The effects of dietary lipid oxidation on farmed fish

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Dissertation submitted in partial fulfilment of a
Philosophiae Doctor Degree in Biology

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Reykjavik, June 2017

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Lipid oxidation in aquafeeds

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Abstract

Although conditions for aquaculture in Uganda and East-Africa are favourable, production remains low. One of the primary limitations to aquaculture development in the region is the lack of economical aquafeeds produced from local raw materials. In this thesis, four experiments were conducted to contribute to the solution of this problem: 1) Defining the optimum experimental design in fish growth studies (i.e., the number of fish and replication level of treatments) and the best statistical methods to analyse the results. Variance estimates from 24 growth studies were similar, hence experiments set in triplicates and with n of 50-100 are recommended for all fish growth studies. A mixed model ANOVA is suitable for analyzing dose response data. 2) Studying the effects of dietary lipid oxidation on farmed fish. The results suggest that lipid oxidation does not affect the growth of Nile tilapia (*Oreochromis niloticus*) in ponds where they have access to plankton rich in vitamin E. 3) Comparing the effects of the commonly used ethoxyquin (EQ) and novel antioxidants in aquafeeds, rosemary oil (RM; *Rosmarinus officinalis*) and bladderwrack (BW; *Fucus vesiculosus*), on farmed fish and their efficacy in preventing oxidation of fish oil. The results suggest that RM can be as effective in preventing oxidation of oils as EQ. Moreover, RM appears to promote better growth than does either EQ or BW. 4) Testing locally available ingredients as alternative protein sources to replace fish meal in aquafeeds. The results suggest that the meal made from the freshwater shrimp *Caridina nilotica*, a by-catch from pelagic fisheries, is economically viable replacement ingredient for *Rastrineobola argentea* fishmeal currently used in aquafeeds. The results of these experiments provide useful information for the future development of aquaculture in Uganda and in other parts of East-Africa.

Útdráttur

Þótt aðstæður til fiskeldis í Úganda og Austur-Afríku séu góðar, þá er fiskeldisframleiðsla á svæðinu ennþá fremur lítil. Helsta hindrun frekari vaxtar fiskeldis er skortur á hagkvæmu fóðri, sem framleitt er úr hráefnum af svæðinu. Í doktorsverkefninu voru gerðar tilraunir sem taka á þessu vandamáli: 1) Með því að skilgreina kjöruppsettningu vaxtartilrauna (heppilegasta fjölda fiska og endurtekninga meðferða) og bestu tölfræðiaðferðir til þess að greina gögnin. 2) Könnuð voru áhrif þránunar á lýsi í fóðri á fiska. Niðurstöðurnar benda til þess að þránun hafi ekki áhrif á vöxt Nílar borra (*Oreochromis niloticus*) í tjörnum þar sem gnægt er af þörungasvifi, ríku af andoxunarefnum. 3) Borin var saman andoxunarvirkni ethoxiquin (EQ), sem mikið er notað í fiskafóðri, og nýrra andoxunarefna: rósmarínolíu (RM; *Rosmarinus officinalis*) og blöðruþangs (BÞ; *Fucus vesiculosus*). Niðurstöðurnar benda til þess að RM geti hindrað þránun lýsis jafn vel og EQ auk þess að hvetja til betri vaxtar fiskanna en EQ eða BÞ. 4) Ný hráefni í fiskifóðri, sem framleidd eru í Úganda, voru prófuð. Niðurstöðurnar benda til þess að hagkvæmt sé að nota rækjuna *Caridina nilotica*, sem er meðafli úr fiskveiðum í Viktoríuvatni, í fóður og skipta þannig út fiskimjöli úr *Rastrineobola argentea*, sem nýta má beint til manneldis. Niðurstöður þessara tilrauna eru mikilvægt framlag til frekari þróunar fiskeldis í Úganda og Austur-Afríku.

Dedication

To my wife and children for the broad-mindedness and stamina during the period I pursued a PhD.

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II. **Kubiriza, G.K.,** Árnason, J., Sigurgeirsson, Ó., Hamaguchi, P., Snorrason, S., Tómasson, T., Thorarensen, H., 2017. Dietary lipid oxidation tolerance of juvenile Arctic charr (*Salvelinus alpinus*) and Nile tilapia (*Oreochromis niloticus*). *Aquaculture* 467, 102–108. doi:10.1016/j.aquaculture.2016.04.006.

III. **Kubiriza**, **K. G**., Akol, M.A., Árnason, J., Sigurgeirsson, Ó., Snorrason, S., Tómasson, T., Thorarensen, H., 2017. Practical feeds for juvenile Nile tilapia (*Oreochromis niloticus*) prepared by replacing *Rastrineobola argentea* fishmeal with freshwater shrimp (*Caridina nilotica*) and mung bean (*Vigna radiata*) meals. *Aquaculture Nutrition* 2017; 00:1–8. doi: 10.1111/anu.12537.

IV. **Kubiriza, G.K.,** Árnason, J., Sigurgeirsson, Ó., Hamaguchi, P., Snorrason, S., Tómasson, T., Thorarensen, H. Efficacy of natural antioxidants in stabilizing lipid oxidation and their effects on growth and antioxidant enzymes in Arctic charr. (*Manuscr*ipt).

Abbreviations and acronyms

AFCR	Apparent Feed Conversion Ratio
AnV	Anisidine Value
AOAC	Association of Official Analytical Chemists
CAT	Catalase
FCR	Feed Conversion Ratio
FFA	Free Fatty Acids
GPx	Glutathione peroxidase
HSI	Hepatosomatic Index
HPCL	High Performance Liquid Chromatography
HUFAs	Highly Unsaturated Fatty Acids
PUFAs	Poly Unsaturated Fatty Acids
ORAC	Oxygen Radical Absorbance Capacity
POV	Peroxide Value
ROS	Reactive Oxygen Species
SGR	Specific Growth Rate
SIDA	Swedish International Development Agency
SOD	Super Oxide Dismutase
TBARS	Thiobarbituric Acid Reactive Substances
UNU-FTP	United Nations University, Fisheries Training Programme
VSI	Visceral Somatic Index

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"It always seems impossible until it's done!" - Nelson Mandela

1 Introduction

Uganda offers natural conditions that are suitable and favourable for aquaculture development. The country has abundant water resources, fairly high and stable tropical temperatures to support fast fish growth, as well as raw materials from various agricultural crops and freshwater bodies for feed formulation and production. Aquaculture in Uganda dates back to the 1950s with pond farming being the oldest and predominant practice (Isyagi et al., 2009a; MAAIF, 2015; Rutaisire, 2007). The estimated number of aquaculture ponds in the country is 25,000-30,000, with about 7,000-14,000 fish farmers (DFR, 2011; Isyagi et al., 2009a; Rutaisire, 2007; UBOS, 2004) although for most of the farmers, aquaculture is only a side-line to other agricultural activities. Pond productivity is low. Nevertheless, supportive government policies continue to fuel the growth of aquaculture in Uganda (Isyagi et al., 2009a; MAAIF, 2015; Mugabira et al., 2013). Surveys indicate, that in recent years, aquaculture has been growing in Uganda with increasing number of ponds and cages and hence, fish farmers (MAAIF, 2014, 2015).

Cage fish farming, that was introduced in Uganda in 2006, has continued to grow and plans are under way to establish communally owned cage farms, known as aqua parks (MAAIF, 2014, 2015; Mugabira et al., 2013). Several companies and organizations/institutions are also establishing new farms not only on Lake Victoria, but also on other water bodies (Dalsgaard et al., 2012). Statistics published in the 2015 Agriculture Sector Performance Review, showed that about 1,700 mt of Nile tilapia were produced in cages in 2013, and about 3,000 mt in 2014 (MAAIF, 2015). Similarly, about 27,000 mt of fish were produced from ponds in 2013 (MAAIF, 2014, 2015). The statistics from the 2015 Agriculture Sector Performance Review are, however, much lower than those published by FAO for aquaculture production in Uganda (FAO, 2017; Fig. 1.1). The production statistics reported by FAO appear to be excessively high, while the numbers from the MAAIF (2015) may be closer to the mark. While there is a general increase in aquaculture production in Uganda, the infrastructure available is not commensurate with the fish production reported by FAO (pers. observation). For example, the estimated fingerling and aquafeed production in Uganda seems to be much

lower than what would be needed to generate the tonnage of farmed fish reported by FAO. Statistics by FAO show a 10 fold increase in the fish farmed in Uganda from about 10,800 tonnes in 2005 to 111,000 tonnes in 2014 (Fig. 1.1). Although these figures may not be accurate, they probably reflect the relative increase in production.

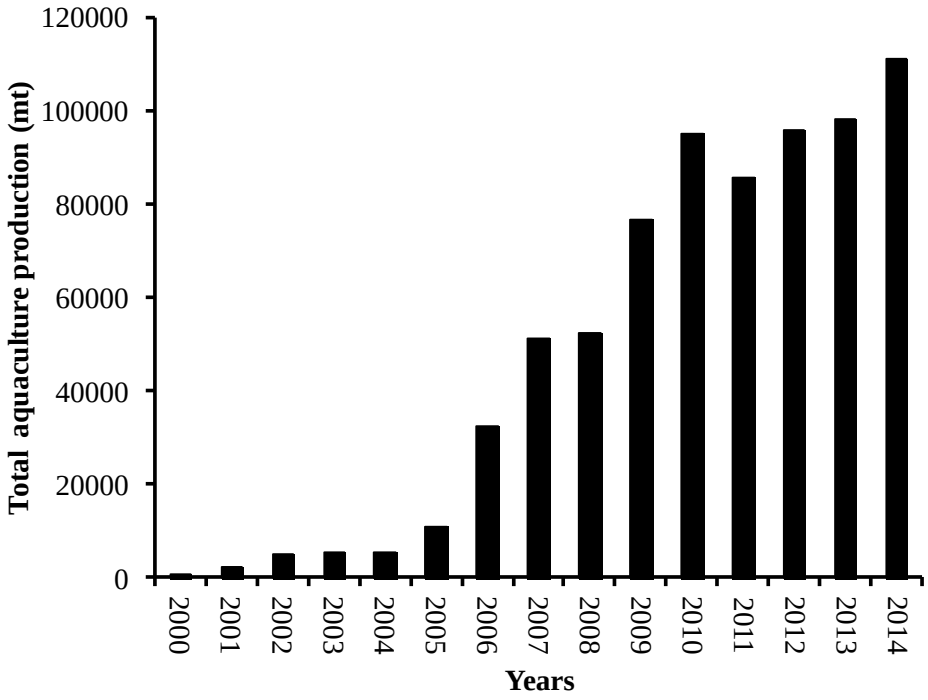

Figure 1.1: Aquaculture production in Uganda from 2000 to 2014 according to FAO (FAO, 2017)

Assuming that the annual aquaculture production in Uganda is about 30 000 mt, it can be estimated that the current need for aquafeed is 54,000 mt (assuming a FCR of 1.8) if the production relied only on prepared feed. Considering an average FCR of 1.8 that is achieved by most fish farmers in Uganda, production of 10,800 tonnes of farmed fish reported in 2005 and 111,000 tonnes in 2014 would respectively require over 19,000 tonnes and 199,800 tonnes of aquafeeds. However, in 2005 only 0.5 tonnes of sinking experimental fish pellets were formulated for the first time in Uganda by Ugachick Poultry Breeders Ltd (UPBL) (Isyagi et al., 2009a; USAID, 2009). During that time only a few farmers were making on-farm feeds. Fish farming in Uganda was mostly at subsistence level and characterised by minimal feeding until in late 2006 (USAID, 2009). Over

time, the amount of sinking pellets produced in Uganda increased, and by 2007 UPBL and Nuvita produced 186 tonnes and 10 tonnes respectively. However, Nuvita stopped producing fish feed within a year while Ugachick has continued to produce formulated fish feeds to this day.

In 2008, SoN fish farm made about 50 tonnes of on-farm fish feed (USAID, 2009), but the venture proved expensive and difficult to manage and sustain. The company then settled for buying locally pelleted feed from UPBL and importing from Mauritius and Brazil. Production of floating pellets by UPBL started in 2009, but their use on farms commenced in 2010 (Damba. pers. comm.). Presently, several farms in Uganda use floating pellets that are either locally manufactured or imported.

While aquaculture expansion in Uganda is evident, the growth of the sector faces several constraints and these include limited access to key inputs such as seed, feed and equipment. The marketing chain for farmed fish is also lacking, and more knowledgeable and skilled man power is needed to support the sector. There are also social issues that slow down the growth of aquaculture in Uganda, such as lack of established institutional structures to deal with conflicting demands of aquaculture and other sectors for access to land and water bodies. In general, the impediments to aquaculture development in Uganda are a blend of biological, environmental and socio-economic issues. This thesis addresses one of the technical challenges affecting the growth of aquaculture in Uganda; i.e., aquafeed formulation and quality preservation.

1.1 Aquafeeds as a constraint to the growth of aquaculture in Uganda

From the 1950s to the mid 2000s, aquaculture in Uganda was mainly at the subsistence level, where fish were fed on some farms to supplement pond productivity (Isyagi et al., 2009a; Rutaisire, 2007). Fresh vegetables, grass, industrial/kitchen waste, insects, cereals, brans, oilseed cakes and occasionally fishmeal (mukene) were among the main supplementary feeds used during this period (Rutaisire, 2007, Fig. 1.2). Over time, some fish farms started formulating on-farm feeds, but with limited considerations given to feed quality, and rarely were feeds prepared according to the known nutrient requirements of fish. The human resource

with expertise in aquafeed formulation was also limited and unable to support farmers.

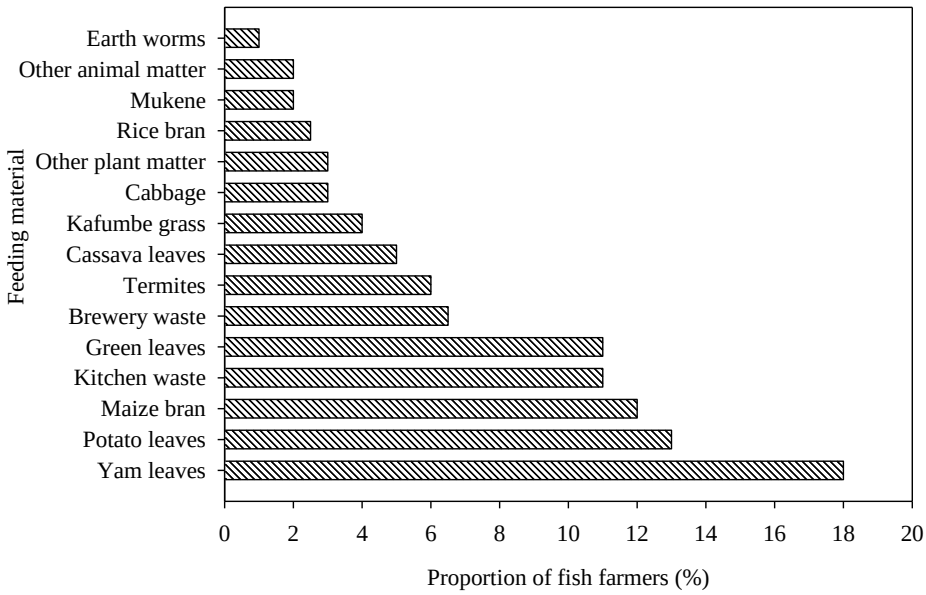

Figure 1.2: Common fish feeding materials and estimated proportion of farmers using them by the late 1990s (Data source: Rutasire, 2007)

Formulated aquafeeds with sinking pellets have only been in common use in Uganda for the last 15 years and extruded pellets for even a shorter time (USAID, 2009, 2012). Ugachick Poultry Breeders Ltd with capacity to produce over 25,000 mt of floating pellets annually is the leading fish feed manufacturer in Uganda. In recent years however, the number of feed mills producing fish feeds has increased and some of these are owned by fish farms (pers. observation).

Agricultural by-products are the main raw materials used in the production of aquafeeds in Uganda. Soybean, cottonseed and sunflower cake/meals are the main protein ingredients used, although oilseed, protein concentrate meals and wheat gluten are occasionally used (Lee, 2014; Nalwanga et al., 2009). Cottonseed and sunflower cakes are more important in backyard and on-farm feed production than in commercial aquafeeds due to their high indigestible fibre content (Dalsgaard et al., 2012; Lee, 2014). Whole and broken maize as well as wheat products such as pollard and bran are important carbohydrate ingredients used to

produce aquafeed in Uganda. Importation of ingredients to Uganda is limited because of the prohibitive costs, except for vitamin and mineral premixes that are obtained from special suppliers (Lee, 2014; Nalwanga et al., 2009).

Despite the growing capacity in feed manufacturing, inconsistency in the nutritional content is a recurring problem in Uganda (Dalsgaard et al., 2012; Nalwanga et al., 2009). Quality issues related to adulteration/contamination, inconsistency of nutrient content and low feed utilization efficiency have been a concern since the inception of aquafeed manufacturing in the mid 2000s (pers. observation). In some cases, locally manufactured aquafeeds do not meet the requirements of farmed fish (Nalwanga et al., 2009), and their prices fluctuate with the availability and cost of raw materials. As a result, some fish farmers resort to importing aquafeeds to supplement the locally produced ones. It is claimed that the imported aquafeeds are of better quality, but they are expensive and therefore inaccessible to most farmers. Some farmers formulate on-farm aquafeeds despite their limited knowledge about the complexity of feeding fish as opposed to terrestrial animals (Dalsgaard et al., 2012; Nalwanga et al., 2009).

Growth of farmed fish and the overall profitability of aquaculture enterprises depend on feed quality and feed cost. Aquafeed palatability and utilization by fish are influenced by feed quality and determine the cost of producing fish. When aquafeed of fairly good quality is used, the average cost per kilogram of fish produced is 50-60% of the total production cost (Cheng and Hardy, 2002). However, when a poor quality aquafeed is used, the cost of feed escalates (Cheng and Hardy, 2002; NRC, 2011; Watanabe, 2002), and makes aquaculture less profitable. In Uganda, feed cost constitutes about 75% of the total production cost in the main aquaculture farms (Osinde. pers. comm.) partly because of poor feed quality (Lee, 2014; Nalwanga et al., 2009). The use of poor quality feeds prolongs the production period, affects fish quality and increases susceptibility of fish to diseases.

1.2 Quality issues in *Rastrineobola argentea* fishmeal

Much as fishmeal is a vital source of protein in the manufacture of aquafeeds, it can be a source of physical and chemical contamination if

poorly processed. For example, oxidized lipids primarily get into aquafeeds through fish oil and fishmeal. In East Africa, *R. argentea*, the primary fishmeal used in aquafeeds constitutes about 53% unsaturated fatty acids (Mwanja et al., 2010) that easily oxidize when exposed to heat, UV-light and/or catalytic metals ions such as copper, iron and/or zinc (Antolovich et al., 2002). Sun dried *R. argentea* is packed in poly-bags that are stacked and stored at ambient temperatures that potentially facilitate lipid oxidation. As a result, dried *R. argentea* is associated with strong odours and off flavours that are characteristic of secondary lipid oxidation (see Antolovich et al., 2002; Frankel, 1984, 1998; Lubis and Buckle, 1990; NRC, 2011). A recent study indicated that lipid oxidation occurs in *R. argentea* processed by different methods (Kubiriza et al. in prep.). This is of concern in the formulation of diets meant for juvenile fish which may constitute up to 40% crude protein, with the biggest proportion derived from *R. argentea* fishmeal. Therefore, juvenile fish that are more susceptible to the detrimental effects of lipid oxidation may be exposed to higher levels of oxidized lipid than adult fish.

The effects of feeding fish on oxidized diets are deleterious and noticeable through retarded growth, impaired feed utilization and high mortalities in extreme cases (Alves Martins et al., 2007; Fontagné et al., 2006, 2008; Tocher et al., 2003). Furthermore, the effects of dietary lipid oxidation on fish are diverse and sometimes inconsistent. This thesis examines lipid oxidation, its effects on farmed fish and the potential mitigation measures in Uganda and East Africa. This information is critical for proper planning and effective management of oil laden raw materials and processed feeds.

There is a growing interest among feed producers and fish farmers of Uganda to improve the quality of both raw materials and aquafeeds (Isyagi et al., 2009b; Mugabira et al., 2013; Nalwanga et al., 2009). Accordingly, aquafeed quality, estimated based on proximate composition, microbial and physical contaminants, have in recent years received some attention. In contrast, dietary lipid oxidation has received minimal attention, even though Uganda is a tropical country where oil laden raw materials and processed feeds are exposed to high and fluctuating temperatures. Moreover the processing protocols and marketing chain for both raw materials and aquafeeds potentially expose them to copper, iron and zinc ions, all of which catalyze lipid oxidation.

1.3 *Rastrineobola argentea* the main source of fishmeal in Uganda

In East Africa, the silver cyprinid (*Rastrineobola argentea*: common names, mukene, daaga, omena; Fig. 1.3) is the primary source of fishmeal used for protein in aquafeeds. *R. argentea* is a small pelagic fish endemic to Lake Victoria that grows to an average standard length of about 9 cm (Wanink, 1999). During the day, adult *R. argentea* stay near the bottom and move close to the surface at night. The juveniles stay at the water surface throughout the day. *R. argentea* feeds primarily on copepods during the day, while at night their diet consists mainly of aquatic insect larvae and pupae (i.e., chaoborids and chironomids) (Wanink, 1988).

Figure 1.3: Dried silver cyprinid (Rastrineobola argentea) used for fishmeal in aquafeeds in East Africa.

R. argentea is harvested by light attraction on moonless nights using mesh sizes of 8 to 10 mm. The harvested *R. argentea* is piled up during overnight fishing in canoes until the following morning when it is sun dried ashore. Artisanal processors dry *R. argentea* on different surfaces including: bare ground, mesh laid on ground, rock, pebbles, and of recent times on air ventilated raised racks (Masette, 2010).

Subsistance fishing of *R. argentea* for animal feed processing dates back to the 1970s (Wanink, 1999). Until the late 1980s, *R. argentea* was of

minor importance in the fisheries of Lake Victoria. It was mostly consumed by the poor people who could not afford tilapia and Nile perch (*Lates niloticus*) and other fish species that were valued then, e.g., the silver catfish (*Bagrus docmak:* local name, semutundu) and African catfish (*Clarias gariepinus:* local name, emmale*)*. Over the years, however, a decline in Nile perch and Nile tilapia catches particularly in the early 1990s boosted the commercial importance of *R. argentea* (LVFO, 2014; Fig. 1.4). Since then, the interest to fish and trade *R. argentea* specifically for human consumption has been on the rise, and this has in turn escalated the beach prices for this fish species.

In the mid 2000s, campaigns were instituted to increase the proportion of *R. argentea* consumed by humans and reduce the quantities used for animal feeds in East Africa (Masette, 2010). As a result, *R. argentea* has become the most traded fish in the regional markets of East and Central Africa, being exported from Uganda to Rwanda, Burundi, DRC, South Sudan and the Central African Republic. Presently, *R. argentea* competes well with tilapia for the second position as the most traded fish species after Nile perch in East Africa.

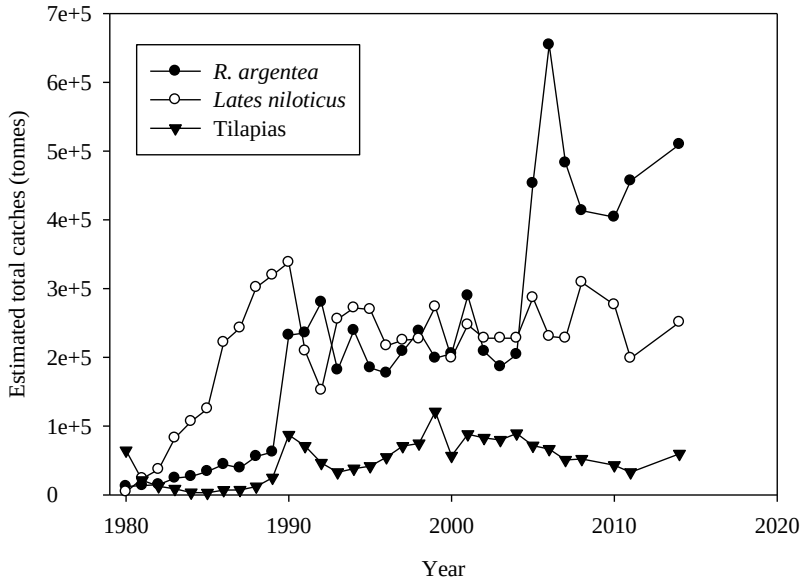

Figure 1.4: Catches of R. argentea, Lates niloticus and tilapias in Lake Victoria from 1980 to 2014 (LVFO, 2017; www.lvfo.org*)*

Since mid 2000s, the volume of *R. argentea* landed from Lake Victoria drastically increased (Fig. 1.4). However, due to high demand (Masette,

2010; pers. observation), the price of *R. argentea* has more than quadrupled in the last decade (LVFO, 2014; Fig. 1.5). Accordingly, *R. argentea* is becoming less available for animal feed production; hence, there is need to search for alternative protein sources that are preferably less competed for.

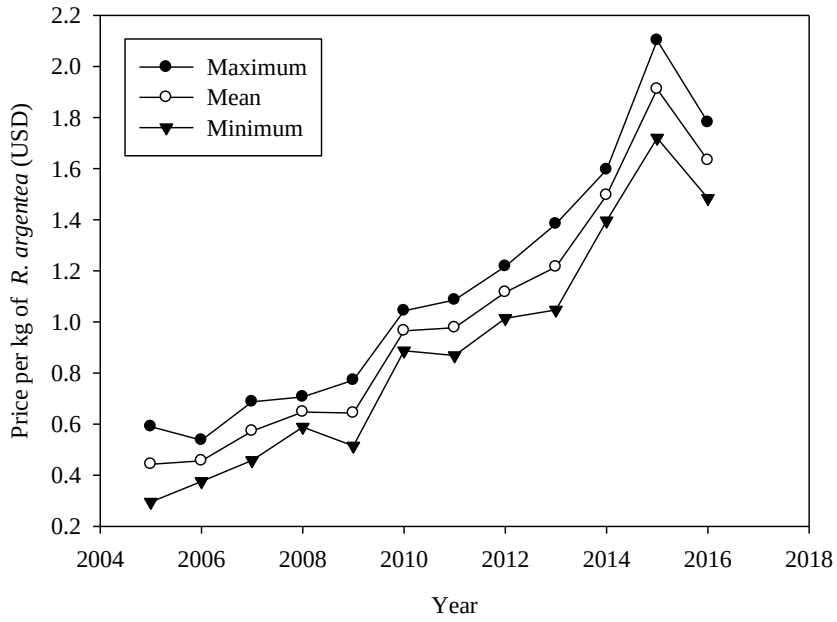

Figure 1.5: Price of dried Rastrineobola argentea on the Ugandan side of Lake Victoria between 2005 and 2016 (Data sources: DFR (2011), and primary data from landing sites)

1.4 Substituting *R. argentea* with other local protein sources

Feeds constitute more than 50% of the total production costs in intensive aquaculture with protein being the most expensive component (Cheng et al., 2010; Watanabe, 2002). Fishmeal is an ideal source of protein in feeds for most fish species (Naylor et al., 2000; NRC, 2011), but due to its limited availability and high price, feed producers are opting for other protein sources. To a large extent, fishmeal prices determine feed cost and the general profitability of fish farming. In E. Africa, *R. argentea* is the main fishmeal used, but the escalating prices (Fig. 1.5) are increasingly

prohibiting its continued use in aquafeeds. For example, the cost of fishmeal in a tonne of commercial aquafeeds containing 20% *R. argentea* was about USD 89 in 2005 and had increased to USD 382 in 2015 (pers. observation). This four (4) fold increase in the price of fishmeal within a decade subsequently led to a further increase in the price of aquafeeds. To ensure affordable aquafeed prices, emphasis needs to be put on finding locally available and/or less competed for protein sources. In addition, raw materials like *R. argentea* that are rich in unsaturated fatty acids (PUFAs) (Mwanja et al., 2010) can easily oxidize. Hence, substituting fishmeal with alternative ingredients serves to reduce aquafeed prices and dietary lipid oxidation.

Uganda has both animal and plant based protein resources (ingredients) that can replace *R. argentea* fishmeal in aquafeeds (Munguti et al., 2012; Nalwanga et al., 2009; Rutaisire, 2007). However, most of the alternative protein sources that have been tested and proved to be effective in replacing fishmeal in aquafeeds are also in demand for other uses. Soybean, one of the most important plant protein sources in aquafeeds, is increasingly in demand for terrestrial livestock feeds and direct human consumption (Cheng et al., 2010; Cheng and Hardy, 2002; Lee, 2014; Watanabe, 2002). Other raw material sources, such as cotton seed and sunflower cakes, are less utilized in commercial aquafeeds due to high indigestible fibre content, but remain important raw materials in backyard on-farm feed production (Dalsgaard et al., 2012; Lee, 2014). There is limited use of imported protein ingredients in Uganda for aquafeed due to the prohibitive costs involved (Lee, 2014; Nalwanga et al., 2009). To achieve profitable and sustainable aquaculture in Uganda, it is imperative to search for cheaper and less utilized protein sources.

A number of plant and animal raw materials that are in limited demand for farm animals or human consumption in Uganda could be used for aquafeeds (Munguti et al., 2012; Nalwanga et al., 2009). For example, different strains of mung beans (*Vigna radiata*) (i.e., the red, yellow and green strains) that contain over 25% crude protein are in little demand for human consumption locally and are not utilized in animal feeds despite being easy to grow in Uganda. Moreover *V. radiata* has been successfully used in diets for broiler chicken (Creswell 1981), Asian sea bass (Eusebio & Coloso 2000) and Nile tilapia (De Silva & Gunasekera 1989).

