic reactions that operate in a highly specific manner on substrates using specific active sites. All digestive enzymes of animals are classified based on the same criteria applied to other protein classifications (Haard & Simpson2000).Fish possess a wide variety of digestive enzymes, which have been investigated in a number of studies and a summary of their general properties is presented in the form of Tables 6.1, 6.2.and 6.3, below according to Rust (2003).

4.2.5.1 Proteases

Proteins are the main organiccomponentin fish tissue, comprising up to 65–75% of the total dry-weight (Wilson 2003). Fish consume protein to obtain amino acids. A regular intake of protein or amino acids is required because amino acids are used continually by the fish, either to build new proteins during growth and reproduction or for maintenance. Inadequate protein in the diet results in a reduction or cessation of growth and a loss of weight due to withdrawal of protein from less vital tissues to maintain the functions of more vital tissues. Fish do not have a true protein requirement but have a requirement for a balanced mixture of essential / indispensable and nonessential / dispensable amino acids (Anderson, *et al.*1993).Size and age, water temperature, and maintenance requirements affect the protein requirements of fishes (Wilson 2003) and these protein requirements are met through ingestion and digestion.

The process of digestion is a co-ordinated combination of physical, chemical, and enzymatic activities that begins as soon as food is taken into the mouth and ends when faeces are excreted from the anus. The development of species-specific off-the-shelf micro particulate diets for small larval fish is one of the most important nutritional technologies needed by aquaculture. A key part of the problem for most species, however, is the mismatch between the current technology being used to

make micro particles and knowledge of the functional development of sensory and digestive systems of larval fish (Rust,2003)

Proteases are enzymes that breakdown peptide bondsandareclassified into two subclasses, exopeptidases and endopeptidases, depending on where the reaction takes place in the polypeptide substrate (Sternlicht & Werb 2001).Endopeptidases are responsible for the cleavage of polypeptide chains at peptide bonds of non-terminal amino acids, whereas exopeptidases hydrolyse one amino acid from the N-terminus (amino peptidases) or the C-terminus (carboxypeptidases) (Klomklao 2008). In addition, based on the nature of the catalytic site, proteases are classified into four main groups: acid or aspartate proteinases, serine proteinases, cysteine proteinases, and metallo-proteinases. (Kim &Dewapriya. 2014). Alkaline proteases are suggested to be more stable at different pH values, because they comprise a greater number of isoforms (trypsin, chymotrypsin, L-aminopeptidase and carboxypeptidase (Martínez-Cárdenas, Álvarez-González, Hernández-Almeida *et al.* 2017).Proteases are often classified according to their substrate specificity, their response to inhibitors or by their mode of catalysis (Simpson,2000). The EC number of the International Union of Biochemists classifies proteolytic enzymes intofour groups namely serine, cysteine, aspartic and metallo proteases with each based ona distinct catalytic group involved in the reaction (Rao *et al.*1998).

However, irrespective of the source, proteases can be classified on the basis of their similarity to previously well-characterised proteases, as trypsin-like, chymotrypsin-like, chymosin-like or cathepsin-like (Klomklao,2008). They may also be classified on the basis of their sensitivity to pH, including acid, neutral or alkaline proteases.

The current chapter examines 4 named enzymes comprising 3 proteases and 1 chitinase, this following from knowledge of key enzymes in fish, the time-frame available for investigation and the need to work initially in the absence of information on the *C.gariepinus* transcriptome.

Table 4-1. The general characteristics of endopeptidase digestive enzymes in fish (adapted from Hardy and Halver 2003 under Nutritional Physiology by Rust 2003 as Table 7.1)

Enzyme	Synthesis Location	Action Location	Optimum pH	Substrate actionAction	Product (s)
Collagenase	Exocrine Pancreas	Lumen of intestine and pyloric caeca	Neutral	Endoprotease activated by Trypsin that primarily cuts collagen	Peptides
Aminopeptidase (several types, acidic and neutral)	Stomach, exocrine pancreas, and enterocytes	Stomach lumen; lumen and brush border of intestine and pyloric caeca	Acid or neutral	Exopeptidases cleaves amino acid from the amino end of peptide chain	Small peptides and free amino acids
Leucine amino- peptidase (example of neutral amino- peptidase)	Enterocytes	Membrane linked in brush border	Neutral	Exopeptidases cleaves leucine from the amino end of peptide chain	Smaller peptides and free Leucine
Carboxypeptidases	Exocrine pancreas and enterocytes	Lumen and brush border of intestine and pyloric caeca	Neutral	Exopeptidases cleaves amino acid from the carboxyl end of peptide chain	Smaller peptides and free amino acids
Non pancreatic lipase or gastric lipase	Gastric glands of stomach Mucosa	Lumen of stomach	Acid	Primarily triacylglycerol lipase of gastric origin	Triacylglycerol lipase and Phospholipase
Pancreatic lipase	Exocrine pancreas	Lumen of intestine and pyloric caeca	Neutral	Includes multiple lipases of pancreatic origin, often bile salt activatedActivated	Triacylglycerol lipase and Phospholipase
Triacylglycerol lipase	Stomach, exocrine pancreas, and enterocytes	Stomach lumen; lumen and brush border of intestine and pyloric caeca	Acid or neutral	Cleaves fatty acids from the 1 and 3 positions of triacylglycerol sometimes with a co lipase,	2-Monoglycerides and free fatty acids

Enzyme	Synthesis	Action	optimum	Substrate	Product (s)

	Location	Location	pH	Action	
Pepsin	Oxyntic peptic cells in the Stomach Mucosa	Stomach lumen	Acid	Nonspecific Endoprotease activated by exposure to acid	Peptides
Trypsin	Exocrine Pancreas	Lumen of intestine and pyloric caeca	Neutral	Endoprotease Activated by enterokinase. Catalyses hydrolysis of peptide bonds next to amino acids with basic side chains	Peptides
Chymotrypsin	Exocrine Pancreas	Lumen of intestine and pyloric caeca	Neutral	Endoprotease Activated by trypsin. Catalyses hydrolysis of peptide bonds next to amino acids with hydrophobic side chains	Peptides
Elastase	Exocrine Pancreas	Lumen of intestine and pyloric caeca	Neutral	Endoprotease activated by trypsin that catalyses hydrolysis of peptide bonds next to glycine or alanine	Peptides

Table 4-1. The general characteristics of exopeptidase digestive enzymes in fish (adapted from Hardy and Halver 2003 under Nutritional Physiology by Rust 2003

Table 4-2. The general characteristics of lipid and carbohydrate digestive enzymes in fish (adapted from Hardy and Halver 2003 under Nutritional Physiology by Rust 2003as table 7.1)

Enzyme	**Synthesis Location**	**Action Location**	**optimum pH**	**Substrate action**	**Product (s)**
Monoglyceride lipase	Enterocytes	Brush border of intestine and pyloric caeca	Neutral	Cleaves fatty acid from 2-monoglycerides	Fatty acids and Glycerol
Phospholipase	Exocrine Pancreas	Lumen of intestine and pyloric caeca	Neutral	Cleaves fatty acids from phospholipids	Fatty acids and Lysophospholipids
Wax ester hydrolase	Exocrine Pancreas	Lumen of intestine and pyloric caeca	Neutral	Hydrolyses wax esters	Fatty alcohols
Amylase	Enterocytes, gut Microflora	Membrane linked in brush border or associated with gut flora	Neutral	Hydrolyses starch	Polysaccharides and monosaccharide's (sugars)
Cellulase	Gut microflora; Endogenous production has not been confirmed	Lumen of intestine	Neutral	Hydrolyses cellulose	Glucose
Chitinase	Stomach and exocrine pancreas	Lumen of stomach, intestine, and pyloric caeca	Acid or neutral	Hydrolyses N-acetyl glucosamine (chitin)	Polysaccharides and Monosaccharides (sugars)

4.2.5.2 Protease classes

4.2.5.2.1.1 Alkaline proteases trypsin and chymotrypsin

Trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) are members of the serine protease family of enzymes, which are commonly found in secretions from the pancreas. Trypsin catalyses the hydrolysis of peptide bonds, breaking down proteins into smaller peptides, which could be further degraded by other proteases and is known to be a sensitive key enzyme supporting growth at both the genetic and environmental level (Rungruangsak-Torrissen*et al.*2006). Chymotrypsin has the ability to break down both proteins and polypeptides, but its catalytic power is relatively low (Klomklao2008). Chymotrypsin is important when growth is restricted or lowered (Rungruangsak-Torrissen *et al.*2006).Trypsinandchymotrypsinhave been shown to decline in their specific activity after the onset of exogenous feeding, thought to be indicativeoftheimportance of alkaline protease enzymes in the cleavage of yolk proteins during the endogenous feeding phaseand the replacement of the larval alkaline-type digestion by a juvenile-type acid digestion (Babaei, *et al.*2011).

Trypsin and trypsin-like proteolytic enzymes have been purified and characterized from the viscera of several fish species. Even though trypsin from marine fish species resembles mammalian trypsin with respect to its physical characteristics, some reports have shown that fish trypsin is more stable under harsh conditions (high temperature and pH) and in the presence of surfactants or oxidizing agents. Generally, the optimum pH of marine-derived trypsin varies from 6 to 11.5, while their optimum temperature for hydrolysis ranges from 35 °C to 65 °C) (Freitas-Júnior, *et al.*2012). Trypsin is considered to be one of the key enzymes associated with growth and feed utilization, as has been observed by Murray *et al.* (2004) and Rungruangsak-Torrissen*et al.* (2006). As well as playing a major role in protein digestion processes, it has also been observed to act as the sensitive key protease under conditions of food deprivation (Einarsson, Davies & Talbot1996; Lehnert & Johnson2002; Rungruangsak-Torrissen *et al.* 2006). Studies of expression of trypsin and chymotrypsin in a number of species, including Atlantic salmon *Salmo salar* L. (Rungruangsak-Torrissen *et al* 2006), suggest that there is interaction between trypsin and chymotrypsin in terms of activity, with trypsin being important in the activation of chymotrypsin (Sunde, Taranger & Rungruangsak-Torrissen2001; Zhou, *et al.*2011) since the trypsin specific activity and protease

ratioof trypsin to chymotrypsin (T/C) aresignificant measures in the digestion processassociating feed conversion efficiency with the growth rate (Rungruangsak-Torrissen et al. 2006).In the absenceof feeding, as is often the case for wildfish, the ratio of trypsin to chymotrypsin value could be used to forecast the present and future growth since it is difficult to determine individal food consumption (Rungruangsak-Torrissen & Male2000; Rungruangsak-Torrissen *et al.*2006).

Chymotrypsin

Definition

Chymotrypsin (EC) Number3.4.21.1 is a serine type, alkaline protease and an endopeptidase enzyme produced in the pancreas and secreted in an inactive form into the lumen of the intestine (Zhou, *et al.*2011). Cleavage of the small peptide chain transforms the inactive zymogen (chymotrypsinogen) to the active form of the enzyme. Chymotrypsin selectively hydrolyses peptide bonds on the carboxyl side of the aromatic side chains of tyrosine, tryptophan, and phenylalanine and of large hydrophobic residues such as methionine (Sunde *et al.*2001)

Chymotrypsin is also sensitive to temperature and pH changes. Additionally, chymotrypsin requires Ca^{2+} ions for structural stability (Applebaum & Holt2003; Applebaum *et al.*2001; Cahu *et al.*2004; Zhou *et al.*2011). In its inactive form as chymotrypsinogen, chymotrypsin can be cleaved into trypsin with the optimum pH activity of chymotrypsin in fish being 8 (Zhou *et al.*2011)

Action of chymotrypsin in different species

Studies in red drum, *Scienops ocellatus*, larvae concerning the temporal evolution of chymotrypsin activity during early ontogeny of laboratory reared larvae, indicated that chymotrypsin activity occurred before the commencement of external feeding. This points to the fact that chymotrypsinexpression is elicited by genetic means or other means but not diet per se (Applebaum *et al.*2001).Chymotrypsinhas been suggested to be a nutritional indicatoralthoughit is suggested that trypsin is in fact a better measurebecauseit plays a role in the activation of other alkaline proteasesin times of starvation (Cara, *et al.*2007; Rungruangsak-Torrissen, *et al.*2006).

Although studies on chymotrypsin activity in freshwater species are few when compared to their counterparts in the marine environment, a recent study has been conducted involving four freshwater species with varying eating

habits:grasscarp,*CtenopharyngodonIdella,* a herbivorous species,silver catfish, *Rhamdiaquelen,* an omnivorous species,Piava,*Leporinusobtusidens,*an omnivorous species and Traira, *Hoplias malabaricus*,a carnivorous species (Gioda, *et al.* 2017). Here, chymotrypsin activity was most evident in the stomach and intestines of *C.idella* and least evident in*Hmalabaricus* (Gioda, *et al.*2017).This may be an indicator that it plays a more important role in herbivores than in omnivores and carnivores.This can be explained once more by the fact that chymotrypsin is important in times of starvation or food scarcity while trypsin protease is more responsive to growth.

4.2.5.2.1.1.1 Trypsin /trypsinogen

Definition

Trypsin,EC Number: 3.4.21.4, is a key proteolytic enzyme influencing the digestive capacity of fish larvae (Ruan *et al.*2010).It is one of the main digestive proteinases detected in the pyloric caeca and intestine of fish (Cai, *et al.*2011; Freitas-Júnior, *et al.*2012), however, it is produced in the pancreas as a proenzyme and kept as trypsinogen in the secretory granules where it can be detected (Gamboa-Delgado, *et al.*2011).Trypsin is a member of a large family of serine proteinases which specifically hydrolyse proteins and peptides at the carboxyl group of arginine and lysine residues and play a critical role in protein digestion.Similar to other proteins, trypsins are synthesized as inactive precursors (zymogens) and subsequently converted to their physiologically active forms by selective enzymatic cleavage of peptide bonds. As ectothermic animals, the survival of fish requires the adaptation of their digestive enzymes to the temperatures of their normal habitats (Cai *et al.*2011).Vertebrate trypsins have been categorised into three forms namely group 1, 11,111.and may be cationic,anionic or psychrophilic enzymes (Manchado, *et al.*2008).

Action of trypsin/trypsinogen in different fish species

Trypsinogen expression during larval development varies in terms of when the onset occursandin terms of days post hatch from species to species (Kurokawa, *et al.*2002). Several studies on the activity of trypsin and chymotrypsin have been conducted.It has been observed that in Senegalese sole, *Soleasenegalensis,*larvae, trypsin activityincreased from 3-4 Dph, while inbullseye puffer, *Sphoeroidesannulatus* (Jenyns, 1842) maternal trypsin mRNA could contribute to the presence of trypsin as early as in the fertilized egg, although

activity was also detected much later at 2Dph when the pancreas and intestines were present,thiscoinciding with a gradual increase in activity and expression (García-Gasca, *et al.*,2006). In many species of fish larvae including rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792) (*Mystus nemerus (Cuv.and Val*.), Green catfish (Srichanun, *et al.*2012),Atlantic bluefin tuna (ABFT) *Thunnus thynnus, (Linnaeus, 1758)* (Mazurais, *et al.*2015), (*Dicentrarchus labrax*) (sea bass) (Tillner, *et al.*2014) Various studies in different fish species includingcarnivorous and herbivorous species have come up with the conclusion that trypsin expression is affected by ontogeny, diet and phylogeny and is genetically programmed,showingincreasingexpression with ontogeny in both the carnivorous and herbivorous species but, in response to high-protein artificial diets, only in carnivores (Gawlicka& Horn2005). This is also evidenced in other speciesincluding Senegalese sole (Gamboa-Delgado, *et al.*2011) where different proteolytic activities of serine proteases are key in the early stages of larval development contributing 75-80 % aminoacids (Moyano, *et al.*1996)and with trypsin and chymotrypsin being the major proteins involved in food conversion efficiency, growth and nutritional condition in larvae but this being highly influenced by age of fish and nutrient composition (Cahu& Infante2001).

In rainbow trout,trypsin has an important function in food digestion and absorption during first feeding withevidence for a range of digestive enzymes, including gastric (pepsin), pancreatic (trypsin, chymotrypsin, α-amylase and lipase) and intestinal (alkaline phosphatase) enzymes being available as early as 1DPh (Golchinfar, *et al.*2011; Zhang, *et al.*2015).

4.2.5.2.2 Acidic proteases

4.2.5.2.2.1 Pepsin

Definition

Pepsin (EC 3.4.23.X) is an enzyme of the aspartic family with a Molecular weight of Da 36 (Zhao *et al* 2011), which plays a major role in protein digestion and is synthesized and secreted by cells in the gastric mucosa. Pepsin, belonging to the aspartic endopeptidase group, is one of three principal protein-degrading enzymes found in the fish stomach. The catalytic activity of pepsin greatly depends on the pH, with pepsin being active and most stable under acidic conditions. Based on its optimal pH, fish-derived pepsin is categorized into two groups, pepsin I and pepsin II. Several pepsin and pepsin-like enzymes have been isolated from fish, including cold- and warm water fish (Kim & Dewapriya 2014). Pepsin is an aspartyl protease and as such it depends on the aspartic acid residue present in its active site for catalytic activity. It is one of the endopeptidases which cleave peptide bonds of proteins at the terminal side of aromatic amino acids including tyrosine and tryptophan, breaking the long polypeptide chains into smaller peptides and free amino acids (Zhao *et al* 2011). As essential digestive enzymes, pepsins are most importantly involved in the digestion of proteins in fish diet. Thus, a study of the role of these proteinases in larval catfish can be helpful to the manufacturing of fish feeds and the development of aquaculture.

Pepsin is derived from pepsinogens which are its precursors. Pepsinogens (PGs), the precursor of the aspartic proteinase pepsin, are normally present in the gastric mucosa of animals. Pepsinogen is the precursor of pepsin and is composed of five hypo types: pepsinogen A (PGA) an active precursor for pepsin A, pepsinogen B (PGB) an active precursor for Pepsin B, pepsinogen C (PGC) an active precursor for gastricsin, pepsinogen F (PGF) an active precursor for Pepsin F and Prochymosin an active precursor for chymosinogen (Kageyama 2002, Olivier*aet al* 2014). Fish are thus far only recognised to synthesize up to 5 types of pepsinogens, normally employing transformations to Pepsin 1 or Aand Pepsin 11 or C. After autolysis of the pepsinogen, the active pepsins are approximately 35kD, this varying according to species (Oliveira *et al* 2014).

4.2.5.3 Chinolytic enzymes

4.2.5.3.1 Definition of Chitinases

Chitinases (EC 3.2.1.14) are enzymes that randomly hydrolyse the β-1, 4 glycosidic bonds of chitin and are important for the enzymatic production of physiologically active GlcNAcn and GlcNAc. They are widely distributed in various living organisms includingfish,microbes,insects, crustaceans *etc*. (Fujitani, *et al.*2014; Ikeda, Kondo & Matsumiya2013; Karthik, Binod & Pandey2017; Ogino, *et al.*2014; Suzuki, *et al.*2014).

4.2.5.3.2 Roles played by chitinases

Chitinases play important roles in the life of vertebrates including fish and humans. They have been identified as being beneficial as defence mechanisms warding off microbes and helping in breaking down of chitin-containing organisms as is the case for fish feeding on insect- /crustacean-containing diets (Gooday1999; Ikeda, Miyauchi & Matsumiya 2012; Matsumiya, *et al.*2006).

Although most studies on chitinases have been conducted on microorganisms, chitinases havealsobeen investigated in many other species, including mammals (Bussink, Speijer, Aerts *et al.*2007; Ohno, Kimura, Miyazaki *et al.*2016) fish (Fänge, Lundblad, Lind *et al.*1979; Gutowska, Drazen & Robison2004) molluscs, insects, plants and fungi (Hamid *et al.*2013).However, the physiological role of chitinases varies in these organisms, in terms of the mechanism and efficiency of chitinase degradation and the substrate specificities, which have been reported to vary. For instance, acidic mammalian chitinase expressed in the stomach of mammals (Kakizaki *et al*. 2015) and chitotriosidase produced by macrophages are thought to function in the digestion and absorption of food and defence against pathogens. In addition, chitinase has been detected at the onset of asthma and allergies, suggesting its involvement in diseases. However, the details of the distribution of chitinases in the body of fish are not fully established.

Chitinolytic enzyme activities vary greatly between fish species and with the various methodologies used to examine them (Lindsay1984). The primary function of chitinolytic enzymes is still debatable and likely varies between species. Along the alimentary tract of fishes, chitinolytic enzymes are believed to have various roles (Brzezinska, Jankiewicz, Burkowska *et al.* 2014; Khoushab & Yamabhai2010).Chitinases are primarily associated with the stomach where they

disrupt exoskeletons allowing other digestive enzymes to access nutrient-rich inner tissues (Gutowska *et al.*2004; Lindsay1984; Lindsay, *et al.*1984; Terahara, Ikeda, Noritake *et al.*2009)). This has been observed in flounder where larvae acquire the ability to synthesize and secrete Chitinase enzymes at first feeding. (Wu et al 2011). Chitinases have also been found in the intestines, where they may aid in removal of fragment blockage (Lindsay1984) Chitobiases are mostly associated with the intestine, and pyloric caeca, where they further break down chitin into single units of N-acetyl-glucosamine (NAG) and could play a role in nutritional breakdown of chitin-containing diets.

4.2.5.3.3 Classification of chitinases

Chitinases are classified as endochitinases (EC 3.2.1.14), which randomly hydrolyse the chitin polymer to generate soluble low-molecular-weight polymers, and exochitinases, which consist of two categories, chitobiosidase (b-Dacetylglucosaminidase, EC 3.2.1.30; now included in EC 3.2.1.52), which catalyses the progressive release of diacetylchitobiose starting at the nonreducing end of the chitin, and b-N-acetylglucosaminidase (EC 3.2.1.52) (Karthik *et al.*2017).

4.2.5.3.4 Action of chitinases in different fish species

Chitinases in fish are hypothesized to be exogenous, originating from feed such as crustaceans *e.g*. zooplankton,artemia, as evidenced from molecular studies (Krogdahl, Hemre& Mommsen2005). It has been revealed that chitinase enzyme reactions do occur in the stomach of fish (Ikeda, Kakizaki & Matsumiya2017).Theyare not onlyrestricted to the stomach but are also present in the area of the pyloric sphincter, intestine, liver, spleen, kidney, ovary, testis, liverspleen, kidney, heartand gills (Gao, *et al.*2017; Ikeda *et al.*2013; Kakizaki, Ikeda, Fukushima *et al.*2015) dependant on species and feeding habits.

Earlier studies on chitinases have intimated that Lindsay 1986 and are known to play vital a role in breaking down chitin taken in with the feed. However later studies with improved techniques including cloning of the genes have indicated that bony fish have an assortment of chitinases present in the stomach while the sarcopterygian coelacanth,*Latimeriachalumnae*,andthe cartilaginous blue shark, *Prionace glauca*, have just one chitinase enzyme playing various roles including breakdown of food and fighting against microbes (Lindsay1986).

Different fish species show various forms of chitinase for example turbot, *Scophthalmusmaximus,*have been found to possess chitinases occurring in three formsnamelyCHIT1, CHIT2 and CHIT3 where theyseem to play a role in fighting microbes (Gao *et al.*2017). In the pike silverside, *Chirostomaestor*, a stomachless fish, both chitotriosidase and chitobiosidase are present,interacting with NAGaseactivityto complete the chinolytic activity (Pohls*et al.*2016). In zebrafish, 6 chitinase enzymes have been identified, namely CHIA.1, CHIA.2, CHIA.3 and CHIA.4, CHIA.5, CHIA.6, each with a distinct pattern of expression with CHIA 4 coming from the maternal DNA and being involved in immunity (Koch, Stougaard & Spaink2014) and CHIA.3 has been implicated in inborn immunity and nutrient degradation (Teng, *et al.*2014). In the threelinegrunt,*Parapristipomatrilineatum*,two forms occur in the stomach namely acidic fish chitinase-1 (AFCase-1) and acidic fish chitinase-2 (AFCase-2) (Ikeda *et al.*2013), which differ from the acidic mammalian chitinase (AMCase) group, InAtlantic cod, *Gadusmorhua*, chitinase and chitobiase are present (Lindsay & Gooday1985)

In the case of Japanese flounder, *Paralichthysolivaceus,*cloning resulted in the discovery of three chitinases, namely (fChi1, fChi2 and fChi3)of which the fChi1 and fChi2 mRNAs were primarily expressed in the gastric glands of the stomach. On the other hand, expression of fChi3 occurred in spleen, pancreas, stomach, intestine, liver, kidney and gonads of adult flounder (Kurokawa, Uji & Suzuki 2004).

In Arctic charr, *Salvelinus alpinus,*substantial chitinase activity is present,mainly in the form of endo- and exo-chitinase iso-forms (Abro, *et al.* 2014). This is an essential attribute and a necessary requisite for efficient utilisation of a diet containing high chitin. Absence of chitinases would have anadverse effect on digestibility, nutrient utilisation and growth performance of fish fed chitin-rich feed.

From the above discussion, the varietyof chitinases present in various fishis an indication that theyhave an excellent chitin-degrading enzymatic system that efficiently breaks down chitin taken in with their diet.

4.2.5.4 Digestive Enzymes produced by the stomach in fish

Fish exhibit different abilities in the usage of nutrients (Hlophe *et al*., 2014) .This is based on the fact that they have different feeding niches,including the fact as

some are omnivores others are carnivores and still others are omnivores .This has underlying availability of the digestive enzymes required for digestion to effectively take place . Various studies have indicated that the enzymes produced in the stomach include proteases, lipases and cellulases (Xiong et al., 2011).

4.2.5.5 Characteristics of the Gastric Secretions

Little is known about gastric secretion in fish, although there is consensus that the presence of only one type of secretory cell in fish which stains positively for indicators of pepsinogen (pepsin precursor) cells.

Other gastric enzymes have been proposed, but not firmly identified. Chitinolytic activity with an optimum at pH 4.5 was claimed for the stomach of *Salmo irideus*, but in most cases is probably from exogenous sources. A notion exists that if fish are like higher vertebrates, then the stomach wall also produces the hormone gastrin which stimulates gastric secretion. A lipase may also be present (Smith in FAO 1980) Chitin is the second most abundant organic compound, next to cellulose, on earth (Danulat&Kausch 1984). It is acted on by the enzyme chitinase which occurs in the digestive tract of many animal species including fish (Fänge, Lundbald&Slettengren 1979). Gastrointestinal chitinases are thought to have multiple roles in digestion including breaking down of the exoskeletons of prey allowing other digestive enzymes to gain entry to the soft inner tissues (Lindsay 1984, Gatswoka 2002).

4.2.5.6 Pepsin /pepsinogen ATP proton pump.

Pepsin is an enzyme of the aspartic family with a Molecular weight Da 36 (Zhao et al 2011) the major role played is digestion of proteins. They occur initially as pepsinogens which are precursors of pepsin. According to Yufera*et al* 2012, two different modes for regulation of stomach acid secretion have been described. Some species exhibit a continuous acid secretion maintaining a low gastric pH during fasting and others, including some teleosts, maintain a neutral gastric pH during fasting (Caruso *et al*., 2016) while the hydrochloric acid is released only after the ingestion of a meal. Fish exhibit 4 different types of pepsinogen namely (PGS 1, 11,111, IV and V) in (Zhao et al 2011)

The appearance of functionally developed gastric glands is commonly considered as the transition from the larval to juvenile stage in fish as it means there is the switch over from the less efficient alkaline digestion to more efficient acid digestion

characteristic of adult fish (Darias et al 2007). Acid digestion takes place by the action of pepsin activity and hydrochloric acid, both secreted by the proton pump.

4.2.5.6.1.1 Digestive Enzymes produced by the pancreas in fish

Pancreatic juice is composed of two secretory products critical to proper digestion: digestive enzymes and bicarbonate. The enzymes are synthesized and secreted from the exocrine acinar cells, whereas bicarbonate is secreted from the epithelial cells lining small pancreatic ducts.The two major pancreatic proteases are trypsin and chymotrypsin, which are synthesized and packaged into secretory vesicles as the (Yoshinakaet al. 1984) inactive proenzymes trypsinogen and chymotrypsinogen. Once trypsin is formed it activates chymotrypsinogen, as well as additional molecules of trypsinogen (Falcón-Hidalgo *et al*. 2011; Mata-Sotres et al., 2016a; Mata-Sotres et al., 2016b; Giyatmi&Irianto 2017).

Trypsin is one of the key enzymes in growth and feed utilization as has been observed by (Rungruangsak-Torrissen et al., 2006; Thongprajukaew et al., 2011; Galaviz et al., 2012; Galaviz et al., 2013; Galaviz et al., 2015).It plays a major role in protein digestion processes. It has been observed to act as the sensitive key protease under conditions of food deprivation.

Chymotrypsin is an endopeptidase enzyme found in the pancreatic tissues of vertebrates (Zhou et al., 2011).In its inactive form as chymotrypsinogen, Chymotrypsin can be cleaved into trypsin The optimum pH activity of Chymotrypsin in fish has been reported as 8 (Zhou et al. 2011).Trypsin is an important protease that is important in activate the action of chymotrypsin (Rungruangsak- Torrissen et al 2006)

Trypsin and chymotrypsin, like most proteolytic enzymes, are synthesized as inactive zymogen precursors (trypsinogen and chymotrypsinogen) to prevent unwanted destruction of cellular proteins, and to regulate when and where enzyme activity occurs. The inactive zymogens are secreted into the duodenum, where they travel the small and large intestines prior to excretion. Zymogens also enter the bloodstream, where they can be detected in serum prior to excretion in urine Zymogens are converted to the mature, active enzyme by proteolysis to split off a pro-peptide, either in a sub cellular compartment or in an extracellular space where they are required for digestion.

Trypsin and chymotrypsin are structurally very similar, although they recognise different substrates. Trypsin acts on lysine and arginine residues, while

chymotrypsin acts on large hydrophobic residues such as tryptophan, tyrosine and phenylalanine, both with extraordinary catalytic efficiency. Both enzymes have a catalytic triad of serine, histidine and aspartate within the S1 binding pocket; although the hydrophobic nature of this pocket varies between the two, as do other structural interactions beyond the S1 pocket.

Trypsin and chymotrypsin digest proteins into peptides and peptides into smaller peptides, but they cannot digest proteins and peptides to single amino acids. Some of the other proteases from the pancreas, for instance carboxypeptidases, have that ability.

In bony fish larvae the pancreas is usually a solid and definite organ also known as the pancreas compactum. The adult pancreas is usually diffuse and dispersed along the gut and seems to develop at the juvenile stage in most fish species.

In larval fish the exocrine pancreas is an acinar gland and based on its morphology it appears to be functional prior to the onset of exogenous feeding as enzymes including trypsin and chymotrypsin have been seen in various studies on fish larvae have resulted first expression in either biochemical methods, histologically the pancreas is present a few days post hatch and also more recently in molecular studies (Mir *et al.*, 2018; Rungruangsak-Torrissen *et al.*, 2006; Savoie *et al.*, 2011; Zhou *et al.*, 2007)

The development of the exocrine pancreas in larva fish in terms of, histology biochemical and some extent molecular has been extensively studied, as exemplified in a study of *(Pleuronectesamericanus)* Winter flounder where the ontogeny of the pancreas was seen from hatching, starting as a compact structure located dorsal and slightly posterior to the liver. As the larval fish turned into juvenile, the pancreas became diffuse, spreading throughout the mesentery surrounding the stomach, the upper intestine and the pyloric caeca (Murray *et al.*, 2004).

4.3 Materials and Methods

4.3.1 Fish Samples for histology

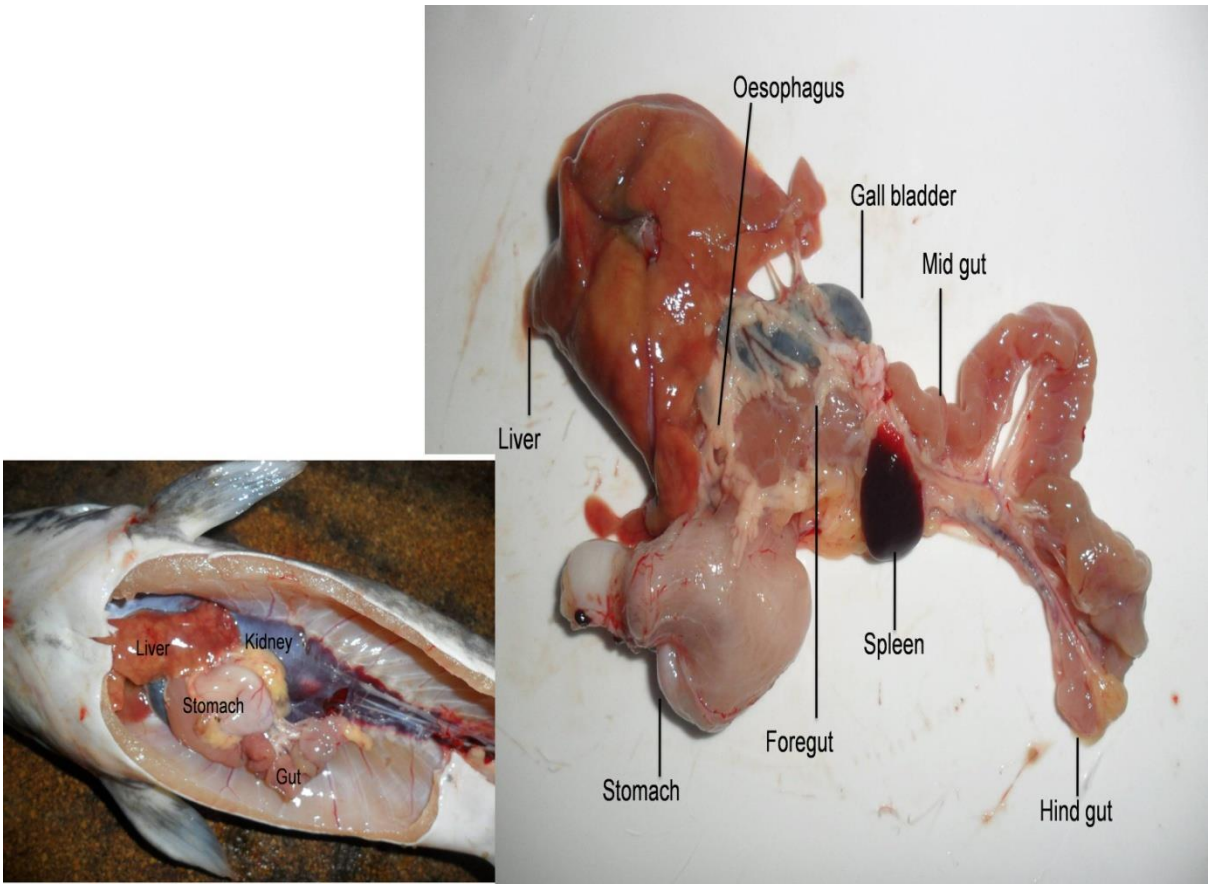


Figure 4-1 showing the open abdominal cavity of adult catfish (left) Dissected digestive system (right).

Fish samples for histology were collected fresh at same time as molecular study samples. The fish were euthanised by an overdose of anaesthetic followed by destruction of the brain. For adult *C.gariepinus*the gut was revealed and dissected out using a pair of scissors and sterile scalpel blade (Solmedia SCL220No.22, UK) and samples of the digestive system and accessory organs (oesophagus, stomach, foregut, midgut, hindgut, spleen, liver, pancreas, gallbladder) were sampled .Whole larvae 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 14, 21, 28 Dph were initially collected and fixed in 10% neutral buffered formalin (NBF).The tissues were then processed as follows.

The tissues were trimmed to a sizeable size using a scalpel blade and were placed in white cassettes and depending on the size of the tissue, small samples (smaller than the size of the pores in the cassette) in this case the larvae of catfish days 0-4 Dph were wrapped in biopsy tissue paper prior to being placed in the cassette. The cassetted samples were then placed in tap water prior to loading to the processor to prevent drying out.

The cassetted samples were then loaded into baskets and placed in the Excelsior Processor overnight where the tissues were impregnated with paraffin wax which is important as a support for the tissue during section cutting. During this stage of processing the first stage was dehydration which involved the removal of water by immersion in a graded series of alcohols; this was followed by clearing which relates to the appearance of the tissues after they have been treated by xylene or chloroform.

The next step was blocking of the tissue samples and involved the removal of the cassettes from the tissue processor and being placed in the auxiliary wax bath on the Leica Histoembedder (model Jung Leica, Germany).The metal lid was removed and the appropriate size of base mould was selected which provided an adequate margin of wax around the tissue to support it during section cutting. The base mould was half filled with molten wax by pushing back gently on the dispenser handle. The base mould was then placed onto the edge of the Histoembedder and the tissues were positioned into the solidifying wax using warmed forceps. Positioning of the tissue depended on the type of section required. For series sections for larval fish, only one specimen was placed per cassette with the dorsal fin upper most and adult catfish digestive system the tissues embedded so that they were at right angles to the blade. The empty cassette was placed on top of the base mould and topped up with wax and placed on the cold plate which allowed the wax to solidify and the sample was held in position. Once solidified (approx. 5min) the block was removed from the base mould.

This was followed by the process of microtomy that involved initially trimming the blocks in which the surface layer of wax was first removed to expose the complete surface of the tissues. This was carried out on the microtome (Leica BioCut 2035 Microtome, USA using an old blade (one which has been used to cut sections previously).The rate of advancement of the block towards the knife was

determined by depressing the manual trimming device to facilitate a 20micron advance section on each rotation of the handwheel.

This was followed by section cutting; this step was carried out by placing the trimmed blocks face down in vessel containing distilled water and soaking for approximately 30min prior to cutting. Blocks were removed and blotted dry then cooled face down on the cold-plate (RA Lamb, Germany) for 5-10 min prior to sectioning. After this time they were turned face up. For serial sections the blocks were trimmed into a trapezium prior to sectioning.

The block was clamped into the block holder and the face of the block was brought as close as possible to the edge of the blade.

The hand wheel was rotated facilitating a (5) five micron advance on each rotation. When a 'ribbon' of sections is obtained it is removed using forceps and floated out onto the surface of the warm water in the water bath. The best section was selected and picked up on a clean Standard 76x26mm Plain glass slide (Solmedia MSS11012,UK) for the gut of adult catfish and, Plus+Frost positive charged slides with white coated end and clipped corners (Solmedia MSS51012WH,UK) for serial sections of larval catfish. Excess water was wiped from around the section using a soft tissue. The reference number was marked onto the frosted end of the slide using pencil, and then the slide was rested section side down against the runners on the hotplate (Model Raymond A Lamb) Slides were then placed into slide racks with the sections all facing in the same direction. Racked slides were then dried in an oven (Windsor Incubator 32-70 UK) at 60°C for at least one hour before staining.

In order to examine sections effectively under the microscope they were stained using Haematoxylin and Eosin stain. There are many different varieties, however the technique favoured in the Institute combined an alum haematoxylin called Haematoxylin 'Z' (similar to Mayer's) with a counter stain of 8 part 1% Eosin (aqueous) to 1 parts Putt's eosin. The haematoxylin component stained the cell nuclei blue to blue/black, whilst the eosin counter stain demonstrated the general histological architecture. Eosin distinguished between the cytoplasm of different types of cells, and between different types of connective tissue fibres, by staining in varying shades of orange, pink and red.

The staining process involved the following steps; Xylene (dewax 1) for 3 min, Xylene (dewax 2) for 2 min, Absolute Alcohol for 2 min, Methylated spirit for 1and

1/2 min, washed in Tap water for 30 seconds, Haematoxylin ‘Z’ for 5 min, washed in Tap water for 30 seconds,1% Acid alcohol for 3 quick dips, washed in Tap water for 3 seconds, Scott’s tap water substitute for 1 minute, washed in Tap water for 3 seconds, Eosin for 5 min, washed in Tap water for 30 seconds, Methylated spirit for 30 sec, Absolute alcohol II for 2 min, Absolute alcohol III for 1 min, Xylene (clearing) for 5 min and Xylene (coverslip).

Slides were left in the Xylene (coverslip) to prevent them from dehydration and coverslipped using Pertex mounting medium.

Slides were left to dry in a fume hood for at least 10-15 minutes prior to microscopy and pictography.Microscopy and photomicrography were conducted by use of Olympus BX51 Microscope connected to an Axiocam Carl Zeiss camera (MRc) and image acquisition for Image Analysis, was performed using the Axiovision program (Carl Zeiss, UK).

4.3.2 Fish Samples for in situ-expression hybridisation

4.3.2.1 General Preparation

All glassware and non-sterile plastic ware were baked in foil at 180°C for 6 hours while plasticware were submerged overnight in 0.1N sodium hydroxide and rinsed in nuclease-free dH_2O.in order to make them RNase-free.

4.3.2.2 Probe Production

RNA was isolated from the organs from which key transcripts were expected to be isolated. For chymotrypsin and trypsin the target was the pancreas, whereas for pepsin the oxy-peptic cells of the stomach walls were used for isolation. For gastric chitinase, the target tissue is considered to be the gastric pits of the stomach. Probes were employed against sections of larvae aged days 2-8Dph, this matching the suggested period of appearance of key enzymes. The RNA was quantified using a NanoDrop 1000 and the quality checked by running 200ng on a 1% agarose gel containing ethidium bromide, to a final concentration of 0.05µg mL^{-1}, alongside a 100bp marker.

4.3.2.3 cDNA Synthesis

cDNA was produced as described in Chapter Two,General Materials and Methods.

4.3.2.4 Standard Polymerase Chain Reaction PCR

PCR was set up using cDNA (diluted 1/10 with nuclease-free dH_2O) and primers containing a T7 promoter sequence (Table 4-1 below) and primers for

chymotrypsin, trypsin. pepsin and gastric chitinase in a total reaction volume of 50µL. Reactions were prepared for all four genes of interest, with the following reaction compositions: 25µL of 2X MyTaq HS mix (Bioline), 1.5µL of 10µM Primer-forward, 1.5µL of 10µMPrimer-reverse, 10µL of cDNA diluted tenfold 1/10 and 1Table 4 1 showing the probes prepared in order to perform in situ expression Hybridisation.2µL of RNase free water.

Primers pairs consisted of (A) sense primer + T7 antisense primer and (B) T7 sense primer + antisense primer (Barker 2009). PCR reaction were also set up as (C) using ordinary sense and antisense primers (i.e. without the T7 promoter) to act as a negative controls as seen in Tables 4-4and 4-5below.

Table 4 4 showing the probes prepared in order to perform in situ expression Hybridisation.

Gene	***Primer/probe***
Chymotrypsin_Forward	*[TGGCAGATCTCCCTCCAGTAT]*
Chymotrypsin _Reverse	*[AGATCCCCACCAGTCAGATTTAGT]*
Chymotrypsin_TP7prom_Forward	*[**TAATACGACTCACTATAGGG**TGGCAGATCTCCCTCCAGTAT]*
Chymorypsin_TP7prom_Reverse	*[**TAATACGACTCACTATAGGG**AGATCCCCACCAGTCAGATTTAGT]*
Trypsin_Forward	*[TGCACTATGGCTACCACTTCTGTG]*
Trypsin_Reverse	*[CAGCGGACTTCCTGCATCTCTA*
Trypsin_TP7prom _Forward	*[**TAATACGACTCACTATAGGG**TGCACTATGGCTACCACTTCTGTG]*
Trypsin_TP7prom_Reverse	*[**TAATACGACTCACTATAGGG**CAGCGGACTTCCTGCATCTCTA]*
Gastric chitinase_Forward	*[TTGACTGGCTGAAGAAAAACAACT]*
Gastric chitinase_Reverse	*[ACTGCTGGAGCCACTGCTGCTA]*
Gast chitinase_TP7prom_Forward	*[**TAATACGACTCACTATAGGG**TTGACTGGCTGAAGAAAAACAACT]*
Gastric chitinase_TP7prom_Reverse	*[**TAATACGACTCACTATAGGG**ACTGCTGGAGCCACTGCTGCTA]*
Pepsin Forward	*[TCCAGCACTTTCCAGACCAACAAC]*
Pepsin_ Reverse	*[CATCCTCCAGAGCAAGCAACAGTC]*
Pepsin_TP7prom_Forward	*[**TAATACGACTCACTATAGGG**TCCAGCACTTTCCAGACCAACAAC]*
Pepsin_TP7prom_Reverse	*[**TAATACGACTCACTATAGGG**CATCCTCCAGAGCAAGCAACAGTC]*

Table 4-5 showing the probes prepared in order to perform in situ expression Hybridisation with their respective TMandamplicon size

Gene	Primer/probe	TM [°C]	Amplicon size
Chymotrypsin_Forward	TGGCAGATCTCCCTCCAGTAT	60° C	450
Chymotrypsin _Reverse	AGATCCCCACCAGTCAGATTTAGT	60° C	
Chymotrypsin_TP7prom _Forward	**TAATACGACTCACTATAGGG**TGGCAGATCTCCCTCCAGTAT	60° C	
Chymorypsin_TP7prom _Reverse	**TAATACGACTCACTATAGGG**AGATCCCCACCAGTCAGATTTAGT	60° C	
Trypsin_Forward	TGCACTATGGCTACCACTTCTGTG	60° C	497
Trypsin_Reverse	CAGCGGACTTCCTGCATCTCTA	60° C	
Trypsin_TP7prom _Forward	**TAATACGACTCACTATAGGG**TGCACTATGGCTACCACTTCTGTG	60° C	
Trypsin_TP7prom_Reverse	**TAATACGACTCACTATAGGG**CAGCGGACTTCCTGCATCTCTA	60° C	
Gastric chitinase_Forward	TTGACTGGCTGAAGAAAAACAACT	60° C	251
Gastric chitinase_Reverse	ACTGCTGGAGCCACTGCTGCTA	60° C	
Gastric chitinase_TP7prom_Forward	**TAATACGACTCACTATAGGG**TTGACTGGCTGAAGAAAAACAACT	60° C	
Gastric chitinase_TP7prom_Reverse	**TAATACGACTCACTATAGGG**ACTGCTGGAGCCACTGCTGCTA	60° C	
Pepsin_Forward	TCCAGCACTTTCCAGACCAACAAC	60° C	446
Pepsin_ Reverse	CATCCTCCAGAGCAAGCAACAGTC	60° C	
Pepsin_TP7prom_Forward	**TAATACGACTCACTATAGGG**TCCAGCACTTTCCAGACCAACAAC	60° C	
Pepsin_TP7prom_Reverse	**TAATACGACTCACTATAGGG**CATCCTCCAGAGCAAGCAACAGTC	60° C	

PCR reactions were then placed in a thermal cycler under the following conditions:

Steps	**Temperature**	
1	95°C	2min
2	95°C	30 sec
3	60°C	30 sec
4	72°C	2min
5	Go to Step 2, 33 more times	
6	72°C	2min
7	10°C	20 sec

2µL of each PCR product was run on a 1% agarose gel with 0.5µL of Ethidium Bromide to confirm the size of the product.

4.3.2.5 PCR Product Purification

PCR products were purified using the QIAquick PCR Purification kit (Qiagen, 28104) as per manufacturer's instructions except that the DNA was eluted in 30µL nuclease-free dH_2O (pre-heated to 55°C). The samples were quantified using the Nanodrop.1000. PCR products are shown below in Figure 4-2a and b

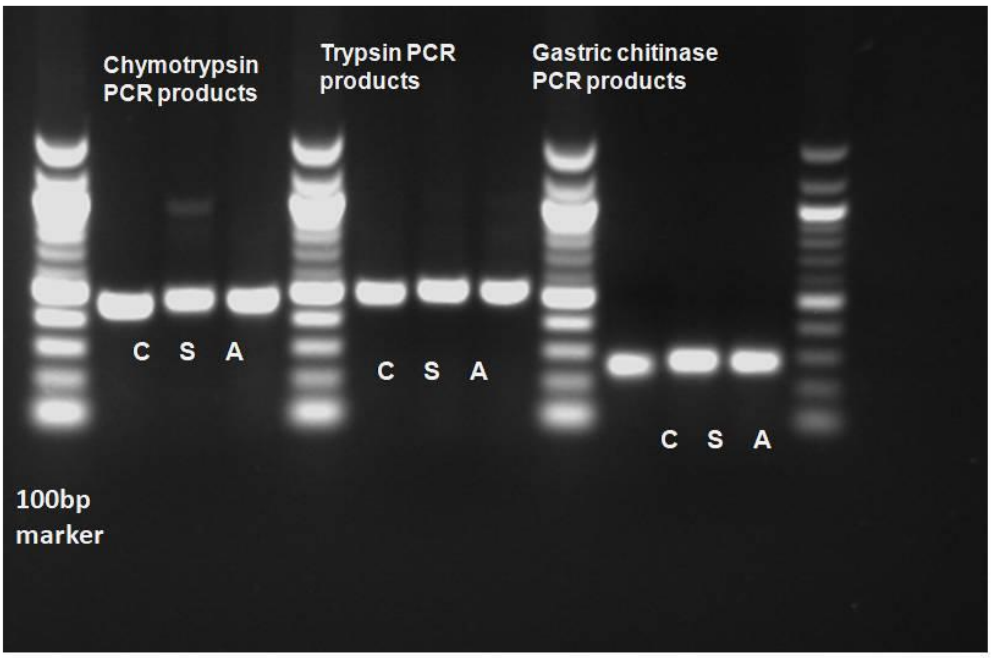


Figure 4-1a showing the 1% agarose gel run to check for the integrity of the PCR products for the four genes of interest namely trypsin, chymotrypsin and gastric chitinase sent off for purification to GATC Company in Germany.C- control,S- sense probeand A –Antisense probe

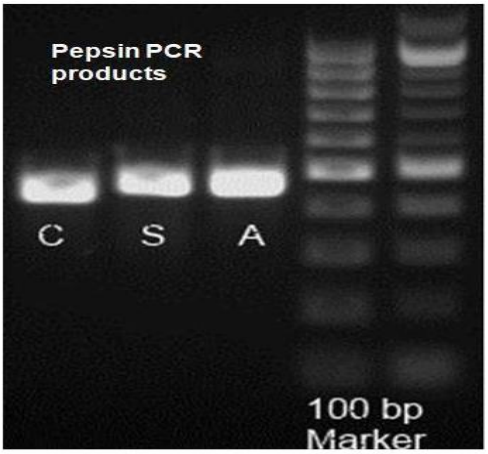


Figure 4-2b showing the 1% agarose gel run to check for the integrity of the PCR products for the gene of interest namely pepsinsent off for purification to GATC Company in Germany.C- control,S- sense probeand A –Antisense probe

4.3.2.6 Sequencing

Samples were processed, as per the requirements of GATC Biotech .com www.gatc-biotech.com and sent off as PCR purified products together with the respective primer sets for sequencing as 20 µL of 30ng/µl concentration using the light run technique and the analysis completed using SeqMan software. The sequence data was entered into the NCBI BLASTn database and resulted in >90% identity between the sample sequences and the target sequences using the EST database. The BLASTn results are shown below as figures 4-3, 4-4, 4-5 and 4-6.

BLAST ®

Basic Local Alignment Search Tool

NCBI/ BLAST/ blastx/ **Formatting Results - 9M05N2ZS01R**

- Formatting options
- Download

Blast report description

12575977.seq - ID: Trypsin PCR product

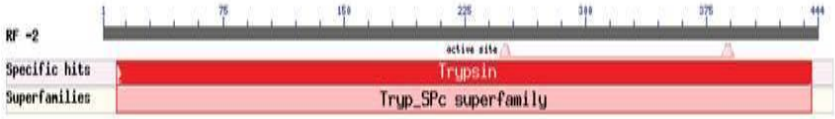
RID 9M05N2ZS01R (Expires on 12-01 22:13 pm)

Query ID lcl|138869
Description 12575977.seq - ID: Trypsin PCR product antisense-Trypsin_TP7prom_R on 2013/11/26-2:29:18 automatically edited with PhredPhrap, start with base no.: 22 Internal Params: Windowsize: 20, Goodqual: 19, Badqual: 10, Minseqlength: 50, nbadelimit: 1
Molecule type nucleic acid
Query Length 444

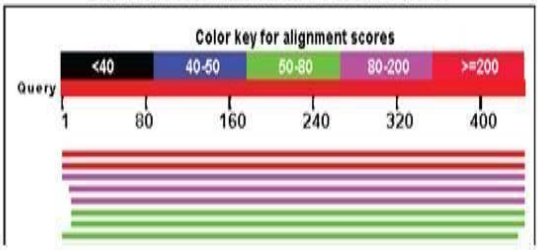
Database Name nr
Description All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects
Program BLASTX 2.2.28+

Graphic Summary

Putative conserved domains have been detected, click on the image below for detailed results.



Distribution of 23 Blast Hits on the Query Sequence



Description	Max score	Total score	Query cover	E value	Ident	Accession
trypsin precursor [Ictalurus punctatus] >gb|ADO28894.1| trypsin [Ictalurus punctatus]	293	293	97%	7e-104	91%	NP_001187619.1
trypsin [Ictalurus furcatus]	292	292	97%	3e-103	91%	ADO28356.1

Figure 4-3 showing the sequenced PCR products checks for trypsin after BLASTn in NCBI.

BLAST ®

Basic Local Alignment Search Tool

NCBI/ BLAST/ blastx/ **Formatting Results - 9KYVUKFW01R**
- Formatting options
- Download

Blast report description

12575973.seq - ID: Chymotrypsin PCR product

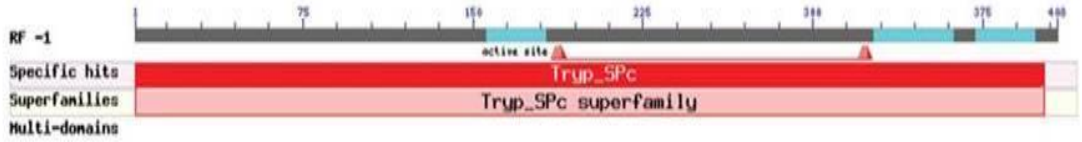
RID 9KYVUKFW01R (Expires on 12-01 21:51 pm)

Query ID lcl|15881
Description 12575973.seq - ID: Chymotrypsin PCR product antisense-ChymoT_is _R T7promoter on 2013/11/26-2:29:20 automatically edited with PhredPhrap, start with base no.: 10 Internal Params: Windowsize: 20, Goodqual: 19, Badqual: 10, Minseqlength: 50, nbadelimit: 1
Molecule type nucleic acid
Query Length 407

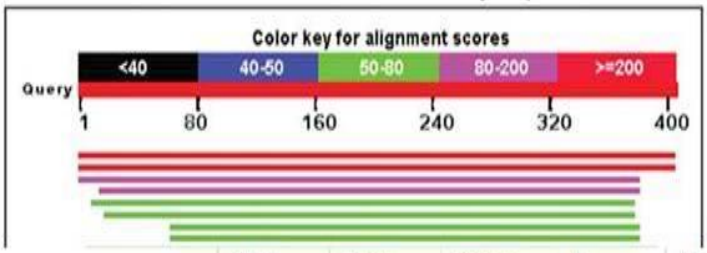
Database Name nr
Description All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects
Program BLASTX 2.2.28+

Graphic Summary

Putative conserved domains have been detected, click on the image below for detailed results.



Distribution of 25 Blast Hits on the Query Sequence



Description	Max score	Total score	Query cover	E value	Ident	Accession
chymotrypsin-like elastase family member 2a [Ictalurus furcatus]	241	241	95%	8e-81	87%	ADO28365.1
chymotrypsin-like elastase family member 2a precursor [Ictalurus punctatus] >gb|ADO28568.1| chymotrypsin-like elastase family member 2a [Ictalurus punctatus]	239	239	95%	4e-80	86%	NP_001188117.1

Figure 4-4 showing the sequenced PCR products checks for chymotrypsin after BLASTn in NCBI.

BLAST ®

Basic Local Alignment Search Tool

NCBI/ BLAST/ blastx/ **Formatting Results - HHZ8WFTC015**
Formatting options
Download
Blast report description

11734079.seq - ID: 72FF30- on 2013/7/2-1:50:50

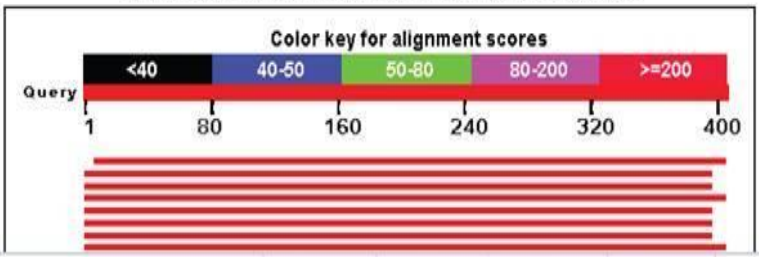
RID HHZ8WFTC015 (Expires on 03-08 05:56 am)
Query ID lcl|20105
Description 11734079.seq - ID: 72FF30- on 2013/7/2-1:50:50 automatically edited with PhredPhrap, start with base no.: 26 Internal Params: Windowsize: 20, Goodqual: 19, Badqual: 10, Minseqlength: 50, nbadelimit: 1
Molecule type nucleic acid
Query Length 408

Database Name nr
Description All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects
Program BLASTX 2.2.29+

Graphic Summary

Putative conserved domains have been detected, click on the image below for detailed results.

