The effect of carbohydrate on dusky kob, *Argyrosomus japonicus*, fed pelleted diets

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ABSTRACT

The dusky kob, *Argyrosomus japonicus*, is an emerging commercial marine aquaculture species in South Africa. Attributes such as market acceptance, fast growth rates, tolerance to sibling density, acceptance of pelleted feed and adaptability to intensive aquaculture conditions make it a good candidate. Feed, the largest running cost in most aquaculture operations, is a limiting factor in dusky kob production as its nutritional requirements are not well understood. The present project formed part of a research program to develop a locally produced, least cost and sustainable feed that will support the growth and health of dusky kob. The approximate protein and energy requirements for kob fed formulated feeds have been established in earlier research, but the ability of kob to utilize carbohydrates as an energy source has not previously been investigated.

The aim of this study was to determine the efficiency of dusky kob to utilize graded levels of carbohydrate (pregelatinized maize starch - PGMS) in pelleted diets. The research objectives were to determine the effect of dietary carbohydrate levels on:

i) growth and feed utilization;
ii) post prandial blood glucose levels; and
iii) general fish health, gut bacterial composition and liver glycogen content of juvenile dusky kob.

Hatchery reared juvenile dusky kob (5 g) were acclimatized in a recirculating

experimental aquaculture system at the Department of Ichthyology and Fisheries Science at Rhodes University. They were fed trout crumble starter feed for three weeks before the start of the feeding experiment. Five diets containing 4.1, 8.2, 16.4 and 24.6% carbohydrate, hereafter referred to as 4.1C, 8.2C, 16.4C and 24.6C respectively, were formulated with pregelatinized maize starch as a carbohydrate source and fishmeal as the main protein source. Fish were fed these diets twice daily at 3.85% body weight per day for three months.

Specific growth rates and feed conversion rations differed significantly between the four dietary treatments. Growth rate increased with increasing carbohydrate up to 16.4%, after which a significant decline was observed. Diet 16.4C produced the highest specific growth rate (SGR) of 1.84 ± 0.05% body weight/day, and the lowest feed conversion ratio of 1.28 ± 0.08. While the lowest specific growth rate and highest feed conversion ratio of 0.79 ± 0.05 and 2.14 ± 0.13 respectively, were found for diet 24.6C. A third order polynomial regression, using SGR, determined the optimum carbohydrate inclusion for dusky kob to be 16.72%.

The general structure of the liver was similar between all fish fed the dietary treatments and all fish liver samples displayed a certain degree of lipid vacuolization of the hepatocytes. Evidence of starvation was observed in the livers of fish fed diet 24.6C. However, no differences in the amount of liver glycogen were observed.

Gut bacterial composition did not differ among the different diets or between the different sections of the gut. Differences were however observed in the diversity of the bacterial community structure at the start (when they were fed commercial trout feed) and end of the experimental period (after being fed a fishmeal-maize diet).

The rate at which glucose was cleared from the blood differed significantly between diets. The low carbohydrate diets (i.e. diet 4.1C and 8.2C) showed a steady rate of glucose removal from the blood over a 48 h experimental period. Sharp increases in blood glucose concentration were observed in diet 16.4C and diet 24.6C, with highest glucose concentrations of 7.18 ± 1.81 and 8.05 ± 2.35 mmol/l respectively, observed 24 h after feeding. The blood glucose concentration of the fish fed diet 16.4C however returned to resting glucose concentration after 48 h, while that of the fish fed diet 24.6C did not.

The results demonstrated that the level of dietary carbohydrate inclusion has a significant effect on the growth and health of the fish. The optimum carbohydrate inclusion in dusky kob diets is indicated to be 16.7%, which gave the best growth rate and maximum protein sparing effect without adverse effects on fish health. These findings are important for diet formulation and producing 'least-cost' diets for dusky kob farming.

TABLE OF CONTENTS

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To my parents, thank you for your support and encouragement through my studies. Inkomu!

CHAPTER 1

GENERAL INTRODUCTION

Argyrosomus japoncus, dusky kob, is an aquaculture species in pilot commercial production in South Africa. Feed is however a limiting factor as there is no locally produced feed for kob that promotes good growth and health and that is affordable. Farmers either have to (a) import high quality feed or (b) pay very high prices for a locally produced feed with very high quality ingredients, or (c) use cheaper local feeds not designed for kob. None of the above are conducive to the success of the local kob farming industry. The local industry needs an affordable feed that promotes fish growth without compromising fish health.

The approximate protein and energy requirement for kob has been established in earlier work (Daniel 2004, Woolley *et al*. 2010). Based on this research, a feed that compares favorably with similar international feeds is marketed in South Africa. However, this feed is expensive and not economically and environmentally sustainable since it relies on a high level fishmeal inclusion (60% fishmeal). The focus now is to develop a least-cost, sustainable kob feed, designed to ensure that fish growth, feed conversion and health.

Studies on the partial substitution of fishmeal with soybean (Daniel 2004) and fish oil with vegetable oil (Rossetti 2011) have been carried out in an attempt to reduce feeding costs and reach fishmeal content targets for sustainability certification. Carbohydrates are a cheaper alternative energy source that could be included in dusky kob diets to spare protein energy and reduce feeding costs. However, not much is known about the utilization of carbohydrates by carnivorous fish. In general carnivorous fish digest raw

starch very poorly as it is not part of their natural diet (Krogdahl *et al*. 2005). However cooked starches are widely used in aquafeeds for predatory fish as they become more available due to the breakdown of the complex starch molecules into simpler sugars (Stone 2003).

Carbohydrate utilization has been tested on fish species with similar feeding habits to dusky kob, such as yellowtail kingfish, *Seriola lalandi* (Booth *et al*. 2013a) and red drum *Sciaenops ocellatus* (Daniel & Robinson 1986). Reports of growth improvement have however been reported at optimum inclusion levels while higher than optimum levels resulted in negative effects on growth and health in fish such as the red bream *Pagrus major* (Furuichi & Yone 1980). Carbohydrate makes up, on average, 15-25% in commercial feed formulated for most marine fish and salmonids (NRC 2011). For dusky kob diets, carbohydrate levels of between 15 – 36% from starch have been used (Daniel 2004, Rossetti 2011) and reduced growth reported when levels exceeded 27.5% (Daniel 2004). This work therefore assesses the effects of graded levels of carbohydrates in pelleted diets on the growth, feed utilization and health of the dusky kob, *Argyrosomus japonicus.*

In the sections below, literature relevant to the utilization of starch in aquafeeds is briefly reviewed in order to contextualize the present project and motivate the research approach and objectives.

Aquaculture and aquafeeds in South Africa

South Africa's aquaculture industry is small compared to major aquaculture producing countries such as China (FAO 2010). It has a total output of approximately 7 700 tons with an estimated value of R0.7 billion (DAFF 2014). Although only limited to a few species, marine aquaculture is a fast-developing sector in South Africa, increasing at an average rate of 11% annually since 2010 (DAFF 2014). It is dominated by marine invertebrates, aquatic plants as well as freshwater fish. During 2013 the sector recorded an increase in production of abalone, mussels, oysters and finfish of 32.25%, 29.82%, 14.75% and 152.90% respectively (DAFF 2014). There is an increasing need for production efficiency, particularly in the marine finfish sector, for the South African aquaculture industry to be competitive.

The marine finfish sector in South Africa has been assessed for feasibility and market access. Several species such as dusky kob (*Argyrosomus japonicus*), silver kob (*Argyrosomus inodorus*), yellowtail (*Seriola lalandi*), red roman (*Chrysoblephus lariceps*) have been identified as potential species for the South African mariculture industry (Hecht 2000), with dusky kob currently being the only species to be commercialized. The sector experienced a substantial increase in production by 152.90% between 2012 and 2013 with dusky kob making up the most of the stock with 147.18 tons (DAFF 2014). Most of the operational dusky kob farms are placed in the Eastern Cape, with some recirculation facilities in the Western Cape as well a pond culture facility in Kwazulu Natal (DAFF 2014). The dusky kob is an economically important line fish targeted by both recreational and commercial fishers (Griffiths 1996). This species has

however been overfished in estuaries and nursing areas as a result of poor stock management (Palmer 2008). The farming of dusky kob is therefore being developed as an option to meet the demand of the market.

In intensive aquaculture farmed fish have limited access to natural feed and rely completely on artificial feed, which makes the need for nutritionally balanced feeds. Feed costs constitute the largest proportion of operating expenses in intensive aquaculture (Halver & Hardy 2002). Feed is also the main route by which pollutants enter a system. Uneaten feed in culture tanks lead to high levels of nitrogenous wastes such as ammonia which deteriorates water quality. It is important that feed provides nutrients for efficient growth with minimal waste released to the environment. Marine finfish such as dusky kob are currently fed imported salmonid feed which is expensive and unsustainable, or fed locally produced trout feed which may not be optimal for dusky kob (Woolley 2009). Below optimal feeding and inefficient feeding regimes result in economic losses and water quality deterioration from waste production (Ginindza 2014). It is therefore crucial that nutritionally sound feed is manufactured locally to assist in the development of the industry. The aquaculture industry needs to formulate diets that meet the nutritional requirements of a fish such as dusky kob without negatively affecting growth or health (Rossetti 2011).

Dusky kob ecology, biology and fisheries

The dusky kob belongs to the family *Sciaenidae*. The family includes 70 genera and 270 species (Nelson 1994). Sciaenids are carnivorous fish widely distributed in estuaries

and near shore coastal waters (<100 m depth) (Silberschneider & Gray 2008). They occur in the eastern Atlantic and Indo-West Pacific regions and are important food fish where they are found (Griffiths & Heemstra 1995). Dusky kob is a sciaenid found on the east coast of the southern Africa from Cape Point to Mozambique (Griffiths & Heemstra 1995). It is most abundant from the Cape Agulhas to the northern parts of Kwazulu Natal. It is also found in other countries such Australia, Hong Kong northwards long the coast of China, South Korea and Japan (Figure 1.1). Dusky kob inhabit near shore waters as well as surf zones and estuaries (Griffiths & Hecht 1995).

Dusky kob is considered to be severely overfished in South Africa. In 1997, biomass levels were reported to be as low as 2.3% of original levels (Griffiths 1997).

It is an estuarine dependent fish which tolerates a wide range of environmental conditions but requires specific temperatures, oxygen and salinity levels for optimal growth. For optimal growth, 25.3°C was estimated to be the best temperature, while 21.4°C was the best temperature for feed conversion ratio (Collett 2007). A salinity of 35 g/l has been found to be the best to allow for maximum dusky kob growth (Bernatzeder *et al.* 2008). Dusky kob juveniles are known to prefer turbid estuaries to clear waters but light intensities in the range of 23-315 lx were however found to have no effect on their growth performance (Collett 2007), suggesting that they can grow well at a wide range of light intensities. The same author also determined that dissolved oxygen levels as low as 4.4 mg/l could be well tolerated by dusky kob.

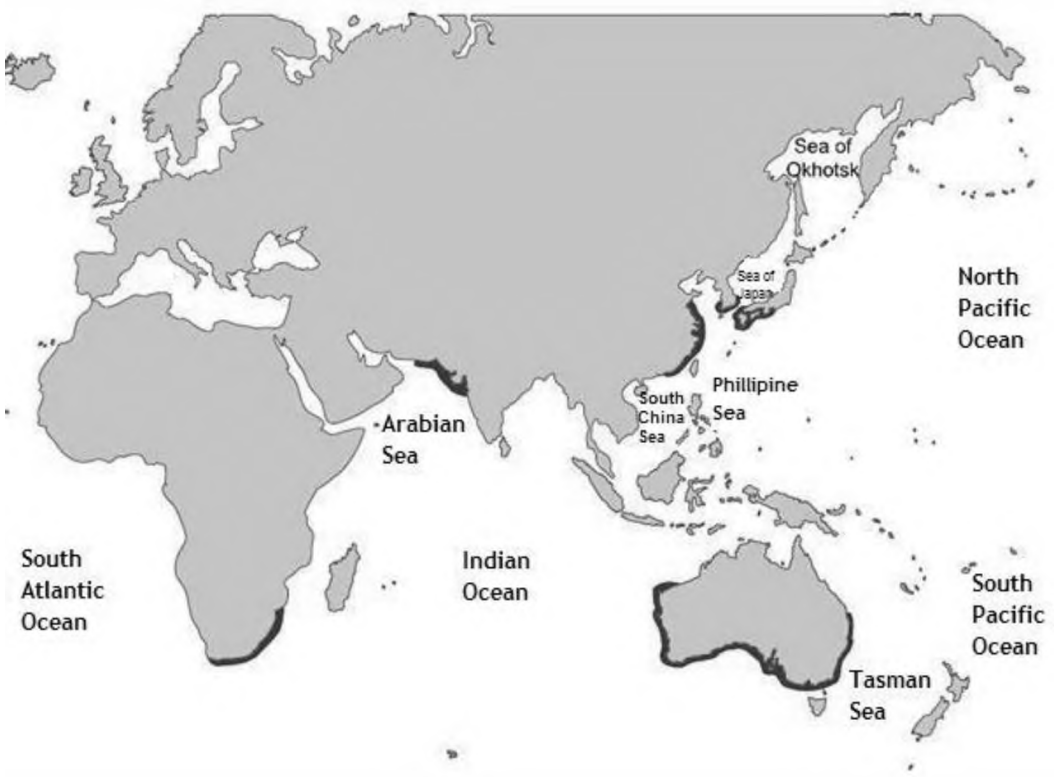

Figure 1.1: Map of global distribution of *Argyrosomus japonicus*, dusky kob (Adapted from Ginindza 2014).

Markets

Dusky kob is a popular table fish and has high market value (Griffiths 1996). It is targeted by both commercial and recreational fishers alike (Mahieu 2015). By 2012, it was estimated that 95% of the wild fish stocks had disappeared due to commercial overexploitation (Otgaar 2012). In recent years, dusky kob has been successfully farmed in South Africa (Burgess 2013), and offers an alternative to wild caught fish. It has been well received as an aquaculture species. Its good flesh quality, high market value and palatability have caused a high demand for the fish. Farmed dusky kob has also been listed as a "green" best choice species by the South African Sustainable Seafood Initiative, while line fish has been listed as "orange" threatened (SASSI 2014).

Kob currently makes up the majority of farmed marine finfish in South Africa (Eastern Cape Development Corporation 2009). High demand of the species has prompted the need for better production efficiencies. The high cost of production leads to the farmers asking for high prices for the product for it to be lucrative. It is however uncertain whether the public is willing to pay more for farmed kob (Mahieu 2015).

Dusky kob nutritional requirements

Dusky kob is a marine carnivorous predatory fish. In the wild it feeds predominantly on crustaceans such as shrimp, as well as smaller fish (Coetzee & Pool 1991). Marine predators typically have high protein and lipid requirements but have limited ability to digest carbohydrate (Wilson 1994).