The freshwater shrimp (*Caridina nilotica*: common name: ochonga) could be a good alternative protein source in aquafeeds (Mugo-Bundi et al., 2015; Munguti et al., 2012). *C. nilotica* is a natural prey of Nile tilapia in

Lake Victoria and a by-catch in the *R. argentea* fishery. Since 1986, the biomass of *C. nilotica* in Lake Victoria has been increasing and was estimated to be over 20,000 metric tonnes by the year 2000 (Cowx *et al.* 2003; Getabu et al., 2003). The decline in Nile perch (*Lates niloticus*) stocks in Lake Victoria (Getabu et al., 2003; Taabu-Munyaho et al., 2013) has further reduced predatory pressure leading to an increase in *C. nilotica* biomass. Presently, *C. nilotica* constitutes about 10% of the catch in *R. argentea* fisheries on Lake Victoria (Kasinga Michael. pers. comm.). Recent estimates show that about 500,000 mt of *R. argeneta* are landed from Lake Victoria annually (LVFO 2014), suggesting that approximately 50,000 mt of *C. nilotica* could be utilized in aquafeeds as alternative protein sources to *R. argentea* annually. A recent study showed that *C. nilotica* can be used to partially replace *R. argentea* fishmeal in Nile tilapia feeds (Mugo-Bundi et al., 2015). *C. nilotica* contains up to 65% crude protein and the amino acid composition is comparable to that of *R. argentea*. When *C. nilotica* was used to replace 25% of the fishmeal, the growth of the tilapia was better than when *R. argentea* fishmeal was the only protein source (Mugo-Bundi et al., 2015). However, this study was carried out in tanks where the environment and food supply were controlled, more so than in cages and particularly in ponds where fish may derive a significant portion of their diet from other sources. Thus, the growth of fish farmed in ponds may be determined by complicated interactions between the natural food sources and the supplemented feed (Tacon and De Silva, 1997). It was, therefore, of interest to test *C. nilotica* on fish fed under semi-intensive conditions similar to those in commercial pond farms in Uganda where the oxygen levels, pH and carbon dioxide fluctuate diurnally. Paper three (III) examines the use of locally available and less competed for protein sources (*V. radiata* and *C. nilotica*) to replace *R. argentea* in practical feeds for Nile tilapia farmed in ponds.

1.5 Aim of the thesis

The overall aim of the study was to lay down more solid foundations for aquafeed production in Uganda and East Africa. Therefore, the studies decribed in this thesis were done with special reference to the fish husbandry conditions in Uganda and Africa in general.

1.5.1 Specific objectives of the study

a) Estimate the minimum detectable difference in growth studies based on the variance of the data and experimental design (Paper I).

b) Compare the fidelity of different statistical methods to the underlying population responses using simulation studies (Paper I).

c) Determine the effect of dietary lipid oxidation on the growth and antioxidant enzyme activities of juvenile Arctic charr (*Salvelinus alpinus*) and Nile tilapia (*Oreochromis niloticus*) (Paper II).

d) Determine the effect of replacing *Rastrineobola argentea* fishmeal with the freshwater shrimp (*Caridina niloticus*) and mung bean (*Vigna radiata*) on the growth of juvenile Nile tilapia (*Oreochromis niloticus*) (Paper III).

e) Compare the efficacy of natural antioxidants (rosemary oil: *Rosmarinus officinalis* and bladderwrack powder: *Fucus vesiculosus*) and the synthetic ethoxyquin in stabilizing lipid oxidation (Paper IV).

f) Compare the effects of natural and synthetic antioxidants on the growth and antioxidant enzyme activities of juvenile Arctic charr (Paper IV).

2 Effective design and analyses of fish growth studies

Fish nutritionists evaluate the suitability of raw materials for aquafeed production by formulating diets according to the nutritional requirements of the target species and by performing growth studies where prospective raw materials are tested. To draw realistic and/or reliable conclusions from such studies, it is imperative that the experiments are properly designed and analyzed. The fidelity of the results from growth studies depends on the nature and attention given to experimental design and analyses (Shearer, 2000; Thorarensen et al., 2015).

In growth studies, the null hypothesis of no effect of experimental treatments is tested and rejected when test statistics (*p*-value) indicate that the probability of the null hypothesis being true is less than 5% (α level less than 0.05). In other words, the probability of rejecting a correct null hypothesis (Type I error) is less than 5%. However, it is also possible that an incorrect hypothesis is not rejected and differences among means are not detected where they truly exist. Failing to reject an incorrect hypothesis is called Type II error. The probability of Type II error is β and the power of a statistical test is defined as 1-β. There is no conventional criterion for statistical power as there is for α, although a minimum of 80% is commonly regarded as suitable (Araujo and Frøyland, 2007). However, statistical power is rarely reported in aquaculture growth studies (Searcy-Bernal, 1994) indicating that researchers are less concerned with Type II error than they are with α and Type I error.

ANOVA is one of the common statistical tools used to analyse data from aquaculture growth studies. Because of the common "tank effect", individual fish within a tank are not independent samples from the population but are instead "pseudo-replicates" as defined by Hurlbert (1984). Hence, ANOVA is performed based on the total biomass or mean body-mass in each tank (Cowey, 1992; Smart et al., 1998) or, using a mixed model ANOVA treatments are considered fixed factors and tanks as nested random factors within treatments. With the latter method, the

information on individual fish is modelled to fully account for the data structure (Ling and Cotter, 2003; Ruohonen, 1998).

Dose-response designs, where treatments are applied at incremental levels of e.g. nutrient content or water quality, are common in aquaculture growth studies. These data can be analysed either with ANOVA or by using different linear and non-linear methods. The latter include broken line analyses, where two straight lines are fitted to the data, polynomial regression and non-linear regression models that fit asymptotic curves to the data (Baker, 1986; Cowey, 1992; Shearer, 2000). When the results are analysed with ANOVA, the critical response is usually determined as the lowest treatment level that gives a response that is not significantly different from the maximum response. However, this approach has been criticised by Shearer (2000) who suggested for example that the ANOVA underestimated the critical response.

The statistical power of mixed hierarchical models depends primarily on five factors: (1) The difference among means caused by the treatment (effect size), (2) the variance of the data, both within tanks and among tanks within the same treatment, (3) the number of replicate tanks, (4) the number of fish within each tank and (5) the number of treatments tested (Deng, 2005; Ling and Cotter, 2003; Nakagawa and Cuthill, 2007). The statistical power increases with increased effect size, number of replications, number of fish per replicate, and reduces with increased variance and number of treatments tested (Ling and Cotter, 2003). Hence acceptable statistical power can be secured by increasing the number of replicates and taking a large sample size. Unfortunately, the number of experimental units and the cost of resources for aquaculture growth studies are usually limiting. Therefore, experimental design must strike a balance between acceptable power and the available resources.

In this thesis, information was accumulated on the variance of data in aquaculture growth studies on different species. Based on this information, the minimum detectable difference at 80% statistical power was estimated. This information was further used to estimate the sensitivity of different experimental designs (with specified replication and sample size). Simulation studies were further generated and used to compare the fidelity of different methods of statistical analysis to the true underlying responses of populations. Ultimately, the optimal experimental design model and analyses in Paper I were adopted and used in the growth studies carried out as part of this thesis.

3 Lipid oxidation and antioxidant defence

3.1 Mechanism of lipid oxidation in oil laden raw materials and aquafeeds

Autoxidative deterioration of lipids/oils is a free radical reaction with initiation, propagation and termination stages (Frankel, 1984; Sargent et al., 2002; Table 3.1). The initiation stage involves formation of free radicals while the propagation stage is characterized by free-radical chain reactions. At the termination stage, non-radical products are formed that are harmless to biological cells (Antolovich et al., 2002; Frankel, 1998; Sargent et al., 2002). The different stages of lipid oxidation and their mechanisms are summarised in Table 3.1

Table 3.1: Lipid autoxidation chain reaction stages and mechanisms

Stage	Chemical reaction	Description
Initiation	$LH + O_2 \rightarrow L\cdot + \cdot OH$ $L\cdot + O_2 \rightarrow LOO\cdot$	L·is a free radical formed by removal of labile hydrogen from a carbon atom adjacent to a double bond Highly reactive ·OH initiates autoxidation reaction Free radical combines with molecular oxygen to form peroxide radical (LOO·)
Propagation	$LOO + LH \rightarrow L + LOOH$ $LOOH \rightarrow LO\cdot + \cdot OH$	LOOH is a lipid hydro peroxide, a product that decomposes to form compounds responsible for off-flavours and odours.
Termination	$L + L \rightarrow LL$ $L + LOO \rightarrow LOOL$ $LOO + LOO \rightarrow LOOL + O_2$	The alkyl and peroxide radicals combine to form stable molecular compounds and end the reaction. i.e., LL and LOOL are stable non-radical products.

LH=unsaturated fatty acid, L·=alkyl radical, LO·= alkoxyl radical, LOO·=lipid peroxyl radical, LOOH=lipid hydro peroxide.

Free radicals include oxygen derivative compounds and other molecules containing one or more unpaired electrons. The most common reactive oxygen species (ROS) responsible for peroxidation include the superoxide ($O_2^{\cdot}$) anion, hydrogen peroxide (H_2O_2), peroxyl (LOO·) and the highly reactive hydroxyl (·OH) radicals. Peroxidation is initiated when highly reactive radicals such as ·OH attack biological molecules and substitute hydrogen, leaving behind unpaired electrons on the atom to which the hydrogen bond was attached (Table 3.1). The ·OH is mainly produced when either transition metal ions react with H_2O_2 or when homolytic fission occurs. Once the ·OH attacks a biological molecule, a self propelling free radical chain reaction starts. Metal ions such as zinc,

copper and iron, lipoxygenase enzymes, light and heme-proteins catalyse lipid oxidation reactions (Antolovich et al., 2002; Frankel, 1984, 1998).

As autoxidation proceeds, different products are generated at primary, secondary and tertiary levels (Table 3.2). Primary autoxidation yields hydrogen peroxides (H_2O_2) that are toxic and very unstable. They decompose rapidly into secondary monomeric and nonperoxidic products, polymers and volatile products such as ketones, aldehydes and alcohols (Antolovich et al., 2002; Frankel, 1998; Table 3.2).

Table 3.2: Lipid autoxidation process, quality attributes affected and techniques used to detect the products formed

Autoxidation level	Product (s)	Negatively affected quality attributes	Detection techniques
Primary	Peroxides	Nutritional value	• Peroxide value (POV) • Chemiluminescence
Secondary	Ketones Aldehydes Alcohols	Odour and taste	• GC-MS • TBARS • Sensory • Anisidine
Tertiary	peroxyacids	Colour and texture	• Fluorescence • Visual analysis • Calorimetry

Note: GC-MS=Gas chromatography with mass spectroscopy detection, TBARS = thiobarbituric acid-reactive substances test.

Secondary volatile products are associated with undesirable flavours, reduced feed palatability and acceptance (Aidos et al., 2001, 2002; Sargent et al., 2002). Secondary lipid oxidation reactions lead to product browning, loss of vitamins and pigments, reduction in protein quality (due to complex formation) and loss of essential fatty acids (Aidos, 2002; NRC, 2011). The products from hydrogen peroxides are decomposed further, into tertiary products and these in turn affect product colour and energy content.

3.2 Natural antioxidant defence mechanisms in fish

Reactive oxygen species (ROS) are produced in many aerobic cellular metabolic processes. If not controlled they will react with various intracellular targets, such as lipids, proteins, DNA and structural proteins/lipids within the cell membrane leading to cell death (Gary, 1991; Weydert and Cullen, 2010). Complex enzymatic and non-enzymatic antioxidant defence systems exist in organisms to curb the effects of ROS such as the free oxygen radical/superoxide ion ($O_2^{\cdot}$) and hydrogen peroxide (H_2O_2) (Frankel, 1998; Weydert and Cullen, 2010).

Anti-oxidative defence mechanisms in fish consist of a number of different pathways (Antolovich et al., 2002; NRC, 2011; Sargent et al., 2002). For example, carotenoids and tocopherols directly react with free radicals while amino acids, ascorbic acid, peptides and tocopherol enhance antioxidant activities. Amines, catalases and peroxidases reduce the concentration of hydroperoxides and retard oxidation. The activity of catalytic metals that facilitate lipid oxidation is inhibited by amino acids, ascorbic acid and peptides. Singlet oxygen radicals are usually quenched and inactivated by the action of ascorbic acid, carotenoids and superoxide dismutase (Antolovich et a., 2002; DeVore et al., 1983).

A number of enzymes are involved in anti-oxidative defence mechanisms such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). SOD, CAT and the substrate specific GPx are the most important primary antioxidant enzymes in all oxygen metabolizing cells (Gary, 1991; Trenzado et al., 2006; Weydert and Cullen, 2010). The metalloezyme superoxide dismutase converts the toxic superoxide ion (O_2^{-}) into harmless oxygen (O_2) and toxic hydrogen peroxide (Sargent et al., 2002; Weydert and Cullen, 2010). The hydrogen peroxide generated may not directly affect most biologically important molecules. However, when it crosses the cell membrane, it oxidizes specific molecules, e.g., protein thiol groups to form products that can affect the cells (Sargent et al., 2002). Hydrogen peroxide may also act as a precursor for more reactive oxidants such as ·OH (DeVore et al., 1983).

In cells, the metal-containing enzyme catalase converts toxic hydrogen peroxide to harmless water and oxygen through a redox reaction. Conversion of hydrogen peroxide to neutral water and oxygen molecules reduces the damage lipid oxidation would exert on cells. However, the

content, activity and efficacy of antioxidant enzymes (SOD and CAT) are associated with the phylogenetic position of fish, being lower in elasmobranchs than in teleosts (DeVore et al., 1983; Martínez-Álvarez et al., 2005). Like CAT, GPx catalyses the reduction of hydrogen in the lipid peroxides (LOOH) to water (H_2O) by reduced glutathione (GSH) (Weydert and Cullen, 2010).

$$2GSH + H_2O_2 \rightarrow 2H_2O + GSSG$$

GSSG is then reduced back to GSH by glutathione reductase at the expense of NADPH:

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

GPx differs from catalase in that it can react with both lipid and hydrogen peroxide. For GPx to function effectively in vertebrates, adequate amounts of selenium must be available and farmed animals mostly obtain selenium from their diets (Decker, 1998). The activity of GPx in chicken muscle was found to increase with dietary selenium content (DeVore et al., 1983). The net effect of the enzyme based natural antioxidant defence of fish, due to SOD, CAT and GPx, is that the two potentially harmful species, superoxide ion and hydrogen peroxide, are converted to water which is harmless.

The interactions among SOD, CAT, and GPx naturally control the impact of the oxidants generated in all aerobic organisms, including fish. Depending on demand, these enzymes are naturally up- or down-regulated. However, the concentration of SOD, CAT and GPx, and hence their effectiveness differ among fish species and can be influenced by age/size within a given species (Fontagné-Dicharry et al., 2014). As a result, the effects of dietary lipid oxidation on fish are diverse, complex and inconsistent among fish species. Moreover the environmental conditions in which studies are conducted also influence the responses fish display towards oxidized diets (Martínez-Álvarez et al., 2005). In Paper II, dietary lipid oxidation tolerance of two aquaculture species, Nile tilapia and Arctic charr, originating from different temperature conditions and having different lipid requirements are examined.

3.3 External antioxidants

Organisms experience oxidative stress when the concentration of oxidants generated within their bodies and those from external sources (e.g., diets) surpass the capacity of the natural defence systems. To support the natural antioxidant defence system, antioxidants are added to raw materials and/or diets to minimize autoxidation of PUFAs and reduce production of ROS that would cause cell damage. Antioxidants are natural or synthetic substances used in low concentrations to either delay or inhibit oxidation (Antolovich et al., 2002). Antioxidants minimize lipid oxidation by either delaying or completely inhibiting the initiation step (Table 1.1) or by reacting with the ROS (Antolovich et al., 2002; Sasse et al., 2009). Hence, varied protection is provided by antioxidants depending on the rate at which they react with the reactive radical and how completely they get used up in the process (Sasse et al., 2009). Some antioxidants work to prevent formation of ROS (Davies, 1995), while others act as reducing agents by scavenging ROS before they can damage cells (Frankel, 1984). Non-enzymatic antioxidants terminate chain reactions by removing free radical intermediates and inhibiting other oxidation reactions (Frankel, 1998). Such antioxidants control the effect of peroxide on cells by themselves being oxidized. These include thiols, polyphenol and ascorbic acid, and are commonly referred to as reducing agents (DeVore et al., 1983).

Ethoxyquin (EQ), butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA) are all synthetic antioxidants used to treat fishmeal and fish oil (Bohne et al., 2008; Hamre et al., 2010; Sasse et al., 2009). However, concerns have been raised about the health of humans consuming fish raised on diets treated with synthetic antioxidants (Bohne et al., 2008; Hamre et al., 2010). The synthetic antioxidants, some with known carcinogenic effects, accumulate in fish muscles and end up being consumed by humans (Bohne et al., 2008). The aromatic amine EQ (1, 2-dihydro-6-ethoxy-2, 2, 4-trimethyl quinoline) is the most common synthetic antioxidant used to prevent lipid oxidation in aquafeeds (Hamre et al., 2010). However, there are fears that EQ could be carcinogenic (Blaszczyk and Skolimowski, 2005a). Fish welfare activists are also concerned about the health effects synthetic antioxidants may have on farmed fish (Antolovich et al., 2002; Hamre et al., 2010; Sasse et al., 2009). Accordingly, there is a growing interest to find natural antioxidants that are safe to both farmed fish and humans (Błaszczyk and Skolimowski, 2005a; Wang et al., 2009, 2015).

In East Africa, antioxidants are seldom used in aquafeeds because most of the locally used raw materials are deficient in PUFAs. Even the *R. argentea* fishmeal, whose fatty acids are 53% PUFAs (Mwanja et al., 2010), is not treated with antioxidants during processing and storage. Moreover, the aquafeeds meant for Nile tilapia and African catfish generally contain low levels of lipids ranging from 6% to 12% (Ng and Romano, 2013; NRC, 2011) and there is very little or no oil added to the feeds. Hence the naturally occurring antioxidants within the raw materials are deemed enough.

Oil extracted rosemary (*Rosmarinus officinalis*) (RM) and powdered bladderwrack (a seaweed, *Fucus vesiculosus*) (BW), are both natural antioxidants with a high potential to stabilize lipid oxidation and replace the synthetic EQ in aquafeeds. BW has recently been studied and found to have high phenolic activity (Wang et al., 2009) but its efficacy at controlling lipid oxidation has not been tested. RM has been found to effectively control lipid spoilage in pork and sausages (Georgantelis et al., 2007), but has not been tested in aquafeeds. Moreover the effects of the two natural antioxidants (RM & BW) on the growth and antioxidant defence of fish have not been studied. Thus, Paper IV compares the relative efficiency of RM and BW to EQ in stabilizing lipid oxidation and their subsequent effects on the growth and antioxidant enzyme activities in Arctic charr.

3.4 The effect of dietary lipid oxidation on farmed fish

Several studies have demonstrated the deleterious effects of dietary lipid oxidation on fish; including reduced feed intake, stunted growth and low survival (Bohne et al., 2008; Fontagné et al., 2006, 2008; Hamre et al., 2010; S argent et al., 1999; Tocher et al., 2003). Species specific responses to dietary lipid oxidation have been cited, such that the natural antioxidant defence system and immunity may be suppressed in some fish species (Alves Martins et al., 2007; Martínez-Álvarez et al., 2005) and unaffected in others (Gao et al., 2012). Different responses have also been found between age groups of the same fish species (Fontagné-Dicharry et al., 2014; Martínez-Álvarez et al., 2005). Environmental factors such as diet composition, daily or seasonal changes in temperature and dissolved oxygen as well as the toxins/pathogens present in the water can influence the response of fish to dietary oxidation (Martínez-Álvarez et al., 2005; Trenzado et al., 2006).

Reponses to dietary lipid oxidation have been documented for several aquaculture species. However, little is known about the effect of dietary lipid oxidation on tilapia, and no studies have tested the effect of lipid oxidation on Arctic charr. Growth, feed conversion ratio and protein efficiency ratio were not affected in juvenile hybrid tilapia (*O. niloticus* x *O. aureus*) fed oxidized oils supplemented with more than 80 IU of vitamin E/kg (Huand and Huang, 2004). Vitamin E is an antioxidant that stabilizes unsaturated fatty acids and prevents lipid oxidation. Hence, the effect of lipid oxidation on fish is suppressed when vitamin E is included in tilapia diet. In this thesis, the effects of dietary lipid oxidation on Nile tilapia and Arctic charr were examined (Paper II).

4 Overview of the methods and main results in the different papers

A summary of the methods (Fig. 4.1) and main results in the four papers (I to IV) contained in this thesis is given in this chapter. Detailed descriptions of the materials and methods, and the results can be found in the original papers.

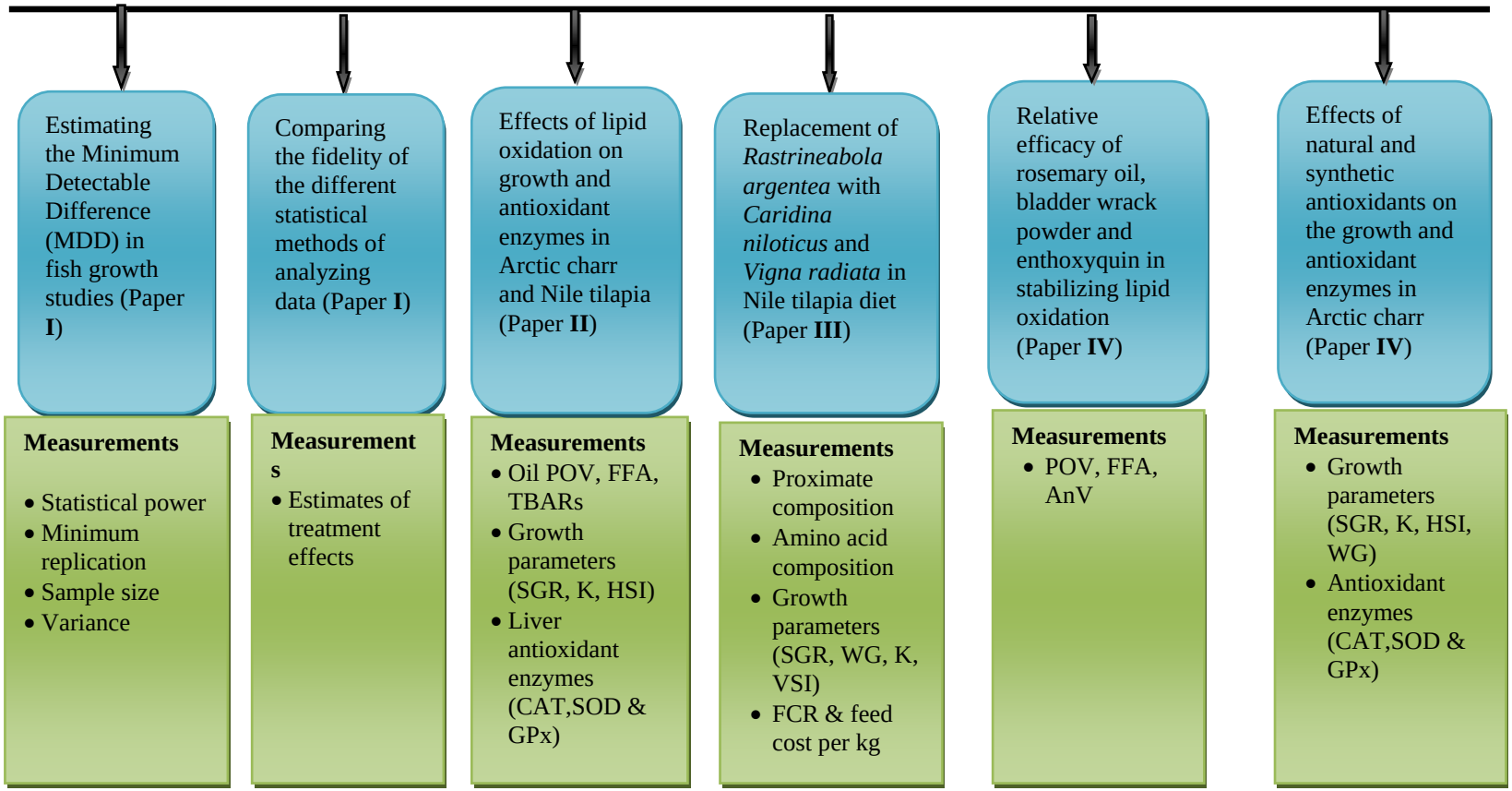

Figure 4.1: An overview of the studies and methods used in this thesis (Roman numerals in bold refer to original papers from the studies).

4.1 Experimental design and statistical analyses of fish growth studies

This chapter is based on Paper I: Thorarensen, H., Kubiriza, G.K., Imsland, A.K. 2015. Experimental design and statistical analyses of fish growth studies. Aquaculture 448, 483–490. doi:10.1016/j.aquaculture.2015.05.018.

Numerous studies comparing the effects of different factors on the growth of farmed fish are published every year. However, comparatively little attention has been given to the experimental design of these studies with regard to in how many rearing units should each treatment be replicated (b), how many fish should be in each tank (*n*) and how the data should be analysed. A survey of recently published aquaculture growth studies suggests that, most commonly, treatments are applied in triplicates (b =3) with an average of 26 fish per tank (range: 4 to 100). Rarely do studies assess statistical power and the probability of detecting a true difference where it exists. In the present study, information was accumulated on the variance of data in aquaculture growth studies on different species to estimate the minimum detectable difference (MDD) and to assist researchers in designing experiments effectively.

Analysis of the growth studies suggests that in most cases, variance increased as the experiments progressed but tended to stabilize when the factorial increase in body mass (i.e. mean body-mass/mean initial body-mass) was about 1.5. However, this pattern was not consistent since in some studies the variance decreased while in others it increased as the experiments progressed (Fig. 4.2).

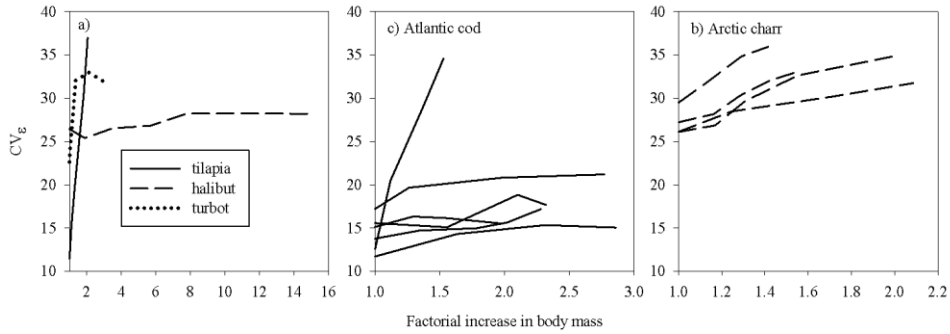

Figure 4.2: Development of CV_ε with increasing body mass in experiments on (a) tilapia, Atlantic halibut and turbot, (b) Atlantic cod and (c) Arctic charr. (The different lines represent separate studies). The increase in body mass is shown as factorial increase (mean body-mass/mean initial body-mass).

Overall, these results suggest that the variance is similar for different aquaculture species. Therefore the same experimental design (level of replication and *n*) is suitable for studies on different species of fish. Mean CV_ε (mean ± SD) was 30.6 ± 4.5% (range: 15% to 56%) while CV_β (Mean ± SD) was 4.5 ± 0.4% (range: 0% to 12%). The minimum detectable difference (MDD) in mean body-mass with power of 80% of different treatment groups in a typical aquaculture study (triplicates, 25 fish in each tank and average variance) with 80% statistical power is around 26% of the grand mean (Fig. 4.3 a).

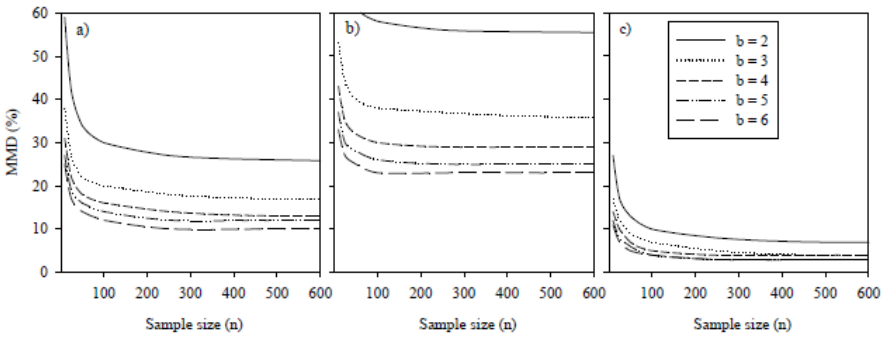

Figure 4.3: Minimum detectable difference (MDD), shown as % of the grand mean in growth studies with five treatments levels when statistical power is 80%. a) Mean CV_ε and mean CV_β. b) High CVε and high CV_β. c) Low CV_ε and low CV_β.

Increasing *n* from 25 to 100 will reduce the MDD to 19% of the grand mean, while a further increase in *n* will have comparatively lesser effect.

Increasing replication to quadruplicates or sextuplicates (with *n* as 100), will further reduce the MDD to 16% and 12% of the grand mean respectively (Fig. 4.3). MDD under 10% of the grand mean with 80% statistical power is only possible when fish for the experiment are selected within a narrow size range to reduce variance.

Simulations were performed where samples (experiments) were repeatedly drawn from artificial populations with identical distribution and with the same experimental design as is commonly used in growth studies. Two of the populations had dose-dependent responses to treatment while one population showed no response to treatment. The resulting data was analysed with a mixed model ANOVA and by fitting either polynomials or asymptotic models to the data. Contrary to earlier suggestions (Shearer, 2000), the critical treatment (minimum treatment to generate maximum response) estimated using ANOVA approached more closely the population responses than did the critical treatments estimated with non-linear models.

Author Contributions

Conceived and designed the study: HT, GKK. Analyzed the raw data and drafted the paper: GKK. Wrote the paper: GKK, HT, IAK. Proof read and fine tuned the paper: IAK.

4.2 The effect of dietary lipid oxidation on farmed Nile tilapia and Arctic charr

This chapter is based on Paper II: Kubiriza, G.K., Árnason, J., Sigurgeirsson, Ó., Hamaguchi, P., Snorrason, S., Tómasson, T., Thorarensen, H. 2017. Dietary lipid oxidation tolerance of juvenile Arctic charr (Salvelinus alpinus) and Nile tilapia (Oreochromis niloticus). Aquaculture 467, 102–108. doi:10.1016/j.aquaculture.2016.04.006.