Distribution of 102 Blast Hits on the Query Sequence



Description	Max score	Total score	Query cover	E value	Ident	Accession
pepsin A [Ictalurus furcatus]	248	248	97%	2e-80	93%	ADO27975.1
pepsin A precursor [Ictalurus punctatus] >gb|ADO29339.1| pepsin A [Ictalurus punctatus]	246	246	97%	4e-78	95%	NP_001187944.1
pepsinogen A2 precursor [Siniperca scherzeri]	210	210	97%	7e-64	77%	ACT35559.1

Figure 4-5 showing the sequenced PCR products checks for pepsin after BLASTn in NCBI

BLAST ®

Basic Local Alignment Search Tool

NCBI/ BLAST/ blastx/ **Formatting Results - HHYXZB1T014**
Formatting options
Download
Blast report description

12575981.seq - ID: Gastric chitinase PCR

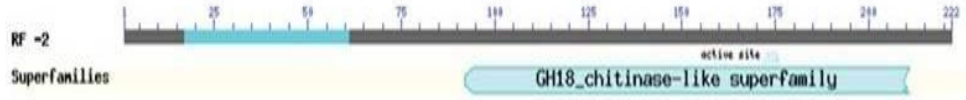
RID HHYXZB1T014 (Expires on 03-08 05:50 am)

Query ID ld|12722
Description 12575981.seq - ID: Gastric chitinase PCR product antisense-Gchit_TP7prom_R on 2013/11/26-2:29:20 automatically edited with PhredPhrap, start with base no.: 13 Internal Params: Windowsize: 20, Goodqual: 19, Badqual: 10, Minseqlength: 50, nbadelimit: 1
Molecule type nucleic acid
Query Length 221

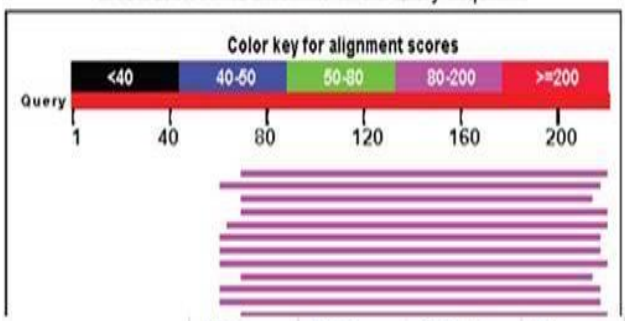
Database Name nr
Description All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects
Program BLASTX 2.2.29+

Graphic Summary

Putative conserved domains have been detected, click on the image below for detailed results.



Distribution of 102 Blast Hits on the Query Sequence



Description	Max score	Total score	Query cover	E value	Ident	Accession
chitinase 1 [Hexagrammos otakii]	89.4	89.4	67%	4e-19	82%	BAK19335.1
PREDICTED: acidic mammalian chitinase-like [Anolis carolinensis]	89.0	89.0	70%	4e-19	77%	XP_003220370.1
chitinase 1 [Scomber japonicus]	89.0	89.0	65%	5e-19	85%	BAL40979.1

Figure 4-6 showing the sequenced PCR products checks for gastric chitinase after BLASTn in NCBI

4.3.2.7 DIG-Labelling

The DIG RNA Labelling kit (Roche, p/n 11175 025 910) was used to transcribe DNA into single-stranded RNA probes using T7 polymerase in the presence of digoxigenin-UTP). Purified PCR products were DIG Labelled as follows:

100ng purified PCR product was added to 0.2mL nuclease-free tubes on ice and the volume made up to 6.5µL using nuclease-free dH_2O. The kit control DNA (4µL) was also employed (*i.e.* Control DNA 1 pSPT18-Neo, 0.25µg/µL, vial 3). The following reagents were then added (per reaction) to make a 10 µL reaction total 1µL of 10x NTP labelling mixture followed by 1µL of 10x Transcription buffer, 0.5µL of Protector RNase Inhibitor and 1µL of RNA polymerase T7. This was followed by gently flicking the tubes to mix the contents. Tubes were then centrifuged briefly and incubated at 37°C for 2h. 2µL DNase was then added to remove template DNA and incubated at 37°C for 15min. Finally 2µL of 0.2M EDTA was added at pH8.0 to stop the reaction).The samples were quantified using the Nanodrop 1000 and then 100ng each of the labelled sample and unlabelled samples (i.e. PCR products) were run on a 1% agarose gel containing 0.5% Ethidium Bromide to verify the size of the transcripts obtained and estimate the labelling efficiency as shown in Figure 4-7a and 4-7b below The DIG-labelled probes were then divided into into 2µL aliquots and stored at -70°C.

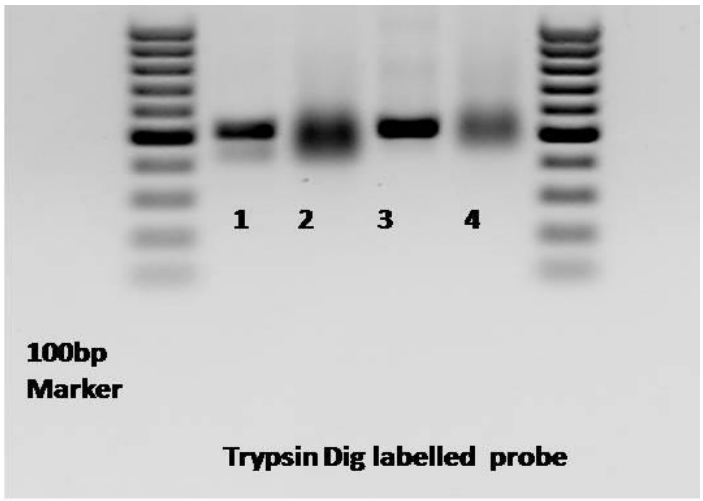


Fig 4-7a Showing Dig labelled products of trypsin:KC –Kit DNA control,1-trypsin sense PCR products,2 trypsin sense DIG product,3 trypsin antisense PCR products,4rypsin Antisense DIG labelled products

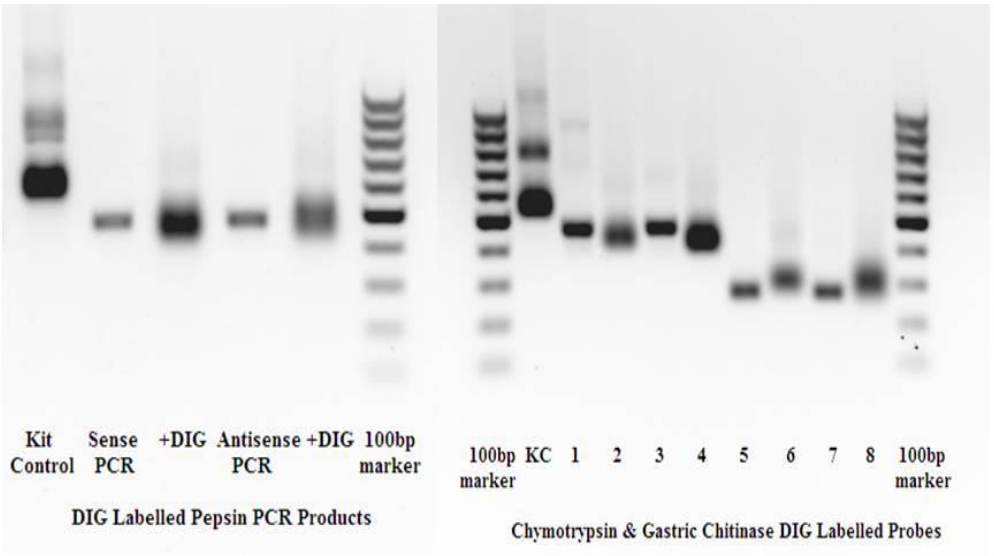


Fig 4-7b Showing Dig labelled products of Pepsin, Chymotrypsin and Gastric chitinase. KC –Kit DNA control,1-Chymotrypsin sensePCR products,2 Chymotrypsin sense DIG product,3 Chymotrypsin antisense PCR products,4 –ChymotrypsinAntisense DIG labelled products,5 Gastric chitinase Sense PCR products,6 -Gastric chitinase sense DIG products,7- Gastric chitinase antisense PCR products and 8- Gastric chitinase antisense DIG products

4.3.2.8 Dot-Blot Analysis

To determine the yield of the DIG-labelled RNA probes a dot-blot analysis was carried out. This required applying a series of dilutions of the RNA probes to a positively-charged nylon membrane along with several known dilutions of the kit control RNA which served as standards.

The Roche protocol suggested that an assumption of the yield of the labelling reaction was that 10µg of labelled RNA per 1µg plasmid or 200ng PCR template should be used. The probe was diluted to a starting concentration of 10ng μL^{-1}. Also diluted was the DIG-labelled RNA kit control 100ng μL^{-1} stock, to 10ng μL^{-1}.Using these starting solutions the following dilution series were prepared as detailed below in Table 4-6

Table 4-6showing the dilutions employed for the dot blot analysis

1	--1	Dilution of probe and vial 5	39---	---	10ng/µL
2	2	1	18	1:10	1ng/µL
3	2	2	198	1:100	10pg/µL
4	15	3	35	1:3.3	3pg/µL
5	5	3	45	1:10	1pg/µL
6	5	4	45	1:10	0.3pg/µL
7	5	5	45	1:10	0.1pg/µL
8	5	6	45	1:10	0.3pg/µL
9	5	7	45	1:10	0.01pg/µL
10	0	---	50	---	0

RNA Dilution Buffer comprised a mix of nuclease-free dH_2O: 20x SSS: formaldehyde in the ratio 5:3:2 and was always prepared fresh.

1µL spots of tubes 3 to 10 were applied onto a square of positively-charged nylon membrane (Roche, 11 209 299001), which had been lightly scored, using the tip of a pair of scissors, to mark out individual boxes. The membrane was held with gloved hands and forceps only. The membrane was placed in a nuclease-free glass container, *i.e*. Pyrex dish previously baked at 180°C for 3 h, covered with foil and incubated at 120°C for 30mins to fix the nucleic acid.

The DIG Nucleic Acid Detection Kit (Roche, 11 175 041 910) was used to determine whether or not the probes had been successfully labelled. This detected the DIG-labelled nucleic acids by enzyme-linked immunoassay using an antibody conjugate (anti-DIG alkaline phosphatase). A subsequent enzyme-catalyzed colour reaction with BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitrobluetetrazolium salt) producing an insoluble blue precipitate which was visualized as hybrid molecules. The DIG Wash and Block Buffer set (Roche, p/n 11 585 762 001) was employed for washing/blocking the membrane.

The membrane was placed into a sterile Petri-dish and washed with 20mL Wash Buffer (1X) on rocking platform (Stuart scientific, Rotator drive STR4,UK) for 5 minutes at Room Temperature (RT).The wash buffer was then poured off and incubated in 20mL Blocking Solution (1X) for 30 minutes on rocking platform at RT. This was followed by removal of the blocking solution and incubation for 30 minutes in 20mL Antibody Solution, on rocking platform at RT. In the meantime the anti-DIG-AP was centrifuged for 5 minutes at 10,000 rpm. The required volume was then pipetted from the surface *i.e*.at a dilution ratio of 1: 5000 in 1X Blocking

Solution *i.e*. I. 4µL antibody in 20mL 1X Blocking solution was used. In the next step the membrane was washed twice for 15 minutes each time (2 x 15 minutes) in 20mL Washing Buffer (1X) on a rocking platform at room temperature. The membrane was then equilibrated for 5 min in 20mL Detection Buffer (1X) on a rocking platform at room temperature (RT). This was then followed by incubation of the membrane in 10-20mL colour substrate solution in the dark at RT without shaking.

The colour precipitate started to form within a few minutes and the reaction completed after 16h-18h.The membrane was left overnight (ca. 18h) and the reaction stopped by adding nuclease-free dH_2O.

The Membrane was then photographed using the Syngene Trans illuminator using the white light source only as shown in Figure 4-8 below.

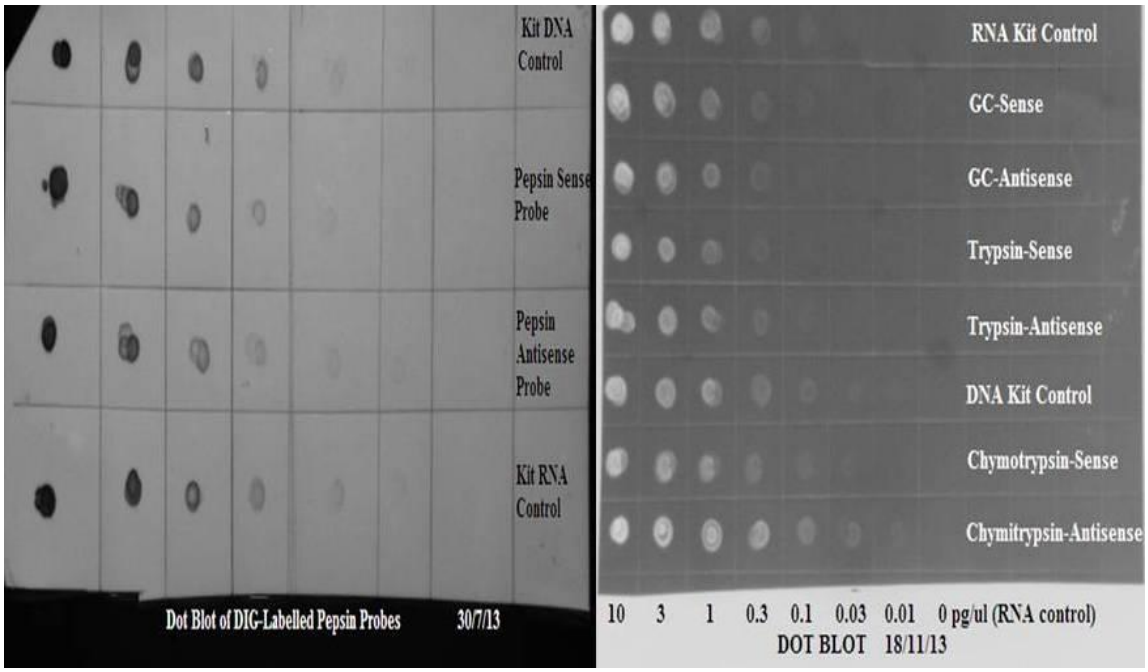


Figure 4-5 showing the Dot blot analysis for the four genes of interest namely trypsin chymotrypsin, gastric chitinase and pepsin in checking for expected amount of labelled RNA probe.

A comparison of the RNA control probe from the kit (vial 5) and the test probes was made. The kit stated that for every 1µg of plasmid template (or 200ng of PCR product), 10µg of labelled RNA was produced. The final volume of the labelling reaction was 24µL so 10µg/24µL = concentration of 416ng/µL. According to the protocol, if the most dilute spot visible on the membrane was the fifth RNA probe spot (tube 7) then the probe contained the expected amount of labelled RNA

probe i.e. 416ng/µL. If the most dilute spot seen was the 4th spot (tube 6) then an adequate amount of labelled RNA probe has been produced i.e. 416ng/mL ÷ 3 = 124ng/µL. If the most dilute spot seen is the 3rd spot (tube 5) then an inadequate amount of labelled RNA had been produced and the labelling reaction had to be repeated.

4.3.3 Hybridisation

5 micron sections were cut from 4% paraformaldehyde-fixed, wax-embedded tissues. Serial sections were taken so that a comparison could be made between similar areas of tissue using the sense and anti-sense probes. A third slide was processed without probe (negative control). The sections were mounted onto a Plus+ Frost, positively charged microscope slides (Solmedia, MSS51012WH). A fourth section was taken onto an ordinary microscope slide to stain using Haematoxylin & Eosin to confirm the presence of the tissue of interest. Slides were baked at 60^0C for one hour in an oven (Windsor Incubator 32-70˚C) and then the *in situ* hybridisation procedure was carried out as detailed below. The protocol is based on one received from Rasmus Skern (Institute of Marine Research, Bergen, Norway) with a few alterations.

Following baking and storage overnight slides were washed twice for 10 minutes each time (2 x 10min) in xylene in nuclease-free staining jars.

This was followed by rehydration: in 95%, 70%, 50% ethanol made using nuclease-free dH_2O for 1 minute in each at RT. Finally, slides were placed in 2x SSC for 1 min at RT. On removal from SSC, the slides were dried as much as possible using a tissue avoiding the sections and using an Imm Edge pen (Vector Labs, p/n H-4000) circles were drawn around the sections and then the tissues were digested using 10µg mL^{-1} Proteinase K in 100mM Tris $50mM^{-1}$EDTA buffer (pre-warmed to 37^0C) made up using the following ingredients for a 1000µL total volume reaction ;100µL of 1M Tris (pH 8), 100µL of 0.5M EDTA,1µL of Proteinase K (10mg/ml) and 799µL nuclease-free dH_2O.

To each Slide was added 150-200µL proteinase K followed by incubation at 37°C for 5 minutes in a humidified box containing tissues soaked in 2x SSC. (Box had been treated with RNAse Zap (Thermo Scientific, 7002) for 10mins and rinsed with nuclease-free dH_2O).

This was followed by the process of post-digestion fixation where Proteinase K was fixed in the tissue sections for 5 minutes in 150mL cold, 4%

paraformaldehyde freshly defrosted from -20^{0}C (Liebherr Comfort Freezer) aliquots in a nuclease-free Easy Dip jar (VWR,720-0791).The slides were then washed twice with 1x PBS in Easy Dip Jar for 2 minutes at RT.

This was followed by the process of *in-situ* hybridisation where the slides were removed from PBS and dried as much as possible using a tissue. A Gene Frame (Fisher Scientific, AB-0578) was placed around the sections on each slide and 150µL of Pre-hybridisation solution (For a total reaction volume made according to this protocol a mixture: 200µL of 20x SSC, 500µL of formamide, 300µL of nuclease-free dH_2O was made)

The slides were then incubated at 37°C for 10mins in a humidified box.

A hybridisation solution master mix for the number of slides required was then prepared: with the mixture per slide comprising 75µL[100% stock solution] of 50% formamide[final concentration],15mg 10% dextran sulphate, 37.5µL[20X stock solution]] of 5x SSC,15µL[50X stock solution] of 5x Denhardts Solution,1.5µL [25mg/ml] of 250µg/mL tRNA,7.5µL [10mg/ml] of 500µg/mL herring sperm DNA*and 15µL [10%] of 1% blocking solution†* herring sperm DNA denatured at 95°C for 5 minutes prior to adding to master mix. Blocking solution was from the DIG Wash and Block Buffer set (Roche, 11 585 762 001).

The master mix was prepared and 150µL aliquoted into the required number of tubes (i.e. three, one for the sense probe, one for the anti-sense probe and one with hybridization mix only negative control}). It was then added to the probes at a final concentration of 300-800ng/mL (0.3-0.8ng/µL) and the samples heated at 80°C for 5 minutes. These were cooled on ice before being added to the appropriate tissue sections. A Gene Frame coverslip was placed on top of the Gene Frame and the slides placed in a humidified box (containing tissues soaked in 2x SSC) and incubated at 42°C overnight (no agitation).

The slides were flushed with 2XSSC at RT to remove coverslips, after removal of cover slips, they were placed 50mL tubes and incubated twice with 2XSSC on rocking platform (Stuart Scientific) at RT for 30 minutes at each Incubation stage, this was followed by addition of a mixture of 200µL of 50% (1ml) formamide + 50% (1ml) 2x SSC inside the ImmEdge pen circle incubated at 65^{0}C for 30min without agitation. This was then followed by washing twice for 10 minutes at 37°C.

The final step carried out was the Immunological Detection in which Roche DIG Nucleic Acid Detection Kit (11 175 041 910) and DIG Wash and Block Buffer Set (11 585 762 001) were used based on the kit protocol below.

The slides were washed with 50mL of the 1x Wash Buffer 1and shaken at Room Temperature for 5 min. The slides were incubated in 50mL,1x Blocking Solution while shaking at RT for 30 min. This was followed by centrifuging the antibody (Anti-digoxigenin-AP) at 10,000 rpm for 5 min. A 1:5000 dilution of the antibody in 1x Blocking Solution was then prepared. From the prepared antibody mix 200µL was pipetted and added to each slide inside the ImmEdge pen circle and incubated without shaking, in a humidified chamber at RT for 2 hours.This was followed by washing the slides with 50mL, 1x Wash Buffer 1 (shaking) RT for 15 min, twice each time replacing the 1x Wash Buffer. Most of the wash buffer was removed and the tissue sections equilibrated by dispensing 200µL of 1x Detection Buffer, inside the ImmEdge Pen circle at RT for 5min.

In the meantime, NBT/BCIP was warmed room temperature to re-dissolve any precipitate. Then 40µL of NBT/BCIP to 2ml, 1x Detection Buffer + 16µL Levamisole [stock = 125mM] (Vector Labs, p/n SP-5000) were added. After thorough mixing, 200µL was dispensed to each slide, incubated at RT in humidified chamber in the dark (without shaking) and viewed using a dissection microscope every 10-15 minutes. Colour developed in the gut using the pepsin probe after 40 minutes, trypsin and chymotrypsin probes for the Pancreas for 45 minutes and gastric chitinase in the gut 55 minutes. The reaction was stopped by adding 200µL of Stop Solution ([10mM Tris (pH 8), 1mM EDTA]) to each slide. Incubated for 1 minute at RT. Removed the buffer using a pipette added another 200µL and incubated at RT for 1 min. The stop buffer was gently flushed from the slides by using with nuclease-free dH_2O.The slides were then dried as much as possible and mounted a coverslip over the sections using Vectamount (Vector Labs, p/n H-5000). The slides were left to dry overnight and sealed the edges of coverslip using clear nail varnish. Allowed to dry overnight before capturing images of the sections using the Olympus BX51 microscope and AxioVision software.

4.4 Results

4.4.1 Histological structuresof the digestive system and accessory organs in adult African catfish (*Clarias gariepinus*)

4.4.1.1 Histology of the Digestive system of Adult *Clarias gariepinus*

In terms of histology the parts of the digestive system studied were as shown below in fig 4-9: oesophagus, foregut, midgut and hindgut as described in 4.4.1.1.1 to 4.4.1.1.4.

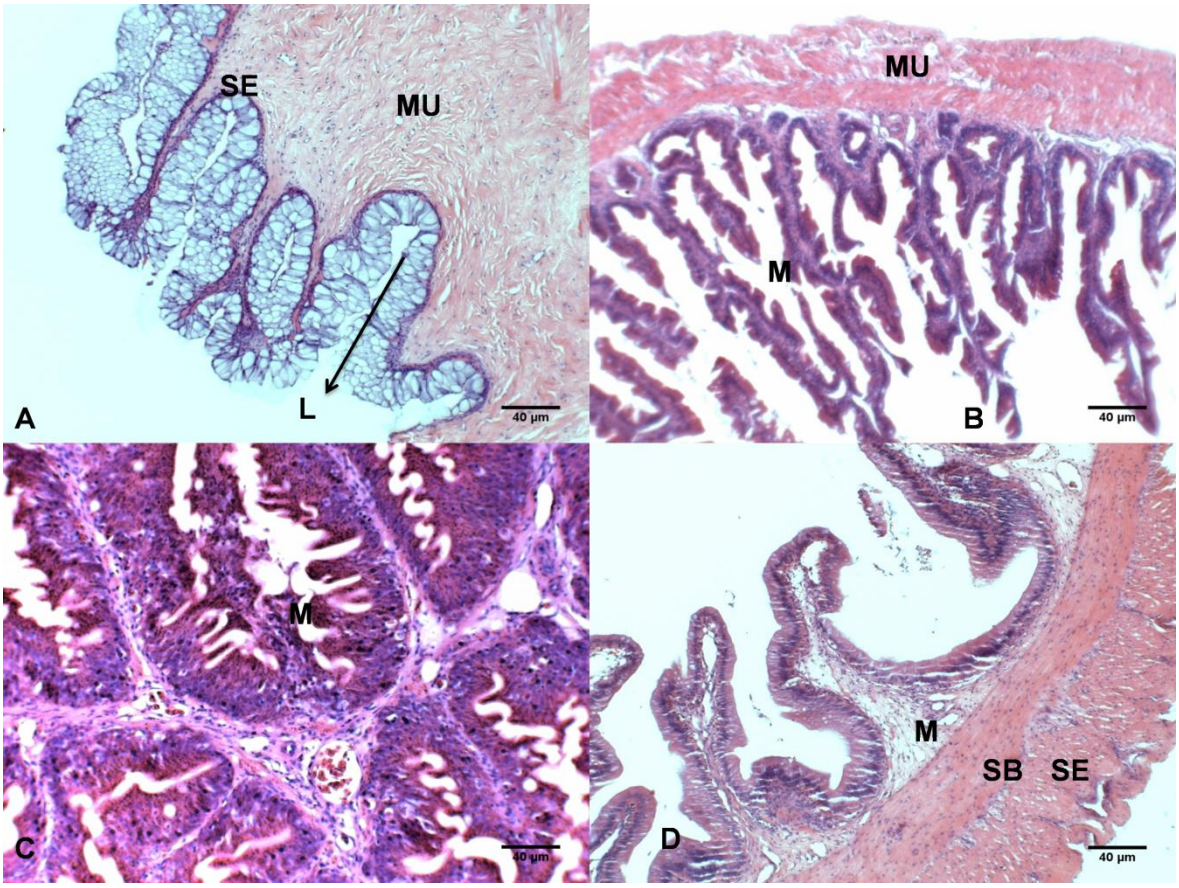


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Figure 4-9 showing A Oesophagus, B Foregut, C Midgut and D hindgut that have been described histologically using H&E stain in 4.4.1.1.1 to 4.4.1.1.4 below. Lamina propria (L), Serosa SE and the Muscularis (MU)

4.4.1.1.1.1 Histo-morphology of the oesophagus in adult *Clarias gariepinus*

In terms of histo-morphology observations, the oesophageal longitudinal folds was lined by stratified mucous epithelium containing eosinophilic club cells, with the lamina propria core containing regular collagen fibres. The oesophageal *tunica muscularis* contained skeletal muscles in an inner longitudinal and outer circular arrangement as seen in Fig 4-9 A above, the oesophagus comprising a mucosa within the thin lamina propria, with numerous goblet cells and an outer thin layer comprising the Serosa SE and the Muscularis (MU).

4.4.1.1.1.2 Histomorphology of the intestine in adult *Clarias gariepinus*

From the morpho-histological observations the intestine possessed simple absorptive epithelium and goblet cells. The mucosal folds of the proximal intestine were modified into a branched labyrinthine anastomosing network resembling a honey comb as seen in fig 4-9B above, showing the branched mucosa in the midgut/intestine. It is more branched than the anterior/fore gut.

4.4.1.2 Histology of the accessory organs and stomach of adult *Clarias gariepinus*

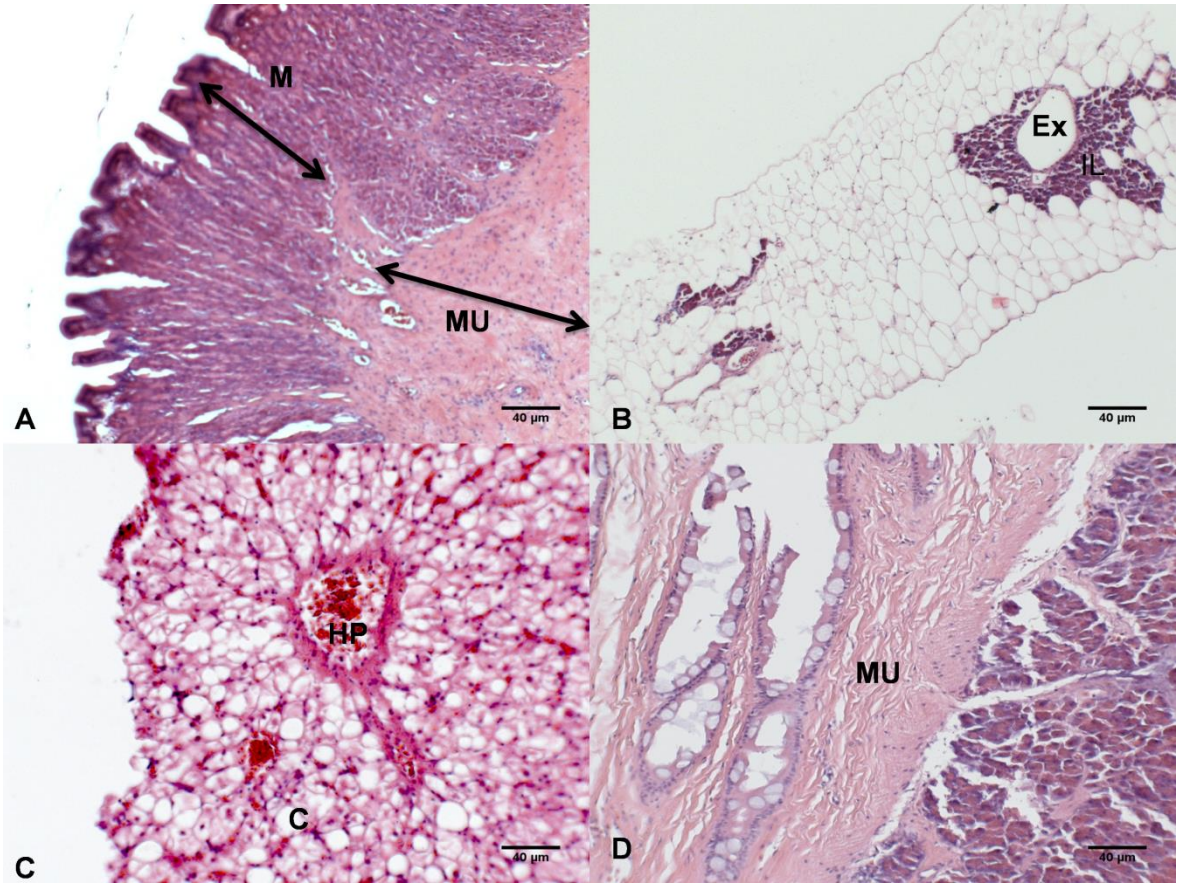

Figure 4-10 showing the histological structures of the accessory glands of the digestive system in adult African catfish (*Clariasgariepinus*).A Cross section of the stomach showing the thick mucosa (M). The sub-mucosa is composed of the fibrous tissue and the Muscularis (MU) with two layers of smooth muscle. B longitudinal section of the pancreas exocrine glands (EX) and islets of Langerhans (IL). C section of the liver showing the hepatocyes, in diffuse pattern. Note the central hepatic portal vein HP.

4.4.1.2.1.1 Histomorphology of the Stomach of adult *Clarias gariepinus*

The stomach was lined by simple columnar epithelium with apical neutral mucin. The gastric epithelium contained intraepithelial lymphocytes. The stomach cardiac and fundic regions contained gastric glands in the lamina propria while none was seen in the pyloric region.As seen in Fig 4-10A there was a thick mucosa with gastric glands. The sub-mucosa was composed of the fibrous tissue and the muscularis with two layers of smooth muscle.

4.4.1.2.1.2 Histomorphology of the pancreas in adult *Clarias gariepinus*

Observation of the pancreas indicated it was diffuse and was histologically located on the borders of the stomach to the proximal intestine. The organ was covered by loose connective tissue The few Islets of Langerhans were seen interspersed in the exocrine cells of the parenchyma.The interlobular duct was lined by simple cuboidal to columnar epithelium with a brush borderasborderas seen in Fig 4-9B and 4-10B.

4.4.1.2.1.3 Histomorphology of the liver of adult *Clarias gariepinus*

In terms of histomorphological observations, the liver was found to be grossly dark brown in colour. Histologically, it was covered by simple squamous cells. The hepatic parenchyma contained hepatocytes. The hepatocytes were polyhedral in shape and each contained a very basophilic central nucleus. The hepatocytes were arranged in a diffused or radial pattern (Fig 4-9C and 4-10C).

4.4.2 Histomorphology of *Clarias gariepinus* larvae aged 0-8days post hatch (Dph)

4.4.2.1 Histomorphology of *Clarias gariepinus* larvae at 0Dph and 1Dph

In Figure 4-11 below, in terms of histology, the yolksac (YS) was observable at 0Dph and 1Dphas seen on the left. Additionally in terms morphology the finfold was present in addition to the yolksac as seen on the right structure.

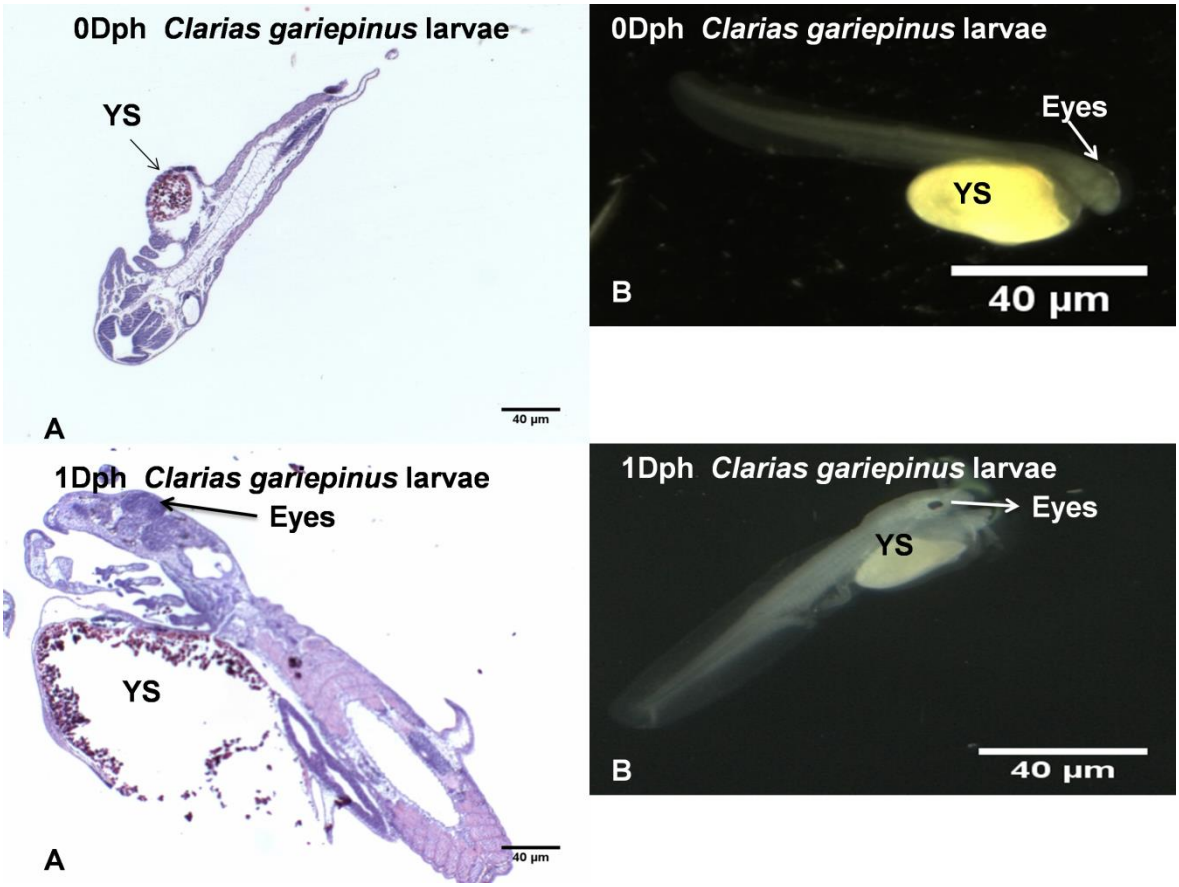

Figure 4--61 showing the histology and morphology of *C.gariepinus* at 0Dph and 1Dph with the visible parts being the (Ys) Yolk sac and finfold.

4.4.2.2 Histomorphology *of Clarias gariepinus* larvae at 2Dph and 3Dph

In Figure 4-12It was observed from the histology (Left) and morphology (Right) of *C.gariepinus* at 2Dph that the visible parts werethe (Ys) Yolk sac and finfold, prominent eye, mouth and beginning of the digestive canal starting to appear and barbels were prominent unlike at 1Dph.

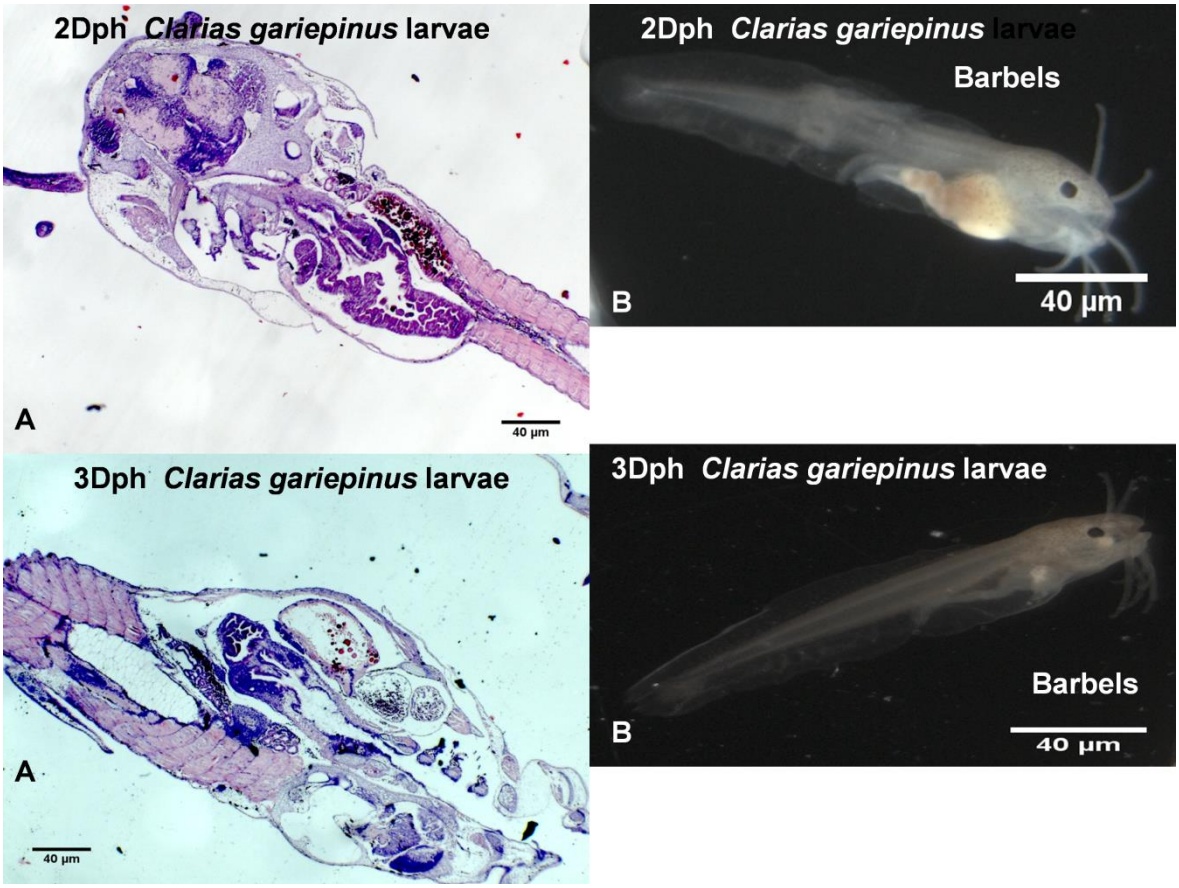

Figure 4-12showing the histology and morphology of *C.gariepinus* at 2Dph and 3Dph with the visible parts being the (Ys) Yolk sac and finfold, prominent eye, mouth and beginning of the digestive canal starting to appear

4.4.2.3 Histomorphology of *Clarias gariepinus* larvae at 4Dph and 5Dph

In terms of the histology and morphology of *C.gariepinus* at 4Dph it was observed that the visible parts were the straight gut, pancreas and stomach beginning to appear histologically as seen in Fig 4-13 below

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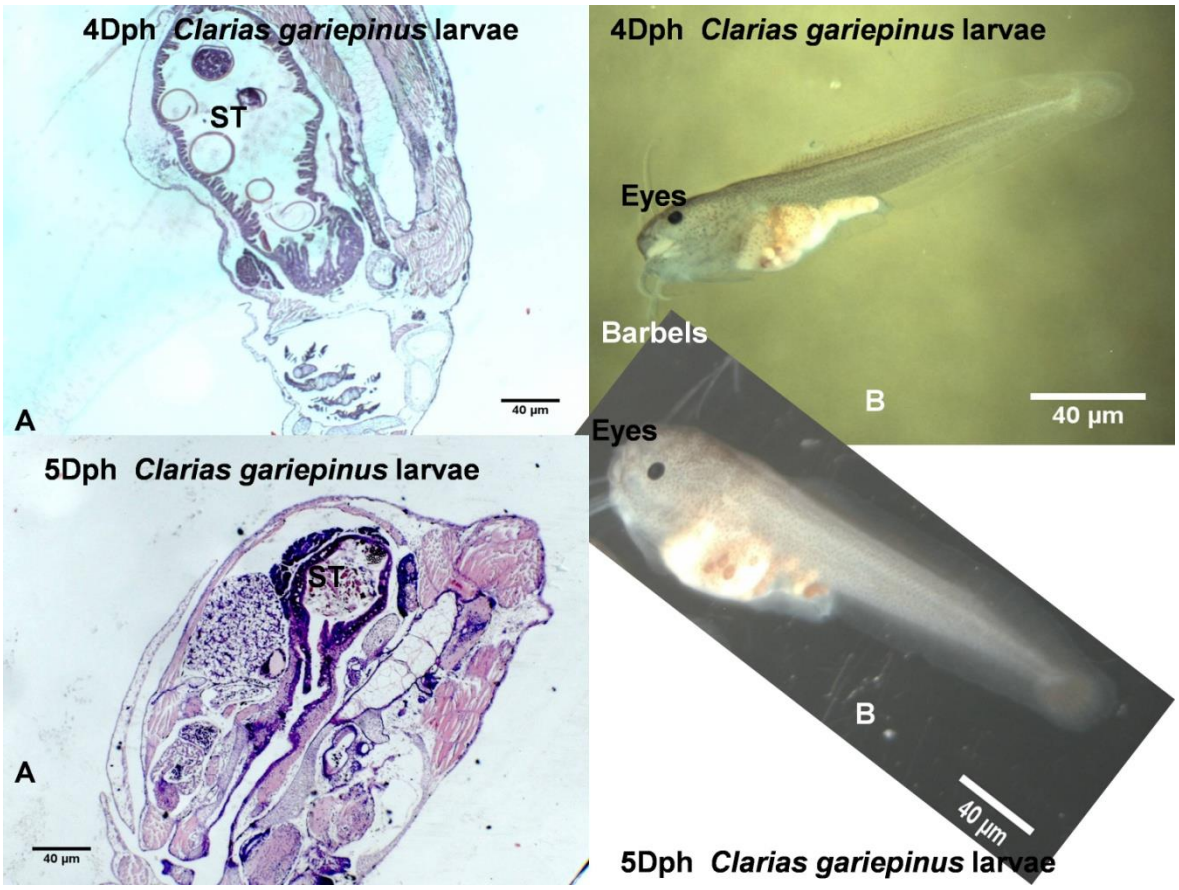

Figure 4-13 showing the histology and morphology of *C.gariepinus* at 4Dph and 5dphwith the visible parts being the straight gut, pancreas and stomach beginnings.

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4.4.2.4 Histomorphology of *Clarias gariepinus* larvae at 6Dph and 8Dph

In terms histology and morphology of *C. gariepinus* at 6Dph it was observed that the visible parts were the stomach, differentiated gastro- intestinal tract (GIT), and oesophagus as seen in Fig 4-14 below.

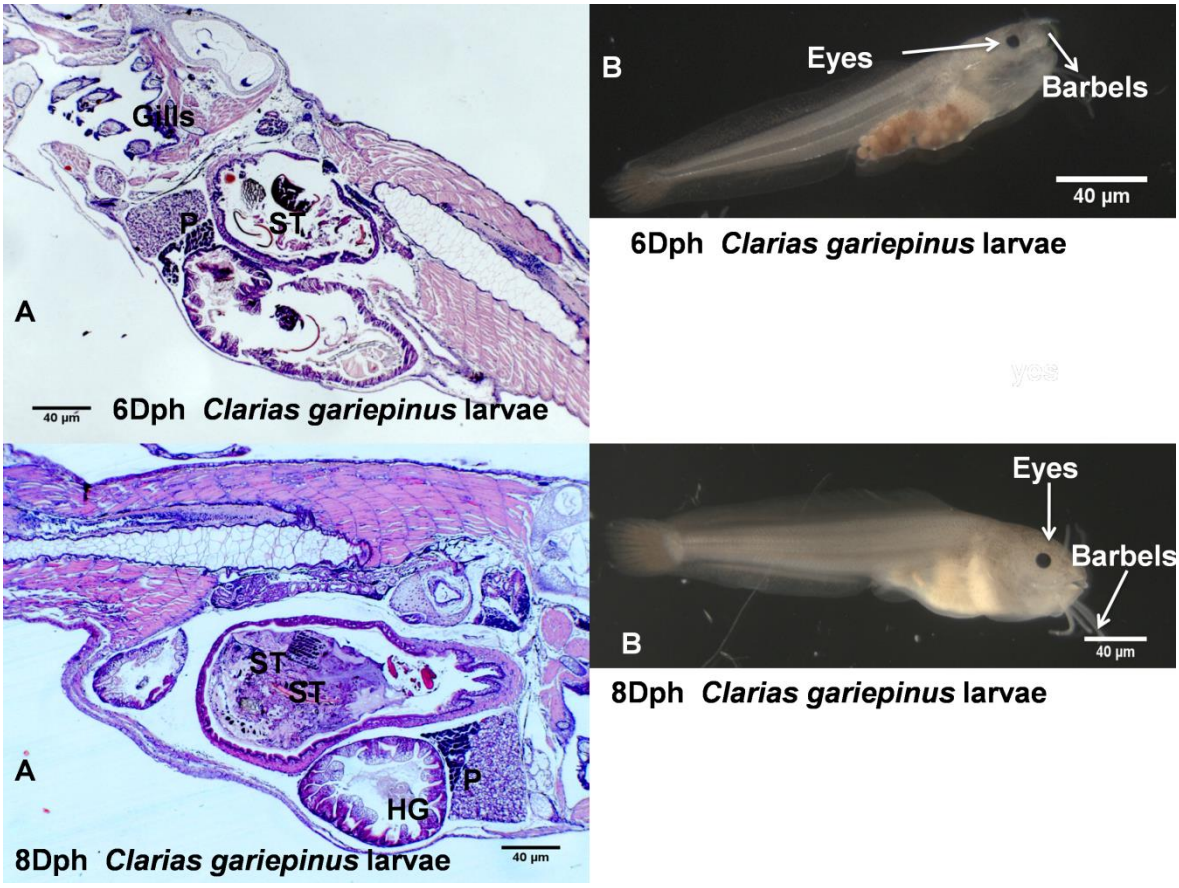

Figure 4-14 showing the histology and morphology of *C. gariepinus* at 6Dph with the visible parts being the stomach, differentiated gastro- intestinal tract (GIT), and oesophagus.

4.4.3 *In situ* expression hybridisation in Adult North African catfish, *Clarias gariepinus*

To better understand localisation of enzyme production in the larvae the researcher initially ran trials of possible target organs as per the literature concerning the site of production and in this case both the pancreas and stomach were thought to be the sites of localisation in adult catfish and in situ labelling was applied to the larvae as they are too small to identify the organs easily.

4.4.3.1 Localisation of chymotrypsin in the pancreas in the GIT of adult *Clarias gariepinus*

In Fig 4-15 chymotryspin was observed to be expressed in the pancreas which was dispersed in the body cavity of the fish around the digestive system.The antisense probe showed labelling in 4-15A and B with positive contro lof sealice D also showing fluorescence. The Control Sense probe C showed no fluorecence. There was also some fluorescence observed in the stomach but this was also present in the sense slide so may be non-specific.There appears to be specific fluorescence (and visible pink staining) in the pancreas (antisense slide) which is not seen in the sense or negative control slides. The negative control slide showed negligible fluorescence.

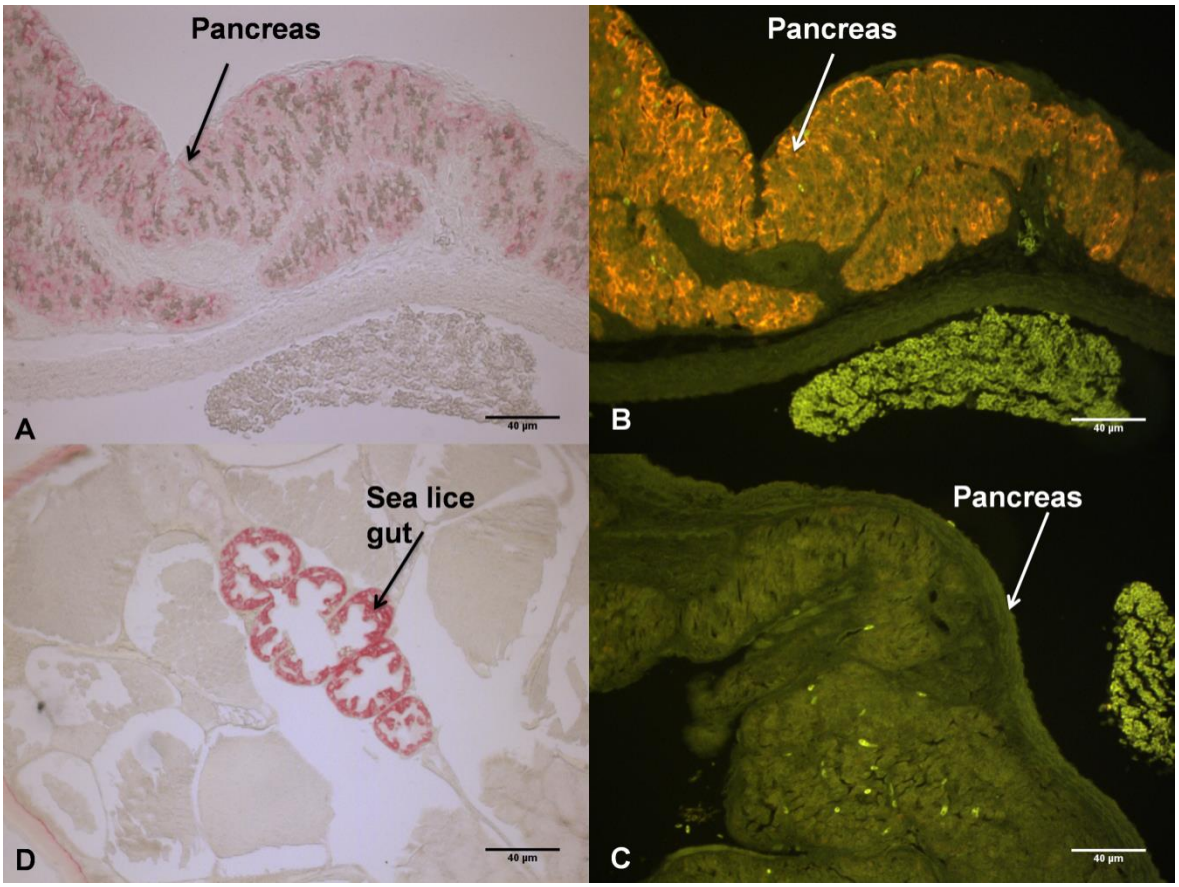

Figure 4-15 showing *in situ* expression hybridisation in the pancreas of adult North African catfish *Clarias gariepinus* of Chymotrypsin. (There appears to be visible pink staining and specific fluorescence in the pancreas (antisense A and B) as seen in the sea lice antisense Positive control (D) but not sense (C) slides.

4.4.3.2 Localisation of trypsin in the pancreas in the GIT of adult *Clarias gariepinus*

In fig 4-16 the site of localisation for trypsin was observed to be the pancreas which was dispersed in the body cavity of the fish around the digestive system.Antisense probe labelling was as seen in A and B andin the sea lice trypsin control tissue C.The control sense probe Dprovided a negative control.

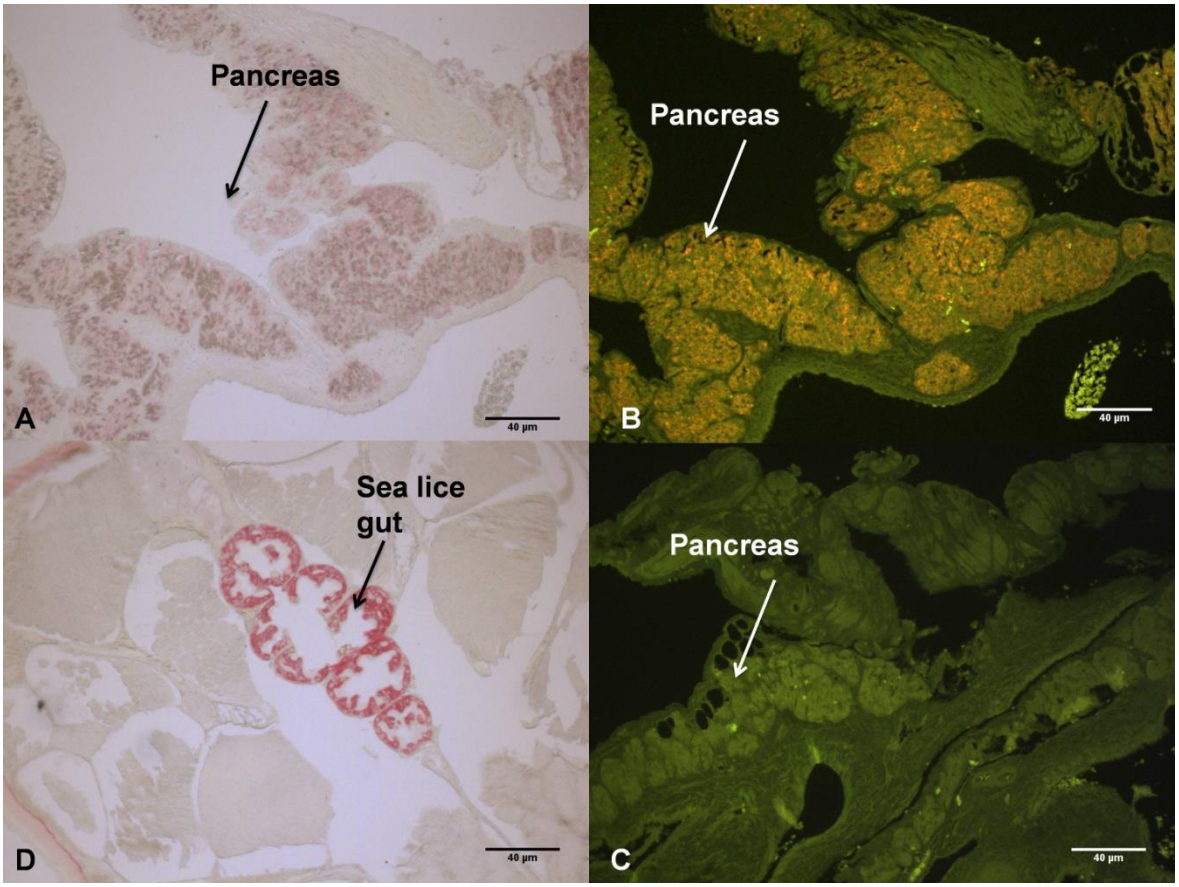

Figure 4-16 showing *in situ* expression hybridisation in the pancreas of adult North African catfish *Clarias gariepinus* for trypsin. A =light microscopy antisense probe; B anti sense probe C sense probe negative control and D = positive antisense control labelling trypsinin sea licegut.

4.4.3.3 Localisation of pepsin in the oxynticopeptic cells of the stomach of the GIT of adult *Clarias gariepinus*

In Fig 4-17 pepsin was localised in the oxynticopeptic cells of the stomach of adult North African catfish *Clarias gariepinus*as signified by the antisense probe labelling seen in A and B, with Dbeing the positive antisense control of sealice and the negative control being the sense probe C (Fig 4-17 below).

