EFFECT OF LIPID INCLUSION LEVELS IN AQUAFEED ON CARCASS COMPOSITION, QUALITY CHANGE DURING STORAGE AND NUTRIENT EXCRETION IN DUSKY KOB (*ARGYROSOMUS JAPONICUS*)

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Nhlanhla Joseph Ginindza

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ABSTRACT

Dusky kob, *Argyrosomus japonicus*, is an aquaculture species in South Africa that is in pilot commercial production. While the major nutrient requirements of the species are known, the advantages of incorporating formulated feeds into the diet of the species has yet to be fully explored. Research on formulated feed composition is required to: minimise input costs; improve the organoleptic properties and meat quality; and minimise nutrient loss, which contributes to environmental pollution. This study sought to test the impact of different lipid levels in aquafeeds fed to dusky kob juveniles by determining:

(i) growth performance, feeding efficiency, proximate and fatty acid composition;
(ii) chemical changes and shelf-life of refrigerated fish fillets; and
(iii) metabolic rates and nitrogen excretion of juvenile dusky kob.

Dusky kob juveniles of 35.12 ± 10.3 g (P = 0.718) and 122.90 ± 11.9 mm (P = 0.062) were sourced from Oceanwise (Pty) Ltd, East London for the growth trial which was run a semi-recirculation system for 106 days. The dusky kob were fed isoenergetic, isonitrogenous diets with lipid levels of 8 %, 12 %, 16 % and 20 % hereafter referred to as 8F, 12F, 16F and 20F respectively. Specific growth rate (SGR) was highest for the 8F treatment (P = 0.001) and decreased with increasing dietary lipid levels. Feed conversion ratios (FCR) increased with increasing dietary lipid levels from 1.24 at the 8F treatment to 1.39 at the 20F treatment (P = 0.034). The deposition of lipids in tissue closely reflected the dietary lipid levels. Lipid levels did not significantly affect the levels of saturated fatty acids in fillets (P = 0.674). Monounsaturated fatty acids were significantly higher in the 8F lipid treatment (P < 0.001), while polyunsaturated fatty acids were significantly higher in the 20F lipid treatment (P = 0.047). Omega 3 fatty acids (n-3) were significantly lower in the 8F treatment (P < 0.001) and increased with increasing dietary lipid levels, while omega 6 fatty acids (n-6) were significantly higher in the 8F treatment (P < 0.001) and negatively correlated ($r = -0.96$, P = 0.044) with dietary lipid levels.

The second experiment investigated the effect of different dietary lipid levels on the biochemical changes in dusky kob fillets refrigerated (6.6 ± 1.5 °C) for 18 days. The total volatile basic nitrogen (TVBN) level, an indicator of total nitrogenous compounds that develop during muscle tissue deterioration, differed significantly across all treatments (Factorial ANOVA: $F_{(12, 140)} = 4.502$, P < 0.001), with the 16F and 20F treatments producing the highest concentrations of volatile bases. Free fatty acids, an indicator of lipid hydrolysis, increased significantly in all treatments with duration of refrigeration (Factorial ANOVA: $F_{(12, 20)} = 924.97$, P < 0.001). The thiobarbituric acid reactive substances (TBARS), indicator of lipid oxidation,

were significantly different among treatments (P = 0.005) and were elevated at higher dietary lipid levels. Our results show that the meat quality of dusky kob fillets fed comparatively low levels of dietary lipids (8F and 12F treatments) was acceptable up to 12 days in refrigerated storage. After this time, the quality of fillet meat became unfit for human consumption. For the higher levels of dietary lipids (16F and 20F treatments), the maximum refrigeration storage time before reaching levels that are unfit for human consumption decreased to 8 days.

The third experiment measured the effect of dietary lipid levels on metabolic rates and nitrogenous excretion rates, and was tested in a closed respirometer run for 25-30 hours. Postprandial metabolic rates (respiration) were not affected by dietary treatments (P = 0.521), but rose by between 47 and 81 % of the basal metabolic rate after 3 hours. Metabolic rates were not correlated to dietary lipid levels. The total excreted NH_4-N was higher in the 8F and 12F diets and decreased with increasing lipid levels (P < 0.001). The 8F treatment registered the highest urea excretion while the other treatments were relatively similar during the first 12-hour interval (P = 0.033). The peak excretion rates of NH_4-N and urea-N were reached between 3 and 5 hours after feeding. In all treatments, a rapid change in pH was observed during feeding, with the 8F and 12F treatments recording the highest rate of change in pH. Ammonium-nitrogen contributed the major share (69 - 86 %) of total excreted nitrogen with urea making up a smaller (14 - 31 %) but significant portion. The results show metabolic rates in dusky kob peaks 3 hours after feeding in all dietary treatments. Higher dietary lipid levels (16F and 20F treatments) results in low NH_4-N and urea excretion rates of dusky kob.