The effects of dietary lipid oxidation on fish appear to be diverse and contrasting. For example, growth, survival, feed intake and liver antioxidant enzyme activities were reduced in juvenile channel catfish (*Ictalurus punctatus*) (Dong et al., 2012) and rainbow trout (*Oncorhynchus mykiss*) fed oils with POV of 10 ± 1 to 144 ± 15 mEqkg^{-1} (Fontagné-Dicharry et al., 2014), but were un-affected in Atlantic cod (*Gadus morhua*) fed different doses of oils with POV 94 mEqkg^{-1} (Zhong et al., 2008) and earlier in rainbow trout fed oils with POV between 2 to 47 mEqkg^{-1} (Cowey et al., 1984). While these responses could be species specific, these studies show that the level of dietary lipid oxidation partly influences the responses of fish. Different responses have also been found between age groups of the same fish species (Fontagné-Dicharry et al., 2014; Martínez-Álvarez et al., 2005). This study investigated the effects of dietary lipid oxidation on the growth of juvenile Arctic charr and Nile tilapia, as well as antioxidant enzyme activity only in Arctic charr.

Fresh and oxidized herring oils were used in the formulation of experimental diets. In the experiment on Arctic charr, a control diet (OX0) formulated with un-oxidized oil (POV = 20.44 mEqkg^{-1} and TBAR 26.60 μmolMDAkg^{-1}) was compared with diets OX1, OX2, OX3 and OX4 that were formulated using oils at four progressively higher oxidation levels. The POV values for oils used in diets OX1 to OX4 were 182.97, 56.12, 33.27, 0.00 mEq kg^{-1} respectively while the TBARs levels were 65.4, 76.60, 131.3, 146.5 μmolMDA kg^{-1}. Juvenile Arctic charr of mean body mass 2.41 ± 0.11 g (mean ± SD) were reared on the five diets for 67 days, at Verid, the experimental station of Hólar University College, Iceland. In a second experiment, juvenile Nile tilapia of average body mass 1.71 ± 0.40 g (mean ± SD) were reared on three diets formulated with fresh oil (OL0) (POV: 19.55 mEqkg^{-1} and TBARs: 46.89 μmolMDAkg^{-1}) and two oxidized herring oils OL1 and OL2 with POV of 447.27 and 32.72

$mEqkg^{-1}$ respectively and TBARs values of 60.91 and 127.51 $\mu molMDAkg^{-1}$, for 90 days. Growth parameters were studied in both species, while antioxidant defence enzyme activities were only studied in Arctic charr (see details in Paper II).

Arctic charr reared on a diet with oil in the primary oxidation state (i.e., with high POV vs. low TBARs) had significantly lower body mass and SGR, but higher hepatosomatic index than the rest of the groups. The activities of catalase and glutathione peroxidase increased with dietary lipid oxidation while that of super oxide dismutase remained fairly stable. In Nile tilapia, both final body mass and condition factor were significantly lower in the group fed on un-oxidized oil than in the groups fed oxidized oil.

The results suggest that Arctic charr are more susceptible to lipid oxidation than are Nile tilapia. These differences may be the result of species differences in tolerance to oxidized lipids, variable intake of oxidized lipids or access to vitamin E/tocopherol in feed.

Author Contributions

Conceived and designed the experiments: GKK, HT, JÁ, ÓS. Conducted experiments GKK. Analyzed the data: GKK, HT. Contributed reagents/materials/analysis tools: PH. Wrote the paper: GKK, HT, ÓS, TT, JÁ, SS.

4.3 Substituting *R. argentea* from Nile tilapia diets with other locally available protein sources

This chapter is based on Paper III: Kubiriza G.K., Akol A.M., Arnason J., Sigurgeirsson Ó., Snorrason S., Tómasson T., Thorarensen H. (2017). Practical feeds for juvenile Nile tilapia (Oreochromis niloticus) prepared by replacing Rastrineobola argentea fishmeal with freshwater shrimp (Caridina nilotica) and mung bean (Vigna radiata) meals. Aquaculture Nutrition, 00:1–8. doi:10.1111/anu.12537.

In Uganda, the freshwater shrimp, *Caridina nilotica* and mung beans, *Vigna radiata* are less utilized protein resources that could be used to replace *R. argentea* in aquafeeds. *C. nilotica* is nutrient rich with a crude protein content of over 60% and amino acid composition close to that of *R. argentea*. A significant volume of *C. nilotica*, landed as a by-catch in the mukene fishery, is discarded on the Ugandan side of Lake Victoria but could be used in aquafeeds. Legumes of the genus *Vigna* have also been tested on Nile tilapia and yielded positive results (Keembiyehetty and De Silva, 1993; Olivera-Castillo et al., 2011). In Uganda, *V. Radiata* legumes are seldom consumed by humans and even though they are easy to propagate. In this paper the quality of *C. nilotica* and *V. radiata* relative to *R. argentea* and the effect of their partial and/or complete utilization in practical diets for juvenile Nile tilapia (*Oreochromis niloticus*) were assessed. The cost of feed needed to produce a kilogram of fish was also estimated.

Dietary ingredients and formulated feeds were analyzed for proximate composition using standard methods (AOAC, 1995). Amino acids content of *R. argentea*, *C. nilotica* and *V. radiata* were also analyzed. A commercial diet and five other diets formulated with varying proportions of *R. argentea* fishmeal, *C. nilotica*, *V. radiata* and full fat soybean meal (SBM) were tested on triplicate groups of 100 Nile tilapia (initial body-mass±sd: 3.30 ± 0.27g), for 90 days. Growth parameters including weight gain, SGR, condition factor and survival were studied.

The protein content of *V. radiata* (238 g/kg) was only about a third of the protein content of *C. nilotica* (650 g/kg) and about half of that of *R. argentea* (531 g/kg). As a result, the essential amino acids (EAA) content of *V. radiata* (g/kg) was lower than in both *C. nilotica* and *R. argentea*,

although the proportional composition of EAA in all raw materials was comparable. The calculated EAA content of the feeds prepared was similar, although compared with the NRC (2011) recommendations; all diets were deficient in methionine. Diets with a combination of *C. nilotica* and *V. radiata* or *C. nilotica* only were also deficient in histidine, threonine and valine. The commercial diet was deficient in histidine and valine, while the diet made of *R. argentea* and *C. nilotica* as primary protein sources was deficient in valine.

The growth of Nile tilapia was highest and comparable amongst groups fed high proportions of either *R. argentea* or *C. nilotica* or a combination of both *R. argentea* and *C. nilotica* as the main protein supplements in addition to soybean. The growth performance of fish fed a commercial diet was intermediate while fish fed diets formulated with *C. nilotica* in combination with *V. radiata* as the primary protein source showed the poorest growth. The apparent FCR was significantly higher in groups fed on a commercial formulation and *R. argentea* with *V. radiata* than in all other groups. The SGR was not significantly different among groups. Average survival rate was above 87% and not significantly different among groups.

The cost of a kg of feed produced by a formulation with high inclusion of *C. nilotica* was lower than that of feeds made with other formulations; being 40% less expensive than feed formulated with high *R. argentea* content which was the most expensive. The cost of feed per kg fish produced was also lowest for the formulation with high inclusion of *C. nilotica*. Similarly, the cost of producing a kg of fish with diet having high *C. nilotica* was 63% less than for commercial diet which was the least economical feed.

Author Contributions

Conceived and designed the experiments: GKK, HT, JÁ, ÓS. Conducted experiments GKK. Analyzed the data: GKK, HT. Wrote the paper: GKK, HT, ÓS, TT, JÁ, SS.

4.4 Efficacy of natural antioxidants and their effects on fish

This chapter is based on paper IV: Kubiriza, G.K., Árnason, J., Sigurgeirsson, Ó., Hamaguchi, P., Snorrason, S., Tómasson, T., Thorarensen, H. Efficacy of natural antioxidants in stabilizing lipid oxidation and their effects on growth and antioxidant enzymes in Arctic charr. (Manuscript).

Several questions have been raised about the health effects of synthetic antioxidants, such as ethoxyquin (EQ), used to stabilize lipids in aquafeeds. It is claimed that antioxidants can affect the welfare and growth of fish and that they accumulate in fish muscles and end up being consumed by humans. Some of the synthetic antioxidants are known to have carcinogenic effects while the natural ones are considered to be safe (Bohne et al., 2008; Hamre et al., 2010).

In Paper IV the efficacy of the natural antioxidants rosemary (*Rosmarinus officinalis*) oil (RM) and powdered bladderwrack (*Fucus vesiculosus*) (BW) were compared to that of the synthetic EQ in stabilizing lipid oxidation of herring oil. The effects of the natural antioxidants on the growth and antioxidant enzyme defence in Arctic charr were studied. The study was done in two experiments. First herring oils were treated with EQ, RM or BW at four different but comparable concentrations. To establish the state of lipid oxidation after two months in storage under room temperature, free fatty acids (FFA), peroxide value (POV), anisidine value (AnV) and fatty acid content were determined using standard methods (AOAC, 1995). Second, juvenile Arctic charr (mean body mass ± SD; 2.41 ± 0.11g) were reared in quadruplicate groups of 60 fish per replicate on 10 diets for 67 days. These diets included the antioxidants (EQ, RM or BW), each tested at three different concentrations and a control diet formulated using herring oil without any antioxidant. Growth parameters and antioxidant defence enzyme activities were studied in Arctic charr, using methods described in Paper II.

All antioxidants were able to reduce lipid oxidation to some extent, and their efficacy increased with application levels. However, EQ was more efficient at stabilizing lipid oxidation, than were RM and BW, although at elevated application levels the stabilizing activity of RM approximated that of EQ. Growth and antioxidant enzyme activities were higher in

Arctic charr fed RM diets compared to those fed EQ or BW diets, suggesting that RM either stimulates appetite and feed intake or contains growth promoters. Accordingly, RM could be used in relatively high concentrations to replace the synthetic EQ whose effects can potentially spread to humans through bio-accumulation.

Author Contributions

Conceived and designed the experiments: GKK, HT, JÁ, ÓS. Conducted experiments GKK. Analyzed the data: GKK, HT. Contributed reagents/materials/analysis tools: PH. Wrote the paper: GKK, HT, ÓS, TT, JÁ, SS.

5 Discussion, synthesis and conclusions

The work in this thesis has provided important information for future aquaculture development in Uganda and East-Africa. In fact, some aspects of the research are applicable to aquaculture in general regardless of the region, production methods/facilities and/or fish species. The work spans a broad spectrum, from experimental design (Paper I) to feed development from local raw materials (Paper III), to the effects of dietary lipid oxidation on farmed fish (Paper II) and potential methods to mitigate it (Paper IV). The results of different studies are discussed below and finally drawn together to derive their significance for African aquaculture.

5.1 Minimum detectable difference in aquaculture growth studies and fidelity of statistical methods used in data analysis

Inadequate experimental design and poor statistical analyses of fish growth studies may, in some cases, mask significant treatment effects and result in wrong conclusions being drawn. The results presented in Paper I provide important information on experimental design and statistical analyses of growth studies. This is the first study to evaluate the variance, statistical power and minimum detectable difference (MDD) in aquaculture growth studies on different species of fish. Mean CV_{ε} (± SD) was 30.6 ± 4.5% (range: 15% to 56%) while CV_{β} (mean ± SD) was 4.5 ± 0.4% (range: 0% to 12%). Earlier, Ling and Cotter (2003) evaluated the variance in growth studies of triploid Atlantic salmon, and reported CV_{ε} in the range of 14% to 41% (mean ± SD: 28 ± 8.6) and CV_{β} ranging from 1% to 7% (mean ± SD: 3.2 ± 1.9%). Thus, the results of the present study and those of Ling and Cotter (2003) are comparable and suggest that the variance in aquaculture growth studies on different species is similar. Therefore, a similar experimental design (replication level and number of

fish in each unit) appears to be appropriate for growth studies on different fish species.

As expected, the result of the present study show that statistical power in growth studies increases with increased replication and sample size (n). Therefore, the ideal design will include as many fish and replications of treatments as possible. However, since in reality the resources (i.e., the number and size of rearing units and the number of fish) are limited, experiments must be designed to strike a balance between the limited resources and the reliability of findings.

In this regard, the results of Paper I, suggest that little is gained in statistical power or MDD when n is increased above 50-100 (Fig. 4.3). For triplicate treatments, the MDD is reduced from about 30% to 19% of the grand mean when *n* is increased from 25 to 100, but a further increase in *n* will have comparatively lesser effect (Fig. 4.3a). For average $CV\varepsilon$ and $CV\beta$, designs in triplicate are required to achieve a MDD of 20% or less (Fig. 4.3a). Increasing replication to quadruplicates or sextuplicates (with *n* as 100), will further reduce the MDD to 16% and 12% of the grand mean respectively (Fig. 4.3). A MDD under 10% is only possible when both $CV\varepsilon$ and $CV\beta$ are low (Fig. 4.3c); reaching 4% to 10% when n is 100. These results suggest that MDD under 10% of the grand mean with 80% statistical power is only possible when fish for the experiment are selected within a narrow size range to reduce variance.

Informal survey of published growth studies (Paper I) suggests that the most common experimental design is to use triplicates and n of 26 (range: 4-100). The MDD with 80% statistical power for this design is around 30%, suggesting that such studies have a high probability of committing Type II error. When experiments are set with fish of differing sizes, the variance is high and therefore higher replication and/or sample size would be needed to increase experimental statistical power and hence, the fidelity of the results. However, when fish within a narrow range is used, variance is usually low and therefore lower replication and relatively small sample size would be adequate. Subsequently, the experimental running costs would also be reduced. The results of the present study provide guidelines for experimental design in terms of replication level and *n* (Paper I) that can optimise resources for experiments.

It has been suggested that ANOVA may lack fidelity and that it can under-estimate the critical treatment effect in dose dependent growth studies for example when different protein levels in diets are compared. When

multiple levels of an independent variable are used, ANOVA treats them as discrete rather than continuous. Thence, the optimum dose is stated as a range between two input levels. Therefore, it has been suggested that, instead of ANOVA, either polynomial or logistic regression techniques may be preferable when responses are dose dependent (Shearer, 2000). The results of the present study however show that this is not necessarily the case. The simulation studies used compared the results of statistical analyses from ANOVA with those of polynomial and non-linear models. The estimate of critical treatment with ANOVA closely matched the critical value of the populations, suggesting that ANOVA does not necessarily underestimate the critical treatment effect. Indeed, the critical treatment (minimum treatment to generate maximum response) estimated with the ANOVA approached more closely the population responses than did the critical treatments estimated with the non-linear models, and this is contrary to earlier suggestions (Shearer, 2000). Besides, ANOVA does not presuppose the shape of the relationship between treatment and effect as the linear and nonlinear methods do.

5.2 Effect of dietary lipid oxidation on juvenile Arctic charr (*Salvelinus alpinus*) and Nile tilapia (*Oreochromis niloticus*)

The effects of dietary lipid oxidation on farmed fish are diverse and in some cases contradictory. The results in Paper II show that dietary lipid oxidation may affect Arctic charr and Nile tilapia differently. The growth of Nile tilapia in ponds was not affected by the levels of lipid oxidation tested. In contrast, the growth of Arctic charr reared in tanks was reduced by primary lipid oxidation. The different responses of Nile tilapia and Arctic charr may reflect species differences in sensitivity to lipid oxidation. However, the contrasting responses could also be due to other factors such as differences in the amount of oxidized lipid consumed (intake), dietary oxidation levels and/or access to antioxidants in diets. Indeed the lipid content per kg of tilapia diets was approx. 70 g while that in Arctic charr diets was 200 g/kg, meaning that Arctic charr ingested much more oxidized oils than did Nile tilapia. In addition, the levels of primary and secondary lipid oxidation in experimental diets for Artcic charr (TBARs range: 26.60 to 146.5 μmolMDAkg^{-1}) and tilapia (TBARs range: 46.89 to 127.51 μmolMDAkg^{-1}) differed slightly. Furthermore,

growth of Artic charr was reduced by primary lipid oxidation (i.e., high POV vs. low TBARs) only, but not by secondary lipid oxidation (i.e., low POV vs. high TBARs).

The food items in stomachs of the Nile tilapia used in these experiments constituted over 60% phytoplankton. Phytoplankton are rich in natural antioxidants (e.g., vitamin E/tocopherol and carotenoids) and they might have offset the effects of lipid oxidation. External antioxidants obtained from phytoplankton support the natural antioxidant defence system of fish and offset the effects of lipid oxidation (NRC, 2011; Tacon and De Silva, 1997). However, under intensive farming conditions, Nile tilapia feed entirely on prepared feeds, with minimal or no access to phytoplankton. Therefore feeding intensively farmed tilapia on oxidized diets may affect their growth and survival. These findings agree with those of Martínez-Álvarez et al. (2005) who suggested that live feeds eaten by Nile tilapia juveniles ameliorate the adverse effects of lipid oxidation. Martínez-Álvarez et al. (2005) further suggested that water quality can influence the responses of fish to dietary lipid oxidation. For example, fish raised in water with low dissolved oxygen (DO) and temperature are more stressed and more suceptible to the effects of lipid oxidation than those raised at higher DO and temperature.

The activity of antioxidant defense enzymes in Arctic charr were also differently affected by the levels of lipid oxidation. CAT and GPx activity increased with dietary lipid oxidation while that of SOD remained fairly stable. Increased CAT and GPx activity with dietary lipid oxidation suggests that Arctic charr can adapt to increased levels of secondary oxidation products in the diet. Boglino et al. (2014) suggested that increased antioxidant enzyme activity enhances both antioxidant defense and detoxification, thus promoting growth. Indeed, in most vertebrates, oxidative stress tolerance has been found to be positively correlated with antioxidant enzyme activity (Pérez-Campo et al., 1993). Accordingly, an increase in CAT and GPx activity in Arctic charr fed oxidized oils affirms increased stress resistance.

The present results mirror the findings of several earlier studies that reported wide variations in the response to dietary lipid oxidation by different fish species. For example, growth and feed utilization were not suppressed by dietary lipid oxidation in Atlantic cod (Zhong et al., 2008), Chinese long snout catfish (*Leiocassis longirostris*) (Dong et al., 2011) or in Senegalese sole (*Solea senegalensis*) larvae (Boglino et al., 2014). In contrast, growth decreased with increasing dietary lipid oxidation in

juvenile channel catfish (*Ictalurus punctatus*) (Dong et al., 2012) and rainbow trout (*Oncorhynchus mykiss*) (Fontagné-Dicharry et al., 2014). While the responses to dietary lipid oxidation could be species specific, differences in age/size and rearing environment can also lead to wide variations even in fish of the same species (Martínez-Álvarez et al., 2005). The differences in the level of lipid oxidation and quantities consumed by fish can also lead to specific responses that are not necessarily species dependent. In rainbow trout, the activity of antioxidant enzymes was much higher in fingerlings fed oxidized diets than in first feeding fry (Fontagné-Dicharry et al., 2014). In Siberian sturgeon (*Acipenser baerii*), high incidence of deformities, poor growth and survival were reported for larvae fed diets with oxidized oils and low vitamin A content (Fontagné et al., 2006), but not for adults fed a similar diet. The phylogenetic position of the fish species can also influence the level and activity of antioxidant enzymes (Martínez-Álvarez et al., 2005; Trenzado et al., 2006); and hence, the response to dietary oxidation. In general, this and earlier studies have demonstrated that the effects of lipid oxidation on the growth and physiology of fish vary among species, rearing environment (including: feed type, dietary oxidation level, quantity of oxidized diet consumed, and water quality) and partly depend on the ontogenic stage of the fish.

5.3 The effect of replacing *Rastrineobola argentea* fishmeal with the freshwater shrimp and mung bean on the growth of juvenile Nile tilapia

Searching for locally available and less competed for protein ingredients to replace more expensive fishmeal is of interest among fish farmers and feed producers. The results in Paper III, demonstrated that *C. nilotica* and *V. radiata* could be suitable alternative protein sources to *R. argentea* fishmeal. Growth was best when fish were fed diets containing *R. argentea* (diet R), *C. nilotica* (diet C) or both (diet RC) as the primary protein sources. Furthermore, growth performance of fish fed these diets was equal to or even better than for fish fed commercial formulation (diet COM). Feed utilization was best and comparable among groups fed high proportion of *R. argentea* or *C. nilotica* or a combination of the two and significantly better than for the groups fed COM. Although Nile tilapia fed *R. argentea*-*V. radiata* (RV) and *C. nilotica*-*V. radiata* (CV) diets showed reduced growth compared with the group fed diet R, these results affirm

that both *C. nilotica* and *V. radiata* are viable alternative protein ingredients. The lower growth in RV and more so in CV based diets was compensated for by the reduced cost of feed, suggesting that *C. nilotica* and *V. radiata* are suitable alternatives for *R. argentea* in Nile tilapia diets. The cost per unit dietary protein and feed cost per kg of fish produced decreased with increased inclusion of *C. nilotica* in diets, to a certain degree, without affecting growth. A formulation with high inclusion of *C. nilotica* yielded a feed that was 40% less expensive than a feed formulated with high *R. argentea* content which was the most expensive. The cost of feed per kg fish produced was also lowest for the formulation with high inclusion of *C. nilotica*; being 63% less expensive than for the commercial formulation which was least economical. Hence, the use of *C. nilotica* in tilapia feeds can reduce the production cost.

These findings are in agreement with those of Mugo-Bundi et al. (2015) who did not observe any negative effects on the growth of Nile tilapia fed diets containing up to 290 g of *C. nilotica* per kg of feed. In fact, a modest inclusion of 100 g of *C. nilotica* per kg resulted in the best growth. In the present study, *C. nilotica* content of up to 270 g/kg did not significantly affect growth of Nile tilapia when compared to a diet where *R. argentea* was the main protein source. Thus both the results of the present study and those of Mugo-Bundi et al. (2015) suggest that *C. nilotica* can at least partially substitute fish meal in diets for Nile tilapia. The present findings further suggest that *C. nilotica* is best utilized by Nile tilapia when minimal quantities are included in a formulation and in combination with a variety of other protein sources. It is likely that the amino acids are well balanced and better utilized by fish when feeds are formulated with both *C. nilotica* and *R. argentea*.

Shrimp meal is nutrient rich, but its digestibility is low when included in diets at high proportions and fish growth is usually compromised (Diop et al., 2013; Fall et al., 2012; Mugo-Bundi et al., 2015; Synowiecki and Al-Khateeb, 2000). When a high proportion of shrimp meal is included in a diet, the indigestible fibres (i.e. chitin and chitosan) build up and make nutrients less accessible to fish (Fall et al., 2012; Leal *et al.*, 2010; Synowiecki and Al-Khateeb 2000). Fibres also interfere with emulsification, absorption and utilisation of fats (Deng et al., 2009; Diop et al., 2013). Consequently, inclusion of high levels of shrimp meal in fish diet can impair growth. Although growth was lowest in the group fed diet CV, a combination of *V. radiata* with *R. argentea* (i.e., diet RV) resulted in better growth. It is probable that when *C. nilotica* and *V. radiata* are combined in a formulation, the combined effects of the high fibre from *C.*

nilotica and antinutrional factors from *V. radiata* retard growth. In contrast, the growth promoters and a well balanced amino acid composition in fishmeal (NRC, 2011) seem to enhance the digestibility and utilization of nutrients from *V. radiata*. Overall, these results show that *C. nilotica* can effectively be used in Nile tilapia diets with or without *R. argentea* fishmeal, and that *V. radiata* may be less suitable for use in Nile tilapia diets, particularly when the formulation also contains *C. nilotica.*

Crude protein content and essential amino acid composition of *R. argentea* and *C. nilotica* matched closely, and much higher than they were in *V. radiata*. However, as raw materials, the proportion of amino acid (AA) in *R. argentea, C. nioltica and V. radiata* were similar; although the CP of *V. radiata* was about 60% lower than in the other two. The CP of *R. argentea* can range between 530 g/kg and 700 g/kg and appears to vary with time of year and processing methods (Kubiriza et al. in prep.). The CP measured by Mugo-Bundi et al. (2015) {Citation}in *R. argentea* (671 g/kg) is within this range. It is not known how variable the CP in *C. nilotica* is, although the levels recorded in the present study (650 g/kg) and that of Munguti et al. (2012) (635 g/kg) and Mugo-Bundi et al. (2015) (561 g/kg) suggest that the CP levels may also be variable. The EAA composition of *R. argentea* measured in the present study and that of Mugo-Bundi et al. (2015) are similar apart from methionine and cysteine that were much lower in the present study. It is not clear if this difference is attributable to variability of the raw materials or measurement errors. However, the variability of the raw materials must be taken into account when formulating the feeds.

5.4 Relative efficacy of rosemary oil, bladderwrack powder and ethoxyquin in stabilizing lipid oxidation and their effects on fish

There is a growing interest in replacing potentially carcinogenic synthetic antioxidants used in fish feed with natural antioxidants (Błaszczyk et al., 2013; Błaszczyk and Skolimowski, 2005). In paper IV, the efficacy of two natural antioxidants, rosemary oil (RM) and bladderwrack powder (BW), was compared to that of the synthetic ethoxyquin (EQ) in controlling lipid

oxidation and their effects on farmed fish (i.e., growth and antioxidant enzyme activity) evaluated.

RM and BW can, to an extent, stabilize lipid oxidation, and of the two, RM is the more effective one. At elevated concentrations, the efficacy of RM approaches that of EQ, suggesting that EQ could be replaced by RM.

An additional benefit of using RM in diets is that it appears to promote growth. In the present study, growth was best in the Arctic charr fed a diet without antioxidants. Growth of Arctic charr fed diets containing EQ was reduced by 23% at 100 ppm, 13% at 300 ppm and by 20% at 500 ppm compared with the control diet. This raises concerns since Arctic charr feeds may contain EQ at 150 ppm (Błaszczyk et al., 2013). The growth of fish fed diets containing RM was comparable to that of the control diet, and more than 20% higher than in the group fed EQ. This suggests that RM could be a suitable antioxidant to replace EQ not only to stabilize lipid oxidation, but also to promote fish growth. Bohne et al. (2008) and Wang et al. (2015) reported decreased assimilation efficiency, increased physiological stress and subsequent poor growth of fish fed diets with elevated concentrations of EQ. In large yellow croaker (*Pseudosciaena crocea*) (Wang et al., 2010) and Japanese sea bass (*Lateolabrax japonicas*) (Wang et al., 2015), growth decreased with elevated concentration of dietary EQ. Therefore, it is possible that the slower growth of Arctic charr fed diets with EQ and possibly those fed BW might have suffered physiological stress. The faster growth in fish fed RM treated diets could be due to minimal physiological stress and improved assimilation compared to EQ and BW treated diets. In addition, RM may have increased diet palatability compared to EQ and BW, leading to increased feed intake.

RM is a natural antioxidant that has not been associated with carcinogenic properties, and a widely used herb in human diets and is considered safe for use in aquafeeds. RM has high concentration of phenolic compounds that contribute to its high antioxidant properties (Huber and Rupasinghe 2009; Sasse et al., 2009). The comparable efficacy of RM to EQ in this study is indeed supported by Babovic et al. (2010), Fukumoto and Mazza (2000) and Frankel (1998) who reported that RM is about four times as effective as butylated hydroxyanisole (BHA) and equivalent to butylated hydroxytoluene (BHT) in preventing lipid oxidation. In addition to promoting fish growth, the present results demonstrated that RM promotes antioxidant enzyme (CAT, SOD and GPx) activity more than do either BW or EQ. These results may suggest that RM stimulates the production

of defence enzymes and offers more effective protection against oxidative stress than EQ or BW. This may, in turn, promote growth.

Rosemary is used to flavour food and it can easily be propagated in the tropical climate of Uganda and East Africa in general. This offers an opportunity for feed processors in Africa to utilize RM to protect fishmeal, oils and aquafeeds from hydrolytic lipid oxidation during processing and storage. The only impediment likely to limit wide use of RM, is the high cost associated with extraction and processing of RM oil which puts the unit price to be about 8 to 12 times higher than that of EQ. However, the significantly higher growth (of over 20%) shown by Arctic charr fed diets treated with RM compared to the group fed EQ in this study could off-set the processing cost. These results have shown that RM and BW could be used to stabilize lipid oxidation in fish oil and aquafeeds, and that RM is more efficient, particularly at elevated concentrations. The results further affirm improved fish growth as an additional advantage of using RM in fish feeds instead of BW. Accordingly, RM is recommended as a more suitable natural antioxidant to replace the synthetic EQ than BW.

5.5 Conclusion

Development of novel fish feeds calls for extensive and focused experimentation, and this is well spelt out in this thesis. Information on effective experimental design and statistical analyses of aquaculture growth studies, and on the development and preservation of aquafeeds in Africa and elsewhere is provided.

The obstacles to aquaculture development in Uganda and East Africa are a blend of biological, environmental and socio-economic issues (Lee, 2014), and are representative of the general situation in Africa. The aquaculture sector is characterised by inadequately trained and therefore poorly skilled individuals who are unable to sustainably support the sector (USAID, 2009, 2012). In addition, acess to key inputs such as seed, feed and equipment is a major limitation. While the global average feed cost ranges between 50% to 60% of the production cost, commercial fish farmers in Uganda spend up to 75% of the production cost on feeds (Osinde. pers. comm.). Moreover feed quality greatly fluctuates due to adulteration and poor ingredient processing methods and storage. These are salient setbacks to aquaculture development that are neglected. Dietary lipid oxidation has for example been given little attention yet its effects on farmed fish could be enormous (Hamre et al., 2010; Kubiriza et al., 2017;

Sargent et al., 1999, 2002; Tocher et al., 2003). In addition, the need to streamline research through experimental design to achieve reliable results at minimal cost for puposes of improving aquaculture production has also achieved little attention (Thorarensen et al., 2015). An outstanding obstacle to aquaculture development in Africa is access to suitable fish feeds at affordable price. Aware that feed cost is greatly influenced by fishmeal prices, research is on-going to identify alternative protein sources. This thesis provides information on the possibility of replacing the expensive *R. argentea* fishmeal with inexpensive and locally available protein sources.

The results suggest that both *C. nilotica* and *V. radiata* are viable alternatives to *R. argentea* or as supplementary protein sources in Nile tilapia diets. Utilizing the two protein sources in formulating feeds for Nile tilapia can greatly reduce the production costs compared to the commercial feed currently used in Uganda. *V. radiata* is in little demand while *C. nilotica* is not used for human consumption in Uganda. These results contribute to the broad agenda of searching for suitable alternative raw materials in fish feeds that are in little demand for human consumpition or less utilized for other animal feeds.

Comparatively little is known about the effects of dietary lipid oxidation on farmed fish species in Uganda. Therefore, this thesis provides new knowledge on the effects of dietary lipid oxidation on Nile tilapia, the commonly farmed species in Uganda and Arctic charr farmed in Iceland where some of the experiments were conducted. The results reflect the complexity and variability in the effects of oxidized lipids on farmed fish that depend on species, fish size and rearing environment. This information can guide feed producers in selecting raw materials for feed formulation, processing methods and storage. Furthermore, fish farmers and researchers in fish nutrition can utilize this information to identify several confounding factors that may lead to peculiar responses in fish fed diets containing oxidized oils/lipids.