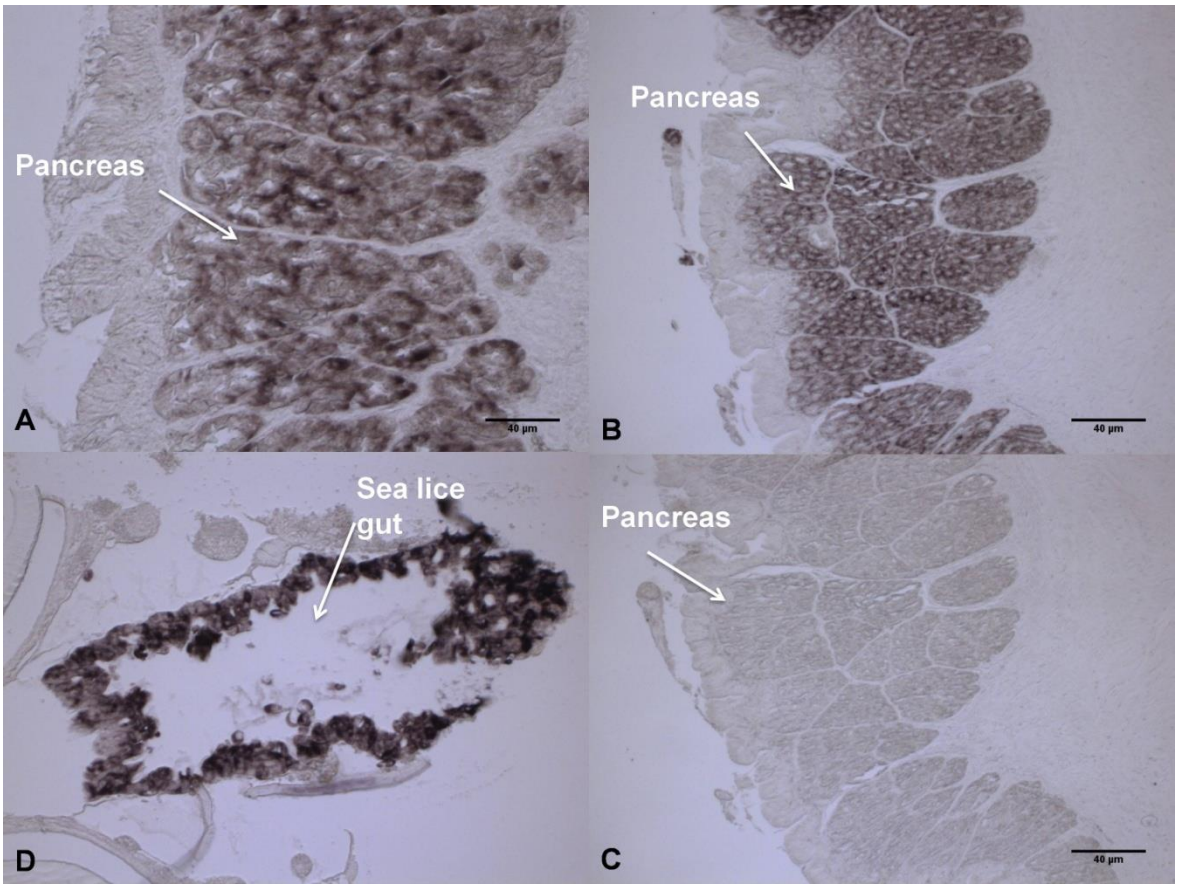

Figure 4-17 showing *in situ* expression hybridisation in the oxynticopeptic cells of the stomach of adult North African catfish *Clarias gariepinus* of pepsin using light microscopy at A and B Antisense probes D Negative control sense probe C – positive control using sea lice trypsin in gut.

4.4.3.4 Localisation of gastric chitinase in the gastric pits of the stomach of the GIT of adult *Clarias gariepinus*

In fig 4-18 gastric chitinase was observed inthe gastric pits of the stomach of adult North African catfish *Clarias gariepinus* as signified by the antisense probe fluorescence seen in A and B, with D being the positive control of sealicetrypsin and D being the negative sense control (Fig 4-18 below).

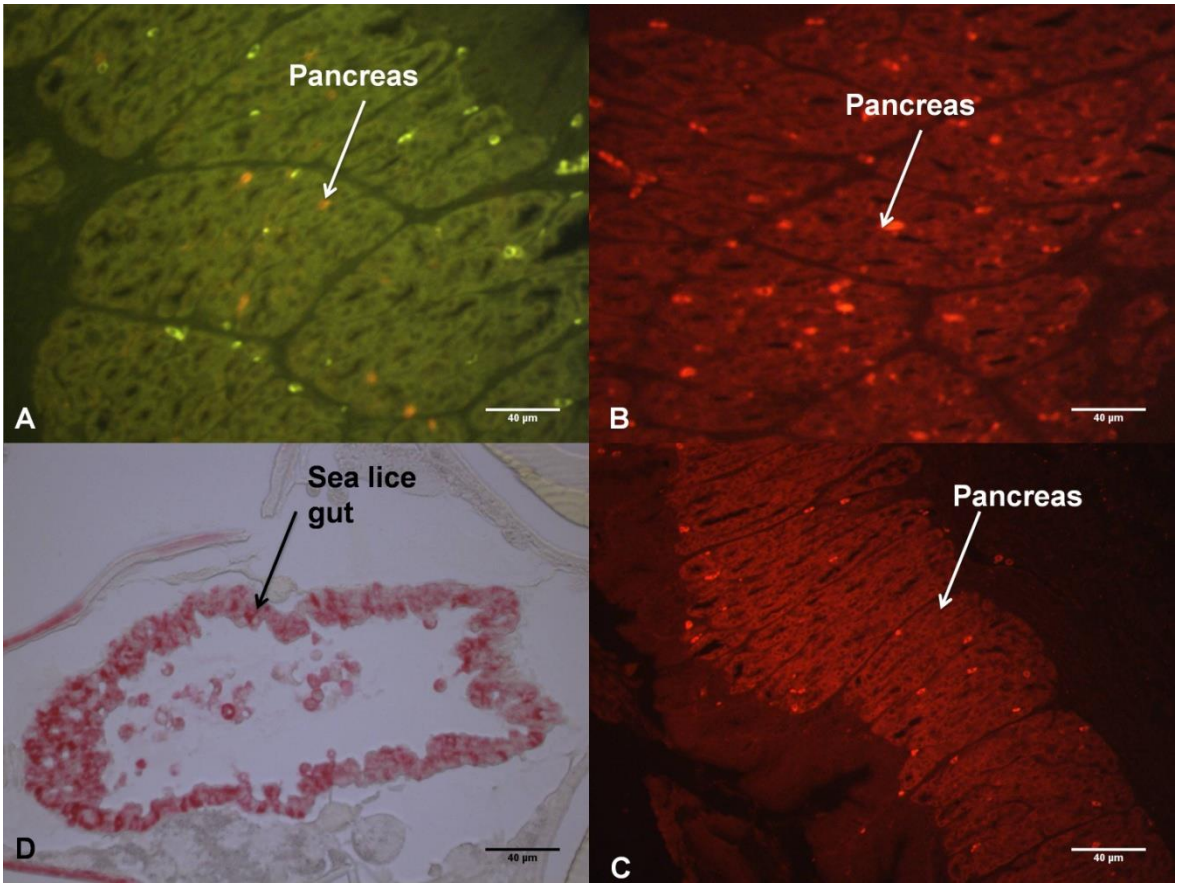

Figure 4-18 showing *in situ* expression hybridisation in the gastric pits of the stomach mucosa of adult North African catfish *Clarias gariepinus* of Gastric chitinase, A with light microscopy of antisense probe; B with green filter light anti sense probe C with light microscopy of sea louse trypsin antisense probe D with blue light filter antisense probe.

4.4.4 In situ expression hybridisation in Larvae of North African catfish, *Clariasgariepinus* for four target genes: chymotrypsin, trypsin, pepsin and gastric chitinase

4.4.4.1 Summary of the localisation of the genes of interest at the site of production using *in situ*-expression hybridisation at 1Dph to 8Dph.

During the study it was observed that the genes start to appear at the site of localization for alkaline digestion, namely trypsin and chymotrypsin, as early as 2 Dph and much later for the acidic digestive process in the larvae at 4Dph as in the case of pepsin and gastric chitinase (Table 4.8).

The localisation at the site of production using expression hybridisation technique for the four genes of interest to the researcher, namely the alkaline digestive enzymes chymotrypsinandtrypsin was successfully accomplished.Gastric chitinase didn't seem to be present by Day 4 as the stomach was either not fully developed in selected specimen or perhaps the specimen used did not yet have the stomach analageN.B:Days1,2,34,6, 8 were selected based on earlier histological studies by Verreth 1992 and Osman 2008

Table 4-8provides a summary showing the localization at sites of production of the genes of interest using*insitu* expression hybridization technique in larvae of *Clariasgariepinus* over 10 Dph.

Gene	**Site of localization**	**1Dph**	**2Dph**	**3Dph**	**4Dph**	**6Dph**	**8Dph**
Chymo-trypsin	Pancreas	Absent	Present	Present	Present	Present	Present
Trypsin	Pancreas	Absent	Present	Present	Present	Present	Present
Pepsin	Oxyntico-peptic cells in the stomach	Absent	Absent	Absent	Present	Present	Present
Gastric chitinase	Gastric glands in the Stomach	Absent	Absent	Absent	Absent*	Present	Present

*may be likely due to the loss of the tissue sample as we see its presence confirmed by the qPCR run in chapter 5

4.4.4.2 Localisation of chymotrypsin at 2 Dph

In figure 4-19below it was observed that the earliest localisation of chymotrypsin in the anlage of the pancreas appears at 2Dph and it was absent at 1Dph.This is signified by the positive results in the antisense probeshown under various filters as seen in A,B and C.The sense probe D was negative.

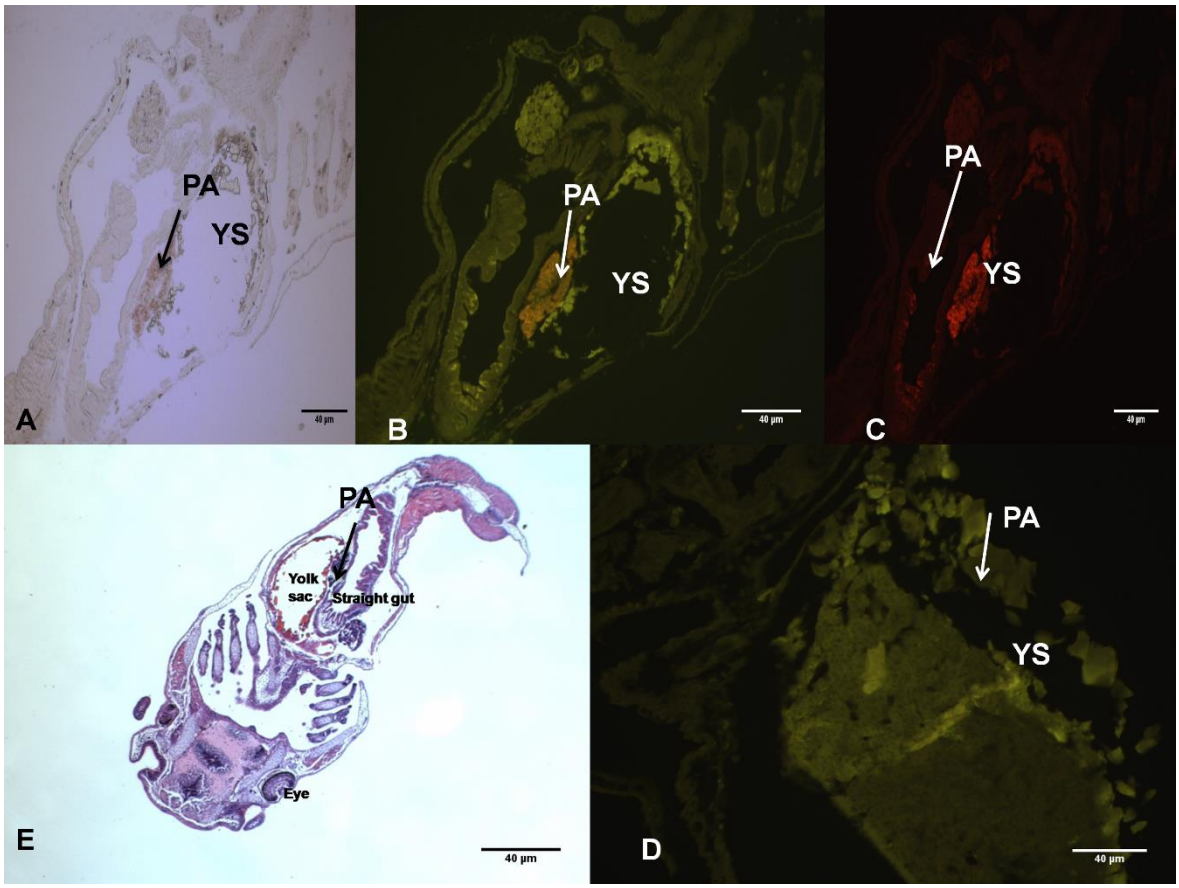

Figure 4-19 showing *in situ* expression hybridisation of Chymotrypsin in the Pancreas for 2Dph larvae of North African Catfish *Clarias gariepinus* A=Blue filter light B=light transmitted C=green filter light is Antisense), D= Sense Probe E =Histology of *Clarias gariepinus* larvae as positive control . E=H&E stain. YS, Yolk sac, PA Pancreatic anlage

4.4.4.3 Localisation of chymotrypsin at 3 Dph

Fig 4-20 belowfurther shows localisation of chymotrypsin in the anlage of the pancreas at the time the yolk sac is reabsorbed at 3Dph.This is signifiedby the positive results in the antisense probeshown under various filters as seen in A,B and C in transmitted white light, blue and greeen light respectively and sense probe D as negative probe.

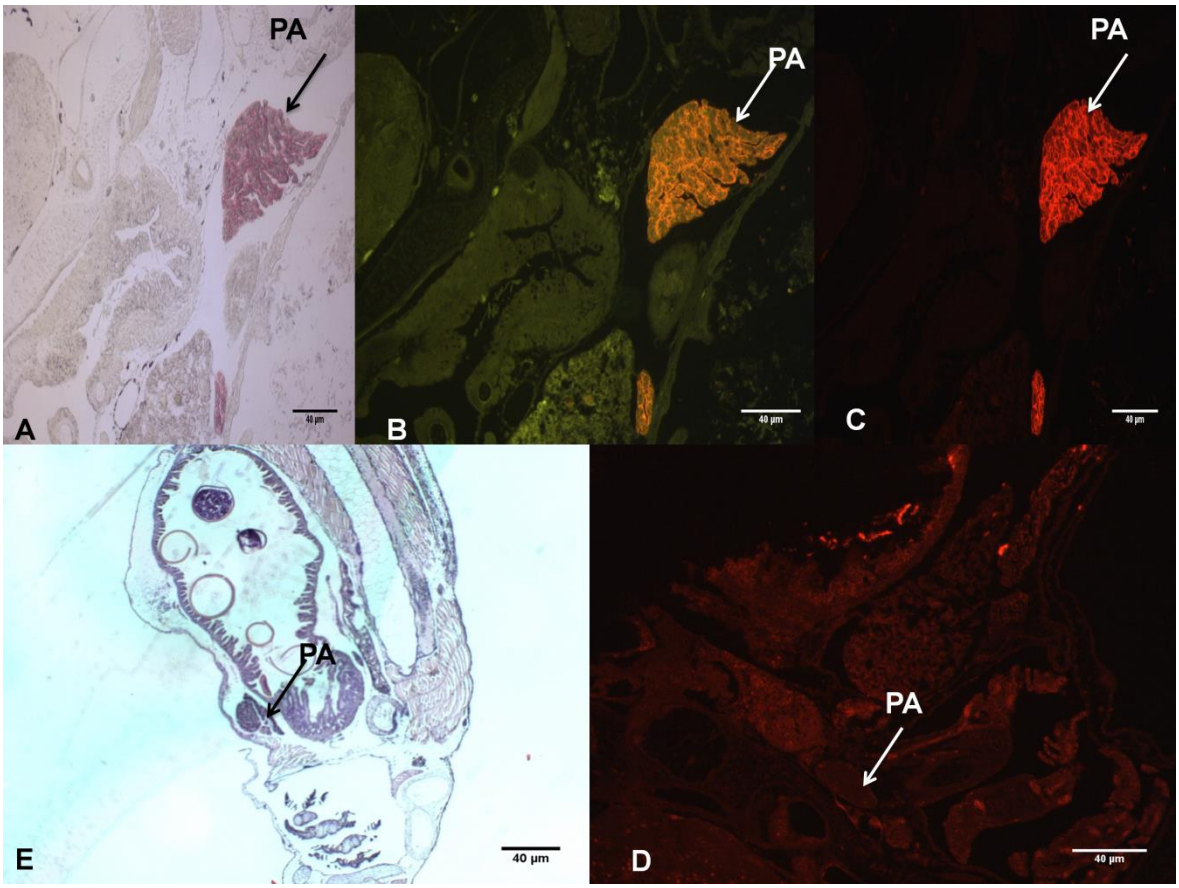

Figure 4-20 showing *in situ* expression hybridisation of chymotrypsin in the pancreatic anlage of 3Dph larvae of North African Catfish, A= antisense probe light transmitted B=bluefilterlight,C is green Antisense probe respectively , D = Negative Sense Probe E=H&E stain. PA is Pancreas analage

4.4.4.4 Localisation of chymotrypsin at 4 Dph

In Figure4-21 below, more intense localisation of chymotrypsin in anlage of the pancreasappears at 4Dph. This is signified by the positive results for the antisense probe shown under various filters as seen in A,B and C.The negative control is provided viathe sense probe D.

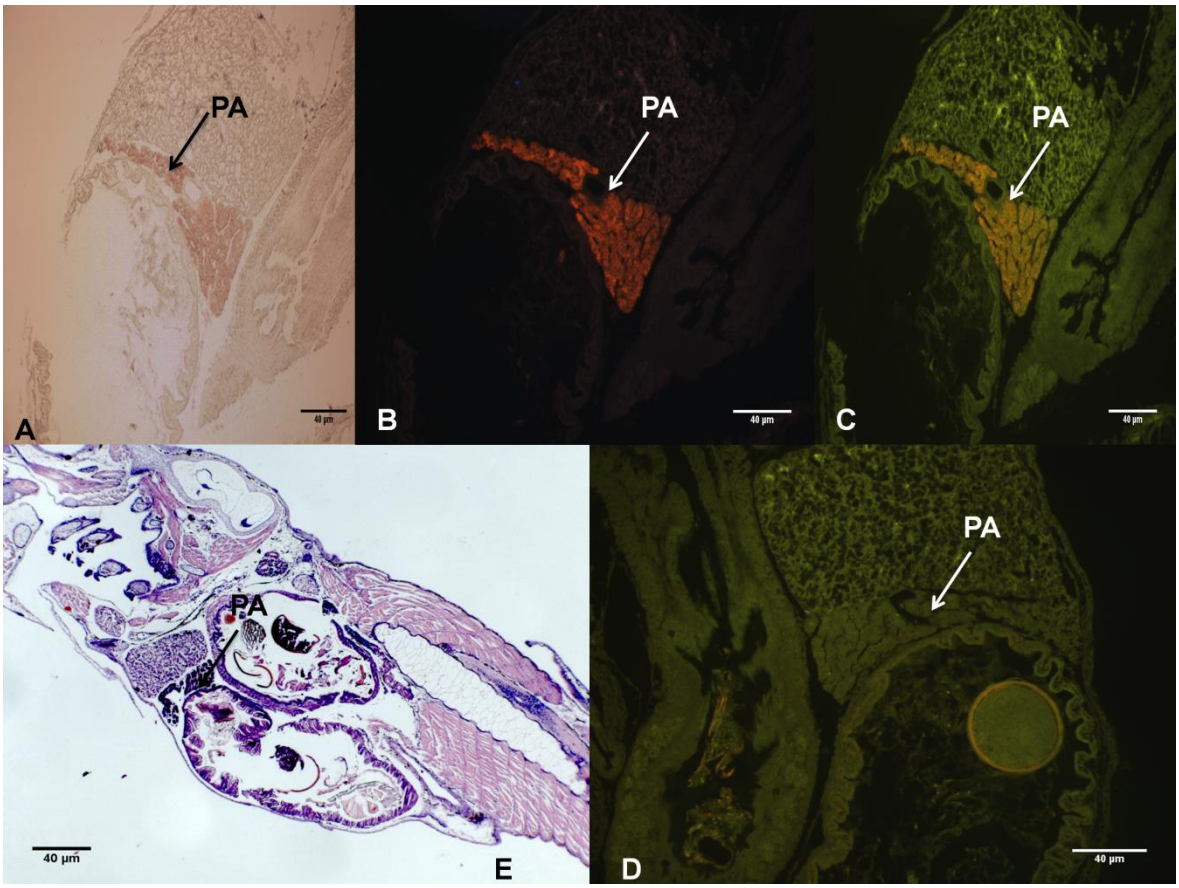

Figure 4-21 showing *in situ* expression hybridisation of Chymotrypsin in the pancreatic anlage of 4Dph larvae of North African Catfish, *Clariasgariepinus* the Pancreas- A=light transmitted B=green filter light =C=Blue filter light is AntisenseD= Sense Probe, E =H&E stain. YS=Yolk sac ;PA= Pancreatic analage

4.4.4.5 Localisation of trypsin at 2 Dph

In Figure 4-22 below, it was observed that the earliestlocalisation of trypsin in anlage of the pancreasappears at 2Dphand it was absent at 1Dph.This is signifiedby the positive results in the antisense probeshown under various filters as seen in A,B and C.D is the negative control sense probe.

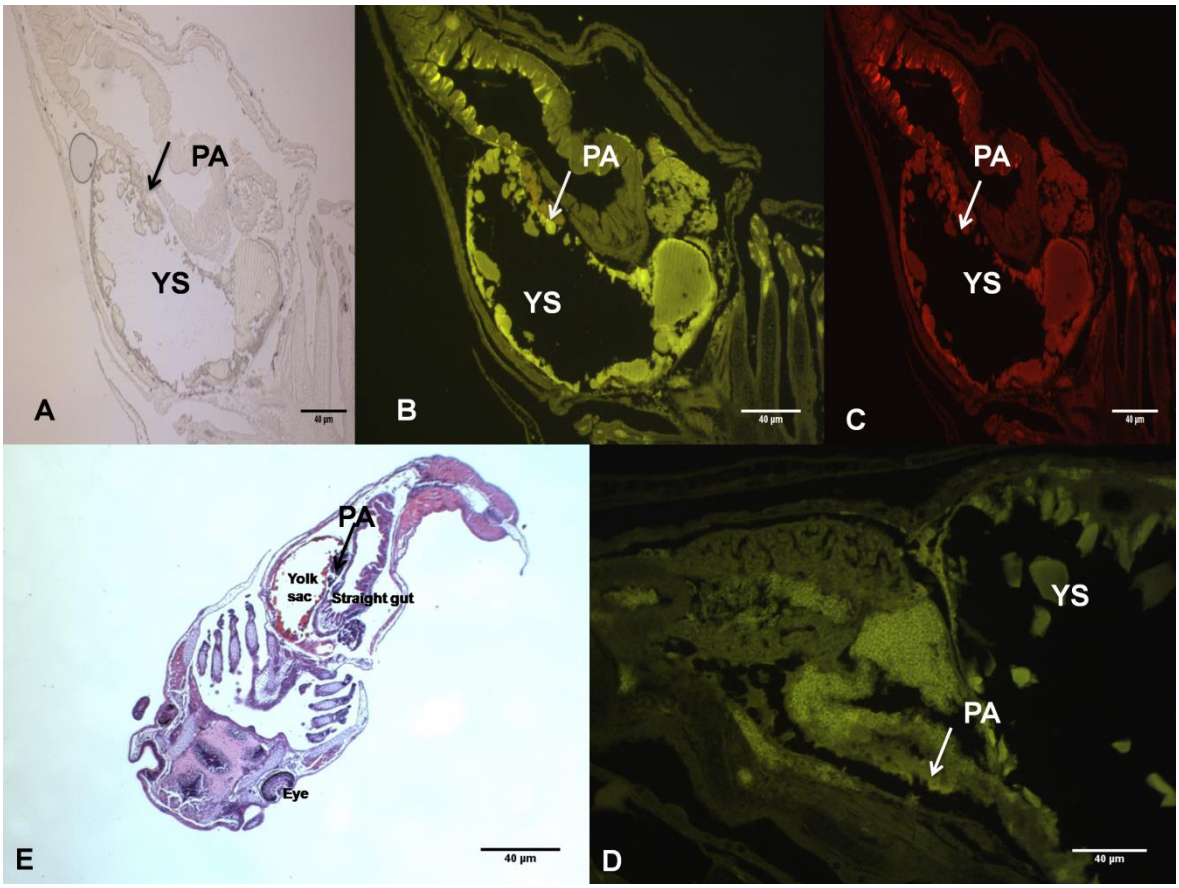

Figure 4-22 shows*in situ* expression hybridisation of trypsin in the pancreatic anlage of 2Dph larvae of North African Catfish, *Clarias gariepinus* Antisense Day 2 Pancreas-in A=light transmitted B=Blue filter light C=green filter light is Antisense D=sense probe & negative control and E= H&E stain. YS Yolk sac, PA =pancreatic analage and GIT Gastrointestinal tract

4.4.4.6 Localisation of trypsin at 3Dph

Fig 4-23 below indicates the continued localisation of trypsin in the anlage of the pancreas at the time the yolk sac is resorpbedat 3Dph .This is signifiedby the positive results in the antisense probeshown under various filters as seen in A B andC.Sense probe Dshows negative control.

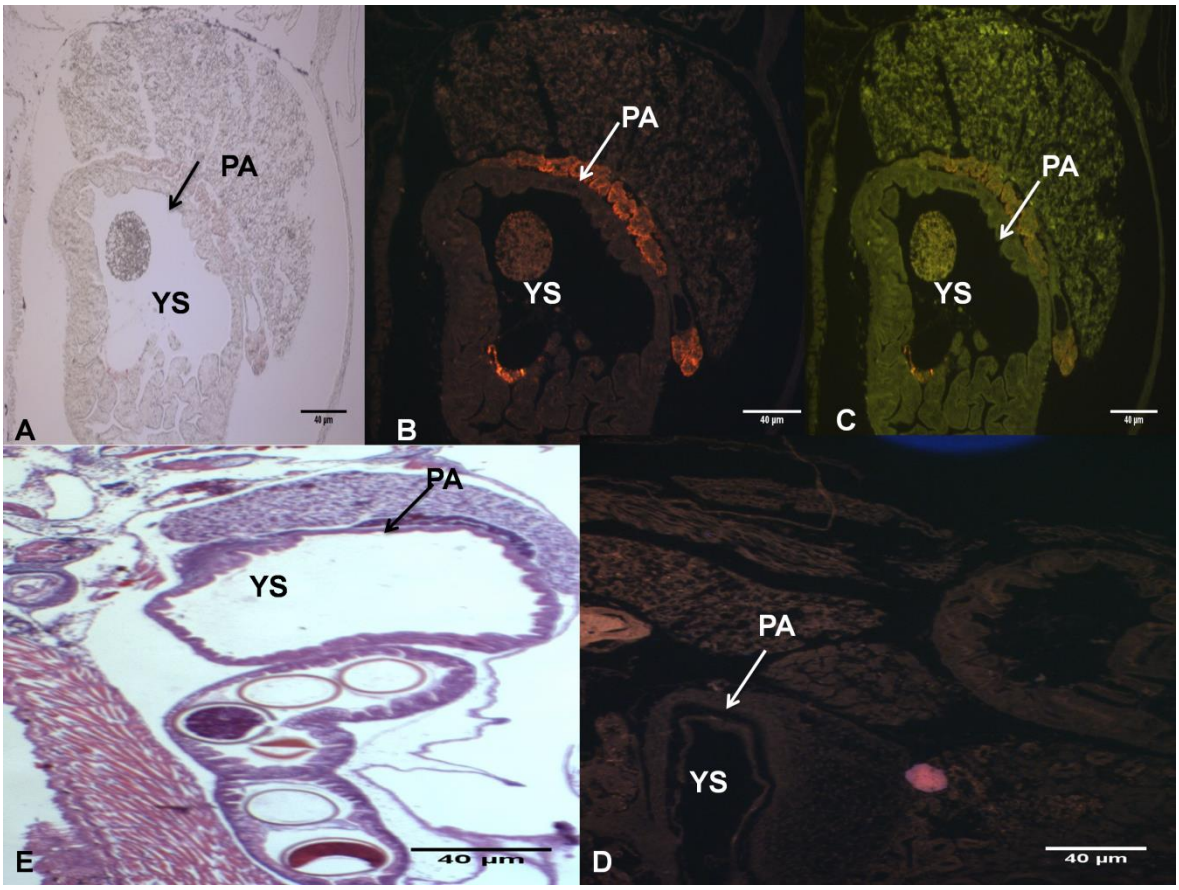

Figure 4-23showing *in situ* expression hybridisation of trypsin in the pancreatic anlage in 3Dph larvae of North African Catfish, *Clarias gariepinus* in - A=light transmitted B=green filter light is Antisense C = Sense Probe D=H&E stain. PA Pancreas analage, YS Yolk Sac

4.4.4.7 Localisation of trypsin at 4Dph

Fig 4-2324 below shows intense localisation of trypsin in a more discrete pancreas at 4Dph. This is signified by the positive results in the antisense probe shown under various filters as seen in A,B and C.The negative control is provided via the sense probe D.

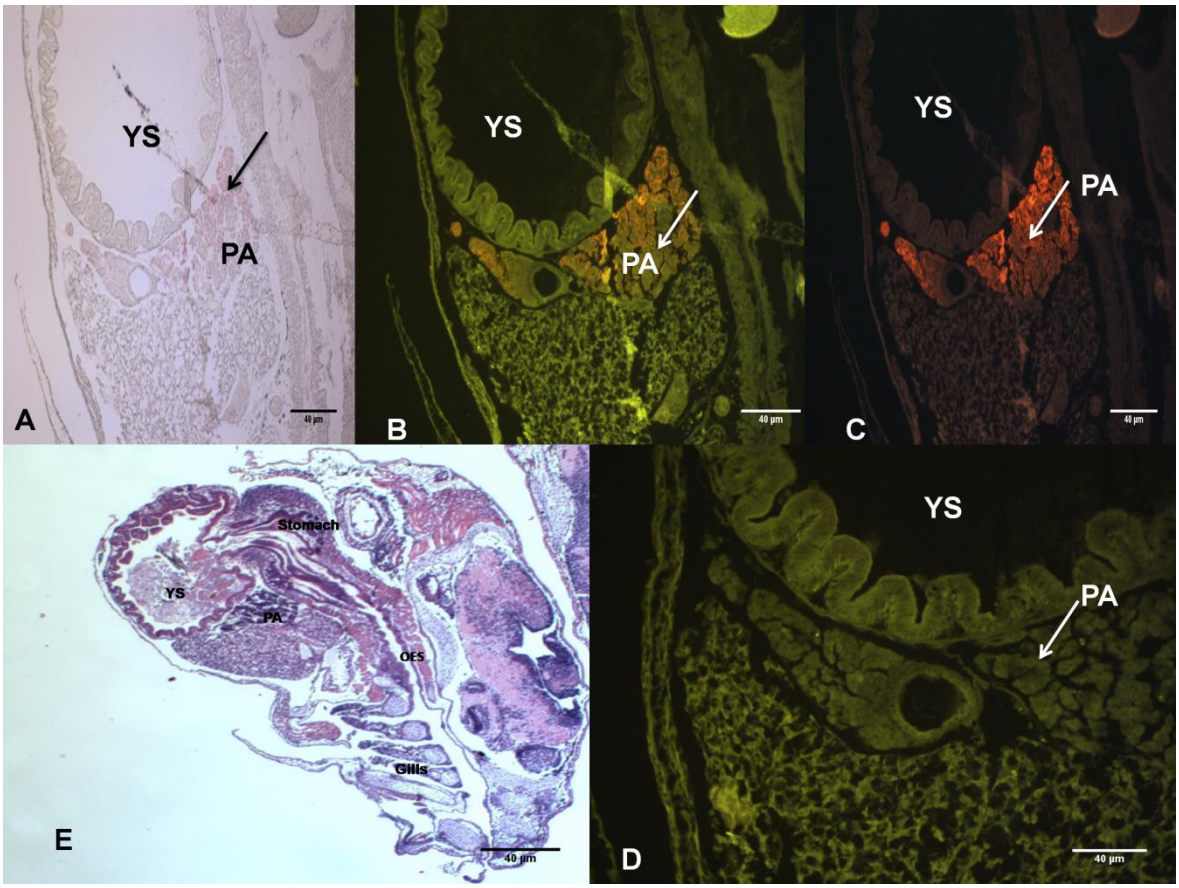

Figure 4-24 showing *in situ* expression hybridisation of trypsin in the pancreatic analage of 4Dph larvae of North African catfish, *Clarias gariepinus* - A=light transmitted B=green filter light =C=Blue filter light is Antisense D= Sense Probe E = H&E stain. YS=Yolk sac ;PA= Pancreatic analage

4.4.4.8 Localisation of pepsin at4Dph

In figure 4-25 below, it was observed that the earlies tlocalisation of Pepsin in the oxynticopepticmcells of the stomach appeared at 4Dph and it was absent at 1Dph,2Dph and 3Dph.This is signified by the positive results in the antisense probe shown under various filters as seen in A,B and C. The negative control is provided by the sense probe D.

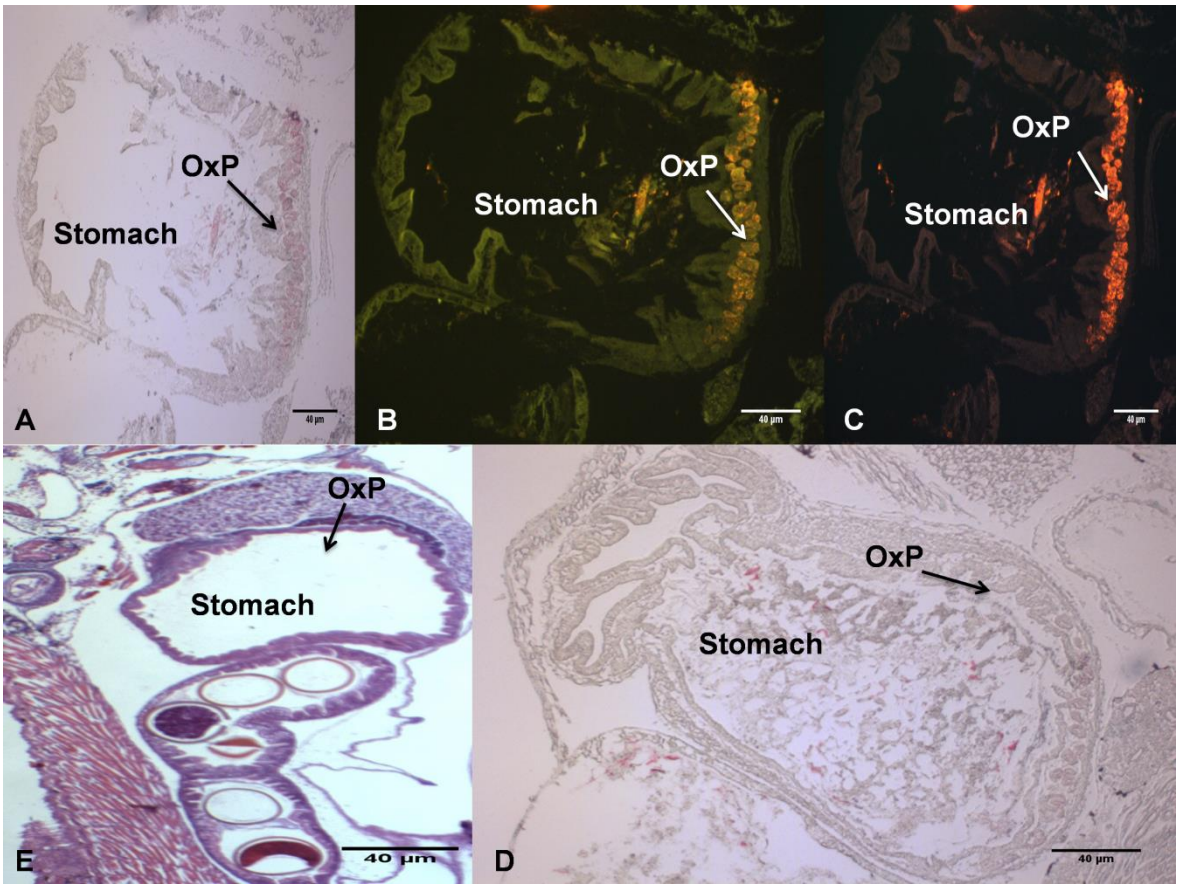

Figure 4-25 showing *in situ* expression hybridisation of Pepsin in the oxynticopeptic cells of the stomach of 4Dph larvae of North African Catfish, *Clarias gariepinus*-A=light transmitted B=green filter light =C=Blue filter light is Antisense. D= Sense Probe E = H&E stain

4.4.4.9 Localisation of pepsin at 6Dph

In Figure4-26 below,it was observed more intense localisation of pepsin in oxynticopeptic cells of the stomach at 6Dph. This is signifiedby the positive results in the antisense probeshown under various filters as seen in A,B and C.The negative control is provided by the sense probe D.

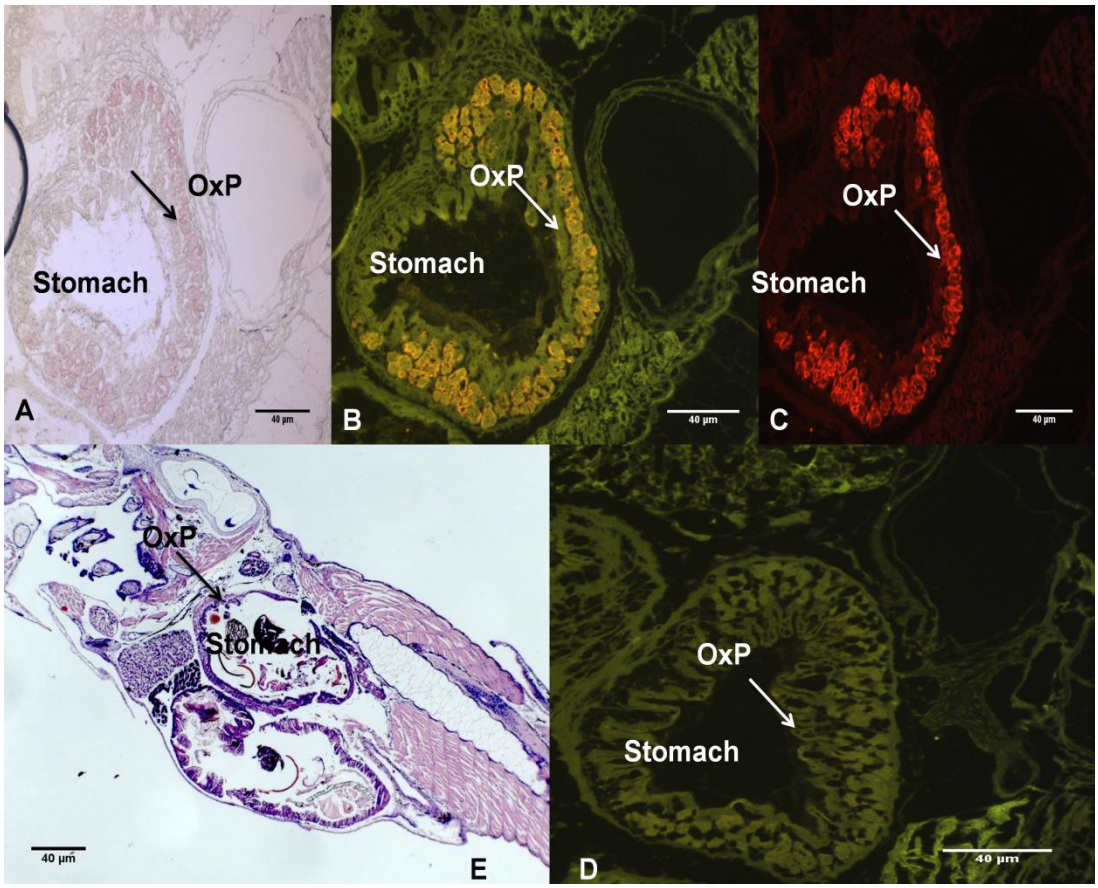

Figure 4-26 showing *in situ* expression hybridisation of Pepsin in the oxynticopeptic cells of the Stomach in 6 Dph larvae of North African Catfish, *Clarias gariepinus*- A=light transmitted B=green filter light =C=Blue filter light is Antisense D= Sense Probe Negative control E=H&E stain

4.4.4.10 Localisation of Pepsin at 8Dph

In figure4-27 below, more intense localisation of pepsin was observed in the oxynticopeptic cells of the stomachat 8Dph. This is signified by the positive results in the antisense probe shown under various filters as seen in A,B and C.The sense probe D provides a negative control.

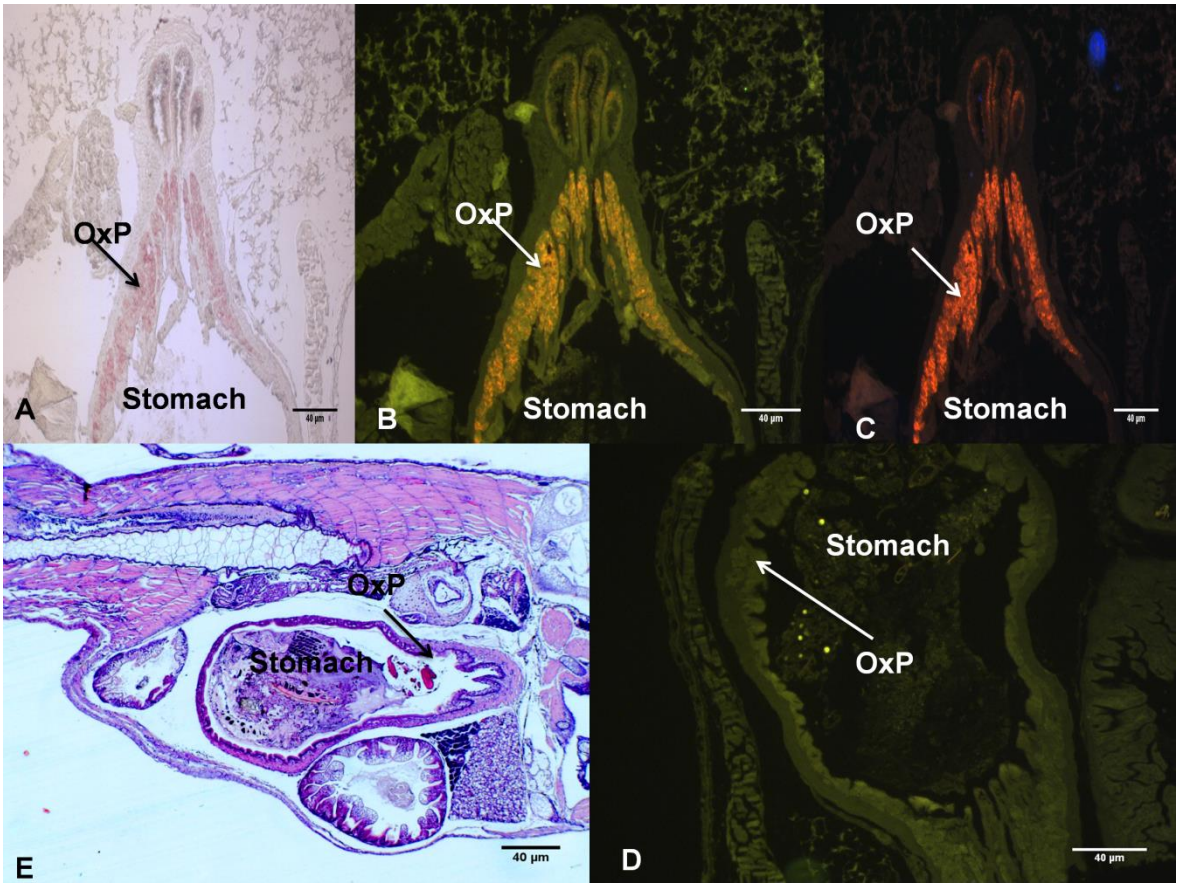

Figure 4-27 showing *in situ* expression hybridisation of Pepsin in the oxynticopeptic cells of the Stomach in Day 8 larvae of North African catfish, *Clarias gariepinus*- A=light transmitted,B=Blue filter light C=green filter light =is Antisense D= Sense Probe E = H&E stain

4.4.4.11 Localisation of gastric chitinase at 4Dph

In figure 4-28 below, gastric chitinase was not present in the gastric pits of the stomach up to Day 4 as the stomach was not yet fully developed for this.This is signified by the negative results in the antisense probe shown under various filters as seen in A,B and C.This contrasts with results of qPCR in chapter 5 where gastric chitinase is switched on by 4Dph

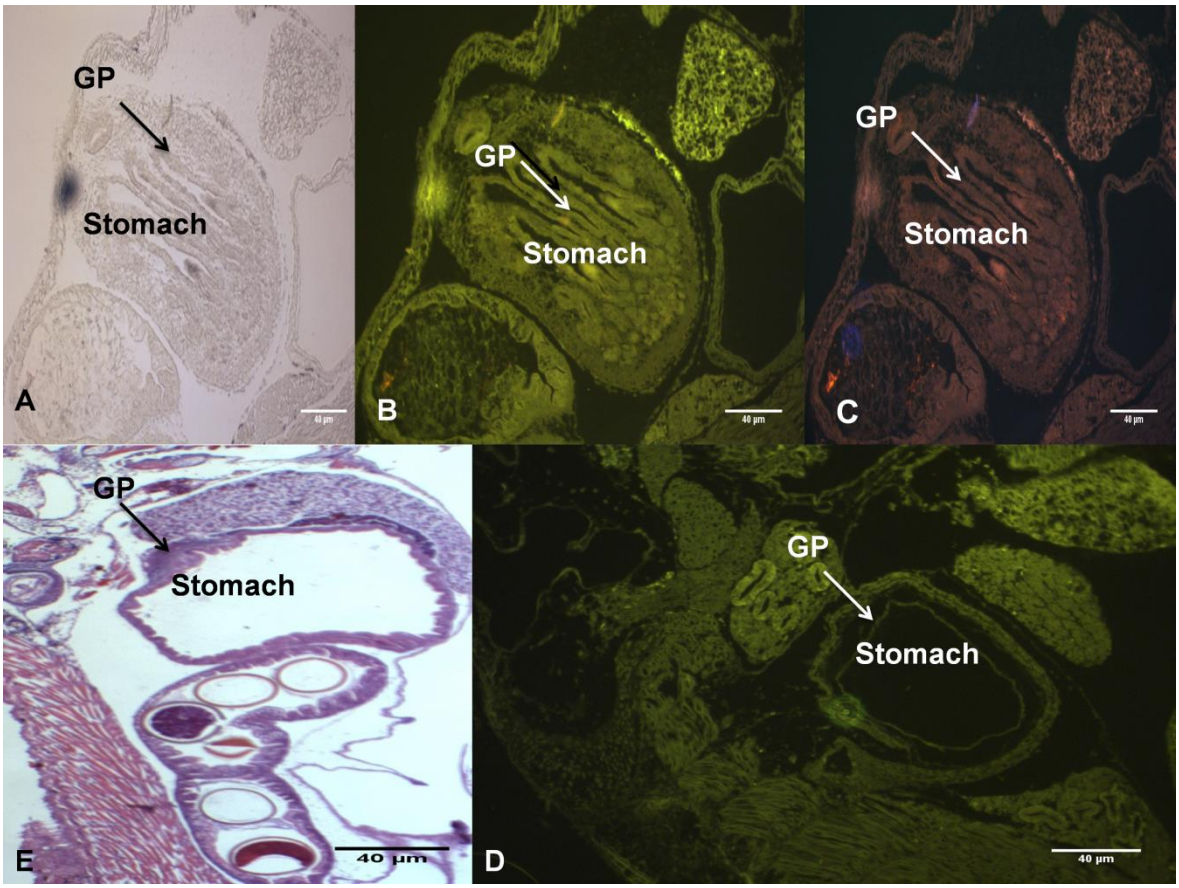

Figure 4-28 showing *in situ* expression hybridisation of gastric chitinase in the gastric pits of the Stomach in 4Dph larvae of North African Catfish,*Clarias gariepinus*. - A=transmitted light B =green filter light =C=Blue filter light is Antisense, D= Sense Probe Negative control E=H&E stain.

4.4.4.12 Localisation of gastic chitinase at 6Dph

In figure 4-29 below, gastric chitinase was seen to be present in the gastric pits of the stomachat 6Dph. This is signified by the positive results in the antisense probe shown under various filters as seen in A,B and C,with the negative control being the he sense probe D.

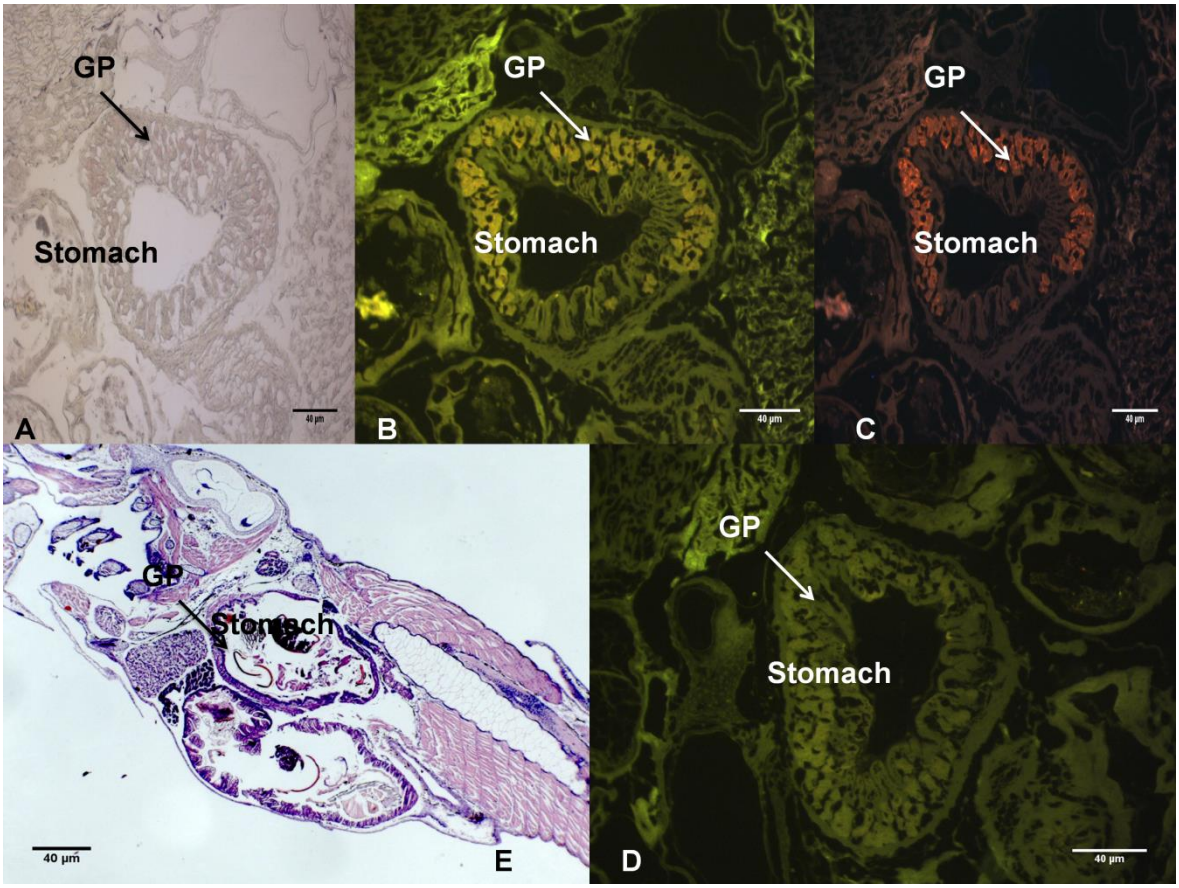

Figure 4-29 showing *in situ* expression hybridisation of gastric chitinase in the gastric pits in the stomach of6Dph larvae of North African Catfish,*Clarias gariepinus*. - A= transmitted light B=green filter light =C=Blue filter light (Antisense), D= Sense ProbeNegative control E=H&E stain

4.4.4.13 Localisation of gastric chitinase at 8Dph

In figure 4-30 below, intense labelling of gastric chitinase was seen in the stomach by 8Dph. This is signified by the positive results in the antisense probe shown under various filters as seen in A,B and C and the absence of labelling in the control sense probe D.

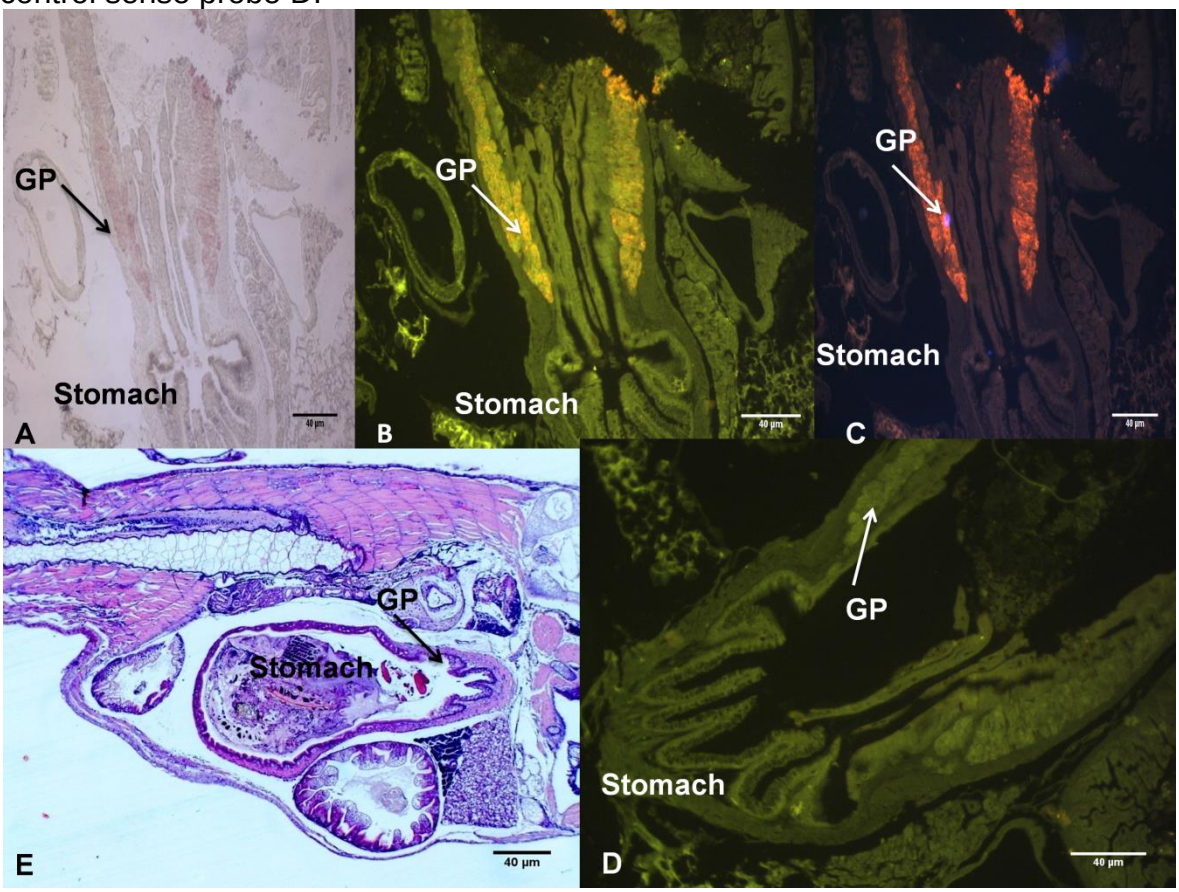

Figure 4-30 showing *in situ* expression hybridisation of gastric chitinase in gastric pits of the stomach mucosa of 8 Dph larvae of North African Catfish, *Clarias gariepinus*- A=light transmitted B=green filter light =C=Blue filter light (Antisense) D= Negative Sense Probe E=H&E stain.

4.5 Discussion

A number of studies have attempted to determine the timing of initiation of exogenous feeding and / or gastrointestinal functionality in *Clarias gariepinus*.The current study is the first to utilize histological and molecular techniques to try and answer this question.

4.5.1 Histomorphology

In terms of gross morphology the stomach appeared as a curved muscular sac, located in the dorso-cranial region of the peritoneal cavity behind the liver. The stomach is a J-shaped sac divided into three regions namely: cardiac; fundic; and pyloric regions. Histologically, its wall is composed of four tunica namely: mucosa; sub mucosa; muscularis externa; and serosa. The mucosa of the three portions showed thick longitudinal folds lined with simple high columnar cells containing oval basally located nuclei. It extended from the oesophagus to intestine. From the histological point of view the results on the development the adult histology of *C.gariepinus* concur with other studies done by (Uys 1989; Segner *et al.,* 1993; Ikpegbu *et al.,* 2012; Ikpegbu *et al.,* 2013; Ekele *et al.,* 2014; Ikpegbu *et al.,* 2014; Ing & Chew 2015; Mokhtar *et al.,* 2015; Moawad *et al.,* 2016).

Although this study didn't go much into depth of the histological analysis of the adult gut except with use of H&E staining, in depth studies on the histology of the GIT of adult North African catfish elsewhere have described in detail the anatomical and histological structures of the stomach of the catfish, using light and electron microscopy of the stomach (Mokhtar *et al*. 2015; Moawad *et al.,* 2017). The stomach is the main site of production of pepsin, produced in the oxyntico-peptic cells, described as polygonal cells with basally located spherical nucleus, and under electron microscopy displaying a prominent central electron lucent nucleolus and with the cytoplasm containing spherical shaped vesicles of different sizes and density, which represent the formation of secretory or zymogen granules. These cells are responsible for production of pepsinogen the precursor for pepsin, the protein / transcript of interest in this chapter.

The gastric *lamina propria* was observed in the fundic region of the stomach of catfish which may help to keep the glands in position and it was rich in collagenous fibres that might form a protective, supporting and strengthening layer in this carnivorous fish with the presence of folded mucosa in fundic region of the stomach and its surface epithelium was lined by simple columnar muco-secretory

cells. The *lamina propr*ia contained simple branched tubular glands. The presence of gastric mucosal folds in the fundic stomach catfish is suggested to allow the fish to accommodate large variations in meal size, by permitting a great deal of distention when excess food is present. The distension serves as a powerful stimulus for gastric digestive secretions including among others gastric chitinase, another of the proteins of interest in this study (Osman & Caceci 1991; Mokhtar et al. 2015).