Nutritional studies done on dusky kob show that it is able to efficiently feed on artificial feed (Daniel 2004, Benjamin *et al.* 2007). Juvenile dusky kob readily accept pelleted feed with high protein levels of 35-55%, with optimal levels between 45-52.3% and that up to 30% fishmeal could be replaced with soybean meal (Daniel 2004). The high protein requirement is consistent with the feeding behavior of carnivorous fish (Sheen 2014). Protein, the most important component in fish feed is also the most expensive and should be kept to a minimum in formulated feeds. Lipids which are the primary source of energy for marine fish (Wilson *et al.* 2001), have been used to spare dietary protein so that it can be utilized for tissue synthesis (Daniel 2004, Woolley 2009, Rossetti 2010, Ginindza 2014). Daniel (2004) found that a lipid level of 9% could spare protein in dusky kob diets and gave an optimal specific growth rate of

1.6 ± 0.2% body weight/day (Daniel 2004). Soybean oil was shown to be able to replace up to 28% of fish oil in formulated diets for dusky kob (Rossetti 2011). The effect of two different levels of protein (i.e. 42 and 46%) on growth and ammonia production were tested on dusky kob (Woolley 2009). Lipid levels (6, 12 or 18%) were also graded to determine the protein sparing effect and ammonia production. Feed utilization was better in diets with 46% protein compared to the 42%. The diet containing 46% protein and 18% lipid with a Protein: Energy of 2.26 g/protein / MJ:kg^{-1} gave the best growth rates suggesting that it was better utilized than the other diets. It was then concluded that this diet was close to optimum for dusky kob (Woolley 2009). The lysine requirement for dusky kob has also been established (2.6% dry diet) (Adesola *et al.* 2017), as well as protein and energy digestibility (Booth *et al*. 2013b) and amino-acid digestibility (Adesola 2016) in dusky kob. Apparent digestibility of most amino acids was found to be high in fishmeal (more than 80%), showing it is a good protein source for dusky kob (Adesola 2016). This information has laid the foundation for the development of a least cost feed.

Practical diets for dusky kob have been formulated in other countries such as Australia and France, based on the nutritional requirements of other marine carnivorous species; red drum *Sciaenops ocellatus* and meagre *A. regius* (Daniel & Robinson 1986, FAO 2004). Knowledge of the nutritional requirements of dusky kob will be important in the formulation of least cost diets which give optimum fish growth.

Investigation into the feeding frequency and intensity on juvenile dusky kob (34.6 ± 17.3 g /fish) found that fish fed once daily grew slower than those fed twice daily at 4% body

weight (Daniel 2004). Similarly, the best growth rate and feed conversion ratios of juvenile dusky kob were in fish fed a restricted diet of 3.6% body weight, two or three times a day (Collett 2007). A study done on two-year old dusky kob also confirmed that frequent feeding resulted in better growth compared to fish that were fasted periodically (Guy *et al*. 2016). The best feeding procedure is one with lowest feeding frequency at which daily ration can be provided without reducing growth and feed utilization (Shipton & Hecht 2013).

Locally produced trout feeds with high quality ingredients that is not specifically designed for kob have been another option available to farmers. However, they have not been optimized for kob and hence have been associated with water quality problems in recirculating systems, poor feed conversion ratios and high feed costs per kilogram of fish produced. A particular research gap is the optimal level of carbohydrate in formulated diets as some farmers have experienced growth and fish health problems with commercial test diets containing high carbohydrate levels (30-32%) (Peter Britz-personal communication).

Dietary carbohydrates in aquaculture

The increase in the diversity of fish feed ingredients such as carbohydrates is expected to increase due to the limited availability of fishmeal and fish oil as dietary protein and lipid sources (Gatlin *et al*. 2007). Carbohydrates constitute a group of substances which include sugars, starches, gums and celluloses, and are included in fish feed as protein sparing energy sources or binding agents for water stable pellets during extrusion (De

Silva & Anderson 1995). Compared to mammals, carbohydrates are not efficiently utilized as an energy source by many fish (Abro 2014).

Physiology and biochemistry of carbohydrate utilization by fish

Raw carbohydrates and simple sugars such as glucose and maltose are also not well utilized by fish compared to complex carbohydrates, especially carnivorous fish. This is due to its low availability in their natural diet (Halver & Hardy 2002). Raw dietary carbohydrates include a mixture of both non-starch polysaccharides and energy yielding polysaccharides such as starch (Stone *at al*. 2003). Non-starch polysaccharides like cellulose are not readily digested by most fish, while less complex carbohydrates like starch are good energy sources for most fish (Krogdahl *et al*. 2005). There are however differences in the carbohydrate metabolism between fish species. Fish that have a natural carbohydrate containing diet such as herbivores and omnivores have a better ability to digest and utilize carbohydrates than carnivorous fish (Moon 2001, Wilson 1994). Warm and freshwater fish are also better at utilizing dietary carbohydrate than marine fish (Wilson 1994). In addition to species specificity, the complexity and amount of the carbohydrate affects the way in which it is utilized by a fish (Moon 2001).

Marine fish in particular have a limited ability to digest carbohydrates therefore diets formulated for them contain mainly protein and lipids (Munilla-Moran 1996). As with most marine finfishes, the ability of dusky kob to utilize carbohydrate as an energy source has not been investigated.

Carbohydrates are a relatively cheap source of energy, however, carnivorous fish have a limited capability to utilize them as an energy source. Raw carbohydrate sources of energy are not well assimilated by carnivorous fish as they lack adequate gut enzymes such as amylases required for efficient carbohydrate digestion (Cuzon *et al.* 2000). Recent studies have however shown that cooking starch using processes such as gelatinization and extrusion makes is better utilized by carnivorous fish (Wilson 1994). Not much is known about the efficiency of dietary carbohydrate utilization as an energy source by dusky kob, however kob has similar nutrient requirements to those of other carnivorous species (Daniel 2004), suggesting it has a similar nutritional physiology.

Compared with mammals, fish are relatively glucose intolerant with a poor ability to regulate their blood glucose levels. Mammals possess two hepatic enzymes glucokinase and hexokinase which play a key role in glucose regulation (Wilson 1994). Hexokinase has low specificity for the sugar substrate and is saturated at normal mammalian glucose levels (about 4.4mM), and cannot respond to small changes in blood glucose. Glucokinase has a higher specificity (10mM) for the sugar substrate and its activity increases with an increase in blood glucose (Wilson 1994). Hexokinase has been isolated in a variety of fish species while glucokinase has been not (Furuichi and Yone 1982, Legate *et al.* 2001). The lack of glucokinase could be the reason fish are better able to utilize complex carbohydrate whereas simple sugars lead to high levels of blood glucose.

The rate at which glucose is cleared from the blood of fish following a carbohydrate rich meal is slow, which has led to them being described as “glucose intolerant” (Furuichi &

Yone 1980, Wilson 1994). Glucose intolerance is usually used to refer to patients with diabetes mellitus (Legate et al. 2001), and it has since been found that fish have insulin independent diabetes (Hertz *et al.* 1989); in other words, insulin does not efficiently reduce blood glucose levels which remain at unhealthy levels. Insulin in fish is more sensitive to amino acids than carbohydrate, as their natural diet is composed of protein which breaks down to give amino acids (Moon 2001, Hemre *et al*. 2002). It has been suggested that the hormone somatosotin, is key to glucose regulation in fish (Navarro *et al*. 2002). The hormone has been found to suppress insulin upon glucose administration. The level of somatosotin was found to rise as insulin decreased with a glucose challenge in *Oncorhynchus mykiss*, rainbow trout (Harmon *et al.* 1991). Glucose administration was also found to stimulate the expression of preprosomatostatin mRNAs *in vitro* and *in vivo* in rainbow trout (Ehrman *et al*. 2000).

Evidence to date suggest that dusky kob has a limited ability to digest cooked starch. Apparent digestibility coefficients (ADCs) of common feed ingredients by dusky kob were determined using indirect methods employing chromic oxide as the marker and faecal collection (Booth *et al*. 2013b). Extruded wheat and 100% pregelatinized unmodified wheat starch (PGWS) were tested at 20 or 30% and 10, 20 and 30% inclusion rates, respectively. Gross energy digestibility decreased significantly from 93.7 to 57.1% when the inclusion of PGWS increased from 10 to 30% (Booth *et al*. 2013b). Organic matter and gross energy ADCs of PGWS were significantly higher at the lower inclusion levels of 10 and 20% than the same parameters in extruded wheat. Extruded wheat gross energy digestibility was found to be 47.6 in the 20% inclusion and 43.3 in the 30% inclusion. Better digestibility of organic matter and energy from PGWS could be

attributed to the high degree of gelatinization and low fiber content of the product (Booth *et al*. 2013b). Linear regression analysis showed a significant negative relationship between inclusion level of pregelatinized wheat starch and organic matter or gross energy ADCs. The results suggest that pregelatinized wheat starch may provide more energy in dusky kob feeds.

Carbohydrate utilization by carnivorous marine fish

Carnivorous fish differ in their ability to digest and utilize different types of carbohydrates at different inclusion levels in formulated feeds. The average carbohydrate inclusion level in most carnivorous fish species is around 20% and anything beyond that compromises fish growth and health (Lee 2011, Shapawi *et al*. 2011). The inclusion level also depends on the fish species and carbohydrate type. Lee *et al*. (2003) investigated the utilization of glucose, maltose, dextrin and cellulose by *Paralichthys olivaceus* (juvenile flounder). Diets containing 15% cellulose, 15% glucose, 15% maltose and 5-25% dextrin were fed to different groups of fish. Fish fed 15% cellulose had the lowest weight gain while that of fish fed diets containing 15% maltose and 15-25% dextrin was higher than those fed diets with 15% maltose and 5% dextrin. Feed utilization and growth increased with increasing dextrin level. The best growth and feed utilization were obtained at 25% dextrin (Lee *et al*. 2003). Dietary dextrin levels that did not reduce fish growth were different for three different species of fish i.e. 10% for yellowtail *Seriola lalandi*, 20% for red sea bream *Pargus major* and 30% for common carp *Cyprinus carpio* (Millikin 1982). The ability of fish to digest carbohydrates also depends on the carbohydrate source and method of processing employed during the

pelleting process (Halver and Hardy 2002). Protein sparing effects of carbohydrates have also been demonstrated in fish such as yellowtail kingfish *Seriola lalandi* (Booth *et al.* 2013a), European seabass *Dicentrarchus labrax* (Peres & Oliva-Teles 2002) and yellowfin bream *Sparus latus* (Wu *et al.* 2007). With this evidence of efficient carbohydrate utilization by carnivorous fish, it is important to determine how efficiently *A. japonicus* can utilize carbohydrates as an energy source.

Aims and objectives

This study aimed at determining the optimum dietary carbohydrate inclusion in dusky kob formulated diets, in an attempt to reduce feeding costs and to contribute to the knowledge base of the nutritional requirements that have been established for kob in previous studies.

The objectives were hence to:

1. evaluate the effect of graded levels of pregelatinized maize starch on the growth and feed utilization in dusky kob; and
2. determine the effect graded levels of pregelatinized maize starch on dusky kob health by assessing (a) the post prandial blood parameters, (b) liver histology and (c) glycogen content, as well as (d) analyzing the change or shift in bacterial community structure in the gut.

CHAPTER 2

EFFECT OF GRADED LEVELS OF CARBOHYDRATES ON GROWTH AND FEED UTILIZATION

2.1 Introduction

The efficiency of fish to utilize carbohydrate as an energy source varies significantly (Stone 2003). The anatomy of the digestive tract of fish has evolved to ensure that assimilation of nutrients is optimized according to feeding habits of the fish species (Rust 2002). Herbivorous and omnivorous fish have more carbohydrate than carnivorous fish in their natural diet, and can hence better utilize dietary carbohydrate as an energy source. In artificial fish feed, carbohydrate utilization in fish is dependent on carbohydrate source, processing technique and inclusion level (Stone 2003). Furthermore, it is dependent on the activity of digestive enzymes such as amylase (Stone *et al*. 2003).

Different sources of carbohydrates are utilized in different ways by carnivorous fish. When fed a diet with wheat middlings as a carbohydrate and energy source, juvenile red drum, *Sciaenops ocellatus* demonstrated that it could utilize up to 24% carbohydrate without any adverse effects on the fish (Ellis and Reigh 1991). Carbohydrate from α-potato starch resulted in optimum growth rates at 20% inclusion in the diets of olive flounder, *Paralichthys olivaceus* (Lee 2011). These fish have similar natural diets to dusky kob (Scharf & Schlight 2000, Sola *et al*. 2007), suggesting similar metabolic pathways and feeding physiologies, indicating that high levels of cooked carbohydrate might be utilized as an energy source by dusky kob.

Various cooked carbohydrates have been included in dusky kob diets in previous feeding trials and commercial prototype diets (Daniel 2004, Woolley 2009). Vegetable starch was used as a binder at a constant level of 16.77%, and no negative effects due to the starch were observed in the fish growth (Woolley 2009). In a different study, pregelatinized maize starch was used as a carbohydrate source and was adjusted to make the diets isocaloric when the protein level was reduced (Daniel 2004). The results showed a decrease in growth rate when the carbohydrate level was included at levels higher than 27.5% (Daniel 2004). The results were not further investigated but it was concluded that the decrease in growth was due to the high level of carbohydrate (> 27.5%) and low protein content (35%). When including carbohydrate in fish diets, it is necessary to include it on a digestible energy basis (Booth *et al.* 2013b). The digestibility of pregelatinized unmodified wheat starch was tested in dusky kob diets at 10, 20 and 30% inclusion rates to study the effect of inclusion level on apparent digestibility co-efficient. The results showed a negative relationship between inclusion level and energy digestibility. Gross energy apparent digestibility for the diets at inclusion levels 10, 20, and 30% were 91.6, 88.9 and 83.6% respectively (Booth *et al.* 2013b). It is therefore necessary to determine the optimum inclusion level of carbohydrate level in dusky kob diets before it can be included as an energy source in commercial diets.

This study was carried out to evaluate the effect of pregelatinized starch and utilization of carbohydrate as a dietary energy source and to determine the optimum inclusion level in juvenile *Argyrosomous japonicus*, dusky kob formulated diets. The specific growth rates, feed conversion ratios and protein efficiency ratios were compared

between kob that were fed diets with graded levels of carbohydrate. In the trial isoenergetic diets with a constant protein energy level were formulated by varying the starch and lipid content.

2.2 Methods and materials

2.2.1 Experimental system

The experiments were conducted at the Rhodes University Department of Ichthyology and Fisheries Science in Grahamstown, South Africa. The experimental system consisted of sixteen rectangular tanks each with a maximum operating volume of 0.35 m^3/tank (Figures 2.1 and 2.2). These tanks formed part of a closed, recirculating water system. Water was drained from each tank through a 50 mm pipe into a common sediment settlement tank (0.35 m^3). It then flowed by gravity through two biological filters. The two biological filters were filled with one half (0.18 m^3) water and one half (0.18 m^3) plastic filter media. From the filters, water flowed by gravity to the sump (0.35 m^3) where it was pumped back to the fish tanks by two submersible water pumps (RESUN, Penguin 8500, 230 W, Baolong, China). The pumps delivered water at a flow rate of 406 L/h/tank. Two weeks after the start of the experiments a protein skimmer (Ultrazap, Johannesburg, South Africa) was fitted and filtered the water of the whole system (Figure 2.3). Water lost through cleaning and siphoning faeces and uneaten feed was replaced whenever necessary. The tanks were covered with one half 80% shade cloth and the other half bird netting (to allow for feeding without lifting the cover).

Water was heated using four 300W submersible heaters (RESUN therm25/300-RH9000, Malsch, Germany) connected to a temperature control unit. An airstone was placed in each tank for aeration. A 12-h dark and 12-h day (06:00 – 18:00) photoperiod was maintained throughout the experiment using a digital timer and fluorescent light.