The thesis provides important information on how raw materials and dietary lipid oxidation can be managed. Presently, antioxidants are seldom used in aquafeeds proroduced in Uganda and East Africa, mainly because feeds formulated for tilapia contain relatively low lipid levels of between 6 to 12%, than that in carnivorous fish diets which can be over 20% (NRC, 2011). However, poorly processed *R. argentea* fishmeal used in East Africa, is a source of oxidized lipids in aquafeeds (Kubiriza et al. in prep.). These results provide an opportunity to feed manufacturers in East

Africa to utilize natural and safe antioxidants to stabilize lipid oxidation in raw materials and aquafeeds. The high efficacy of rosemary oil at elevated concentrations provides an opportunity for Ugandan fish farmers to retard dietary lipid oxidation without using the potentially carcinogenic ethoxyquin. Furthermore, rosemary oil may promote fish growth relative to the synthetic antioxidants. While the cost of extracting rosemary is higher than that of processing ethoxyquin, the additional growth in rosemary fed fish can compensate for the cost. Besides, rosemary can be easily cultivated in Uganda and other parts of Africa. Once rosemary is considered for use in aquafeeds, as recommended by this study, business opportunities can be opened to potential commercial farmers, processors, and traders or marketers of rosemary products.

Overall, the multifaceted impediments to aquaculture development in Africa, ranging from poor feed quality and high prices, seed, inaccessible equipment and unskilled human capital, require multidisciplinary approach to mitigate them. The wide scope of studies in this thesis offer useful information on effective experimental design, raw material quality preservation, use of novel antioxidants and production of cost effective aquafeeds from locally available protein sources. Application of these results can boost aquaculture development in Uganda and in other parts of Africa where natural conditions for fish farming are favourable. This thesis constitutes baseline and novel studies that have examined key quality issues in aquafeeds processed in Uganda and East Africa in general. Further research towards improving feed quality and reduction of production cost needs to be embraced and continued. The experiments in this thesis were performed on juvenile tilapia and Artic charr, and the smaller the fish the higher is the requirement for quality protein in their diets to ease digestibility. Additionally, juvenile fish diets are formulated to constitute high levels of crude protein. Therefore, the studies presented in this thesis need to be repeated using different life-history stages of the two fish species herein.

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Paper I

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Review

Experimental design and statistical analyses of fish growth studies

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ABSTRACT

Every year, numerous studies are published that compare the effects of different factors on the growth of aquaculture fish. However, comparatively little attention has been given to the experimental designs of these studies — in how many rearing units should each treatment be replicated, how many fish should be in each tank (n) and how should the data be analysed. The reliability of the results increases with increased replication and n. In reality, however, the experimental design must strike a balance between limited resources and the reliability of the statistical analysis. A survey of recent publications in Aquaculture suggests, that most (83%) aquaculture growth studies apply each treatment in triplicates with an average of 26 fish in each tank (range: 4 to 100). The minimum difference that can reliably be detected with statistical analyses is determined by the number of replications of each treatment, n, the variance of the data and the number of treatments applied. In the present study, we accumulated information on the variance of data in aquaculture growth studies on different species to estimate the minimum detectable difference and to assist researchers in designing experiments effectively. These results suggest that the variance is similar for different aquaculture species and, therefore, the same designs (level of replication and n) are suitable for studies on different species of fish.

The minimum difference (MDD) in mean body-mass of different treatment groups that can be detected in a typical aquaculture study (triplicates, 25 fish in each tank and average variance) with 80% statistical power (less than 20% chance of Type II error) is around 26% of the grand mean. Increasing the n from 25 to 100 will reduce the MDD to 19% of the grand mean, while a further increase in n will have comparatively lesser effect. Increasing replication to quadruplicates or sextuplicates (with n as 100), will further reduce the MDD to 16% and 12% of the grand mean respectively. MDD under 10% of the grand mean is only possible when fish for the experiment are selected within a narrow size range to reduce variance.

Simulations were performed, where samples (experiments) were repeatedly drawn from artificial populations with identical distribution and with the same experimental design as is commonly used in growth studies. Two of the populations had dose-dependent responses to treatment while one population showed no response to treatment. The resulting data was analysed with a mixed model ANOVA and by fitting either polynomials or asymptotic models to the data. Contrary to earlier suggestions, the critical treatment (minimum treatment to generate maximum response) estimated with the ANOVA approached more closely the population responses than did the critical treatments estimated with the non-linear models.

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1. Introduction

Information on the effect of feed ingredients, physical environment and other factors on the growth of fish are important for the development of aquaculture. Therefore, growth studies are common in aquaculture research where the mean sizes of different groups are compared following various treatments; the objective being to predict the performance of populations (all fish of the same species/strain) under different conditions.

The design of aquaculture growth experiments usually includes replication of treatments in two or more rearing units (e.g. tanks, ponds or net pens) where the replicates are considered independent samples from the populations. How accurately the results of experiments reflect the mean responses of the populations depends primarily on the number of fish sampled (within each replicated unit), the number of replicates and the variance of responses, both among individual fish within a replicated unit and among replicates.

A number of approaches have been used to analyse the results of growth studies, but the method most commonly used is analysis of variance (ANOVA). A cursory examination of growth studies (Table 3) published during the last year in the journal Aquaculture (29 in total) suggests that ANOVA is used in some capacity in all studies although 24% of the studies complement the analysis of dose response data with linear or non-linear methods.

In growth studies where treatments are replicated, individual fish should not be considered the experimental units. The fish within a tank are all exposed to the same "tank effects" (differences between tanks independent of treatment effects) and complicated interactions among the fish may contribute to variability within the tank that are not caused by the treatment (Gardeur et al. 2001; Imsland, 2001; Kozlov and Hurlbert, 2006). In fact, it can be argued that because of the common "tank effect", individual fish within a tank are not independent samples from the population but are instead "pseudoreplicates" as defined by Hurlbert (1984). A better approach is to perform ANOVA based on the total biomass or mean body-mass in each tank (Cowey, 1992; Smart et al., 1998) or, better still, to use a mixed model ANOVA where treatments are fixed factors and tanks are nested as random factors within treatments. With the latter method, the information on individual fish is modelled to fully account for the data structure (Ruhonen, 1998; Ling and Cotter, 2003). If the design of the experiment is balanced, i.e. the number of fish in all tanks and the number of tanks in all treatments are the same, the results of the simple and mixed model ANOVA will be the same. However, in long term growth studies the design may not be balanced, since mortality can vary among rearing units and all fish from single rearing units may be lost due to mishaps. When the design is not balanced, a mixed model should be used since the risk of type I error (rejecting a correct hypothesis) is increased when a simple ANOVA is used for the analysis of unbalanced data (Ruhonen, 1998).

In recent years, methods for mixed model analysis have developed rapidly and now many software packages such as SAS (SAS Institute Inc., Cary, NC, USA) and R (R Core Team, 2014) offer the possibility of linear mixed models with the Kenward–Roger modification of *F*-tests (Kenward and Roger, 1997, 2009). The Kenward-Roger modification adjusts the *F* values and degrees of freedom depending on the size of the "tank effect" and thus increases statistical power when the "tank effect" is small. The method has been used in aquaculture growth studies (Tobin et al., 2006; Schram et al., 2014). Over 83% of the growth studies published last year in Aquaculture use the mean body-mass or total biomass in each tank as the unit of analysis while only 11% used a mixed model analysis (Table 3).

In ANOVA, the null hypothesis of no effect of experimental treatments is tested and the means of the treatment groups are considered significantly different when the test statistics (*p*-value) indicates that the probability of the null hypothesis being true is less than 5% (α level less than 0.05). In other words, the probability of rejecting a correct null hypothesis (type I error) is less than 5%. However, it is also possible that an incorrect hypothesis is not rejected and differences among means are not detected where they truly exist. Failing to reject an incorrect hypothesis is called Type II error. The probability of Type II error is β and the power of a statistical test is defined as $1-\beta$. There is no conventional criterion for statistical power as there is for α, although a minimum of 80% is commonly regarded as suitable (Araujo and Frøyland, 2007). Statistical power is rarely reported in aquaculture growth studies (Searcy-Bernal, 1994) indicating that researchers are less concerned with Type II error than they are with α and Type I error.

The statistical power of mixed models depends on five factors: (1) the difference among means caused by the treatment (effect size), (2) the variance of the data, both among fish within a tank and among tanks receiving identical treatments, (3) the number of replicate tanks, (4) the number of fish within each tank and (5) the number of treatments tested (Ling and Cotter, 2003; Sokal and Rolf, 2012). Statistical power increases with increased effect size, the number of replicate tanks and the number of fish within each replicate tank while statistical power is reduced with increased variance and number of treatments tested (Ling and Cotter, 2003). Hence, to secure acceptable statistical power, replications and sample size per replicate should be maximized. However, the number of tanks available and the cost of resources for aquaculture growth studies are usually limited. Therefore, experimental design must strike a balance between acceptable power and the available resources.

The issue of the minimum detectable difference (MDD) in aquaculture studies, i.e. the minimum difference that is likely to be detected with 80% statistical power, has received little attention. Ling and Cotter (2003) shed important light on this subject when they compiled information on the coefficient of variation within tanks (CV_ε) and the coefficient of variation among tanks within treatment ($CV_{\beta 3}$) for triploid Atlantic salmon. In the present study, we compiled information on variance in body-mass in growth studies on different fish species to be able to estimate statistical power and the MDD. This information was then used to calculate the expected statistical power and effect size for experimental designs with different levels of replication and number of fish in each replicate tank.

Dose–response designs, where treatments are applied at incrementing levels of e.g. nutrient content or water quality, are common in aquaculture growth studies. These data can be analysed either with ANOVA or by using different linear and non-linear methods. The latter

include: broken line analyses, where two straight lines are fitted to the data, polynomial regression or non-linear regression models that fit asymptotic curves to the data (Baker, 1986; Cowey, 1992; Shearer, 2000). When the results are analysed with ANOVA, the critical response is usually determined as the lowest treatment level that gives a response that is not significantly different from the maximum response. However, this approach has been criticised by Baker (1986) and then later by Cowey (1992) and Shearer (2000). After reviewing a number of published growth studies with dose-dependent relationship, Shearer (2000) concluded that ANOVA may underestimate the critical treatment level by as much as 50% due to the inability of the method to detect small differences. Instead several authors (Baker, 1986; Cowey, 1992; Shearer, 2000) recommend the use of linear or non-linear methods and suggested that they provided more accurate results. However, fitting lines of different shapes assumes that there is a certain underlying structure to the data. Moreover, due to the inherent variability in aquaculture growth data it may be difficult to determine visually if the response is polynomial or asymptotic. Therefore, it is questionable if this approach is more appropriate than ANOVA. A second objective of this study was to use simulation studies to compare the fidelity of different methods of statistical analysis to the true underlying responses of populations and the conclusions drawn based on their results.

2. Methods

2.1. Data acquisition

Original raw data from 24 independent growth studies on Arctic charr (*Salvelinus alpinus*), Atlantic halibut (*Hippoglossus hippoglossus*), Atlantic cod (*Gadus morhua*), turbot (*Scophthalmus maximus*) and tilapia (*Oreochromis shiranus*) were analysed in this study. Data on Arctic charr (Ólafur Sigurgeirsson and Jón Árnason, unpublishEd.), Atlantic halibut (Thorarensen et al., 2010), Atlantic cod (Edelsparre, Pálsson and Steingrímsson, unpublished; Thorarensen, unpublished), and *turbot* (Imsland et al., 2013) were from growth studies conducted at Verið research station, Sauðárkrókur, Iceland. The studies examined different treatment effects (dietary ingredients, oxygen saturation, light regimes and temperature) on the growth performance of fish. Rearing conditions and fish size varied between experiments (Table 1). The data for tilapia were from a study conducted at Bunda College, University of Malawi on the effect of temperature on *O. shiranus* (Ssebisubi, 2008).

2.2. Data analysis

Data were analysed using mixed model ANOVA in SPSS to obtain the mean sums of square for tanks nested within treatments (MS_{within}) and the error mean square (MS_{error}), which constituted the error variance ($\hat{\sigma}^2_\varepsilon$). The coefficient of variation of the error term (CV_ε) was calculated as $CV_\varepsilon = \frac{\hat{\sigma}_\varepsilon}{\overline{X}}$ where $\overline{X}$ is the grand mean. The variance among tanks within treatments ($\hat{\sigma}^2_\beta$) was calculated as $\hat{\sigma}^2_\beta = \frac{(MS_{\text{within}}) - \hat{\sigma}^2_\varepsilon}{n}$, where n is the number of fish in each tank. The coefficient of variation for tanks within treatments (CV_β) was calculated as $CV_\beta = \frac{\hat{\sigma}_\beta}{\overline{X}}$. The statistical power was estimated as described by Ling and Cotter (2003). Briefly, the mean variance of treatment groups ($s^2_{\bar{Y}}$) was estimated as: $s^2_{\bar{Y}} = \frac{MS_{\text{within}}}{nb}$, where b is the number of replicate tanks within treatments. The $s^2_{\bar{Y}}$ was used to compute Tang's parameter (ϕ) (Tang, 1938) as $\phi = \sqrt{\frac{d^2}{2as^2_{\bar{Y}}}}$; where d is the difference between means and a is

Table 1
Variance and power in 24 independent growth studies on fish.

Study	Species	Treatment levels[a]	No. of tanks[b]	N[c]	Average final body mass (g)[d]	d (% of grand mean)[e]	CV_ε[f]	CV_β[g]	Observed power[h]	Minimum detectable difference at 80% power[i]
1	Halibut	5	3	47	122	24	0.32	0.00	99	11
2	Turbot	3	3	36	330.3	30	0.28	0.09	44	36
3	Tilapia	3	6	16	11.3	56	0.37	0.04	100	33
4	Arctic charr	7	4	50	4.7	30	0.25	0.07	100	22
5	Arctic charr	7	4	39	10.9	17	0.28	0.08	49	28
6	Arctic charr	6	4	50	90	12	0.21	0.09	23	32
7	Arctic charr	6	3	35	230.8	11	0.24	0.04	34	21
8	Arctic charr	6	3	132	672.8	4	0.15	0.02	40	8
9	Arctic charr	6	3	64	1067.9	4	0.18	0.00	20	9
10	Arctic charr	6	3	60	1437.5	10	0.17	0.00	98	15
11	Arctic charr	6	3	96	886.7	17	0.39	0.06	55	27
12	Arctic charr	16	3	30	2.3	37	0.26	0.06	100	33
13	Arctic charr	6	3	90	1082.9	6	0.16	0.03	23	12
14	Arctic charr	16	4	151	4.7	19	0.26	0.06	97	23
15	Atlantic cod	5	3	13	800	18	0.36	0.00	41	31
16	Atlantic cod	5	3	12	1497.3	13	0.33	0.00	60	6
17	Atlantic cod	5	3	46	248.7	7	0.32	0.05	12	24
18	Atlantic cod	6	3	15	791.8	20	0.35	0.00	46	32
19	Atlantic cod	6	3	32	105.2	37	0.32	0.12	37	55
20	Atlantic cod	3	6	56	1.9	16	0.36	0.07	38	17
21	Atlantic cod	2	9	105	1.8	17	0.39	0.10	92	14
22	Atlantic cod	2	5	31	0.23	13	0.48	0.11	29	28
23	Atlantic cod	2	5	35	0.52	8	0.36	0.00	44	12
24	Atlantic cod	2	5	14	0.08	13	0.56	0.00	13	31

Data from: 1—Thorarensen et al. (2010); 2—Imsland et al. (2013); 3—Ssebisubi (2008); 4–14—Ólafur Sigurgeirsson and Jón Árnason, unpublished results; 15—Ólafur Sigurgeirsson and Jón Árnason, unpublished results; 16–21—Arnason et al. (2010); 22–24—Allan Edelsparre and Stefan Oli Steingrimsson, unpublished.

[a] Number of treatments tested in the experiment.
[b] Number of tanks tested for each treatment.
[c] Number of fish in each tank.
[d] Mean body-mass of fish (g) in a study.
[e] Maximum difference between treatments means (% of grand mean).
[f] Error coefficient of variation (CV_ε).
[g] Coefficient of variation for tanks within treatment (CV_β).
[h] Retrospective power (%) at the end of studies.
[i] Effect size (% of grand mean) at 80% power.

the number of treatments tested. This value was then used to compute the non-centrality parameter (λ) as: $\lambda = a\varphi^2$.

The statistical power of each study was then calculated with the programme G*Power (Faul et al., 2007) using the λ and degrees of freedom with the α-level set at 0.05. This protocol was repeated to model the MDD for different values of CV_ε and CV_β (Table 2) using levels of replications (b) from 2 to 6 and number of fish in each tank (n) from 10 to 1000.

2.3. Simulation studies

Simulations were performed to compare three different methods for statistical analysis of growth studies with a graded response: ANOVA, a second order polynomial and a three parameter logistic growth model. The simulations were performed with R (R Core Team, 2014). The datasets used for the analysis represent random samples from three different populations:

Res45%: A population with a saturation type relationship to treatment where the response increased with treatment level until it plateaued with a response of 100% at treatment levels over 100%. The response to the minimum treatment was 45% lower than the maximum response (100%) (Fig. 1).

Res 11%: A population with saturation type relationship to treatment where the minimum response was 11% lower than the maximum response. The maximum response was 100% and reached when the treatment level was 100% (Fig. 1).

Res0%: A population with no response to treatment (Fig. 1).

The population responses to the treatments were normally distributed at each treatment level and the same variance was assumed for all responses regardless of treatment level.

The simulations were performed on 1000 datasets generated from each population. The simulations were made for experiments with 18 tanks and 50 fish in each tank. The datasets were random samples, generated based on the mean responses of the population at different treatment levels with equal variance for the means of tanks within all treatment levels. The means of tanks within treatments were normally distributed with a standard deviation equal to 4.5% of the grand mean for tanks within treatments. The residual variance within each tank was normally distributed with a standard deviation equal to 30.6% of the grand mean. These standard deviations are the same as the mean CV_β and CV_ε for all species found in this study (Table 1). In the data sets generated, the treatment levels tested were in arbitrary units expressed in percentages and could range between 85% and 121%. To reflect the strengths of different statistical approaches, tanks were allocated differently for mixed model ANOVA, polynomial models and non-linear models. In the mixed model simulations, six levels of treatments were tested, each in triplicate. In each sample, the lowest treatment levels tested ranged at random between 85% and 90% and then successive treatment levels were applied in 5% increments. The samples for the polynomial and non-linear models were in duplicate at nine treatment levels. In each sample, the lowest treatment level tested ranged at random between 85% and 89% and then successive treatment levels were applied in 4% increments covering a range of treatment levels of 32%.

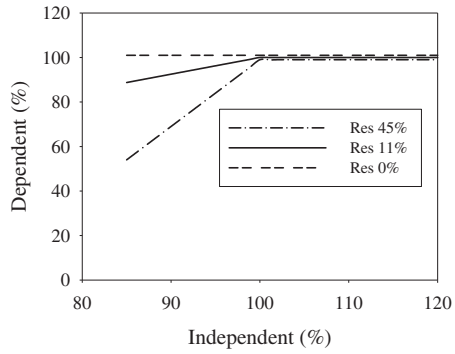

Fig. 1. The three populations used in the model simulations: Res45% where the minimum treatment gave a response that was 45% less than the maximum; Res11% where the minimum treatment gave a response that was 11% less than the maximum; and Res0% where treatment had no effect on response. The units for treatment and response are shown as percentages. For Res11% and Res45%, a treatment level of 100% will produce a 100% response.

Three methods were used to analyse the data:

1) Mixed model ANOVA with tanks as random factors nested within treatments and measurements of individual fish in each tank using the lme function within the nlme package (Pinheiro et al., 2014) in R. All designs were balanced with the treatment degrees of freedom as 5 (treatment levels − 1) and the residual degrees of freedom as 12 (treatment levels × (tanks within treatments − 1)).
2) Second order polynomial using the lm function in R.
3) Non-linear three parameter logistic growth model using a self-starting logistic function in R (SSlogis).

Table 2
Summary of analyses from simulation studies on data sampled from artificial populations, two with graded responses (Res11% and Res45%) and one population with no response to treatment (Res0%). Randomized normally distributed data with equal variances was generated based on the population responses assuming that CV_ε was 30.6 and CV_β was 4.5. The treatment level required to give a maximum response was 100% for all artificial populations and the maximum response was 100%.

	ANOVA			Second order polynomial			Three parameter logistic regression		
	Res45%	Res11%	Res0%	Res45%	Resp11%	Resp0%	Resp45%	Resp11%	Resp0%
Mean critical treatment (±95% range)[a]	99.7 (96–104)	95.0 (90–103)	92.3 (90–101)	110.7 (107–113)	108.8 (102–113)	96.5 (85–108)	101.5 (97–107)	97.0 (88–128)	–
Median critical treatment (%)	100	95	92	111	108	98	101	92	–
Mean maximum response (95% range)[b]	100 (95–105)	101 (97–106)	100 (94–105)	103 (99–106)	102 (99–106)	105 (101–109)	96.8 (93–101)	97 (93–112)	–
Mean effect size as % of grand mean (95% range)	18.3 (10.2–26.9)	13.1 (8.8–17.5)	9.0 (5.8–12.0)	–	–	–	–	–	–
Mean square residual deviation[c]	8.4	12.0	23.4	9.6	10.2	16.5	8.9	3.8	–
Proportion of analyses showing a significant effect of treatment	100%	36%	1%	100%	51%	5%	–	–	–
Analysis producing an error message	–	–	–	–	–	–	0.1%	20%	67%

[a] The treatment effect required to give maximum response.
[b] Estimated maximum effect.
[c] The mean square residual deviation between predicted responses and population responses.

Three approaches were used to compare the analysis methods:

1. The critical treatment levels, the minimum treatment level required to generate a maximum response were estimated for all the models:
   a. For the ANOVA, the highest treatment level did not generate a response significantly different from those of the two highest treatment levels.
   b. For the polynomial model, the critical level was the estimated treatment level that caused the maximum response.
   c. In the logistic growth model, the treatment level causing a response that was 98% of the asymptote was arbitrarily chosen as the critical treatment.
2. The residual variance of the predicted values for each model from the population values: $\frac{1}{t}\sum_{i=1}^{t}\left(\hat{Y}_i - Y_i\right)^2$ where t represents the treatment levels tested, $\hat{Y}$ is the predicted response and Y is the population response.
3. The maximum responses, estimated from the predicted values of the ANOVA and the second order polynomial and from the asymptote of the logistic regression model.

3. Results

3.1. Coefficient of variation for fish within tanks (CV_ε)

In most studies, CV_ε increased as the experiments progressed but tended to stabilise when the factorial increase in body mass (mean body-mass/mean initial body-mass) was about 1.5 (Fig. 2a, b, c). However, this pattern was not entirely consistent: In the study on Atlantic halibut, the CV_ε was nearly constant throughout and in the study on tilapia the CV_ε increased progressively (Fig. 2a). At the end of the experiments, the mean CV_ε was 30.6 ± 4.5% (mean ± SD) and ranged from 15% to 56% (Table 1). There were no clear differences in the final CV_ε for different species and the CV_ε varied between different studies on a single species. Thus the final CV_ε for Atlantic cod ranged from 32 to 56% (Fig. 2b; Table 1) and from 15 to 39% for Arctic charr (Fig. 2c; Table 1).

3.2. Coefficient of variation for tanks within treatments (CV_β)

The mean CV_β at the end of all studies was 4.5 ± 0.4% (Mean ± SD; range: 0–12). The CV_β increased initially in many studies but stabilised as the experiments progressed (Fig. 3a, c). However, this pattern was not consistent in all studies and in some, the CV_β decreased as the experiments progressed (Fig. 3a, b). Of the 24 studies investigated, eight had a final CV_β of zero; five had CV_β ranging from 2% to 5%, while 11 had CV_β of above 5%, the highest being 12% (Table 1).

3.3. Correlation between initial and final CV and body mass

In 20 studies (Table 1), information was available on both initial and final variance in body-mass. The final CV_ε in these studies was significantly correlated with initial CV_ε ($r = 0.621$; $p < 0.003$; $N = 20$). Similarly, final CV_β in different studies was significantly correlated with the initial CV_β ($r = 0.657$; $p < 0.002$; $N = 20$).

Information was available from several studies on Arctic charr and Atlantic cod (Table 1). These data were used to compare the variance in studies on the two species. The final CV_ε and CV_β in experiments on both species ($p < 0.05$) decreased with increasing final body mass (Fig. 4a, b). Adjusting for body mass, CV_ε was significantly lower ($p < 0.0001$) in Arctic charr than in Atlantic cod (Fig. 4a); while CV_β was not significantly different (Fig. 4b). However, the initial CV_ε in the studies on Atlantic cod was higher than in the studies on Arctic charr and, when the initial CV_ε is included as a variable in the model, the difference between the species was no longer significant.

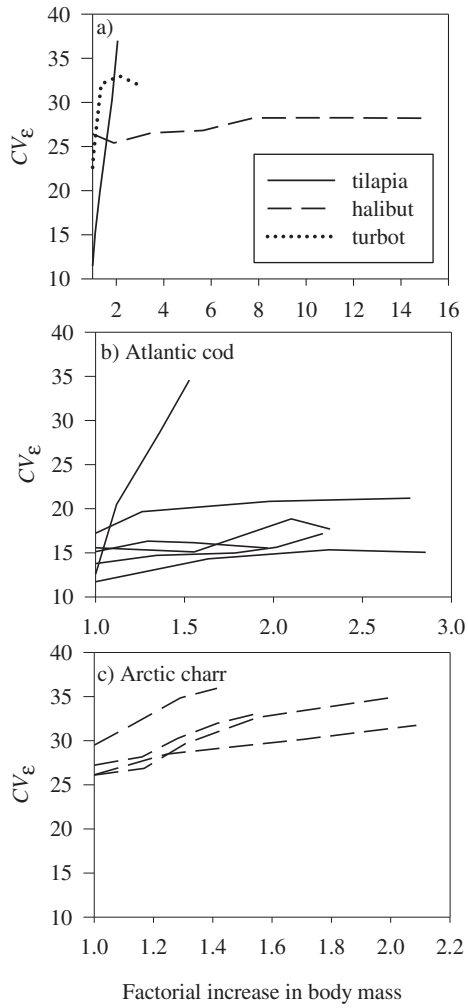

Fig. 2. Development of CV_ε with increasing body mass in experiments on (a) tilapia, Atlantic halibut and turbot, (b) Atlantic cod and (c) Arctic charr (The different lines represent separate studies). The increase in body mass is shown as factorial increase (mean body-mass/mean initial body-mass).

3.4. Statistical power and minimum detectable difference with 80% statistical power

When experiments are designed it is recommended that statistical power is 80%. In the experiments analysed (Table 1), the mean statistical power estimated post hoc was 53.9 ± 0.3% (mean ± SD) and ranged from 12% to 100%. The MDD was 18.1 ± 12.8% (range: 4% to 56%) of the grand mean (Table 1).

To show how experimental design is likely to affect the MDD, we modelled MDD using different number of replications and numbers of fish within each tank. The MDD was modelled for medium, high or low CV_ε and CV_β using the average, maximum and minimum CV_ε and CV_β encountered (Table 1). For the purpose of the modelling, it was assumed that five different treatments were being tested.

The level of replication and the number of fish in each tank affects the MDD (Fig. 5a, b, c). For all levels of replication, the MDD decreases

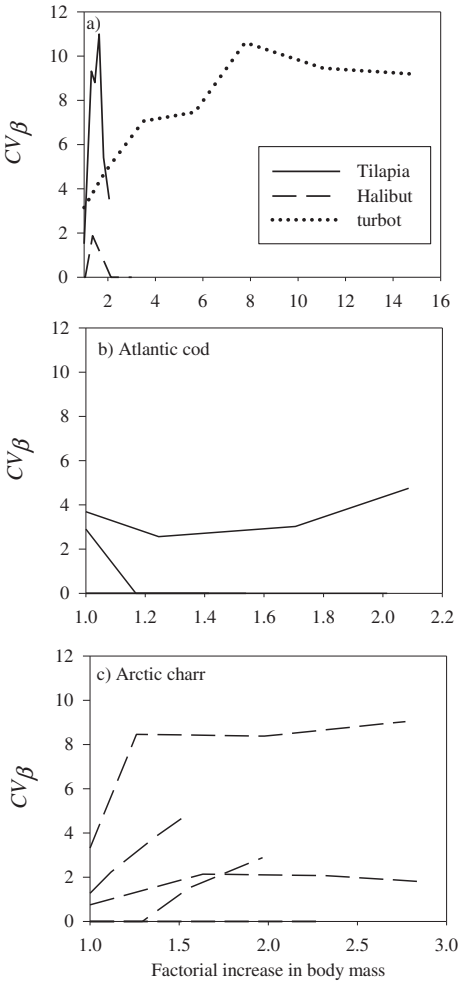

Fig. 3. Development of CV_β with increasing body mass in experiments on (a) tilapia, Atlantic halibut, and turbot, (b) Atlantic cod and (c) Arctic charr (The different lines represent separate studies). The increase in body mass is shown as factorial increase (mean body-mass / mean initial body-mass).