The pancreas was diffuse in adult *Clariasgariepinus* from the present study and the researcher had to repeat histology several times to be able to identify the pancreas. This concurs with (Uys 1989; Ikpegbu 2012) who also indicated that adult *Clariasgariepinus* has a non-discrete form of pancreas located along the borders of the stomach and proximal intestine. This diffuse pancreas with basophilic zymogen containing cells of the exocrine pancreas is for production and storage of pancreatic enzymes including chymotrypsin trypsinogen, elastase and amylase.

The histology described for the digestive tract in larvae of *Clariasgariepinus*is in agreement with earlier studies by (Verreth et al. 1992; Verreth 1994; Osman et al. 2008; Ing & Chew 2015) with the pancreas being present at 1Dph while the stomach starts to appear between the 3Dph and 5Dph.

4.5.2 Localisation of the sites and timing of enzyme production using *in situ* expression hybridisation

The ability to follow the expression of digestive enzymes at the transcriptional level allows an accurate estimate of GI functionality free from interfering signals from live prey enzymes that would be detected in biochemical assays. In the course of this study the researcher was able to clone and sequence portions of genes that encode four key digestive enzymes in adults and later use developed probes on larval *Clariasgariepinus* for examining expression of chymotrypsin, trypsin, pepsin and gastric chitinase. Trypsin and chymotrypsin were localised in the pancreas, pepsin was localised in the oxyntic peptic cells and gastric chitinase was localised in the gastric pits.

For trypsin the localisation in the pancreas has also been observed in other species, notably Haddock (Perez-Casanova *et al*., 2004) the Winter flounder (Murray *et al*., 2003) and Halibut . (Murray *et al*, 2006).

Results for localisation of the site of production as indicated by *in situ* expression hybridisation, showed that both trypsin and chymotrypsin were found in the pancreas which was diffuse in the adults and was more discrete in the larva .This concurs with other studies The pancreas consists of an exocrine portion that secrets enzyme rich fluid required for digestion and an endocrine portion secreting hormones essential for the control of carbohydrate metabolism (Saadatfar*et al*., 2007). Morphologically, the pancreas of most fishes has differences to that found in mammals. Some fish have their pancreatic tissue formed into discrete organs, some have it scattered as clumps of parenchyma in the mesentries that support intestine. There is pancreatic tissue scattered diffusely in the mucosal lining of the intestine. In Zebrafish the larval exocrine pancreas development occurs over two phases whereby during the first stage, the exocrine anlage forms from rostral endodermal cells and the second stage, proto differentiated progenitor cells undergo terminal differentiation followed by acinar gland and duct morphogenesis (Yee et al., 2005). Results presented here showed that the site was active by 2 Dph in the larvae. At 1Dph there was no indication of the localisation at the site of production, although from chapter 6, the researcher observed that in fact the production of both trypsin and chymotrypsin was present as early as 1Dph.

Results also indicated that Pepsin is localised in the Oxyntic peptic cells of the stomach and gastric chitinase is localised in the gastric pits of the stomach as also noted by Danulat (1987) and Smith (1989). Further, the results indicated that pepsin seemed to be localised by 4 Dph and gastric chitinase seemed to be localised by 6Dph as per the selected protocol meaning that the Oxyntic peptic cells are formed earlier than the gastric pits in the stomach.

4.6 Conclusion

The histomorphology of both Adult and larval *Clarias gariepinus* has been examined and showed similar patterns to those indicated by early researchers, with the pancreas being dispersed throughout the instestinal region in the adults however being more discrete in the larval stages.The stomach was present by 4 to 5 Dph indicating that alkaline protease digestion is the first to take place before the stomach is formed.

From the point of view of molecular localisation, trypsin and chymotrysin are localised in the pancreas as early as 2 Dph while pepsin and gastric chitinase are localised in the stomach but more specifically pepsin in the Oxyntic peptic cells

and gastric chitinase in the gastric pits, with the former being localised by 4Dph and the latter being localised by 6Dph.

5 CHAPTER FIVE

Gene expression of four key digestive enzymes during the larval development of North African catfish (*Clarias gariepinus*, Burchell 1822)Introduction

In Uganda, the generation and processing of fish and fisheries products employs over 1.2 million people and provides up to 50% of animal dietary protein consumed (UBOS2013). A number of factors, which include increasing population (2.88% per annum) (UBOS 2014), declining natural fish stocks due to increased fishing pressure, growing fishing malpractice, habitat destruction and high costs for fisheries regulation have led to a steady decline in the *per capita* fish consumption from 10.2 kg per person per annum between 2003 and 2005 to 5.7 kg in 2010/11 (MAAIF, 2012). This is far below the FAO and World Health Organization recommended *per capita* fish consumption of 17kg and 24kgs respectively (*Global Program on Fisheries: strategic vision for fisheries and aquaculture* 2011). The fast-growing human population of Uganda is further widening the gap between fish demand and supply. It is now widely accepted that it will be difficult to increase fish production much above its current level on a sustainable basis based only on improvement in management of capture fisheries in the country. There is therefore a need to learn from experiences of countries where insufficient fish production has been addressed through adoption of commercial aquaculture.

The species which have demonstrated aquaculture yield potential in Uganda include; Nile tilapia (*Oreochromis niloticus*), African catfish (*Clarias gariepinu*s) and the common carp (*Cyprinus carpio*). The African catfish (*C. gariepinus*) has become a popular species for aquaculture in sub-Saharan Africa and from a biological perspective, is one of the most suitable species for aquaculture globally (Safina, *et al.* 2012). It is mainly being cultivated for food, but is also used in tilapia ponds to control over population and grown as bait fish for the lake Victoria Nile perch fishery. *C. gariepinus*has been found to be one of the fish species that are abundant in almost all the waters of Uganda, especially those linked to swamps and wetlands and has traditionally been a primary target for a good segment of the fishing community. It has a number of useful biological traits, including omnivorous feeding habits, ability to feed on both natural and supplemental feeds, resistance to disease, tolerance of low oxygen levels, crowding and fluctuating pH levels,

high fecundity and easy spawning under captive conditions (Nyina-wamwiza, *et al.* 2010) These factors make it a highly desirable candidate for aquaculture for most farmers on the African continent.

Because of its hardiness and ability to withstand adverse environmental conditions (Safina *et al.* 2012), African catfish comprise a critical standing crop in aquaculture and is particularly amenable to the farming practices of small scale holders who comprise the majority of farmers in developing countries like Uganda. Seed production in African catfish, particularly rearing of larvae and fry, are the most critical steps in aquaculture (Herath& Atapaththu 2013). Among other factors, lack of the most reliable diets, which provide essential nutrition for growth and survival of fish, especially at fry level, strongly affects aquaculture production both in hatcheries and grow-out facilities.

Uganda's highest producing commercial catfish hatcheries produce about 500 to 30,000 larvae per female brood stock with an average earning of UGX 100,000-6,000,000UGX (approx 29-1700 USD) from each broodstock per year. This can be compared to Nigeria, where on average the hatchery operator produces 60,000 fingerlings per annum, earning an equivalent of 12,000,000/=UGX (approx 3400 USD) per broodstock per year (Owori-Wadunde & Kityo 2014).In the case of Ugandan hatcheries, high larval mortality is reported from newly hatched eggs through larval stages to juvenile stages, primarily due to poor feeding, for example on dry diets. However, these early catfish stages require a live feed, since in terms of embryology and early development they are not fully developed, with the fertilized eggs of African catfish hatching into larvae at a size of 3.5-4.00mm in length with no eyes and no gut. Without a developed gut, catfish larvae rely on limited yolk supplies before development of the digestive system, hence can'tfeed on an artificial diet. Two or three days following hatching the yolk sac is depleted but the digestive system is not fully developed (Owori-Wadunde & Kityo2014; Verreth, *et al.*1992) It is perhaps not surprising therefore that larval nutrition, and in particular that of the sensitive first-feeding larvae, has become one of the major bottlenecks preventing the full commercialization of African catfish and many other farmed fish species (Conceição *et al.*2003; Conceição, *et al.*1998; Conceição, *et al.*1998; Hamre, *et al.*2013; Olurin & Oluwo 2010). Moreover, formulated feeds do not generally meet all these requirements and usually result in poor growth and survival of small fish larvae as well as fry. The natural diet of most cultured fish

consists of a wide diversity of phytoplankton species (diatoms, flagellates, *etc*.) and zooplankton organisms (copepods, cladocerans, rotifers, *etc*.), found in great abundance in natural waters. This abundance and high diversity of food organisms of different sizes and nutritional composition provide maximal chances for meeting all the requirements of the predatory larvae and fry. Hence, there is an urgent need to address the issue of development of digestive capacity / nutrition and one approach to this is the use of molecular tools, including transcriptomics, to enable us to understand what enzymes are initially available post-hatch. Such knowledge could be used in supplementing larval requirements and providing high quality off - the-shelf feeds, which farmers could access in quantity and at a favourable cost, thereby promoting aquaculture development.

In raising African catfish, live feed confers a better specific growth rate for larvae compared to the use of decysted Artemia cysts (Abaho, *et al.*2016; Chepkwemoi, *et al.* 2013).Many studies on the nutrition of larval catfish have centred on the use of live feeds in the first few days post-hatching including decystedArtemia, Artemia enriched by ascorbic acid, rotifers, mixed live feed and dry diet (Kwikiriza, *et al.*2016; Nyina-wamwiza, Wathelet & Kestemont 2007) use of lyophilized egg white augmented with cod liver oil (Bukola, Bombata & Elegbede 2015) and more recently probiotics (Dennis & Uchenna 2016)as first feed for the larvae of *Clarias gariepinus*.

Complete substitution of live feed for larval fish is next to impossible because of the exceptional nourishment that live feed provides (People Le Ruyet, Alexandre, Thébaud *et al.* 1993) However, many disadvantages follow when using live feed, including the need for demanding labour in the case of raising copepods where there are no current production protocols for the hatchery production of larval fish available (Rasdi & Qin 2016).In the case of algal cultures and *artemia* there is need for very good knowledge in order to sustain their production (Roy & Pal 2015).Rotifers as a source of food for fish require high costs and experience to manage when they attain the maximum growth, as well as suitable facilities for production in large numbers, which is also costly in terms of time and money. It may involve having to go the wild to collect the initial stock which is problematic if one needs to culture enough stock for sustainable maintenance of the enterprise. Given projected requirements for live feed, in the case of Uganda, the costs and

techniques involved can't easily meet the increased need for commercialization of aquaculture. (Bwanika Pers Com 2010).

5.1.1 Aims and Objectives

The principal objectives of the work described in this chapter originate in the hypothesis that: The key digestive enzymes pepsin, chymotrypsin, trypsin and gastric chitinase are present at hatching in catfish larvae, and can be described as follows:

5.1.1.1 Major objective

To characterise the ontogeny of gene expression of two key alkaline proteolytic, one acidic proteolytic and one chinolytic enzyme using three reference housekeeping genes namely beta actin, elongation factor 1 (EF1) and tubulin (Tuba).

5.1.1.1.1 Specific objectives

To establish whether the key enzymes, chymotrypsin, trypsin, pepsin and gastric chitinase are expressed during early development of C. gariepinus larvae

To examine the ontogeny of transcript (mRNA) expression for the above enzymes during early development

To determine the quantity available at the sites of enzyme expression in developing larvae

5.1.2 Justification

The development of an off-the-shelf micro particulate diet for small larval fish is one of the most important nutritional technologies needed by the aquaculture sector (Rust 2003) and therefore development of microdiets is urgently needed for replacement of live feeds, as this will result in increased seed production, improve the consistency of hatchery production and lower the costs of production. The major hurdle to overcome is improvement of the acceptability and digestibility of the diet. It has been observed, however, that a major mismatch occurs between the current technology being used to make microdiets and the functional development of the sensory and digestive system in larval fish (Conceição, *et al.*2011; Rønnestad, Tonheim, Fyhn *et al.*2003; Rust 2003; Rust, Hardy & Stickney1993).

As described above, the culture of North African catfish in Africa is critically dependent upon the provision of sufficient, high quality fry, and this is particularly true for Uganda, which provides fry for internal and extra-national culture. The

importance of catfish production for subsistence farming and small local businesses in Uganda means that a better understanding of larval physiology is urgently needed as a pre-requisite for development of feed methodologies and technologies that may be used successfully for both subsistence and commercial culture.

Knowledge of differentiation of the digestive tract and accessory glands during larval development is essential for understanding the digestive and nutritional physiology of larval fish so that feeding practices and rearing protocols may be synchronised with the physiological stage of development of the fish. In terms of producing initial baselines, it is clear that ontogenetic patterns and biochemical characteristics of larval digestive capacity need to be determined. In this respect, the determination of changes in enzyme activity during larval development may be useful in assessing the optimal moment for weaning as well as the dependence of larvae upon exogenous sources of enzymes (*i.e.* live feed) (Applebaum,. Lazo *et al.* 2001; Verreth & Van Tongeren 1989). Although the biochemical quantification of digestive enzymes has proven a very widespread and useful tool for assessment of the digestive capabilities of fish larvae, molecular tools have gained increasing importance over the last decade for studying the ontogeny of digestive enzyme gene expression, allowing understanding of the mechanisms underlying the digestive physiology of young fish (Lazo, Darias & Gisbert 2010; Zambonino-Infante, *et al.*2008).

The work described in this chapter aimed to examine aspects of the expression of two alkaline proteolytic enzymes (chymotrypsin and trypsin) one acidic proteolytic enzyme (pepsin) and one chitinolytic enzyme (gastric chitinase) during the first ten days of post-hatch larval development, these being key functional components of the digestive system of *C.gariepinus* larvae. In particular, special attention was paid to the question of what happens during the critical period spanning 2nd to 4th days of post-hatch larval development, this following the recommendations of (Verreth,1994) to provide deeper understanding of early development. In order to contextualise expression of the selected enzymes, a broader scale transcriptomics study was also conducted to examine wider gene expression during early development (see Chapter 6).

5.2 Review of related literature

5.2.1 Introduction

Digestion in larval fish is related to feeding activity and therefore larval fish need a highly dynamic digestive system to be able to process the enormous amount of food taken in as a means to support ontogeny of the larval fish (Conceição, Morais & Rønnestad 2007; Murashita, Fukada, Takahashi *et al.* 2015; Yúfera, Moyano & Martínez-Rodríguez 2018).At the functional level, enzymatic studies of larval fish have focussed more on pancreatic enzymes including amylase andbile-activated lipase (BAL),trypsinand chymotrypsin have also been studied in various fish species as has pepsin. However, there seems to more emphasis on some aspects than others, for instance not much has been done to study changes of enzyme along the intestine and brush bordered epithelium at the mRNA expression level when compared to enzymatic activity (German, Horn & Gawlicka 2004; Yúfera *et al.* 2018). The relationship between gene expression and enzyme activity is not well known since expression and translation to stored products may occur substantially in advance of enzyme activity.

Usually, live feeds, most commonly zooplankton, are used as the first diet for larval fish and these are thought to ease the process of digestion by incorporating their own digestive enzymes by autolysis and or zymogens. Live feeds are also known to impart neuropeptides and nutritional growth factor to improve larval nutriment during formulation of inert diets for larval fish. (Kolkovski 2001).

5.2.2 Gene expression studies in relation to enzymes

Phenotypic expression reflects the interactions of genes and the ambient environment (Kruk & Bottema 2005). In the case of fish, nutrients, defined as any compound required for the sustenance of life, are critical to survival and key life outcomes *e.g.* reproductive contribution.

In farmed fish species, it has been observed that gene expression can be controlled nutritionally (Panserat& Kaushik 2010). This has been the case for rainbow trout, *Oncorhynchus mykiss*,where amylase expression is responsive to increased carbohydrates (Geurden, *et al.* 2007) and for European sea bass,*Dicentrarchuslabrax,*where gene expression of trypsin is responsive to increased protein intake (Cahu & Zambonino-Infante 2001; Zambonino-Infante & Cahu 2001),although in the latter case the transcription remains constant in at the start but increases with later development of the larvae (Cahu, *et al.* 2004).

In the past few years a lot of scientific works have been done on digestive activities of fish larvae (Yúfera *et al.* 2018).However, most of these studies have concentrated on the ontogeny and hydrolysing effects with not so much on the functional aspects of feed and feeding. A relationship exists between feeding behaviour and the digestive process at genomic and biochemical levels and elucidating these links is a much needed step towards attaining a more complete solution to current larval fish feeding protocol problems faced in use oflive and inert feeds.This will improve feed uptake by larvae allowing them to attain better growth during early development.

In recent years, high level molecular techniques have provided an important breakthrough in developing an understanding of the mechanisms for uptake and metabolism of nutrientsandfor resolving their rolesinorganisms (Lall & Dumas 2015).Nutrients can also serve to modulate metabolic processes and can act virtually at various molecular and cellular sites, including direct involvement in the transcriptional process and the expression of genes (Lall & Dumas 2015).

The ability to measure profiles and levels of transcript expression for digestive enzyme precursors has provided a valuable tool that complements information about the nutritional condition of an organism that may be obtained through enzymatic indicators (Lazo *et al.* 2010).This may prove particularly helpful in aquaculture where nutritional requirements for fish larvae need to be optimized and the basis for suboptimal larval growth and performance derived from food supply is often unknown. With the increasing knowledge being generated by gene cloning and genome and transcriptome sequencing programs for different aqua cultured species, researchers and producers have thus been able to obtain a wider view of how nutritional factors influence animal health and productivity (Conceição, *et al.* 2010).

Young (2000) has redefineda nutrient as: "*a fully characterized (physical chemical, physiological) constituent of a diet, natural or designed, that serves as either (i) a significant energy yielding substrate, (ii) a precursor for the synthesis of macromolecules and/or compounds needed for normal cell differentiation, growth, renewal, repair, defence and/or maintenance, (iii) a required signalling molecule, cofactor and/or determinant of normal molecular structure/function and/or (iv) a promoter of cell and organ integrity.*"This therefore means that nutrients as dietary constituentsare considered to be physiologically important incellular and animal

metabolism and essential for optimum growth, reproduction, andhealth (Lall& Dumas2015).Nutrients are classified into macronutrients (protein, lipid, carbohydrate) and micronutrients (vitamins, minerals).The metabolism of essential nutrients is continuously under physiological control within cellsor between cellsunderhormonalcontrol. It has also been noted thatnutrient requirements across the key stages of development during the life cycleof fish of various species vary, with alterationin diet composition also influencingthe nutrient makeup of flesh and product quality (Lall & Dumas 2015).

In most larval fish, after mouth opening, fish larvae are exposed to variable food quantities and qualities and exhibit remarkable phenotypic plasticity in response to external factors including diet type and food availability, which is expressed through adaptation of digestive enzyme activities in terms of levels of secretion and or gene expression (Pittman, *et al.*2013; Zambonino-Infante *et al.* 2008). Most studies on mRNA expression in response to diet, however, have been conducted with marine fish species, with only a few studies having involved freshwater or anadromous fish*e.g.*salmon,rainbow trout, carp and tilapia,withno studies having covered North African catfish in terms of digestive enzyme expression.The current study on the digestive physiology of North African Catfish *C.gariepinus*larvae will provide a resource for one of the most important fish species in aquaculture in Sub-Saharan Africa.

The stomach of *C. gariepinus larvae* is not functional at the start of exogenous feeding (Stroband& Kroon 1981) and only becomes functional on day 5 after the start of exogenous feeding (Verreth*et al*. 1992). In fish larvae with a digestive system that is still in development, different food consumption and evacuation might be expected because of the effect of developmental and physiological processes during food digestion. In catfish larvae, the role of the stomach during food digestion might be limited to storage of food before it becomes completely functional, as is the case for most cultured catfish species worldwide. The ability to follow the expression of digestive genes over time by transcriptional profiling has allowed scientists to accurately estimate digestive system functionality (Murray, *et al.* 2006; Ruan, Li, Gao *et al.* 2010).

5.2.3 Action of alkaline proteolytic enzymes trypsin and chymotrypsinin different fish species

Trypsin acts as an activator of pancreatic digestive zymogens, as well as of its own inactive precursor, trypsinogen. To avoid an accidental activation of the zymogens in the pancreas, trypsinogens need to be activated by enterokinase (Manchado *et al*., 2008)).

The pancreas in fish has been associated with production of alkaline leaning digesting proteolytic enzymes, particularly trypsin, Trypsin is especially important during the early life stages of altricial fish, due to the absence of a functional stomach (Manchado et al., 2008) (Gilannejad *et al*., 2019). This serine alkaline protease, produced as the inactive precursor trypsinogen (try), has two major functions namelythe hydrolytic function, and playing an important role in activation of other pancreatic enzymes in the gut lumen (Ueberschär 1993; Rønnestad *et al.* 2013). In most studies, trypsin activity and/or expression pattern is characterized by a sharp peak, at different ages during larval ontogeny, followed by a decrease, normally attributed to its partial replacement by the activity of pepsin in developing stomach (Rønnestad *et al.* 2013; Yúfera *et al.* 2018). Acidification capacity and pepsin-like enzyme activities develop during metamorphosis (Yúfera et al. 2004; Darias et al. 2007, 2012). Gastric H+/K+-ATPase or proton pump, exchanges proton with potassium using energy from ATP to generate hydrochloric acid, which is necessary for converting the inactive precursor pepsinogen (pga) into pepsin (Gawlicka 2001; Darias et al. 2005). Unlike pancreatic proteases, pepsin has a broad range of active sites and allows a more efficient digestion.

5.2.4 Action of pepsin in different fish species

Fish with a stomach exhibit variable pepsinogens within species as exemplified in various studies, for example in seabream,*Sparus latus Houttuyn*, 4 pepsinogens (PGS)have been characterised (Q. Zhou, Fu, Zhang, Su, & Cao, 2007), mandarin fish,S*iniper cachuat*si,with 4 PGs (Q. Zhou *et al*., 2008),Snakehead;*Channa argus* has 3PGs (Chen *et al.,* 2009),European eel,*Anguilla Anguilla* with 3 PGs (Wu, Sun, Du *et al.*2009), albacore tuna,*Thunnus alalunga* has 1PG (Nalinanon, Benjakul, & Kishimura, 2010),Japanese seabass,*Lateolabrax japonicus* has 3 PGs (Cao *et al*., 2011),largemouth bass, *Micropterus salmoides* has 6PGs (Miura, Kageyama, & Moriyama, 2015).Pepsinogens are produced in the fish stomach in

the gastric glands specifically in the oxynpeptic cells. However, in some fishes like puffer fish, *Takifugu rupbripes,*lack gastric glands and in this species pepsinogen is expressed in the skin and gills because they are without stomachs and enzyme production is not related to nutrient intake but rather to protectionof the skin (Kurokawa, Uji & Suzuki 2005).

Larval fish gene expression studies have been conductedon key digestive enzymes including trypsin, pepsin, bile activated salt lipase (BAL),Lipase, alkaline phosphatase (AP) and a-amylase in larvae of haddock,*Melanogrammus aeglefinus*, and Atlantic cod, *Gadus morhua*,where all enzymes are detectable except lipase.Trypsin was seen to be present at hatch when the pancreas is detectable and pepsin is available when the stomach is present, however, in haddock, trypsin was higher at hatch while in Atlantic cod it was less and increased with age (Perez-Casanova, *et al.*2006). Similar trends of gene expression of the enzymes pepsinogen (pg), trypsinogen (try), chymotrypsinogen (ctr), bile salt-activated lipase (bal), α-amylase (amy), leucine aminopeptidase (lap) and alkaline phosphatase (alp) were observed in Asian seabass, *Lates calcarifer*,

Studies on pepsinogens and pepsins from rainbow trout (*Oncorhynchus mykiss)* (Antunnes*et al* 2013), Pacific blue fin tuna (*Thunnusthynuus*orientalis) North (Serra *et al* 2000, Serra Diaz *et al* 1992), (*Gadus morhua*) Atlantic cod (Rojo *et al* 2002), and Portuguese dogfish (*Centroscymnus coelolepsis*), which have been well documented. More recently, pepsinogens and pepsins from Antarctic rock cod (*Trematomus bernacchii*) (Hassan *et al* 2010), African coelacanth (*Latimeria chalumnae*) (Foster *et al* 2004), and giant grenadier (*Albatrossia pectoralis)* (Gilbert, 1892)) (Richter *et al* 1998) have been reported. However, all these studies have been performed on marine fish. Only a few studies concerning pepsinogens and pepsins from freshwater fish have been reported. Notable among the studies Pepsinogens and Pepsins in fresh water fishes from Mandarin Fish (Siniper cachuatsi) (Zhou et al., 2008)and in 2 snakehead fish species namely *Channa punctata* and *Channa striata* (Borman et al., 2015) which indicated 4 Pepsinogens occurring in the former namely (PG-I, PG-II, PG-III (a), and PG-III (b),determined by biochemical means, and observed histomorphologically in the latter.

Most fish larvae possess a complete digestive tract, which fully becomes functional inclusive of gastric digestion, and is completely developed in weeks to

months and varies from species on species. (Rønnestad, *et al.*2007).The larval digestive system is capable of supporting growth as long as there is natural food supply in the aquatic environment.

Gastrointestinal development in larval fishis thought to be genetically programmed (Zambonino Infante & Cahu 2001).The stomach, pancreas and intestine are not fully developed in most larval fish species and this is so for the case of the stomach especially from the structural point of view.it has been observedtooccurmuch later than inthe pancreas as evidenced in many fish species including sole,Europeanseabass and Atlantic salmon.It has been suggested that initially, fish exhibitalkaline protein digestion based on trypsin and chymotrypsin,which is less efficient than the acidic protein digestion of pepsin, with a transition occurring between larval and juvenile stages of development (Darias, *et al.*2007).

According to Yufera *et al* 2012, two different modes for regulation of stomach acid secretion have been described in vertebrates. Some species exhibit a continuous acid secretion maintaining a low gastric pH during fasting and others, including some teleosts, maintain a neutral gastric pH during fasting while the hydrochloric acid is released only after the ingestion of a meal. (Cao, *et al.*2011; Chen, *et al.*2009; Miura, Kageyama & Moriyama2015; Nalinanon, Benjakul & Kishimura 2010; Zhou *et al.* 2007; Zhou, *et al.* 2008). The appearance of functionally developed gastric glands is commonly considered as a sign of the transition from the larval to the juvenile stage in fish as it means there is a switch over from the less efficient alkaline digestion to the more efficient acid digestion characteristics of adult fish (Zambonino Infante & Cahu 2001, Darias *et al* 2007).

Freshwater fish undergo both major morphological and cellular changes during their life, just like their marine counterparts. The development of the gastrointestinal tract (GIT) inyellow catfish, *Pelteobagrus fulvidraco,*larvae at the histological level shows serial growth of oxynticopeptic cells,important in acid-secreting and pepsinogen-secreting functions (Yang, *et al.*2010).In white seabass, *Atractoscion nobilis*,larvae it has been observed that GIT is present at 3 Dph, however, the stomach is fully functional between 16 to 18Dphtherefore the switch to an inert diet is only possible then when pepsin is produced in increased in amounts (Galaviz, *et al.*2011).

Gene expression studies of trypsinogen and pepsinogen in Indian walking catfish, (*Clarias magur*), showed that at 3Dph mRNA expression of trypsin was present,peaking at 9 Dph and 22 Dph, while mRNAs of pepsin were present at 4 Dphpeaking inexpression at 19 and 22 Dph. However the specific activity of trypsin increasedfrom 11 dph onwards up to 16 Dph showing that the pancreas was present (Mir, *et al.*2018). This has implications for all studies conducted so far,int hat, from the molecular point of view, the enzymes nominally appear to be expressed before functional activity can measured.

5.3 Materials and methods

Bioinformatics using Channel catfish *Ictarulus punctatus* used as the initial resource. The initial step involved data mining for expressed sequence tags (ESTs) from the National Center for Biotechnology Information (NCBI) website accessed on URL: http://www.ncbi.nlm.nih.gov/unigene more specifically the unigene project: http://www.ncbi.nlm.nih.gov/UniGene/lbrowse2.cgi?TAXID=7998

In studies involving quantitative real-time reverse transcription-PCR (RT-PCR) usually reference genes or housekeeping genes are a requirement (Andersen, Jensen & Ørntoft 2004; Small, Murdock, Bilodeau-Bourgeois *et al.*2008).This is to provide standardisation through constitutively expressed control genes (Andersen *et al.*2004). For this study suitable reference genes were sought that were in line with the generally accepted criterion,*i.e.* genes that exhibited little expressional variation across the samples and states under study (Andersen *et al.*2004).

Three reference genes, namely beta actin (ACTB), elongation factor-1 alpha (EFF1A) and alpha tubulin (TUBA)were selected (Small *et al.*2008).Four target genes of interest, namely pepsin, chymotrypsin, trypsin and gastric chitinase were also identified through homology searching and downloaded and saved as FASTA files. The selected genes were aligned using bioedit (ver. 7.0.5) and Seqman™ (DNASTAR ver 6.1) software, followed by blasting using BLASTx and BLASTn in NCBI to find conserved regions based on the knowledge that channel catfish, *I. punctatus* and African catfish *C. gariepinus* are related, albeit distantly, in evolutionary terms. Primers of size range 320-650 base pairs (bp) were designed to amplify the identified targets as shown in table 5.4 and 5.6 below. These primers were designed using the program Lasergene® Primer Select™ (DNASTAR Inc. ver. 6.1). Primers were designed to amplify conserved regions in *I. punctatus* transcripts and were then tested using complementary DNA (cDNA)

prepared from the digestive system of the adult catfish *Clarias gariepinus* using standard polymerase chain reaction techniquesq PCR Data Using Double Delta Ct Analysis - The scheme of work is shown in Figure 5.1 and tables 5.4-5.7 below for use during Standard PCR (300-650 bps) and qPCR (.100-200bps).

Table 5-1 showing Standard PCR primers prepared using Channel catfish EST sequences and amplified using Standard PCR using African catfish cDNA

Gene	**Primer/probe**	**TM [°C]**	**Amplicon size (bp)**	**Accession Number**
Chymotrypsin Forward	TGTAAAGAGGGTTGTGGGTGGTGA	60° C	644	NP_ 001188117.1
Chymotrypsin _Reverse	TCAGGCCAGAGCCAAAGCTCACA	60° C		
Trypsin _Forward	GCCATGGATGGTGTCTCTGCACT	60° C	574	NP_ 001187619.1
Trypsin _Reverse	GCCTGGTTGGCCACAGCCTT	60° C		
Gastric chitinase_Forward	CAGCCAGAACGTTTGGGTTGGC	60° C	327	NP_001153946.1
Gastric chitinase_Reverse	CTTGCCAGCACAGAAGCCGC	60° C		
Pepsin _Forward	GCCAGGCTTGCCAGAACCA	60° C	515	NM_001201015.1
Pepsin_ Reverse	GGAAGTGCCCGTGTCAATAATAGC	60° C		

Table 5-2 showing African Catfish species-specific primers for amplification use in Real time qPCR after sequencing for species specific genes.

Gene	**Primer/probe**	**TM [°C]**	**Amplicon size (bp)**	**Accession Number**
Chymotrypsin_Forward	GCCCCATAATGCTCCTTGCTAC	60° C	134	NP_ 001188117.1
Chymotrypsin _Reverse	ATCCCCACCAGTCAGATTTAGTGC	60° C		
Trypsin _Forward	GGCACCCCAACTACGACTACAGA	60° C	177	NP_ 001187619.1
Trypsin _Reverse	AAGAGACCCCCATCCAGAGACAG	60° C		
Gastric chitinase_Forward	CAACCAGGGCAAATATCCTCTGA	60° C	107	NP_001153946.1
Gastric chitinase_Reverse	CTGGGGCAGCAGTCACAGG	60° C		
Pepsin _Forward	TTGCTTCTGATAATGCCACTCC	60° C	101	NM_001201015.1
Pepsin_ Reverse	CACTGCCCTGCTGACCAT	60° C		

Table 5-3 Standard PCR primers selected for reference genes designed from Channel catfish ESTs used for sequencing to make African catfish species specific primers.

Gene	Primer/probe	TM [°C]	Amplicon size (bp)	Accession Number
Beta actin_Forward	AAAGCCAACAGGGAGAAGATGAC	60° C	555	V988275.1
Beta actin_Reverse	AGTGTTGGCATACAGATCCTTACG	60° C		
Elongation factor -1 Alpha _Forward	CAAGCCTGGCATGGTTGTGACCTT	60° C	413	CB940917
Elongation factor 1 Alpha_Reverse	TGGGCTTGCCAGGAACCATTTC	60° C		
Alpha tubulin_Forward	GGAGCCATACAATTCCATCCTG	60° C	511	CB938582.1
Alpha tubulin _Reverse	CTTGAATCCAGTGGGACACCAG	60° C		

Source: (Small *et al.*2008)

Table 5-4 showing the African catfish species specific referencing gene primer sets used for real-time qPCR.

Gene	Primer/probe	TM [°C]	Amplicon size (bp)	Accession Number
Beta actin_Forward	TGTGCCCATCTATGAAGGTTATGC	60° C	132	V988275.1
Beta actin_Reverse	CTCTCGGCTGTGGTGGTGAAG	60° C		
Elongation factor -1 Alpha _Forward	GAAGGACATCCGCCGTGGTAAC	60° C	159	CB940917
Elongation factor 1 Alpha_Reverse	GCAGGCAATGTGAGCAGTGTGG	60° C		
Alpha tubulin_Forward	AACCTGGTGCCATATCCCAGAA	60° C	156	CB938582.1
Alpha tubulin_Reverse	ATGGCGTGGGTCACACTTCAC	60° C		

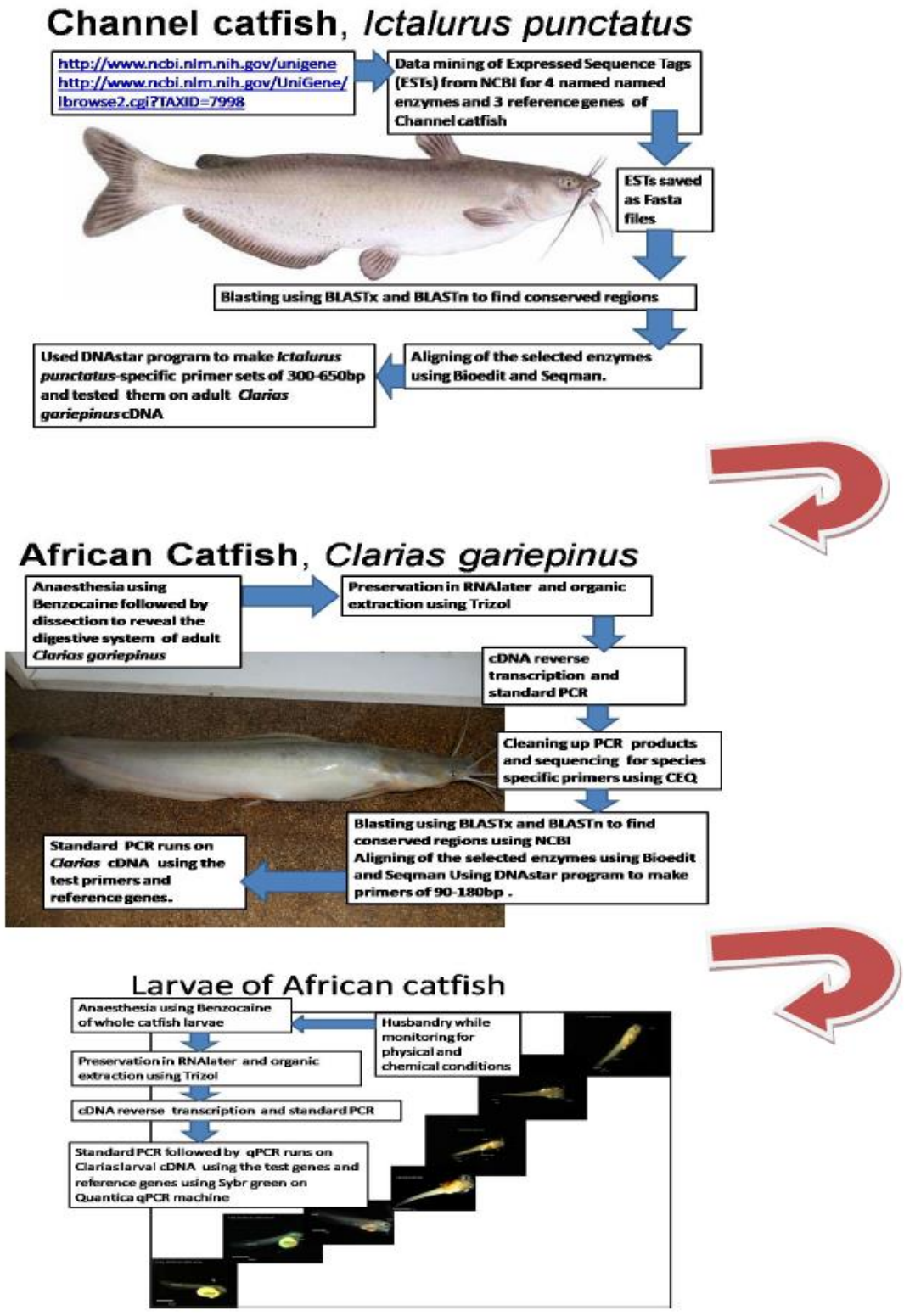

Figure 5-1 Schematic representation of analytical processes involved in determination of digestive enzyme expression

5.3.1 Standard polymerase chain reaction (standard PCR)

Following the design of the specific North African catfish primers for standard PCR and checking primers for amplification of appropriate products, primers were then designed for use in a real-time quantitative polymerase chain reaction (RT qPCR), which amplified a smaller product of 100-200 base pairs (bp) as shown in Tables 5-5 and.5-7 as seen above.

Laboratory preparations of adult *C. gariepinus* for initial primer checks for later use on Larval *C.gariepinus*

In order to obtain RNA to provide a mixed tissue sample for a catfish transcriptome as well as downstream RT qPCR studies of expression of key enzyme transcripts, tissue samples were obtained from a single adult 2kg 3 year old catfish. Samples taken comprised of anterior gut, midgut,posterior gut, stomach, pancreas, liver,oesophagus,gall bladder,eye,gills,pseudo lung, posterior heart, anterior heart, ovary, brain, gall bladder, eggs,, testis and seminal vesicles,barbels, anal opening. Larvae, which were too small to obtain individual tissue samples from, were processed as whole fish after ascertaining that the genes of interest could be detected in adult catfish. Larvae aged 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 14, 21 and 28 days post-hatch were sampled. Adult and larval fish were euthanized according to U.K. Home Office Schedule 1 methods, using terminal benzocaine overdose and, for adults, destruction of the brain. The adult fish was dissected and ~5 x 5 mm samples were excised using sterile scalpel blades and preserved in RNAlater® (Life technologies, Ambion®, UK) at a tissue: fixative ratio of 1:10. Whole larvae were also preserved in RNAlater®. Samples were maintained in RNAlater® for 24 hours at 4°C and RNAlater® was then drained according to manufacturer's instructions. Samples were placed in 3.6 mL Nunc Cryo tubes (Thermofisher Scientific, Denmark) and stored until use at -70°C (New Brunswick Scientific low temperature U570).

5.3.2 RNA extraction

RNA extraction was carried out as described in Chapter 2, General Materials and Methods.

5.3.3 cDNA Synthesis by reverse transcription for RT qPCR

Reverse transcription was carried out using the SuperScript® III Reverse Transcriptase kit (Invitrogen, Life technologies). It was performed in the following order to obtain a 20µL total reaction volume the first reaction mix comprised of 1µL of 1 µg of Total RNA, 1 µL of 10mMl dNTPs, 1 µL of 20µM oligoDt Random Primers, 9 µL RNase free water. The mixture was heated in a PCR machine at 90°C for 5 minutes and placed on ice to chill. In the meantime, reaction mix 2 comprising of 4 µL of 5x buffer, 1 µL of 0.1M DTT, 1 µL of 200U μL^{-1}SuperScript® III Reverse RT; 2 µL of RNase-free water was prepared and added to reaction 1 above and heated in a PCR Machine at 50°C for 1 hour and annealed at 70°C for 15 minutes and stored at -60°C for long-term use.

Alongside this negative reverse transcription (Minus -RT) control samples where no Superscript® III Reverse Transcriptase was added were employed to control for DNA contamination for use in the Real Time qPCR analysis at the next step.

5.3.4 Standard PCR and RT qPCR experimental set up

To perform qPCR initially the samples were tested starting with Standard PCR

5.3.4.1 Standard polymerase chain reaction

The PCR was performed in 5 µL total volume comprising 2.5 µL MyTaq™ HS Mix (Bioline), 1.2µLRNAse-free water, 0.3µL of 0.5 µM primer mix comprising of each of the forward and reverse primer set and 1 µL of 5ng μL^{-1} of cDNA Template and runon the Thermocycler under the following conditions 95˚C for 2minutes followed by 33 cycles at 95˚C for 30seconds followed by 60˚C for 30 seconds, 72˚C for 2minutes and an annealing step of 72˚C for 2minute with a final cooling step of 10˚C for 20seconds.. A no-template control (NTC) was run alongside samples to monitor contamination. The size of PCR product was verified by electrophoresis in 1.5% agarose gel using Gene Ruler 100 bp DNA Ladder marker (Thermo Scientific).

5.3.5 Sequencing of samples

At the start of the study, sequencing was performed at the Institute of Aquaculture using the following procedure but at a later stage the samples were sent off to a commercial company GATC Biotech (www.gatc-biotech.com, Lake Constance, Germany).Sequencing in the Institute involved the use of the Beckman-Coulter GenomeLab™ Dye Terminator Cycle Sequencing with Quick Start Kit-1. The technique involved preparation of standard PCR products in a total reaction

volume of 50 µL for all four genes of interest and three reference genes. The total reaction volume comprised 25 µL of 2X MyTaq HS mix (Bioline), 1.5 µL of 10 µM forward-primer, 1.5 µL of 10 µM reverse-primer, 10 µL of 10ngµL^{-1} cDNA diluted tenfold 1/10 and 12µl of RNase free water.This was followed by placing the samples on thermal cycler under the following conditions

Steps	**Temperature**	
1	95°C	2min
2	95°C	30 sec
3	60°C	30 sec
4	72°C	2min
5	Go to Step 2, 33 more times	
6	72°C	2min
7	10°C	20 sec

2 µL of each PCR product was then run on a 1% agarose gel with 0.5 µL of ethidium bromide to confirm the size of the product.

The samples were then purified using the QIAquick PCR Purification kit using a micro centrifuge (Qiagen, 28104) as per manufacturer's instructions except that the DNA was eluted in 30µl nuclease-free dH_2O (pre-heated to 55^{o}C). The samples were quantified using the Nanodrop 2000 and 50 ng of the DNA was run on a 1% agarose gel with 0.5 µL of ethidium bromide to confirm the size of the product.

This was then followed by preparation of the sequencing reaction in a 0.2 mL thin-wall tube with 2 tubes per template DNA for a forward sequencing primer and a reverse sequencing primer reaction. All reagents were kept on ice while preparing the sequencing reactions and were added in the order listed below. One µL of sterile water and 1 µL of DNA (at 100 ng µL^{-1}) were added to each sequencing tube. Tubes were spun briefly to allow collection at the bottom of the tube. Then the DNA and water mix was preheated at 96°C for 1 min in the thermo cycler and then cooled on ice. 2 µL DTCS Quick Start Master Mix was added to each tube followed by 1 µL of forward sequencing primer to one of the tubes for a template and 1µL of reverse sequencing primer to the other tube were added in the order

mentioned. This was followed by thorough mixing of the reaction components and centrifugation before thermal cycling to allow for consolidation of the liquid at the bottom of the tube well.

This was followed placing in a thermal cycler programmed at 96°C for 20 seconds followed by 60°C for 20 seconds, 60°C for 4 minutes, for 30 cycles followed by holding at 4°C

Sample preparation for sample loading in Beckman-Coulter Genome Lab™ Dye Terminator Cycle Sequencing with Quick Start Kit- 2

Initially, as mentioned, sequencing was carried out at the Institute of Aquaculture using the following procedures:

The sample from the previous step was prepared in the following manner. To a labelled sterile 0.5 mL microfuge tube with 5 µL of freshly prepared Stop Solution / Glycogen mixture (per sequencing reaction) comprising 2 µL of 3M Sodium Acetate (pH 5.2), 2 µL of 100mM Na2-EDTA (pH 8.0) and 1 µL of 20 mg mL^{-1} of glycogen (supplied with the kit). This was then mixed thoroughly this followed by addition of 60 µL cold 95% (v/v) ethanol / dH2O from -20°C freezer, thorough mixing and immediate centrifugation at 21000g at 4°C for 15 minutes. The supernatant was then carefully removed with a micropipette leaving the pellet visible. The pellet was rinsed 2 times with 200 µL 70% (v/v) ethanol / dH2O from -20°C freezer. Following each rinse, it was centrifuged immediately at 21000g at 4°C for a minimum of 2 minutes. After centrifugation,all of the supernatant was carefully removed with a micropipette. The sample was then vacuum dried for 10 minutes and re-suspended in 40 µL of the sample loading solution provided in the kit.

The sample was then prepared for sequencer loading by transfer of the re-suspended samples to the appropriate wells of a 96 well sample plate (P/N 609801)and each re-suspended sample was overlaid with one drop of light mineral oil (provided in the kit or Sigma Cat # M 5904).and the sample plate loaded into the instrument and theCEQ 8800 program for sequencing run with the resultant sequence contigs presented in the results .

5.3.6 Real-time RT-qPCR using fluorescent dyes

Real time quantitative polymerase chain reaction (RT-qPCR) is a universally employedtechniquefor detecting and quantifying RNA expression in the form of complimentary DNA (cDNA) (Dijkstra, Kempen, Nagtegaal *et al.*2014; Nolan,

Hands & Bustin 2006). RT-qPCR is usually fluorescence assay based technique and this study used Sybr green fluorescence to determine and quantity in real time the expression of the genes under study, with data collection beingin accordance with MIQEs laid down by Bustin *et al.* (2009)

After designing primers of between 100-200bp using PrimerSelect primer design software for the four target genes and three reference genes as shown in Tables 6.5 & 6.7, complementary DNA (cDNA) was synthesized from single stranded mRNA as per the earlier outlined protocol. The cDNA from pooled samples was analysed using the TaqMan RT kit (p/n N808-0234.) according to the flow diagram shown in Figure 5-2.

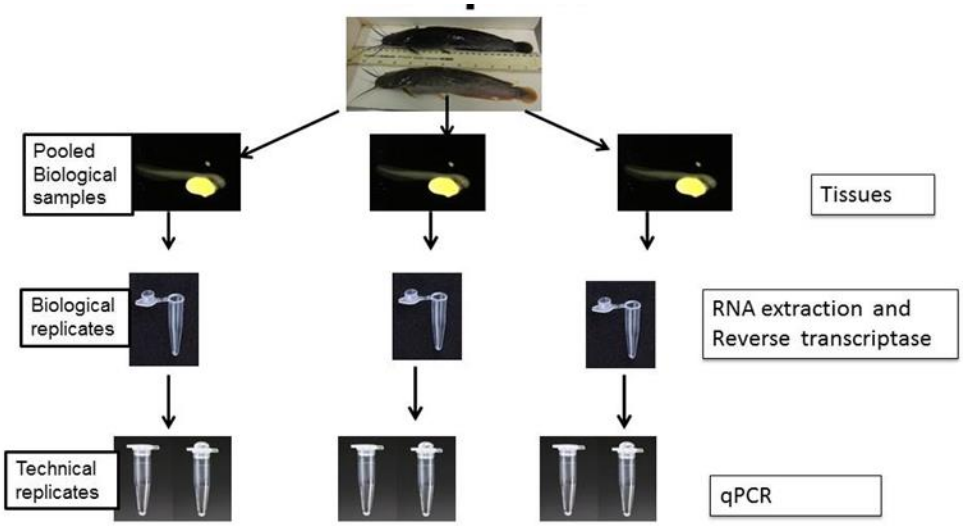

Figure 5-2 Flow diagram of optimizations for qPCR

5.3.7 cDNA Synthesis of North African Catfish larvae for Real-time PCR Study

cDNA was produced using 2µg of RNA with TaqMan Reverse Transcription (Applied Bio systems, N808-0234) for a total reaction volume of 20µL.

RNA was diluted to make 2µg μL^{-1} stock in a 10 µL volume placed in a 0.2 mL nuclease-free tube (Thermo Scientific). This was then followed by denaturing at 70° C for 5 minutes on a thermo cycler (Biometra T gradient) and placing on ice.The next step was the preparation of a master mixture of 2 µL of 10XRT buffer, 0.8 µL of 25XDNTP (100mM) 1.5 µL of 10X random primer, 0.5 µL of 20µM Oligo (dT), 1 µL Multiscribe Reverse Transcriptase and 4.2 µL of nuclease-free water.

The 10 µL mix was added to the sample on ice, flick-mixed and briefly centrifuged. Samples were placed on the thermal cycler under the following conditions: 25^{o}C for 10min followed by 48^{o}C for 60min then 95^{o}C for 5min followed by a final holding step of 25^{o}C for 2s

Samples without enzymes acted as negative controls (*i.e*. to determine whether or not the samples contained genomic DNA) and were similarly prepared using 2 µg of RNA with TaqMan Reverse Transcription (Applied Bio systems, N808-0234) for a total reaction volume of 20µL. Control samples were prepared and processed as above, save for the omission of the Multiscribe Reverse Transcriptase and the use of 5.2µL of nuclease-free water to make up the full reaction volume.

Once the cDNA has been prepared, standard samples were prepared according to Table5-8 below to obtain a standard curve.

Table 5-5 showing the dilution curve prepared for the cDNA to be used on the qPCR run.

cDNA µg	RNASE free water µl	Total µl	Dilution factor 1:20
2	38	40	
16*	64	80	1:20
40	40	80	1:100
40	40	80	1:200
40	40	80	1:400
40	40	80	1:800
40	40	80	1:1600

* Taken from previous total cDNA

Samples based on: 1, 2, 3, 4, 5, 6, 7, 8, 14 and 21 days post hatch (Dph) were selected.

3µl of each sample was pooled and aliquoted into a 1.5 mL microfuge tube (positive standard). Fourteen negative control samples were also prepared. The melt curve / dissociation graph was obtained for each reference gene and allowed us to check for primer dimer / non-specific amplification.as seen in Table 5-9 below.

Table 5-6 Showing the slope and cycle over which qPCR occurred in the target genes and house keeping genes

Gene	R^2	E	Slope	Cycles at appearance.
Reference genes				
Beta actin	0.952	1.983	y=-3.362x+34.044	10-25
Alpha tubulin	0.990	2.073	y=-3.931x+34.168	10-25
Elongation factor	0.996	1.996	y=-3.427x+18.968	10-25
Target genes				
Pepsin	0.997	2.097	y=-3.301x+21.576	10-25
Gastric chitinase	0.988	2.054	y=-3.198x+20.853	15-25
Trypsin	0.989	2.009	y=-3.301x+22.863	15-25
Chymotrypsin	0.984	2.013	y=-3.292x+20.198	10-25

Dilution factors were selected to provide CT values for all primers in the range 16-25. Reaction efficiency (E) of PCR was sought to be between 1.90 & 2.10 (ideally 1.95-2.05) and the R2 was aimed for >0.980 for each primer set. To ensure that there was no non-specific amplification and that the product was the correct size, the real-time PCR product was run on a gel for 30 minutes and denatured at 95° for 5 minutes.

5.3.8 qPCR reaction preparation

Each qPCR reaction contained the following reaction mix: 10.0 µL of 1X ABgeneSybr Green 0.6 µL of 300 nM forward-primer (10µM), 0.6 µL of 300nM reverse-primer (10µM), 3.8 µL of dH2O making 15.0 µL and topped with 5.0 µL of cDNA of varying dilutions starting with (1/20 dilution). The prepared samples were then run on the Quantica under the following conditions 95°C for 15 minutes, 94°C for 15s, 60°C for 15s for 40 cycles, 72°C for 30s and melt from 55°C-95°C. The samples were laid out as shown in Table5-10.

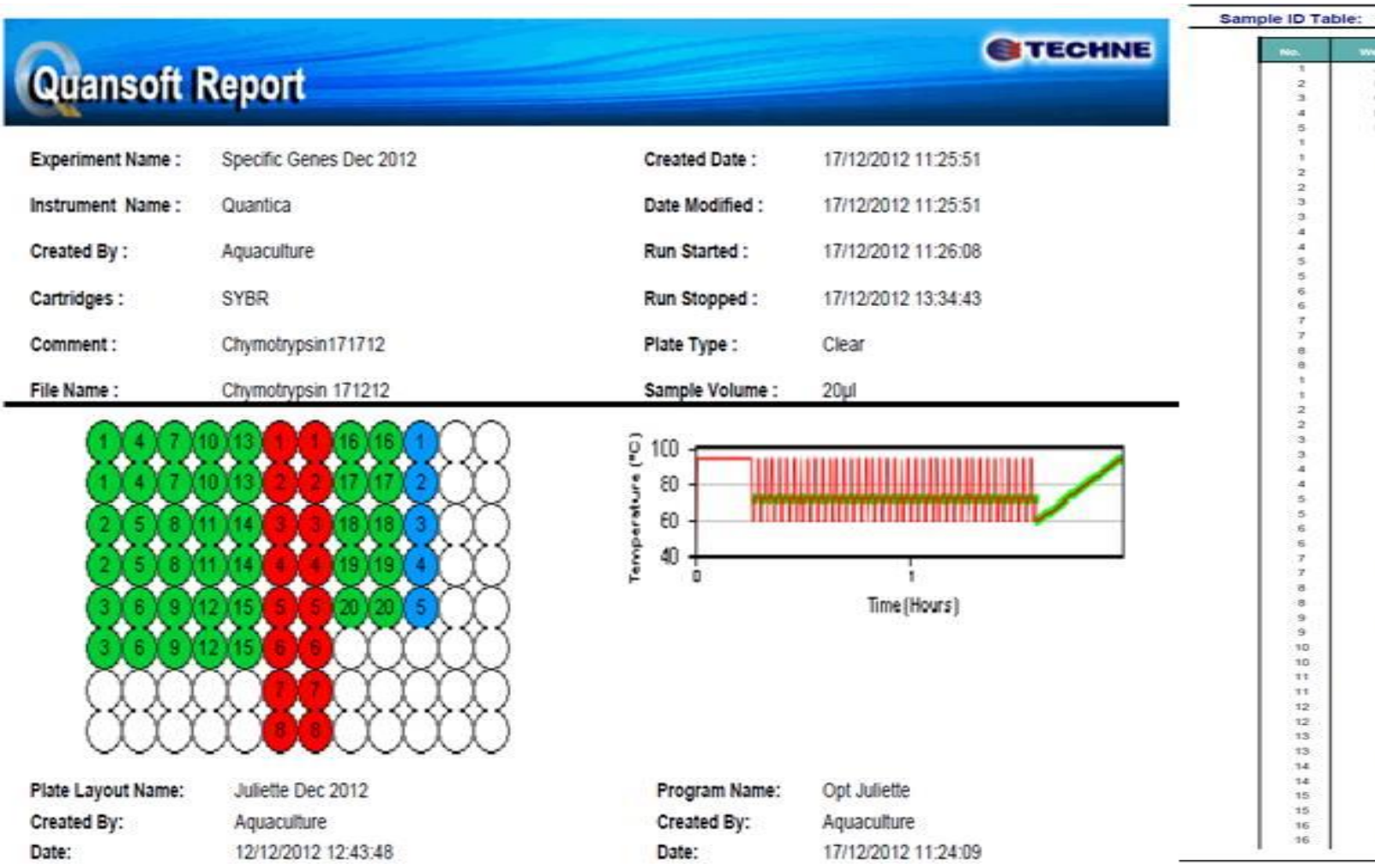

Table 5-10. Example sample layout on plate for the qPCR run on the Quantica machine

5.3.9 Data analysis

Data were analysed using GenEx Enterprise program and mean expression of beta actin (ACTB) elongation factor 1 alpha and alpha tubulin (TUBA) were used as normalisation factors. Mean normalised expression of the target genes was calculated from Raw Cq values by relative quantification (Muller, Janovjak, Miserez *et al.*2002) and graphs plotted. Statistical analysis was conducted using one wayAnova and Kruskal-Wallis test after testing for normality and homogeneity of variance.

5.4 Results and discussion

5.4.1 Species-specific sequences from *C. gariepinus* standard **PCR**

Having used Channel catfish sequences as a basis for primer design, the following species-specific sequences were successfully obtained for *C. gariepinus* from Becker coulter sequenced samples. Figures 5-3 to 5-6 give the consensus sequences obtained for the enzyme transcripts of interest and from which North African catfish species-specific primers were designed for use in the subsequent qPCR analyses.

>African_Catfish_chymotrypsin_consensus_sequence

CAGCTGGCCCTGGCAGATCTCCCTCCAGTATACCAGTAGTGGTAACTGGTACCACACCTG
TGGAGGAACTTTGATCTCTGACCAGTGGGTTCTGACTGCTGCTCACTGTATCGGAAGCAG
AACTTACAGAGTCTACCTTGGCAAACACAACCTGAAGGTGGAAGAGAATGGTTCTTTGGC
TGTCCCAGTTGAAAAAATCATTGTCCATGAGAAGTGGAATTCTTTTTTTATCCGTAACGA
CATTGCCCTGGTGAAGCTCCAGACTCCTGTGGCTTTCAGTGAAACCATCACACCTGCATG
CCTTCCCGAGGACGGTTTCATCTTGCCCCATAATGCTCCTTGCTACGTCACTGGCTGGGG
ACGCATGAAGACTGGAGGTCCCATTGCAGATATCCTGCAGCAGGCTCTGCTCCCTGTGGT
TGACTATGCTACCTGCACTAAATCTGACTGGTGGGGATCTCAAGTTAAGGACACCATGGT
CTGTGCTGGAGGAGATGGCATTGTGTCTGGCTGCAATGGTGATTCTGGTGGcCCCCTGAA
CTGCCAAAATGCTGTTGGTGCCTGGGGAAGTT

Figure 5-3. Consensus sequence for chymotrypsin, for the North African catfish species sequenced at the Institute of Aquaculture molecular laboratory facility using Beckman-Coulter Genome Lab™ Dye Terminator Cycle Sequencing.