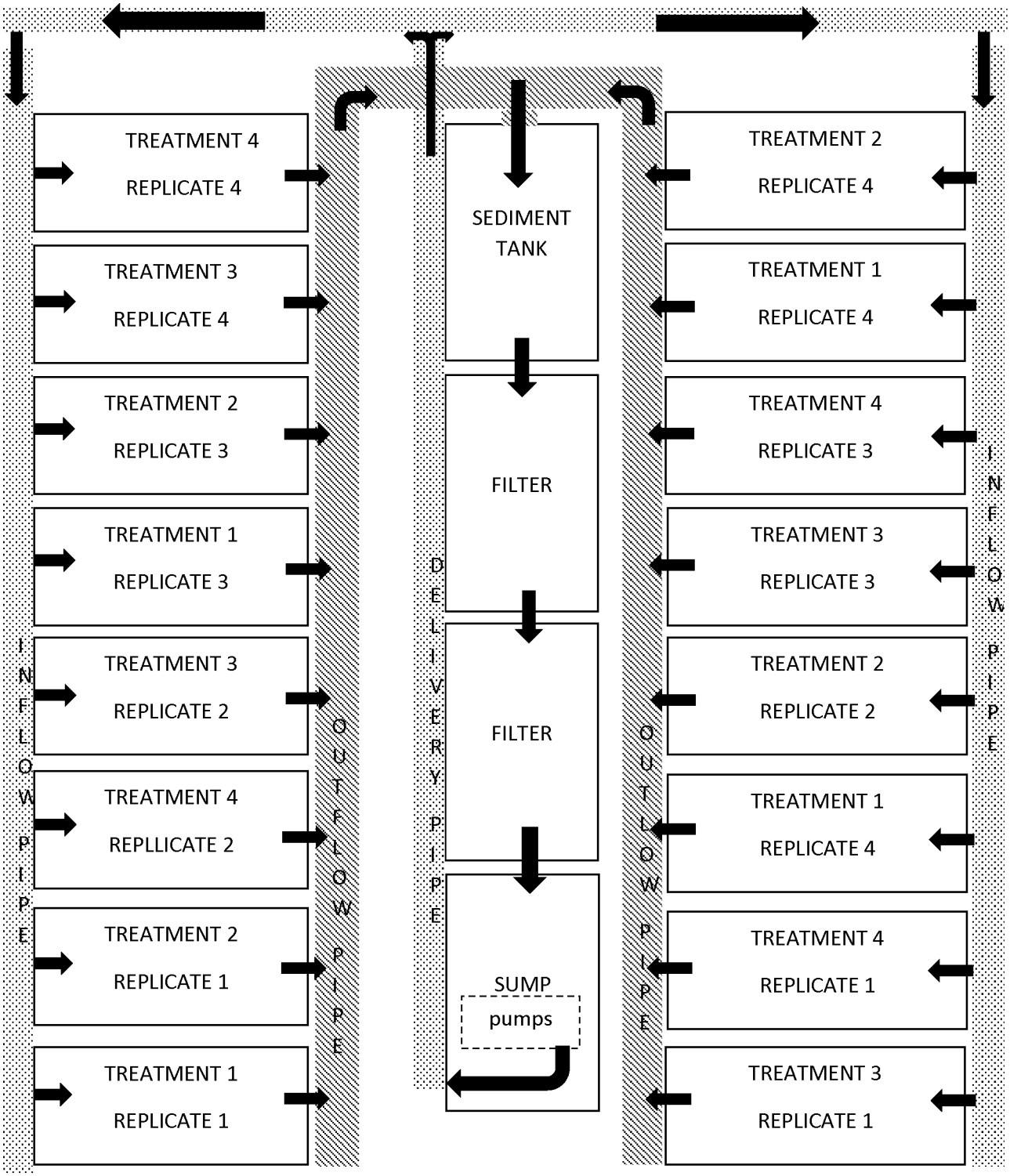

Figure 2.1: Schematic areal view of the recirculating system used for the experiments. Block arrows show the direction of the water flow in the delivery pipes (stippled) to the fish tanks and effluent outflow (horizontal lines) from the tanks. Rectangles labeled with treatment and replicate numbers represent the experimental fish tanks.

Figure 2.2: Rectangular tanks used for the growth experiments.

Figure 2.3: Protein skimmer in the sump installed to filter water throughout the experimental system.

2.2.2 Experimental fish and acclimation

Juvenile *A. japonicus* weighing about five grams each were obtained from a commercial hatchery (Pure Ocean Pty Ltd, East London, South Africa), and transported to the Rhodes University Department of Ichthyology and Fisheries Science (DIFS) in Grahamstown. The fish were acclimatized for three weeks before the experiment was started. During this period, they were fed crumble starter trout feed (50% protein, 14% fat; Aqua plus, Avi Products (Pty) Ltd, Linkhills, South Africa), and then weaned on to trout finisher two millimeter pellets (48% protein, 14% fat; Aqua plus, Avi Products (Pty) Ltd, Linkhills, South Africa). They were considered weaned when they readily accepted the dry pellets. They were fed to apparent satiation three times a day (at 08:00, 12:00 and 16:00) for the first two weeks and twice daily (09:00 and 16:00) for the last week of acclimation to acquaint them with the experimental feeding procedure.

2.2.3 Starting weight, stocking density and feeding

After the acclimation period, fish were purged for 24 h, anesthetized with 2-phenoxyethanol at 0.2 ml/l (Deacon *et al*. 1997). The fish were then weighed to the nearest gram on an electronic scale (ADAM, Highland HCB 3001, Kingston, UK), and standard length was measured to the nearest millimeter using a measuring board. The fish now with an average weight and length (± standard error) of 9.55 ± 0.24 g/fish and of 89.95 ± 1.73 mm respectively, were randomly distributed among the 16 tanks at a stocking density of 45 fish per tank which equated to 1.2 kg fish/m^3.

2.2.4 Experimental diets and treatments

Four isonitrogenous (45% protein) and isoenergetic (17.9 MJ/kg) diets were formulated according to the previous requirements established for dusky kob (Daniel 2004, Woolley *et al*. 2010, Rossetti 2011, Ginindza 2014). All the diet formulations had the same basic composition on a dry weight basis (Table 2.1). Pregelatinized starch was included at different rates (5 – 30%) that resulted in carbohydrate levels of 4.1 – 24.6% and other ingredients were adjusted to maintain protein levels between treatments, with fishmeal being the main source of protein (Table 2.1). Fish oil content was adjusted to standardize energy between the diets and to maintain a constant protein:energy ratio (Table 2.1). The maximum lipid level was limited to 18% as negative health effects have been recorded above this level (Woolley *et al*. 2010).

Pregelatinized maize starch was used as the carbohydrate source and also served as a binding agent. Marine fish oil was used as a lipid source and carboxymethyl cellulose (CMC) as a non-nutritive filler where necessary (Table 2.1).

The experimental diets were manufactured in the experimental feed laboratory (Department of Ichthyology and Fisheries Science, Rhodes University). The dry ingredients were weighed and mixed with an industrial food mixer (Macadams baking systems, SM, 201, Cape Town, South Africa). The marine fish oil and water were subsequently introduced to the dry ingredients and mixed into a homogenous dough. The dough was extruded (ICME Motor electri Bologna, Italy), cut into pellets and placed on trays and dried overnight in an oven at 38°C. The feed was placed into sealed packets, which were stored in buckets at -20°C.

Table 2.1: Raw ingredients and formulated proximal composition of the experimental diets with different inclusion of pregelatinized maize starch that resulted in varying carbohydrate contents (%C).

	4.1C	8.2C	16.4C	24.6C
Ingredient (%)				
Pregelatinized maize starch	5.00	10.00	20.00	30.00
Fishmeal	68.21	67.73	66.75	65.77
Vitamix	1.00	1.00	1.00	1.00
Marine fish oil	11.54	9.44	5.26	1.07
Carboxymehtyl cellulose	14.25	11.83	6.99	2.16
TOTAL	100.00	100.00	100.00	100.00
Formulated proximal composition (%)				
Carbohydrate	4.10	8.20	16.40	24.60
Protein	45.00	45.00	45.00	45.00
Lipid	18.20	16.20	12.20	8.20
Energy ($MJ.kg^{-1}$)	17.90	17.90	17.90	17.90
Protein:Energy ratio	2.51	2.51	2.51	2.51

Each diet was fed to fish in one tank in each of four blocks of a randomized block design, so that each replicate was represented in each block of four tanks closest or furthest from the door. Feed was limited to a maximum of 3.85% of their body weight/day (Kaiser *et al*. 2011), which was split into two meals/day (09:00 and 16:00). Any uneaten feed was removed, dried and weighed to determine feed consumption. The treatments will be referred to as 4.1C, 8.2C, 16.4C and 24.6C for 4.1, 8.2, 16.4 and 24.6% carbohydrate respectively.

2.2.5 Data collection

The fish weights and lengths were measured at the start of the trial using the methods described in Section 2.2.3 and was repeated every six weeks for a total of three months.

Dead fish were removed from the tanks on a daily basis and mortality was recorded and used to calculate the rate of survival between treatments.

2.2.6 Response monitoring

The following growth and nutritional indices were used to evaluate the performance of the fish during the growth experiment:

Specific growth rate (SGR; Ricker 1979):

SGR = [(Ln (final weight g) - Ln (Initial weight g) / no of days] x 100

Food conversion ratio (FCR; Cowey 1992):

FCR = food intake (dry weight g) / body weight gain (wet weight g)

Protein efficiency ratio (PER; Wilson 1989):

PER = body weight gain (wet weight g) / protein ingested (g)

Condition factor (CF; Bolger and Connolly 1989):

$CF = [\text{body weight (wet weight g)} / \text{total length}^{2.84}]$

2.2.7 Proximate analysis of feed

One hundred grams of each batch of formulated feed was sent to the Agricultural Research Council, Department of Poultry Science for proximate analysis. Crude protein was determined using the Kjeldahl method (AOAC Official Method 954.01) using a digester block. Fat was extracted using Soxhlet extraction method with diethyl ether

(AOAC official Method 920.39). For the determination of ash, the samples were burned in a furnace for four hours at 550 ºC (AOAC Official Method 942.05). For moisture, the samples were dried in an air circulated hot oven at 95 ºC for 72 h (AOAC Official Method 934.01). Energy was calculated following the guidelines described by Atwater and Bryant (1900), and Greenfield and Southgate (2003) (ASM 076):

$$ME = (GE_p - 7.9/6.25)\, D_p + GE_f D_f + GE_{cho} D_{cho}$$

where, GE_p, GE_f and GE_{cho} represent the gross energy of protein, fat and carbohydrate, respectively and D_p, D_f and D_{cho} represent the digestibility coefficient of protein, fat and carbohydrate, respectively.

2.2.8 Water quality

Water temperature (°C), pH and dissolved oxygen (DO, mg/l) were measured twice weekly with a hand held electric probe (Hach HQ30d Multimeter probe, Loveland, USA). Total ammonia (mg/l), nitrite (mg/l) and nitrate (mg/l) were also monitored twice weekly using commercial, calorimetric water quality test kits (Sera aquarium; NH_3 NH_4+, NO_2, NO_3 test kits, Heinsburg, Germany). Salinity (g/l) was measured weekly with a refractometer (H196822 Seawater refractometer, Hanna, Limena, Italy).

Water quality parameters were kept within the following limits: pH ranged between 7.0 and 7.8 and temperature was kept between 22 – 24°C. The salinity ranged between 33 – 35 pp, ammonia ranged between 0.0 – 0.10 mg/l, nitrite also remained below 0.10 mg/L and nitrate averaged at 20 mg/l.

2.2.9 Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) followed by a Tukey's multiple range post hoc analysis if a significant difference was found between treatments, at $p<0.05$. Kruskal-Wallis ANOVA ($p<0.05$) was used if data did not meet the assumptions of ANOVA, i.e. Shapiro-Wilk's test for normality of residuals and Levene's test for homogeneity of variance. All ANOVA analyses were performed using STATISTICA® software, version 16.0 (Statasoft, Tulsa, OK, USA) and results presented as mean ± standard error. A correlation was run to determine the relationship between SGR and FCR using STATISTICA® software, version 16.0 (Statasoft, Tulsa, OK, USA). The optimum dietary carbohydrate inclusion was estimated by third order polynomial regression analysis ($Y = ax^3 + bx^2 + cx + d$) using SGR.

2.3 Results

2.3.1 Growth performance

The mean weight, length and condition factor did not differ significantly between the fish at the start of the trial ($p > 0.05$; Table 2.2). Weight gain and SGR increased with increasing carbohydrate level in the diet up to 16.4% carbohydrate inclusion and then dropped sharply for fish fed the 24.6% carbohydrate treatment (Table 2.2). The highest SGR of 1.84%/day was observed in the fish fed diet 16.4C. The value was significantly higher than that in all the other treatments (ANOVA; $F_{(3,12)}$, $p < 0.001$; Table 2.2). The specific growth rate of fish fed diet 4.1C was significantly higher than those fed 24.6C

but was not significantly different to those given the 8.2C treatment. A third order polynomial was used to estimate the optimum dietary carbohydrate inclusion by estimating the asymptote which was at 16.72% (Figure 2.4).

The condition factor was not significantly affected by the difference in the carbohydrate contents of the treatments (ANOVA; $F_{(3,12)} = 1.28$, $p = 0.33$, Table 2.2).

Table 2.2: Mean (± standard error) weight, length and condition factor (CF) of dusky kob *A. japonicus* at the start and end of the experiment, and mean weight gain (MWG), condition factor (CF), specific growth rate (SGR) and feed intake of fish fed diets with varying carbohydrate levels (%C) for 84 days. Values with different superscripts within each row are significantly different (ANOVA, $P < 0.05$, N=4).

	4.1C	8.2C	16.4C	24.6C	$F_{(3,12)}$	Pvalue
Initial weight (g/fish)	9.32 ± 0.21	9.74 ± 0.87	9.54 ± 0.31	` 9.63 ± 0.41	0.11	0.95
Initial length (mm)	87.37 ± 1.57	91.58 ± 6.50	88.40 ± 0.65	92.43 ± 2.94	0.44	0.72
Initial CF	1.36 ± 0.05	1.44 ± 0.03	1.37 ± 0.02	1.32 ± 0.02	2.25	0.13
Final weight (g)	34.53 ± 2.15^{a}	34.89 ± 3.50^{a}	44.86 ± 1.02^{b}	18.75 ± 1.36^{c}	23.54	<0.001
Final length (mm)	131.37 ± 2.43^{a}	129.44 ± 4.25^{a}	138.55 ± 1.87^{a}	106.34 ± 2.11^{b}	24.35	<0.001
SGR (%/day)	1.55 ± 0.06^{a}	1.52 ± 0.06^{a}	1.84 ± 0.05^{b}	0.79 ± 0.05^{c}	68.48	<0.001
MWG (%)	269.47 ±17.25^{a}	258.57 ± 17.60^{a}	372.07 ± 19.86^{b}	94.20 ± 7.94^{c}	49.60	<0.001
Final CF	1.50 ± 0.02	1.54 ± 0.01	1.64 ± 0.05	1.50 ± 0.10	1.28	0.33

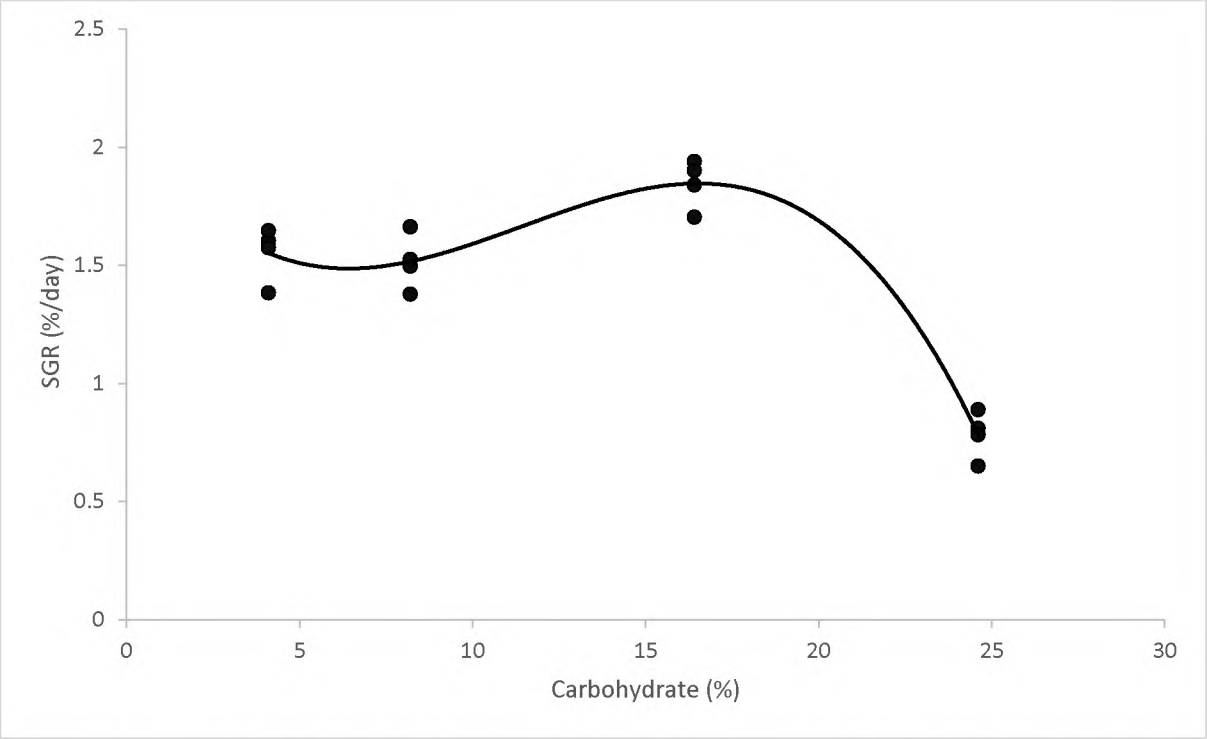

Figure 2.4: Relationship between specific growth rate (SGR, %/day) and dietary carbohydrate level (%) in juvenile dusky kob, *A. japonicus* ($y = -0.0007x^3 + 0.0239x^2 - 0.2213x + 2.1046$, $R^2 = 0.94$, $p < 0.001$).