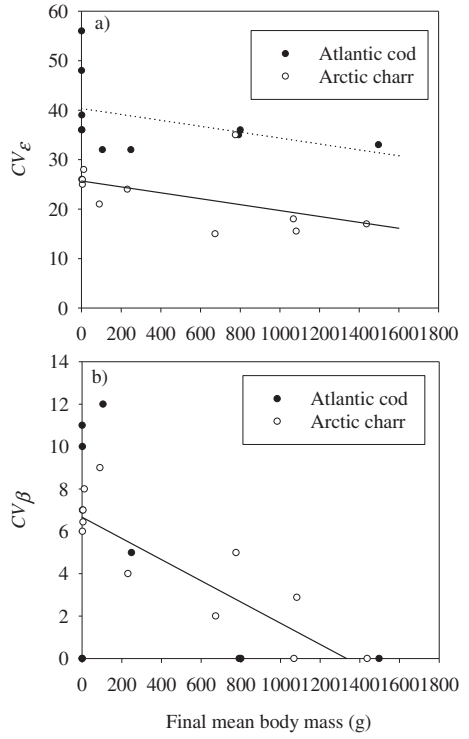

Fig. 4. Coefficients of variation in growth studies of Atlantic cod and Arctic charr at different final mean body masses. a) CV_ε and mean final body-mass. The intercepts for the two species were significantly different ($p < 0.0001$) while the slopes of the regression lines for the two species were not significantly different. The regression lines (interrupted for the Atlantic cod, continuous for Arctic charr) with a common slope were CV_β = Intercept − 0.006 × body mass with the intercepts being 25.7 and 40.6 for the Arctic charr and Atlantic cod respectively. b) CV_β and mean final body-mass. Neither slopes nor intercepts were significantly different. The common regression line was: $CV_\beta = 6.67 - 0.005 \times$ body-mass (R^2: 0.38).

markedly with increasing n until it reaches about 100. There is comparatively little gained in reduced MDD by increasing n over 100. For average CV_ε and CV_β, designs in triplicate are required for reaching an MDD of 20% or less. Similarly, four to six replications can give a MDD of 1014% (Fig. 5a). A MDD under 10% is only possible when both CV_ε and CV_β are low (Fig. 5c); reaching 4 to 10% when n is 100.

3.5. Comparison of different methods to analyse graded treatment growth data

Datasets were generated from random samplings of three different populations (Fig. 1) based on the average CV_ε and CV_β (Table 1). In total, 1000 datasets were generated for each population and analysed using a mixed model ANOVA, a second order polynomial and logistic regression. The logistic regression failed to converge on average in 0.1%, 20% and 67% of trials for the Res45%, Res11% and Res0% populations respectively.

With the ANOVA, the estimated mean treatment level required to create a 100% response for the Res45% population was 99.7%, matching closely the critical treatment of the population (100%) with 95% of estimated values being between 96% and 104% (Table 2). The second order polynomial overestimated the critical treatment of the population with more than 95% of the estimates being higher than 107% (Table 2). The critical treatment estimated through the logistic regression (Table 2) was 101% (95% range 97%–107%). However, it should be stressed that the critical treatment was arbitrarily chosen to be where the response reached 98% of the estimated maximum. Obviously the response level chosen will affect the estimate of the critical treatment value.

Analysis of the Res11% population showed a significant treatment effect in 36% of tests with ANOVA and 51% with the polynomial tests. The mean critical treatment estimate from the ANOVA was 95% (range 90%–103%) while statistical analysis with the polynomial estimated the critical treatment values as 109% (range: 102%–113%) (Table 2). The mean critical treatment estimate from the logistic regression was 103% (range: 93%–113%).

For the Res0% population, where treatment had no effect (all responses were 100%), the polynomial showed significant effects in 5% of tests while the mixed model ANOVA only showed significant differences in 1% of the analyses. As described above, the logistic regression analysis did not converge in most of the analyses of samples from the 0% population.

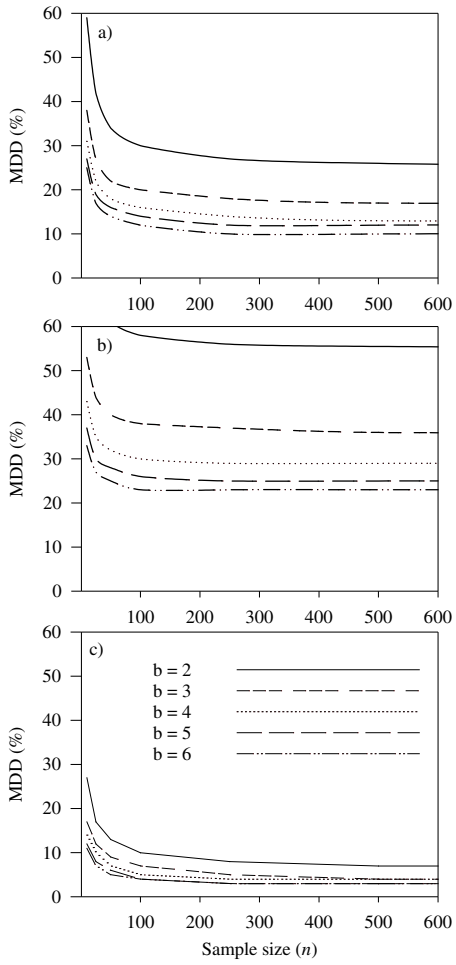

Fig. 5. Minimum detectable difference (MDD), shown as % of the grand mean in growth studies with five treatments levels when statistical power is 80%. a) Mean CV_ε and mean CV_β, b) High CV_ε and high CV_β, c) Low CV_ε and low CV_β.

The estimated maximum responses were similar for all methods of analysis with the 95% range of responses covering the population maximum response of 100%. For the Res45% population, estimates from all statistical methods show a similar mean square residual deviation from the population response (Table 2), while at Res11% and Res0% the residual values for the ANOVA were slightly higher than for either the polynomial or the logistic regression. The mean MDD in the ANOVA was 18.3% and 13.1% for the Res45% and Res11% populations respectively.

4. Discussion

This is the first study to evaluate the variance, statistical power and MDD in growth studies of various aquaculture species. Earlier, Ling and Cotter (2003) evaluated the variance in growth studies of triploid Atlantic salmon, finding a mean CV_ε of 28 ± 8.6% (range: 14–41%) and CV_β of 3.2 ± 1.9% (range: 1–7%). In 29 growth studies on 24 species published during the last year in Aquaculture (Table 3), the estimated mean CV_β was 5% (range: 0–49%) while the mean CV_ε was 28%. All these values are in accord with the results of the present study where the

Table 3
A summary of variability of final body mass and experimental design in 29 growth studies of 24 species of fish published in 2013 and 2014 in Aquaculture. The CV_β was estimated based on reported standard errors and levels of replication in studies where simple ANOVA was used for statistical analysis.

	Mean	Range	Mean factorial increase[b]
CV_ε (%)[a]	27.9	23–36	1.78
CV_β (%)	4.9	0–49	1.75
Level of replication (rearing units/treatment)	3	2–6	
Number of fish in each rearing unit	25.7	4–100	

[a] Information on CV_ε was only available in 4 studies.
[b] Final divided by the initial CV_ε and CV_β.

CV_ε and CV_β (mean ± SD) were 30.6 ± 4.5% (range: 15%–56%) and 4.5 ± 0.4% (range: 0–12%) respectively. Both the present study and that of Ling and Cotter (2003) show that CV_ε and CV_β for a single species can range widely among different studies. The only indication of species differences in variance in body mass is the apparent difference in CV_ε between the Atlandic cod and Arctic charr (Fig. 4a). However, this may not reflect species specific variance, but instead higher initial CV_ε in the former studies. Fish were selected for these studies to be within certain size ranges and, therefore, the CV_ε does not reflect the natural variation of the species, but rather the abundance of fish available. Combined, these results suggest that the variance encountered in growth studies of different species of fish is similar, suggesting, that similar experimental designs are appropriate for all these species.

The model calculation conducted in this study shows, as expected, that both the number of fish in each treatment and the level of replication affect the MDD. Increasing n up to 100 decreases the MDD considerably, while increasing n over 100 has a limited effect (Fig. 5a, b, c). Increasing the level of replication from duplicates to triplicates reduces the MDD by about 30%. Further increases in the level of replication will reduce the MDD, although the gain in reduced MDD is progressively decreased with each increase in level of replication.

The MDD is of particular interests for researchers. The average expected MDD for mixed model ANOVA (for statistical power of 80%) in the experimental data analysed from the different growth studies (Table 1) was 23% of the mean (range: 6–55%). In studies published in Aquaculture during the last year (Table 3), treatments in triplicate were the most common (83% of studies), with duplicates (10%) and quadruplicates (3%) being less common. One study used six tanks per treatment. The mean number of fish in each tank in these studies was 25.7 (range: 4–100). For triplicates, n of 26 and statistical power of 80%, the expected minimum detectable difference is 26% when variance is average. These results suggest that in most growth studies published, differences smaller than about 25% of the grand mean are not reliably detected (i.e. in least 80% of trials) and half of studies will fail to detect reliably true differences under 20%.

Researcher can take active measures to increase the resolution of statistical tests by increasing the level of replication and the n. Furthermore, when CV_ε and CV_β are low the MDD is also reduced. Both CV_ε and CV_β tend to increase as the experiments progressed (Fig. 2 and 3) and this was also the case in 74% of the growth studies published in Aquaculture during the last year (Table 3). However, the initial variance and final variance are positively correlated and, therefore, our results suggest that it is possible to reduce the MDD further by selecting fish for experiments within a narrow size range. By using stochastic models Imsland (2001) suggested, that there were two main causes for size variation seen in laboratory studies with turbot: (a) Individual genetical growth rate variation, this trait is stochastic in the population and changes with time (stochastic growth with memory) (b) Combination of individual genetical growth rate and size-related dominance hierarchies. By selecting fish within a narrow size range both a) and b) above will be minimized which makes it possible to reduce MDD.

However, if the responses to treatments are size specific, i.e. treatment effect depends on size, selection of fish within a narrow size range may produce a bias in the results.

When the differences among treatments in growth studies are small, the duration of the experiment is also important. As most of the growth experiments evaluated in this study progressed, both CV_ε and CV_β tended to level off (Fig. 2a,b,c). If CV_ε and CV_β are stable while the difference in mean size of treatment groups increases with time, statistical power will increase. Furthermore, both CV_ε and CV_β are reduced as size increases (Fig. 4a,b). Therefore, in order to avoid type II errors, the duration of experiments must be extended where differences between effects of different treatments are small.

Another possibility to increase statistical power is to include data from the entire study rather than analysing only the final size of the fish. This can be done with mixed model ANOVA by including time either as a categorical factor (Ling, 2007), as a covariate or using repeated measures ANOVA (Imsland, 2001). When time is included as a covariate the growth performance is compared as the slopes of the growth curves rather than the final size. However, when there are large differences in the size of the fish at different times, the variances may not be equal and then one of the assumptions of the ANOVA may be violated. Therefore, it may be necessary to use statistical procedures such as GLM in R which allows data with gamma distribution or PROC MIXED in SAS where variance and covariance structures can be directly modelled.

The results of the present study are an interesting contribution to the discussion of which is the most appropriate statistical method to analyse data from growth studies. Analysing published data on feed studies, Shearer (2000) suggested that ANOVA, in dose–response studies, might under-estimate the critical treatment effect required to produce a maximum response due to the inability of ANOVA to detect small differences. Instead he recommended using regression techniques, either polynomial or logistic. However, the results of the simulations performed in the present study directly contradict his conclusion. They suggest that ANOVA does not necessarily underestimate the critical treatment effect. In fact, the estimate of critical treatment with ANOVA most closely matched the critical value of the populations. Polynomials tended to overestimate the critical treatment level by 11% on average. With the logistic asymptotic function, it is difficult to decide when the maximum response is reached and this will limit its usefulness. Furthermore, the logistic regression procedure failed in many cases to fit the model, especially when the treatment effect was small. Moreover, the advantage of using ANOVA rather than the linear and nonlinear methods is that it does not presuppose the shape of the relationship between treatment and effect. Therefore, we suggest that a mixed model ANOVA is the most appropriate statistical method to analyse data from growth studies.

4.1. Conclusions

The results of this study suggest that the variance in aquaculture growth studies on different species is similar and, therefore, a similar experimental design (replication level and number of fish in each unit) can be employed in growth studies regardless of the species of fish. The results of the study suggest that most aquaculture growth studies cannot reliably (with 80% power) detect a difference in weight that is less than 26%. However, researchers can take measures to reduce the minimum detectable difference by selecting fish within a narrow size range for experiments. This may reduce the MDD to 5% with adequate replication.

The results of the present study suggest, that in contrast to the suggestions of Baker (1986), Cowey (1992) and Shearer (2000), a mixed model ANOVA is the best approach to analyse growth data with graded responses and superior to non-linear models.

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Dietary lipid oxidation tolerance of juvenile Arctic charr (*Salvelinus alpinus*) and Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

The effects of dietary lipid oxidation on fish are diverse and contradictory. In this study, herring oil was oxidized to five different levels (peroxide values (POV): 20.44, 182.97, 56.12, 33.27 and 0.00 meq kg^{-1}) and used to formulate five experimental diets. Juvenile Arctic charr (*Salvelinus alpinus*, mean body mass ± SD: 2.41 ± 0.11 g) were reared on the five diets for 67 days. In another experiment, juvenile Nile tilapia (*Oreochromis niloticus*) (1.71 ± 0.40 g) were reared on three diets formulated with oxidized oils having POV of 19.55, 447.27 and 32.72 respectively, for 90 days. Growth responses were monitored in quadruplicate groups of 60 individuals of Arctic charr and 100 for Nile tilapia, respectively. The activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were only studied in Arctic charr.

At the end of the study, the Arctic charr group reared on a diet with oils in the primary oxidation state had significantly lower body mass and specific growth rate, but higher hepatosomatic index compared to the rest of the groups. The activities of CAT and GPx increased with dietary lipid oxidation while that of SOD remained fairly stable. In Nile tilapia, both final body mass and condition factor were significantly lower in the group fed fresh oil than in the groups fed oxidized oils. Fillet lipid content decreased with lipid oxidation while ash and protein did not differ amongst tilapia groups. The results suggest that Arctic charr are more susceptible to lipid oxidation than are Nile tilapia. These differences may be the result of species differences in tolerance to oxidized lipids, variable lipid intake or access to vitamin E/tocopherol in feed.

Statement of Relevance: Important for Arctic charr and Nile tilapia feed development.

1. Introduction

Fishmeal and oils used in commercial aquafeeds contain high levels of polyunsaturated n-3 fatty acids (PUFAs) and are, therefore, susceptible to oxidation (Trenzado et al., 2006; Laohabanjong et al., 2009; Hamre et al., 2010; NRC, 2011). PUFAs tend to oxidize on exposure to oxygen, transition metal ions (mainly copper and iron), lipoxygenase enzymes, light and hemo-proteins. Oxidized diets are characterized by off-flavours, vitamin deficiency and low essential fatty and amino acids content (Sargent et al., 2002; Sutton et al., 2006; NRC, 2011). Complex formation due to lipid oxidation reduces the availability of dietary nutrients (Marmesat et al., 2009) and is associated with reduced digestibility and suboptimal dietary concentrations of n-3 fatty acids (Forster et al., 1988; Sutton et al., 2006). Furthermore, fish fed on oxidized diets may be stressed, have reduced immunity and high incidences of disease and mortality (Tocher et al., 2003; Fontagné et al., 2006, 2008; Alves Martins et al., 2007). Accordingly, dietary lipid oxidation is of concern to fish feed manufacturers and fish producers.

Results from studies on the effects of oxidized dietary lipids on fish are inconsistent and probably species and/or size/stage dependent. In juvenile channel catfish (*Ictalurus punctatus*) (Dong et al., 2012) and rainbow trout (*Oncorhynchus mykiss*) (Fontagné-Dicharry et al., 2014), growth decreased with increasing dietary lipid oxidation. Liver and kidney cellular deformations were found in channel catfish fed dietary oxidized fish oils (Dong et al., 2012). In rainbow trout, age specific responses to lipid oxidation were shown by a significant increase in the activity of antioxidant enzymes at fingerling stage as opposed to the first feeding fry stage (Fontagné-Dicharry et al., 2014). Increased deformities, poor growth and survival were reported in Siberian sturgeon (*Acipenser baerii*) larvae fed diets with oxidized oils and low vitamin A content (Fontagné et al., 2006). In contrast, growth and feed utilization were not suppressed by dietary lipid oxidation in Atlantic cod (*Gadus*

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morhua) (Zhong et al., 2008), Chinese longsnout catfish (*Leiocassis longirostris*) (Dong et al., 2011) and in Senegalese sole (*Solea senegalensis*) larvae (Boglino et al., 2014). Furthermore, no changes were reported in the heapato and visceral somatic indices and heamatocrit levels of Atlantic cod fed oxidized oils with a POV of 94 meq kg^{-1} oil regardless of vitamin E content (Zhong et al., 2008). Cowey et al. (1984) also reported no difference in growth and glutathione peroxidase activity in rainbow trout fed oxidized oils with or without DL-α-tocopheryl acetate. As such, the effects of dietary lipid oxidation on fish are inconsistent and are seemingly species and/or age specific and require closer examination.

Autoxidation of lipids occurs in fish under natural conditions (Martínez-Álvarez et al., 2005; Fontagné et al., 2006, 2008; NRC, 2011), but its subsequent effects are minimized by an elaborate antioxidant system consisting of vitamins E and C, carotenoids and an array of antioxidant enzymes (Martínez-Álvarez et al., 2005; Fontagné et al., 2006, 2008; Alves Martins et al., 2007; NRC, 2011; Boglino et al., 2014; Fontagné-Dicharry et al., 2014). As in other vertebrates, the main antioxidant enzymes in fish are superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidase (GPx). SOD converts superoxide radicals to hydrogen peroxide and molecular oxygen while CAT and GPx, break down hydrogen peroxide into water. A combination of these enzymes renders the toxic superoxide radical and hydrogen peroxide harmless (Aebi, 1984; Gary, 1991; Weydert and Cullen, 2010). Depending on demand, these enzymes are naturally up or down regulated.

The present experiments were conducted to examine the effects of diets containing oxidized herring oils on the growth and survival of juvenile Arctic charr and Nile tilapia, as well as antioxidant defenses in Arctic charr. Dietary oxidation is likely to be a problem in tropical and developing countries such as Uganda where feed reaches the producers via a marketing chain that involves several middlemen. Furthermore, the processed feed is not stored under conditions that minimize lipid oxidation (humidity, light or temperature), but rather is exposed to ambient conditions. In temperate environments like Iceland, Arctic charr feeds may not necessarily be exposed to high temperature fluctuations, but they contain high levels of polyunsaturated fatty acids that are prone to autoxidation. Ultimately, this study provides important information on how the two important aquaculture species (Arctic charr and Nile tilapia) respond to dietary lipid oxidation.

2. Materials and methods

2.1. Preparation of experimental oils

Herring or capelin oil is typically used in Arctic charr diets, but not in tilapia feeds. In this study, herring oil was used because it had not been treated with antioxidants and has high proportion of highly unsaturated fatty acids (HUFAs), making it easy to oxidize. Bottled fresh herring oil (POV 20.44 ± 1.78) was oxidized by bubbling pure oxygen into a corked bottle through a needle. The bottle was shaken for 10 min to ensure thorough distribution of the injected oxygen before it was incubated at 50–70 °C, for two to seven days depending on the targeted oxidation level. The oxidation state of experimental oils was monitored by assaying the peroxide (POV), anisidine (AnV), free fatty acid (FFAs) and thiobarbituric acid reactive substances (TBARs) levels every 24 h. All analyses followed standard protocols outlined by the Association of Official Analytical Chemists (AOAC, 1995). Once the required oxidation level was achieved, the oils were fixed with 300 ppm ethoxyquin antioxidant and stored at −25 °C until experimental diets were prepared. Four categories of oils (Table 1), oxidized to different levels, were produced (OX1, OX2, OX3, OX4) and the fifth category (OX0) contained only un-oxidized oil (Table 1). Using the above treatment protocols and facilities, three other separate categories of oil were prepared for the tilapia study done in Uganda (Table 2). These oils did not have exactly the same lipid oxidation characteristics as those used on Arctic charr, but were prepared to have the three main oxidation levels as used in making Arctic charr trial feeds. That is, the un-oxidized or fresh (OL0), moderately oxidized (OL1) and highly oxidized oils (OL2) (Table 2). The OX1 and OL1 oils were exposed to heat for 48 h, while the OX2, OX3 and OL2 were exposed for 72–84 h, and OX4 for 148 h. The oils used in tilapia study were also fixed with 300 ppm ethoxyquin before they were shipped to Uganda. In Uganda, they were kept in a blast freezer (range −30 to −25 °C) at Ugachick Poultry Breeders Limited for two weeks until the experimental diets were formulated.

2.2. Feed formulation and preparation

2.2.1. Arctic charr growth trial diets

A single un-extruded diet was formulated to meet the crude protein (45%) and crude lipid (20%) requirements of Arctic charr (Table 3). This diet was then used to produce five separate experimental diets, each of which was mixed with equal volumes of oils OX0, OX1, OX2, OX3 and OX4 (Table 1). The diets were pelletized and air dried. The feed ingredients used in the formulation of experimental diets were fish meal (NSM-715/85), fresh or oxidized herring oil (obtained from Síldarvinnslan hf, Hafnarbraut 6, 740 Fjardabyggd, Iceland), soy bean meal 47/5 Brasil, wheat flour, wheat gluten, monocalcium phosphate and vitamin and mineral premix (Laxá Feed mill, Akureyri, Iceland). The formulated feeds were kept in a freezer at −22 °C to prevent further oxidation as the experiment progressed.

2.2.2. Tilapia growth trial diets

In the tilapia study, experimental diets were made from solvent extracted soya, wheat bran, freshwater shrimp (*Caridina nilotica*), whole maize, wheat pollard, salt, vitamin and mineral premix (Ugachick Feed Mill, Uganda) and either fresh or oxidized herring oil (obtained from Iceland). For optimal growth, Nile tilapia requires 28–30% crude protein and 6–12% crude lipid (NRC, 2011). Hence, all diets were formulated to contain 28% crude protein and 7% crude lipid (Table 4). Each experimental diet was thoroughly mixed with equal volumes of oil, pelleted and air dried to produce diets OL0 (fresh oil), OL1 (moderately oxidized oil) and OL2 (highly oxidized oil) (Table 4). The prepared diets were stored in a freezer at −22 °C to hinder further oxidation as the experiment progressed.

2.3. Fish husbandry

2.3.1. Arctic charr growth trial

Juvenile Arctic charr were obtained from the Hólar breeding farm, Iceland and brought to Verið, Sauðárkrókur, the research facility of

Table 1
Peroxidation characteristics of the oils used in Arctic charr experimental diets.

Dietary oil	POV (meq·kg^{-1})	AnV	Totox	FFA (%)	TBARs (μmolMDA·kg^{-1})
OX0	20.44 ± 1.78^{a}	12.62 ± 0.23^{a}	53.49 ± 3.80^{a}	4.09 ± 0.21^{a}	26.60 ± 0.28^{a}
OX1	182.97 ± 16.47^{c}	28.52 ± 0.14^{b}	394.46 ± 32.81^{c}	4.21 ± 0.05^{a}	65.4 ± 0.85^{c}
OX2	56.12 ± 5.17^{b}	37.62 ± 0.47^{c}	149.85 ± 10.82^{b}	4.09 ± 0.02^{a}	76.60 ± 0.001^{d}
OX3	33.27 ± 2.57^{a}	51.1 ± 0.42^{d}	182.98 ± 5.10^{b}	5.12 ± 0.03^{b}	131.3 ± 0.14^{e}
OX4	nd*	116.43 ± 0.03^{e}	116.43 ± 0.03^{a}	5.50 ± 0.001^{b}	146.5 ± 3.81^{b}

*not detected. OX0: un-oxidized oil; OX1: primary oxidation state; OX2, OX3: secondary oxidation state; OX4: tertiary oxidation state. Values in the same column with different superscripts are significantly different ($P < 0.05$).

Table 2
Peroxidation characteristics of the oils used in Tilapia experimental diets.

Dietary oil	POV (meq·kg^{-1})	FFA (%)	Acid value	TBARs (μmolMDA·kg^{-1})
OL0	19.55 ± 5.39[a]	1.41 ± 0.02	2.81 ± 0.04	46.89 ± 0.78[a]
OL1	447.27 ± 3.08[c]	1.47 ± 0.02	2.93 ± 0.04	60.91 ± 4.85[b]
OL2	32.72 ± 2.79[b]	1.52 ± 0.01	3.02 ± 0.02	127.51 ± 4.87[c]

OL0: un-oxidized oil; OL1: primary oxidation state; OL2: secondary oxidation state. Values in the same column with different superscripts are significantly different at ($P < 0.05$).

Hólar University College where the experiment was conducted. The fish were distributed at random amongst twenty cylindrical flow-through tanks (20 L) and allowed one week of acclimation before the experiment commenced. During acclimatization, fish were fed on a weaning diet from Laxá Feed mill (Akureyri, Iceland) and any fish that died in the same period were replaced. Sixty fish of average initial body mass 2.41 ± 0.11 g, (mean ± SD) were stocked per tank. Each diet was fed to quadruplicate tanks in excess using automated belt feeders set to feed at five minute intervals. Feeding was continuous under a 24-hour light regime. Excess feed was siphoned off and any dead fish removed and recorded for survival estimation at the end. The duration of the experiment was 67 days. Throughout the experimental period, approximate water flow was 2 L min^{-1} in each tank while water temperature and dissolved oxygen saturation were maintained at 9.65 ± 0.67 °C and 101.72 ± 8.71%, respectively.

2.3.2. Tilapia growth trial

The Nile tilapia experiment was conducted for 90 days at the facilities of Kireka fish farm in Uganda. Nile tilapia juveniles were obtained from Namuyenje mixed farm in Mukono, Central Uganda. The fish were acclimated in a 200L concrete tank using Ugachick-35% CP diet for two weeks, after which they were distributed amongst 12 hapas (1.2 × 1.2 × 1.5 m with 0.5 m freeboard) that were installed in the same pond. Each experimental diet was fed to quadruplicate groups of 100 fish. The average body mass at the start of the experiment was 1.71 ± 0.40 g. All groups were fed to satiation three times each day. Satiation was assumed when fish showed no further interest in pellets supplied. Whenever it rained, the fish were either fed once or twice each day, depending on temperature and fish activity. The daily feed given to fish in each hapa was recorded. Cleaning was done fortnightly to remove waste and algae from the hapa nets. Measured dissolved oxygen ranged from 1.66 ± 0.13 to 6.81 ± 0.52 mg L^{-1}, temperature from 18.92 ± 0.28 to 29.90 ± 1.15 °C and pH from 5.43 ± 0.04 to 7.43 ± 0.17 at dawn and dusk, respectively

Table 4
Formulation and proximate composition of the tilapia trial diets.

Ingredient (g kg^{-1})	Experimental diet		
	OL0	OL1	OL2
Solvent extracted soya	354.0	354.0	354.0
Wheat bran	200.0	200.0	200.00
Caridina nilotica meal	132.8	132.8	132.80
Whole maize	118.0	118.0	118.0
Wheat pollard	110.0	110.0	110.0
Fish oil[a]	6.20	76.20	76.20
Mineral premix[b]	5.00	5.00	5.00
Vitamin premix[c]	2.00	2.00	2.00
Salt	2.00	2.00	2.00
Chemical composition (%)			
Dry matter	86.41 ± 0.36	88.11 ± 0.20	85.42 ± 0.18
Crude protein	28.76 ± 0.02	28.72 ± 0.27	27.88 ± 0.08
Crude lipid	7.07 ± 0.28	7.47 ± 0.42	7.19 ± 0.50
Ash	6.63 ± 0.22	6.53 ± 0.13	6.47 ± 0.01
Crude fibre	6.29 ± 0.02	6.77 ± 0.20	6.12 ± 0.14

[a] Herring fish oil was oxidized in the rest of the diets except OL0.
[b] Mineral premix (mg/kg diet): $Ca(H_2PO_4)\cdot 2H_2O$, 180,000; $CaCO_3$, 140,000; $MgSO_4\cdot 7H_2O$, 5100; $FeSO_4\cdot 7H_2O$, 1000; $NaHCO_3$, 6880; $MnSO_4\cdot 4H_2O$, 350; KIO_3, 10; $CoCl_2\cdot 6H_2O$, 20; $Na_2MoO_4\cdot 2H_2O$, 80; $NaSeO_3$, 20; $KH_2PO_4\cdot 2H_2O$, 11,996; $ZnCO_3$, 150; $CuSO_4\cdot 5H_2O$, 30; NaCl, 2470; $Al_2(SO_4)_3$, 20.
[c] Vitamin premix (mg/kg of diet): thiamine, 1000; pyridoxine, 1000; folic acid, 250; ascorbic acid, 20,000; pantothenic acid, 3000; myo-inositol, 2000; biotin, 100; niacin, 7500; cyanocobalamin, 10; riboflavin, 1000; retinol acetate, 400; tocopheryl acetate, 2000; menadione, 4000; Cholecalciferol, 30; dextrin, 93971.

2.4. Measurements

All fish in a tank/hapa were weighed in at least three subsample batches at the commencement of the studies and at every sampling interval, i.e., every three weeks in Arctic charr and once a month in Nile tilapia. The fish were starved for 24 h before measurement. At the end of the experiments, the body mass and length of all fish were recorded. Fish were anaesthetized using phenoxyethanol at 0.3 mL L^{-1} of water before measurements.

The specific growth rate (SGR) of fish in different tanks/hapas was calculated as $SGR = 100 \times \frac{\ln(w_2) - \ln(w_1)}{t_1 - t_2}$, where w_1 is biomass at time (t_1) and w_2 is biomass at time (t_2). The condition factor (K) was calculated as:

$K = 100 \times \frac{\text{Body mass}}{\text{Length}^3}$, length being in cm and body mass in g.

Given the role of the liver in lipid metabolism, liver mass was weighed and the hepatosomatic index (HSI) calculated as follows.

$HSI = 100 \times \frac{\text{Liver mass}}{\text{Body mass}}$.

Table 3
Formulation and proximate composition of the Arctic charr trial diet.

Ingredient (g kg^{-1})	Experimental diet				
	OX0	OX1	OX2	OX3	OX4
Wheat flour	139.8	139.8	139.8	139.8	139.8
Soy 47/5 Brasil	70.0	70.0	70.0	70.0	70.0
Mono calcium phosphate	2.5	2.5	2.5	2.5	2.5
Fish oil[a]	152.7	152.7	152.7	152.7	152.7
Wheat gluten	100.0	100.0	100.0	100.0	100.0
Fish meal (NSM-715/85)	524.9	524.9	524.9	524.9	524.9
Premix Laxá[b]	10.0	10.0	10.0	10.0	10.0
Chemical composition (%)					
Dry matter	90.75 ± 0.12	83.29 ± 0.05	84.79 ± 0.23	87.21 ± 0.22	90.05 ± 0.27
Crude protein	48.16 ± 0.60	46.17 ± 0.15	47.10 ± 0.39	46.91 ± 1.22	47.22 ± 0.78
Crude lipid	20.14 ± 0.31	20.10 ± 0.11	20.53 ± 0.38	20.51 ± 0.63	21.51 ± 0.10
Ash	7.92 ± 0.04	7.27 ± 0.01	7.42 ± 0.03	7.59 ± 0.27	7.74 ± 0.03

[a] Herring fish oil was oxidized in the rest of the diets except OX0.
[b] Premix Laxa (mg/kg diet): biotin, 3000; calcium, 32,000; Cl, 26; Co, 1; crude ash, 91,600; Fe, 300; folic acid, 30; I, 30; Mn, 100; NaCl, 25; P, 130; K, 14.5; Na, 428.3; Zn, 2000; Se, 0.3; Vit A, 25; vit B1, 70; vit B2, 150; vit B3, 400; vit B6, 150; Vit B12, 50; vit C, 1000; vit E, 1000; Vit D3, 15; niacine, 300.