>African_catfish_trypsin_consensus_sequence

CATGGATGGTGTCTCTGCACTATGGCTACCACTTCTGTGGAGGAGTGCTTATCAGTGAAC
AGTGGGTGCTCTCTACTGCTCAGTGCTGGTACAACCCCTATAGTATGCAGCTTATTTTGG
GAGATCATGATGTGCGTGTGTTTGAGGGAACTGAACAGCTTCTGAAGACAGAAAATATTA
TCTGGCACCCCAACTACGACTACAGAACTCTGGACTATGATATTATGCTAATTAAGCTGT
TYCACCCCGTGAAAGTGACTAATGCTGTACGGCCAATATCTCTGCCAACTGGCTGTCCCT
TTGAAGGAATGCCCTGTACTGTCTCTGGATGGGGGTCTCTTTACGCTGATTCCTTATTCA
TGCCTTTTCGTCTTCAGTGTGCTGATATCCCAATTGTGGGTGAGCAGGAGTGTGAAAAGT
CTTATCCTGGTATGCTAACTCGTAGAATGGTGTGTGCTGGCCTTAAGGAGGGTGGAACGG
ATGCCTGCTCTAGAGATGCAGGAAGTCCGCTGGTGTGCTACGGAGAGGTGCATGGGCTA

Figure 5-4. Consensus sequence for trypsin for the North African catfish species sequenced at the Institute of Aquaculture molecular laboratory facility using Beckman-Coulter Genome Lab™ Dye Terminator Cycle Sequencing.

>African_Catfish_PepsinA_consensus_sequence

CAATAAATTCAACCCAACACAGTCCAGCACTTTCCAGACCAACAACCAGGCTCTGTCCAT
TCAGTATGGCACAGGCAGCATGACTGGATACCTGGGTTATGACACTGTGACGGTTGGTGG
AATCTCAGTGCAAAATCAGATCTTTGGACTGAGTGAGACTGAGGCGCCCTTCATGGCCAG
CATGACAGCAGATGGCATCCTGGGTCTGGCCTATCAATCCATTGCTTCTGATAATGCCAC
TCCCGTCTTTGACAACATGATGAGTCAGGGCTTGGTCTCCCAGGATGTCTTCTCCGTCTA
CCTGAGCAGCAATGGTCAGCAGGGCAGTGAGGTACTGTTTGGTGAGATTGACACCTCCTA
CTACACTGGTAGCATCTACTGGATCCCTCTGTCCTCAGAGAGCTACTACCAGGTCACCAT
GAACAGTGTTACTATCAATGGCCAGACTGTTGCTTGCTCTGGAGGATGCCAG

Figure 5-5. Consensus sequence for pepsin A, for the North African catfish species sequenced at the Institute of Aquaculture molecular laboratory facility using Beckman-Coulter Genome Lab™ Dye Terminator Cycle Sequencing

>African_Catfish_gastric_chitinase_consensus_sequence

TATGACAACATCAAGAGCTTCCAGATTAAGATTGACTGGCTGAAGAAAAACAACTTTGGA

GGTGCTATGGTGTGGTCTCTGGACCTGGATGACTTCAGTGGCACCTTCTGCAACCAGGGC

AAATATCCTCTGATCAACACCATCAAGAGTGGCCTGGGAACTGGCCAAAGTTGCTCAGCT

CCTTCTCAGCCTCTGGCTCCTGTGACTGCTGCCCCAGCTACTGCTGCTCCTGCTCCTAGT

GGTGGTGGCAGCAGTGGTGGTAGCAGCAGTGGCTCCAGCAGTGGCA

Figure 5-6 Consensus sequence contigs for gastric chitinase for the North African catfish species sequenced at the Institute of Aquaculture molecular laboratory facility using Beckman-Coulter Genome Lab™ Dye Terminator Cycle Sequencing

5.4.2 Onset of gene expression of interest from preliminary results for standard PCR

Primers designed using conserved transcript regions from Channel catfish, *I. punctatus,* successfully amplified products for all four target genes (pepsin, chymotrypsin, trypsin and gastric chitinase) using cDNA templates derived from RNA extracted from African catfish tissue samples (Figure 5-7).

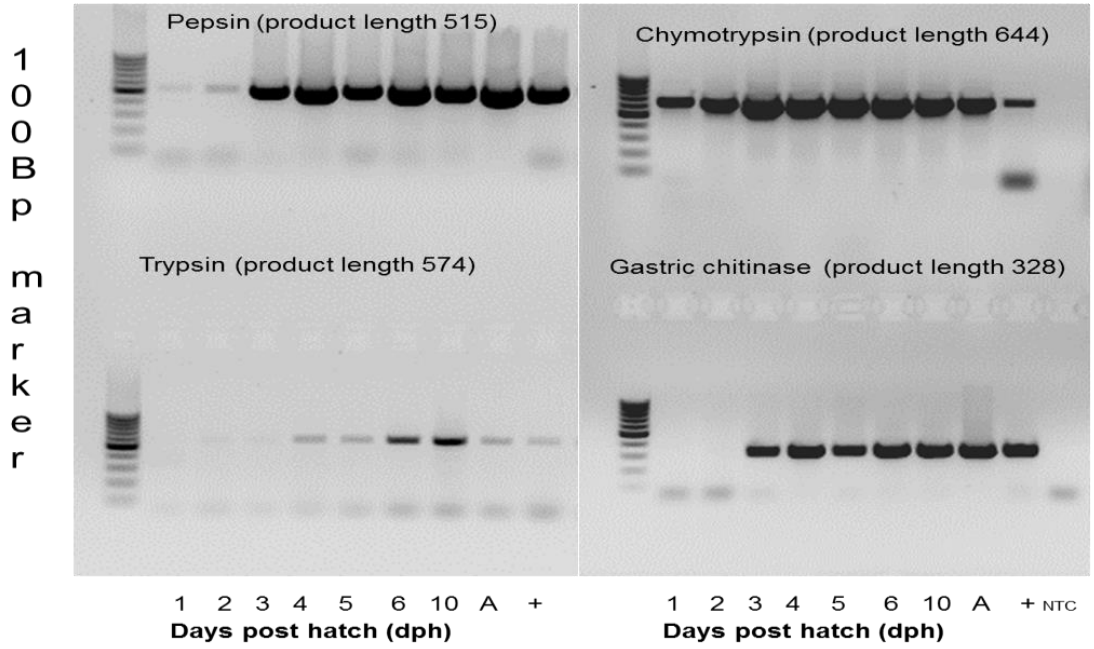

Figure 5-7 Preliminary PCR results for the 4 target genes (A=Adult, NTC= Non Template control)

The PCR results suggested that chymotrypsin was highly expressed from 1dph, however gastric chitinases and pepsin were highly expressed only from 3dph while trypsin seemed only to show significant expression from day 1.

Preliminary PCR investigation using the North African catfish specific primers (Fig.5-8) indicate that pepsin appears to switch on at 2 dph, increasing throughout development while trypsin seems to switch on around 4 dph increasing considerably at 6 dph. Chymotrypsin is present from the onset while gastric chitinase appears at 3 dph and is present throughout thereafter.

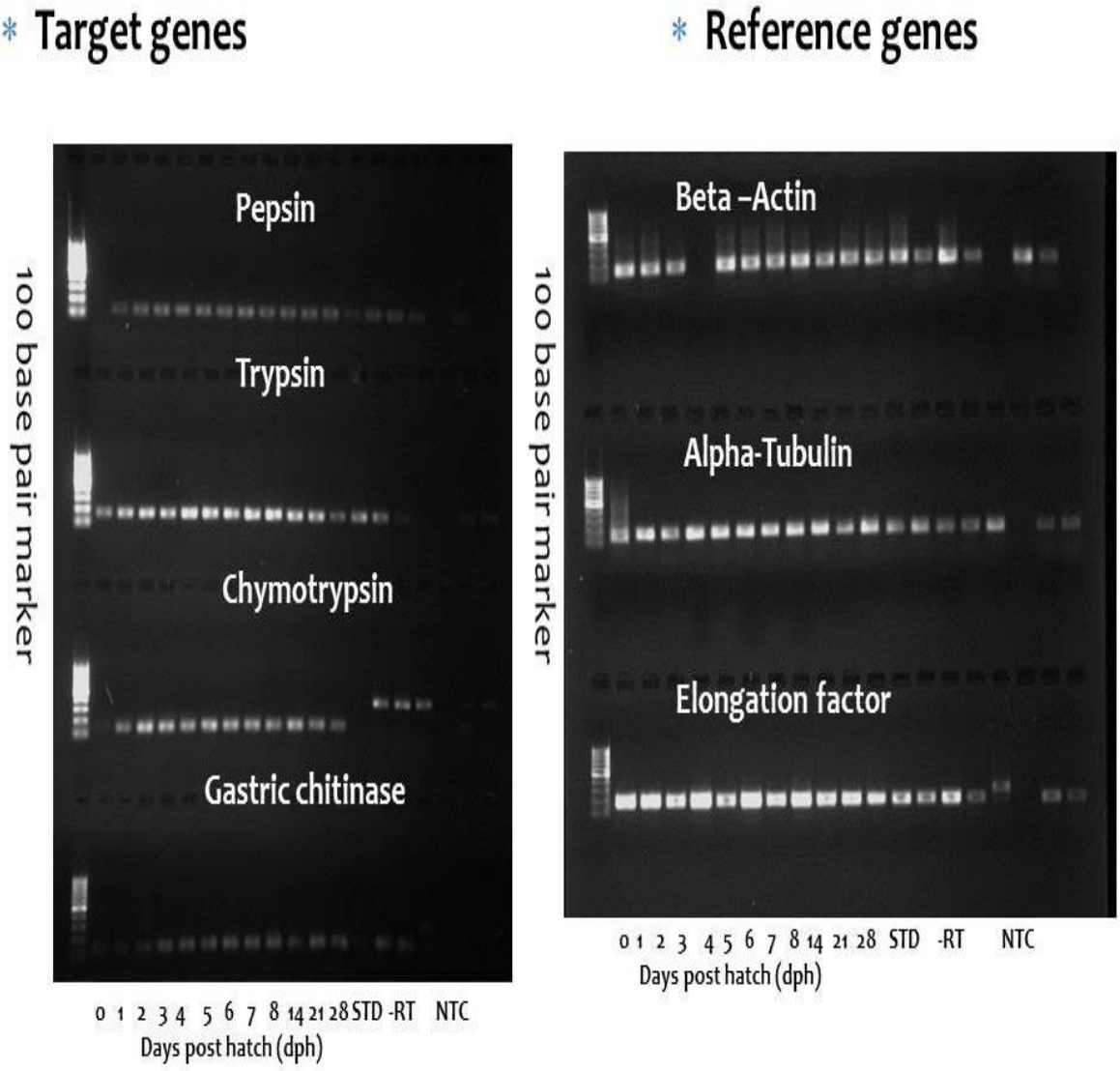

Figure 5-8. Preliminary run of the North African catfishspecific primers on 1.5% agarose gel for both the target genes and housekeeping / reference genes in preparation for qPCR run. (STD=Standard, -RT=Negative Reverse Template, NTC=No Template Control

5.4.3 Results of pancreatic enzyme gene expression assessed by RT qPCR

5.4.3.1 Results for trypsin and chymotrypsin mRNA gene expression

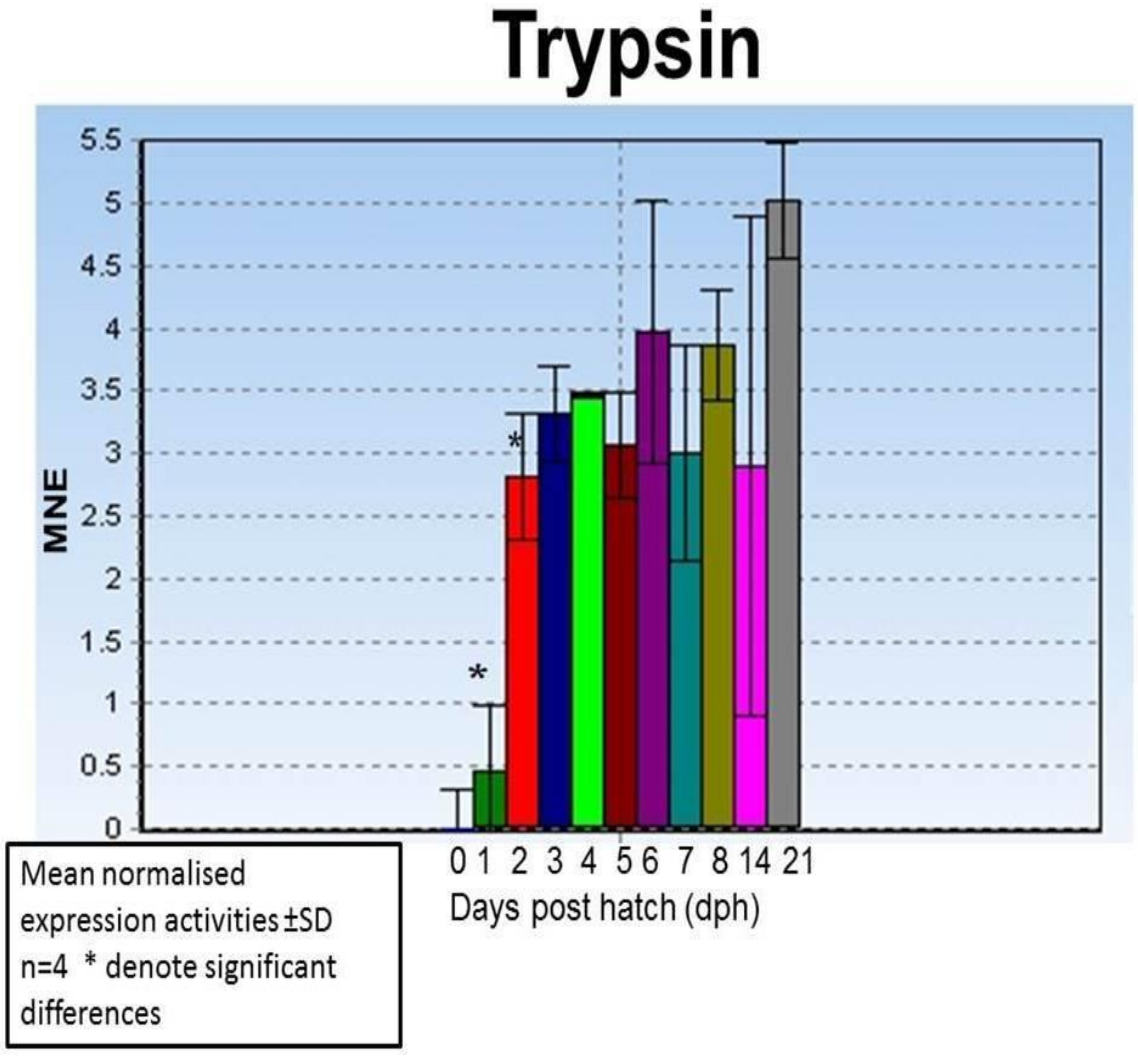

Figure 5-9 Mean normalised expression activities of trypsin from days 0, 1, 2, 4, 5, 6, 8, 14, 21 Dph, nominally produced by the pancreas

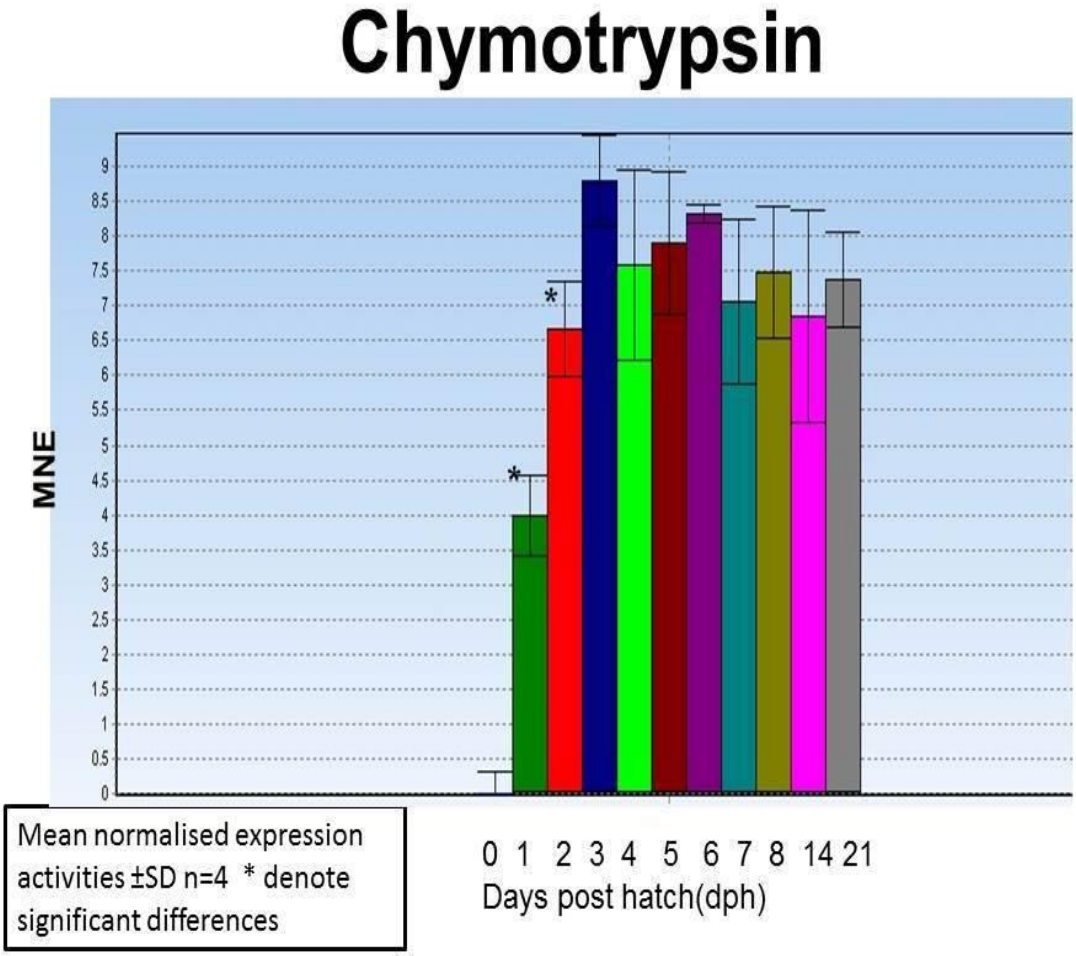

Figure 5-10. Mean normalised expression activities of chymotrypsin from days 0, 1, 2, 4, 5, 6, 8, 14, 21 Dph, nominally produced by the pancreas

Trypsin and chymotrypsin expression was detectable from 1 dph, with clear expression by 2dph, as shown in Figures 5-9 5-10 . This coincides with the appearance of the first pancreas anlage in histology (Verreth 1992, Osman 2008). Chymotrypsin was expressed at higher levels when compared to trypsin, as seen in Figure 5-9, with chymotrypsin being almost twice trypsin levels by 3 dph when trypsin expression was only about 3.5. However, trypsin continues to increase, being highly expressed at 21 dph while chymotrypsin had decreased slightly by 21dph.

5.4.4 Results of Stomach /gastric gland enzyme gene expression assessed by RT qPCR

5.4.4.1 Results for mRNA expression for Pepsin

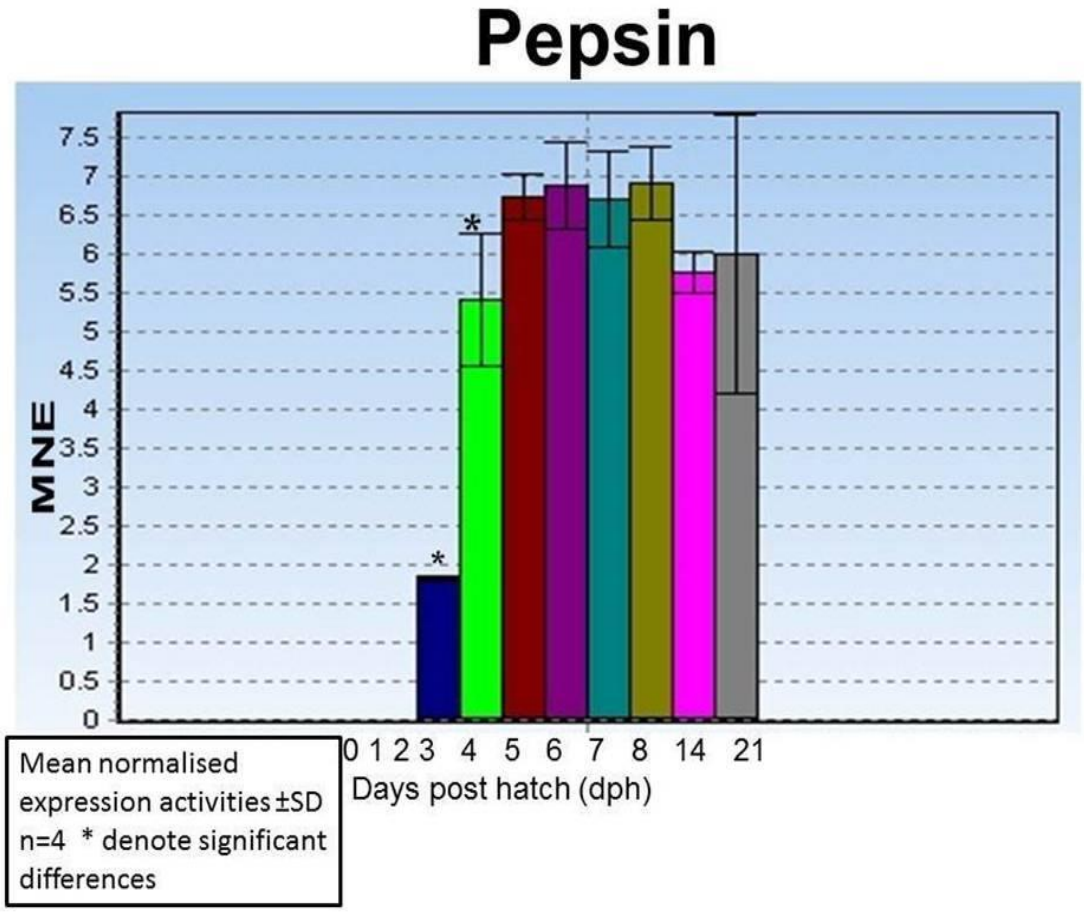

Figure 5-11 Mean normalised expression activities of pepsin from days 0, 1, 2, 3, 4, 5, 6, 8, 14, 21 Dph produced in the stomach oxynticopeptic cells

Results for mRNA expression for the gene pepsin are shown in Figure 5-11. Pepsin was present from 3 Dph with a big increase in expression seen between 3 Dph and 4 Dph. Expression increased from that point, peaking at 6 Dph, however, it subsequently decreased between 14 Dph to 21 Dph.

5.4.5 Results for mRNA expression of gastric chitinase in the gastric glands of larval *Clariasgariepinus*

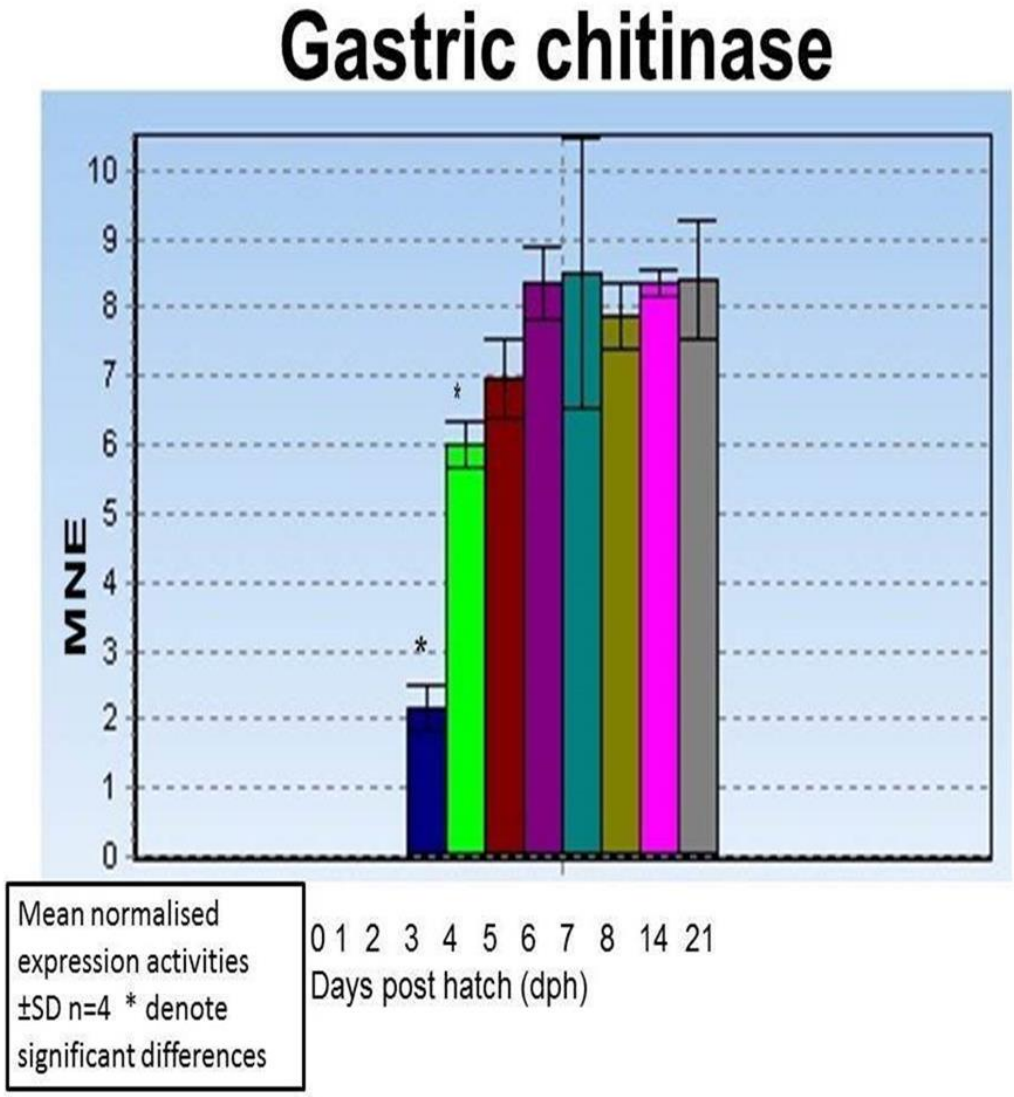

Figure 5-12. Mean normalised expression activities of gastric chitinase from days 0, 1, 2, 3, 4, 5, 6, 8, 14, 21 Dph produced in the gastric pit in the mucosa

Gastric chitinase in larval *C. gariepinus* first appeared at 3 Dph and seems to have been completely switched on by 4Dph just like its counterpart pepsin, produced by the stomach, increased and reached its maximum at 7 Dph, thereafter being relatively stable. The mean normalised ratio was however higher when compared to Pepsin mRNA expression over the period of study.

5.5 Discussion

5.5.1 Transcript expression of pancreatic enzymes trypsin and chymotrypsin

Expression of the pancreatic enzymes trypsin and chymotrypsin in *C. gariepinus* larvae was detected at low levels at hatching and levels increased markedly during the first week of life. In general, these results agree with previous reports in a wide range of marine fish species. (Darias, *et a*l. 2007; Darias, Zambonino-Infante *et al*. 2008; Mir *et a*l. 2018; Srichanun*et al*. 2013). During early larval stages in most marine fish, there is no acid protease (pepsin) activity due to the undeveloped gastric glands, and pancreatic alkaline proteases are, initially, the main proteolytic enzymes (ZamboninoInfante and Cahu, 2001). The pancreatic trypsinogen and chymotrypsinogen are proenzymes for the serine proteases trypsin and chymotrypsin, respectively. Trypsin plays a key role in the digestive capacity of fish larvae. In this study, trypsin mRNA levels showed an increasing pattern from 1 to 21 dph. This is different from the expression profiles described for studies of winter flounder, *Pleuronectus americanus*, where 3 trypsins were detectable namely Try 1,Try 2 and Try 3, with Try 2 appearing 5 Dph, Try3 appearing at 20 Dph and Try 1 appearing much later. However, in terms of histology the fish showed a clear developmental sequence, with the pancreas initially compact but transforming into a diffuse system when the fish developed into young adults (Murray *et al.* 2004). In red porgy, trypsin gene expression in larvae was observed from hatching (Darias et al., 2007).

Studies of trypsin mRNA gene expression in Indian catfish,*Clarias magur,* a close relative of *C. gariepinus* (Darias, *et al.* 2007) have shown comparable results with those of the present study, in that the gene switches on at 3 DPh with a sharp increase between 9 Dph and 11 Dph (Mir *et al.* 2018) with enzyme expression coming much later at 11Dph with the appropriate switch to a dry diet possible at 26 Dph.

Expression profiles of trypsin and chymotrypsin were quite similar; however mRNA expression for chymotrypsin was higher than for trypsin. However, there appeared to be a shift in gene expression, since the highest mRNA expression of trypsin was detected on 21 Dph. The observed decrease in mRNA levels of chymotrypsin from 14 dph may be due to the increased contribution from acid digestion and development of a functional stomach, as reflected in strongly increased pepsin

expression. This concurs with other studies which suggest that trypsin plays a vital role in protein metabolism in the early life of fish when the stomach is not fully developed and for which expression is a precursor to the activation of other pancreatic enzymes including chymotrypsin, BAL and amylase up until acidic pepsin based metabolism commences when the stomach is fully developed (Chi, *et al.* 2015; Darias *et al.* 2007; Darias, *et al.* 2008; Mata-Sotres, *et al.* 2016).

5.5.2 Transcript expression for pepsin

The appearance of gastric glands is taken to indicate functional development of the stomach and is generally considered as forming a part of the transition from the larval to the juvenile stage in fish (Galaviz *et al.* 2011; Galaviz *et al.* 2012; Galaviz, *et al.* 2015; Miura, Kageyama & Moriyama 2015; Nalinanon *et al.* 2010; Perez-Casanova *et al.* 2006; Richard, *et al.* 2015). Functional development of gastric glands in the stomach has been studied previously in larval *C. gariepinus* by histological examination (Verreth *et al.* 1992).

From the results of this study a sharp increase in pepsin was observed between 3 Dph and 4 Dph, this coinciding with the time when anatomically the stomach is complete and also coinciding with the time when exogenous feeding normally commences in this species (Verreth *et al.* 1992). These results also suggest that the switch on is genetically programmed as this timing is also seen in the close relative Indian catfish, *Clarias magur,* where the mRNAs of pepsin switch occurred 4 Dph peaking in expression at 19 and 22 Dph (Mir *et al.* 2018). These results also support observations of a similar trend of acidic protein metabolism occurring later in post-hatch, as has been observed in other fish species both marine and fresh water and as evidenced in Asian seabass, *Lates calcarifer*, where measurable Pepsinogen mRNA levels were present but barely discernible during 0–15 dph, but were expressed in increased proportions from 18 dph and onwards (Srichanun *et al.* 2013).

Pepsin gene expression was detected from 20 dph and coincided with the appearance of gastric glands in winter flounder (Douglas et al., 1999), and in red porgy, pepsinogen expression coincided with an increase in proton pump gene expression and decreased pH levels in the stomach (Darias *et al.* 2007). In the present study, the highest expression of pepsinogen was observed at 6 dph. From this study it can be suggested that pepsin, an acidic proteolytic enzyme, comes much later, conforming to studies of red drum (Lazo 2007) in which alkaline

proteases played a major role in digestion during the first days of feeding, while acid proteases became more important toward the end of the larval period, concomitant with the appearance of a functional stomach. Martínez-Cárdenas *et al.* (2017), in their studies on characterization of digestive proteases in the juvenile green cichlid, *Cichlasoma beani*, confirm that both alkaline and acid proteases are involved in the digestion of *C. beani*, reflecting the fact that this species is omnivorous, with carnivory a tendency, which is also true of larvae of catfish in which the studied alkaline proteases are more abundant.

In the case of spotted rose snapper, *Lutjanus guttatus*, studies have indicated that the stomach becomes adapted to function between days 20 and 25 post-hatch however the pepsinogen gene is available at 18 Dph before 20Dph when its activity and the presence of the gastric glands histologically, means that weaning could practically be performed much earlier than is usually the case (Galaviz, et al. 2012).

5.5.3 Discussion of mRNA expression of gastric chitinase

The expression of gastric chitinase showed a similar trend to pepsin, appearing at 3 dph as seen in figure 6-9, shortly before the first anlage stomach histologically appears (Verreth 1992).This seems to point to the fact that timing is genetically controlled. It is interesting to see it decreasing at 7Dph, when farmers in Uganda report high mortality rate (Isyagi per com 2014) and this may in part be due to microbial attack and chitinase playing a role of defence as in other species reported earlier on. It is clear that in adults, chitinase was expressed in the stomach but since this study employed whole larvae, it is not known whether, in the early stages of *C. gariepinus* larvae or juvenile fish, it occurs in other organs as is the case for pike silverside, *Chirostoma estor,* where in juveniles, chitinase is found in the hepatopancreas (Pohls *et al.* 2016).

Chitinase has been implicated in many roles in fish including breakdown of chitin based diets and also as part of the defence mechanism against microbes and parasites. Most of the studies look at the types of chitinase possessed by fish and the roles played, with less work on the gene expression with respect to involvement in the breakdown of diets. This is one of the few studies which has highlighted the expression of the gene in terms of development of the larva and can be useful in examining the importance of chitinases in nutrient breakdown in

more detail. This can inform decisions made when making an inert diet based on insects since chitin will be a key part of feed composition.

5.6 Conclusions

For all the genes investigated, we observed first expression at a time when the site of production was first observable histologically, with increasing levels of transcript expression with time post-hatch up to a maximum value. It was not possible to conclude whether all genes were constitutively expressed or responsive to an element in the live feed that, if detected and isolated, could be incorporated into formulated feeds to induce a more natural production of these enzymes in larvae. It is also not possible to say if the translated proteins were deployed immediately or stored for use. We envisage that studies of larval fish digestive physiology at the molecular *i.e.* transcriptomic level can serve to improve our capability to wean larval catfish on to microdiets early and also to help to provide an off-the-shelf diet leading to increased seed production, improved consistency in hatchery production and lower costs of production.

5.7 Recommendations

As this study was not exhaustive, coverage of a greater proportion of digestion-associatedenzymes is still required.Further studies should therefore concentrate on expression and activity of other enzymes including carboxypeptidases, lipases, α-amylase, carboxyl esterase,bile salt-activated lipase (bal) and neutral lipase (Saele *et al*., 2010). Additionally, expression and enzymatic activities of leucine aminopeptidase (lap) and alkaline phosphatase (alp), intestinal enzymes located at the brush border membrane which relate to the adult mode of digestion by enterocytes should be established (Cahu& Infante 2001, Infante & Cahu 2001, Cahu et al. 2003, Alvarez-Gonzalez et al. 2006, Henning et al. 1994). Such studies will help to give a complete picture of the expression or lack of expression of these enzymes in catfish larvae in order to help develop a complete inert diet for them.

6 CHAPTER SIX

Application of a mixed-tissue oligonucleotide DNA microarray for the study of larval development in African Catfish, Clarias gariepinus, (Burchell 1822)

6.1 Introduction:

Fish are the most speciose of the vertebrates. They have evolved to inhabit a high diversity of habitats and conditions including among others freshwater, sea water, tropical, temperate, diurnal and nocturnal (Sarropoulou *et al.*, 2016).Catfish (order Siluriformes) has been identified as one of the largest orders of teleosts, containing about 4100 species, representing about 12% of all teleosts and about 6.3% of all vertebrates (Eschmeyer & Fong, 2014; Jin *et al.*, 2016). In terms of genomic studies, considerable work, especially concerning the catfishes of North America, has been conducted over the last two decades (Jin *et al.*, 2016). This has led to better understanding of the biological characteristics of catfish, their evolution and capacity for genetic improvement as aquaculture broodstock. Although North American catfish (*Ictalurus punctatus)* and Blue catfish (*Ictalurus furcatus)* have been investigated in some detail, providing an important repository of Expressed Sequence Tag (ESTs) data, little work had been conducted, at the inception of the research described in this thesis, on North African catfish species such as *C. gariepinus*, the subject of this work.

Transcriptomic study of early development of larval fish is very important in gaining an understanding of the processes controlling development, which in turn are dependent on nutritional aspects of the organism (Song *et al.*, 2016). In terms of evolution, many organisms are driven by cyclic patterns, which structure the way they respond to the external environment (Boyle *et al.*, 2017; Yúfera *et al.*, 2017a). These patterns are thought to be regulated by gene expression and are also known to be self–regulated on a diurnal basis. Feeding and physiology for farmed fish species, in terms of growth, health, and well-being are dependent on the interaction between endogenous, genetically programmed patterns and external factors that include those imposed by production practices. Gaining improved knowledge of early developmental biology and physiology for farmed species through transcriptomic analysis can help determine the capacity of fish to adapt to changing environments, including those exerted by farming practices, to recognise and apply indicators of health and performance, and to come up with more

acceptable feeding practices by matching diet composition or the time of diet delivery to the developmental / physiological requirements of the organism. Both microarray and RNA-Seq gene expression profiling of whole-body larvae of other teleost fishes have been used to study key issues of development, domestication, and the effects of different environmental factors such as, diet, and stress (Boyle *et al.*, 2017; Patton & Mistlberger, 2013; Yúfera *et al.*, 2012; Yúfera *et al.*, 2017a).

High-throughput sequencing technologies, also known as next-generation sequencing (NGS) technologies, have altered the way in which genomic research is conducted. A lot of work has been done at the level of genome sequence and structure, however, transcriptomic analysis of larval development has been more sparsely conducted, with the exception of zebrafish, and particularly for farmed species in tropical and sub-tropical habitats. While a range of broad-scale transcriptomics technologies are now available, RNA sequencing. (RNA-seq) has proven particularly valuable as a readily deployed, high-throughput technology. Broad scale profiling tools, including RNA-seq, have therefore provided a powerful tool for mapping and annotating fish transcriptomes and improving our understanding of many biological processes in fish, such as development, adaptive evolution, host immune response, and stress response (Qian, *et al* 2014).

6.2 Transcriptomics

The term “transcriptome” refers to the complete set of transcripts in a specific cell, tissue or organism, which serve to regulate gene expression and maintain cellular homeostasis. Unlike the relatively stable genome, the transcriptome varies with cell-type, tissue, developmental stage, physiological condition and external environment.Transcriptomics nominally covers all types of transcripts, including messenger RNAs (mRNAs), microRNAs (miRNAs), and different types of long non-coding RNAs (lncRNAs) (Lindberg & Lundeberg, 2010). Modern transcriptomic analysis uses high-through put methods to analyse the expression of multiple transcripts in different physiological or pathological conditions and this is rapidly expanding our understanding of the relationships between the transcriptome and the phenotype across a wide range of organisms (Shahandeh *et al.*, 2016).

6.3 Mixed tissue / stage oligo microarray studies

Expressed sequence tags (ESTs) are partial sequences of mRNA molecules generated from complementary DNA sequences that may be used as a proxy identifier for the larger molecule. EST information is particularly useful for gene expression studies and genome mapping. As Type I markers or markers with known functions, ESTs are useful for comparative genome mapping between species. (Liu & Dunham 1999; Liu & Cordes 2004). While RNAseq provides an increasingly powerful tool for transcriptomic analysis it remains expensive, particularly for the analysis of statistically valid sample numbers. Microarray on the other hand provides a cost-effective tool for transcriptomic analysis of a defined panel of transcripts and has been applied in numerous studies to give an idea of the expression of thousands of genes for any given organismal state or states. When used appropriately, transcriptomic analysis can provide broad-brush understanding of an organism's state under particular conditions and provides an important tool for hypothesis generation, subject to downstream testing across a range of analytical modalities. Microarrays can be employed to visualize which genes are likely to be used in a particular tissue at a particular time under a particular set of conditions. The output of a microarray experiment is called a "gene expression profile. Microarray technology has been applied to study mechanisms of disease, which is turn has led to improved discovery of new drugs across short timescales.

Prior to microarray, only a few genes could be studied at a time using the Northern Blot Analysis. Gene Chip microarrays use the natural chemical attraction, or hybridization, between DNA on the array and RNA target molecules from the sample based on complementary base pairing. Only RNA target molecule that have exact complementary base pair bind to the probe are capable of being detected. This has allowed us to measure tens of thousands of genes at a time, and it is this quantitative change in the scale of gene measurement that has led to a qualitative change in our ability to understand regulatory processes that occur at the cellular level. The short probe on the microarray measures the expression of the complete transcript by sampling only a small section of the transcript. In some instances, as little as one RNA molecule out of 100,000 different RNAs in an original sample may be detected which has enabled the technology to be used for

a broad variety of research (Roy 2017). Functional analysis of the catfish genome through transcriptomics is useful for the identification of genes controlling traits of economic importance, especially growth, disease resistance and nutritional status. The use of microarrays for the study of various aspects of fish physiology has seen a spectacular increase in recent years. From early studies with model species, such as zebrafish, to current studies with commercially important species, such as salmonids, catfish, carp, and flatfish, microarray technology has emerged as a key tool for understanding developmental processes as well as basic physiology. In addition, microarrays are being applied to the fields of ecotoxicology and nutrigenomics. A number of different platforms are now available, ranging from microarrays containing cDNA amplicons to oligomers of various sizes. Commercial high-density microarrays containing hundreds of thousands of distinct oligomers have been developed for zebrafish for instance. As this exciting technology advances, so will our understanding of global gene expression in fish. Furthermore, lessons learned from this experimentally tractable group of organisms can also be applied to more advanced organisms such as humans. (Douglas 2008; Murray *et al* 2010; Roy 2017).

No transcriptome had been established for *C. gariepinus* when the current work commenced and neither was there an available genome for *Clarias* as of 2012 when this work commenced. A complete mitochondrial genome has, however, now been sequenced (Han *et al.*, 2015). The work described in the current chapter documents the development of transcriptomic resources for *C. gariepinus* and examines the transcriptomic changes associated with key aspects of early development in larvae of *C. gariepinus* under different dietary regimes.

6.3.1 Objectives

The key objectives emphasised in this part of the chapter were ;

1. To sequence, assemble and annotate a mixed-tissue transcriptome for the North African catfish *C. gariepinus*
2. To determine the effects of introduction of dry diet (Coppens Standard diet for Catfish larvae during the yolk sac period i.e. days 1,2,3 post hatch (Dph) on growth and onset of enzyme expression microarray analysis of transcripts during early larval development
3. To contextualise enzyme expression within larger developmental transcriptomic events and examine whether dietary composition / timing affects expression of these enzymes

6.4 Materials and Methods

6.4.1 Fish samples for transcriptome studies

6.4.1.1 Sample collection and preservation in RNAlater

Adult female and male cat fish aged 3 years and weighing 2kg-3.5Kg, juvenile male and female catfish aged 3 months and weighing 100-150g were euthanised by terminal anaesthesia and destruction of the brain according to UK Home Office schedule one methods. Larval catfish aged 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 14, 21, 28 days old were also euthanised using terminal anaesthesia. Adult fish were dissected to reveal the digestive, reproductive, circulatory, muscular, nervous, metabolic and immune system tissues. Using a sterile scalpel blade, fresh 0.5 x 0.5 cm samples of the heart, liver, alimentary canal, gall bladder, accessory breathing organs, gills, testis, eggs, fins, skin were excised and then preserved in RNAlater® (Life technologies, Ambion®, UK) at a volume ratio of 1:10, as were whole catfish larvae, perforated to allow preservative ingress.The samples were then left for RNAlater permeation and placed in the fridge at 4°C.

24 hours after keeping the samples at 4°C in a fridge, the RNAlater was drained off and samples placed in 3.6 mL Nunc Cryo tubes (Thermo fisher Scientific, Denmark) and the samples stored in a freezer at -70°C (New Brunswick Scientific low temperature U570). This was done to prepare RNA for sequencing later in order to obtain mixed tissue for a catfish transcriptome.

6.4.1.2 RNA extraction

RNA was extracted using an organic extraction analysis protocol as follows:

30 mg tissue was placed in 2mL screw cap micro tubes (Alpha laboratories,UK) containing two 3mm glass beads (Merck KGaA,USA) and homogenised in 1mL of Trizol (TRI)Reagent (Sigma Aldrich USA) for 30-45 seconds in the mini bead beater (Thistle Scientific,UK)., until tissue was significantly disrupted. The sample was incubated at room temperature for 5 minutes.

100µL 1-bromo-3-chloropropane [BCP] (Sigma Aldrich USA) was added and tube was shaken vigorously by hand for 15 seconds. It was then left to incubate at room temperature for 15 minutes. The sample was then centrifuged (SciQuip 4K15 Sigma) at 20,000 × g for 15 minutes; at 4ºC.Then 400µL of the upper aqueous phase was transferred to a nuclease-free 1.5mL microfuge tubes (Axygen fisher Scientific, UK).

200µL of RNA precipitation solution followed by an equal volume of isopropanol (Fluka) was added to the aqueous phase. The sample was then gently inverted 4-6 times. This was followed by Incubation for 10 minutes at room temperature. The sample was then centrifuged at 20,000 × g for 10 minutes at 4°C. A precipitate of RNA formed a gel like pellet on the side/bottom of the tube. This was followed by the washing phase during which stage the supernatant was removed and then the pellet was washed with 1 mL of 75% ethanol (Fisher Scientific). This was followed by brief flick-mixing of the sample, then centrifugation at 2000 × g for 5 minutes at room temperature. The supernatant was carefully removed using a 1mL (Gilson) Pipette followed by re-spinning down the sample and further removal of the supernatant using 20µL (Gilson) pipette. Finally the RNA pellet was air dried at room temp for 3-5 minutes, until all visible traces of ethanol were evaporated.

The RNA was resuspended 30µL of RNase free water and then incubated overnight on ice. The sample concentration was then measured using a Nanodrop 1000.

Integrity checks were then performed by heating an aliquot of RNA 1.0 µg for 5 minutes at 75ºC,, chilling briefly on ice and running on a 1% agarose gel containing Ethidium Bromide, to a final concentration of 0.05µg mL^{-1}) along with a 100bp marker. Photography of the gel was then done on a Syngene Bio imaging (USA) machine.

6.4.1.3 Transcriptome sample analysis

Total RNA samples were sequenced by University of Edinburgh. High thoroughput Illumina sequencing was performed and the transcriptome Miseq results were initially analysed using R-genomics resulting in over 300,000,000 reads each with a mean read size of 100 bp**,** this was followed by removal of Low quality sequences and primers. *De novo* assembly was performed using Velvet \ Oases programs: Oases version 0.2.01, and Velvet 1.1.06 9. Assembled contigs were annotated and best annotated transcripts were employed for microarray design.

6.4.1.4 Preparation of total RNA samples for hybridisation

6.4.1.4.1 Source of tissue samples for transcriptional analysis

Larval tissue samples were obtained from the experiment described in Chapter 3, Trial 4. In brief, treatment 1 (T1) larvae were fed on 100% Coppens diet from day 1 dph, T2 were fed on 100% Coppens diet from day 2, T3 were fed on 100% Coppens diet from day 3, T4 were fed on 50% *Artemia* and 50% Coppens diet

from day 1, T5 were fed on 50% *Artemia* and 50% Coppens diet from day 2, T6 were fed on 50% *Artemia* and 50% Coppens diet from day 3. The fish were then fed on the dry diet until day 10. This trial was selected on the basis that it gave promising results in terms of growth performance, as seen in figure 6-0 below.

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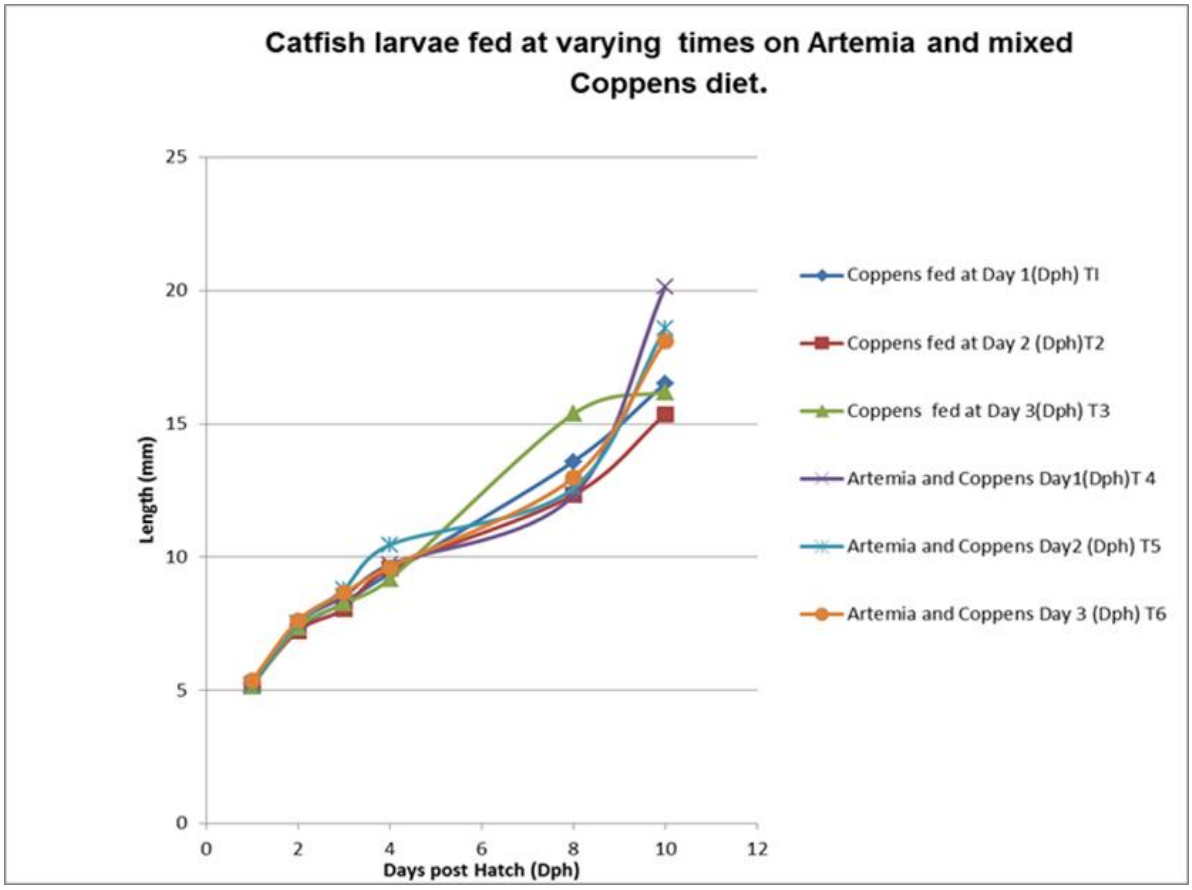


Figure 6-0a showing growth of larvae of African catfish *Clarias gariepinus* under different feeding regimes

Based on the results obtained from the growth curve, larvae from two feed states were selected (see Figure 6-0b), and were then subject to transcriptomics analysis: Feed state T2 fed on 100% Coppens diet from day 2 (“Dry diet”), which was the poorest performing diet and Feed state T5 fed on 50% *Artemia* and 50% Coppens diet from day 2 (Normal / standard feeding) (see Figure 6-0b). Both treatments were examined over 4 days of development *i.e.* days 1, 2, 3, 4, with post hatch (0 hr fish) also being selected.

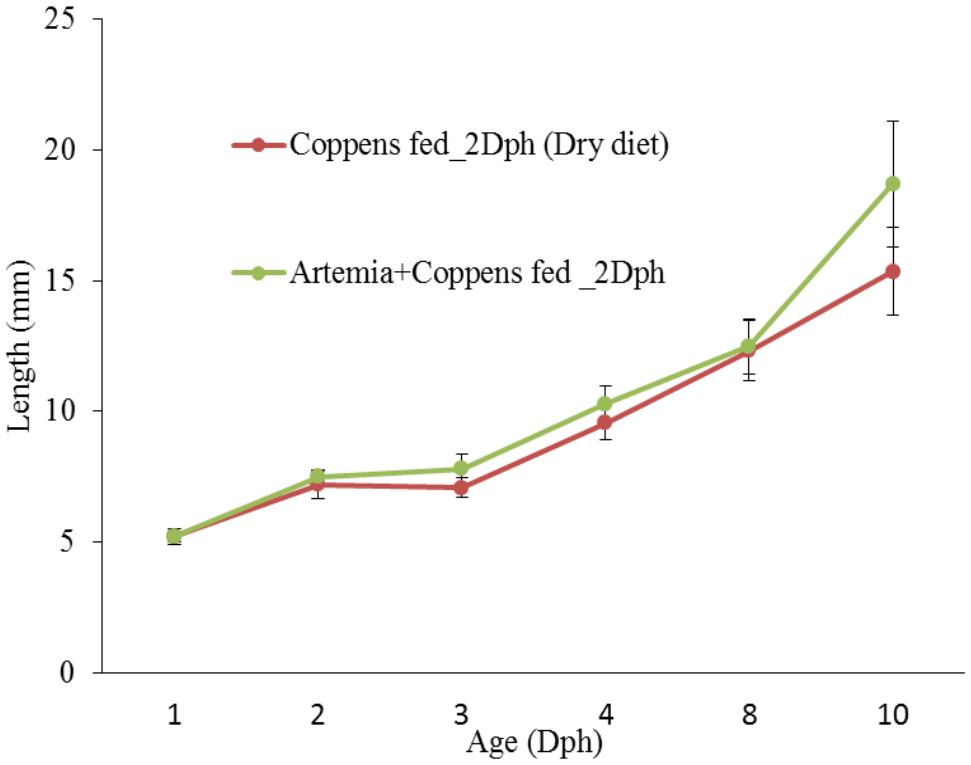


Figure 6-0b Growth of African catfish *Clarias gariepinus* larvae selected for use on the mixed oligo-microarray (only days 1-4 used for microarray).

For each day and feed state, 6 pools of fish were sampled into RNAlater following euthanisation and for total RNA extraction 4 individuals were taken from each pool. This resulted in 42 sample pools as day 1 had no treatment. Pooled sample concentrations are shown in Appendix 4.The gel (as seen in Appendix 4) was run to ensure that RNA for microarray analysis was of good quality.

6.4.2 Design and construction of a mixed-tissue *C. gariepinus* oligo microarray

6.4.2.1 Custom oligo microarray design

A custom oligo microarray was designed and constructed that allowed characterization of different states during larval digestive development / digestion in order to improve understanding of early development and nutrition. The microarray design comprised a 15K feature Agilent oligo microarray which was employed in a two-colour, pooled reference design experiment. Samples were labelled with the Cy3 and Cy5 dyes, hybridised and scanned with an Axon microarray scanner. The process occurred in accordance to the following steps:

A Two colour microarray was prepared by amplification and fluorescence hybridisation of Complimentary RNA, cRNA. Sample preparation employed the Low Input Quick Amp Labelling Protocol v6.6 (Agilent Technologies, USA). For

sample preparation, 150ng of total RNA from each sample was used for cDNA synthesis. The cDNA was then primed with an Oligo dT T7 RNA polymerase promoter. The total RNA was mixed with 0.8 µL of primer and water in total volume of 5.3 µL. The primer and the template were denatured by incubation at 65 °C for 10 min and subsequently placed in ice. cDNA mix was prepared by combining 2 µL of 5 × First Strand Buffer, 1 µL of 0.1 M DTT, 0.5 µL of 10 mM dNTP mix and 1.2 µL of Affinity Script RNase Block Mix after which 4.7 µL of the mix were added to reaction and incubated at 40 °C for 2 hr with final inactivation achieved by incubation at 70 °C for 15 min. Subsequently, T7 RNA polymerase incorporated CTPs (Cyanine 3 (Cy3) or Cyanine 5 (Cy5)) for the experimental or reference samples respectively, into a growing chain of antisense cRNAs. Six µL of the mix containing 0.75 µL of nuclease-free water, 3.2 µL of 5 × Transcription Buffer, 0.6 µL of 0.1 M DTT, 1 µL of NTP mix, 0.21 µL of T7 RNA polymerase Blend and 0.24 µL of Cyanine 3-CTP or Cyanine 5-CTP, were added to the tubes and incubated for another 2 hr at 40 °C. Amplified cRNA was purified using the GeneJET RNA Purification Kit (Thermo Scientific, UK) following the manufacturer's RNA Clean-up Protocol and was further quality-checked by spectrophotometry (NanoDrop ND-1000, Thermo Scientific, USA) and agarose gel electrophoresis. Three hundred nanograms of each Cy3 labelled test and 300 ng of Cy5 labelled reference pool cRNA were mixed together and incubated with 5 µL of 10 × Gene Expression Blocking Agent, 1 µL of 25 × Fragmentation Buffer and sterile water to reach total reaction volume of 25 µL. Samples were incubated at 60 °C for 30 min in the dark to fragment the RNA and cooled on ice for 1 min. Twenty-five µL of 2 × Hi-RPM Hybridization Buffer pre-warmed at 37 °C were added to reactions. Tubes were centrifuged at 13,000 × g for 1 min at RT, placed on ice and immediately loaded on the arrays. Competitive hybridizations were carried out in a rotary oven (Agilent Technologies, UK) over 17 hours at 65°C and 10 rpm. Following Agilent standard protocols the slides were subsequently washed in Wash Buffer 1 (at RT and 10 s at 31 °C), Wash Buffer 2 (1 min at 31 °C), acetonitrile (10 s) and Agilent Stabilization and Drying solution (30 s), the latter to reduce ozone-induced decay of Cy5 signal. Slides were stored in light proof box and scanned within 1 hr of washing.