2.3.2 Feed intake, feed conversion ratio (FCR) and protein efficiency ratio (PER)

Feed intake per fish differed significantly among the dietary treatments (ANOVA; $F_{(3,12)} = 21.47$, $p < 0.001$, Table 2.3). Diet 24.6C had the lowest feed intake of 19.16 g/fish, and the fish also appeared thin and emaciated. The feed intake in this group of fish was 80% less than the highest intake of 44.99 g/fish observed in the 16.4C. The fish fed diets 4.1C, 8.2C and 16.4C did not differ significantly in feed intake (Table 2.3).

Carbohydrate level had a significant effect on the FCR of dusky kob (ANOVA; $F_{(3,12)} = 8.38$, $p = 0.003$, Table 2.3). The lowest FCR value of 1.28 was observed in fish fed diet 16.4C followed by diets 4.1C and 8.2C and the highest was FCR value of 2.14 was found in the 24.6C diet. Protein efficiency ratio (PER) ranged from 1.04 to 1.76.

Significant differences were observed between diet 16.4C and diet 24.6C but diet 4.1C and 8.2C were similar ($H_{(3,12)}$ = 10.26, p = 0.02; Table 2.3).

Table 2.3: Mean (± standard error) feed intake, feed conversion ratio (FCR) and protein efficiency ratio (PER) of dusky kob, *A. japonicus* fed diets with varying carbohydrate levels (%C) for 84 days (ANOVA/Kruskal Wallis). Values with different superscripts within row are significantly different (P < 0.05, N=4).

	4.1C	**8.2C**	**16.4C**	**24.6C**	**F/$H_{(3,12)}$**	**P value**
Feed intake (g/fish)	42.02 ± 3.05^{a}	39.66 ± 2.78^{a}	44.99 ± 2.71^{a}	19.16 ± 1.14^{b}	21.47	< 0.001
FCR	1.67 ± 0.04^{ab}	1.62 ± 0.19^{b}	1.28 ± 0.08^{b}	2.14 ± 0.13^{ac}	8.38	< 0.001
PER	1.33 ± 0.03^{c}	1.42 ± 0.16^{c}	1.76 ± 0.12^{d}	1.04 ± 0.06^{e}	10.25	0.02

2.3.3 Specific growth rate (SGR) and feed conversion ratio (FCR) correlation

The correlation between SGR and FCR showed a significantly inverse relationship between the values (correlation coefficient: -0.89, p < 0.05), where FCR decreased with increasing SGR (Figure 2.5).

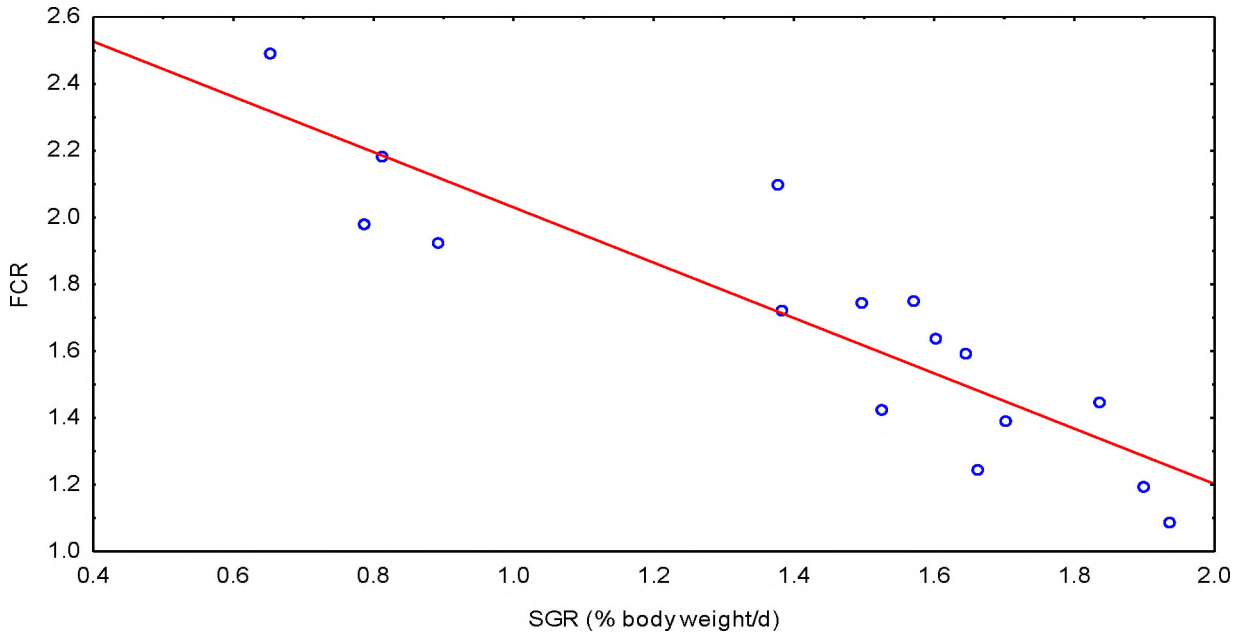

Figure 2.5: Correlation between specific growth ratio (SGR) and feed conversion ratio (FCR) of dusky kob, *A. japonicus* fed diets with graded levels of carbohydrates (FCR = 2.86 – 0.83 * SGR, r = -0.89).

2.3.4 Survival

The fish survival rates ranged between 34.13 and 90.67%. The rates of survival in fish fed diets 4.1C, 8.2C and 16.4C were all similar (83.05 ± 2.39 – 90.67 ± 1.41% survival), and all differed significantly from those fed diet 24.6C ($F_{(3,12)} = 72.49$, $p < 0.001$). Fish fed diet 24.6C had the lowest survival rate where only 34.13 ± 4.03% of the initial population of 45 fish survived.

3.2.5 Proximate composition of the diets

The proximate analysis of the diets showed a decline in protein and dietary energy content with increasing carbohydrate content in the diets (Table 2.4).

Table 2.4: Proximate composition of four experimental diets with graded levels of pregelatinized starch that resulted in varying carbohydrate contents (%C) as analyzed after the diets were manufactured in the laboratory.

	4.1C	8.2C	16.4C	24.6C
Composition				
Protein (%)	41.21	39.53	38.98	38.78
Lipid (%)	16.13	13.22	9.54	5.75
Moisture (%)	11.82	15.19	17.13	18.86
Ash (%)	12.66	11.75	10.79	10.14
Energy (KJ/kg)	18.79	17.73	17.22	16.13
Protein:Energy ratio	2.19	2.23	2.26	2.40

2.4 Discussion

The present study showed that growth, feed conversion ratio and protein efficiency ratio in dusky kob were significantly affected by dietary carbohydrate with the best performance in respect of growth, SGR, FCR and PER obtained for the 16.4%

carbohydrate content diet. The optimal inclusion level was predicted to be 16.7% carbohydrate, with growth performance, FCR and SGR declining sharply above this level. This suggests that inclusion of pregelatinized carbohydrate in juvenile kob diets up to 16.7% can produce optimal growth and feed utilization while sparing protein and lipid energy. The assumption underpinning this recommendation is that the starch energy efficiently substituted lipid energy and that both were equivalently digestible and available. Further trials will be required to validate the extent to which this is true. The sharp decline in performance at higher levels of starch suggest that the ability of the fish to digest and assimilate the starch breaks down above 20% dietary starch content (Moon 2001). The dietary protein content could have influenced the results as the dietary proximal analysis revealed that the actual protein content of the diets varied from the formulation, with a 3% decline between the lowest and highest carbohydrate diets. The protein content of the 16.4C and 24.6C was however very similar and thus the performance decline can be ascribed to the high starch level (Halver & Hardy 2002).

The present results are similar to those obtained for other cultured marine fish. For example, humpback grouper, *Cromileptes altivelis*, was found to have better growth when fed a diet containing 20% maize compared to a diet with a lower 10% maize (Shapawi *et al.* 2011). Research on other carnivorous marine fish has also found that inclusion of a certain amount of carbohydrate in diets helped improve fish growth. Up to 10% pregelatinized wheat starch supported growth of yellowtail kingfish, *Seriola lalandi* and any amount beyond that point compromised growth (Booth et al. 2013a). Contrary to yellowtail, red drum, *Sciaenops ocellatus*, which is also a carnivorous fish showed an inverse relationship between carbohydrate inclusion and growth; the best growth rate

was found when carbohydrate was included at 24% which was the lowest inclusion level used in the study (Ellis and Reigh 1991). Growth performance and feed utilization were inversely related to carbohydrate content in the feed and protein was better spared by lipid rather than carbohydrate, showing that red drum has a limited ability to utilize carbohydrate as an energy source (Ellis and Reigh 1991). These contrasting results demonstrate the different abilities of the different carnivorous (freshwater and marine) fish to utilize carbohydrate as an energy source (Table 2.5).

Table 2.5: Optimum inclusion levels of carbohydrate from different sources for different fish species.

Fish species	Carbohydrate source	Optimum inclusion level (%)	Reference
Clarias gariepinus	Corn fibre	5	Orire and Sadiku (2014)
	Corn starch	15	Orire and Sadiku (2014)
Chianna striata	Dextrin + wheat flour	12	Arockiaraj *et al*. (1999)
Paralichthys olivaceus	Dextrin	15-20	Lee *et al*. (2003)
	Starch	20	Lee *et al*. (2003)
Mystus montanus	Wheat flour	9.48	Raj *et al*. (2008)
Oncorhynchus mykiss	Gelatinized corn starch	12	Gumus and Ikiz (2009)
Cromileptes altivelis	Maize	20	Shapawi *et al*. (2011)
Seriola lalandi	Pregelatinized wheat starch	10	Booth *et al.* (2013a)
	Extruded wheat	10	Booth *et al.* (2013a)
Sciaenops ocellatus	Wheat middlings	24	Ellis and Reigh (1991)

The results obtained in this study show that, like most carnivorous fish, dusky kob can digest cooked starch. The results are in line with results obtained in other fish species that were fed diets with carbohydrate, where the optimum inclusion levels ranged from 10 - 20% (Table 2.5).

Fish in this study attained a SGR of 1.8%/day when fed a diet containing 16.4% carbohydrate. Marine carnivorous fish are known to be better able to utilize dietary lipid, compared to carbohydrate (Castro *et al.* 2016). Dusky kob fed diets containing graded

amounts of lipid could obtain a highest SGR of 1.5%/day when fed a diet containing 6.0% lipid (Ginindza 2014). This suggests that dusky kob can utilize carbohydrate as well as lipid in their diet. The ability of dusky kob and cultured marine predators to efficiently utilize cooked carbohydrate is probably related their digestive enzymes. Amylase, the enzyme responsible for carbohydrate digestion has been found to be high in marine fish during larval development and thereafter decreases (Zouiten *et al*. 2008). The activity of amylase in the digestive system of marine predatory fish is stimulated by the presence of carbohydrate in their diet (Hunter 2015). Raw starch however inhibits amylase activity (Milikin 1982). The process of gelatinization which involves the partial breakdown of non-starch polysaccharides improves the apparent digestibility of carbohydrate (Stone *et al.* 2003). Gelatinization makes carbohydrate chains more accessible to enzymes, consequently increasing the activity of amylase (Baks *et al*. 2007). Carnivorous fish such as red drum have been found to have enzymes involved in carbohydrate digestion from larval stage such as amylase (Lazo 2000). Other marine fish such as kingfish, *Scomberomorus cavalla* (Chen *et al*. 2006) and yellowtail, *Seriola lalandi* (Shimeno *et al*. 1979) also have been found to have amylase activity. Amylase activity has been characterized in dusky kob larvae (Hunter 2015), and this amylase activity could be the reason dusky kob in this study could utilize a high amount of carbohydrate.

The best protein efficiency ratio obtained in this study was at 16.4% carbohydrate level in the feed. The observed decline in the efficiency of protein utilization in the high carbohydrate diet suggests that there was a limit to the protein sparing effect, which was predicted to occur at 16.7% in the present experiment. This could be attributed to

nutritional restrictions considering that the diet was not well consumed by dusky kob. Unpalatability of feed could have led to a physiological nutritional feedback loop where feed was consumed at low quantities. The overall decline in all performance indicators is indicative of a physiological breakdown of fish not being able to handle high starch content despite all diets being formulated to be isonitrogenous and isocaloric.

A strong positive relationship was observed between SGR and carbohydrate level in the feed up to 16.7% carbohydrate content. This suggests that kob can efficiently utilize cooked carbohydrate of up to 16.7% as an energy source, this was also evidenced by the protein sparing effects indicated by PER. The optimal carbohydrate inclusion of 16.7% does not differ greatly from the optimal carbohydrate of 20% in the marine humpback grouper (Lee *et al.* 2003). It is however higher than the 5% optimum of *Clarias gariepinus* diets, which is a warmwater fish and would normally be expected to have a higher optimum dietary carbohydrate than marine fish (Orire and Sadiku 2014) (Table 2.5). The strong correlation observed between SGR and FCR show that FCR improved with increasing SGR in the fish.

Feed conversion and protein efficiency ratios obtained in the current study were inferior to those which have been found for dusky kob in previous growth trials (Woolley 2009, Rossetti 2011). The study by Woolley (2009) found FCR and PER values of 1.05 and 1.89 respectively, while that of Rossetti (2011) found values of 1.11 and 1.90 respectively. The FCR of 1.28 and PER of 1.76 were the best obtained in the current study. The protein levels used in the current study (38.78 - 41.21%) were however lower than those used in the previously mentioned studies (46 and 48%).

In the present study, reduced growth in the fish fed the diet with 24.6% carbohydrate inclusion could be due to reduced feed intake which then led to fish starvation. A study done by Woolley (2009) reported that acceptance of pellets by fish was affected by texture and hardness. The diet with 24.6% carbohydrate had a harder texture than the other diets, due to the high amount of carbohydrate which acted as an additional binder. Dusky kob in the present study were often observed biting onto a pellet and then spitting it out. Similar behavior was observed in dusky kob by in a different study (Benjamin *et al*. 2007). Ejection of whole, partially chewed or crushed pellets was seen when the fish were fed pellets with extra binder. Similarly, softer pellets reduced the chance of chewing and ejection in *S. aurata (*Andrew *et al.* 2004). The texture of the pellets in the current study probably played a role in feed intake of fish fed diet 24.6C.