Body composition was analyzed in 10 fish from each experimental treatment at the end of the experiments. Dry matter, ash, crude protein and crude lipids were analyzed following standard procedures outlined by AOAC (1995).

2.5. Determination of antioxidant enzyme activities

Antioxidant enzyme activity was determined in Artic charr. Liver samples were homogenized in 0.05 M Phosphate buffer pH 7.8 (Tissue:buffer ratio 1:3), after which samples were placed in a 1.5 mL Eppendorf tube for sonication. Homogenization and sonication were both done on ice. Sonication was done three times for 90 s separated by 30 s intervals for cooling. Treated samples were kept at −80 °C until enzyme assays were performed. Catalase and GPx activities were assayed according to Weydert and Cullen (2010), while total SOD was determined using a kit obtained from Sigma Aldrich (19,160 SOD determination kit). Protein concentration in the supernatant solutions was determined by modified method of Lowry et al. (1951) following degradation of NaOH/sodium dodecyl sulphate (SDS).

2.6. Statistical analysis

All statistical analyses were performed with SPSS version 18 (SPSS, Chicago, IL, USA). To determine whether significant differences occurred amongst fish groups fed diets at different oxidation states, a one-way mixed model analysis of variance (ANOVA) was used with tanks/hapas nested as random factors within treatments. Significant differences in group means were compared using Bonferroni test. *P*-values < 0.05 were taken to indicate significant differences.

3. Results

3.1. Oxidation levels of oils

The AnV and TBARs values of the oxidized oils increased progressively with time of exposure to oxygen and high temperature (50–70 °C; Tables 1 and 2). There was a nine fold and 23 fold increase in POV of OX1 and OL1 respectively compared with OX0. The increase in POV of OX2, OX3 and OL3 was 2–3 fold (Tables 1 and 2). Peroxides in OX4 were completely converted to secondary products and could not be detected. A reduction in the POV content was succeeded by an increase in the secondary oxidation products as indicated by the high AnV in OX2-OX4 oils for Arctic charr and OL2 for Nile tilapia diets.

3.2. Arctic charr growth trial

The oxidation level of the diets significantly ($P < 0.05$) affected the growth of the fish (Fig. 1). The final body mass of fish fed diet OX1 with the highest POV was 13% lower ($P < 0.05$) than in groups fed fresh (OX0) and very highly oxidized oils OX4 (Fig. 1). Compared to the groups reared on diets OX0, OX2, OX3 and OX4, final body mass of OX1 was less ($P < 0.05$) by about 14.5, 10.7, 8.0 and 14.0%, respectively (Fig. 1). Specific growth rate was also significantly lower in the group fed diet OX1 than in other groups (Fig. 2a). The HSI was significantly higher ($P < 0.05$) in the OX1 group than in the rest of the groups (Fig. 2b). Condition factor was significantly lower in fish fed unoxidized oil ($P < 0.001$; Fig. 2c). Fillet protein and lipid content as well as dry matter and ash did not differ across groups (Table 5). Survival was above 97% in all treatment groups and did not differ.

3.3. Liver antioxidant enzyme activities in Arctic charr juveniles

The oxidation levels of the diet affected the hepatic antioxidant enzyme activities in Arctic charr. Catalase and GPx activities were significantly higher ($P < 0.05$) in the group fed highly oxidized dietary oils (i.e., OX4, with trace peroxide) than in the other groups (Table 6). No differences were found in the liver catalase activities amongst groups fed diets OX0, OX1, OX2 and OX3 dietary oils. GPx activity in the groups fed diets OX0 and OX3 was similar and significantly lower ($P < 0.05$) than in the groups fed diets OX1 and OX2. The activity of GPx in groups

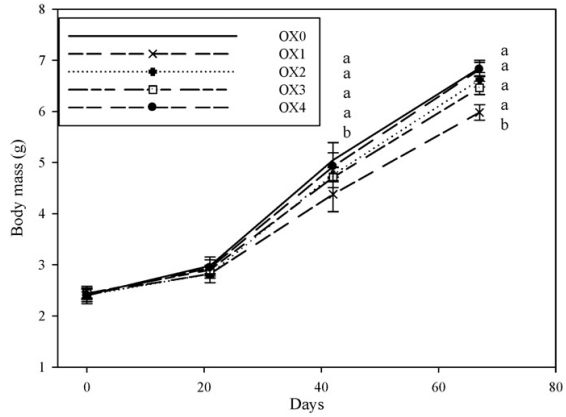

Fig. 1. Mean body-mass (±SEM) of Arctic charr fed a diet with different oxidation levels (Bonferroni, $P < 0.05$, $n = 4$).

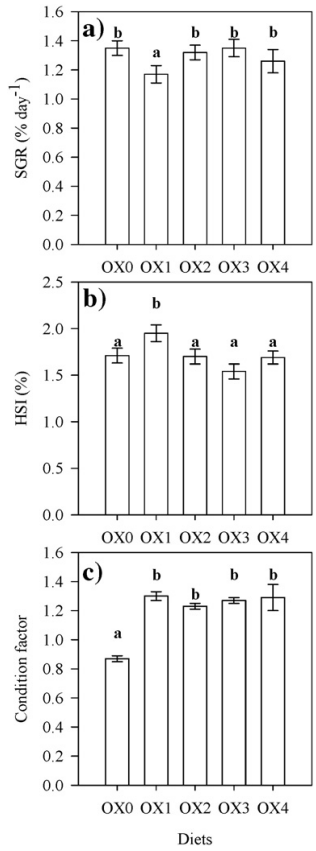

Fig. 2. Specific growth rate (SGR) (a), hepatosomatic index (b), and final condition factor (c), in Arctic charr fed a diet with different oxidation levels (Bonferroni, $P < 0.05$; $n = 4$).

Table 5
Proximate composition of juvenile Arctic charr fish fillets at the end of the trial*.

Parameter	Dietary oxidation level (% ± SE, n = 3)				
	OX0	OX1	OX2	OX3	OX4
Protein	18.80 ± 0.36	16.74 ± 0.72	17.09 ± 0.12	16.56 ± 0.28	17.05 ± 0.08
Lipid	21.07 ± 3.02	18.54 ± 1.00	20.60 ± 1.15	24.01 ± 1.85	27.31 ± 1.60
Dry matter	28.27 ± 0.60	25.39 ± 0.24	24.78 ± 0.34	25.09 ± 1.02	26.07 ± 0.43

*The mean values for each group are based on three samples, each pooled from eight fish.

fed on OX1 and OX2 did not differ. In contrast, the activity of SOD was significantly lower ($P < 0.05$) in the group fed the highly oxidized dietary oil (OX4) than in the rest of the groups (Table 6).

3.4. Tilapia growth trial

Throughout the experiment, growth of tilapia fed fresh herring oils remained lower than in those fed moderately and highly oxidized oils (Fig. 3). By the 30th day (Fig. 3), the body mass of the tilapia group fed on OL0 diet (4.48 ± 0.34 g) was significantly lower ($P < 0.05$) than that of the groups fed OL1 (6.44 ± 0.61 g) and OL2 diets (6.04 ± 0.78 g). There were no significant differences between groups fed OL1 and OL2 by day 30. However, the growth rate of groups fed oxidized oils (OL1 and OL2) decreased between days 30 and 60 while that of the group fed fresh oil (OL0) was maintained similar as during the previous period (Fig. 3). By day 60, the body masses of groups fed highly oxidized (6.81 ± 0.50 g) and fresh oils (6.71 ± 0.59 g) were comparable. After day 60, the growth rate of fish fed oxidized oils increased again such that by day 90, the body mass of fish fed fresh oils was 26% and 23% lower ($P < 0.05$, Fig. 3) than in the groups fed OL1 and OL2 oxidized oils respectively.

At the end of the experiment, both SGR and HSI (Table 7) did not differ amongst the three groups. However, the mean condition factor of fish reared on diet OL0 (2.95 ± 0.18) was significantly lower ($P < 0.05$) than in groups fed diets OL1 (3.34 ± 0.18) and OL2 (3.39 ± 0.16). As in Arctic charr, the condition factor of the tilapia increased with lipid oxidation level (Table 7). The fillet lipid content decreased ($P < 0.05$) with increased oxidation level (Table 8) being 76% lower in the OL2 than in the OL0 group. Nile tilapia survival was above 90% in all treatment groups and did not differ.

4. Discussion

The oxidation level of the experimental herring oils increased progressively with prolonged exposure to heat and oxygen. The longer the oils were exposed to heat, the higher were the AnV and TBARs values and the lower was POV (Tables 1 and 2). Hence, the concentration of secondary lipid oxidation products (AnV and TBARs) were comparatively low in oils used in diets OX1 and OL1 that were treated for shorter periods (Tables 1 and 2), while the primary products (POV) were high, indicating that these oils had mainly gone through primary oxidation. In oils OX2, OX3 and OX4 used in Arctic charr diets, POV decreased while the AnV and TBARs values progressively increased (Table 1), suggesting an increasing level of secondary oxidation. Similarly, the TBARS values were higher and POV lower in the OL2 compared with the OL1 oil in the tilapia diets.

The oxidation level of the diet affected the growth performance of the fish. The growth of the Arctic charr was poorest in the group fed the OX1 diet (Fig. 1) which contained the highest amount of primary oxidation products (Table 1). However, there was no difference in growth of the control group (OX0) and the groups fed diets containing oils that had progressed into secondary oxidation. Therefore, increased levels of secondary oxidation products appeared to have limited effects on the growth of Arctic charr. The increased activities of CAT and GPx with dietary lipid oxidation suggest that the Arctic charr can adapt to increased levels of secondary oxidation product. Boglino et al. (2014) suggested that increased antioxidant enzyme activity enhances both antioxidant defense and detoxification thus promoting growth. Pérez-Campo et al. (1993) also reported a positive correlation between antioxidant enzyme activities and oxidative stress tolerance in vertebrates. In contrast, the activity of SOD was not increased in Arctic charr fed diets containing higher levels of products of primary oxidation (OX1).

The growth of tilapia was also affected by the oxidation level of the diets although the responses were quite different from those of Arctic charr. The body mass was lowest in the group fed a diet with unoxidized oil (low POV and low TBARs), but increased with lipid oxidation; indicating that increased oxidation levels may stimulate growth and/or appetite in juvenile Nile tilapia. An increase in fish length, SGR, and condition factor (Table 7) affirms that tilapia fed oxidized lipids increases both in girth and length. Furthermore, the dietary oxidation also affected the flesh lipid content of the tilapia and was lower in groups fed oxidized diets. The decline in flesh lipid content in groups fed oxidized oils (Table 8) suggests that lipid oxidation affects the energy allocation in Nile tilapia.

The results from different studies on the effects lipid oxidation in fish are variable and yield contrasting responses (Alves Martins et al., 2007; Lewis-McCrea and Lall, 2007; Dong et al., 2011), similar to those of Arctic charr and Nile tilapia in this study. In some instances, the effects of feeding fish on oxidized diets are positive while in other fish, the effects are negative. For example, Chen et al. (2012) reported improved growth in juvenile largemouth bass (*Micropterus salmoide*) fed oxidized diets. On contrary, growth of turbot (*Scophthalmus maximus*) and Atlantic halibut (*Hippoglossus hippoglossus*) was progressively reduced with increased dietary lipid oxidation (Lewis-McCrea and Lall, 2007). In

Table 6
Antioxidant enzyme activity in juvenile Arctic charr fed oils at different oxidation levels.

Antioxidant enzyme	Dietary lipid oxidation level (mean ± SE, n = 3)				
	OX0	OX1	OX2	OX3	OX4
CAT (U/mg protein)	132.32 ± 33.04^{a}	165.96 ± 8.35^{a}	182.78 ± 10.76^{a}	175.19 ± 16.77^{a}	250.02 ± 7.25^{b}
GPx (U/mg protein)	159.77 ± 11.78^{a}	235.13 ± 17.25^{b}	236.15 ± 37.27^{b}	246.48 ± 13.05^{a}	342.08 ± 19.59^{c}
SOD (U/mg protein)	65.66 ± 1.14^{b}	66.56 ± 1.42^{b}	62.38 ± 3.57^{b}	63.58 ± 2.37^{b}	51.84 ± 2.74^{a}

CAT: Catalase; GPx: glutathione peroxidase; SOD- superoxide dismutase.
Values with different superscripts within a row are significantly different ($P < 0.05$).

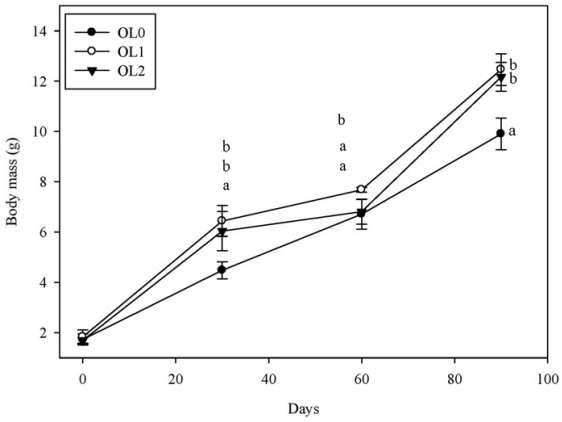

Fig. 3. Mean body-mass (±SEM) of tilapia fed a diet with three oxidation levels. Points indicated by different letters represent significantly different values (Bonferroni, $P < 0.05$; $n = 4$).

halibut (Lewis-McCrea and Lall, 2007) and black seabream (*Spondyliosoma cantharus*) fed oxidized oils with POV of up to 45 meq kg^{-1}, HSI increased with dietary oxidation (Peng et al., 2009).

Liver antioxidant defense enzyme activities was found to increase in sea bream and turbot fed peroxidised oil (Sargent et al., 2002; Tocher et al., 2002, 2003; Lewis-McCrea and Lall, 2007).

Diet acceptability by fish is also affected by dietary oxidation levels. In Atlantic salmon (*Salmo salar*) for example, feed intake was high in the group fed less oxidized diets (with high concentration of POV) and low in the group fed highly oxidized diet (Hamre et al., 2001), suggesting high sensitivity of Atlantic salmon to secondary oxidation. However, in the present study, the highest HSI and reduced body mass were observed in Arctic charr fed a diet with POV of 182 meq kg^{-1} oil, indicating high sensitivity of Arctic charr to primary oxidation. In some fish species like gilthead seabream (*Sparus aurata*) (Maurente et al., 2000), Atlantic cod (Zhong et al., 2008) and Chinese longsnout catfish (Dong et al., 2011), growth was not affected by dietary lipid oxidation. The reasons for these contradicting results may be complicated and relate to species and/or size differences in tolerance. Moreover, these differences may also be a result of dietary factors such as the amount of lipid consumed and the amount of antioxidants included in the diet.

Addition of vitamin E to the diet may ameliorate the negative effects of oxidized oils on fish. For example in juvenile hybrid tilapia (*O. niloticus* × *Oreochromis aureus*), oxidized dietary oils did not affect growth, feed conversion ratio and protein efficiency ratio when more than 80 IU of vitamin E kg^{-1} was added to diet (Huang and Huang, 2004). Similarly, in turbot, sea bream and halibut fed oxidized diets, survival was better in groups where vitamin E was supplemented (Tocher et al., 2003). Growth, feed conversion efficiency and hematocrit values were not affected when coho salmon (*Oncorhynchus kisutch*) were reared on oxidized diets supplemented with 30 and 1030IU of vitamin E kg^{-1} in diet (Forster et al., 1988). Vitamin E effectively protected largemouth bass juveniles fed oxidized dietary oils (Chen et al., 2013). In contrast, Tocher et al. (2002) found reduced growth, poor survival and increased liver antioxidant enzyme activity in sea bream fed peroxidized diets containing low vitamin E. In Atlantic halibut fed oxidized diets with POV in the range of 0.6 to 15 meq kg^{-1} oil (Lewis-McCrea and Lall, 2007) and juvenile Atlantic cod fed oils oxidized to POV of 94 meq kg^{-1} of oil (Zhong et al., 2008), with or without α-tocopherol, growth, survival, hepatosomatic indices and hematocrit were not affected (Lewis-McCrea and Lall, 2007).

Table 7
Specific growth rate (SGR), hepatosomatic index (HSI) and condition factor (K) in tilapia fed dietary oxidized fish oils for 90 days.

Oxidation level	SGR(% day^{-1})	HSI (%)	Condition factor (K)	Length (cm)
OL0	2.17 ± 0.15	1.19 ± 0.05	2.95 ± 0.18^{a}	7.18 ± 1.80^{a}
OL1	2.43 ± 0.03	1.32 ± 0.07	3.34 ± 0.18^{ab}	7.84 ± 1.50^{b}
OL2	2.42 ± 0.21	1.35 ± 0.04	3.39 ± 0.16^{b}	7.91 ± 1.73^{b}
P-value	0.224	0.195	0.023	0.001

Values with different superscripts within a column are significantly different ($P < 0.05$).

Table 8
Proximate composition of juvenile Nile tilapia fish fillets at the end of the trial.

Parameter	Dietary oxidation level (% ± SE, n = 3)		
	OL0	OL1	OL2
Protein	14.01 ± 0.04	14.55 ± 0.29	14.52 ± 0.18
Lipid	14.38 ± 0.79^{c}	8.36 ± 0.83^{b}	3.40 ± 0.83^{a}
Dry matter	24.76 ± 0.71	25.78 ± 0.70	25.09 ± 0.22
Ash	18.13 ± 0.01^{c}	15.47 ± 0.35^{a}	16.50 ± 0.07^{b}

Values with different superscripts within a row are significantly different at ($P < 0.05$).

The concentration of vitamin E in diets fed to the Arctic charr (approx. 10 IU vitamin E kg^{-1}) in the present study was comparatively low and clearly inadequate to counteract the effects of lipid oxidation in the diets. The amount of vitamin E in the diets for the Nile tilapia was even lower (approx. 4 IU vitamin E kg^{-1}). However, the Nile tilapia were reared in a semi-natural pond system where they may have ingested phytoplankton that contain natural antioxidant compounds. Furthermore, the lipid content of the tilapia diets was approx. 6% while that in Arctic charr diets was 20%, meaning that Arctic charr ingested much more oxidized oils than the Nile tilapia.

4.1. Conclusion

The effect of lipid oxidation on growth and physiology varies amongst different fish species. This is clearly demonstrated in this study where lipid oxidation reduced the growth of Arctic charr but increased the growth of Nile tilapia when compared to fish fed diets with un-oxidized lipids. These differences may be the result of species specific differences in tolerance to oxidized lipids, variable lipid intake or access to vitamin E/tocopherol in feed.

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ORIGINAL ARTICLE

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Practical feeds for juvenile Nile tilapia (*Oreochromis niloticus*) prepared by replacing *Rastrineobola argentea* fishmeal with freshwater shrimp (*Caridina nilotica*) and mung bean (*Vigna radiata*) meals

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Abstract

This study examined the potential of using the freshwater shrimp, *Caridina nilotica* (CNM), and mung beans, *Vigna radiata* (VRM), to replace *Rastrineobola argentea* fishmeal (RAF) as the primary protein source in fish feeds in East Africa. Six diets with varying proportions of RAF, CNM, VRM and full fat soybean meal were tested on Nile tilapia (initial body mass ± *SD*: 3.30 ± 0.27 g). The growth performance was best in groups fed diets containing either *R. argentea* or *C. nilotica* or a combination of the two. Growth was poorest in the fish fed a combination of *C. nilotica* and *V. radiata*, and intermediate in the groups fed the commercial formulation and a diet containing a combination of *R. argentea* and *V. radiata*. The cost of feed per kg of fish produced decreased with increasing inclusion of *C. nilotica*. The price of feed per kg fish produced was comparatively high in feeds containing *V. radiata* due to poor feed conversion ratio. Of all the diets tested, the price of feed per kg of fish produced was highest in the commercial formulation. *Caridina nilotica* is a good candidate to substitute *R. argentea* fishmeal in practical diets for Nile tilapia.

KEYWORDS
feed formulation, growth, Nile tilapia, practical diets, protein sources, reduced cost

1 | INTRODUCTION

Feeds constitute more than half of the total production costs in intensive aquaculture with protein being the most expensive component (Cheng et al., 2010; Watanabe, 2002). Fishmeal (FM) is an ideal source of protein in feeds for most fish species (Naylor et al., 2000; NRC, 2011). However, due to limited availability and high price of FM, feed producers are opting for other protein sources. Moreover, there is an increasing demand from consumers and retailers for reduced use of low-tropic-level pelagic fish for FM production (Naylor et al., 2000; NRC, 2011). In fact, sustainable protein acquisition is seen as a limiting factor to aquaculture development (Cheng, Hardy, & Usry, 2003; Cheng et al., 2010; Deng et al., 2009; Watanabe, 2002).

In East Africa, dried silver cyprinid (*Rastrineobola argentea*: common names: mukene, dagaa, omena), an indigenous pelagic fish species from Lake Victoria, is used as fishmeal. However, there is an increasing demand for *R. argentea* for human consumption. Furthermore, sun-dried *R. argentea* FM undergoes peroxidation during storage (Kubiriza et al. in prep.) which may create off flavours, reduce growth and in extreme cases cause mortalities (Fontagné-Dicharry et al., 2014; NRC, 2011; Sargent, Tocher, & Bell, 2002). Consequently, it is imperative to search for alternative protein sources to replace *R. argentea* FM in aquafeeds in E. Africa.

A number of alternative protein sources for Nile tilapia feed have been tested. Most of these raw materials are derived from different plant sources (Abowei & Ekubo, 2011; Al-Hafedh & Siddiqui, 1998; De Silva & Gunasekera, 1989; Deng et al., 2009; El-Saidy & Gaber, 2003; El-Sayed, 1999; Fall, Tseng, Ndong, & Sheen, 2012; Keembiyehetty & de Silva, 1993; Liti, Cherop, Munguti, & Chhorn, 2005; Nahid, Perumal, & Klaus, 2003; Olivera-Castillo et al., 2011) or animal by-products (El-Sayed, 1998, 1999; Hernã Ndez et al., 2010; Mugo-Bundi et al., 2015; Munguti, Waidbacher, Liti, Straif, & Zollitsch, 2009). Several of these are available in E. Africa, but are sought after for human consumption, livestock production and other industrial use which raises their prices (Cheng et al., 2003, 2010; Deng et al., 2009; Luo et al., 2012; Watanabe, 2002).

Although popular for human consumption in many parts of the world, mung beans (*Vigna radiata*) are in limited demand for human consumption in Uganda and are presently not used in animal feeds although they grow well in the area. Elsewhere, *V. radiata* has been successfully used in diets for broiler chicken (Creswell, 1981) and Asian sea bass (Eusebio & Coloso, 2000). Other legumes of the genus *Vigna* have also been tested on Nile tilapia and yielded positive, albeit slightly contradicting results (Keembiyehetty & de Silva, 1993; Olivera-Castillo et al., 2011). Results from a study by De Silva and Gunasekera (1989) suggest that *V. radiata* can partly replace fishmeal in the diets of juvenile Nile tilapia. In their study, fish fed diets where 250 g kg^{-1} of the fishmeal was replaced by *V. radiata* grew well, while growth rate was compromised when the inclusion levels were higher (i.e., when 500 g kg^{-1} of fishmeal per kg of diet was replaced by *V. radiata*). These results suggest that *V. radiata* may be a good protein source to partially replace fishmeal in Nile tilapia feed.

The freshwater shrimp *Caridina nilotica* (common name: Ochonga) may be a suitable protein source in Ugandan aquafeeds. It is a natural prey of Nile tilapia and a bycatch that constitutes 10% of *R. argentea* landed (Michael Kasinga personal communication). The decline in Nile perch (*Lates niloticus*) stocks in Lake Victoria (Getabu, Tumwebaze, & MacLennan, 2003; Taabu-Munyaho et al., 2013) has led to increased abundance of *C. nilotica*. The annual catch of *R. argentea* is about 120,000 MT on the Ugandan side of Lake Victoria (LVFO, 2014; Taabu-Munyaho et al., 2014), suggesting that as much as 12,000 MT of *C. nilotica* may be available annually for fish feeds in Uganda. In fact about 50,000 MT of *C. nilotica* could be accessed for fish feed production from the estimated 500,000 MT of *R. argentea* landed from the whole of Lake Victoria annually (LVFO, 2014).

Caridina nilotica and *R. argentea* are dried together and are separated later by hand. Considerable quantities of *C. nilotica* are discarded during the processing because the interest and emphasis are put on *R. argentea*. Therefore, suitable harvesting and processing protocols for *C. nilotica* should be developed, if it is to be used as an alternative protein source to *R. argentea* in large-scale feed formulation.

The results of a recent study suggest that *C. nilotica* can be used to partially replace *R. argenta* FM in feed for Nile tilapia (Mugo-Bundi et al., 2015). The growth of the tilapia was best when *C. nilotica* replaced 250 g kg^{-1} of the FM and better than when *R. argentea* FM was the only protein source. In contrast, the growth was reduced and feed conversion ratio increased when all the FM was replaced by *C. nilotica* (Mugo-Bundi et al., 2015). This study was performed in tanks under controlled conditions where water quality was maintained at a fairly constant level. However, it is of interest to test *C. nilotica* in feed under semi-intensive conditions similar to those in commercial fish farms using ponds in Uganda where the oxygen levels, pH and carbon dioxide fluctuate diurnally. Moreover, fish in ponds may derive a significant portion of their diet from plankton, and, thus, the growth of the fish may be determined by complicated interactions between the natural food sources and the supplemented feed (Tacon & De Silva, 1997).

This study was conducted to test locally available protein ingredients, *V. radiata* and *C. nilotica*, in diets to replace *R. argentea* FM in practical diets for Nile tilapia reared in ponds in Uganda. Additionally, the economic feasibility of using these raw materials in feed was examined.

2 | MATERIALS AND METHODS

2.1 | Experimental feed formulation

All diets were formulated using WinMix supplied by WinMix Soft Ltd (http://www.winmixsoft.com), Ukraine. Dried *C. nilotica*, *V. radiata* and *R. argentea* were used in varying proportions with full fat Soybean meal (SBM) as protein sources to formulate six different diets for Nile tilapia (Table 1). Diets were formulated to approximate crude protein (CP: 300 g kg^{-1}) and crude lipid (EE: 70 g kg^{-1}) content of the commercial feeds used to raise juvenile Nile tilapia on farms in Uganda. For comparison, one of the diets tested (COM) was made following a commercial formula for the feeds available on the market in Uganda which is produced with SBM (405 g kg^{-1}) and *R. argentea* (163 g kg^{-1}) as the primary protein sources (Table 1). A second diet (R) contained more *R. argentea* (233 g kg^{-1}) and less of SBM than COM. The third diet (RV) contained *V. radiata* (150 g kg^{-1}) and less *R. argentea* (174 g kg^{-1}) than diet R. The fourth diet (RC) contained *C. nilotica* (130 g kg^{-1}) and *R. argentea* (140 g kg^{-1}). In the fifth diet (RC), *C. nilotica* (145 g kg^{-1}) and *V. radiata* (129 g kg^{-1}) replaced all the *R. argentea*. The sixth diet (C) was formulated with *C. nilotica* (270 g kg^{-1}) as the primary protein source. To maintain the protein content at target level of 300 g kg^{-1}, the amount of SBM was reduced in diets RC and C. All diets were pelleted using a simple kitchen pelletizing machine (NIKAI, NMG-744, China) and air-dried prior to feeding the fish.

2.2 | Fish husbandry

The experiment was performed at Kireka fish farm, central Uganda. Juvenile Nile tilapia were obtained from the National Aquaculture Research and Development Center (ARDC), Kajjansi. The fish were acclimatized in a 200-L concrete tank using a commercial feed from Ugachick Poultry Breeders Ltd containing 350 g of CP per kg of diet for 1 week, before they were distributed among 24 hapas (1.2 m by 1.2 m by 1 m deep) installed in one pond. Each experimental diet was tested in quadruplicate groups of 100 fish per hapa. The average body mass at the commencement of the experiment was 3.30 ± 0.27 g. Throughout the experimental period (90 days), all groups were hand-fed to

TABLE 1 Ingredients, their cost per kg and crude protein content, formulations and proximate composition of the diets tested

Ingredient	Cost per kg (USD)	CP content (g kg^{-1})	Inclusion level of each ingredient (g kg^{-1}) in trial diets					
			COM	R	RV	RC	CV	C
Toasted full fat soy	0.62	383	405	300	310	193	307	137
Wheat bran	0.13	157	200	200	200	200	200	200
*Caridina nilotica**	0.58	650	–	–	–	130	145	270
Vigna radiata	0.77	238	–	–	150	–	129	–
Rastrineobola argentea	1.35	531	163	233	174	140	–	–
Whole maize	0.31	83	114	148	46.9	218	100	274
Wheat pollard	0.15	155	110	110	110	110	110	110
Salt	0.23	0	2	2	2	2	02	2
Ugachick vitamin pemix[1]	2.31	0	2	2	2	2	2	2
Ugachick mineral premix[2]	3.08	0	5	5	5	5	5	5
Chemical analysis (g kg^{-1})								
Dry matter			887.7 ± 0.9	891.5 ± 0.05	892.1 ± 3.4	878.0 ± 1.0	898.1 ± 3.8	893.7 ± 11.5
Crude protein			305.5 ± 5.1	301.0 ± 0.3	295.4 ± 4.2	294.8 ± 6.5	293.3 ± 4.9	297.8 ± 3.5
Crude lipid			69.6 ± 0.4	74.7 ± 0.3	77.0 ± 6.1	709 ± 1.0	73.8 ± 0.7	67.8 ± 5.6
Ash			83.2 ± 1.5^{b}	84.3 ± 0.2^{b}	99.0 ± 3.4^{c}	84.4 ± 0.2^{b}	75.4 ± 2.3^{a}	75.4 ± 1.6^{a}
Crude fibre			48.5 ± 0.3^{c}	38.2 ± 0.4^{a}	42.7 ± 0.6^{b}	38.1 ± 0.3^{a}	435 ± 0.3^{b}	48.0 ± 0.1^{c}
Gross energy (kJ g^{-1})			16.9	17.4	17.0	16.3	16.8	17.2

[1]Vitamin premix (mg kg^{-1} of diet): thiamine = 1,000; pyridoxine = 1,000; folic acid = 250; ascorbic acid = 20,000; pantothenic acid = 3,000; myo-inositol = 2,000; biotin = 100; niacin = 7,500; cyanocobalamin = 10; riboflavin = 1,000; retinol acetate = 400; tocopheryl acetate = 2,000; menadione = 4,000; Cholecalciferol = 30; dextrin = 93,971.