6.4.2.2 Microarray data analysis

Scanning was conducted using an Axon Genepix 4200A scanner with Genepix Pro 6.1 image acquisition software (Molecular Devices, UK) with 60 % red and 90 % green laser power and 5 µm resolution. Saturation tolerance was set to 0.05 % and automatic photo-multiplier tube (auto-PMT) gain used to achieve similar mean intensities of Cy3 and Cy5 signals. Acquired data were exported and processed using the Agilent Feature Extraction software (v9.5.3.1) to obtain background-subtracted signals, as well as other spot statistics and quality metrics. Scan data were analysed using GeneSpring GX v12 (Agilent Technologies, UK). Baseline transformation was not employed and data were normalized using a Lowess model. Principal Component Analysis (PCA) was conducted to visually inspect the distribution of gene expression variance among arrays within and between experimental conditions. Following removal of Agilent control features; quality filtering was done which involved removal of saturated and non-uniform features, population outliers and features that were not significantly positive with respect to the local background intensity. Statistical analyses were carried out in Genespring (v.13) (Agilent Technologies, UK) and R (v. 3.01 using a range of downstream analytical tools including Go annotation, KEGG pathway analysis, Heat maps, Venn diagrams and Principal component analysis.

6.4.2.3 Profiling of tissue-specific gene expression signatures

Functional gene set analyses were performed on the entire list of quality filtered entities based on KEGG Orthology (KO) identifiers. During the analyses, expression profiles were collapsed into unique KOs. Using the median method, Tests were run to identify significantly up - and down- regulated pathways for each tissue and their pairwise combinations against all other samples. A default false discovery rate (FDR) q-value of 0.1 was used as a cut-off. Pathways were subsequently grouped into KEGG functional categories and the difference between the counts of up and down-regulated pathways in each category hierarchically clustered and displayed as a heatmaps, GO ontologies, Venn diagrams and Principal component analysis.

6.5 Results and Discussion

6.5.1 Annotation of the mixed tissue transcriptome of North African catfish, *C. gariepinus*

Sequencing, assembly and annotation of transcriptome data were successfully achieved, with 301,407,872 reads being generated in total (see Table 6-1). Assembly of the mixed tissue transcriptome yielded a total number of 201,487 assembled contigs / transcripts, which, after quality filtration was reduced to 58,830 transcripts, covering 52,744 genes. N50 was 688, shortest transcript was 271, longest transcript was 3,664 mean sizeswere 678, median size was 615, Mean GC% was 44.8% and N% was 0.4%. These transcripts were annotated and from the annotated transcripts 46,830 unique probes were designed using the Agilent technology probe design platform as seen in table 6-1 below.

Table 6-1 showing the Statistical summary of the annotation of the mixed tissue transcriptome of North African catfish, *C. gariepinus* (Burchell 1822)

Raw data	**Base pairs**
Number of reads:	301,407,872
Mean read size:	100
After assembly	
Number of transcripts	201,487
Mean size	437
Total size of transcripts	87,996,714
N50	546
After filtering	
Number of transcripts	58,830
N50	688
Total size of transcripts	39,906,473
Shortest transcript	271
Longest transcript	3,664
Mean size	678
Median size	615
Mean GC%	44.80%
N%	0.40%

Once obtained, the RNA sequence data were deposited with the accession number ERP006668. The transcriptome was submitted to NCBI database in 2015 at https://www.ncbi.nlm.nih.gov/bioproject/298976 as Project Accession number PRJEB6987 on 16th October 2015, copyright of Institute of Aquaculture (IOA), University of Stirling. Submission details are given in Table 6-2.

6.5.2 Accession of the transcriptome into NCBI database

Table 6-2North African catfish, *C. gariepinus,*transcriptome profile submitted to NCBI

Accession	PRJEB6987
Scope	Monoisolate
Submission	Registration date: 16-Oct-2015 IOA

Project Data

Resource Name	Number of Links
SEQUENCE DATA	
SRA Experiments	1
OTHER DATASETS	
BioSample	1

SRA Data Details

Parameter	Value
Data volume, Gbases	30
Data volume, Mbytes	17952

6.5.3 Oligo microarray results

Microarray data were analysed using Genespring v13 and R Bioconductor scripts to produce heat maps, KOs and KEGG maps respectively.

6.5.4 Heat maps

Two-way ANOVA was conducted to identify the most significantly differentially expressed transcripts associated with time and diet+diet x time. The top 100 most-significantly differentially regulated transcripts over time are shown in Figure 7-1. A common method of visualising gene expression data is to display it as a heat map. In these, transcript expressions under different conditions are clustered by profile similarity across conditions. This visualisation technique can be useful for identifying genes that are commonly regulated, or biological signatures associated with a particular condition (e.g. a disease or an environmental condition) (Chipman *et al.,* 2003).

In heat maps the data is displayed in a grid where each row represents a transcript and each column represents a sample. The colour and intensity of the boxes is used to represent changes relative to the pooled reference control (not absolute values) of gene expression. In the heatmap below, red represents up-regulated genes and green represents down-regulated genes. White represents unchanged expression.

6.5.4.1 Significant differential expression over time

A heat map of the 100 most significantly differentially regulated transcripts over time for oligo-microarray analysis for catfish larvae fed on standard diet and dry diet at days 2, 3 and 4 Days post hatch (Dph) is shown in Figure 7-1 below. Results can be resolved into 5 clustered expression patterns, namely cluster 1 (1-8); Cluster 2 (9-33); Cluster 3 (34-42); cluster 4 (43-60) and cluster 5 (61-100).

In cluster 1, two important expression patterns are observed, one presumed to relate to embryonic development and the other to digestion, and these occurring when the larvae are fed on the both standard and dry diet over 2, 3, 4 Dph. Up-regulation of hyaluronidase is observed at 4 Dph and is believed to play a role in various potential roles in the developing larvae including, among others, embryonic development, cell migration / differentiation, wound healing, inflammation and growth (Kreil, 1995). These processes are important to the developing larvae and were significantly up regulated at 4Dph.

In the case of digestive activities, an important enzyme encountered in chapters 5 and 6, chitinase, is significantly up-regulated by 4Dph. This concurs with the earlier results observed in chapters 5 using *in situ* expression hybridisation, with site of production being the stomach and transcript presence observed from 3Dph and being prominent at 4Dph. Chitinases are thought to function in the digestion and absorption of food and in terms of immune defence against pathogens (Di Rosa *et al.*, 2016), both of which roles are important to developing fish larvae, particularly as their immune systems are likely to be poorly developed in early life requiring a reliance on innate immunity.

In cluster 2 various transcriptional activities are represented, prominent among which, in terms of digestive development of the larvae, is intestinal tubulin, alpha 1, which may be indicative of growth of the developing intestinal wall during this period. In both feeding states it has been observed that expression of this cluster of transcripts is slightly up-regulated at 2 Dph, however, is down-regulated by 4Dph in the larva. These transcripts may thus be important during the stage when the stomach is not fully developed as seen earlier in chapters 5 and 6 when only pancreatic digestion is present but before acid digestion takes place.

In cluster 3 a set of transcripts geared towards growth in the form of protein production, nervous system development and muscle tissue components. In this cluster it has been observed that the transcript expression is slightly up-regulated by 2Dph in both the standard diet and dry diet.

2Dph	3Dph	4Dph	2Dph	3Dph	4Dph	
Standard diet			Dry diet			**Description**
-4.01	-3.67	1.88	-4.29	-3.60	1.00	hyaluronidase PH20like [Pundamilia nyererei]
-3.26	-2.81	1.52	-3.79	-2.99	1.25	F_IpSto_13_b17 Acidic mammalian chitinase precursor, mRNA sequence
-3.64	-2.93	1.40	-3.60	-2.42	1.34	F_IpSto_13_k22 Stomach Acidic mammalian chitinase precursor, mRNA sequence
-3.42	-2.65	1.26	-3.03	-2.58	1.18	acidic mammalian chitinaselike [Anolis carolinensis]
-2.68	-2.20	1.59	-2.46	-1.47	1.58	solute carrier family 26, member 9 [Oreochromis niloticus]
-2.88	-2.69	1.54	-2.70	-2.26	1.53	F_IpSto_12_e19 Stomach Acidic mammalian chitinase precursor, mRNA sequence
-3.69	-1.46	1.36	-3.92	-2.61	0.64	nattectinlike [Maylandia zebra]
-3.26	-1.34	1.44	-3.35	-1.52	1.29	cochlinlike [Pundamilia nyererei]
0.71	-0.18	-0.39	0.32	-0.06	-0.49	Lix1 homolog (chicken) [Danio rerio]
0.56	-0.17	-0.48	0.33	0.04	-0.44	keratin, type 1, gene c5 [Danio rerio]
0.26	-0.26	-0.42	0.35	-0.54	-0.82	hyaluronan and proteoglycan link protein 1b [Danio rerio]
0.30	-0.16	-0.45	0.12	-0.13	-0.44	zgc:113210 [Danio rerio]
0.22	-0.27	-0.63	0.29	0.03	-0.59	hyaluronan and proteoglycan link protein 3 [Mesocricetus auratus]
0.05	-0.31	-0.56	0.06	-0.10	-0.55	
0.06	0.39	-0.23	0.19	-0.34	-0.60	putative LOC568911 [Danio rerio]
-0.12	0.18	-0.34	-0.05	-0.45	-0.60	histone H3like centromeric protein A [Danio rerio]
-0.02	0.13	-0.53	0.06	-0.34	-0.68	CHK1 checkpoint homolog (S. pombe) [Danio rerio]
-0.02	0.03	-0.35	-0.01	-0.29	-0.61	m7GpppX diphosphataselike [Oreochromis niloticus]
0.09	-0.10	-0.21	0.13	-0.03	-0.36	highmobility group box 2a [Danio rerio]
0.15	-0.24	-0.07	0.10	-0.22	-0.46	keratocanlike [Oryzias latipes]
0.25	0.10	-0.26	0.20	-0.24	-0.38	F_IpInt_58_k11 Intestine tubulin, alpha 1, mRNA sequence
0.16	0.21	-0.20	0.32	-0.02	-0.34	protein phosphatase 1, regulatory (inhibitor) subunit 8a [Danio rerio]
0.29	-0.23	-1.04	0.32	-1.67	-2.01	carbohydrate (chondroitin 6) sulfotransferase 3a [Danio rerio]
0.15	-0.26	-1.28	0.29	-0.50	-1.33	collagen, type XXVIII, alpha 1 [Oreochromis niloticus]
0.23	-0.06	-1.15	0.53	-0.05	-0.97	ATPsensitive inward rectifier potassium channel 1like [Takifugu rubripes]
-0.09	-0.31	-1.05	0.55	0.04	-1.24	glycolipid transfer protein domain containing 2 [Danio rerio]
0.33	-0.65	-1.31	0.12	-0.77	-0.52	5,6dihydroxyindole2carboxylic acid oxidase-like [Pundamilia nyererei]
0.05	-0.55	-0.84	0.00	-0.26	-0.78	tenascinXlike [Danio rerio]
-0.06	-0.65	-0.69	0.16	-0.15	-0.47	forkhead box I3b [Danio rerio]
0.30	-0.55	-0.84	0.16	-0.35	-1.11	beta1,4Nacetylgalactosaminyltransferase 3-like [Danio rerio]
0.41	-0.66	-1.09	0.37	-0.34	-0.80	retinol dehydrogenase 5 (11cis/9cis) [Danio rerio]
-0.97	0.99	-0.09	-0.64	0.07	0.17	farnesyl diphosphate synthase, geranyltranstransferase) [Danio rerio]
-0.39	-0.04	0.75	-0.20	0.41	0.79	vitronectin a [Danio rerio]
-0.31	-0.02	0.60	-0.29	0.65	0.80	interalpha (globulin) inhibitor H5 [Oreochromis niloticus]
-0.56	-0.17	0.22	-0.31	0.49	0.59	calcium channel, voltagedependent, gamma subunit 6a [Danio rerio]
-0.50	0.01	0.46	-0.42	0.37	0.77	si:dkey32e6.6 [Danio rerio]
-0.41	0.02	0.51	-0.31	0.48	0.57	claudin6like [Takifugu rubripes]
-0.30	0.29	0.47	-0.26	0.25	0.47	lysyl oxidaselike 3a [Danio rerio]
-0.30	0.23	0.50	-0.43	0.50	0.57	zgc:162509 [Danio rerio]
-0.26	0.38	0.54	-0.33	0.42	0.50	endoplasmic reticulum resident protein 27like [Oreochromis niloticus]
-0.14	0.39	0.62	-0.04	0.54	0.52	lysyl oxidase homolog 4like [Takifugu rubripes]
-0.14	0.40	0.69	-0.20	0.23	0.37	lysyl oxidase [Danio rerio]
-0.44	0.00	0.53	-0.69	0.27	0.60	Gprotein coupled receptor 64like [Danio rerio]
-0.53	-0.04	0.79	-0.61	-0.07	0.59	F_IpInt_52_c11 Intestine cDNA library Ictalurus punctatus cDNA 5' similar to desmin, mRNA sequence
-0.40	0.09	0.52	-0.53	-0.10	0.70	uroplakin 3l [Danio rerio]
-0.45	-0.30	0.22	-0.53	-0.04	0.51	Parkinson disease (autosomal recessive, juvenile) 2, parkin [Danio rerio]
-0.39	-0.22	0.40	-0.39	-0.01	0.42	si:ch211106h4.12 [Danio rerio]
-0.27	0.06	0.31	-0.37	0.32	0.44	interferon induced transmembrane protein 5 [Danio rerio]
-0.44	0.01	0.60	-0.36	0.14	0.59	serine (or cysteine) proteinase inhibitor, clade A (alpha1 antiproteinase, antitrypsin) [Danio rerio]
-0.37	0.07	0.39	-0.27	0.10	0.53	grainyheadlike 2 (Drosophila) [Ornithorhynchus anatinus]
-0.54	-0.05	0.36	-0.28	0.15	0.44	pannexin 3 [Danio rerio]
-0.59	-0.02	-0.02	-0.45	-0.05	0.20	im:7158179 [Danio rerio]
-0.34	-0.09	0.14	-0.43	0.13	0.15	zgc:158296 [Danio rerio]
-0.36	-0.16	0.08	-0.46	0.32	0.16	putative LOC102203580 [Pundamilia nyererei]
-0.14	-0.13	0.24	0.11	0.08	0.45	cathepsin B, a [Danio rerio]
-0.08	-0.02	0.38	-0.09	0.03	0.22	collagen, type IV, alpha 2 [Danio rerio]
-0.31	-0.07	0.28	-0.20	0.02	0.34	regucalcin [Ictalurus punctatus]
-0.24	-0.07	0.22	-0.23	0.17	0.30	lysosomal ProX carboxypeptidaselike [Oreochromis niloticus]
-0.17	-0.07	0.26	-0.25	0.10	0.25	A_IpInt00346 IntestineRibonuclease angiogenin inhibitor 1, mRNA sequence
-0.13	-0.06	0.32	-0.27	0.05	0.25	integrin, alpha 10 [Danio rerio]
-0.63	0.15	1.02	-0.50	0.02	1.29	follistatinrelated protein 5like [Danio rerio]
-0.49	0.00	0.58	-0.63	0.53	1.08	connexin 31.7 [Danio rerio]
-0.65	-0.15	0.68	-0.90	0.42	1.38	F_IpSto_13_g07 Chain A, Alcohol Dehydrogenase, mRNA sequence
-0.78	-0.26	0.59	-1.06	0.14	1.09	alcohol dehydrogenase 1like [Takifugu rubripes]
-1.35	0.21	0.75	-1.41	0.22	0.91	vitelline membrane outer layer protein 1 homolog [Maylandia zebra]
-1.08	-0.10	0.75	-1.27	0.12	0.83	chymotrypsinlike elastase family member 2Alike [Maylandia zebra]
-0.97	0.16	0.84	-1.29	0.36	0.99	si:dkey32e23.4 [Danio rerio]
-0.91	-0.15	0.05	-1.10	0.53	0.92	cathepsin L [Ictalurus punctatus]
-1.19	0.54	0.51	-1.18	0.64	1.17	kinectinlike [Oreochromis niloticus]
-0.61	0.40	0.54	-1.23	0.38	0.56	solute carrier family 30 (zinc transporter), member 10 [Danio rerio]
-0.53	-0.81	0.92	-0.28	0.41	1.03	cytochrome P450 [Ictalurus punctatus]
-0.63	-0.60	0.54	0.00	0.48	0.82	zgc:103710 [Danio rerio]
-0.42	-0.40	0.60	0.22	0.64	0.90	zgc:103710 [Danio rerio]
-0.74	-0.51	0.40	-0.36	0.41	0.69	si:ch211149b19.3 [Danio rerio]
-0.85	-0.23	0.39	-0.38	0.47	0.52	creb3 regulatory factor [Danio rerio]
-0.87	-0.35	0.37	-0.58	0.39	0.50	creb3 regulatory factor [Danio rerio]
-0.77	-0.46	0.53	-0.59	0.38	0.54	sorting nexin 14 [Geospiza fortis]
-0.83	-0.10	0.71	-0.98	0.23	0.55	alpha1A adrenergic receptorlike [Danio rerio]
-0.85	-0.29	0.42	-0.85	0.20	0.57	cleavage and polyadenylation specific factor 1 [Danio rerio]
-0.99	-0.19	1.14	-0.87	-0.13	0.86	microfibrillarassociated protein 4 [Salmo salar]
-1.12	-0.37	0.49	-0.73	-0.15	0.66	ceruloplasmin [Ictalurus punctatus]
-0.63	-0.40	0.77	-0.71	-0.21	0.67	si:ch211186e20.2 [Danio rerio]
-1.42	-0.87	0.92	-1.47	-1.55	1.04	putative LOC101886976 [Danio rerio]
-0.91	-0.83	0.85	-1.74	-1.08	0.91	glucagon receptorlike [Danio rerio]
-2.03	-1.91	1.32	-2.02	-1.71	0.99	synemin, intermediate filament protein [Danio rerio]
-1.40	-1.10	1.84	-1.97	-1.01	1.74	glucagon receptorlike [Danio rerio]
-1.96	-1.00	1.01	-1.59	-0.83	1.04	si:ch1073473i7.3 [Danio rerio]
-1.93	-1.36	1.06	-1.70	-1.37	1.16	transmembrane emp24 protein transport domain containing 6 [Gallus gallus]
-1.73	-0.92	1.22	-2.08	-0.93	1.48	connexin 34.4 [Danio rerio]
-2.09	-1.05	1.20	-2.31	-1.26	1.14	transmembrane emp24 domaincontaining protein 6like [Takifugu rubripes]
-1.73	-0.77	1.11	-2.06	0.07	1.17	phospholipase A2like [Maylandia zebra]
-1.92	-0.79	1.10	-2.25	-0.27	1.12	saxitoxin and tetrodotoxinbinding protein 2like [Danio rerio]
-1.19	-1.17	0.99	-1.60	0.01	1.15	complement component 7b [Danio rerio]
-1.41	-0.43	0.63	-1.43	-0.35	0.95	transmembrane protein 86A [Danio rerio]
-1.64	0.21	0.38	-2.39	0.38	0.61	natterin3like [Takifugu rubripes]
-1.17	0.11	0.65	-1.93	-0.04	1.08	Sin3Aassociated protein a [Danio rerio]
-1.76	0.20	0.59	-1.88	0.10	0.98	proactivator polypeptidelike [Maylandia zebra]
-1.44	0.82	0.76	-1.37	-0.24	0.58	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)like 2 [Danio rerio]
-1.54	-0.15	1.17	-1.15	-0.78	0.21	putative LOC567193 [Danio rerio]
-1.60	-0.11	1.34	-1.39	-0.27	0.65	keratin, type I cytoskeletal 13like [Oreochromis niloticus]

Figure 6-1 Heatmap showing 100 most significantly differentially regulated transcripts over time when fish larvae are fed on standard diet and dry diet at 2, 3 and 4 Dph. Red = up-regulated; Green = down-regulated.

In cluster 4 up-regulation of transcript expression was observed from 2Dph increasing in intensity to 4Dph in both instances when the larvae are fed on the standard or dry diet. Notable among the activities include proteins production and carbohydrate metabolism and controls to the pancreatic cells involved in growth and development. This set may reflect the demands of the rapid development previously noted for North African catfish larvae.

In cluster 5 most of the activities were down-regulated at 2Dph, switching on 3Dph and were highly up-regulated by 4 Dph. Notable among these activities are metabolic enzyme activity for example farnesyl diphosphate synthase a condensing enzyme that catalyses the synthesis of polyunsaturated very long chain fatty acids, which is part of a key isoprenoid pathway generating products that are crucial for cellular processes; namely, cholesterol synthesis, protein glycosylation, growth control and synthesis of several hormones (Martín *et al.*, 2007). These are vital activities for the development of the growing larval and can be seen to occur on both the diets over the same period and at similar expression levels, which could mean that dry diet, Coppens, is suitable for growth and development of larvae of *C. gariepinus* up to 4 Dph.

Another one observed is beta1,4,N-acetylgalactosaminyltransferase 3-like, which initiates apoptosis, which is an important regulator of growth and development and has been known to act as part of quality control and repair based on genetic mechanisms allowing for plasticity in growth and development of organisms (Meier *et al.*, 2000; Ulukaya *et al.*, 2011; Zeng *et al.*, 2014). It has been observed that apoptosis occurs from early development to adult stages and is key to the homeostasis of multicellular organisms, particularly during disease events, development and in response to different stimuli in many different systems. This is therefore very relevant for the developing larvae and is now seen to occur as early 2 Dph when fed on both diets. The similarity between diets suggests that both were equally suitable in terms of allowing the same pattern and intensity of expression. In summary, there seems to be a gradual switch on of genes from day 2 after the start of feeding in both larvae fed on the dry diet and standard diet for the first cluster of 8 transcripts, however, the next cluster of 23 transcripts are switched on at the start but begin to switch off towards the fourth day in both the larvae fed on the dry diet and standard diet. For the remainder of the 69 genes

there is gradual switch on of the genes for both the larvae fed on dry diet and standard diet.

A number of KEGG pathways were observed to be over-represented in terms of transcript expression with respect to diet at 2 days post-hatch (Dph) and 4 Dph Some transcripts showed consistent differential expression among replicates, however, a number of transcripts showed heterogeneous expression between replicates. These included ko04610 Complement and coagulation cascades, ko04974 Protein digestion and absorption, ko04142 Lysosome ko04210 Apoptosis and ko03320 PPAR signalling pathway. These denote significant differential expression in elements of growth, digestion, and the immune system.

In addition during development, the following pathways were observed to be over-represented in terms of transcript expression between 1day post-hatch (Dph) and 4 Dph Some transcripts showed consistent differential expression among replicates, however, a number of transcripts showed heterogeneous expression between replicates. These included ko04974 Protein digestion and absorption, ko04976 Bile secretion, Complement and Coagulation cascades ko04610 Glycolysis gluconeogenesis, ko00010 PPAR signalling pathway Ko03320, Ribosome Ko3010, calcium signalling pathway ko034020, Phagosome ko04145, Serotonergic synapse ko04726, cell adhesion ko04514 oxidative phosphorylation ko00190,Cholinergic synapse ko04725,Lysosome ko04142, Starch and sucrose metabolism ko00500, Vascular smooth muscle contraction ko04270 Long term potentiation. These denote changes in elements of growth, digestion, the immune system and synaptic transmission.

During larval development of North African catfish, various proteins come together to perform different functions and at this stage expressional changes are dominated by transcripts involved in promoting or enacting rapid growth in the larvae and in facilitating the digestive and metabolic events that support it.

6.5.4.2 Significant differential expression between diets and between diets over time

It was observed that when the larvae were fed on a standard diet by day 2 as seen in cluster 1 (1-4) the larvae show up-regulated expression of transcripts 1-4 at day 2, with these being down-regulated by day 3 and 4. However, this did not seem to be the case in the larvae fed on the dry diet as these transcripts remained down-regulated as seen in figure 6-2. The major activities identified as being up-regulated notably included cyclic AMP dependent transcription factor ATF4-like which plays various roles including, among others, anti-apoptosis activity, involvement in cell growth and DNA damage response (Kawasaki *et al.*, 2000). It is also noted to be involved in the cellular responses to amino acid and glucose starvation and endoplasmic reticulum stress, which could suggest that its up-regulation may be in response to a poorer starter-diet. Another notable expressed transcript is that of zinc finger protein 706-like, this suggested to have a role in the exit of embryonic stem cells (ESCs) from self-renewal (Laity *et al.*, 2001; Lau *et al.*, 2012; Meier *et al.*, 2000). These are vital processes in the developing larvae, however, they are up-regulated in the larvae fed on standard diet, which may have consequences for developing larvae. This concurs with the results for larval growth in chapter 3 where larvae fed on standard diets initially showed less growth in regards to weight and length.

Calponin.is another of the observed present by 2 Dph, this is a vital enzyme in the bio mineralization process giving rise to bones important in locomotion and otoliths important in hearing and balance for the larval fish (Addadi& Weiner, 2014; Hu *et al.*, 2011). Failure to develop can result in deformed fish larvae and its lower expression in larvae fed on dry diet may have underlying effects on the development of the growing larvae.

Various other clusters of expression are observed and in Cluster 2 (5-6) the transcripts were down-regulated. These are then followed by cluster 3 (7-36), Cluster 4 (35-49) cluster 5 (50 -100)

In Cluster 2 the genes were down-regulated in both feeding states, this is in part a response to an external stimulus. This was observed for the ATG16L1 transcript, which codes for autophagy related 16-like 1. This protein is a member of a larger family of proteins that are required for autophagy. Cells use this process to recycle worn-out cell parts and break down certain proteins when they are no longer

needed. Autophagy also plays an important role in controlled cell death (apoptosis). We see that during development this process may be important and that in this case, the standard feed decreases expression, albeit minimally, during early stages.

	Day 2	Day 3	Day 4	Day 2	Day 3	Day 4	
	Standard Diet			Dry Diet			Description
1	0.34	-0.50	-1.87	-3.06	-3.34	-3.76	cyclic AMPdependent transcription factor ATF4like [Acyrthosiphon pisum]
2	0.91	-0.75	-2.46	-2.51	-2.26	-2.53	calponinlike protein Chd64 [Apis mellifera]
3	0.83	-0.09		-2.13	-2.06	-1.79	cytochrome bc1 complex subunit 6, mitochondriallike [Aplysia californica]
4	0.25	-0.11	0.22	-1.80	-1.60	-1.99	zinc finger protein 706like [Acyrthosiphon pisum]
5	-1.13	-1.14	-1.74	-1.45	-1.41	-1.03	si:dkey253i9.4 [Danio rerio]
6	-0.75	-1.10	-2.40	-1.45	-0.98	-1.60	ATG16 autophagy related 16like 1 (S. cerevisiae) [Danio rerio]
7	0.33	-0.65	-1.31	0.12	-0.77	-0.52	5,6dihydroxyindole2carboxylic acid oxidase-like [Pundamilia nyererei]
8	-0.42	-1.27	-1.27	-0.04	-0.16	-0.81	ATG16 autophagy related 16like 1 (S. cerevisiae) [Danio rerio]
9	-0.85	-1.61	-0.70	-0.82	0.25	-1.07	TBC1 domain family member 2Alike [Oreochromis niloticus]
10	-0.48	-0.84	-0.15	-0.19	-0.05	-1.09	kinetochoreassociated protein DSN1 homolog [Danio rerio]
11	-0.73	-0.34	1.03	-0.72	0.37	0.04	Ctype mannose receptor 2like [Danio rerio]
12	-1.10	0.10	0.61	-0.08	0.32	0.58	proteasome subunit beta type9a like protein [Ictalurus punctatus]
13	-1.11	-0.93	0.21	0.88	-0.50	1.10	gastric intrinsic factorlike [Oryzias latipes]
14	-0.53	-0.81	0.92	-0.28	0.41	1.03	cytochrome P450 [Ictalurus punctatus]
15	-0.69	-0.81	0.22	-0.26	-0.07	1.35	putative LOC100697633 [Oreochromis niloticus]
16	-0.87	-0.30	-0.92	-0.01	0.17	-0.12	acylCoA synthetase longchain family member 1a [Danio rerio]
17	-0.62	-0.38	-0.36	0.39	0.14	0.28	putative LOC101732979 [Xenopus (Silurana) tropicalis]
18	-0.03	-0.23	-0.48	0.37	0.15	-0.01	guanine nucleotide binding protein (G protein), gamma transducing activity p
19	-0.32	-0.74	-0.56	0.10	0.00	0.10	tribbles homolog 1like [Takifugu rubripes]
20	-0.24	-0.39	-0.39	-0.03	0.36	0.06	trinucleotide repeat containing 6A [Microtus ochrogaster]
21	-0.27	-0.61	-0.30	0.03	0.25	-0.25	hypoxanthineguanine phosphoribosyltransferase [Ictalurus punctatus]
22	-0.59	-0.40	0.12	-0.35	0.46	0.58	chemokine (CC motif) ligand 2 [Sarcophilus harrisii]
23	-0.40	-0.34	0.22	-0.22	0.17	0.37	cholinergic receptor, nicotinic, alpha polypeptide 1 [Danio rerio]
24	-0.56	-0.17	-0.29	-0.14	0.01	0.39	Golgi apparatus protein 1like [Maylandia zebra]
25	-0.55	-0.41	-0.32	-0.48	-0.02	-0.03	epithelial mitogen [Bos taurus]
26	-0.79	-0.93	-0.37	-0.45	0.65	0.17	inositol polyphosphate5phosphatase Jb [Danio rerio]
27	-0.91	-1.06	-0.57	-0.74	0.01	0.00	Eselectinlike [Oreochromis niloticus]
28	-0.09	-0.09	-0.23	0.17	0.63	-0.05	unknown
29	-0.46	-0.44	-0.06	0.23	0.54	0.23	arylsulfatase family, member K [Salmo salar]
30	-0.18	-0.25	0.03	0.32	0.60	0.16	poly(A) binding protein, cytoplasmic 1like [Danio rerio]
31	-0.12	-0.23	0.10	0.34	0.71	0.21	procollagen galactosyltransferase 1like [Oreochromis niloticus]
32	-0.44	-0.20	0.05	0.11	0.25	0.08	choline kinase beta [Danio rerio]
33	-0.21	-0.07	0.13	0.13	0.46	0.24	apolipoprotein Bediting catalytic subunit 2b [Danio rerio]
34	-0.31	0.05	0.02	0.02	0.24	0.28	G2/M phasespecific E3 ubiquitinprotein ligaselike [Maylandia zebra]
35	0.06	-0.40	0.16	0.10	0.21	-0.05	zgc:162883 [Danio rerio]
36	0.11	-0.35	-0.11	0.14	0.33	0.04	protein FAM69Alike [Danio rerio]
37	-0.02	-0.33	0.08	0.08	0.47	0.32	serine active site containing 1 [Danio rerio]
38	-0.13	-0.36	-0.03	0.04	0.25	0.15	WD repeat domain 91 *[Danio rerio]*
39	-0.18	-0.18	0.01	0.05	0.37	0.01	protocadherin 10b [Danio rerio]
40	-0.06	-0.21	-0.02	0.13	0.35	0.13	SWI/SNF related, matrix associated, actin dependent regulator of chromatin,
41	-0.13	-0.15	0.01	0.35	0.15	0.05	ubiquitin associated protein 1 [Danio rerio]
42	-0.17	-0.10	0.17	0.29	0.15	0.30	Fbox and WD40 domain protein 11b [Danio rerio]
43	-0.05	-0.03	0.16	0.04	0.14	0.03	synapse associated protein 1 [Zonotrichia albicollis]
44	0.11	-0.17	0.08	0.13	0.21	0.22	cathepsin F [Danio rerio]
45	0.03	-0.21	0.00	0.09	0.12	0.21	mesothelinlike [Oryzias latipes]
46	-0.08	-0.12	1.02	0.62	0.42	0.21	serine/threonineprotein kinase tousledlike 1Blike [Maylandia zebra]
47	-0.46	-0.03	0.28	0.77	-0.26	0.20	hypocretin (orexin) neuropeptide precursor [Danio rerio]
48	-0.33	-0.42	-0.04	0.85	0.03	0.32	transportin2like [Takifugu rubripes]
49	-0.16	0.08	0.05	-0.24	0.85	0.04	vascular endothelial zinc finger 1b [Danio rerio]
50	0.35	-0.22	0.00	-0.16	0.78	-0.26	mitochondrial fission factorlike [Danio rerio]
51	0.41	-0.04	0.11	-0.32	0.84	-0.33	unknown
52	-0.34	0.24	0.40	0.02	-0.01	-0.04	aldehyde dehydrogenase 9 family, member A1b [Danio rerio]
53	-0.45	-0.03	0.37	-0.11	0.20	-0.01	ATPase, Class VI, type 11C [Danio rerio]
54	0.21	-0.08	-0.13	-0.26	-0.16	0.09	RAD50 homolog (S. cerevisiae) [Danio rerio]
55	0.32	0.06	-0.13	-0.47	-0.16	-0.13	mutS homolog 6 (E. coli) [Danio rerio]
56	-0.23	0.08	0.21	-0.42	-0.04	-0.04	kelchlike 41b (Drosophila) [Danio rerio]
57	0.12	-0.03	0.19	-0.21	-0.16	-0.22	NADPH:adrenodoxin oxidoreductase, mitochondriallike [Danio rerio]

Figure 6-2 Heatmap showing 100 most significantly differentially regulated transcripts between diets+between diets over time when fish larvae are fed on standard diet and dry diet at 2, 3 and 4 Dph. Red = up-regulated; Green = down-regulated.

58	-0.22	-0.46	0.15	-0.42	0.35	-0.20	tetranectin [Ictalurus punctatus]
59	-0.10	-0.23	-0.28	-0.06	0.09	-0.17	troponin T2e, cardiac [Danio rerio]
60	-0.09	-0.15	-0.14	-0.02	-0.01	0.01	ubiquitin specific peptidase 48 [Danio rerio]
61	-0.13	-0.19	-0.06	-0.12	0.00	0.02	baculoviral IAP repeatcontaining protein 2like [Maylandia zebra]
62	-0.97	0.99	-0.09	-0.64	0.07	0.17	farnesyl diphosphate synthase [Danio rerio]
63	-0.62	0.90	-0.01	-0.30	0.03	0.22	Diphosphomevalonate decarboxylase [Salmo salar]
64	-1.44	0.82	0.76	-1.37	-0.24	0.58	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)like 2
65	-1.22	0.76	0.11	-1.13	-0.37	0.22	lanosterol 14alpha demethylaselike [Oreochromis niloticus]
66	-0.74	-0.55	0.08	-0.86	-1.03	-0.92	alpha2macroglobulinPlike [Danio rerio]
67	-0.54	-0.11	1.07	-0.74	-0.63	-0.05	alpha2macroglobulinPlike [Danio rerio]
68	-0.48	0.21	0.30	-1.00	-1.05	-0.13	titin b [Danio rerio]
69	-0.08	0.21	0.34	-0.31	-0.33	-0.38	angiopoietinlike 3 [*Danio rerio*]
70	-0.17	0.34	0.59	-0.24	-0.51	-0.25	tumor protein 63like [*Oreochromis niloticus*]
71	-0.12	0.00	0.26	-0.36	-0.75	-0.06	preBcell leukemia homeobox interacting protein 1b [Danio rerio]
72	-0.05	0.23	0.20	0.03	-0.86	-0.38	apoptosisinducing factor, mitochondrionassociated 1 [Danio rerio]
73	0.30	0.19	0.13	-0.71	-1.17	-0.69	anoctamin 10a [Danio rerio]
74	-0.23	0.40	-0.12	-0.59	-0.56	-0.60	NADPH dependent diflavin oxidoreductase 1 [Salmo salar]
75	-0.29	0.36	-0.37	-0.27	-0.70	-0.73	UPF0160 protein MYG1, mitochondriallike [Oreochromis niloticus]
76	-0.11	0.17	-0.21	-0.27	-0.86	-0.54	proline, glutamic acid and leucinerich protein 1-like [Danio rerio]
77	-0.11	0.36	-0.44	-0.16	-1.06	0.20	Janus kinase and microtubule interacting protein 3 [Danio rerio]
78	0.50	0.33	-0.99	-0.16	-0.22	0.32	copine8like [Danio rerio]
79	0.01	0.29	-0.06	0.11	-0.33	-0.67	glutathione peroxidase 7 [Danio rerio]
80	0.28	0.19	-0.08	-0.09	-0.55	-0.58	si:dkeyp117h8.4 [Danio rerio]
81	0.09	0.08	-0.32	-0.37	-0.48	-0.40	suppressor of var1, 3like 1 (S. cerevisiae) [Xenopus (Silurana) tropicalis]
82	-0.08	-0.12	-0.09	-0.32	-0.45	-0.27	NADPH:adrenodoxin oxidoreductase, mitochondriallike [Danio rerio]
83	0.01	0.09	-0.05	-0.19	-0.39	-0.36	regulator of telomere elongation helicase 1 [Danio rerio]
84	0.11	0.04	-0.17	-0.22	-0.21	-0.27	premRNAprocessing factor 39like [Oreochromis niloticus]
85	0.06	0.09	-0.29	0.00	-0.17	-0.37	KRR1 small subunit processome component homolog [Oryzias latipes]
86	0.04	-0.05	-0.10	-0.03	-0.31	-0.34	wu:fb17g07 [Danio rerio]
87	-0.04	0.02	-0.21	0.08	-0.29	-0.28	transaldolase [Ictalurus punctatus]
88	0.16	0.21	-0.20	0.32	-0.02	-0.34	protein phosphatase 1, regulatory (inhibitor) subunit 8a [Danio rerio]
89	0.31	0.14	-0.07	0.16	-0.16	-0.28	tubulin alpha3 chainlike [Sarcophilus harrisii]
90	0.24	0.23	-0.10	0.15	-0.12	-0.28	prolyltRNA synthetase (mitochondrial) [Danio rerio]
91	0.02	0.41	-0.11	0.24	-0.40	-0.29	pseudouridylate synthase 10 [Danio rerio]
92	0.29	0.24	0.09	0.21	-0.29	0.00	upstream transcription factor 1 [Ictalurus punctatus]
93	0.02	0.21	0.00	0.11	-0.18	-0.17	argininetRNA ligase, cytoplasmiclike [Oryzias latipes]
94	0.16	0.17	-0.03	0.07	-0.15	-0.18	zgc:66484 [Danio rerio]
95	-0.02	0.93	0.74	-0.30	0.39	-0.21	glutamate receptor, ionotropic, Nmethyl Daspartate 1a [Danio rerio]
96	0.10	0.64	0.64	-0.54	0.15	-0.30	pleckstrin homology domain interacting protein [Danio rerio]
97	0.18	0.46	0.36	-0.44	-0.06	-0.25	fibromodulinlike [Takifugu rubripes]
98	0.75	0.84	0.54	0.11	-0.07	0.41	farnesyl diphosphate synthase [Danio rerio]
99	0.88	-0.04	0.91	0.35	-0.23	-0.49	Foxl2 ortholog [Oncorhynchus mykiss]
100	0.69	0.49	0.83	-0.09	-0.46	-0.36	serine protease inhibitor 5 [Acyrthosiphon pisum]

Figure 6-2 (contd.) Heatmap showing 100 most significantly differentially regulated transcripts between diets+between diets over time when fish larvae are fed on standard diet and dry diet at 2, 3 and 4 Dph. Red = up-regulated; Green = down-regulated.

For Cluster 3 the genes and behaviour patterns are different for larva fed in dry diet from standard, in the former there is observed increasing up- regulation of the genes notable among which include acyl CoA synthetase, important in the production of long-chain fatty acids for synthesis of cellular lipids. It may also play an important role in fatty acid metabolism in brain and the acyl-CoAs produced may be utilized exclusively for the synthesis of brain lipids. In animal tissues and cells, long chain acyl-CoA synthetase is believed to play a major role in supplying

acyl-CoA, since non ester free fatty acids are produced by fatty acid synthase (Faergeman& Knudsen, 1997; Neess *et al.*, 2015; Tomoda *et al.*, 1991; Tuohetahuntila *et al.*, 2015)

In Cluster 4 there was a slight up-regulation of expression in fish larvae fed on standard diet at 2Dph and 4 Dph while these transcripts are down-regulated at 3 Dph, on the other hand in the fish larvae fed on dry diet there was up-regulation which is observed more at 3Dph when there is downward regulation in fish larvae fed on standard diet. However, most of the transcripts show gradually increasing expression by day 4. The genes involved are mostly immune genes for example associated ubiquitin protein known to play a role in autophagy of proteins selectively (Kirkin *et al.*, 2009; Pickart & Eddins, 2004) and metabolic enzymes such as protein phosphatase 1 (PP1), which belongs to a class of phosphatases known as protein serine/threonine phosphatases (Cheng *et al.*, 2017; Cohen, 2002; Davé *et al.*, 2014). PP1 has been found to be important in the control of glycogen metabolism, muscle contraction, cell progression, neuronal activities, splicing of RNA, mitosis cell division apoptosis, protein synthesis, regulation of membrane receptors and channels and DNA replication (Davé *et al.*, 2014). This is very important for the developing larvae being differentially expressed across the two diets. This may be due in part to the dietary composition and could explain the difference in growth observed earlier on in chapter 4.

In Cluster 5 it was observed that in the fish larvae fed on standard diet expression was up-regulated from 2Dph through to 4 Dph, while for those fed on dry diet there was down-regulation of expression. Notable among these are transcripts mainly involved in growth and immunity as exemplified by the presence of suppressor var 1 which plays a role in the RNA surveillance system in mitochondria; regulates the stability of mature mRNAs, the removal of aberrantly formed mRNAs and the rapid degradation of non-coding processing intermediates. It is also capable of protecting cells from apoptosis and associates with mitochondrial DNA. Another notable transcript was lanosterol 14alpha demethylase, which acts specifically toward polyunsaturated acyl-CoA with the higher activity toward C20:4 acyl-CoA.

6.5.4.3 Significant differential expression over 1, 2, 3 and 4 Dph when fed on standard diet.

During development catfish larvae show a variety of transcripts being differentially expressed as shown in Figure 7-3. It was observed that the first 18 transcripts

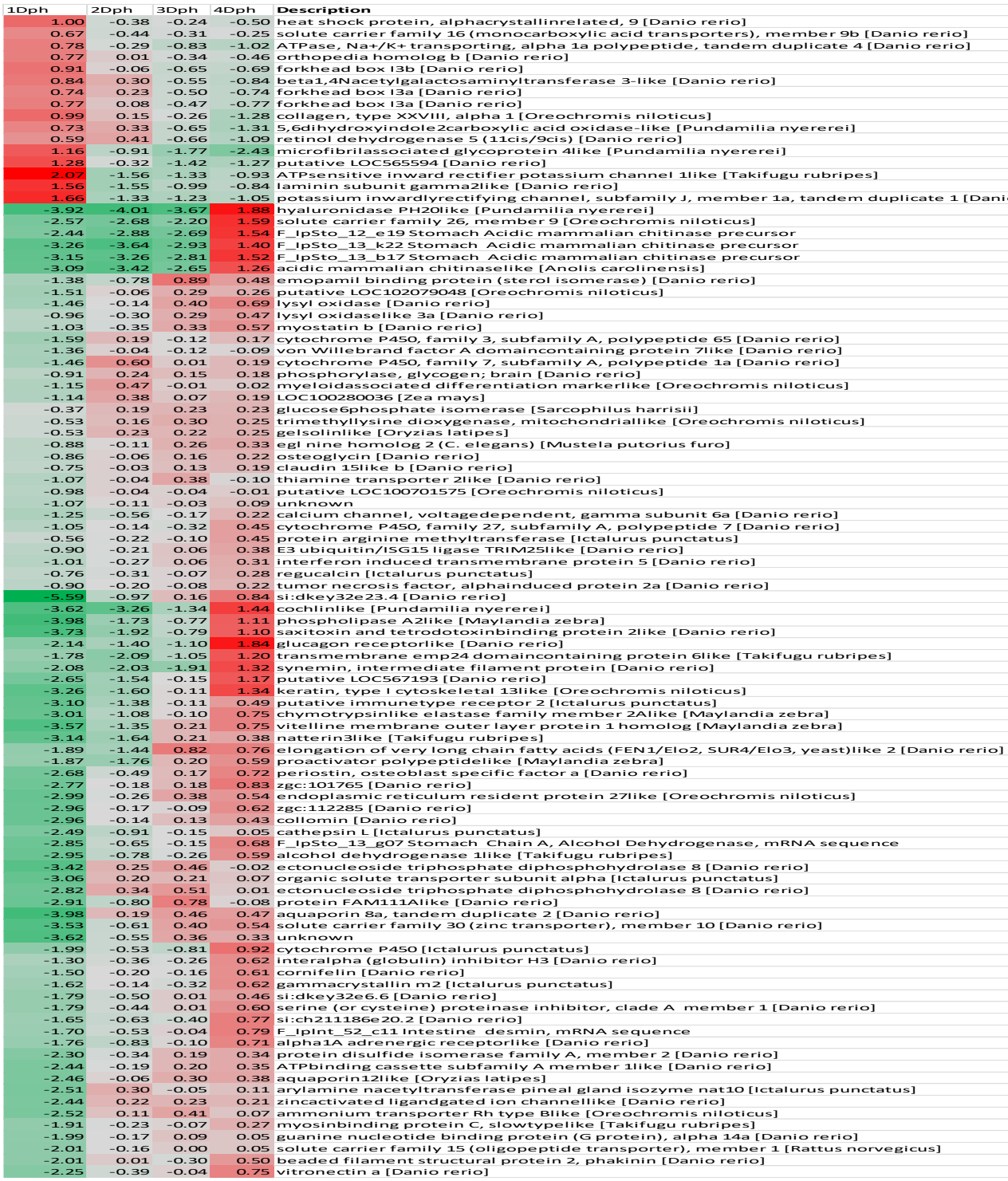
were up-regulated on day one but then down-regulated by day 4. All the 82 genes are switched on by day 4, performing various functions including among others immunity *e.g*. aquaporin, nutrition as seen by expression of digestive enzymes, development as seen through transcripts associated with apoptosis and growth. In terms of development 3 clusters have been observed namely cluster one (1-15), cluster 2(16-21) and cluster 3 (22-100).

For Cluster 1 it was observed that at 1Dph several transcripts were up-regulated, however, they are progressively declining between 2Dph and 3Dph and are almost fully down-regulated by 4 Dph in the developing larvae of *C. gariepinus.* Notable among them include the heat shock proteins (Hsps) or chaperones which perform a range of biological functions including among them transcription, translation and post-translational modifications, protein folding, and aggregation and disaggregation of proteins (Tiwari *et al.*, 2015). They also are observed to play a role in regulating immune responses (Bogart *et al.*, 2012),HSPs have been found not to act in heat shock feedback but also have developmental roles in embryonic or juvenile stages of mammals, teleost fish and some lower vertebral genomes. hspb1 (HSP27) is expressed during stress and during the development of embryo, somites, mid-hindbrain, heart and lens in zebrafish (Marvin *et al.*, 2008). Another of the notable transcripts is fork box which has been described as a transcription factor that play important roles in regulating the expression of genes involved in cell growth, proliferation, differentiation, and longevity (Zaret& Carroll, 2011).

In cluster 2 there was down-regulation of the activities from 1Dph up to 3Dph and up-regulation by 4 Dph in terms of development in the larvae. Notable transcripts included chitinase, one of the target proteins in this study, and this observation fits in well with the results for gastric chitinase observed in chapters 5 and 6, where the site of production, the gastric glands, are present and in terms of time of production it fits in well as by 4Dph gastric chitinase is fully switched on.

1Dph	2Dph	3Dph	4Dph	Description
1.00	-0.38	-0.24	-0.50	heat shock protein, alphacrystallinrelated, 9 [Danio rerio]
0.67	-0.44	-0.31	-0.25	solute carrier family 16 (monocarboxylic acid transporters), member 9b [Danio rerio]
0.78	-0.29	-0.83	-1.02	ATPase, Na+/K+ transporting, alpha 1a polypeptide, tandem duplicate 4 [Danio rerio]
0.77	0.01	-0.34	-0.46	orthopedia homolog b [Danio rerio]
0.91	-0.06	-0.65	-0.69	forkhead box I3b [Danio rerio]
0.84	0.30	-0.55	-0.84	beta1,4Nacetylgalactosaminyltransferase 3-like [Danio rerio]
0.74	0.23	-0.50	-0.74	forkhead box I3a [Danio rerio]
0.77	0.08	-0.47	-0.77	forkhead box I3a [Danio rerio]
0.99	0.15	-0.26	-1.28	collagen, type XXVIII, alpha 1 [Oreochromis niloticus]
0.73	0.33	-0.65	-1.31	5,6dihydroxyindole2carboxylic acid oxidase-like [Pundamilia nyererei]
0.59	0.41	-0.66	-1.09	retinol dehydrogenase 5 (11cis/9cis) [Danio rerio]
1.16	-0.91	-1.77	-2.43	microfibrilassociated glycoprotein 4like [Pundamilia nyererei]
1.28	-0.32	-1.42	-1.27	putative LOC565594 [Danio rerio]
2.07	-1.56	-1.33	-0.93	ATPsensitive inward rectifier potassium channel 1like [Takifugu rubripes]
1.56	-1.55	-0.99	-0.84	laminin subunit gamma2like [Danio rerio]
1.66	-1.33	-1.23	-1.05	potassium inwardlyrectifying channel, subfamily J, member 1a, tandem duplicate 1 [Danio rerio]
-3.92	-4.01	-3.67	1.88	hyaluronidase PH20like [Pundamilia nyererei]
-2.57	-2.68	-2.20	1.59	solute carrier family 26, member 9 [Oreochromis niloticus]
-2.44	-2.88	-2.69	1.54	F_lpSto_12_e19 Stomach Acidic mammalian chitinase precursor
-3.26	-3.64	-2.93	1.40	F_lpSto_13_k22 Stomach Acidic mammalian chitinase precursor
-3.15	-3.26	-2.81	1.52	F_lpSto_13_b17 Stomach Acidic mammalian chitinase precursor
-3.09	-3.42	-2.65	1.26	acidic mammalian chitinaselike [Anolis carolinensis]
-1.38	-0.78	0.89	0.48	emopamil binding protein (sterol isomerase) [Danio rerio]
-1.51	-0.06	0.29	0.26	putative LOC102079048 [Oreochromis niloticus]
-1.46	-0.14	0.40	0.69	lysyl oxidase [Danio rerio]
-0.96	-0.30	0.29	0.47	lysyl oxidaselike 3a [Danio rerio]
-1.03	-0.35	0.33	0.57	myostatin b [Danio rerio]
-1.59	0.19	-0.12	0.17	cytochrome P450, family 3, subfamily A, polypeptide 65 [Danio rerio]
-1.36	-0.04	-0.12	-0.09	von Willebrand factor A domaincontaining protein 7like [Danio rerio]
-1.46	0.60	0.01	0.19	cytochrome P450, family 7, subfamily A, polypeptide 1a [Danio rerio]
-0.91	0.24	0.15	0.18	phosphorylase, glycogen; brain [Danio rerio]
-1.15	0.47	-0.01	0.02	myeloidassociated differentiation markerlike [Oreochromis niloticus]
-1.14	0.38	0.07	0.19	LOC100280036 [Zea mays]
-0.37	0.19	0.23	0.23	glucose6phosphate isomerase [Sarcophilus harrisii]
-0.53	0.16	0.30	0.25	trimethyllysine dioxygenase, mitochondriallike [Oreochromis niloticus]
-0.53	0.23	0.22	0.25	gelsolinlike [Oryzias latipes]
-0.88	-0.11	0.26	0.33	egl nine homolog 2 (C. elegans) [Mustela putorius furo]
-0.86	-0.06	0.16	0.22	osteoglycin [Danio rerio]
-0.75	-0.03	0.13	0.19	claudin 15like b [Danio rerio]
-1.07	-0.04	0.38	-0.10	thiamine transporter 2like [Danio rerio]
-0.98	-0.04	-0.04	-0.01	putative LOC100701575 [Oreochromis niloticus]
-1.07	-0.11	-0.03	0.09	unknown
-1.25	-0.56	-0.17	0.22	calcium channel, voltagedependent, gamma subunit 6a [Danio rerio]
-1.05	-0.14	-0.32	0.45	cytochrome P450, family 27, subfamily A, polypeptide 7 [Danio rerio]
-0.56	-0.22	-0.10	0.45	protein arginine methyltransferase [Ictalurus punctatus]
-0.90	-0.21	0.06	0.38	E3 ubiquitin/ISG15 ligase TRIM25like [Danio rerio]
-1.01	-0.27	0.06	0.31	interferon induced transmembrane protein 5 [Danio rerio]
-0.76	-0.31	-0.07	0.28	regucalcin [Ictalurus punctatus]
-0.90	-0.20	-0.08	0.22	tumor necrosis factor, alphainduced protein 2a [Danio rerio]
-5.59	-0.97	0.16	0.84	si:dkey32e23.4 [Danio rerio]
-3.62	-3.26	-1.34	1.44	cochlinlike [Pundamilia nyererei]
-3.98	-1.73	-0.77	1.11	phospholipase A2like [Maylandia zebra]
-3.73	-1.92	-0.79	1.10	saxitoxin and tetrodotoxinbinding protein 2like [Danio rerio]
-2.14	-1.40	-1.10	1.84	glucagon receptorlike [Danio rerio]
-1.78	-2.09	-1.05	1.20	transmembrane emp24 domaincontaining protein 6like [Takifugu rubripes]
-2.08	-2.03	-1.91	1.32	synemin, intermediate filament protein [Danio rerio]
-2.65	-1.54	-0.15	1.17	putative LOC567193 [Danio rerio]
-3.26	-1.60	-0.11	1.34	keratin, type I cytoskeletal 13like [Oreochromis niloticus]
-3.10	-1.38	-0.11	0.49	putative immunetype receptor 2 [Ictalurus punctatus]
-3.01	-1.08	-0.10	0.75	chymotrypsinlike elastase family member 2Alike [Maylandia zebra]
-3.57	-1.35	0.21	0.75	vitelline membrane outer layer protein 1 homolog [Maylandia zebra]
-3.14	-1.64	0.21	0.38	natterin3like [Takifugu rubripes]
-1.89	-1.44	0.82	0.76	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)like 2 [Danio rerio]
-1.87	-1.76	0.20	0.59	proactivator polypeptidelike [Maylandia zebra]
-2.68	-0.49	0.17	0.72	periostin, osteoblast specific factor a [Danio rerio]
-2.77	-0.18	0.18	0.83	zgc:101765 [Danio rerio]
-2.99	-0.26	0.38	0.54	endoplasmic reticulum resident protein 27like [Oreochromis niloticus]
-2.96	-0.17	-0.09	0.62	zgc:112285 [Danio rerio]
-2.96	-0.14	0.13	0.43	collomin [Danio rerio]
-2.49	-0.91	-0.15	0.05	cathepsin L [Ictalurus punctatus]
-2.85	-0.65	-0.15	0.68	F_lpSto_13_g07 Stomach Chain A, Alcohol Dehydrogenase, mRNA sequence
-2.95	-0.78	-0.26	0.59	alcohol dehydrogenase 1like [Takifugu rubripes]
-3.42	0.25	0.46	-0.02	ectonucleoside triphosphate diphosphohydrolase 8 [Danio rerio]
-3.06	0.20	0.21	0.07	organic solute transporter subunit alpha [Ictalurus punctatus]
-2.82	0.34	0.51	0.01	ectonucleoside triphosphate diphosphohydrolase 8 [Danio rerio]
-2.91	-0.80	0.78	-0.08	protein FAM111Alike [Danio rerio]
-3.98	0.19	0.46	0.47	aquaporin 8a, tandem duplicate 2 [Danio rerio]
-3.53	-0.61	0.40	0.54	solute carrier family 30 (zinc transporter), member 10 [Danio rerio]
-3.62	-0.55	0.36	0.33	unknown
-1.99	-0.53	-0.81	0.92	cytochrome P450 [Ictalurus punctatus]
-1.30	-0.36	-0.26	0.62	interalpha (globulin) inhibitor H3 [Danio rerio]
-1.50	-0.20	-0.16	0.61	cornifelin [Danio rerio]
-1.62	-0.14	-0.32	0.62	gammacrystallin m2 [Ictalurus punctatus]
-1.79	-0.50	0.01	0.46	si:dkey32e6.6 [Danio rerio]
-1.79	-0.44	0.01	0.60	serine (or cysteine) proteinase inhibitor, clade A member 1 [Danio rerio]
-1.65	-0.63	-0.40	0.77	si:ch211186e20.2 [Danio rerio]
-1.70	-0.53	-0.04	0.79	F_lpInt_52_c11 Intestine desmin, mRNA sequence
-1.76	-0.83	-0.10	0.71	alpha1A adrenergic receptorlike [Danio rerio]
-2.30	-0.34	0.19	0.34	protein disulfide isomerase family A, member 2 [Danio rerio]
-2.44	-0.19	0.20	0.35	ATPbinding cassette subfamily A member 1like [Danio rerio]
-2.46	-0.06	0.30	0.38	aquaporin12like [Oryzias latipes]
-2.51	0.30	-0.05	0.11	arylamine nacetyltransferase pineal gland isozyme nat10 [Ictalurus punctatus]
-2.44	0.22	0.23	0.21	zincactivated ligandgated ion channellike [Danio rerio]
-2.52	0.11	0.41	0.07	ammonium transporter Rh type Blike [Oreochromis niloticus]
-1.91	-0.23	-0.07	0.27	myosinbinding protein C, slowtypelike [Takifugu rubripes]
-1.99	-0.17	0.09	0.05	guanine nucleotide binding protein (G protein), alpha 14a [Danio rerio]
-2.01	-0.16	0.00	0.05	solute carrier family 15 (oligopeptide transporter), member 1 [Rattus norvegicus]
-2.01	0.01	-0.30	0.50	beaded filament structural protein 2, phakinin [Danio rerio]
-2.25	-0.39	-0.04	0.75	vitronectin a [Danio rerio]

Figure 6-3 Heatmap showing 100 most significantly differentially regulated transcripts over time for 1, 2, 3 and 4 Dph. Red = up-regulated; Green = down-regulated.