Low survival rates in the fish fed diets containing 24.6% carbohydrate could also be attributed to low feed intake. Starvation mortalities have been reported in fish such as *Perca flavescens*, yellow perch juveniles (Manning *et al*. 2014). The survival of fish between the four dietary groups were generally high except for fish fed the diet containing 24.6% carbohydrate. The fish appeared thin and emaciated. The high mortality rate in this group of fish is thus likely due to starvation, which was also evidenced by the significantly low final weights. Smaller size fish have been reported to be more vulnerable to death due to starvation (Miller *et al.* 1988). The small size of this group of fish rendered them more susceptible to starvation mortalities.

High levels of carbohydrate in fish feed also lead to reduced apparent digestibility of feed ingredients (Booth *et al*. 2013b), where the digestibility of dietary ingredient generally decreases with an increase in the carbohydrate dietary component. Low

digestibility could have then resulted in diffusion being impaired, as well as transport of digestive enzymes (Amin *et al*. 2014). These reasons could explain the low weight gain, slow growth rate and poor nutrient utilization and low survival in the fish fed the diet containing 24.6% carbohydrate.

The results obtained in this study show that cooked carbohydrate has potential use in dusky kob formulated feeds both as a binder and energy source. This information will be important in assisting with the formulation of least cost diets for this fish. Carbohydrate inclusion of up 16.7% will improve growth and feed utilization and anything beyond that point will have negative effects on dusk kob growth.

CHAPTER 3

THE EFFECT OF GRADED LEVELS OF DIETARY CARBOHYDRATE ON HEALTH

3.1 Introduction

High levels of digestible carbohydrates in in the diet of carnivorous fish are unfavorable to health. Formulated diets usually contain high levels of carbohydrate used as binding agents or energy sources, while the natural diet of carnivorous fish is completely devoid of carbohydrate (Halver & Hardy 2002). Undesirable carbohydrate levels places stress on the digestive system, which results in certain physiological responses in the fish (Hemre *et al*. 2002). Health effects such as deposition and accumulation of fat in the visceral organs and flesh, fatty liver tissue, high levels of glucose and triglycerides in the blood as well as reduced liver function can occur as a result (Wilson 1994, Krogdahl *et al*., 2005, Zhou *et al.* 2015). Comprehensively assessing these health effects is required before a formulated diet can be used in commercial aquaculture. The aim of this study was thus to assess the health status of dusky kob fed diets with graded levels of pregelatinized starch.

Various health indices and tests can be used to assess the health status of a fish. Two of the commonly used health indices are the hepatosomatic index (HSI) which is the relationship between liver and body weight, and the visceral index (VSI) which is the ratio between visceral organ and body weight. Unhealthy amounts of carbohydrates in the diet may lead to fat deposition in the internal organs, thereby increasing the visceral and liver weights which will then result in high HSI or VSI (Ahmad *et al*. 2012).

Histological investigation of cellular structures for disturbance can be a reliable indicator of whether fish have been subjected to environmental or nutritional stress (Mekkawy 2012, Osman 2012). Histological structures in the liver is dependent on the age, temperature, food and lipid content (Genten *et al*. 2009). The liver is the central organ of metabolism and acts in defense against toxins (Genten *et al*. 2009). Nutritional imbalances and environmental conditions of a fish can be reflected in the fish liver. Histology is particularly useful for assessing the condition of the liver, which is a key indicator of a fish's health status. The liver is a large gland in the body and is responsible for metabolism, catabolism and excretion of toxic substances (Genten *et al*. 2009). In some teleost fish the liver is a compound organ known as hepatopancreas, unlike in some fish where the pancreas is a separate organ (Roberts & Ellis 2001). It is usually reddish brown in healthy fish, darker in carnivorous fish and light brown in herbivores (Bruslé & Anadon 1996). The livers of cultured fish are usually very light in colour due to the high lipid levels in formulated diets (Bruslé & Anadon 1996). The functional cells of the liver are hepatocytes which make up about 80% of the liver cells and the remainder is made up of spindle shaped macrophages (Genten *et al*. 2009). Hepatocytes are spherical in shape with a centrally located single nucleolus. They are arranged into polygonal shaped structures separated by sinusoids where blood movement takes place (Osman 2012). The pigments containing macrophage cells usually aggregate in melano-macrophage aggregates in the stroma of hemopoietic tissue, around blood vessels and lymphatic structures (Roberts 1975). Melanomacrophage centers increase in number and size when a fish has been exposed to unfavorable or stressful conditions (Dezfuli *et al.* 2007). Excess

energy in the diet can lead to fatty deposition in the liver which distorts the normal structure and arrangement of hepatocytes. Lipid deposits are often seen as clear round structure in the hepatocytes (Rossetti 2011). It can also lead to cell shrinkage or even lysis. Bile ducts also occur in the liver parenchyma. Small and large bile ducts can be observed and differ in the amount of epithelial cells around them (Genten *et al*. 2009). The hepatic structure varies considerably with age, gender, food availability and environmental conditions (Genten *et al*. 2009). Excess carbohydrate can also be stored in the liver as glycogen which may lead to a reduction in liver function, decreasing its ability to handle stress (Amoah *et al.* 2008). Liver glycogen can therefore be an important indicator of fish health (Peters *et al*. 1987). Stored glycogen is used as an energy source in critical stress situations, monitoring change in glycogen can assist in determining the health status of a fish at any point (Bruslé 1990).

The study of haematological parameters is another useful tool for evaluating diet related stress in fish, as blood parameters are sensitive to changes in the health of fish (Blaxhall & Daisley 1973). They are patho-physiological indicators of whole body condition. Blood parameters such as glucose, triglycerides and cholesterol can assist in determining the health status of a fish (Moon 2000, Woolley 2009, Zhou *et al.* 2015). Glucose and triglyceride levels in the blood tend to rise in fish subjected to stress and conditions such as hyperglycemia and hypertriglyceridemia occur as a consequence (Parks 2001, Kandepaan 2014, Zhou 2015).

Ingestion of feed containing high amounts of carbohydrate results in high blood glucose in the fish. Fish have a poor ability to clear glucose from the blood and this then results in post prandial hyperglycemia (Moon 2001). Spikes in blood glucose are observed

following a meal which often places metabolic stress on the fish (Zhou *et al.* 2013). The magnitude and duration of hyperglycemia are species dependent (Moon 2001). Glucose tolerance tests have been conducted in kingfish *Seriola lalandi* (Booth *et al.* 2013a), yellowtail *Seriola quinqueradiata* and red sea bream *Sparus aurata* (Furuichi & Yone 1982). In all cases, oral administration of glucose resulted in hyperglycemia. Hyperglycemia has also been observed in juvenile flounder (*Paralichthys olivaceus*), fed diets containing dextrin and maltose (Lee *et al.* 2003).

High blood glucose and triglycerides can cause excessive stress in the fish liver, which is the center of metabolic processes. Excess energy from diet is stored in the liver as triglycerides in some fish species (Corraze 2001). Hepatic cells are incapable of oxidizing fatty acids when there is a surplus of energy; this then leads to deposition of triglycerides (Caballero *et al.* 2004).

Bacterial diversity in the gut of a fish can provide insight into the health status of a fish (Cahill 1990). The presence of carbohydrate in the diet of dusky kob can influence gut microbiota, as unlike omnivores carbohydrates are not in the natural diet of carnivorous fish (Silva *et al.* 2005). Bacterial fermentation of carbohydrate has been demonstrated in some species such as sea bass (Leenhouwers *et al.*, 2008). Such kinds of bacterial host interaction can provide information about carbohydrate digestion in carnivorous fish, and this has not been investigated in dusky kob.

The aims of this chapter were to describe the changes that occur in the body of dusky kob fed diets with graded levels of carbohydrate. This was done by comparing various blood parameters, liver glycogen, liver histology, gut bacterial community and whole

body composition of dusky kob fed carbohydrates that ranged from 4.1 to 24.6% of the diet.

3.2 Materials and methods

3.2.1 Experimental treatments

The data presented in this chapter were collected from the fish in the growth experiment (Chapter 2). As such, the data presented here were collected from the same numbers of replicate tanks of fish, which were subject to the same experimental treatments presented in Section 2.2.4 (Chapter 2), and for the same period of 84 d.

3.2.2 Hepatosomatic index (HSI) and visceral index (VSI)

After the final weigh and measure (Chapter 2) two fish were randomly selected from each tank and were euthanized using 0.4 ml/l 2- phenoxyethanol (Brown 2003). The liver of each fish was removed and weighed (0.1 g) and the hepatosomatic index (HSI) was calculated using the following equation:

HSI = liver mass/eviscerated body mass x 100

Eviscerated body mass was calculated by multiplying the body mass was by a conversion factor, since only whole body mass was recorded at the time that the livers were weighed. This conversion factor was determined by euthanizing 30 randomly selected fish from the same size range of fish in this trial, removing and weighing their viscera and dividing this visceral weight by the body weight.

3.2.3 Macroscopic evaluation of the liver

At the end of the growth trial (Chapter 2), and additional two fish per tank were sampled and given a lethal dose of 2-phenxyethanol (0.4 ml/l) (Brown 2003), dissected aseptically on ice using scissors to expose the liver which was then removed using forceps. The liver colour and condition were recorded. Dissecting equipment was sterilized in 70% alcohol in between dissections.

3.2.4 Liver histology and glycogen

The same fish livers sampled in 3.2.3 were either fixed in 10% buffered formalin for histological examination or frozen at -80ºC for glycogen analysis (after examination in Section 3.2.3).

Histology

The fish liver samples were dehydrated in a graded ethanol series and then embedded in paraffin wax. Sections of four micrometers were cut and stained with hematoxylin and eosin (H&E) (Bancroft & Cook 1994).

Examination of histological slides was performed using a light microscope (Olympus, BX50, Tokyo, Japan). Pictures were taken using a digital camera (Olympus, U-CMAD3, Tokyo, Japan) mounted on the microscope.

Glycogen analysis

Glycogen analysis was carried out using the methods described by Woodcock and Bekendorff (2008): approximately 0.250 g of liver sample was homogenized, 0.6 M

perchloric acid (PCA) was added at a ratio of 1:5 and samples were incubated at room temperature for 30 min. Samples were centrifuged for 10 min at 10 000 rpm. Six hundred microliters of supernatant removed following initial centrifugation were centrifuged for 5 minutes at 10 000 rpm to separate particles from mixture. Seventy seven microliters of the supernatant was added to 500 µL iodine reagent and 77 µL of the supernatant was added to 500 µL triple distilled water, to use as the blank sample. Samples were incubated at room temperature for 20 min then two microliters of each sample were used to determine the absorbance. Absorbance of the solution was recorded using a spectrophotometer (Thermo Scientific, Nanodrop 2000, Massachussetes, USA) at a wave length of 460 nm. The absorbance readings were converted to glycogen concentrations using a standard curve prepared with oyster glycogen (Sigma Chemicals, USA).

3.2.5 Gut microbial communities

Sample collection

An initial six gut samples were obtained from dusky kob that had been fed trout finisher two millimeter pellets (48% protein, 14% fat; Aqua plus, Avi Products (Pty) Ltd, Linkhills, South Africa). The fish were fed this diet for two weeks during the acclimation period of the growth trial described in Chapter 2, prior to the introduction of the experimental diets and after their digestive tracts had been purged for 48 hours.. After three months, gut samples from two fish per tank for each of the four replicates per diet treatment (i.e. eight fish per diet) were collected. These fish were euthanized (0.4 ml/l 2-phenoxyethanol; Brown 2003), submerged in 70% ethanol for one minute to remove

surface bacteria (Sawabe *et al.* 1995), and dissected aseptically on ice. A foregut (the intestine posterior to the pyloric caeca and anterior to the first intestinal loop) and a hindgut region (posterior to the second intestinal loop, extending to the anus) was sectioned from each fish using a sterilized scalpel. Gut samples were scraped free of excess feed and then stored at - 20ºC before deoxyribonucleic acid (DNA) extraction.

Fore and hindgut samples were combined for each replicate tank (N= 2) to obtain a maximum of 250 mg tissue. The composite intestinal region samples were digested through incubation with Proteinase K and total genomic DNA was extracted using a PowerFecal® DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA 92010, USA). Cell lysis was performed in microcentrifuge tubes containing small (~1 mm diameter) garnet beads, lysis buffer, and a SDS-detergent, during mechanical aggravation (vortexing) for tubes heated to 65°C. The tubes were centrifuged and the lysate was mixed with a reagent to precipitate organic and inorganic contaminants. The DNA-containing lysate was transferred to a spin filter to allow DNA to bind with a silica membrane in the presence of a high salt concentration. The membrane-bound DNA was subsequently washed and DNA was released from the membrane with the addition of an elution buffer and stored at -20°C.

The DNA was submitted to MR DNA (www.mrdnalab.com, Shallowater, TX, USA) for sequencing. Barcoded 16S ribosomal ribonucleic acid (rRNA) gene polymerase chain reaction (PCR) primers 515/806 for the variable V4 region were used with a HotStarTaq Plus Master Mix Kit (Qiagen, USA) in a standard PCR reaction protocol (MR DNA). Sequencing was performed on a MiSeq sequencer. Sequence data were processed using the MR DNA analysis pipelines: small (< 150 bp) and ambiguous sequences were

removed and sequences were denoised. Operational taxonomic units (OTUs) were generated by clustering sequences with a 97% similarity and classified using BLASTn against RDPII and NCBI databases (www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu).

3.2.6 Whole body proximate analysis

At the start of the trial, a composite sample of six randomly selected fish from the original population from which the fish in the trial were selected, were sacrificed in 0.4 mg/ml 2-phenoxyethanol and immediately frozen at -20°C for whole body proximate analysis. Two composite samples, each sample consisting of two fish that were fed each of the experimental diets (i.e. two samples from a total of four fish per treatment, where each fish originated from a different tank) were taken at the end of the growth trial. The samples were sent to the Agricultural Research Council, Department of Poultry Science for whole body proximate analysis. Crude protein was determined using the Kjeldahl method (AOAC Official Method 954.01) using a digester block. Fat was extracted using Soxhlet extraction method with diethyl ether (AOAC official Method 920.39). For the determination of ash, the samples were burned in a furnace for four hours at 550ºC (AOAC Official Method 942.05). For moisture, the samples were dried in an air circulated hot oven at 95ºC for 72 h (AOAC Official Method 934.01). Energy was calculated following the guidelines described by Atwater and Bryant (1900), and Greenfield and Southgate (2003) (ASM 076):

$$ME = (GE_p - 7.9/6.25)\, D_p + GE_f D_f + GE_{cho} D_{cho}$$

where, GE_p, GE_f and GE_{cho} represent the gross energy of protein, fat and carbohydrate, respectively and D_p, D_f and D_{cho} represent the digestibility coefficient of protein, fat and carbohydrate, respectively.

3.2.7 Postprandial glucose and triglycerides

At the end of the feeding trial, after the final weigh and measure (Chapter 2), fish were fasted for 72 h. On the fourth day, they were fed their respective diets at 09:00. The exact time of feeding was recorded for each tank. Blood samples were collected from one fish per replicate, per sample time, which included a sample before the fish were fed and 1, 3, 6, 12, 24 and 48 h postprandial. These samples were taken from the caudal peduncle of the fish using a 23 gauge, 1.5 inch needle and 3.0 cc syringe. A drop of blood was placed onto a test strip (Accutrend Plus, Roche Diagnostics, Vokietjia, Germany) for either glucose or triglyceride analysis. The strips were then inserted into a blood glucose and triglyceride analyzer (Accutrend Plus, Roche Diagnostics, Vokietjia, Germany). After the blood sample was drawn, the fish was placed into a separate holding basket to avoid one fish being samples more than once.