[2]Mineral premix (mg kg^{-1} diet): $Ca(H_2PO_4).2H_2O$ = 180,000; $CaCO_3$ = 140,000; $MgSO_4.7H_2O$ = 5,100; $FeSO_4.7H_2O$ = 1,000; $NaHCO_3$ = 6,880; $MnSO_4.4H_2O$ = 350; KIO_3 = 10; $CoCl_2.6H_2O$ = 20; $Na_2MoO_4.2H_2O$ = 80; $NaSeO_3$ = 20; $KH_2PO_4.2H_2O$ = 11,996; $ZnCO_3$ = 150; $CuSO_4.5H_2O$ = 30; NaCl = 2,470; $Al_2(SO_4)_3$ = 20.

*While *Caridina nilotica* is currently discarded, its cost per kg in this study was not zero (0). A cost of USD 0.58/kg was derived from the cost of collecting and transporting it from the landing site to the experimental area. Values with different superscripts in a column are significantly different ($p < 0.05$).

near-satiation three times a day. The hapas were cleaned fortnightly to remove residue feed and filamentous algae attached to the walls. Every other day, dissolved oxygen (DO) and water temperature (WT) were measured with YSI DO 200 m, and pH with EC500 pH/Conductivity ExStik® II meter. Dissolved oxygen ranged from 1.66 ± 0.13 at dawn to 6.81 ± 0.52 mg L^{-1} in the afternoon, temperature from 18.92 ± 0.28 to 29.90 ± 1.15°C and pH from 5.43 ± 0.04 to 7.43 ± 0.17. All these parameters did not differ across treatments ($p > .05$).

2.3 | Sampling and growth assessment

Total fish biomass was measured at the commencement of the study and subsequently at 30-day intervals. At the end of the study, individual fish body mass and length were measured and used to estimate the growth indices: mean weight gain (WG), specific growth rate (SGR) and condition factor (k) as follows:

WG = Final mean body mass (FBM) − Initial mean body mass (IBM),

SGR = 100 × [ln (w_2)−ln (w_1)]/(t_2−t_1), where w_1 is biomass at time (t_1) and w_2 is biomass at time (t_2),

K = 100 × (Body mass/Length^3), length being in cm and body mass in g.

Survival was calculated as follows:

$$\text{Survival} = 100 \times \frac{\text{Number of live fish per replicate}}{\text{Number of fish stocked per replicate}}$$

A total of 40 fish were sampled from each diet treatment (10 from each replicate) for measuring visceral somatic index (VSI) and hepatosomatic index (HSI) calculated as follows:

$$\text{VSI} = 100 \times \frac{\text{Visceral mass (g)}}{\text{Fish body mass (g)}}$$

$$\text{and HSI} = 100 \times \frac{\text{Liver mass (g)}}{\text{Body mass (g)}}$$

2.3.1 | Estimation of feed efficiency

Data on the weight of feed given and fish weight gain were collected and used to estimate the apparent feed conversion ratio (AFCR) in the respective treatments as follows:

$$\text{AFCR} = \frac{\text{Weight of dry feed given (g)}}{\text{Fish weight gain (g)}}$$

2.3.2 | Protein cost and cost of feed per kg of feed

Using ingredient cost and the inclusion levels per diet (Table 1), the practical cost of each diet was estimated.

Furthermore, protein cost per kg of feed and feed cost per kg of fish produced were computed as follows:

$$\text{Protein cost per kg of feed(USD/kg)} = \frac{\text{Cost of protein in a kg of feed (USD)}}{\text{Weight of protein in a kg of feed (kg)}}$$

$$\text{Feed cost per kg of fish produced(USD/kg)} = \frac{\text{Cost of dry feed given (USD)}}{\text{Fish weight gain (kg)}}$$

2.4 | Chemical analyses

Dietary ingredients and formulated feeds were analysed for proximate composition using standard methods (AOAC, 1995). Moisture was determined by drying samples in an oven at 105°C for 24 hr. Crude protein was estimated as Nx6.25, after determining nitrogen (N) content of the sample using micro-Kjeldahl (AOAC, 1995). Lipids were extracted using soxhlet apparatus (Soxtec TM 2050 Avanti Extraction Unit), while ash was determined by combustion of dry samples in a muffle furnace at 550°C for 8 hr. The amino acid composition of *R. argentea*, *C. nilotica* and *V. radiata* was analysed by the South African Grain Laboratory (SAGL), the Willows, Pretoria, South Africa. Subsequently, the essential amino acid (EAA) composition (g kg^{-1} of diet) of the different diets tested were calculated using the amino acid content analysed for *R. argentea*, *C. nilotica* and *V. radiata* and those from NRC (2011) for other raw materials used.

2.5 | Statistical analysis

All statistical analyses were performed using PASW version 18 (SPSS, Chicago, IL, USA). Biometric data among fish groups reared on different diets were compared using a one-way mixed model analysis of variance (ANOVA). Normality was assessed by comparing histograms with normal curve, while equal variance was assessed by Bartlett's test. Group means were compared using Bonferroni technique at *p*-values <.05.

3 | RESULTS

The protein content of *V. radiata* (238 g kg^{-1}) was only about a third of the protein content of *C. nilotica* (650 g kg^{-1}) and about half of *R. argentea* (531 g kg^{-1}) (Table 1). As a result, the EAA content of *V. radiata* (g kg^{-1}) was lower than in both *C. nilotica* and *R. argentea* (Table 2). However, the proportional composition of EAA in all raw materials was comparable (Table 2).

The calculated EAA content of the feeds prepared was similar (Table 3). However, compared with the NRC (2011) recommendations for Nile tilapia, all diets were deficient in methionine. Diets CV and C were further deficient in three EAA, histidine, threonine and valine. Diet COM was deficient in histidine and valine, while diet RC was deficient in valine (Table 3).

Growth performance was significantly ($p < .05$) affected by diet from day 60 when the body mass of fish fed diet R (with high proportion of FM) was significantly higher than in all other groups (Figure 1). Similarly, at the end of the experiment, the FBM and WG were significantly affected by diet treatment ($p < .001$). They were highest and not significantly different among groups fed diets R, RC and C (Table 4). The FBM and WG in the group fed the COM diet were intermediate and not significantly different from that of groups fed RV, RC and C (Table 4). The FBM and WG were lowest in the group fed diet RV (Table 4) and significantly different from that of all other groups (Table 4).

The AFCR was higher in groups fed diets COM and RV than in all other groups (Figure 2). The SGR was not significantly different among groups. The average survival rate was 91% and not significantly different among groups (Table 4).

The condition factor was significantly ($p < .001$) different among groups. The *K* was highest in the group fed diet RV and significantly different from that of all groups. The *K* was lowest in groups fed diets COM and C being 23% less than in RV. The VSI was highest in the groups fed diets COM and RC and significantly ($p < .001$) higher than in other groups. The HSI was not significantly different among groups (Table 4).

The costs of feed (per kg) produced with formulation C were lower than the costs of feed made with other formulations (Table 5). It was 40% less expensive than feed R which was the most expensive formulation (Table 5). However, the price of diet R was only 3% higher than

TABLE 2 Amino acid composition (g kg^{-1}) of dried *Rastrineobola argentea*, *Caridina nilotica* and *Vigna radiata*. Values in parentheses show the proportion of each amino acid within each raw material source

Amino acid	*R. argentea*	*C. nilotica*	*V. radiata*
Essential			
Arginine	53.5 (8%)	55.7 (10%)	20.2 (7%)
Histidine	23.0 (3%)	14.2 (3%)	9.9 (3%)
Isoleucine	29.0 (4%)	24.5 (4%)	11.8 (4%)
Leucine	52.9 (8%)	42.3 (8%)	22.6 (8%)
Lysine	62.2 (9%)	43.2 (8%)	22.3 (8%)
Methionine	14.8 (2%)	10.9 (2%)	4.0 (1%)
Phenylalanine	29.4 (4%)	24.9 (4%)	17.3 (6%)
Threonine	29.8 (4%)	22.3 (4%)	10.2 (4%)
Tryptophan	6.10 (1%)	5.6 (1%)	2.8 (1%)
Valine	32.9 (5%)	27.2 (5%)	15.2 (5%)
Non-essential			
Alanine	42.0 (6%)	36.3 (6%)	13.1 (5%)
Aspartic acid	60.8 (9%)	53.3 (10%)	33.0 (12%)
Cystine	8.00 (1%)	7.4 (1%)	3.10 (1%)
Glutamic acid	100.8 (15%)	82.1 (15%)	52.9 (18%)
Glycine	43.9 (7%)	33.0 (6%)	12.4 (4%)
Proline	29.6 (4%)	22.7 (4%)	12.8 (4%)
Serine	30.9 (5%)	28.6 (5%)	16.0 (6%)
Tyrosine	25.6 (4%)	25.3 (5%)	7.2 (3%)

TABLE 3 Calculated essential amino acid (EAA) composition (g kg^{-1} of diet) for the diets tested

		Experimental diet					
Amino acids	**NRC, (2011)[a]**	**COM**	**R**	**RV**	**RC**	**CV**	**C**
Arginine	12.0	21.9	23.1	22.8	22.8	21.3	21.9
Histidine	10.0	8.9	11.6	15.2	10.9	6.2	8.1
Isoleucine	10.0	13.1	13.6	13.5	12.6	11.9	11.2
Leucine	19.0	23.7	24.8	24.4	23.2	21.3	20.7
Lysine	16.0	20.1	22.9	22.5	20.5	17.6	16.8
Methionine	7.0	5.4	5.9	5.5	5.5	4.5	4.8
Phenylalanine	11.0	14.5	14.8	15.4	13.7	13.7	12.2
Threonine	11.0	12.2	12.9	12.5	11.7	10.4	10.0
Tryptophan	3.0	3.6	3.6	3.6	3.3	3.3	3.0
Valine	15.0	14.6	15.3	15.4	14.2	13.4	12.7

[a]NRC (2011)-recommended amino acid requirements for *Oreochromis spp*.

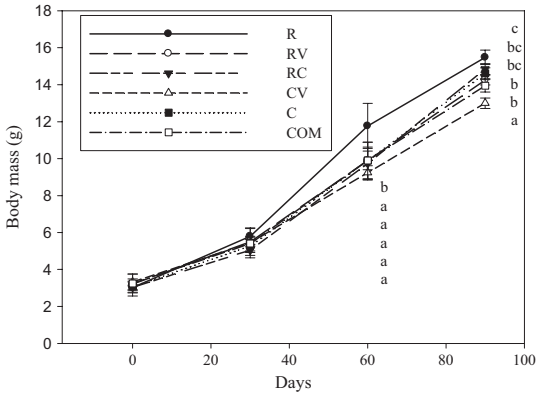

FIGURE 1 Mean body mass (±*SEM*) of Nile tilapia reared on six formulations

that of diet COM. The cost of other feeds was intermediate. The cost of feed per kg fish produced was also lowest for diet C. The cost of producing a kg of fish with diet C was 63% less expensive than for diet COM which was the least economical feed (Table 5).

4 | DISCUSSION

The present study demonstrates the potential of using *C. nilotica* to replace most or all of the *R. argentea* FM in practical feeds for juvenile Nile tilapia grown in ponds. Growth was best when the fish were fed diets containing *R. argentea* (diet R), *C. nilotica* (diet C) or both (diet RC) as the primary protein sources (Table 4). Furthermore, the growth performance of fish fed these diets was equal to or even better than for fish fed commercial formulation (diet COM). Feed utilization was best and comparable among groups fed high proportion of *R. argentea* or *C. nilotica* or a combination of the two (Figure 2) and significantly better than for the groups fed COM. Finally, the price of the feed and the feed cost per kg of fish produced was reduced with increasing inclusion of *C. nilotica*, being lowest in diet C (Table 5). In fact, the price of feed made from formulation C was 38% lower than for COM, and the feed cost for each kg fish produced was 63% less. Taken together, these results suggest that using *C. nilotica* for practical feed formulations could reduce the cost of producing tilapia significantly compared with using the most common brand of commercial fish feeds in Uganda.

The results of the present study concur with those of Mugo-Bundi et al. (2015) in that *C. nilotica* can substitute *R. argentea*

TABLE 4 Growth indices (mean ± *SE*) in *Oreochromis niloticus* reared on diets with different proportions of *R. argentea*, *C. nilotica* and *V. radiata*

Parameter/Diet	COM	R	RV	RC	CV	C	*p*-Value
IBM	3.40 ± 0.90	3.02 ± 0.26	3.21 ± 0.23	3.77 ± 0.71	3.32 ± 0.87	3.07 ± 0.59	.628
FBM	13.94 ± 0.34^{b}	15.48 ± 0.39^{a}	14.25 ± 0.26^{b}	14.85 ± 0.29^{ab}	13.00 ± 0.28^{c}	14.62 ± 0.29^{ab}	<.001
WG	10.54 ± 0.93^{b}	12.46 ± 0.29^{a}	11.05 ± 0.19^{b}	11.08 ± 0.22^{ab}	9.68 ± 0.21^{c}	11.55 ± 0.25^{ab}	.048
SGR	1.58 ± 0.18	1.85 ± 0.07	1.66 ± 0.13	1.86 ± 0.25	1.53 ± 0.20	1.80 ± 0.08	.586
K	1.86 ± 0.03^{a}	2.06 ± 0.03^{b}	2.45 ± 0.03^{d}	2.03 ± 0.04^{b}	2.24 ± 0.03^{c}	1.90 ± 0.02^{a}	<.001
VSI	7.65 ± 0.18^{b}	6.71 ± 0.26^{a}	6.07 ± 0.22^{a}	7.78 ± 0.24^{b}	6.42 ± 0.19^{a}	6.32 ± 0.17^{a}	<.001
HSI	0.66 ± 0.10	0.59 ± 0.09	0.63 ± 0.07	0.58 ± 0.06	0.75 ± 0.07	0.69 ± 0.09	.696
Survival	90.75 ± 13.32	88.75 ± 13.00	95.00 ± 4.76	94.50 ± 7.77	90.75 ± 17.84	87.25 ± 8.54	.920

Values are represented as quadruplicate mean ± *SE*, with 100 fish in each replicate. Different superscripts within a row indicate significant differences among means ($p < .05$, one-way mixed model ANOVA).

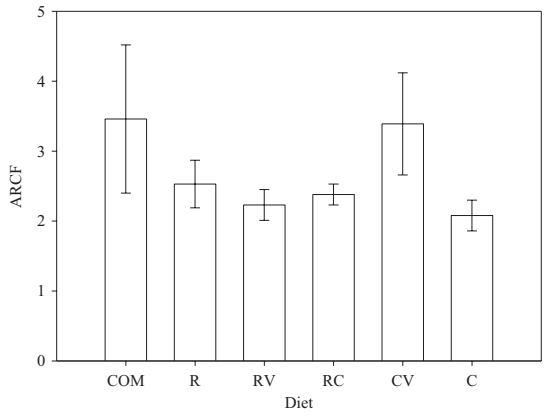

FIGURE 2 Apparent feed conversion ratio of juvenile tilapia fed different experimental diets

TABLE 5 Estimated cost of feed and feed cost per kg of feed and fish produced using the different test diets

Diet	Parameter		
	Cost per kg of feed produced (USD/kg)	Unit protein cost (USD/kg)	Feed cost per kg of fish produced (USD/kg)
COM	0.512	0.017	1.771
R	0.528	0.018	1.336
RV	0.49	0.016	1.093
RC	0.432	0.015	1.028
CV	0.37	0.012	1.254
C	0.319	0.011	0.663

USD 1 = 2,600 UGX.

fishmeal in diets for Nile tilapia. However, comparison of the two studies is complicated because of the differences in composition of the diets used, fish sizes tested and culture conditions (tanks versus ponds). The CP of the diets in the present study (290–310 g kg^{-1} of feed) was slightly higher than that of Mugo-Bundi et al. (2015) of 280 g kg^{-1} of feed; however, their feed contained less of the plant-based protein sources than were used in the present study. Mugo-Bundi et al. (2015) did not observe any negative effects on the growth of Nile tilapia fed diets containing up to 290 g kg^{-1} of *C. nilotica*, although a modest inclusion (100 g kg^{-1}) of *C. nilotica* resulted in the best growth. These findings are consistent with the results of the present study where *C. nilotica* was included up to 270 g kg^{-1} (Diet C) without any significant effect on growth, except when *C. nilotica* and *V. radiata* were added in combination. Thus, both the results of the present study and that of Mugo-Bundi et al. (2015) suggest that *C. nilotica* can partially substitute fishmeal in diets for Nile tilapia.

Although shrimp meal is nutrient rich, several studies have shown that diet digestibility and fish growth are compromised when high proportions are used (Diop et al., 2013; Fall et al., 2012; Mugo-Bundi et al., 2015; Synowiecki & Al-Khateeb, 2000). Shrimp meals made from shrimp shells may contain relatively high amounts of indigestible fibres (chitin and chitosan), which build up when high proportions are used in diet (Fall et al., 2012; Synowiecki & Al-Khateeb, 2000) and make nutrients less accessible by fish (Leal, Castro, Lima, Souza Correia, & Souza Bezerra, 2010). Furthermore, the fibres interfere with emulsification, absorption and utilization of fats by fish (Deng et al., 2009; Diop et al., 2013). The chitin absorbs fats and bile in fish intestine (Tharanathan & Kittur, 2003) and reduces their availability for usage by fish. Therefore, meals from whole shrimp, as used in the present study, may be preferable to shrimp shell meal.

In contrast, inclusion of *V. radiata* in diets for Nile tilapia results in reduced growth performance both when added in combination with *R. argentea* (diet RV) and even more so with *C. nilotica* (diet CV). The results of an earlier study indicated no significant effect of inclusion of *V. radiata* (up to 500 g kg^{-1}) on the growth of Nile tilapia (De Silva & Gunasekera, 1989) at similar levels of CP (i.e., 300 g kg^{-1}), although the final body mass of the fish fed no *V. radiata* was the highest, albeit not significantly so. At a lower CP level (250 g kg^{-1}), the growth of the fish fed no *V. radiata* was significantly better than where it was included (De Silva & Gunasekera, 1989). Thus, the results of the present study, where inclusion of *V. radiata* was 0–170 g kg^{-1}, and that of De Silva and Gunasekera (1989), where inclusion was tested at 0–500 g kg^{-1}, both suggest that *V. radiata* in the diet of Nile tilapia may retard growth. Moreover, the unit cost of protein in *V. radiata* is comparatively high due to the low protein content relative to the price of the raw material (Table 2). That, and the reduced growth rate, suggests that *V. radiata* is not a good candidate raw material for tilapia feed.

The amino acid composition of the diets (Table 3) matched fairly well the recommendations from NRC (2011). However, all diets were deficient in methionine, and diets COM, CV and C were deficient in histidine and valine. Diets CV and C were also slightly deficient in threonine. The fish fed diet R grew best, and this diet was only deficient in methionine, while other diets were also deficient in other essential amino acids (Table 3). However, apart from diet R, differences in the growth of fish fed different diets (Table 3) can hardly be attributed to differences in EAA composition of the diets. Thus, diet C was deficient in the same EAA as diet CV, although the fish fed the latter were significantly smaller at the end of the experiment. The levels of EAA in the diets of the present study were similar to (or slightly higher than) those of Mugo-Bundi et al. (2015). This may be due to the wider range of protein sources used in the present diets than were used in that of Mugo-Bundi et al. (2015).

As raw materials, *R. argentea*, *C. nilotica and V. radiata* all have similar proportional amino acid composition although the CP of *V. radiata* was about 60% lower than in the other two. The CP of *R. argentea* may range between 530 and 700 g kg^{-1} and appears to vary with time of year and processing methods (Kubiriza et al. in prep.). The CP measured by Mugo-Bundi et al. (2015) in *R. argentea* (671 g kg^{-1}) is within this range. It is not known how variable the CP in *C. nilotica* is, although the levels recorded in the present study (650 g kg^{-1}) and that of Munguti et al. (2012) (635 g kg^{-1}) and Mugo-Bundi et al. (2015) (561 g kg^{-1}) suggest that the CP levels may be variable. The EAA composition

of *R. argentea* measured in the present study and that of Mugo-Bundi et al. (2015) are similar apart from methionine and cysteine that were much lower in the present study. It is not clear whether this difference is attributable to variability of the raw material or measurement errors. However, the variability of the raw materials must be taken into account when formulating the feeds.

The AFCR observed in the present study is comparatively high, ranging between 2.2 and 3.5. However, these values are similar to those observed in the study of Liti et al. (2005) (2.6–3) and Liti et al. (2006) on tilapia in ponds (3.4–4.04) but higher than those observed by Liti, Waidbacher et al. (2006) under similar conditions & Mugo-Bundi et al. (2015) for fish reared in tanks (1.19–2.03). The high AFCRs could suggest that the fish were overfed. In general, conditions appear to have been fairly good in the ponds as survival rates were above 87%, even though growth was fairly slow. The overall slow growth rate of fish farmed on different diets could have been due to the daily fluctuation in water quality, particularly dissolved oxygen which fell to as low as 1.66 ± 0.13 mg L^{-1} and temperature to about 19°C at dawn. The diurnal changes in dissolved oxygen and temperature were outside the recommended range for the optimal growth of Nile tilapia and are likely to have exerted prolonged stress and retarded growth. However, if a farming system exhibits the minimum water quality requirements for Nile tilapia, it is likely that faster growth can be achieved using the best experimental feed from this study.

In conclusion, the amino acid composition of fishmeal reflects better requirements of fish than plant sources in general. However, our results suggest that the proportion of amino acid composition of the *V. radiata* meal was similar to that of fishmeal. The present study confirms that *C. nilotica* is a suitable protein source in diets for Nile tilapia. The cost per unit dietary protein and feed cost per kg of fish produced decrease with increasing inclusion of *C. nilotica* in diets, at least to a certain degree, without affecting growth. The use of *C. nilotica* in feeds can reduce the cost of production in tilapia farming. Moreover, *C. nilotica* can easily be accessed locally by fish farmers to formulate on-farm diets for Nile tilapia. *R. argentea* and soybean are expensive protein sources for on-farm and backyard feed formulation; hence, replacing them with the inexpensive *C. nilotica* that is currently in less demand can reduce feed cost. In contrast, *V. radiata* does not appear to be a suitable raw material for Nile tilapia feeds.

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Paper IV

Growth and antioxidant enzyme activity of juvenile Arctic charr (*Salvelinus alpinus*) reared on diets treated with ethoxyquin, rosemary (*Rosmarinus officinalis*) oil and bladderwrack (*Fucus vesiculosus*) powder

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Abstract

In this study, the efficacy of the natural antioxidants rosemary oil (RM) and powder from the seaweed Bladderwrack (BW) were compared to that of the synthetic ethoxyquin (EQ) (which is commonly used in fish feeds) in stabilizing lipid oxidation in herring oil. Furthermore, their effects on the growth and antioxidant enzyme activities in Arctic charr were studied. Lipid oxidation stability was monitored by assaying free fatty acids (FFA), peroxide value (POV) and anisidine value (AnV) of herring oil treated with RM, BW and EQ at five concentrations (100, 200, 300, 400, 500 ppm EQ equivalent). The growth and activities of catalase (CAT), glutathione peroxidise (GPx) and superoxide dismutase (SOD) were studied in Arctic charr fed diets containing RM, BW and EQ at three different concentrations (100, 300 and 500 ppm EQ equivalent). The results show that RM and BW can, to an extent, stabilize lipid oxidation in herring fish oils and that RM is more effective in stabilizing lipid oxidation than BW. At elevated concentrations, RM is similar to EQ in performance. Growth was significantly affected by antioxidant type and concentration, being fastest in the group fed antioxidant free and RM diets, and poorest in those fed EQ and BW diets. CAT, GPx and SOD activities increased with dietary concentration of RM compared to the antioxidant free, BW and EQ groups. These results suggest that RM could be used to replace EQ at relatively high concentration to stabilize lipid oxidation and also to enhance the growth in fish.

Key words: antioxidant enzymes, Arctic charr, bladderwrack, ethoxyquin, rosemary oil, growth, lipid oxidation.

1. Introduction

Antioxidants are substances used in low concentrations to either delay or inhibit lipid oxidation (Antolovich et al., 2002). Antioxidants minimize lipid oxidation by either delaying or completely inhibiting the initiation step or by reacting with the reactive oxygen species (ROS) (Antolovich et al., 2002; Sasse et al., 2009). Hence, varied protection is offered by antioxidants depending on the rate at which they react with the reactive radical or how completely they get used up in the process (Sasse et al., 2009). Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and ethoxyquin (EQ) are synthetic antioxidants that have been used for decades to prevent lipid oxidation in fish feeds and primary raw materials such as fish oil and fishmeal (Bohne et al., 2008; Hamre et al., 2010; Lundebye et al., 2010). The aromatic amine EQ (1, 2-dihydro-6-ethoxy-2, 2, 4-trimethyl quinoline) is the most commonly used synthetic antioxidant to prevent lipid oxidation (Hamre et al., 2010).

The use of EQ has raised concerns related to animal welfare and food safety (Antolovich et al., 2002; Bohne et al., 2008; Hamre et al., 2010; Raza et al., 2009; Sasse et al., 2009), prompting the need to find alternative antioxidants. EQ used in aquafeeds tends to bio-accumulate in flesh and ends up being consumed by humans (Bohne et al., 2008). Apoptosis and cytotoxic effects have been reported in animals fed EQ treated feeds (Błaszczyk and Skolimowski, 2005a, 2005b); and these raise concern for human consumption (de Koning, 2005). Concerns have been raised about the potential carcinogenic effects that could arise from consuming fish contaminated with EQ (Błaszczyk and Skolimowski, 2005a). The utilization of alternative antioxidants to prevent lipid oxidation depends on how well they compare with the synthetic preparations currently available on market (Antolovich et al., 2002; Dauqan et al., 2011; Hamre et al., 2010). Furthermore, these antioxidants should have minimal adverse effects on the colour, flavour and odour of the substrate; not to affect acceptance, palatability and digestibility (Antolovich et al., 2002; NRC, 2011).

The primary objective of the present study was to seek alternative antioxidants that could replace EQ in fish feeds and raw materials used for fish feed production. The novel antioxidants tested were rosemary oil (RM) and dried bladder wrack powder (BW), a brown sea weed common in coastal zones around the North Atlantic Ocean. Oil-extracted rosemary has been found to stabilize lipid oxidation in several substrates, including sunflower oil (Babovic et al., 2010) and pork sausages (Georgantelis et al., 2007). Bladder wrack was identified as a good antioxidant due to high concentration of polyphenolic compounds (Wang et al., 2009). However, to our knowledge, no studies have investigated the potential of RM at stabilizing lipid peroxidation in oil/fishmeal as well as its effects on fish. Moreover, there are no studies that have investigated the potential effects of RM or BW on growth and antioxidant enzyme defense in fish. Specifically, we compared the efficacy of RM and BW to EQ in stabilizing lipid oxidation in herring oil. Secondly, the effects of feeding Arctic charr on EQ, RM and BW treated diets were also compared.

2. **Materials and methods**

2.1. Antioxidant application

Antioxidants activities are reported in different units; making it difficult to compare their effectiveness on a unified scale. In the present study, an attempt was made to establish a comparable scale by calculating a ratio based on the Oxygen Radical Absorbance Capacity (ORAC) value (*µmol* of TE/g) of each antioxidant obtained from literature (Ho et al., 2008; Thorisson, 1987; Tsai et al., 2008; Wang et al., 2009, Table 1). The ORAC value expresses the ability of an antioxidant to scavenge oxygen radicals and stop oxidation. Thus, the ORAC value for the synthetic antioxidant EQ (considered to be 100% active) was used as a reference for the other two natural antioxidants RM and BW. The concentrations of RM and BW that could generate comparable activity as EQ of a given concentration were computed using their ORAC value (*µmol* of TE/g): First, the ORAC-ratio was obtained as follows:

$$\text{Antioxidant ORAC ratio} = \frac{\text{EQ ORAC value}}{\text{Natural antioxidant ORAC value}}$$

Then, the concentration of each natural antioxidant, in ppm added to oil sample, was derived as shown below:

$$\text{Natural antioxidant concentration (ppm)} = \frac{\text{Concentration of EQ (ppm)}}{\text{Natural antioxidant ORAC ratio}}$$

Five different concentrations of EQ (100, 200, 300, 400 and 500 ppm) and the equivalent concentrations of RM and BW based on their ORAC ratios were tested (Table 2). A fresh oil sample without any antioxidant was included, and two separate experiments were then set up.

2.2. Choice and treatment of herring fish oil

Herring oil was used to assess the relative efficacy of the different antioxidants in stabilizing lipid degradation. Herring oil was chosen because it is highly susceptible to lipid degradation and it is commonly used in aquafeeds (NRC, 2011). The herring oil without any antioxidant added was obtained from Síldarvinnslan hf, Hafnarbraut 6, 740 Fjardabyggd, Iceland. The oil was transported in a 20 L dark closed container and stored at -25 ^{o}C at the laboratories of MATIS, Iceland, before the experiments commenced.

2.3. Experiment 1

The first experiment was a laboratory study that evaluated the relative efficacy of the natural antioxidants (RM and BW) to the synthetic EQ in stabilizing lipid oxidation. Five concentrations (100, 200, 300, 400 and 500ppm) were prepared with all antioxidants (Table 2). RM and EQ were liquids while BW was a powder. To every 200 g of herring oil was added a specified antioxidant concentration (Table 2). The oil-antioxidant mixtures were sealed and electrically shaken for 10 minutes to achieve thorough mixing, after which they were stored at room temperature (22-25 ^{o}C) for two months. A fresh oil sample without any

antioxidant was prepared and stored under similar experimental conditions. At the end of two months, samples were analyzed for free fatty acids (FFA), peroxide value (POV), Anisidine value (AnV) and fatty acid profiles at MATIS, Iceland. To ascertain the initial oxidation state of herring oil, a fresh sample of untreated herring oil was stored at -80 ^{o}C and analyzed together with the rest of the samples.