1Dph	2Dph	3Dph	4Dph	Description
1.00	-0.38	-0.24	-0.50	heat shock protein, alphacrystallinrelated, 9 [Danio rerio]
0.67	-0.44	-0.31	-0.25	solute carrier family 16 (monocarboxylic acid transporters), member 9b [Danio rerio]
0.78	-0.29	-0.83	-1.02	ATPase, Na+/K+ transporting, alpha 1a polypeptide, tandem duplicate 4 [Danio rerio]
0.77	0.01	-0.34	-0.46	orthopedia homolog b [Danio rerio]
0.91	-0.06	-0.65	-0.69	forkhead box I3b [Danio rerio]
0.84	0.30	-0.55	-0.84	beta1,4Nacetylgalactosaminyltransferase 3-like [Danio rerio]
0.74	0.23	-0.50	-0.74	forkhead box I3a [Danio rerio]
0.77	0.08	-0.47	-0.77	forkhead box I3a [Danio rerio]
0.99	0.15	-0.26	-1.28	collagen, type XXVIII, alpha 1 [Oreochromis niloticus]
0.73	0.33	-0.65	-1.31	5,6dihydroxyindole2carboxylic acid oxidase-like [Pundamilia nyererei]
0.59	0.41	-0.66	-1.09	retinol dehydrogenase 5 (11cis/9cis) [Danio rerio]
1.16	-0.91	-1.77	-2.43	microfibrilassociated glycoprotein 4like [Pundamilia nyererei]
1.28	-0.32	-1.42	-1.27	putative LOC565594 [Danio rerio]
2.07	-1.56	-1.33	-0.93	ATPsensitive inward rectifier potassium channel 1like [Takifugu rubripes]
1.56	-1.55	-0.99	-0.84	laminin subunit gamma2like [Danio rerio]
1.66	-1.33	-1.23	-1.05	potassium inwardlyrectifying channel, subfamily J, member 1a, tandem duplicate 1 [Dani
-3.92	-4.01	-3.67	1.88	hyaluronidase PH20like [Pundamilia nyererei]
-2.57	-2.68	-2.20	1.59	solute carrier family 26, member 9 [Oreochromis niloticus]
-2.44	-2.88	-2.69	1.54	F_IpSto_12_e19 Stomach Acidic mammalian chitinase precursor
-3.26	-3.64	-2.93	1.40	F_IpSto_13_k22 Stomach Acidic mammalian chitinase precursor
-3.15	-3.26	-2.81	1.52	F_IpSto_13_b17 Stomach Acidic mammalian chitinase precursor
-3.09	-3.42	-2.65	1.26	acidic mammalian chitinaselike [Anolis carolinensis]
-1.38	-0.78	0.89	0.48	emopamil binding protein (sterol isomerase) [Danio rerio]
-1.51	-0.06	0.29	0.26	putative LOC102079048 [Oreochromis niloticus]
-1.46	-0.14	0.40	0.69	lysyl oxidase [Danio rerio]
-0.96	-0.30	0.29	0.47	lysyl oxidaselike 3a [Danio rerio]
-1.03	-0.35	0.33	0.57	myostatin b [Danio rerio]
-1.59	0.19	-0.12	0.17	cytochrome P450, family 3, subfamily A, polypeptide 65 [Danio rerio]
-1.36	-0.04	-0.12	-0.09	von Willebrand factor A domaincontaining protein 7like [Danio rerio]
-1.46	0.60	0.01	0.19	cytochrome P450, family 7, subfamily A, polypeptide 1a [Danio rerio]
-0.91	0.24	0.15	0.18	phosphorylase, glycogen; brain [Danio rerio]
-1.15	0.47	-0.01	0.02	myeloidassociated differentiation markerlike [Oreochromis niloticus]
-1.14	0.38	0.07	0.19	LOC100280036 [Zea mays]
-0.37	0.19	0.23	0.23	glucose6phosphate isomerase [Sarcophilus harrisii]
-0.53	0.16	0.30	0.25	trimethyllysine dioxygenase, mitochondriallike [Oreochromis niloticus]
-0.53	0.23	0.22	0.25	gelsolinlike [Oryzias latipes]
-0.88	-0.11	0.26	0.33	egl nine homolog 2 (C. elegans) [Mustela putorius furo]
-0.86	-0.06	0.16	0.22	osteoglycin [Danio rerio]
-0.75	-0.03	0.13	0.19	claudin 15like b [Danio rerio]
-1.07	-0.04	0.38	-0.10	thiamine transporter 2like [Danio rerio]
-0.98	-0.04	-0.04	-0.01	putative LOC100701575 [Oreochromis niloticus]
-1.07	-0.11	-0.03	0.09	unknown
-1.25	-0.56	-0.17	0.22	calcium channel, voltagedependent, gamma subunit 6a [Danio rerio]
-1.05	-0.14	-0.32	0.45	cytochrome P450, family 27, subfamily A, polypeptide 7 [Danio rerio]
-0.56	-0.22	-0.10	0.45	protein arginine methyltransferase [Ictalurus punctatus]
-0.90	-0.21	0.06	0.38	E3 ubiquitin/ISG15 ligase TRIM25like [Danio rerio]
-1.01	-0.27	0.06	0.31	interferon induced transmembrane protein 5 [Danio rerio]
-0.76	-0.31	-0.07	0.28	regucalcin [Ictalurus punctatus]
-0.90	-0.20	-0.08	0.22	tumor necrosis factor, alphainduced protein 2a [Danio rerio]
-5.59	-0.97	0.16	0.84	si:dkey32e23.4 [Danio rerio]
-3.62	-3.26	-1.34	1.44	cochlinlike [Pundamilia nyererei]
-3.98	-1.73	-0.77	1.11	phospholipase A2like [Maylandia zebra]
-3.73	-1.92	-0.79	1.10	saxitoxin and tetrodotoxinbinding protein 2like [Danio rerio]
-2.14	-1.40	-1.10	1.84	glucagon receptorlike [Danio rerio]
-1.78	-2.09	-1.05	1.20	transmembrane emp24 domaincontaining protein 6like [Takifugu rubripes]
-2.08	-2.03	-1.91	1.32	synemin, intermediate filament protein [Danio rerio]
-2.65	-1.54	-0.15	1.17	putative LOC567193 [Danio rerio]
-3.26	-1.60	-0.11	1.34	keratin, type I cytoskeletal 13like [Oreochromis niloticus]
-3.10	-1.38	-0.11	0.49	putative immunetype receptor 2 [Ictalurus punctatus]
-3.01	-1.08	-0.10	0.75	chymotrypsinlike elastase family member 2Alike [Maylandia zebra]
-3.57	-1.35	0.21	0.75	vitelline membrane outer layer protein 1 homolog [Maylandia zebra]
-3.14	-1.64	0.21	0.38	natterin3like [Takifugu rubripes]
-1.89	-1.44	0.82	0.76	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)like 2 [Danio rerio]
-1.87	-1.76	0.20	0.59	proactivator polypeptidelike [Maylandia zebra]
-2.68	-0.49	0.17	0.72	periostin, osteoblast specific factor a [Danio rerio]
-2.77	-0.18	0.18	0.83	zgc:101765 [Danio rerio]
-2.99	-0.26	0.38	0.54	endoplasmic reticulum resident protein 27like [Oreochromis niloticus]
-2.96	-0.17	-0.09	0.62	zgc:112285 [Danio rerio]
-2.96	-0.14	0.13	0.43	collomin [Danio rerio]
-2.49	-0.91	-0.15	0.05	cathepsin L [Ictalurus punctatus]
-2.85	-0.65	-0.15	0.68	F_IpSto_13_g07 Stomach Chain A, Alcohol Dehydrogenase, mRNA sequence
-2.95	-0.78	-0.26	0.59	alcohol dehydrogenase 1like [Takifugu rubripes]
-3.42	0.25	0.46	-0.02	ectonucleoside triphosphate diphosphohydrolase 8 [Danio rerio]
-3.06	0.20	0.21	0.07	organic solute transporter subunit alpha [Ictalurus punctatus]
-2.82	0.34	0.51	0.01	ectonucleoside triphosphate diphosphohydrolase 8 [Danio rerio]
-2.91	-0.80	0.78	-0.08	protein FAM111Alike [Danio rerio]
-3.98	0.19	0.46	0.47	aquaporin 8a, tandem duplicate 2 [Danio rerio]
-3.53	-0.61	0.40	0.54	solute carrier family 30 (zinc transporter), member 10 [Danio rerio]
-3.62	-0.55	0.36	0.33	unknown
-1.99	-0.53	-0.81	0.92	cytochrome P450 [Ictalurus punctatus]
-1.30	-0.36	-0.26	0.62	interalpha (globulin) inhibitor H3 [Danio rerio]
-1.50	-0.20	-0.16	0.61	cornifelin [Danio rerio]
-1.62	-0.14	-0.32	0.62	gammacrystallin m2 [Ictalurus punctatus]
-1.79	-0.50	0.01	0.46	si:dkey32e6.6 [Danio rerio]
-1.79	-0.44	0.01	0.60	serine (or cysteine) proteinase inhibitor, clade A member 1 [Danio rerio]
-1.65	-0.63	-0.40	0.77	si:ch211186e20.2 [Danio rerio]
-1.70	-0.53	-0.04	0.79	F_IpInt_52_c11 Intestine desmin, mRNA sequence
-1.76	-0.83	-0.10	0.71	alpha1A adrenergic receptorlike [Danio rerio]
-2.30	-0.34	0.19	0.34	protein disulfide isomerase family A, member 2 [Danio rerio]
-2.44	-0.19	0.20	0.35	ATPbinding cassette subfamily A member 1like [Danio rerio]
-2.46	-0.06	0.30	0.38	aquaporin12like [Oryzias latipes]
-2.51	0.30	-0.05	0.11	arylamine nacetyltransferase pineal gland isozyme nat10 [Ictalurus punctatus]
-2.44	0.22	0.23	0.21	zincactivated ligandgated ion channellike [Danio rerio]
-2.52	0.11	0.41	0.07	ammonium transporter Rh type Blike [Oreochromis niloticus]
-1.91	-0.23	-0.07	0.27	myosinbinding protein C, slowtypelike [Takifugu rubripes]
-1.99	-0.17	0.09	0.05	guanine nucleotide binding protein (G protein), alpha 14a [Danio rerio]
-2.01	-0.16	0.00	0.05	solute carrier family 15 (oligopeptide transporter), member 1 [Rattus norvegicus]
-2.01	0.01	-0.30	0.50	beaded filament structural protein 2, phakinin [Danio rerio]
-2.25	-0.39	-0.04	0.75	vitronectin a [Danio rerio]

Figure 6-4Heatmap (contd.) showing 100 most significantly differentially regulated transcripts over time for 1, 2, 3 and 4 Dph. Red = up-regulated; Green = down-regulated.

In cluster 3 it was observed that there was down-regulation progressing through 2Dph and finally up-regualtion by 4 Dph in terms of development. The activity included the gene chymotrypsin, a protein of interest in this study and appears to

be up-regulated much later than in the former chapters where it is observed to be at least present by 1Dph.

6.5.5 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways

The KEGG reference maps for metabolic pathways provide a visual representation of biochemical knowledge, containing all well-characterised reaction pathways. Lists of transcripts may be associated with their respective pathways and a visual indication of key transcript parameters (*e.g*. expression levels, significance of expression, up- / down-regulation) may be displayed on the KEGG pathway maps (Ogata *et al.*, 1999).

To reconstruct the metabolic pathways involved in *C. gariepinus* development, annotated sequences were mapped to KEGG) using the Kaas KEGG automatic annotation platform (Kanehisa *et al.*, 2011; Moriya *et al.*, 2007). Significantly differentially expressed transcripts coding for proteins represented in KEGG pathways in *C. Gariepinus* were linked to their respective pathways. Pathways that were significantly overrepresented in the list of differentially expressed transcripts were then identified and are listed in Tables 7-3 and 7-4. While *C. gariepinus* is not one of the KEGG pathway species, the coherence of the association of transcripts with pathways suggests that EC numbers have been correctly assigned to a high proportion of annotated sequences (Rismani-Yazdi *et al.*, 2011). It is important to remember, however, that the small scale of the microarray (15K features) coupled with the fact that most transcripts have not been subject to significant differential expression, means that the majority of proteins in any given KEGG map are not represented.

6.5.5.1 Transcript expression with respect to diet at 2 days post-hatch (Dph) and 4 Dph

A number of KEGG pathways were observed to be significantly over-represented in terms of up-regulation of expression between 2 days post-hatch (Dph) and 4 Dph. Some transcripts showed consistent differential expression among replicates (signified by uniform colouring of transcript on KEGG map), however, a number of transcripts showed heterogeneous expression between replicates (signified by different coloured bars on the KEGG map). Significantly represented pathways included ko04610 Complement and coagulation cascades, ko04060 Cytokine-cytokine receptor interaction, ko03320 PPAR signalling pathway ko04974 ko04514 Cell adhesion molecules (CAMs), Protein digestion and absorption, ko04270

Vascular smooth muscle contraction, ko04142 Lysosome, ko04210 Apoptosis. Involvement of these pathways denotes changes in elements of growth, digestion, the immune system and synaptic transmission. As shown in Table 6-3.

Table 6-3 showing the most over represented upregulated pathways with heterogeneous expression between replicates.

1	ko04610 Complement and coagulation cascades
2	ko04060 Cytokine-cytokine receptor interaction
3	ko03320 PPAR signalling pathway
4	ko04514 Cell adhesion molecules (CAMs)
5	ko04974 Protein digestion and absorption
6	ko04270 Vascular smooth muscle contraction
7	ko04721 Synaptic vesicle cycle
8	ko04142 Lysosome
9	ko04210 Apoptosis

6.5.5.2 Transcript expression (Up-regulated) transcripts with heterogeneous expression between replicates.

6.5.5.2.1 Complement and coagulation cascades pathway

One of the over-represented pathways was that comprising complement and coagulation cascades pathway (ko04610) shown in figure 6-5 below Within this pathway there was significant up-regulation of C3, C5, C7 C15, A2M, PROC, F8, SERPINA 5 and SERPINA 1.as seen in fig 6-5 above in all the six (6) pools on the oligo microaaray between 2Dph and 4Dph for the developing larva of North African catfish (*C. gariepinus* Burchell 1822).

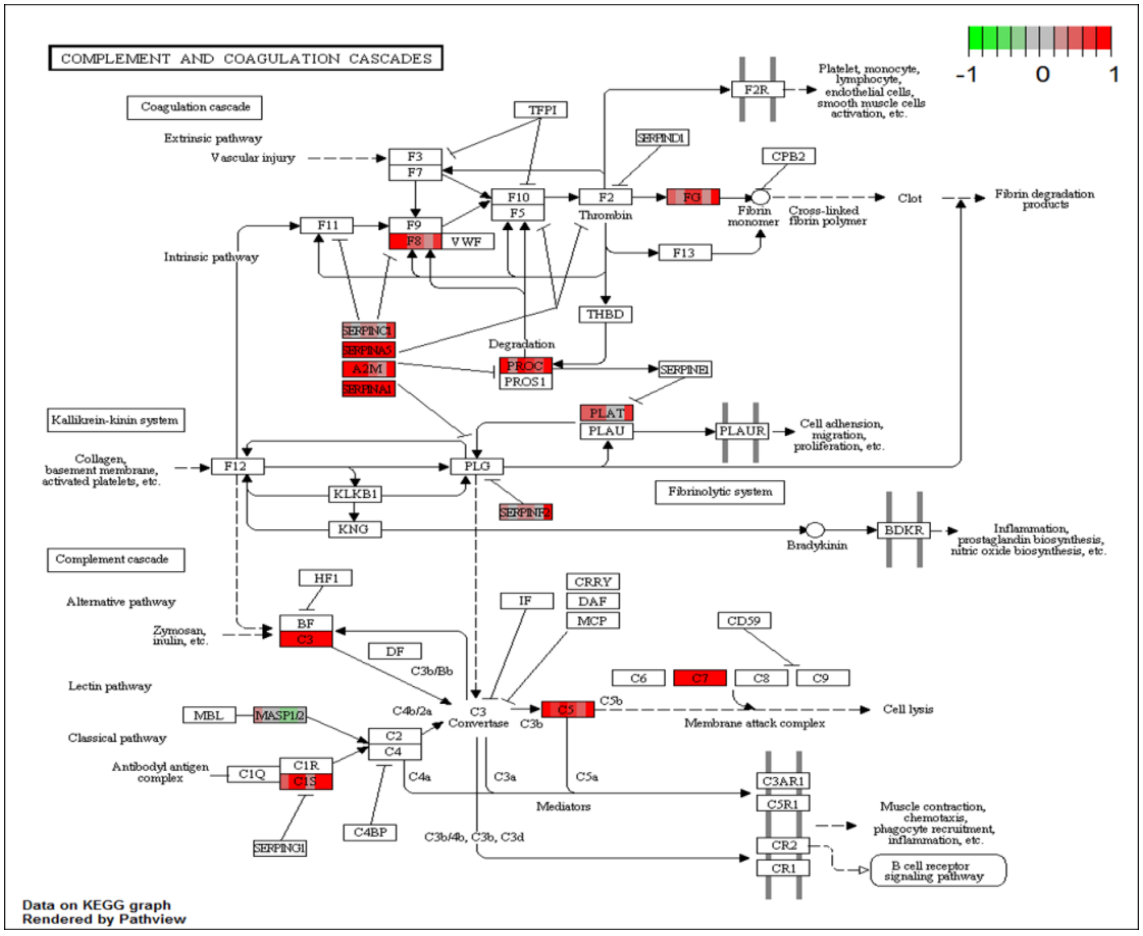


Figure 6-5 showing the KEGG graph for the Complement and coagulation cascades pathway plotted using Pathview package for R/Bioconductor. Red = up-regulated transcript, Green = down-regulated transcript. Six pooled replicates highlighted for each transcript.

Bony fish innate immune pathways comprise several mechanisms by which they maintain balance in terms of homeostasis. These include coagulation, comprising clotting cascades involving thrombin and which require the additional control provided by SERPIN A5, SERPIN A1 and Protein C (PROC), a vitamin K–dependent SERPIN with potent anticoagulant and anti-inflammatory properties (Rebl& Goldammer, 2018) and which together are also seen to be up-regulated in the larvae of *C. gariepinus* larvae between 2Dph and 4Dph. Coagulation is often accompanied by the opsonisation process, which involves elements of the complement system and plays a role in bacterial detection through pattern-recognition receptors as well as cytokine-mediated inflammation in which A2M has been identified to play a role. The complement system is made up plasma proteins that can play a role in the direct activation by pathogens or indirectly by pathogen-bound antibodies, leading to a cascade of reactions that occur on the surface of pathogens and generates active components with various effector functions (Martínez-Barricarte *et al.,* 2010; Ichiki *et al.,* 2012; Jaguva Vasudevan *et al.,* 2013; Zipfel *et al.,* 2015). All three pathways can be initiated independently of antibodies as an aspect of innate immunity.

These interlinked pathways provide two of the cornerstones of innate immunity and are thus very important for the developing larvae. Their up-regulation in early development is likely to be an indicator of the early stages of maturation of the immune system, providing larvae with increasing immune competence particularly in terms of key mechanisms of innate immunity, which is essential for larvae to successfully fight off initial infection by pathogens. In fish, innate immunity is the key mode of defence in the first 12 weeks of development, after which the adaptive immune response system starts to take a greater role (Magnadottir, 2006).

6.5.5.2.2 Cytokine-cytokine interacting pathway

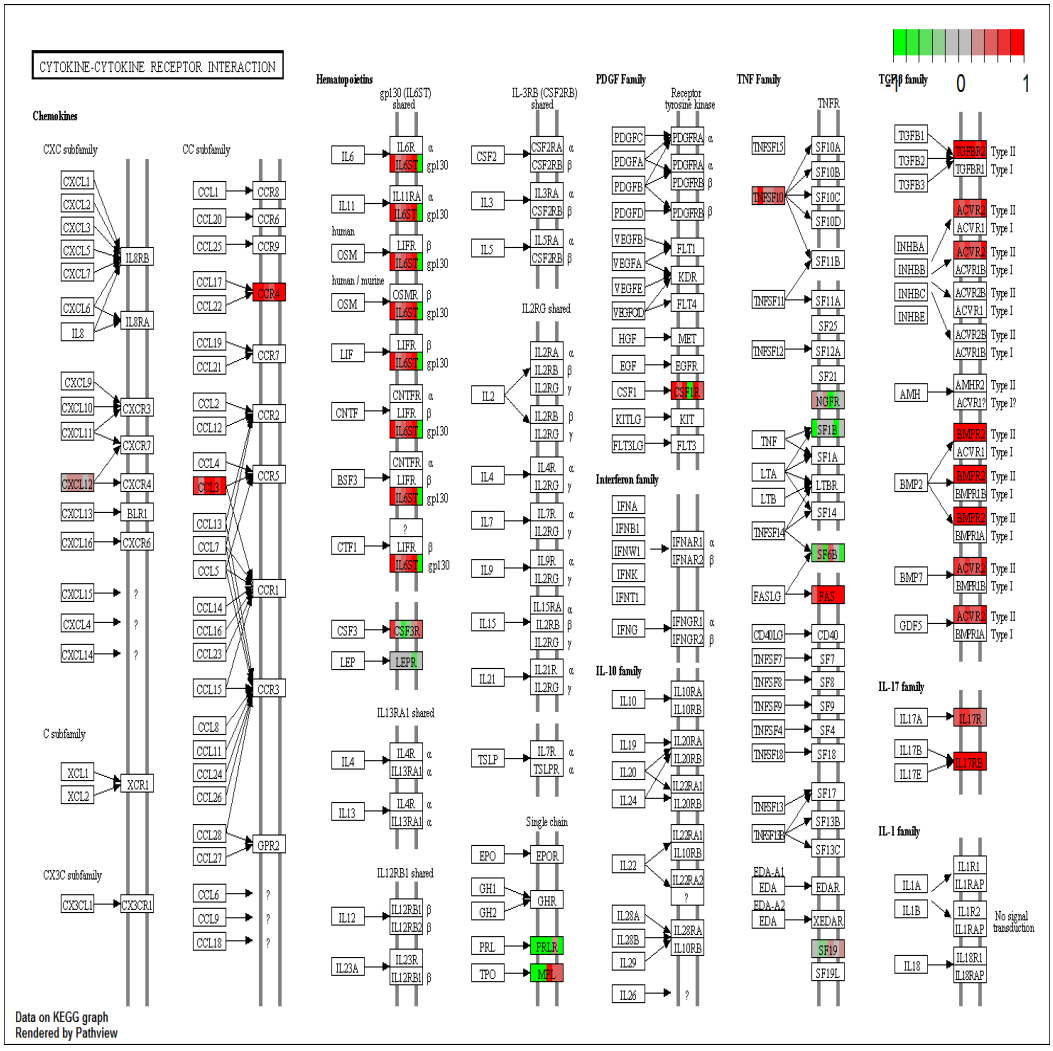


Figure 6-6 KEGG graph for (ko04060). Cytokine-cytokine interacting pathway plotted using Pathview package for R/Bioconductor.

CCR4,IL1TR and CCL3 are referred to as chemokines and are known to play vital roles in the development, homeostasis, and function of the immune system. They may also have underlying effects on cells of the central nervous system as well as on endothelial cells involved in angiogenesis. This latter process is vital for growth and development as well as for wound repair. Several studies in fish, including zebrafish (*Danio rerio),* Rainbow trout (*Oncorhynchus mykiss*) and Channel catfish (*Ictalurus punctatus)* have highlighted their role in immune pathways and was the case for coagulation and complement pathways above, larvae between 2Dph and 4Dph show up-regulation of this pathway, presumably giving increasing capacity for protection from bacterial infection and therefore increased survival. Another of the up-regulated genes in this pathway was BMPR2 which is a serine/threonine receptor kinase. BMPs are involved in a number of cellular functions including

osteogenesis, cell growth and cell differentiation, all key aspects of early development.

Activin signals and its receptors namely ACVR1 and ACVR2, are important in cell proliferation, differentiation, apoptosis, metabolism, immune response, wound repair, and endocrine function. These pathways are important in larvae of *C. gariepinus* at 2Dph and 4Dph as they are at the stage when they are growing at a prolific rate as well as experiencing the new environment where they are prone to bacterial infection thereby affecting survival.

6.5.5.2.3 PPARγ signalling pathway (ko03320) pathway

One of the over-represented pathways was the PPAR signalling pathway (Figure 6-7). Within this pathway there was significant up-regulation of retinoid X receptor (RXR), Lipoprotein lipase (LPL), glycerol kinase (GyK) and acyl-coenzyme A synthetase in all the six (6) pools on the oligo microarray between 2Dph and 4Dph.

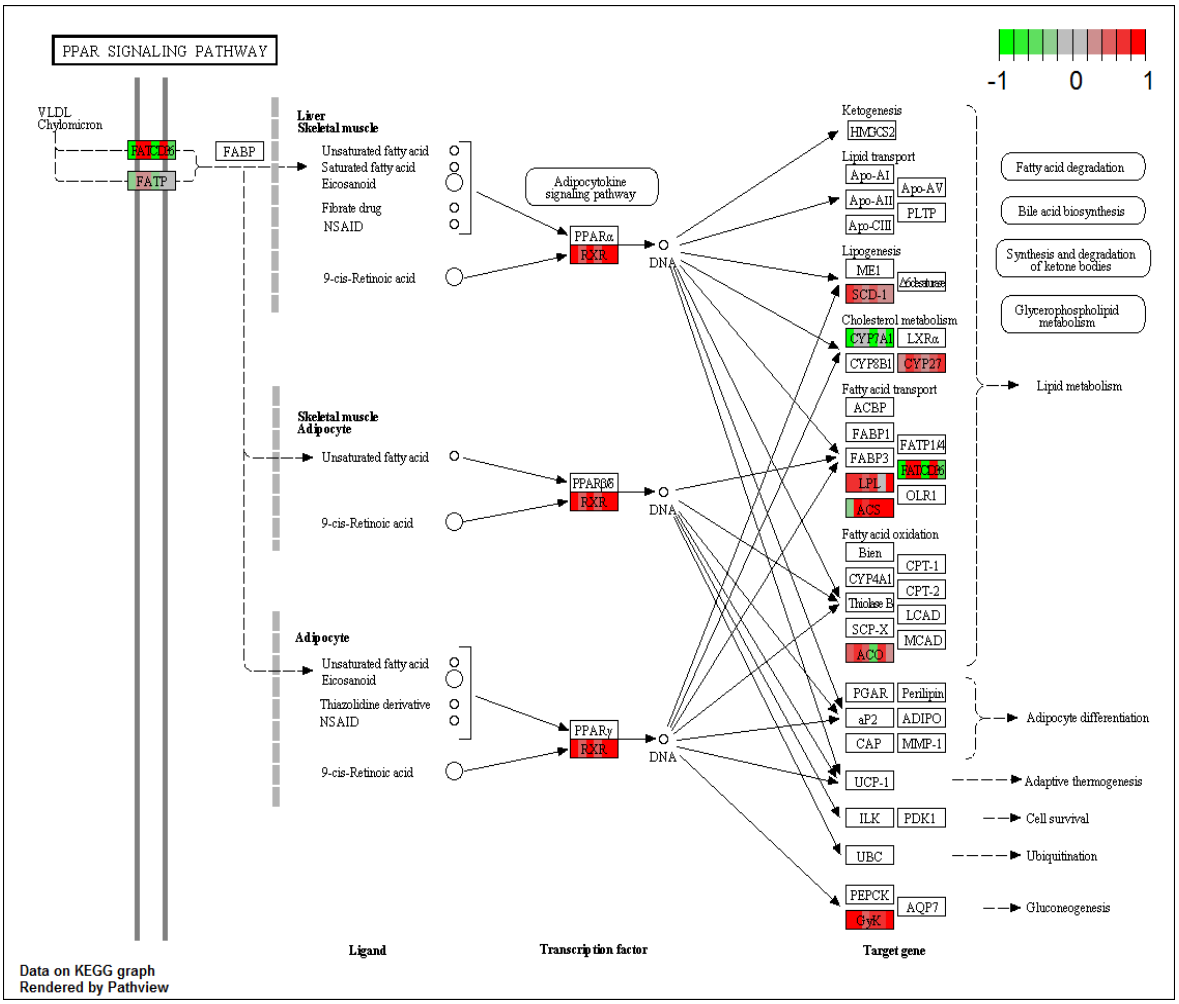


Figure 6-7 showing the KEGG graph for PPARγ signalling pathway (ko03320) pathway plotted using Pathview package for R/Bioconductor

Upregulated transcripts are largely concerned with lipid metabolism, gluconeogenesis and utilisation of circulating chylomicrons.

LPL encodes lipoprotein lipase, which plays a vital role in hydrolysis of triacylglycerol in circulating chylomicrons providing fatty acids for tissue utilisation and AcCoA, known as acyl CoA synthetase, is important in the activation of long-chain fatty acids for both synthesis of cellular lipids, and degradation.

This pathway is therefore essential in providing energy reserves for the developing larvae between 2Dph and 4 Dph.

6.5.5.2.4 Cell Adhesion molecules CAMS) pathway

One of the over-represented pathways was that comprising Cell Adhesion molecules (CAMS) pathway (ko04514) (Figure 6-8). Within this pathway there was significant up-regulation of MHC1, platelet/endothelial cell adhesion molecule 1 (PECAM1), ITA6, MAG in all the six (6) pools on the oligo microarray between 2Dph and 4Dph.

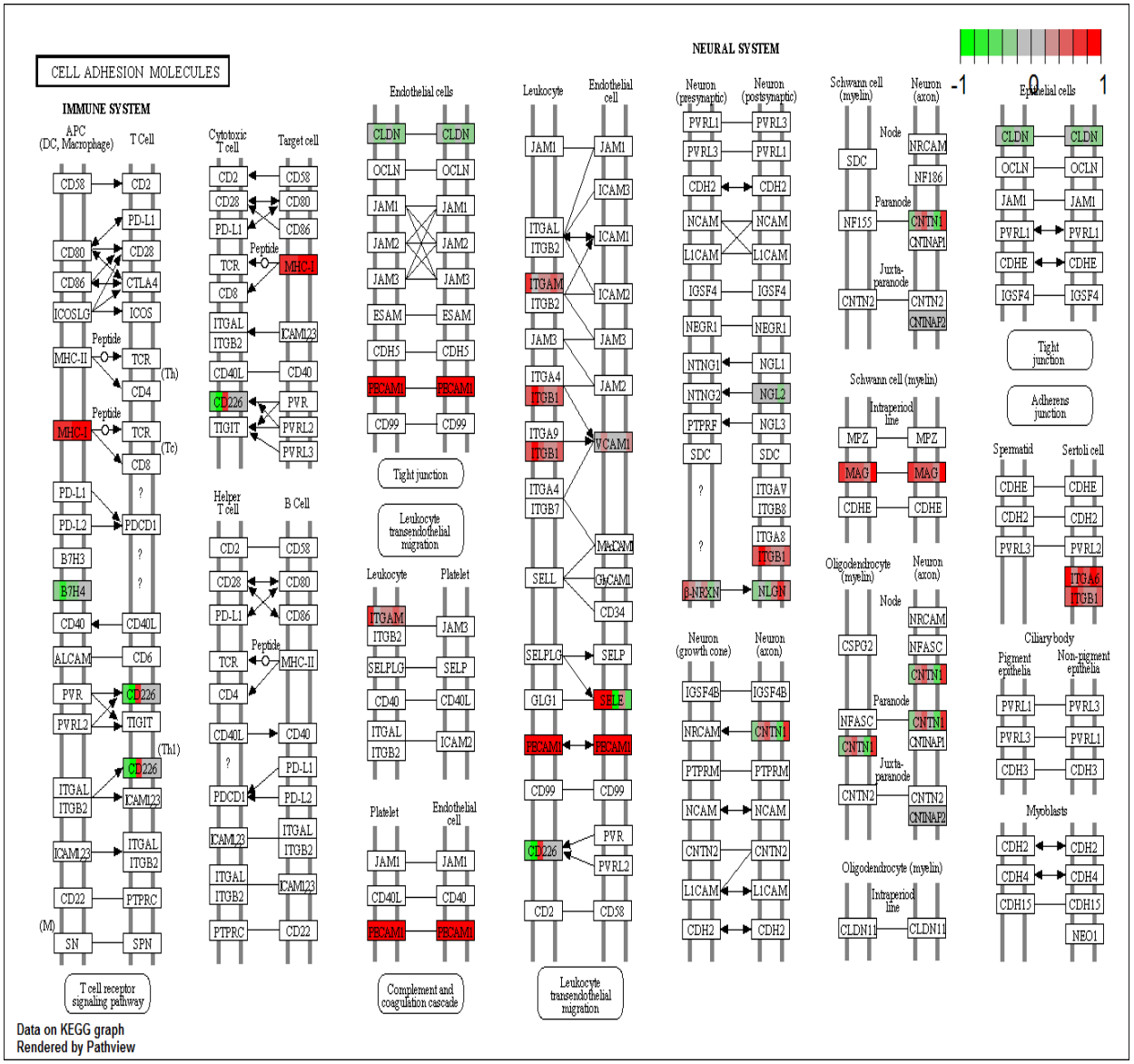


Figure 6-8 KEGG graph for (ko04514). Cell Adhesion molecules CAMS) pathway plotted using Pathview package for R/Bioconductor.

MHC class I molecules are one of two primary classes of major histocompatibility complex (MHC) molecules, the other being MHC class II that help to provide disease resistance in vertebrates, including teleost fish, as a component of

adaptive immunity (Omote *et al.*, 2017) and can also affect sexual selection and mate choice which in turn has a bearing on immunity (Xu *et al.*, 2011). Platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) is a molecule expressed on all cells within the vascular compartment and plays roles in angiogenesis, platelet function, thrombosis, mechano-sensing of endothelial cell response to fluid shear stress, and regulation of multiple stages of leukocyte migration through venular walls (Woodfin *et al.,* 2007). For the growing larvae of *C. gariepinus,* both MHC I and PECAM provide key elements of larval development and are also important as markers of the presumed developing immune competence of the larvae.

6.5.5.2.5 protein digestion and absorption pathway

One of the over-represented pathways was that comprising the protein digestion and absorption pathway (Figure 6-9 below). Here there was significant up-regulation of peptidase, pepsin and CELA, however; ATPase was observed, to be down-regulated between 2Dph and 4Dph.

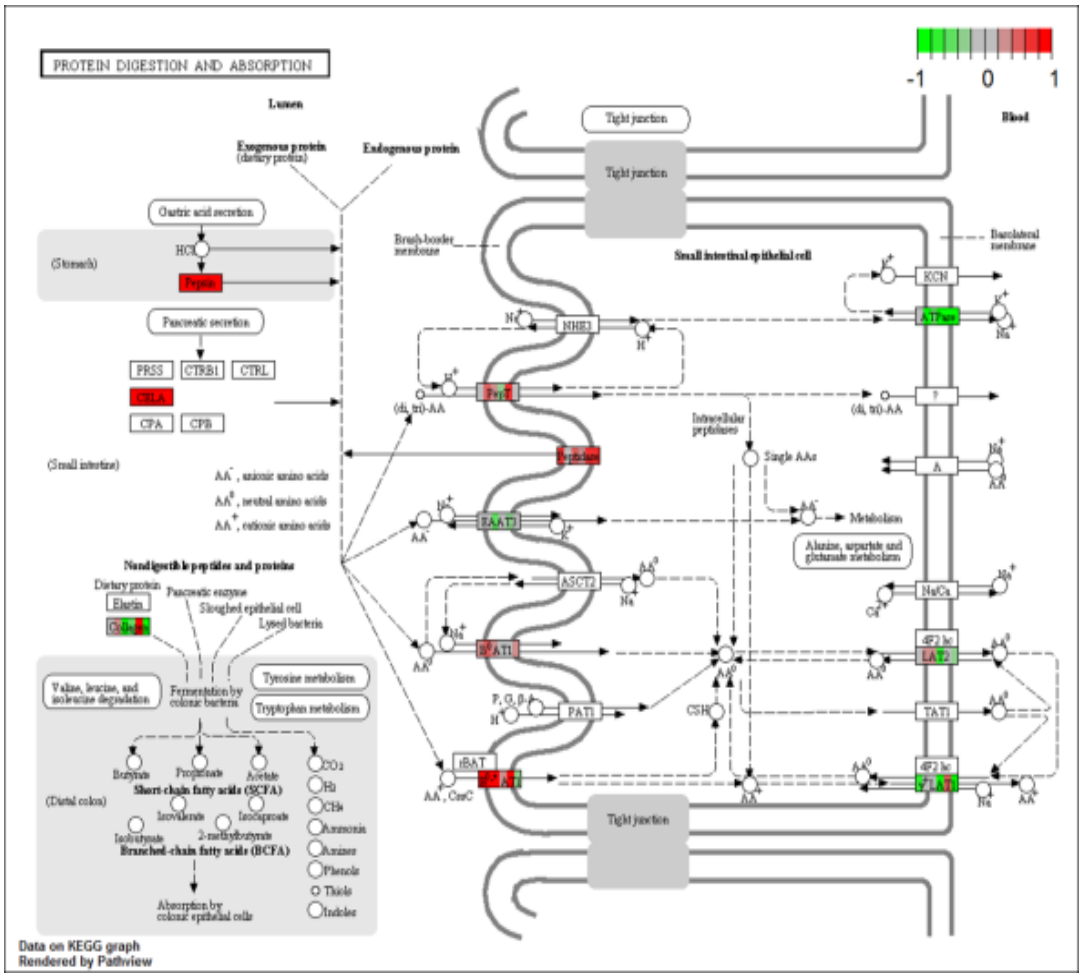


Figure 6-9 KEGG graph for (ko04974) protein digestion and absorption pathway plotted using Pathview package for R/Bioconductor.

Pepsin is a protease that breaks down proteins into smaller peptides. It is produced in the stomach and is a key digestive enzyme for vertebrates. Its upregulation toward 4DPh seen here in figure 6-8 concurs with earlier findings in chapters 4 and 5 where the site of production was highlighted and supports the expression of this transcript from at least 4 Dph not the 5 Dph of Verreth *et al.* (1992).

Increased expression of CELA (Chymotrypsin-like elastase) may be suggestive of the increased contribution of pancreatic secretions to digestion as this protein is a relatively non-specific protease used in digestion and therefore potentially important to first-feeding larvae. There is a slight caveat, however, that applies to all of the transcriptomics described here, in terms of the fact that whole larvae were processed such that this chymotrypsin-like elastase may represent a different family member with non-pancreas associated activities.

6.5.5.2.6 Lysosome pathway

One of the over-represented pathways was that comprising (Ko04142) Lysosome pathway*)*. Within this pathway there was significant up-regulation of LGMN, mannose 6-phophate receptors (MPR) and Saposin and down-regulation of lysosomal alpha-glucosidase (GAA) and GM2A (Figure 6-10).

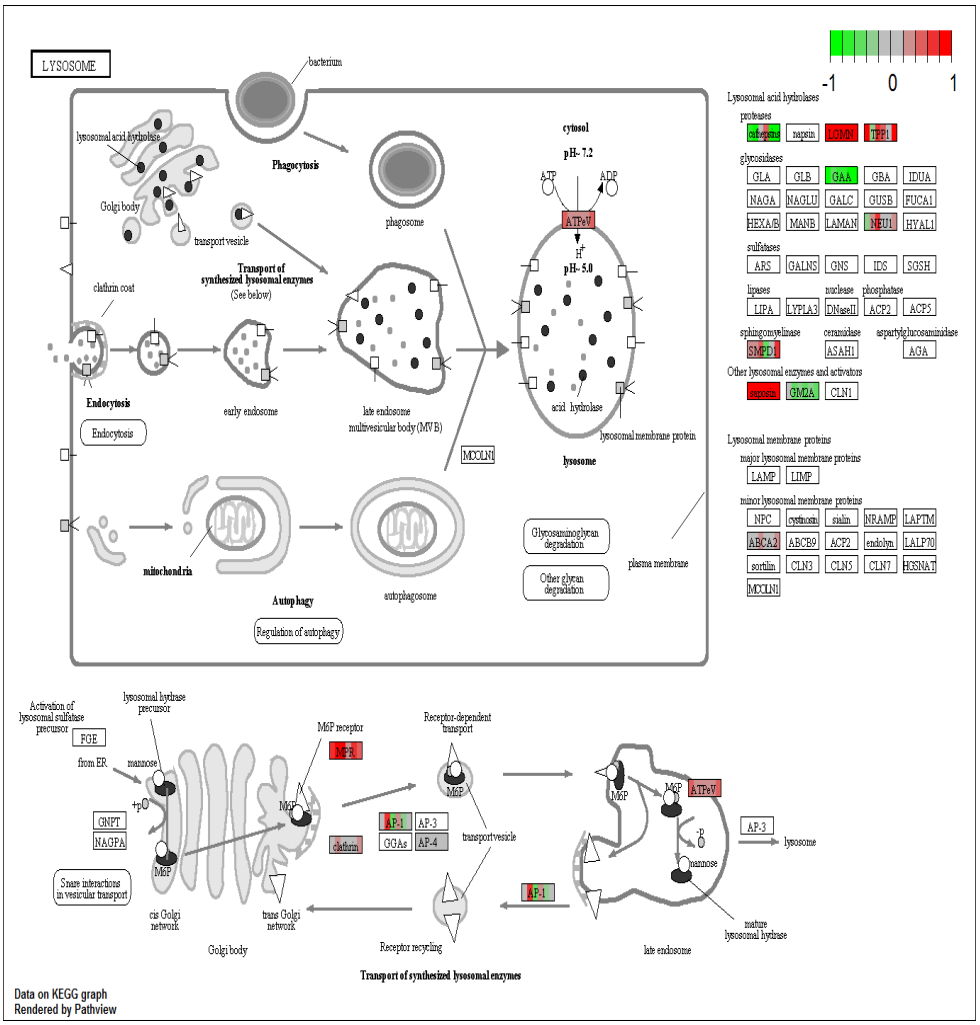


Figure 6-10 KEGG graph for the (Ko04142) Lysosome pathway plotted using Pathview package for R/Bioconductor.

The lysosome is an organelle in fish that plays an essential role in metabolism of proteins, carbohydrates, lipids and nucleic acids as they are involved in the storage and deployment of acid hydrolases (Fukuda, 2016; Lübke *et al.*, 2009; Lunardi *et al.*, 1995; Martinez *et al.*, 2000). They are also implicated in cell signal transduction, plasma membrane repair, homoeostasis, and autophagy (Yu *et al.*, 2016).

The mannose 6-phophate receptors (MPRs) are trans-membrane glycoproteins that target enzymes to lysosomes in vertebrates. In the case of the fish under study, MPRs were up-regulated in 5 out of the 6 pools supporting a consistent role in development. LGMN, Legumain, on the other hand may be involved in the processing of proteins for MHC class II antigen presentation in the lysosome system (Fukuda, 2016) and is up-regulated in these larvae suggesting that their ability to present antigens is either increasing or has been stimulated by potential pathogens during development. Lysosomal alpha-glycosidase GAA is important for the degradation of glycogen to glucose in lysosomes and has been reported to be down-regulated.

6.5.5.2.7 Apoptosis pathway

One of the over-represented pathways was the Apoptosis pathway (ko04210). Within this pathway there was significant up-regulation of FAS, Caspase 7 (CASP7) and down-regulation of CASP3 (Figure 6-11 below)

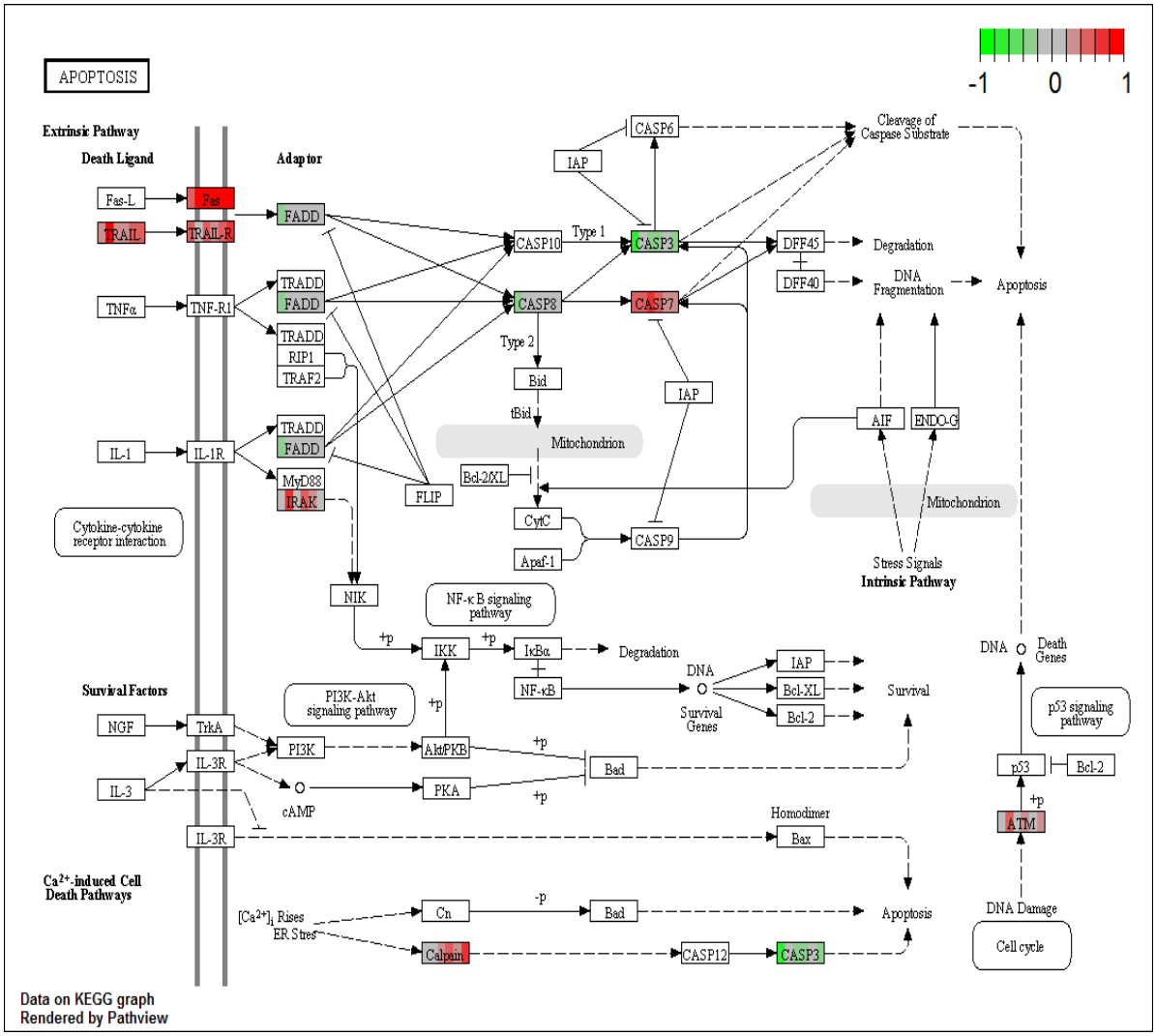


Figure 6-11 KEGG graph for the (ko04210) Apoptosis pathway plotted using Pathview package for R/Bioconductor.

Apoptosis, programmed cell death, is key to development and maintenance of homeostasis including aspects of immunity (*e.g.* during viral infection) (Pérez-Garijo, 2017).During apoptosis, cells of multicellular organisms undergo both cell division as well as cell death. If cells are no longer needed or infected by a virulent pathogen they may commit suicide by activating an intracellular death program (Romano *et al.*, 2013; Rong *et al.*, 2011; Sagulenko *et al.*, 2018). Caspase 7 and Caspase 3 showed different trends in the developing larvae between 2Dph and

4Dph and have been documented to play a vital role during apoptosis. However, their role is not limited to this function only as they are also involved in inflammation control activities and non-apoptotic activities such as osteogenesis and tooth formation, particularly caspase 7 (Svandova *et al.*, 2014).Caspase-containing cells (both the ones destined to die and the ones that are able to overcome caspase activation) have been designated as important signalling centres, with numerous non apoptotic roles including bone formation and lymphocyte proliferation and therefore vital in immunity and tissue repair. Again, increased expression of these transcripts may be suggestive of increasing capacity for immune defence and tissue repair in developing larvae, although these are also features of early development.

6.5.5.3 Transcript Pathways with consistent down-regulated differential expression between 2 Dph and 4Dph

A number of pathways were observed to be over-represented in terms of down-regulated transcript expression between 2 days post-hatch (Dph) and 4 dph (Table 6-4), showing consistent differential expression among replicates. These included ko03010 Ribosome, ko00190 Oxidative phosphorylation, ko04111 Cell cycle – yeast,ko04110 Cell cycle, ko04141 Protein processing in endoplasmic reticulum, ko04918 Thyroid hormone synthesis, ko04114 Oocyte meiosis, ko03030 DNA replication and ko00230 Purine metabolism.

Table 6-4 showing the over expressed transcripts with consistent down-regulated differential expression among replicates.

1	ko03010 Ribosome
2	ko00190 Oxidative phosphorylation
3	ko04110 Cell cycle
4	ko04141 Protein processing in endoplasmic reticulum
5	ko04918 Thyroid hormone synthesis
6	ko04114 Oocyte meiosis
7	ko03030 DNA replication
8	ko00230 Purine metabolism

6.5.5.3.1 Ribosome pathway

One of the over-represented pathways was that comprising Ribosome (ko03010). Within this pathway there was significant up- down-regulation of S11e, L3e, L23ae, L17e, L19E, L5e, and L10e (Figure 6-12).

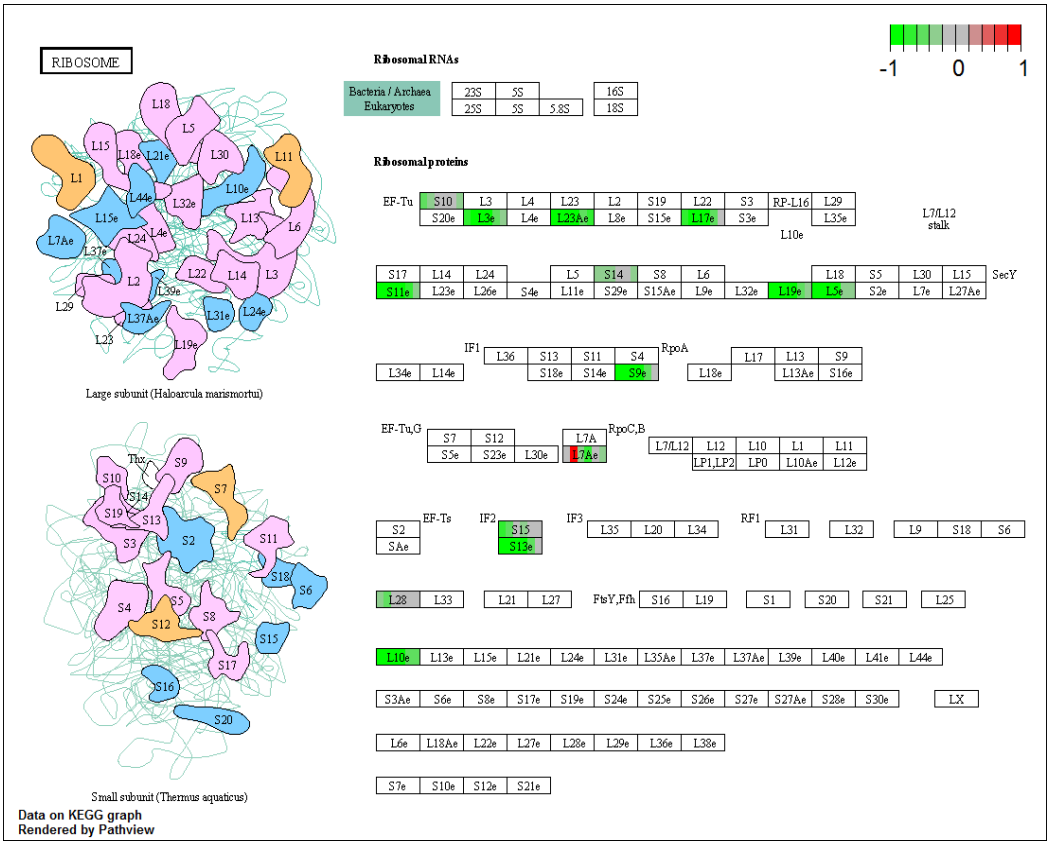


Figure 6-12 KEGG graph for (ko03010) Ribosome plotted using Pathview package for R/Bioconductor.

The pathway above plays a vital role in protein synthesis from mRNA (Zhou *et al.*, 2015).This process is conducted via the mechanism referred to as ribosomal biogenesis together with the process of protein translation .These processes are vital for cell growth, proliferation, differentiation and development. Failures within this pathway may lead to retarded cell growth thereby affecting the animals' development. In the case of the growing larvae these processes are down-regulated, possibly correlating to the fact that the stomach is not fully developed yet and the acidic digestion in the larvae is still not yet functional. This fits in well with the earlier chapters 5 and 6 when we see the stomach appearing at about 4Dph or shortly after and acidic digestion is then assumed to begin. The slowdown in ribosomal processes may also reflect a tail-off in speed following an initial growth / developmental spurt and the shift from yolk-based to exogenous feeding.

Two of the down-regulated enzymes L3 and L23 between 1DPh and 4Dph have been observed to change during the development of the pancreas in zebrafish (Provost *et al.*, 2012; Provost *et al.*, 2013). This also ties in with chapter 5 and 6 results where the pancreas is seen to be the developed organ playing a vital role in alkaline digestion with the help of the enzymes trypsin and chymotrypsin.

6.5.5.3.2 Oxidative phosphorylation pathway

One of the over-represented pathways was that comprising oxidative phosphorylation (ko00190) (Figure 6-13). Within this pathway there was significant up- regulation of complex v 36310 and down-regulation of Cox 4, Ndufu9, Ndufa13, alpha, gamma, and complex v 3.6.3.14, Ndufv1.