3.2.8 Statistical analysis,

Data were subjected to one-way analysis of variance (ANOVA) followed by a Tukey's multiple range post hoc analysis if a significant difference was found between treatments, at $p<0.05$. Kruskal-Wallis ANOVA ($p<0.05$) was used if data did not meet the assumptions of ANOVA, i.e. Shapiro-Wilk's test for normality of residuals and Levene's test for homogeneity of variance. Repeated measure ANOVA was used to

determine the differences between glucose levels over time. All ANOVA analyses were performed using STATISTICA® software, version 16.0 (Statasoft, Tulsa, OK, USA) and results presented as mean ± standard error.

Shannon diversities (*H'*) and differences in bacterial operational taxonomic units (OTUs) composition between treatments were analysed with a non-metric multidimensional scaling (n-MDS) plot, one-way analysis of similarity (ANOSIM) and similarity percentages (SIMPER) analyses with a Bray-Curtis similarity measure using PAST® statistical software (Hammer et al. 2001).

3.3 Results

3.3.1 Postprandial blood glucose and triglycerides

There was a significant interaction between time and diet on post meal blood glucose concentration (ANOVA: $F_{(6,72)} = 4.24$, $p < 0.001$; Figure 3.1). Fasting blood glucose concentration of fish fed the four dietary treatments had a mean of 2.79 ± 0.34 mmol/l. Glucose concentration of fish fed diets 16.4C and 24.6C increased to 7.18 ± 1.81 and 8.05 ± 2.35 mmol/l, respectively around 24 h after feeding (Figure 3.1). This returned to the resting glucose concentration after 48 h in fish fed diet 16.4C, whereas the blood glucose did not return to resting concentration in this period for fish fed the highest (26.4C) carbohydrate amount (Figure 3.1). The glucose level of the fish fed diets 4.1C and 8.2C remained more constant over the entire period (Figure 3.1).

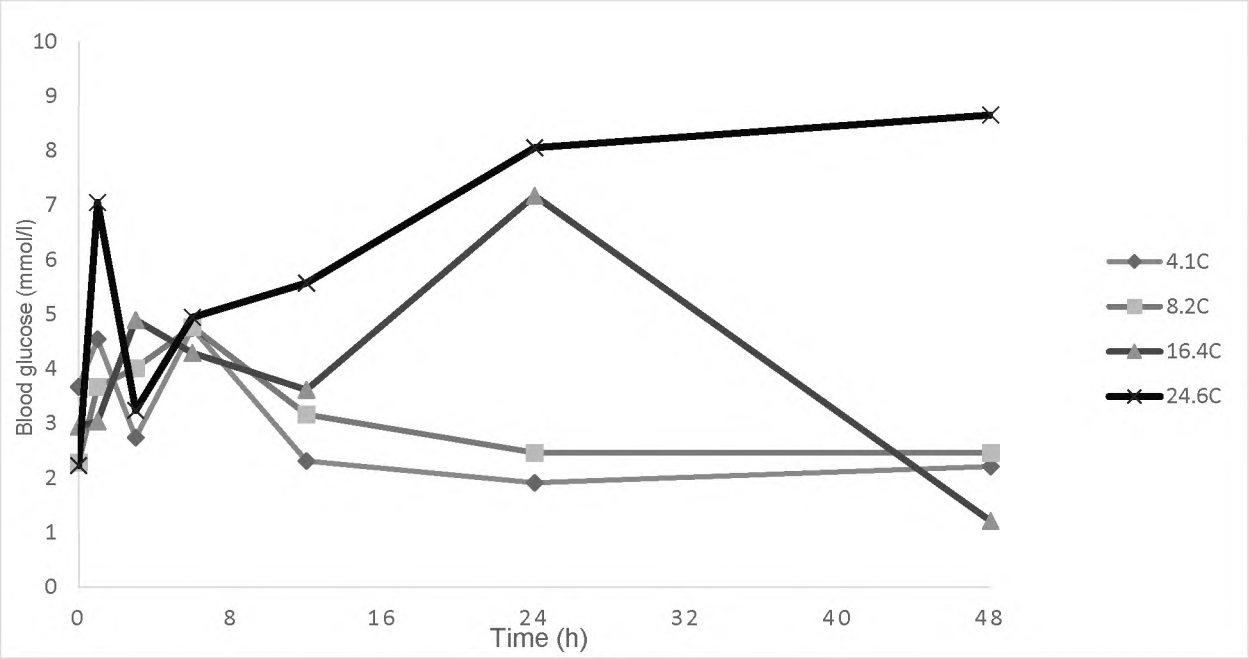

Figure 3.1: Mean pre- and post-prandial glucose concentration in the blood of dusky kob *A. japonicus* fed diets with graded levels of carbohydrates (%C) for 84 days.

The mean fasting triglycerides concentration between all dietary treatments was 1.39 ± 0.22 mmol/l Carbohydrate level in the diet had a significant effect on the blood triglycerides (ANOVA: $F_{(6,72)} = 10.46$, $p = 0.001$; Figure 3.2). Diet 24.6C was significantly lower than all the other diets (Figure 3.2). There was no significant difference due to time or interaction between time and diet (repeated measures ANOVA: $F_{(6,72)} = 2.25$, $p = 0.05$ and $F_{(6,72)} = 1.13$, $p = 0.34$ for time and an interaction of time and diet, respectively).

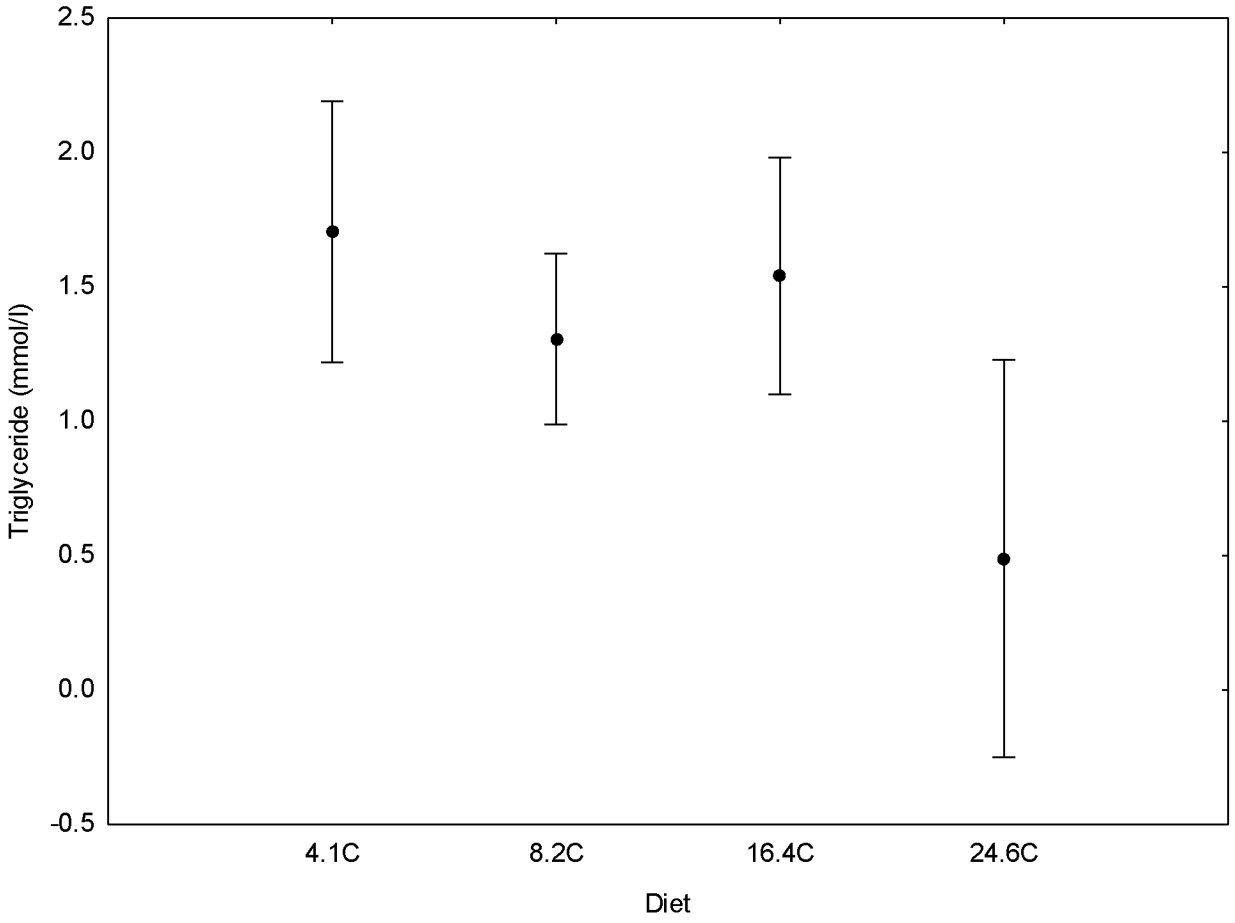

Figure 3.2: Mean triglyceride concentration (mmol/l) in the blood of *A. japonicus* dusky kob fed diets with graded levels of carbohydrate (%C) for 84 days. Error bars represent standard error of treatments means.

3.3.2 Hepatosomatic index (HSI) and visceral index (VSI)

The HSI values ranged from 0.93 ± 0.19 to 1.34 ± 0.16 but there was no trend observed between the groups of fish fed the different diets. There were no significant differences in the HSI of dusky kob fed different diets after 84 days of feeding (ANOVA: $F_{(3,12)}$ = 0.49, p = 0.49, Table 3.1). Mean VSI was also similar between the four dietary groups (ANOVA: $F_{(3,12)}$ = 0.76, p = 0.54, Table 3.1).

Table 3.1: Mean (± standard error) hepatosomatic index (HSI) and visceral index (VSI) of fish fed diets with graded levels of carbohydrate (%C) for 84 days (ANOVA, $P < 0.05$, $N = 4$).

	4.1C	8.2C	16.4C	24.6C	$F_{(3,12)}$	P value
HSI	1.25 ± 0.38	0.93 ± 0.19	1.34 ± 0.16	1.18 ± 0.19	0.49	0.69
VSI	4.44 ±0.09	4.06 ± 0.27	3.96 ± 0.58	3.62 ± 0.43	0.76	0.54

3.3.3 Macroscopic and morphologic evaluation of the liver

Fish livers from all dietary treatments showed an unhealthy, pale colour. They also a very soft texture and broke easily. The livers of fish fed diets 4.1C and 8.2C also appeared swollen (Figure 3.3).

A B C D

Figure 3.3: Livers taken from dusky kob, *A. japonicus* fed diets (A) 4.1C, (B) 8.2C, (C) 16.4C and (D) 24.6C for 84 days. All livers were pale (A, B, C and D) and some were swollen (A and B).

3.3.4 Liver histology and glycogen content

Histology

The general architecture of the liver, bile ducts and hepatopancreas showed no major abnormalities between all treatments and histological characteristics were similar (Figure 3.4).

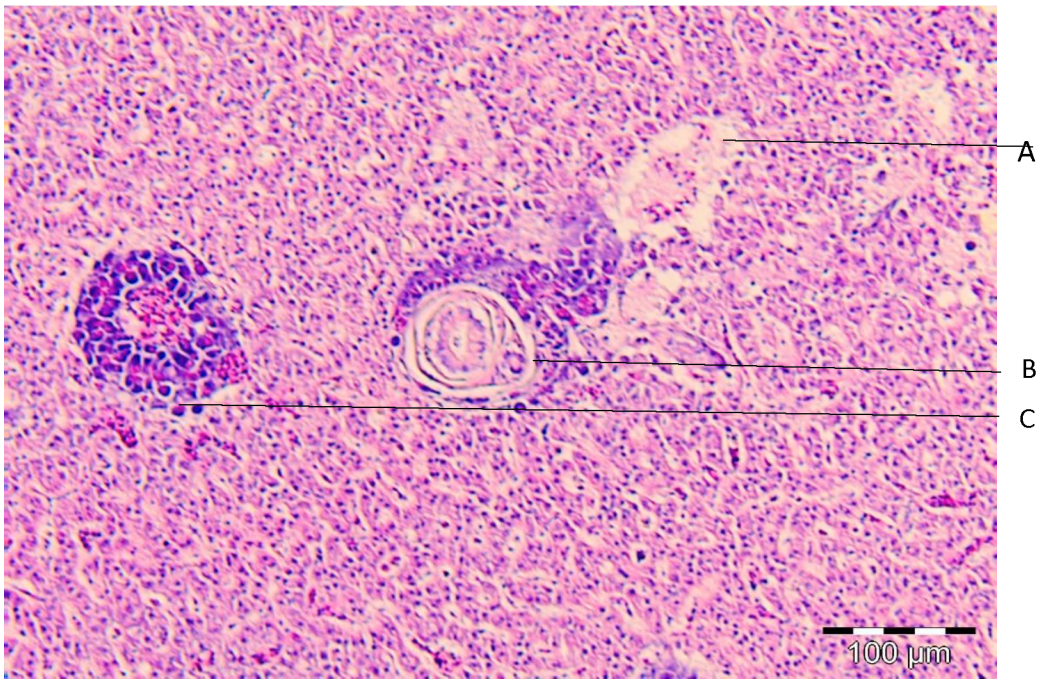

Figure 3.4: Section of liver (hematoxylin and eosin, 10x) from dusky kob, *A. japonicas* fed a diet 4.1C for 84 days. The photograph shows a blood vessel containing hemolyzed blood (A), a bile duct surrounded by connective tissue (B) and a hepatopancreas structure with acinar cells (C).

The hepatocytes of fish in all the dietary treatments showed a certain degree of lipid vacuolation (Figure 3.5). The lipid droplets in the livers of fish fed diets 4.1C, 8.2C and 16.4C were larger than those fed diet 24.6C, and there were also more lipid droplets in these livers. (Figure 3.5, A-C), compared to those fed diet 24.6C (Figure 3.5, D). Spherical cells with centrally located nucleus and a homogenous shape were observed in all dietary treatments (Figure 3.5). Prominent spindle shaped erythrocytes were observed in fish fed diet 24.6C (figure 3.5, D).