2.4. Determination of the different oil parameters

2.4.1. Free fatty acids (FFA)

In total, 100 mL of neutralised 2-propan (HPCL) were added to a weighed amount (5-10 g) of oil sample and titrated against 0.1018N sodium hydroxide (NaOH), with phenolphthalein as an indicator. Free fatty acids (%) as oleic acid were then estimated as: %FFA = (volume of NaOH used (mL) xNx28.2)/mass of oil sample (g). Where N is the normality of NaOH and 28.2 is a constant (AOCS, 1990).

2.4.2. Lipid hydroperoxide (POV)

Lipid hydroperoxides were determined according to AOAC (1995). To about 5 g of oil sample in a conical flask, 50 mL of iso-octane (2,2,4-trimethyl pentane) were added, followed by 3-5 drops (about 0.5 mL) of saturated potassium iodide (KI). The reaction was allowed to occur for one minute after which it was stopped by adding about 30 mL of water. The liberated iodine was titrated immediately with 0.010N sodium thiosulphate, and the titre value obtained was then used to calculate the POV as:

POV =(V_{thio} x Nx1000)/m, where: V_{thio}-volume of thiosulphate used; N-is the normality of sodium thiosulphate and m is mass of the oil used.

2.4.3. Anisidine value (AnV)

A sample of oil was centrifuged at 2000-3000 rpm (TS-25-centrifuge: Beckmann Coulter, California, USA) for 10 minutes to settle water molecules that could interfere with the reaction. A sample of 0.5 g was then drawn from the top layer into a 25 mL volumetric flask, which was filled to the mark with glacial acetic acid/iso-octane (3:2; v/v) mixture. In two separate tubes, 1 mL of p-anisidine was then added separately to 5 mL of the mixture and to 5 mL of the solvent. The reaction was allowed to occur for 10 minutes, after which the absorbences of both the fat-solvent mixture and solvent were read at 350 nm, in a UV-1800 spectrophotometer, (Shimadzu, Kyoto, Japan). These procedures were repeated in triplicates and the absorbence values obtained were used to compute AnV as:

AnV $=(25 \times (1.2A_s - A_b))/m$, where: A_s is absorbence of the fat solution after reaction with p-anisidine, A_b is absorbance of the unreacted fat solution and m is the mass (g) of the oil sample.

2.5. Experiment 2

A growth trial was conducted to examine the effects of antioxidant type and concentration on the growth and antioxidant enzyme activities of Arctic charr. In this trial, EQ, RM and BW were added to herring oil at EQ equivalent of 0, 100, 300 and 500 ppm and mixed with experimental diets.

2.5.1 Feed formulation and preparation

A single un-extruded diet (approx. Crude protein (CP) = 46% and crude lipid (EE) = 20%, on dry matter basis; Table 3) was formulated and divided into 10 portions. To each portion was added herring oils treated with either EQ or RM or BW at three different levels (Table 3). Fresh herring oil without any antioxidant was mixed with one portion of the diet, to constitute a control. Each experimental diet was thoroughly mixed with the oil and pelleted using a

simple pelletizing machine. The pellets were air dried. The feed ingredients used in the formulation of diets (Table 3) were fish meal (NSM-715/85), herring oil, soybean meal 47/5 Brasil, wheat flour, wheat gluten, monocalcium phosphate and vitamin and mineral premix (Laxa Feed mill, Akureyri, Iceland). To prevent any further oxidation, the formulated feeds were kept in a freezer at -22 ^{o}C as the experiment progressed.

2.5.2 Fish husbandry

Juvenile Arctic charr were obtained from Hólar breeding farm, Iceland and brought to Verið, Sauðárkrókur; the research facility of Hólar University College where the experiment was conducted. The fish were distributed at random amongst forty tanks for acclimation. The fish were fed a weaning diet from Laxá Feed mill, Akureyri, Iceland for one week before the experiment commenced. Fish that died during the acclimation week were replaced. The study was set with 60 fish of average initial body mass 2.41±0.11g per 20L flow-through cylindrical plastic tank. Each diet was fed to quadruplicate groups of fish using automated belt feeders set to feed at five minute intervals. Feeding was continuous under a 24-hour light regime for a total of 67 days. Excess feed was siphoned off and any dead fish removed and recorded for survival estimation at the end. Growth parameters and antioxidant enzymes were studied. Throughout the experimental period, water temperature and dissolved oxygen saturation (relative to air saturated water) were maintained at about 9.65±0.67 ^{o}C and 101.72±8.71%, respectively.

2.5.3 Measurements

All fish in a tank were weighed in at least three sub-sample batches at the commencement of the study and at every sampling interval following 24 hours of starvation, i.e., every three weeks. At the end of the experiments, the body mass and length of all individual fish were recorded. Fish were anaesthetized using phenoxyethanol at 0.3ml/l of water before measurements.

The specific growth rate (SGR) of fish in different tanks was calculated as: $SGR = 100 \text{ x} \frac{\ln(w_2) - \ln(w_1)}{t_2 - t_1} SGR = 100 \text{ x} \frac{\ln(w_2) - \ln(w_1)}{t_1 - t_2}$, where w_1 is biomass at time (t_1) and w_2 is biomass at time (t_2).

The condition factor (K) was calculated as: $\text{K} = 100 \text{ x } \frac{\text{Body mass}}{\text{Length}^3}$, length being in cm and body mass in g.

Given the role of the liver in lipid metabolism, liver mass was weighed and the hepatosomatic index (HSI) calculated as: $\text{HSI} = 100 \text{ x} \frac{\text{Liver mass}}{\text{Body mass}}$.

Body composition was analyzed in 10 fish from each experimental treatment at the end of the experiments. Dry matter, ash, crude protein and crude lipids were analyzed following standard procedures outlined by AOAC, (1995).

2.5.4 Determination of antioxidant enzyme activities

Liver samples were homogenized in 0.05M Phosphate buffer pH 7.8 (Tissue: buffer ratio 1:3), after which samples were placed in a 1.5 ml Eppendorf tube for sonication. Homogenization and sonication were both done on ice. Sonication was done in 30 second intervals for 90 seconds. Treated samples were kept at -80 $^{\circ}$C until enzyme assays were performed. Catalase and GPX activities were assayed according to (Weydert and Cullen, 2010), and total SOD was determined using a kit obtained from Sigma Aldrich (19160 SOD determination kit). Protein concentration in the supernatant solutions was determined by modified method of Lowry *et al.* (1951) following degradation of NaOH/sodium dodecyl sulfate (SDS).

2.5.5 Determination of fatty acid composition

The fatty acid composition of antioxidant treated and free herring oils were determined by gas chromatography (Varian 3900 GC, Varian, Inc., Walnut Creek, CA, USA), after

derivatization to fatty acid methyl esters (FAME; (AOCS, 1998)). The procedure was based on AOAC (2000). The result of each fatty acid was expressed as percentages of the total FAME.

2.6 Statiatical analysis

All statistical analyses were performed with SPSS version 18 (SPSS, Chicago, IL, USA). The data (body-mass, FFA, POV, AnV and fatty acid composition) were analyzed with a two-way mixed model ANOVA with concentration and antioxidant as main effects and replicate tanks nested within treatments. The assumptions of normality were assessed by histogram, while equal variance was assessed by Bartlett's test. Using Bonferroni test, significant differences among means were considered at P-value < 0.05.

3. Results from experiment 1

3.1. Lipid oxidation stability

The efficacy of antioxidants in stabilizing primary and secondary lipid oxidation of herring oil was based on FFA content and POV and AnV values. Fatty acid composition and proprotions were also used to understand the extend of lipid degredation.

All antioxidants inhibited lipid degradation. The concentration of FFA was significantly higher ($P < 0.05$) in untreated oil samples than in those treated with antioxidants. However, the FFA content of the treated oils was not significantly affected by antioxidant concentration (Table 4).

There were significant effects of both antioxidant type ($P < 0.0001$) and concentration levels ($P < 0.0001$) on the POV (Fig. 1a) and AnV (Fig. 1b) contents of the treated oils. Regardless of the antioxidant type, considerable reduction in POV and AnV content of treated oils was at 100 ppm EQ equivalent (Fig. 1a & b). Further increase in antioxidant concentration up to 500 ppm EQ equivalent resulted in smaller reduction in POV and AnV for both EQ and RM treated oils. In BW treated oils, however, there was an increase in POV and AnV content at

500 ppm EQ equivalent (Fig.1a & b). The EQ treated oils had significantly lower POV (Fig. 1a) and AnV (Fig. 1b) content ($P < 0.05$) than those treated with RM and BW. The POV and AnV content of EQ and RM treated oils significantly decreased ($P < 0.001$) with increasing antioxidant concentration (Fig.1a & b).

The polyunsaturated fatty acid (PUFA) content, the proportion of monounsaturated fatty acids (MUFA) and saturated fatty acid (SFA) of treated herring oil were fairly stable, and were not significantly affected by antioxidant type or concentration levels, hence mean values are presented (Table 5a). The proportion of EPA (C22:5n-3) and DHA (C22:6n-3) fatty acids were not significantly affected by antioxidant type and application levels (Table 5b).

3.2. Growth of fish raised on diets containing oils with different antioxidants

The best growth performance was observed in the group fed a diet without any antioxidants (Fig. 2a,b,c) while the second best growth was observed in the group fed diets with RM treated oils. The average final body mass of the fish fed diet without antioxidants was 23% higher ($P < 0.0001$) than in the groups fed either EQ or BW and 7.3% higher than the group fed RM (Fig. 2). The final body mass of the group fed diet without antioxidants and that fed RM at 300 ppm EQ equivalent (Fig. 2b) were not significantly different. The final body mass of the groups fed diets EQ and BW were about 13% lower ($P < 0.001$) than in the group fed RM (Figs. 2a,b,c). Growth of fish fed diets treated with EQ and BW was similar and the lowest at all application levels.

The SGR of the fish group fed a diet without antioxidants was significantly higher ($P < 0.005$) than in groups fed diets treated with EQ or BW (Table 6). However, the final body mass and SGR of the group fed antioxidant-free and that fed RM diets were not significantly different (Table 6), although the latter were consistently smaller. At all concentration levels, the SGR of Arctic charr fed RM was significantly higher than in the groups fed EQ and BW

diets. There were no differences in the SGR of fish fed diets treated with EQ and BW (Table 6). Condition factor and HSI did not differ among fish groups fed different antioxidants.

3.3. Antioxidant enzyme activity

CAT and GPx activity were affected by antioxidant type ($P < 0.05$) but not by concentration levels (Table 7), being significantly higher in fish fed RM ($P < 0.001$) than in the groups fed diets treated with EQ and BW. SOD activity significantly differed among fish groups fed different antioxidants ($P < 0.001$) and application levels ($P < 0.001$). Regardless of the application level, SOD activity was significantly lower ($P < 0.05$) in fish fed BW diets than in those fed RM and EQ (Table 7). There were no significant interaction effects of antioxidant type and application levels on CAT, GPx and SOD activity, although it was significantly higher ($P < 0.01$) at 500 ppm than at 100 and 300 ppm.

SOD activity generally increased with antioxidant application level in fish fed EQ and RM treated diets, although at 500 ppm the activity was higher in fish fed EQ than in those fed RM (Table 7). Fish fed diets with BW had significantly lower CAT, GPx and SOD activity ($P < 0.01$) than those fed EQ or RM at all concentration levels. Compared to the group fed antioxidant free diet, CAT, GPx and SOD activity significantly increased ($P < 0.001$) with the dietary concentration of RM and generally decreased in groups fed EQ and more so BW treated diets (Table 7).

4. Discussion

The results of the present study show that both RM and BW could be used as antioxidants in fish oils. The concentrations of FFA, HUFAs and PUFAs in oils treated with all three antioxidants were comparable, suggesting that the quality of the oils treated by RM and BW was comparable to that treated with EQ. However, the indicators of lipid oxidation (POV and AnV; Fig. 1a & b) suggest that slightly more oxidation occurred in RM and BW treated oils

than in those treated by EQ. The higher levels of POV and AnV in the RM and BW treated oils may suggest that the ORAC values for RM and BW reported in literature may be overestimated (Ho et al., 2008; Tsai et al., 2008; Wang et al., 2009) or that RM and BW cannot suppress oxidation to the same level as EQ can. Nonetheless, the similar levels of POV and AnV in RM and EQ treated oils (Fig. 1a & b) when the concentration of RM was high, indicate that at elevated concentrations RM is as effective as EQ in stabilizing lipid oxidation.

The relatively high efficacy of RM in stabilizing lipid oxidation is attributed to the high concentration of phenolic compounds which increase its antioxidant properties (Huber and Rupasinghe, 2009; Sasse et al., 2009). Indeed studies have shown that the antioxidant activities of RM are comparable to that of Butylated hydroxytoluene (BHT) (Fukumoto and Mazza, 2000) and some times higher than that of butylated hydroxyanisole (BHA) (Babovic et al., 2010). RM consists of high proportion of rosmanol, rosmarimic acid, carnosic acid and carnosol that are believed to be about four times as effective as BHA and equivalent to BHT at preventing lipid oxidation (Thorisson, 1987; Frankel, 1998). In the present study, RM was slightly less effective than EQ, particularly at low concentrations (Fig. 1a & b). However, at elevated concentrations RM can prevent oxidation almost to the same level as does EQ, suggesting that it could replace the synthetic BHA, BHT and EQ.

While lipid oxidation in herring oil decreased with increasing concentration of EQ and RM, it was fairly constant in samples treated with BW at EQ equivalent between 100 and 400 ppm, but increased at 500 ppm. This could be attributed to the observed clogging of the powdery BW at high concentration leading to reduced surface area of the antioxidant exposed to scavenge the oxygen radicals responsible for lipid oxidation (see Antolovich et al., 2002).

Effect of antioxidants on growth and antioxidant enzymes of Arctic charr

Growth was best in the Arctic charr group fed a diet without antioxidants and reduced in groups fed diets treated with antioxidants. Two issues are of particular interest in the context. First, the growth of Arctic charr fed diets containing EQ was reduced by 22.9% at 100 ppm, 13.2% at 300 ppm and 20.1% at 500 ppm compared with the group fed antioxidant free diet. It is likely that when EQ is applied at 100 ppm, the lipids are not well stabilized, and as a result, the products of lipid oxidation generated can stress fish and subsequently reduce growth (see Kubiriza et al., 2017). However, application of EQ at elevated concentrations may directly affect fish and also reduce growth (Błaszczyk et al., 2013). This is of concern for fish farmers because EQ is commonly used as an antioxidant in fish feeds at 150 ppm (Błaszczyk et al., 2013), and sometimes at 300 ppm when fishmeal or feeds are to be stored for some time or when in transit. Secondly, the growth of fish fed diets containing RM was nearly as good as in those fed a diet without antioxidants (Fig 2a,c). The good antioxidant activity of RM and the good growth of the charr suggests, that RM is a good candidate to replace EQ as antioxidant.

The results of the present study suggest that RM does not suppress fish growth quite as much as do EQ and BW. In a recent study by Kubiriza et al. (2017), growth was reduced in Arctic charr fed diets mixed with oils in primary oxidation state (i.e., high POV, 182.97 $meq \cdot kg^{-1}$ and low AnV, 28.52 $meq \cdot kg^{-1}$). However, in the present study, the natural antioxidants maintained the levels of POV and AnV much lower than those that affected Arctic charr. Therefore, the differences in the growth of fish fed diets containing RM, EQ and BW is unlikely due to different oxidation status, but entirely on antioxidant type. The fast growth in Arctic charr fed RM could be due to diet palatability, stimulated appetite and, as a result,

increased feed-intake. Further, the fast growth amongst groups fed RM treated diets compared to those fed EQ and BW could be a result of improved assimilation and reduced physiological stress. In Japanese seabass (Wang et al., 2014) and in Atlantic salmon (*Salmo salar* L.) (Bohne et al., 2008), EQ reduced nutrient assimilation efficiency and tended to accumulate in muscles as dietary concentration increased. The accumulated EQ is associated with physiological stress, particularly inhibiting energy metabolism and ATP production (Błaszczyk et al., 2013; Blaszczyk and Skolimowski, 2005; Eun et al., 1993), and increased number of apoptotic cells (Blaszczyk and Skolimowski, 2007). Accordingly, the poor growth seen in Arctic charr fed EQ treated diets and possibly in those fed BW might have been due to increased physiological stress and reduced nutrient assimilation. The reduced growth in Arctic charr fed EQ and BW treated diets in this study are similar to the observed decrease in growth of large yellow croaker (*Pseudosciaena crocea*) (Wang et al., 2010) and Japanese seabass (*Lateolabrax japonicas*) (Wang et al., 2014), fed elevated levels of dietary EQ. Therefore, the result of the present study and those by Wang et al. (2010; 2014) suggest that EQ suppresses fish growth.

The Arctic char fed diets containing RM showed increased CAT, GPx and SOD activities compared to the groups fed antioxidant-free, EQ and BW diets (Fig. 4a-c). These results may suggest that RM stimulates the defense enzymes and offers more effective protection against oxidative stress than do EQ or BW. This may, in turn, promote growth.

In conclusion, the present study has demonstrated that RM and BW can, to an extent, stabilize lipid oxidation in fish oils, and that RM is more efficient than BW, approximating the efficacy of EQ at elevated concentrations. Further, the findings suggest that EQ and BW suppress fish growth. The growth of fish fed diets containing RM is slightly reduced compared with groups fed no antioxidant but significantly better than in fish fed either EQ or BW. Antioxidant enzyme protection is enhanced in fish fed RM, particularly at elevated

concentrations. Interestingly, RM is widely consumed by humans as a herb; and no negative health effects have been reported. Accordingly, using elevated concentrations of RM to stabilize lipid oxidation in aquafeeds is less likely to affect humans. Moreover, RM is easy to propagate, although a bit costly to extract the active phenolic antioxidant compounds; hence being expensive (about 8-12 times higher) than EQ. However, our results suggest that significant gains in growth can be incurred when fish are fed diets containing RM compared with EQ. The cost of RM must be weighed against these gains.

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Tables and Figures

Table 1: Oxygen Radical Absorbance Capacity (ORAC) values for ethoxyquin (EQ), Rosemary (RM) and Bladder wrack (BW)

Antioxidant	ORAC-Value (*μmol* of TE/g)	ORAC-ratio	Reference
EQ	50255	1	Thorisson, 1987
Ethanol extracted RM	2800	0.0712	Ho et al., 2008
	4360		Tsai et al., 2008
	Mean: 3580		
Ethyl acetate extracted BW	1840	0.0366	Wang et al., 2009

Table 2: Concentration levels of RM and BW relative to EQ used in the study

EQ concentration level (ppm)		No antioxidant	100.0	200.0	300.0	400.0	500.0
Equivalent (ppm) of:	RM	0.0	1403.8	2807.5	4211.3	5615.1	7018.9
	BW	0.0	2731.3	5462.5	8193.8	10925.0	13656.3

Table 3: Formulation and proximate composition of the trial diets (g/kg diet)

	Experimental diet			
Ingredient dry weight (g/kg)	No antioxidant	100	300	500
Wheat flour	139.8	139.8	139.8	139.8
Soy 47/5 Brasil	70.0	70.0	70.0	70.0
Mono calcium phosphate	2.5	2.5	2.5	2.5
Oxidised fish oil	152.7	152.7	152.7	152.7
Wheat gluten	100.0	100.0	100.0	100.0
Fish meal (NSM-715/85)	524.9	524.9	524.9	524.9
Premix Laxa	10.0	10.0	10.0	10.0
Chemical composition (%)				
DM	90.75±0.12	83.29±0.05	84.79±0.23	87.21±0.22
Crude protein	48.16±0.60	45.17±0.15	47.10±0.39	46.91±1.22
Crude lipid	20.14±0.31	20.10±0.11	20.53±0.38	20.51±0.63
Ash	7.92±0.04	7.27±0.01	7.42±0.03	7.59±0.27

*The proximate feed composition is expressed as % of dry matter in the feed.

Table 4: Free fatty acid content (g/100g of oil) of herring oils treated with three antioxidants at five concentration levels and stored at room temperature for two months (mean ±SEM, n=3)

	EQ equivalent concentration levels (ppm)					
Antioxidant	No antioxidant*	100	200	300	400	500
EQ		4.22±0.01^{ab}	4.26±0.03^{ab}	4.21±0.04^{a}	4.22±0.01^{ab}	4.30±0.01^{b}
RM	4.51±0.01	4.29±0.04^{b}	4.28±0.02^{b}	4.07±0.04^{a}	4.21±0.01^{b}	4.38±0.05^{b}
BW		4.34±0.01^{bc}	4.27±0.01^{bc}	4.27±0.02^{a}	4.23±0.01^{b}	4.22±0.01^{cd}

*Oil sample maintained at room temperature without any antioxidant. Values with different superscripts in a row are significantly different at $P < 0.05$.

Table 5: Mean concentration of a) saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA) and b) the proportion of highly oxidizable fatty acid (C20:5n3: EPA & C22:6n3: DHA) expressed in peak area% of total fatty acid methyl esters (FAME) in herring oil stored for two months at room temperature

a) Antioxidant	Fatty acids	**Concentration**	
		Without antioxidant*	Treated sample mean ± SE
EQ	SFA	22.44 ± 4.41	22.79±0.14
	MUFA	48.74 ± 6.34	49.57±0.18
	PUFA	21.05 ± 2.12	20.07±2.00
RM	SFA	22.44 ± 4.41	22.53±0.23
	MUFA	48.74 ± 6.34	48.84±0.54
	PUFA	21.05 ± 2.12	20.92±0.19
BW	SFA	22.44 ± 4.41	22.23±0.19
	MUFA	48.74 ± 6.34	49.01±0.57
	PUFA	21.05 ± 2.12	29.93±0.32

b) Antioxidant	Highly oxidizable fatty acid (EPA/DHA)	**Proportion in herring oil**		
		Initially	Without antioxidant*	Treated sample mean ± SE
EQ	C20:5n3	5.76	6.33	6.02±0.08
	C22:6n3	5.47	6.12	7.70±0.08
RM	C20:5n3	5.76	6.33	6.35±0.08
	C22:6n3	5.47	6.12	6.04±0.05
BW	C20:5n3	5.76	6.33	6.33±0.11
	C22:6n3	5.47	6.12	6.15±0.10

*Oil sample maintained at room temperature without any antioxidant.

Table 6: Growth and condition indicators of fish reared on a diet without antioxidants and those treated with three antioxidants at three different application levels

		EQ equivalent concentration levels (ppm)				
Antioxidant	Parameter	No antioxidant	100	300	500	*P*-value
EQ	IBM[1](g)	2.45±0.10	2.51±0.08	2.46±0.12	2.50±0.12	0.931
	FBM[2](g)	7.51±0.74[b]	5.79±0.43[a]	6.52±0.17[ab]	6.0±0.61[a]	0.002
	SGR[4]	1.67±0.04[b]	1.25±0.07[a**]	1.44±0.12[a*]	1.30±0.05[a**]	<0.0001
	K[5]	1.71±0.11	1.58±0.09	1.82±0.06	1.74±0.06	>0.05
	HSI[6]	1.26±0.02	1.37±0.15	1.33±0.03	1.23±0.02	>0.05
RM	IBM[1](g)	2.45±0.10	2.59±0.13	2.54±0.13	2.61±0.11	0.383
	FBM[2](g)	7.51±0.74	6.88±0.58	7.24±0.12	6.89±0.66	0.212
	SGR	1.67±0.04	1.46±0.12***	1.57±0.06**	1.44±0.05***	0.045
	K	1.71±0.11	1.6±0.1	1.72±0.07	1.9±0.06	>0.05
	HSI	1.26±0.02	1.32±0.02	1.34±0.03	1.33±0.03	>0.05
BW	IBM[1](g)	2.45±0.10	2.62±0.18	2.57±0.09	2.58±0.07	0.397
	FBM[2](g)	7.51±0.74[b]	5.84±0.16[a]	6.51±0.58[ab]	5.86±0.83[a]	0.001
	SGR	1.67±0.04[b]	1.18±0.12[a*]	1.39±0.09[a*]	1.21±0.07[a*]	<0.0001
	K	1.71±0.11	1.68±0.06	1.63±0.06	1.7±0.03	>0.05
	HSI	1.26±0.02	1.33±0.01	1.29±0.02	1.29±0.03	>0.05

[1]Initial body mass (IBM), [2]Final body mass (FBM), [3]Specific growth rate (SGR), [4]Condition factor (K), [5]Heapato somatic index (HSI). *Indicates significant differences in the SGR of Arctic charr fed RM, EQ and BW at the different concentration levels.

Table 7: Antioxidant enzyme activity (U/mg protein: mean ± SE) in juvenile Arctic charr fed diets treated with different antioxidants types and quantities. Significant differences in enzyme activities among application levels within each antioxidant (in a row) are shown by different lowercase superscripts, while significant differences among antioxidants at each application level are shown by different uppercase superscripts within a column. Significant differences are considered at $P < 0.05$.

		EQ equivalent concentration levels (ppm)			
Enzyme	Antioxidant	No antioxidant*	100	300	500
CAT	EQ		191.39±15.78^{bB}	195.29±5.17^{bB}	161.03±13.67^{aA}
	RM	225.01±8.18	264.01±32.7^{C}	282.85±39.41^{C}	268.18±24.12^{C}
	BW		167.98±6.88^{bA}	145.15±8.18^{aA}	133.51±14.91^{aB}
GPX	EQ		159.25±8.25^{aA}	176.15±46.48^{cB}	168.78±11.2b^{B}
	RM	200.31±21.79	235.85±26.02^{C}	251.23±34.06^{C}	238.26±18.31^{C}
	BW		204.48±21.62^{cB}	161.87±28.31^{bA}	136.38±10.98^{aA}
SOD	EQ		64.82±1.16^{aB}	64.01±1.05^{aB}	71.41±0.62^{bB}
	RM	61.68±1.54	64.83±0.94^{aB}	65.66±1.47^{aB}	68.69±2.32^{bB}
	BW		59.76±1.45^{bA}	57.19±1.49^{aA}	61.75±1.64^{bA}

*Diet made using herring oil not treated with any antioxidant. CAT: Catalase; GPx: Glutathione peroxidase, SOD: Super oxide dismutase.

Figures legend:

Figure 1: a) Peroxide value and b) Anisidine value (mean±SEM, n=3 replicates) of herring oils treated with three antioxidants at five different concentrations following 60 days of storage at room temperature.

Figure 2: Mean body mass (±SEM) of fish reared on a diet containing oils treated with one of three different antioxidants applied at three different levels. The control diet contained fresh oil without any antioxidant. Significant differences are indicated with superscripts letters (Bonferroni test, $P<0.05$).

Figure 1:

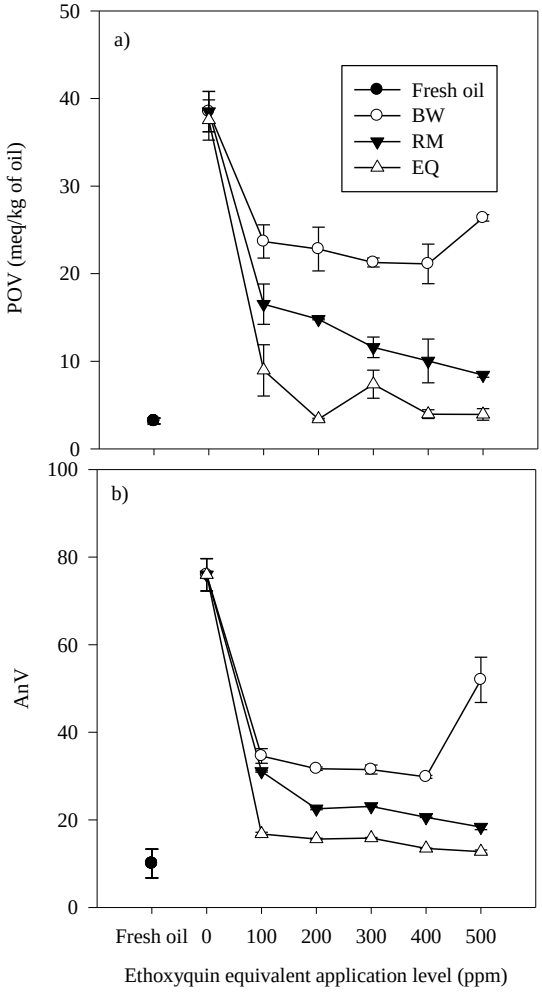
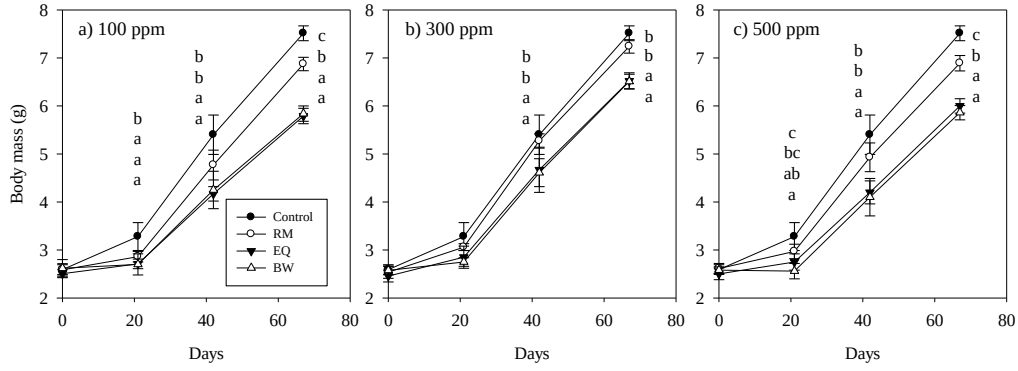

a)
b)
Fresh oil
BW
RM
EQ
POV (meq/kg of oil)
AnV
0
10
20
30
40
50
0
20
40
60
80
100
Fresh oil
0
100
200
300
400
500
Ethoxyquin equivalent application level (ppm)

Figure 2:


a) 100 ppm
b) 300 ppm
c) 500 ppm
Body mass (g)
Days
Control
RM
EQ
BW
b a a a
b b a a
c b a a
b b a a
b b a a
b b a a
c bc ab a
b b a a
c b a a