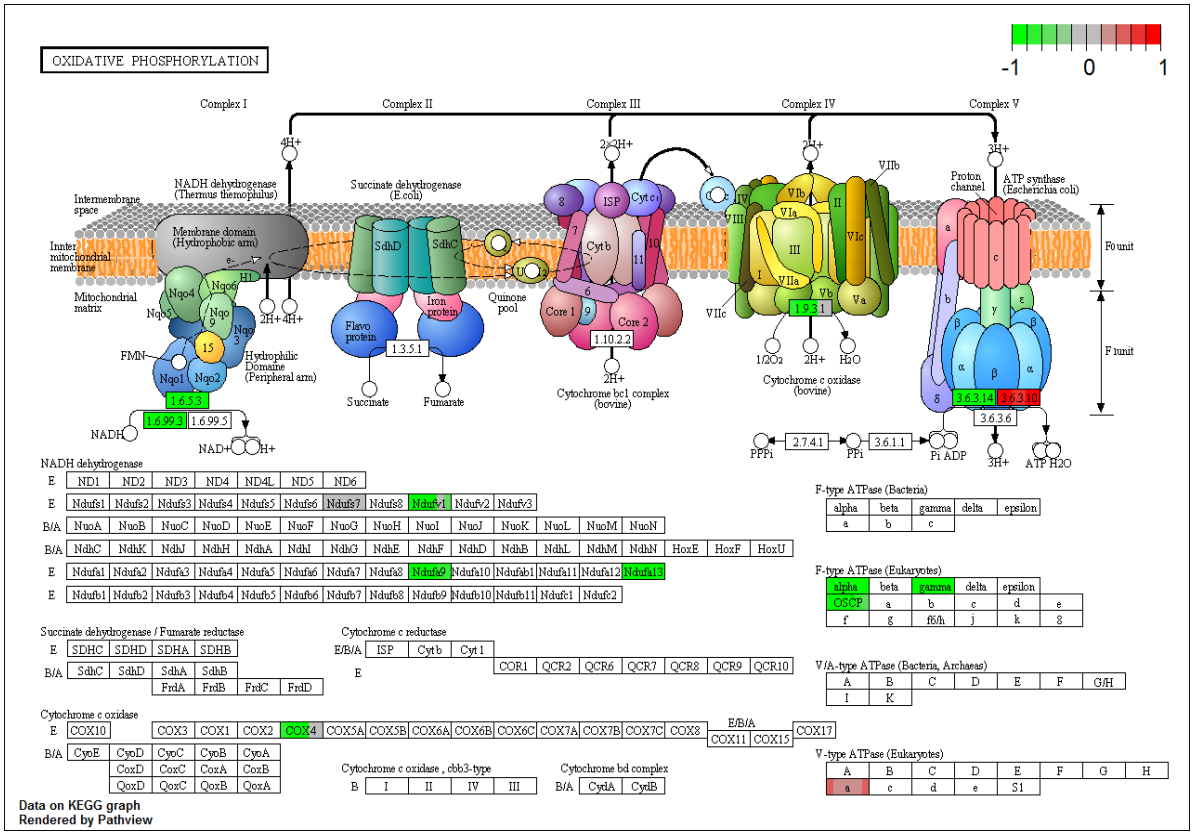


Figure 6-13 KEGG graph for the oxidative phosphorylation (ko00190) pathway plotted using Pathview package for R/Bioconductor

NADH:ubiquinone oxidoreductase subunit A13 and 9 are accessory subunits of the mitochondrial membrane respiratory chain NADH dehydrogenase Oxidative phosphorylation (OXPHOS). This is the metabolic process that generates most of the energy required for proper functioning by cells. OXPHOS plays two roles namely respiration and ATP synthesis (Fernández-Vizarra *et al.*, 2009; Nsiah-Sefaa & McKenzie, 2016). As with the ribosomal pathway, a fall in energy generation / demand may reflect a slower pace of development or the change from yolk to exogenous feeding and a consequent change in energetic substrate.

6.5.5.3.3 Cell cycle pathway

One of the over-represented pathways was that comprising cell cycle pathway as shown in figure 6-14 below with Origin Recognition Complex (ORC), transforming growth factor (TGF)-beta,

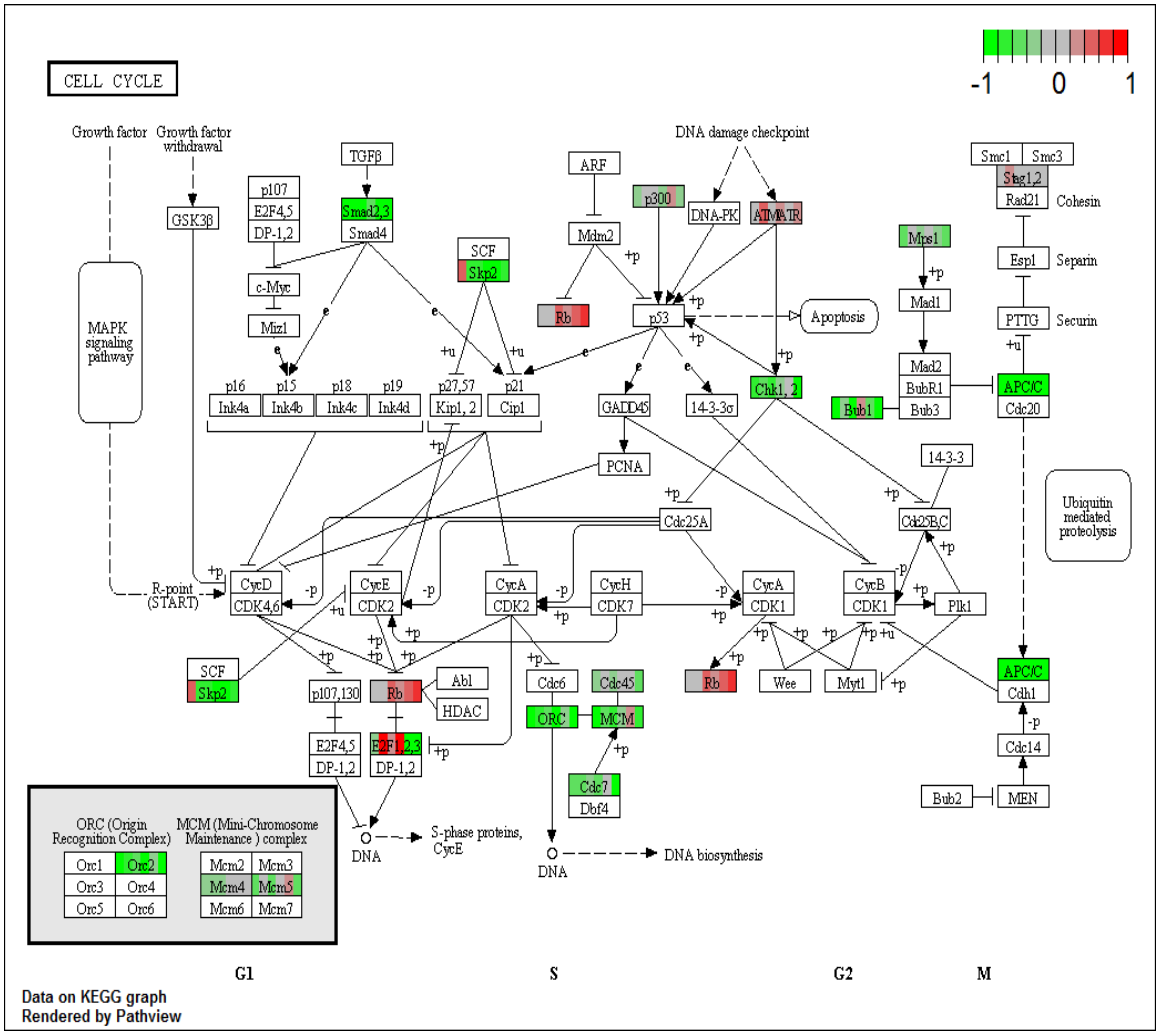


Figure 6-14 KEGG graph for the (ko04110).Cell cycle pathway plotted using Pathview package for R/Bioconductor.

(SKP1-cullin-F-box), functions in phosphorylation-dependent ubiquitination and thus in cell protein turnover, presumably reflecting rapid changes during tissue modelling / re-modelling in larval development. Origin Recognition Complex (ORC) is a central component for eukaryotic DNA replication, and remains bound to chromatin at replication origins throughout the cell cycle (Matsuda *et al.,* 2007) ORC directs DNA replication throughout the genome and is required for its initiation (Zheng et al. 2002) and is hence important in development. Similarly, Mini-chromosome maintenance proteins (MCM) has been implicated in being

involved in the initiation of eukaryotic genome replication. SMAD proteins are signal transducers and transcriptional modulators that mediate multiple signalling pathways including the signal of the transforming growth factor (TGF)-beta, and thus regulates multiple cellular processes, such as cell proliferation, apoptosis, and differentiation. The down-regulation of processes in this pathway therefore seems to reflect a reduction in the rate of cell growth and turnover in early larval development.

6.5.5.3.4 Thyroid hormone pathway

Expression of transcripts in the Thyroid hormone pathway ko04918 (Figure 6-15 below) has shown that the most dominant gene expressed is Thyroglobulin (TG) and is down-regulated in the pathway as is ATPase. The gene PLC is not uniformly expressed among the 6 pools of fish indicating that either the samples were not homogenous or the fish were at different stages of development

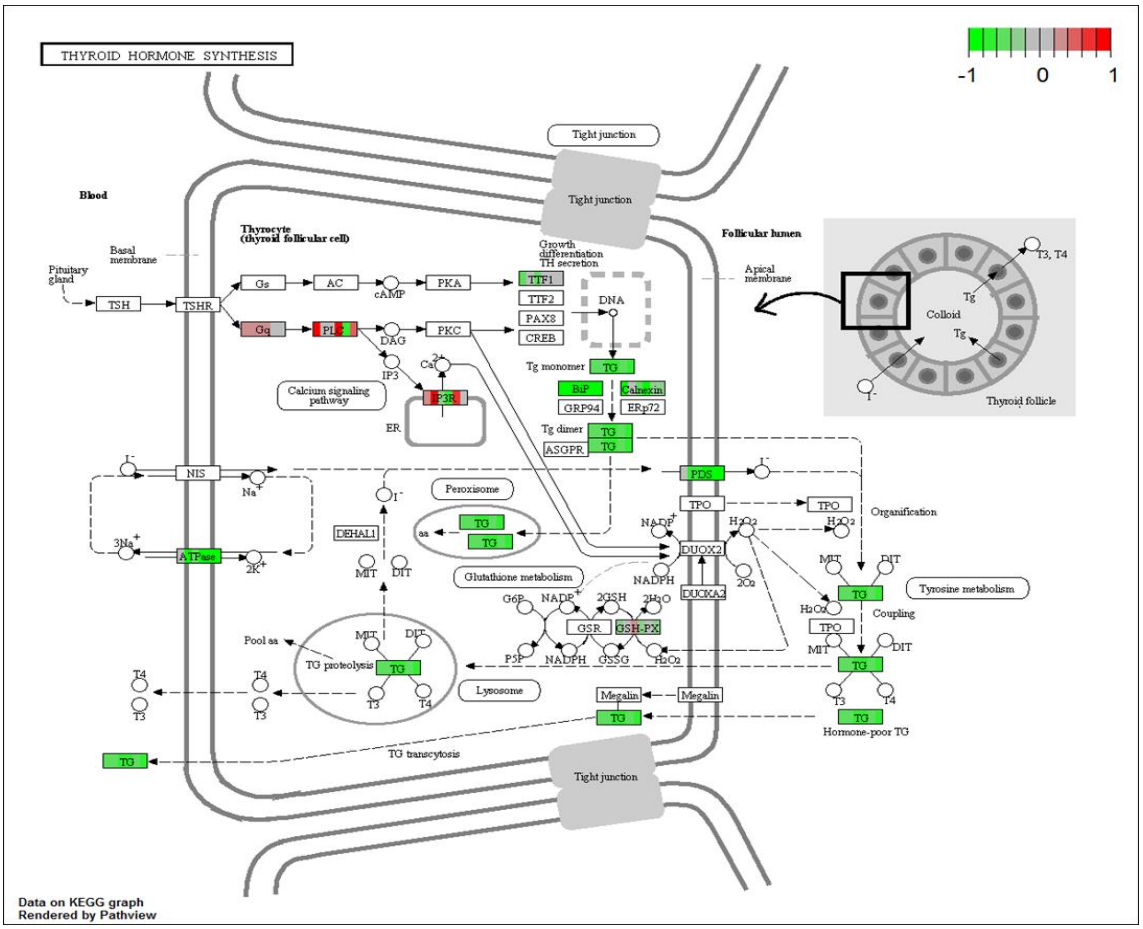


Figure 6-15 KEGG graph for ko04918 Thyroid hormone pathway plotted using Pathview package for R/Bioconductor.

Thyroglobulin (TG) has been implicated in embryogenesis in developing fish larvae as it has been found in eggs of various fish species including zebrafish and Japanese flounder (Yamano, 2005). Down-regulation may reflect the switch from embryonic to larval developmental requirements.

6.5.5.3.5 DNA replication pathway

Another of the over-represented pathways was that comprising DNA replication (ko03030).as seen in Figure 6-15. Within this pathway there was significant down-regulation of DNA polymerase complex subunit 83, RFC1, Pn1

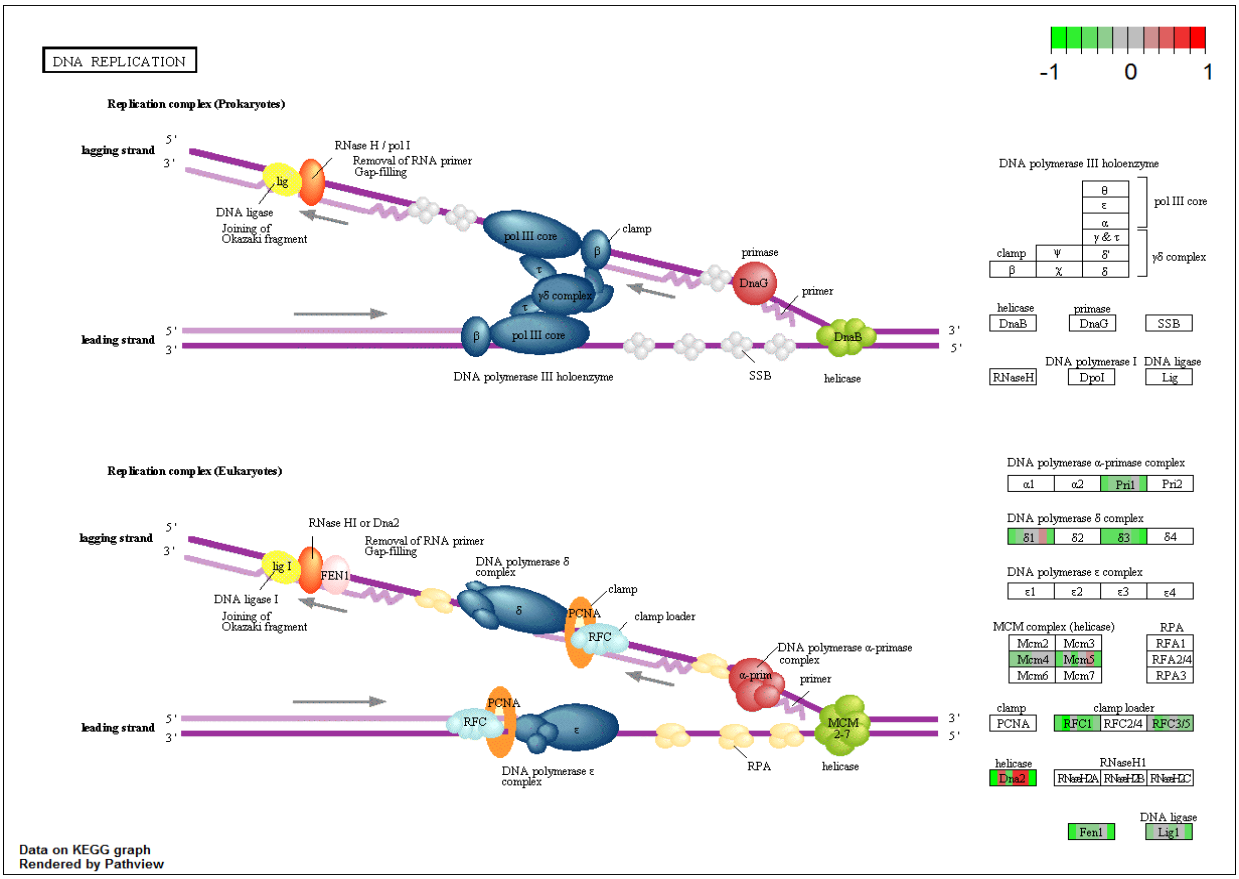


Figure 6-15 showing the KEGG graph of the (ko03030).DNA replication pathwayplotted using Pathview package for R/Bioconductor.

In the formation of a complex organism and the differentiation of specific cell types, there are often demands for high levels of particular gene products. These demands can be met by increasing transcription or translation, or by decreasing the rate of mRNA or protein turnover. Although these are the most common means to increase expression levels, there is another mechanism: gene amplification. Developmental gene amplification is a DNA replication-based process whereby specific genes are replicated above the copy number of

surrounding sequences, resulting in an increase in the template available for transcription (Yúfera *et al.*, 2017b).

6.5.5.4 Transcript expression during development between 1Dph (Dph) and 4 Dph

Some transcripts showed consistent differential expression among replicates, however, a number of transcripts showed heterogeneous expression between replicates. These included (ko04974) Protein digestion and absorption, ko04976 Bile secretion, Complement and Coagulation cascades ko04610 Glycolysis / gluconeogenesis, ko00010 PPAR signalling pathwayKo03320, Ribosome Ko3010, calcium signalling pathway ko034020, Phagosome ko04145, Serotonergic synapse ko04726, cell adhesion ko04514 oxidative phosphorylation ko00190,Cholinergic synapse ko04725,Lysosome ko04142, Starch and sucrose metabolism ko00500, some of which have already been discussed. This section will look some of the pathways that have not thus far been discussed.

6.5.5.4.1 Glycolysis /gluconeogenesis pathway

For the Glycolysis /gluconeogenesis pathway several enzymes were expressed including EC.1.2.1.12 as shown in Figure 6-16. Some are up -regulated in all the six (6) pools on the oligo microarray between 1Dph and 4Dph.

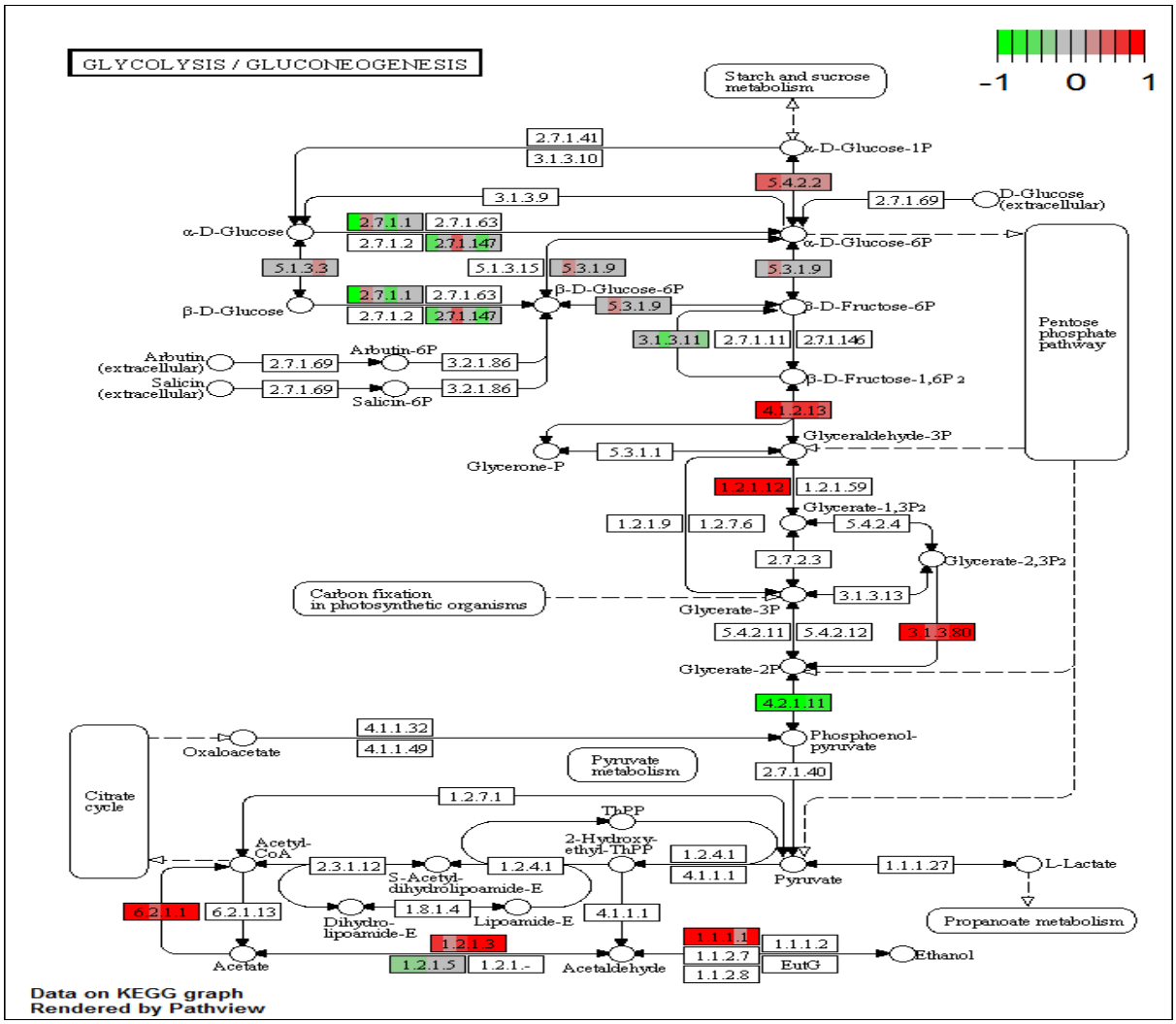


Figure 6-16 Kegg graph for the ko00010.Glycolysis /Gluconeogenesis pathway plotted using Pathview package for R/Bioconductor

Glycolysis is the first pathway used in the breakdown of glucose to extract energy (Katz *et al.*, 1954; Louis, 1987). The process does not use oxygen and is, therefore, anaerobic. While further data is needed to support the suggestion, the down-regulation of a number of transcripts in this pathway may reflect the transition from a more anaerobic to a more aerobic metabolism post-hatching, moving from embryonic to larval development.

6.5.5.4.2 Protein processing in the endoplasmic reticulum pathway

The Kegg pathway for Ko04110 Protein processing in the endoplasmic reticulum pathway is shown in Figure 6-17. The following were down-regulated: ASK, WFSI, BiP, PDIS, TRAP, IREI, PERK and sec61 while Parkin and Calpsin were up-regulated.

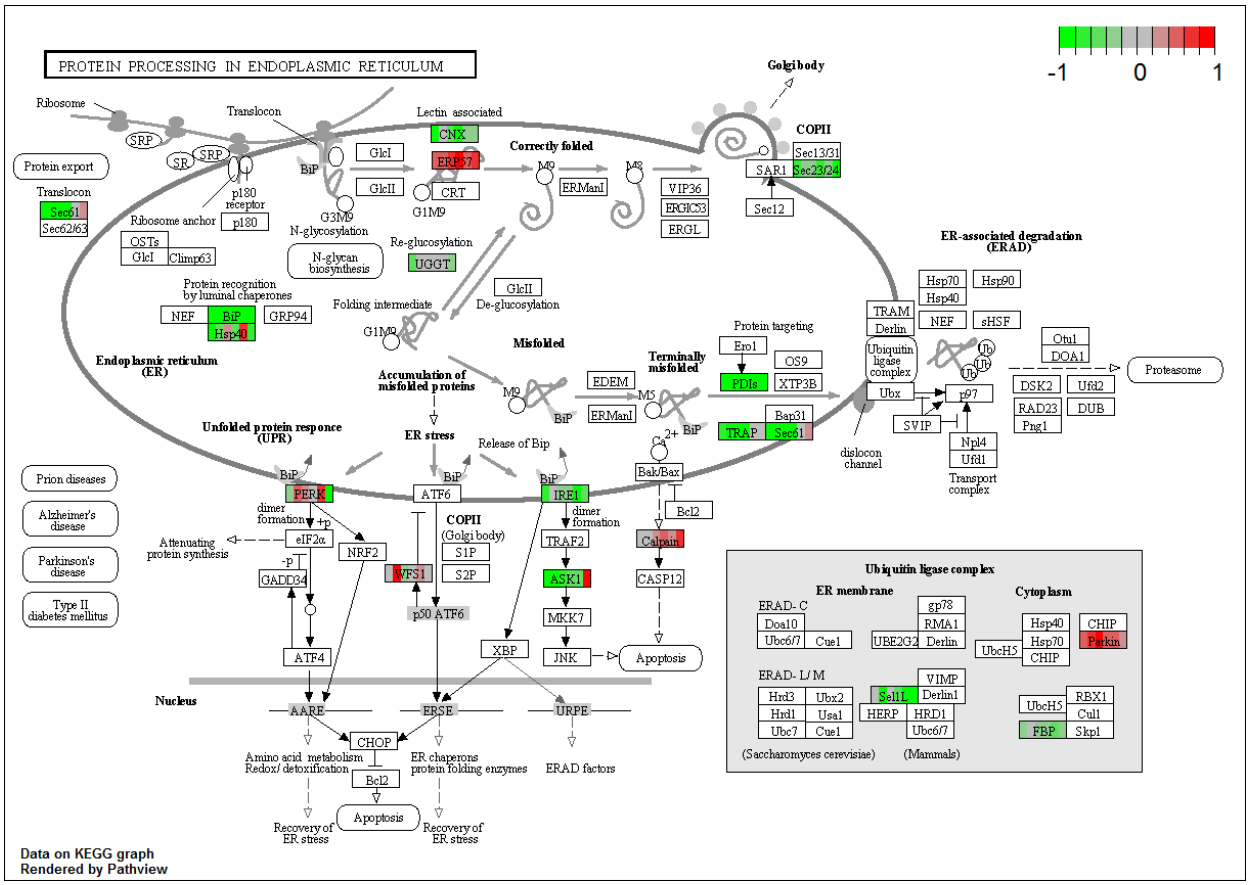


Figure 6-17 Kegg graph ko04110.Protein processing in endoplasmic reticulum pathwayplotted using Pathview package for R/Bioconductor

Parkin and Calpsin were up-regulated.and are known to play a role in lysosomal activities and apoptosis respectively and this may suggest that they may be important in the immunity of the developing larvae.Overall, however, the upregulation of transcripts in this pathway may be indicative of increased protein production and trafficking as an aspect of rapid larval development.

6.5.5.4.3 Bile secretion pathway

The Bile secretion pathway (Figure 6-18) is associated with the production of bile, which helps to emulsify fats in the small intestine, playing a key role in dietary lipid digestion and absorption, and also serves as a route to eliminate unwanted substances from the liver. Many of the genes are up-regulated all through the six pools of the samples between 1Dph and 4Dph, including OSTβ,OSTα,CYP7A1,SHP,AQRA and MDRI.

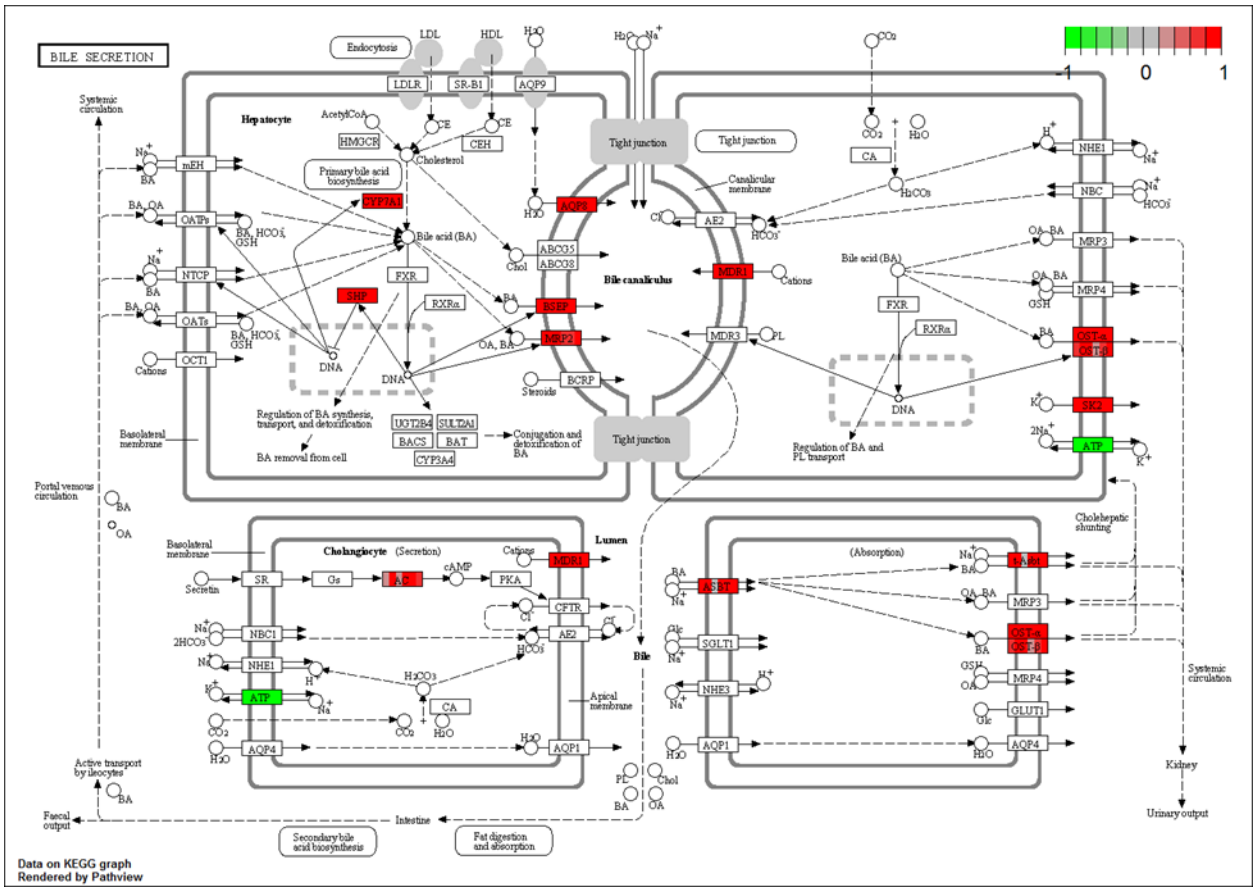


Figure 6-18 KEGG graph for ko04976 Bile secretion pathway plotted using Pathview package for R/Bioconductor.

This pathway is essential to the hepatic synthesis of bile acids, a primary pathway for cholesterol catabolism carried out by the cholesterol 7α-hydroxylase enzyme (CYP7A1). As well as their role in dietary lipid digestion and absorption, bile acids also act as signalling molecules and through their endocrine function, activate TGR5 signalling pathways that are associated with the control of immune function, liver and gall-bladder physiology and glucose and energy homeostasis (Li & Chiang, 2014; Mertens *et al.*, 2017).

This pathway is therefore critical to the developing larva in terms of regulating energy use and equipping the larva for lipid digestion and absorption. It is therefore notable that this pathway appears to be largely down-regulated between 2 and 4Dph, as normally this might be expected to be a period when larval lipid absorptive capacity should increase (Izquierdo *et al*., 2000). One possible explanation is that increase in absorptive capacity has been observed to be delayed in larval fish fed on formulated rather than live prey diets (Izquierdo *et al*., 2000) hence the diets employed here may be delaying or impeding this aspect of digestive development.

6.6 Conclusions and Recommendations

A number of pathways were observed to be over-represented in terms of transcript expression in regards to diet at 2 days post-hatch (Dph) and 4 Dph Some transcripts showed consistent differential expression among replicates, however, a number of transcripts showed heterogeneous expression between replicates. These included ko04610 Complement and coagulation cascades, ko04974 Protein digestion and absorption, ko04142 Lysosome ko04210 Apoptosis, ko03320 PPAR signalling pathway denoting changes in elements of growth, digestion, and the immune system.

In addition during development, the following pathways were observed to be over-represented in terms of transcript expression between 1 day post-hatch (Dph) and 4 Dph. Some transcripts showed consistent differential expression among replicates, however, a number of transcripts showed heterogeneous expression between replicates. These included (ko04974) Protein digestion and absorption, ko04976 Bile secretion, Complement and Coagulation cascades ko04610 Glycolysis .gluconeogenesis, ko00010 PPAR signalling pathwayKo03320, Ribosome Ko3010, calcium signalling pathway ko034020, Phagosome ko04145, Serotonergic synapse ko04726, cell adhesion ko04514 oxidative phosphorylation ko00190, Cholinergic synapse ko04725, Lysosome ko04142, Starch and sucrose metabolism ko00500, Vascular smooth muscle contraction ko04270 and denoting changes in elements of growth, digestion, the immune system and synaptic transmission.

During larval development various genes come together to perform different functions recognised as important were growth related genes which were more expressed at this time as there is rapid growth during this time.

The results of the this work are very important to the feed industry in that it could be used as a baseline to make diets for early weaning in larval fish with knowledge of which enzymes and when individual enzymes are produced by the fish as this is the first study of its kind on *C. gariepinus*.

This work has brought out the fact that in the early stages of development many activites in terms of the different organ systems are taking place and are under control of some menchanismlike they are genetically programmed and neither be be influenced externallyespecially in terms of nutrition as seen in the Heat mapsthere seem to be a similar trend in action visa vie the developing larvae

feeding on different feeding regimes .However it was notable that immunity seems to be very vital to the growing larvae as they seem to show variuos pathways more geared to survival in the new environment.

7 CHAPTER SEVEN

7.1 General Discussion, Conclusions and Recommendations

7.1.1 Introduction

As noted in Chapter 1, the major constraints to the culture of catfish in Uganda include a lack of sufficient quality and quantity of catfish larvae required by grow-out farmers (FAO 2012), low catfish larval survival rates in part due to cannibalism, nutritional problems and high costs of live feed. Provision of protein for increasing populations worldwide, in the face of climate change and other challenges, is a growing priority and the North African catfish can make an important contribution in this respect.

The work undertaken and described in this thesis sought to improve understanding of larval feeding and development, particularly during the critical first four days post hatch (Dph), in an effort to help improve early larval survival and growth and thereby to assist aquaculture stakeholders, both subsistence farmers and commercial growers, in Uganda and more widely. The key objectives, which have all been successfully realised, were:

- To confirm the catfish species raised at the Institute of Aquaculture to allow for optimization of the knowledge gained back to the species of interest namely *Clarias gariepinus* in the bid to formulate a micro-diet for the larvae and improve seed quantity and quality from the nutritional physiological point of view
- To establish whether the key enzymes pepsin, chymotrypsin, trypsin and gastric chitinase are expressed during early development of *C. gariepinus* larvae
- To examine the ontogeny of transcript (mRNA) expression for the above enzymes during early development
- To determine the sites of enzyme expression in developing larvae

The digestive systems of fish larvae are different from those of adults in the way that they utilize their diets, as they have go through a series of changes in terms of feeding ontogeny, moving through a size-coupled trophic food chain that entails initially feeding on a live diet which may be zooplankton in the wild and Artemia in the case of cultured fish larvae, to bigger diets including zoobenthic species. The adult fish then switch to omnivorous, carnivorous or herbivorous diet (Govoni et al., 1986)

At first feeding, the larval digestive system is functional, but is structurally and functionally less complex than that of adults. However, it has been observed that the larval digestive system remains unchanged histologically during the larval period before changing to an adult. The ontogeny of the digestive system differs from species to species, which is why it has been important to study the digestive physiology of larvae of *C. gariepinus*, as all evidence suggests that functional differences exist between them and other cultured fish species, and that this may have underlying effects on the assimilation efficiency of the current formulated microdiets and the inability of the fish larvae to effectively use them from the onset of exogenous feeding, as the observable and functional stomach in larvae is poorly developed initially. A holistic understanding of feeding ecology and digestive functions is important for designing fish diets for fish larvae and the adaptation of rearing conditions to meet requirements for the best presentation of prey and microdiets.

7.1.2 Main findings

7.1.2.1 Chapter 2

In Chapter 2, in addition to reporting on aspects of the general material and methods, a definitive species identity was sought for the catfish population held at the Institute of Aquaculture, University of Stirling. From the morphological and meristic characterisation, in addition to the molecular taxonomic classification conducted, the species held at the Institute of Aquaculture and employed for the present study was confirmed to be the North African Catfish, *Clarias gariepinus*, Burchell 1822 rather than a hybrid with *Clarias anguillaris*, which is a situation that is common in African populations. This is the first time that the identity of this population has been fully verified. This finding was critical as the work reported in this thesis is targeted for application to aquacultural practices in Uganda, where *C. gariepinus* remains one of the most important cultured species.

7.1.2.2 Chapter 3

In Chapter 3. the reported studies were undertaken to determine the point at which when exogenous feeding should be commenced and when an inert formulated diet could be introduced in order to improve growth during larval rearing. From the growth studies conducted, findings revealed that at 2Dph a mixed diet of (live) *Artemia* and (inert) Coppens diet gave better growth than both Coppens and *Artemia*fed alone, which gave similar growth rates. Application of these findings in

Ugandan aquaculture, even without further optimisation, would improve larval growth by introducing the larvae to a mixed dietoflive feed in our case artemia and dry diet comparable to CATCocoppens would go a long way thelping farmers tosustainably produce seed in quantity though the costs have not been evaluated at this point in time and would in a way reduce on cannibalism as opposed to the current regime where the larvae are switched to food after yolk re-absorption

7.1.2.3 Chapter 4

In Chapter 4., the histomorphology of both adult and larval *Clarias gariepinus* was examined in order to determine the course of development of digestive structures in the larva. In addition, new in situ labelling procedures were developed to highlight the localisation of expression of transcripts for key digestive enzymes. Larval development followed a time-course similar to that described by other researchers with the pancreas being discrete in the larval stages but being more dispersed throughout the intestinal region in the adults. The stomach was present by 4 to 5 Dph, supporting a suggestion that alkaline protease digestion dominates early in development, switching to a more acidic digestive mode following development of the stomach.

In situ labelling protocols having been successfully developed, from the point of view of molecular localisation, trypsin and chymotrypsin were found to be localised in the pancreas by as early as 2 Dph, while pepsin and gastric chitinase were localised in the stomach. More specifically, pepsin was localised to oxynticopeptic cells and gastric chitinase to the gastric pits with the former being present by 4Dph and the latter being present by 6Dph.

These findings can help to better formulate inert feeds and optimise timing of introduction to larval stages to maximise growth and survival.

7.1.2.4 Chapter 5

In Chapter 5, the expression of four key digestive genes, namely trypsin, chymotrypsin, pepsin and gastric chitinase were investigated in order to more accurately determine the first evidence for transcript expression. First expression was observed, perhaps unsurprisingly, to occur largely at a time when the tissue representing the site of production was first observable by histological examination and expression increased from this time. Trypsin and chymotrypsin expression wasdetectable from 1Dph, with clear expression by 2Dph, was coinciding with the appearance of the first anlage of the pancreas in histology (Verreth et al., .1992,

Osman et al., 2008). Pepsin and gastric chitinase mRNA was detected at 3Dph and was highly expressed from 4dph onwards. This similarly coincided with the appearance of the first anlage of the stomach in histology (Verreth 1992).

These findings confirm the observations made in Chapter 4 but provide a more accurate estimate of timing of transcript expression for the four key digestive enzymes. It is, however, critical to restate the fact that transcript expression is not the same as protein deployment and it should be kept in mind that proteins may first be stored as inactive pre-proteins and then may need to be activated to be employed such that transcript expression does not equal protein concentration.

As for Chapter 4., the findings of this chapter can help farmers to optimise larval feeding and feed producers to improve diet formulation.

7.1.2.5 Chapter 6

The work described in Chapter 6 set out to build new transcriptomic resources for *C. gariepinus* for the first time and to employ them, in the form of an oligo-microarray, to investigate larval development and the impact of the presentation of different feeds to larval transcript expression.

A mixed tissue transcriptome for *C. gariepinus*, the first of its kind, was produced and from the annotated transcripts, 46,830 unique probes were designed and used to make a mixed tissue oligo microarray and examine differential larval transcript expression occurring in the critical window between 2Dph and 4Dph.

Findings revealed that a number of processes were taking place in quick succession which included among others pathways involved in controlling immunity, growth, digestion and muscle development.

Using next-generation RNA-seq technologies on an Illumina HiSeq 2000 platform, we assembled a de novo transcriptome and tested for differential expression in response to stress. A total of 65,129 unigenes were generated, the mean unigene size and N50 were 607 bp and 813 bp, respectively. Of the assembled unigenes, we identified 2,990 genes that were significantly up-regulated, while 3,416 genes were significantly down-regulated in response to loading stress. Pathway enrichment analysis based on loading stress-responsive unigenes identified significantly stress related pathways. “Metabolism” and “immunity” were the two most frequently represented categories. In the “metabolism” category, “glucose metabolism” and “lipid metabolism” were major subclasses. The transcriptional

expression of rate-limiting enzymes in “glucose metabolism” and “lipid metabolism” was detected by RT-qPCR; all were significantly increased after stress. Apoptosis associated proteins tumour necrosis factor alpha (TNF-α), caspase 9, cytochrome c and caspase 3 in the stress group were significantly elevated.

7.1.3 Discussion and conclusion.

This study is the first of one to provide a deeper understanding of the development of digestive capacity in larval *C. gariepinus*. Using a range of classical and molecular techniques it has been made clear that transcripts for the enzymes involved in pancreatic alkaline digestion, namely trypsin and chymotrypsin, are produced in larvae of *C. gariepinus* as early as 1Dph, even though the pancreas is still not fully developed, and increase significantly at 2Dph when the pancreas anlage first appears with more chymotrypsin being present. The acid digestive enzymes namely pepsin and gastric chitinase produced by the stomach were shown to first appear at 3Dph as opposed to the findings of earlier studies by biochemical methods coupled with histology which indicated 5 Dph. From this study all these enzymes are significantly present from 4 Dph. This seems to point to the fact that initial enzyme transcript expression is largely genetically programmed suggests that while histologically cryptic the early stomach tissue may be functionally active despite its apparent histological absence.

One of the observed promising activity was that for the growing larvae presence of innate immunity which was prominent is therefore vital in the larviculture of fish and any efforts to assist the innate immune system through use of immunostimulants in the diet which already have proven important, particularly for marine larvae could be further developed for larvae of *Clarias gariepinus*. With current needs to intensify aquaculture practices in the face of increased population and the need for higher quality and quantities of seed for a sustained production cycle, there is a clear need to reduce the mortality of larval fish due to bacterial infection, which can be achieved in part by improving innate immunity.

7.1.4 General Recommendation

This study covered the two important enzymes involved in pancreatic alkaline digestion namely trypsin and chymotrypsin and two other important enzymes produced in the stomach namely pepsin an important enzyme involved in acidic digestion of proteins at the latter stage of development and an indicator for

transition from larvae to juvenile stage in this species .Other enzymes studied were gastric chitinase and pepsin whose roles are diverse in the fish larvae.

As the study was not exhaustive of all the digestive enzymes, more studies should concentrate on other enzymes including carboxypeptidase, lipases and α-amylase, carboxyl esterase and bile salt-activated lipase (bal). Additionally, enzymatic activities of leucine aminopeptidase (lap) and alkaline phosphatase (alp); intestinal enzymes located at the brush border membrane which represents the adult mode of digestion by enterocytes should also be studied in order to give an a complete picture of the expression. A better understanding of the timing and physiology of digestion in *C. gariepinus* should help in the formulation and deployment of inert feeds.

Furthermore, from the microarray results it is evident that larvae of *Clarias gariepinus* are ramping up immune capacity from the earliest stages of development, particularly in order to cope with the threat of bacterial and viral pathogens in early life. Knowledge of the immunology of this species should be further improved and can be exploited to formulate diets that boost innate immunity and support the development of adaptive immunity as larvae and juveniles develop. This may reduce losses incurred at the early life stages, for example Isyagi pers. comm. (2014) suggested that farmers in Uganda incur significant losses as a result of death of many developing fish between 7 Dph and 8Dph. Using broad scale transcriptomic methods, particularly newer sequencing approaches such as Illumina RNAseq, Oxford nanoprobe and PacBio sequencing can rapidly improve knowledge of a range of key processes in developing larvae that can be confirmed by more direct studies.

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Appendix 1Composition of diets fed to the fish

This section provides the composition of diets fed to the fish in the various stages of the research

Completed feed for Trout small shaped pellets for trout 150 EWOS dietary composition fed to the brood stock in the final experimental setup 2013

Additives	concentration
Vitamins	
E671 vitamin D3	200IU/Kg
Trace elements	
E1 Iron(ferrous sulphate)	50mg/Kg
E2 Iodine (Calcium Iodate)	3mg/Kg
E4 Copper (cupric sulphate)	6mg/Kg
E5 Manganese (mangenese sulphate)	36mg/Kg
E6 Zinc (Zinc sulphate)	120mg/Kg
3b8.12 Selenium (selenised yeast)	0.2mg/Kg

2a (ii) Paracoccuscaronificatians (Panferd AX) 2000

Analytical constituents	%
Oil	27.6
Protein	41.6
Fibre	2.5
Ash	7.9
Calcium	1.2
Sodium	0.1
Phosphorus	1.2
Composition	
Fishmeal	
vegetable oil	
Soya protein concentrate	
Wheat	
Sunflower seed meal	
Fish oil	
Horse beans	
Wheat gluten	
Rape seed	
Mono-Ammonium Phosphate	
DL-Methionine	

Completed feed forTroutSkretting expanded 40/60 (Preston UK)

Additivesinitially fed to the brood stock

E1 Iron (Ferrous sulphate,monhydrate)	40mg/kg
E2Iodine (Calcium iodate anhydrous)	2mg/kg
E4 Copper (Cupric sulphate,pentahydrate)	5mg/kg
E5 Manganese (Manganese sulphate	15mg/kg

monohydrate)	
E6 Zinc(Zincsulphate,monohydrate)	100mg/kg
Antioxidants	
E8 10 propyl gallate	2mg/Kg
E8 20 BHA Butylatedhydrotourene	7mg/kg
Vitamins	
E8 71 Vitamin D3	1500IU/Kg
E8 72 Vitamin A	4000IU/Kg

Analytical constituents	%
Crudefat &Oil	80
Protein	39/37.2
Fibre	4.9
Ash	5.4
Calcium	0.7
Sodium	0.2
Phosphorus	0.9
Composition	
Wheat	
Soya bean (GM product)	
Sun flower expeller(organicproduct)	
Vital wheat gluten	
Fish meal	
Fish oil	
Rape seed oil	
Vitamis	
Minerals	

Appendix 2 : Preparing 4% Paraformaldehyde

This section provides a description of the preparation the stock solution used to preserve the samples for in situ –expression hybridisation.

450 mL of 1X PBS was added to a nuclease-free glass bottle and warmed up slightly in the microwave. Weighed out 20g of paraformaldehyde (taking necessary safety precautions i.e. wearing gloves, face mask and protective eye goggles) and added to the PBS in the fume-hood. Stirred on a heated stirrer using a nuclease-free magnetic stirrer to 60^0C and added 1-2 drops of 5M NaOH to clear the solution. Allowed to cool to room temperature, made up the volume to 500mL and filtered through Whatman No. 1 filter paper (using a filter funnel treated with RNAZap) into a fresh nuclease-free bottle. Aliquoted into 50mL centrifuge tubes and stored at -20^0C until use. (The pH of the solution should be ca. pH 7.4. This can be checked using pH strips but is not necessary).

Appendix 3 Samples selected for microarray - Nanodrop ND 1000 concentration measurements

This section describes the selected samples used in the microarray.

Final selected samples RNA Extraction Nanodrop concentrations and purity estimates					
SNR	**Age (days)**	**treatment**	**ng µL-1**	260/280	260/230
1	1	None	213.56	1.86	2.03
2	1	None	248.67	1.84	1.98
3	1	None	257.26	1.79	1.44
4	1	None	254.19	1.74	1.45
5	1	None	224.86	1.83	1.60
6	1	None	288.78	1.80	1.65
13	2	2	359.24	1.88	0.63
14	2	2	297.47	1.98	1.04
15	2	2	558.94	2.05	1.25
16	2	2	214.53	1.88	1.70
17	2	2	431.39	1.92	0.70
18	2	2	326.04	2.02	0.93
49	3	2	671.82	2.06	1.87
50	3	2	216.76	1.95	2.09
51	3	2	369.51	1.96	1.86

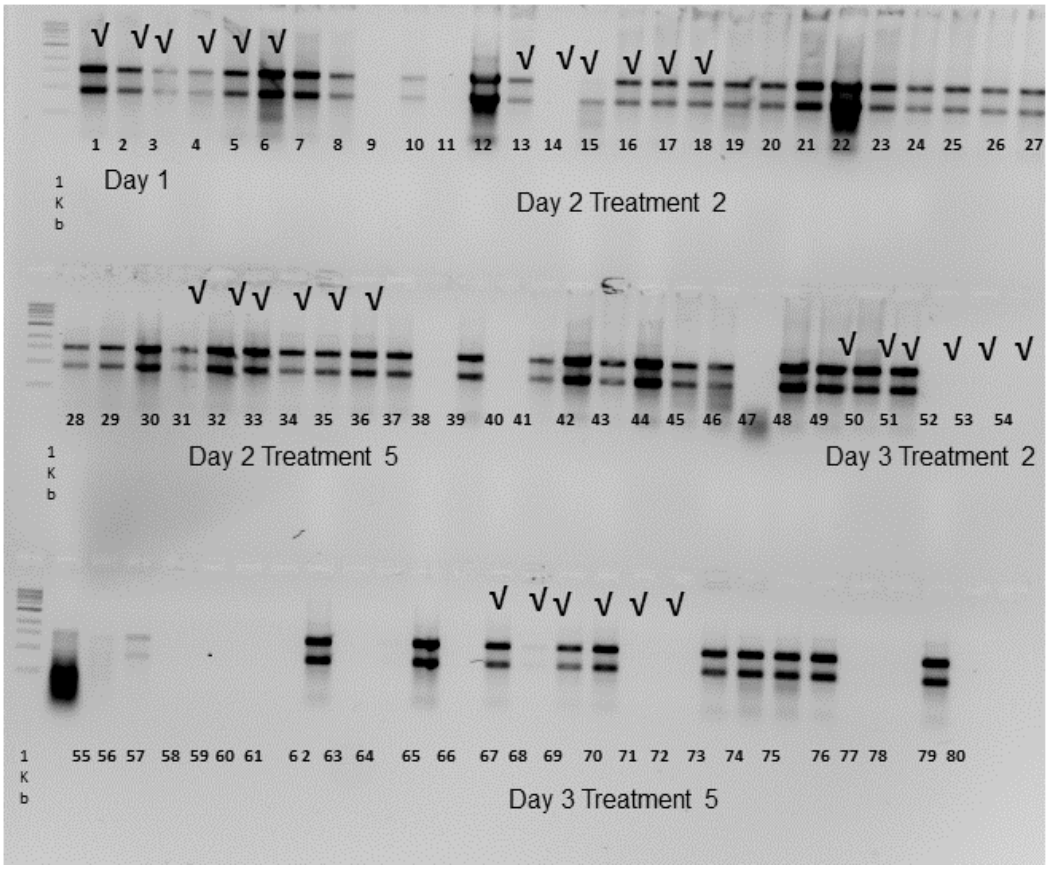
52	3	2	514.3	2.08	1.66
53	3	2	322.1 4	2.02	1.39
54	3	2	650.5 9	2.07	2.01
85	4	2	794.9 4	2.03	1.68
86	4	2	923.9 6	2.07	2.12
87	4	2	785.6 4	2.06	1.99
88	4	2	647.6 4	1.95	2.19
89	4	2	700.6 5	2.07	1.96
90	4	2	666.8 5	2.04	1.81
31	2	5	487.1 8	2.03	1.75
32	2	5	597.9 7	1.93	2.00
33	2	5	415.1 4	1.99	2.15
34	2	5	297.4 3	1.95	0.81
35	2	5	212.3 9	1.91	0.56
36	2	5	301.9 9	2.01	0.71
67	3	5	440.4 4	2.00	1.63
68	3	5	526.8 6	2.06	0.88
69	3	5	314.2 3	2.00	1.82
70	3	5	180.2 2	1.97	1.48
71	3	5	758.1	2.13	1.73

			6		
72	3	5	582.05	2.14	1.67
103	4	5	867.72	2.05	1.95
104	4	5	862.49	2.07	1.82
105	4	5	1073.37	2.08	1.98
106	4	5	929.17	2.03	1.71
107	4	5	811.78	2.05	2.02
108	4	5	984.14	2.09	2.20

Appendix 4 Gel checks for selected samples to include in the Microarray.

Ticks represent selected samples.

- Samples 1 -80



Appendix 5 Description of procedure forcapillary electrophoresis using Beckman Coulter sequencer

Critical factors
Excess salt/impurities in sample matrix
Sample loading volume
Voltage
Some comments
Always read the status of the machine in the top LHS dialogue box and only move to next step when it reads “idle”
Always store gel in the fridge
Store capillary arrays in fridge
Gel cartridges contain 20ml (if there is not enough gel for the run requested then the computer will flag this)
Always replace gel with empty cartridge– referred to as plug in dialogue box
Replace black cap onto gel cartridge and replace in fridge

Instructions for operation of sequencer
Switch on computer and leave for a few minutes
Then the computer and enter password
Click on CEQ system icon – password is CEQ8800

Changing wetting tray
Click on direct control
Select replenish/replace wetting tray
Yes
Wait till idle in top LHS dialogue box
Open lid and replenish wetting tray and replace
Done

Installing Gel cartridge
Direct control
Click on gel
Fill in details if new cartridge set to new button
Open gel door
Release plug – the lever does not require to be turned here
Turn lever to release old cartridge or plug
Remove black cap and insert new cartridge ensuring it is firmly in place
Done
If not enough gel for amount of samples machine will flag this

Sample Sheet set up

Sequence and fragment samples can be loaded on same plate
They run at different temperatures and fragment samples are recommended to be loaded first
Create new sample plate
Click plate icon from toolbar
Sample plate selection – either click on new plate or open existing sample plate
This can be one prepared and saved earlier from excel spreadsheet - you need to transfer to swap area as the computers are not networked

If new then blank sample plate format will open
Enter name of sample in Sample Name text box and press enter
File save as – CEQ
You need to create a personal file and export to swap area to access the analysed data as the CEQ computers not networked

Assigning a method (the programme of events that the system uses to collect data)
Frag Methods used to collect data for fragment analysis
LFR methods used to collect data for sequence analysis

FRAG - 3 for 400 size standard
FRAG - 4 for 600 size standard
Click on Method tab
A method selection must be made for each column
A method selection must be made before the sample plate can be saved

Assigning Analysis Parameters
For sequence analysis select default sequence analysis parameters
For Fragment analysis select 400PA or 600PAdepending on size standard being used

Click Analysis tab to choose parameters from drop down menu in parameter set
Exporting data
Highlight data to be exported and tick export data from analysis tab
Select destination folder and file type
Choose CEQ if data is to be viewed using CEQ software
Choose SCF (Ver 3) if to be viewed using other software
File and save as
OK

Accessing plates

It is important that sample and buffer plates are prepared at this point
Direct control or mouse over plates on diagram
The window warning referring to capillaries being exposed to air will appear
There is a 15 min time limit which is critical so that the capillaries are not adversely affected by exposure to air
Clickstart
Wait until capillaries have been removed from wetting tray before opening the sample door – the top LHS dialogue box will read “ idle” and there will be a message giving you permission to open the sample door and load the plates
If only running single plate then load right hand sample plate holder
The notched corner of each plate should be to back of instrument on RHS
At this point also ensure that the wetting trays are filled to the mark with dis water
Run sample plate
Sample plate confirmation window appears
If two [plates are being run tick run plate first for RHS and also tick plate loaded here
Then tick LHS plate loaded
Type in operator name
Select sample plate name from bottom of plate window (good idea to have given date for this when saving plate details)

Click start
The progress of the run can be viewed by clicking on log where you can read the progress
Before running check the following
Capillary array has been installed
Gel cartridge has been in stalled
Wetting tray has been filled

Closing down machine
Direct control
Click on wetting tray
Wait till instruction to open door
Remove plate
Remove wetting tray and refill with dist water and replace
Click done
Click on gel image
Click Remove gel
If required wipe any excess gel off the cartridge and store in fridge
Install/Replace with plug
Switch off machine

Retrieving data

Go into data manager to check that samples have been done

Removing/exporting data can be done either by exporting to swap area or to saving on to memory stick

If the data hasn't been saved to swap area then once the programme has finished the results may be accessed through data manager

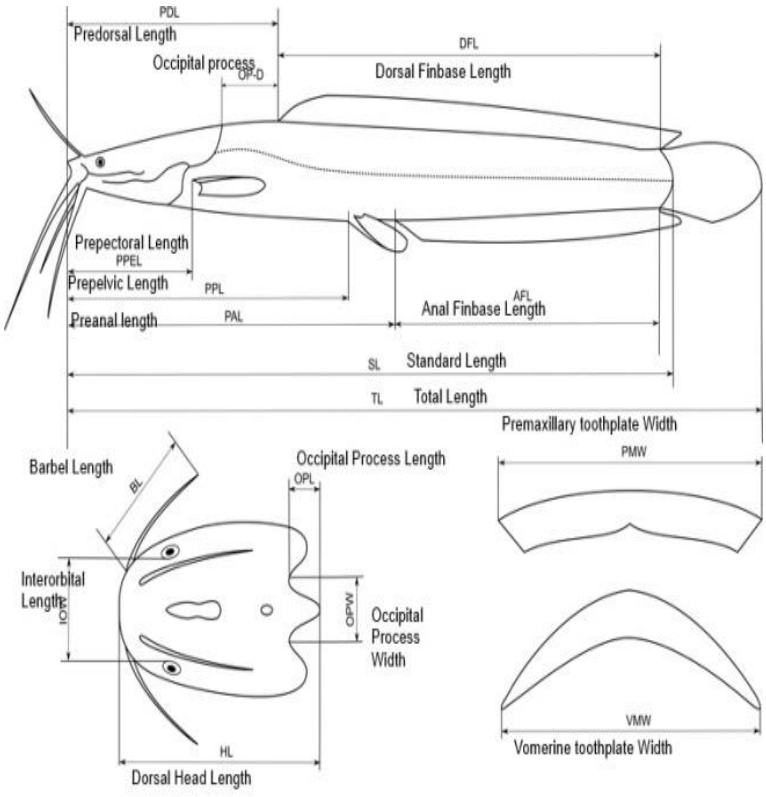
Find your data on either sequence results/fragment results

Highlight

Export to swap area

Appendix 6 Morphometrics of *Clarias gariepinus*

Table showing morphometric data for *C. gariepinus*



Diagrammatic representation of morphometric measurements taken from *C. gariepinus* and key to table above