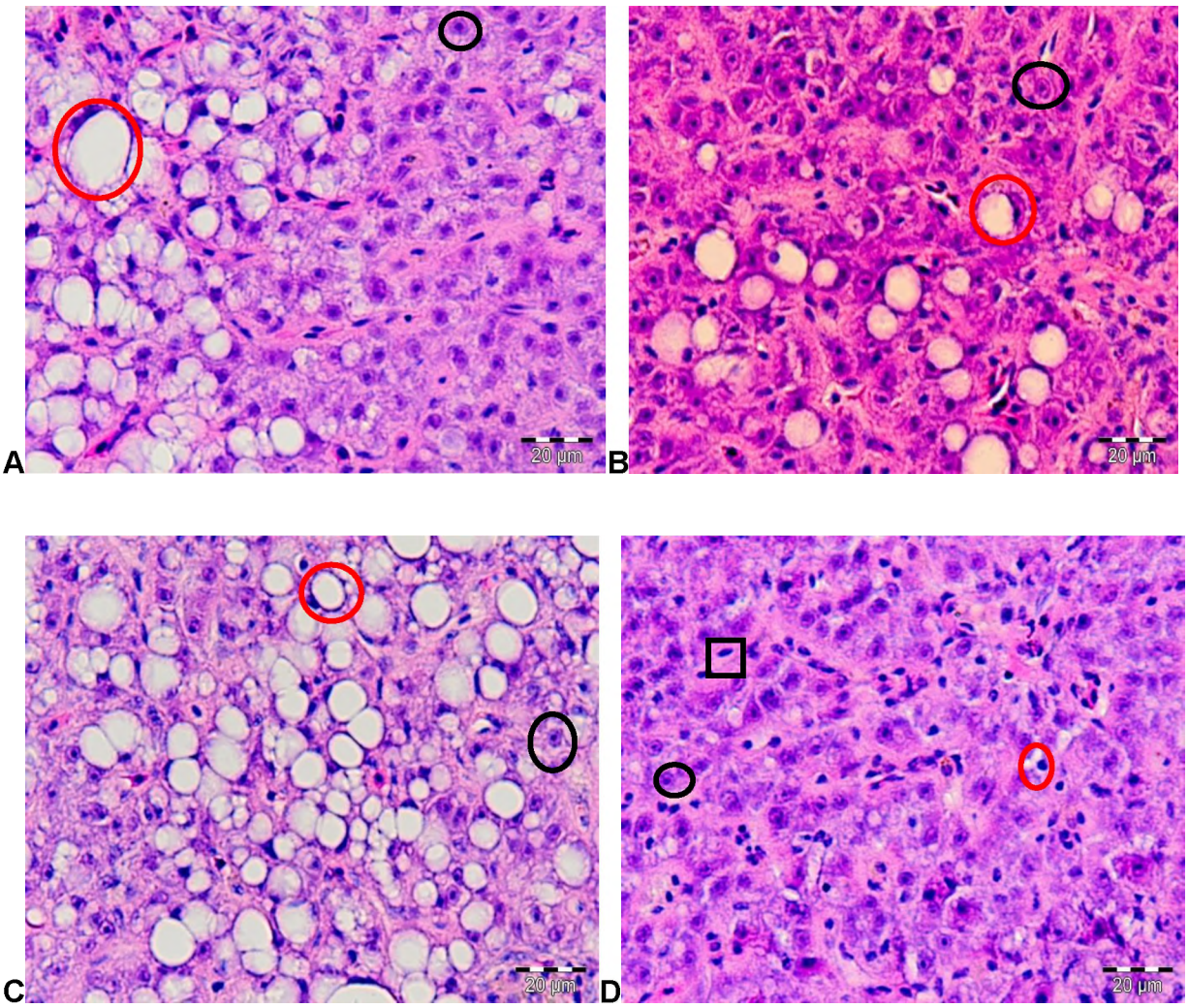

Figure 3.5: Liver section (hematoxylin and eosin, 20x) of dusky kob *A. japonicus* fed diets; (A) 4.1C, (B) 8.2C, (C)16.4C and (D) 24.6C for 84 days, showing different number and sizes of lipid vacuoles (red circles). Black circles show hepatocytes. Squares in (D) show spindle shaped erythrocytes.

Melamomacrophage aggregates were observed in all liver samples. They appeared randomly distributed in the liver parenchyma. Diet 24.6C however had more than one per section whereas the other did not (Figure 3.6).

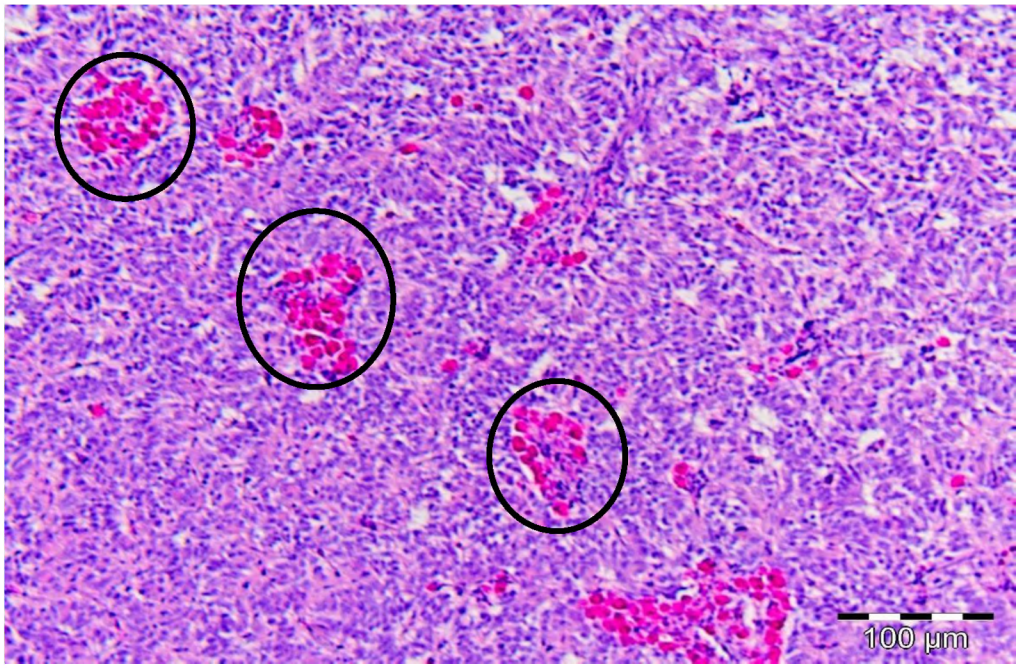

Figure 3.6: Liver section (hematoxylin and eosin, x 10) of dusky kob, *A. japonicus* fed diet 24.6C for 84 days showing melanomacrophage aggregates (black circles).

Glycogen

There was no significant difference in the hepatic glycogen between the four dietary treatments (ANOVA: $F_{(3,12)} = 0.58$, $p = 0.64$,). The values ranged from 28.35 ± 1.01 to 30.13 ± 1.24 mg/liver with an overall mean of 29.52 ± 0.52 mg/liver.

3.3.5 Gut microbial communities

The foregut and hindgut bacterial diversity increased between the start of the trial at which time all the fish had been fed a trout starter diet (Shannon diversity indices of 3.37 and 3.21 respectively) and the end after the fish had been subject to the experimental diets for 84 d (Shannon diversity indices of 4.20 and 4.11 respectively). Foregut samples of kob at the start of the trial displayed a significant dissimilarity from

the foreguts of all the dietary treatments at the end of the growth trial (SIMPER: 84.16% dissimilarity, Figure 3.7). Similarly, hindgut samples at the start of the trial showed a significant dissimilarity to the hindgut samples of all dietary treatments at the end of the trial (SIMPER analysis: 75.89% dissimilarity, Figure 3.7). There was a significant difference in the gut bacterial composition of fish at the start and end of the growth trial (One way ANOSIM: $p = 0.02$, $R^2 = 0.88$). No significant differences were observed between the foregut and hindgut bacterial communities of all experimental diets ($p = 0.11$, $R^2 = 0.28$).

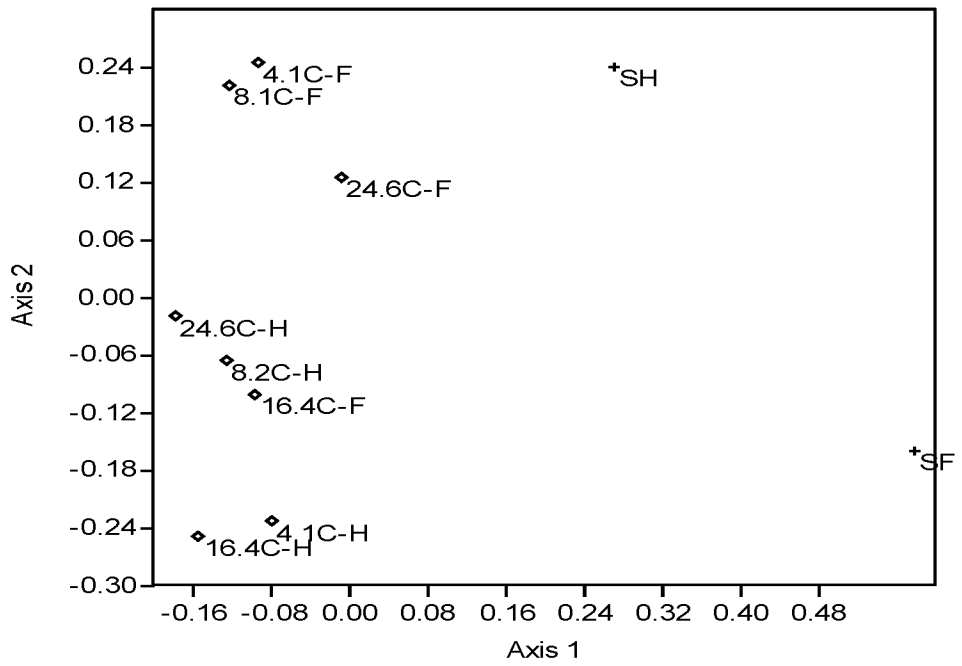

Figure 3.7: A n-MDS plot with a stress value of 0.15 and R^2 values of 0.74 and 0.034 for axis 1 and 2 respectively, are displayed to illustrate the similarity in gut samples of dusky kob, *A. japonicus* fed diets with graded levels of carbohydrate (%C) using a Bray-Curtis similarity measure. Numbers with "C" are the treatments the dietary treatments with different carbohydrate levels. Samples at the start of the trial are denoted by "S". "F" and "H" denote foregut and hindgut respectively.

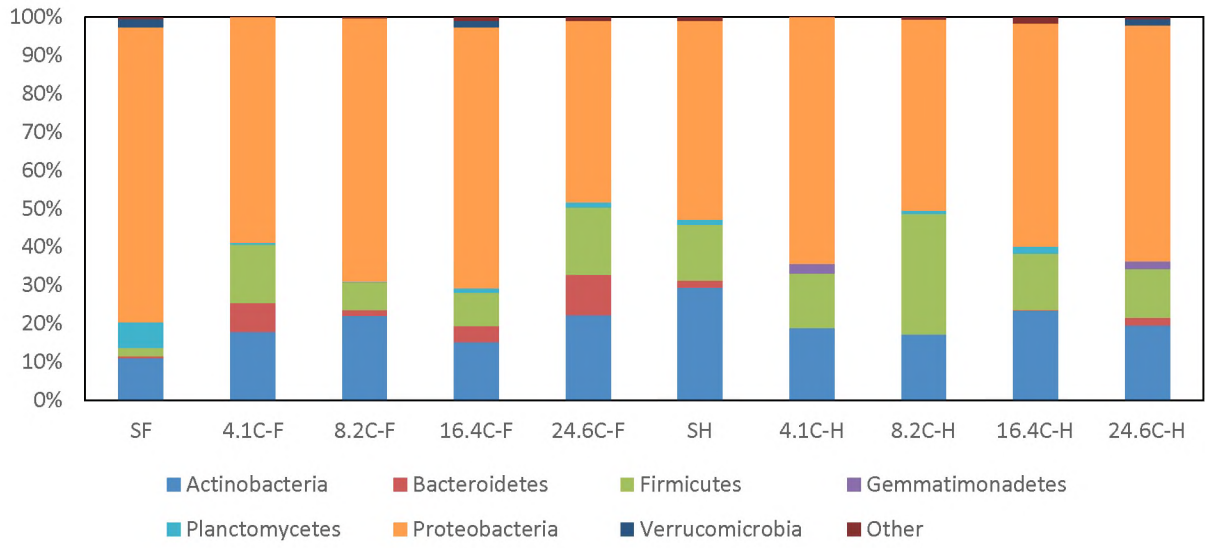

Figure 3.8: Relative abundances (%) of different phyla of hindguts of dusky kob, *A. japonicus* fed diets with graded levels of carbohydrates. Numbers with "C" are the treatments the dietary treatments with different levels of carbohydrate (%C). The start sample hindguts are denoted by "S", the fore and hindguts for the different experimental diets are denoted by "F" and "H" respectively.

The dominant identified bacterial phyla in all samples were Proteobacteria, Actinobacteria, Firmicutes and Plactomycetes, whereas bacteria from Bacteriodetes, Gemmatimonadetes and Verrumicrobia constituted less than 10% of the gut bacterial communities in each sample. Phyla grouped under other included Tenericutes, Chloroflexi, Chlamydiae and Deinococcus thermos contributed less than 2% of the gut bacterial communities in dusky kob and therefore not displayed (Figure 3.8). Identified bacterial genera from the main phyla included *Coxiella, Phaeobacteria* and *Mycobacterium* (Table 3.2).

Table 3.2: Relative contribution (%) of gut bacteria in dusky kob, *A. japonicus* fed diets containing graded levels of carbohydrate (%C) for 84 days at the start and end of growth trial.

		Relative contribution (%)	
Phylum	Genus	Start guts	Experimental guts
Proteobacteria	*Coxiella*	11.41	4.93
	Phaeobacteria	8.36	-
	Stenoxybacteria	6.56	-
	Oceanicola	3.87	-
	Ruegeria	3.54	-
	Skermanella	-	6.71
	Stenotrophomonas		5.05
Actinobacteria	*Mycobacterium*	-	13.90
	Propionibacterium	-	3.76

3.3.6 Proximate composition of the fish

There were no significant differences in the fat, ash, moisture and energy in fish fed diet 24.6C from those at the start of the trial; whereas the ash and energy content of the fish fed all the other dietary treatment levels increased significantly and the moisture content decreased significantly, compared with fish at the start of the trial (Table 3.3).

Table 3.3: Whole body proximal analysis of dusky kob, *A. japonicus* before and after being fed diets with graded levels of carbohydrate (%C) for 84 days (ANOVA/Kruskal Wallis). Values with different superscripts within row are significantly different ($P < 0.05$, N=4).

	Initial sample	4.1C	8.2C	16.4C	24.6C	$F/H_{(3,12)}$	P value
Crude protein (N x 6.25)	14.26	16.06 ± 0.42	15.81 ± 0.23	16.19 ± 0.14	15.09 ± 0.77	1.52	0.26
Fat (%)	2.44^{b}	3.67 ± 0.29^{a}	3.41 ± 0.27^{a}	3.34 ± 0.37^{a}	1.11 ± 0.09^{b}	14.14	0.0002
Ash (%)	4.05^{c}	4.57 ± 4.57^{d}	4.43 ± 0.06^{d}	4.35 ± 0.06^{d}	5.26 ± 0.05^{c}	17.96	<0.0001
Moisture (%)	76.79^{e}	73.55 ± 0.55^{f}	73.65 ± 0.37^{f}	73.62 ± 0.49^{f}	76.44 ± 0.82^{e}	5.48	0.009
Energy (kJ/kg)	3.75^{g}	4.45 ± 0.15^{k}	4.41 ± 0.10^{k}	4.41 ± 0.15^{k}	3.33 ± 0.16^{g}	1.50	0.0005

3.4 Discussion

The fasting glucose concentration of about 3 mmol/l is similar to the fasting glucose obtained for yellowtail kingfish, *Seriola lalandi* (Booth *et al*. 2013a). Similar fasting glucose concentrations of 3.6 mmol/l have been reported for other carnivorous fish such as gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) (Peres *et al.* 1999). Dusky kob showed a similar response pattern to other carnivorous fish after ingestion of a meal containing carbohydrate (Booth *et al*. 2013a, Castro *et al*. 2016). Only slight increases were observed in the lower (4.1 and 8.2%) carbohydrate levels in the blood glucose after meal ingestion and remained relatively constant 48 hours post feeding. Peaks in blood glucose were observed in the fish fed the higher carbohydrate levels 24 h after meal ingestion. The levels at which the peaks were observed were similar to those observed when kingfish was fed diets with 40% pregelatinized wheat starch (Booth *et al.* 2013a). The rate at which the peak levels were reached was however slower than in kingfish, where it was reached in only nine hours. A faster peak time of 5 – 6 h was observed in the European seabass, *Dicentrarchus labrax* fed diets with 20% gelatinized starch (Enes & Peres 2011). The slower plasma glucose peak observed in dusky kob could be indicative of the slower absorption rates of glucose from diet (Furuichi and Yone 1982).

The feeding of diets with 16.4 and 24.6% carbohydrate resulted in post-prandial hyperglycemia, but it was prolonged in the 24.6% carbohydrate diet suggesting kob were not able to clear this glucose. The prolonged hyperglycemia shows a glucose excess which is not efficiently utilized (Lee *et al.* 2008). The duration of hyperglycemia is dependent on factors such as enzyme activity (Enes *et al.* 2008,

Yengkokpam *et al*. 2007), hormonal regulation of glucose metabolism and dietary composition (Moon 2001). Spikes in plasma glucose could mean that glucose entered the blood at a rate the fish could not control via normal metabolic processes (Booth *et al*. 2013a). Increase in glucose levels due to high carbohydrate diet have been observed in dusky kob (Woolley 2009). High plasma glucose impairs efficient utilization of dietary carbohydrate as an energy source (Enes & Peres 2011). This result corresponds to the slow growth and poor feed utilization observed for the 24.6% carbohydrate in (Chapter 2). These results confirm that the fish were subjected to metabolic stress due to the unfavorable amount of carbohydrate in the diet.

A sharp increase in blood glucose was followed by a rapid decline in the fish fed the 16.4% carbohydrate diet, indicating the removal of glucose from the blood. It has been suggested that the removal could be due to an unknown excretory mechanism rather than metabolism by fish (Hutchins *et al*. 1998). More detailed examination of the processes involved in glucose metabolism may be important to determine its action in dusky kob. A similar glycemic response has been observed in kingfish, *Seriola lalandi* dosed with 1 g glucose kg.BW^{-1} (Booth *et al*. 2013a). It was then concluded that kingfish was unable to adequately control blood glucose by upregulation of insulin (Booth *et al*. 2013a). Dusky kob produces digestive amylase (Hunter 2015). Starch can absorb amylase in the chyme which indirectly reduces its ability to hydrolyze starch (Spannhof & Plantikow 1983). This could explain the difference in the glucose response of the fish fed the different starch diets. Higher levels of starch carbohydrate could have absorbed more amylase leading to a significant reduction in its activity. This could explain the prolonged hyperglycemia in the fish fed diets with a high 24.6% carbohydrate.

Hexokinase and glucokinase are key hepatic enzymes involved in intermediary glucose metabolism (Wilson 1994). Hexokinase which has low specificity for the sugar substrate has been measured in certain fish species, whereas glucokinase has not been (Wilson 1994). The absence of glucokinase could explain why fish cannot utilize carbohydrates which are digested and slowly absorbed. High levels of digestible carbohydrates in the diet could release too much glucose that it saturates hexokinase thereby decreasing its activity (Tung & Shiau 1991).

The variation in triglyceride levels suggests that it has an important role in the regulation of blood glucose in dusky kob (Peres *et al*. 1999). Hypertriacylglycerolemia is usually associated with high carbohydrate diets in humans (Peres *et al*. 1999). The opposite was observed with the fish in this experiment. The diet with the lowest amount of carbohydrate and highest amount of lipid had the highest amount of triglycerides and vice versa. Similar results have been observed in gilthead seabream, where higher amounts of triglycerides in the blood were found in the fish fed no carbohydrate diets compared to those fed a high carbohydrate diet (Castro *et al.* 2016). Triglycerides in the current study were not affected by time. The dietary treatments utilized in this study had the same amount of energy; however dietary lipid varied. The differences in triglycerides could be due to the difference in the lipid levels that were used to standardize the energy which led to different levels of triglyceride deposition.

Stored triglycerides are utilized during periods of fasting or between meals (Petterson 2010). The enzyme lipase is responsible for the breakdown of triglycerides stepwise into the final product, glycerol and 3 fatty acids (Parks 2001). Glycerol then gets further broken down or is subject to glucose synthesis (Castro *et al.* 2016). The high

carbohydrate level in the diet with 24.6% carbohydrate could have resulted in nutritional deficiencies, and this could have been worsened by the low intake of the feed (Chapter 2, Table 2.3). This may have led to fish starvation, leading to utilization of stored triglycerides which could explain the lower triglycerides levels recorded in the fish fed the high triglyceride diet compared to the lower 4.1-16.4% carbohydrate diets.

Different amounts of carbohydrate in the diet had no effect on the HSI and VSI of dusky kob. The results obtained in this study are consistent with those obtained in largemouth bass, *Micropterus salmoides*, where graded levels of carbohydrate in the diets did not influence the HSI (Amoah *et al.* 2008). This however differs from results reported for *Salmo salar* Atlantic salmon, *Oncorhynchus mykiss* rainbow trout and *Hippoglossus hippoglossus* Atlantic halibut where HSI increased with increasing carbohydrate in the diet (Brauge *et al*. 1994, Hamre *et al.* 2003). The VSI and HSI are associated with the amount of fat in the viscera and liver respectively and are sensitive to fish health (Bruslé & Anadon, 1996). Increased HSI in fish is associated with deficiencies of essential nutrients such as fatty acids (Montero *et al*. 2001). These reasons can be used to suggest that the diets in the current study provided the same amount of nutritional value to all groups of fish, hence the similar health indices.

The colour of healthy fish liver is usually reddish brown (Bruslé & Anadon 1996). The pale colour observed in the livers in this study could be due to the lipid content (8.20 - 18.20%) used to standardize energy across diets. Pale livers have been observed in fish fed diets containing high lipid content (Rossetti 2011, Sellami *et al*. 2014). The swollen livers could also be explained by lipid accumulation (Hardy 2001).

There was histological evidence of lipid accumulation in the livers of all fish in this study. There was more hepatocyte vacuolation in the livers of fish fed the 4.1-16.4% carbohydrate diets. A similar occurrence was seen in Malaysian mahseer fingerlings (*Tor tambroidesl*) fed diets containing graded levels of carbohydrate (Jimoh *et al*. 2015). The size and number of lipid vacuolation in the Malaysian mahseer increased with increasing dietary carbohydrate, whereas dusky kob in the present study showed decreased lipid content in the livers of fish fed the high (24.6%) carbohydrate diet. This could be explained by the low feed intake as presented in the previous chapter (Chapter 2, Section 2.3.2). The low intake of feed could mean that ingested feed was metabolized completely and no excess dietary energy required storage (Enes *et al*. 2008). This suggestion is confirmed by the low body fat in the proximal analysis of the fish fed this diet (discussed later).

Melanomacrophage aggregates were found in all livers sampled. There was however a higher number observed in the specimens of the fish fed the 24.6% diet. The number and size of melanomacrophage aggregates is associated with nutritional status of a fish, with an increase in overall number and the size of the aggregates when fish were starved (Agius 1981). Since a higher occurrence was observed in the fish fed the diet with the highest carbohydrate inclusion, it can be concluded that the fish were subjected to starvation (Montero *et al*. 1999).

Excessive carbohydrates in fish diets lead to glycogen accumulation in the hepatocytes (Amoah *et al*.2008). In the present study, the level of liver glycogen was not affected by the different levels of carbohydrates in diets. The results are similar to those obtained in largemouth bass *Micropterus salmoides*, fed diets containing carbohydrates of up to

25% (Amoah *et al*. 2008) and juvenile flounder, *Paralichthys olivaceus* fed diets containing dextrin (Lee *et al*. 2003). They however differed from those obtained in other carnivorous fish such as stripped seabass *Morone saxatilis*, rainbow trout *Oncorhynchus mykiss* and Atlantic salmon, *Salmo salar* which showed an increase in liver glycogen with increasing dietary carbohydrate (Bergot 1979, Millikin 1982, Brauge *et al*. 1994). Similar amount of glycogen storage in the liver could suggest that glucose energy was readily available to all groups of fish and glycogen was stored in equal amounts.

Gut bacterial community diversity indicated by the Shannon diversity was relatively high for the fish fed all experimental diets compared to when the experiment was initiated. Although there were dissimilarities in the bacterial diversities before and after fish were subjected to experimental treatments, the dominant bacterial phyla were the same. Proteobacteria, actinobacteria and firmicutes were the three most dominant phyla in the dusky kob gut bacteria, making up about 90% of the total phyla. Resident bacteria prevailed and were not affected much by the change in diet. Similar resident bacteria were found in the shrimp *Neocaridina denticulate* (Cheung *et al*. 2015). Evidence has been found in trout and other species that some core intestinal microbiota resists change (Wong & Rawls 2012, Heikkinen *et al*. 2006, Ringo *et al*. 2006). This could explain the observed dominance of the same bacterial phyla in dusky kob even when a change in diet was introduced, however this hypothesis requires further investigation. Bacteriodetes and Firmicutes have also been found to contribute to carbohydrate fermentation in the fish intestine (Spor *et al*. 2011). *Aeromonas* of the phylum

Proteobacteria has also been isolated from Arctic charr (*Salvelinus alpinus* L.) fed diets containing carbohydrates (Ringo & Olsen 1999).

The main operational taxonomic units (OTUs) contributing to the dissimilarity between the guts samples at the start and end of growth trial included those belonging to the genus *Coxiella, Phaeobacteria* and *Mycobacerium.* All the bacteria belonged to the most abundant phyla in the fish guts. There increase in bacterial diversity was not accompanied by any evidence of carbohydrate level related effects. A change in gut bacteria has been observed in fish such as Atlantic salmon, (*Salmo salar* L.) when fed diets with different protein sources (Hartviksen *et al*. 2014). The increase observed here could therefore be a dietary effect due to the change in diet as the fish were switched from the trout feed which contained carbohydrate, fishmeal and various terrestrial protein products, to the experimental diets which only contained carbohydrate and fishmeal.

Though dusky kob is a carnivorous fish, this study showed that the fish can be fed carbohydrate diets of up to 16.7% without detrimental effects. The different levels of carbohydrate did not influence the protein content of the whole fish but presented differences in the fat content and ability to clear glucose from the blood. The inability of dusky kob to clear glucose from the blood was an indication of a breakdown in the ability to digest high carbohydrate levels. A consequence was reflected in the low carcass lipid of the fish fed diet with 24.6% carbohydrate, showing a poor assimilation of dietary energy. It would be important to determine the fatty acid composition as well as suitability for human consumption. The diversity in gut bacterial composition suggested that bacteria contribute to the digestive process. The flexibility of gut bacteria has been

suggested to show fish digestive adaptability (Karasov *et al.* 2011). This could be further investigated to determine its contribution to nutrient digestion in dusky kob.

CHAPTER 4

GENERAL DISCUSSION

Dusky kob aquaculture is developing in South Africa but is limited by the availability of a commercial feed formulated for the specie's optimal requirements. This study therefore aimed to investigate the suitability of carbohydrate as an economically and environmentally sustainable alternative energy source in dusky kob, *Argyrosomus japonicus* diets. This was done by assessing the effects of graded levels of pregelatinized starch (4.1-24.6%) (and consequently decreasing the amount of fish oil to maintain a standard energy of 17.9 kJ/kg) on the growth, feed utilization, proximate composition and various health parameters in dusky kob.

The present study demonstrated that juvenile dusky kob diet can contain up to 16.7% carbohydrate before adverse effects were noted. Higher dietary carbohydrate level resulted in starvation and it induced prolonged hyperglycemia; liver condition was however not affected by the increase. The gut bacterial composition was not affected by carbohydrate level, but was probably influenced by the change/switch in protein source in diet. Therefore, it is recommended that a maximum of 16.7% carbohydrate from starch be included in the diets, with no adverse health effects.

The diets in this study were formulated to be isonitrogenous (45% protein) and isocaloric (17.9 kJ/kg). Starch substituted lipid in the formulations and thus it was assumed that the varying lipid content was equally available as energy and did not affect the performance of the fish. The proximal analysis of the diets however showed a decrease in the protein and energy levels with increasing carbohydrate in the diet, while

the protein to energy ratio remained relatively constant. Therefore it was concluded that the poor performance in the higher (24.6%) carbohydrate was most likely due to the inability of the fish to digest high carbohydrate content in the feed.

The poor performance in the fish fed the diet with 24.6% carbohydrate might have been due to the decrease feed intake, which was attributed to unpalatability due to the hard texture of the pellet. Furthermore, growth inhibition could be a sign of decreased nutritional value of the diet. Inclusion of high content of carbohydrate could have resulted in a less nutritionally balanced diet (Watanabe 1982). The high blood glucose and prolonged hyperglycemia showed the inability of the fish to utilize high levels of carbohydrate resulting in insufficient energy available for growth. There was evidence of fish starvation in the group of fish fed this high carbohydrate diet, which supports the suggestion that inclusion of 24.6% carbohydrate diet did not provide sufficient energy for the fish. If the provision of energy is compromised, stored amino acids in the muscle then gets broken down to supply energy for the fish which then reduces the mass of the flesh (Johnston 1981). There was also poor nutrient utilization reflected in the feed conversion and protein efficiency ratios. The poor feed utilization was attributed to the inability of dusky kob to digest high levels of carbohydrate. In this study, metabolic stress was evident in the prolonged hyperglycemia in following a carbohydrate dense meal, suggesting that nutrients were not being efficiently metabolized and were not sufficiently available for growth. It also means that protein sparing was minimal. It is therefore necessary to include carbohydrate at 16.7% in dusky kob diets for optimum growth and efficient feed utilization.

The livers of fish fed all dietary treatments looked unhealthy and showed a pale colour. Histological analysis revealed lipid vacuolation of the hepatocytes. Similarly, vacuolation of the hepatocytes in the livers of dusky kob fed different lipids was also observed (Rossetti 2011). High lipid vacuolation could be a sign of the onset of fatty tissue build up in the liver (Caballero *et al*. 2004). There was however no evidence of lipid deposition in the current study as the lipid levels used in this study were within the limits that dusky kob can handle (6 - 18%) (Woolley 2009). The carcass lipid was also very low (1.11 - 3.67%), further supporting that there was no lipid accumulation in the fish due to diet.

There were no differences in the bacterial community structures of the four dietary treatments. The only difference observed was in the increase in bacterial diversity when the fish were switched from the weaning (trout feed) to the experimental feed. While there was no evidence of any positive or negative effects on gut bacteria with a change in the amount of carbohydrate in the diet, the results show that the gut bacteria can withstand a wide range of carbohydrate level in the diet.

Future research should also focus on enhancing the carbohydrate tolerance of this aquaculture species, and this will help reduce food costs and decrease the demand for fish meal. For example, promoting growth of gut bacteria that produce extracellular enzymes for carbohydrate hydrolysis is a strategy that could be used to deal with non-nutritional factors associated with plant feed ingredients (Serra *et al*. 2016). Intermediary metabolism programming is a technique which has been used to attempt to establish a glucose tolerant phenotype in rainbow trout, *Oncorhynchus mykiss* (Panserat *et al*. 2016). Upregulation of glucose metabolism related genes was observed

in alevins fed a high carbohydrate diet, and showed a similar pattern observed in juveniles (Marandel *et al*. 2015). It was then concluded that timing of gene expression at ontogenesis and application of the relevant stimulus could be key in achieving a glucose tolerant phenotype. This programming could as well be applied in dusky kob to achieve glucose tolerant fish.

It has generally been accepted that carnivorous fish cannot efficiently utilize dietary carbohydrate (Wilson 1994). Recent studies have however shown that carbohydrate utilization is species specific and that the optimum carbohydrate requirement of most carnivorous species in below 20% (Enes *et al.* 2008). This work has laid down a foundation for carbohydrate utilization by dusky kob by showing that up to 16.7% of juvenile dusky kob diet can be made up of carbohydrate with no negative effects on fish growth and health. This result was based on the inclusion levels that were used in this study. More work is needed to further refine levels between 16.4 and 24.6% to refine the optimum dietary carbohydrate level further. It is also necessary to optimize dietary protein to energy ratios in diet formulations. This allows for maximum growth and feed utilization while keeping nutrient losses at a minimum. The experimental design of the current study did not allow for this. Future work should focus on optimizing protein to energy ratio to allow for maximum nutrient utilization. More work should be done to better understand the processes involved in carbohydrate utilization which could help even further with least cost diet formulation to reduce costs of feeding in dusky kob aquaculture. It will also be useful to repeat this study on different size dusky kob to determine the optimal carbohydrate inclusion of various developmental stages of the fish.

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