The experiments showed that low dietary lipid levels increase growth rate and improve feed conversion ratio. An increase in dietary lipid levels significantly increased the concentration of tissue polyunsaturated fatty acids, and lowered NH_4-N and urea excretion rates. However, higher dietary lipid levels tended to slow growth rates and reduced refrigeration shelf-life. The three experiments provided preliminary data on the effect of dietary lipid on growth performance, assimilation efficiency, carcass composition, meat quality deterioration and nutrient excretion. The demonstrated methodologies employed during this study may prove useful tools for laying a foundation for future studies

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Dedicated to my grandmother

CHAPTER 1

GENERAL INTRODUCTION

The globally increasing demand for seafood combined with maximally exploited capture fisheries has resulted in a rapid expansion of aquaculture production to alleviate this supply deficit (FAO, 2012). Global fisheries production (fish, aquatic plants and non-food products) from capture and aquaculture reached 181.7 million tonnes in 2012 (FAO, 2014b), with aquaculture (fish, aquatic plants and non-food products) contributing 90.4 million tonnes. Over the past decade, aquaculture in Africa grew from 1.2 % to 2.2 % of world aquaculture production, mainly as a result of development of freshwater aquaculture (FAO, 2014a). Egypt contributed 69 % percent of Africa's aquaculture production in 2012 and this trend is likely to be maintained. In terms of top producers in Africa, South Africa slipped from being ranked number 6 in 2000 to number 14 in 2012 (FAO, 2014a, b).

South African aquaculture production primarily comprises of marine invertebrates (abalone, mussels and oysters), seaweed and freshwater fish. Production in 2012 was estimated at 5 927 tonnes, consisting mainly of 2 000 tonnes of seaweed, 1 428 tonnes of trout, 1 111 tonnes of abalone, and around 860 tonnes of Mediterranean mussel (Department of Agriculture Forestry & Fisheries, 2014b). There is a need for growing marine aquaculture production efficiency in South Africa, particularly in the marine finfish sector so that the South African aquaculture industry can be competitive. In view of the comparatively low productivity of the South African aquaculture sector, policies have been developed in recent years to stimulate the sector's development.

During the period between 2007 and 2014, the South African government passed the National Aquaculture Policy Framework, the National Aquaculture Strategic Framework, Agriculture Policy Action Plan, and the Policy for the Development of a Sustainable Marine Aquaculture Sector. These policy instruments are aimed at alleviating the burden of over-fishing on some marine resources and also addressing socio-economic challenges (Department of Agriculture Forestry & Fisheries, 2012, 2013, 2014a; Department of Environmental Affairs & Tourism, 2007). As South African aquaculture is still in a developmental stage, there are huge research gaps that need to be addressed before production can reach globally competitive levels. Central in these policies are measures for stimulating aquaculture research and development on indigenous species that can be taken to pilot stage prior to commercial production. The government's aquaculture development policies identify environmental factors, fragmented legislation and inadequate investment in research and development as the major challenges

limiting aquaculture growth in South Africa. Most indigenous species have not been thoroughly researched; hence aquaculture research has been identified as a key element that needs to be addressed.

There are several aquaculture candidate species that have been identified for possible commercialisation. Of these, dusky kob (*Argyrosomus japonicus*), silver kob (*Argyrosomus inodorus*), white stumpnose (*Rhabdosargus globiceps*), geelbek (*Atractoscion aequidens*), yellowtail (*Seriola lalandi*), red roman (*Chrysoblephus lariceps*), turbot (*Scophthalmus maximas*) and white margined sole (*Synaptura marginata*) have been identified as potential finfish species for the South African mariculture industry (Hecht, 1999). However, the only marine fish species that is currently being developed for commercial aquaculture is the dusky kob. The dusky kob is an economically important linefish to both recreational and commercial anglers in South Africa. However, poor stock management has resulted in overfishing of this species and hence stricter measures have been put in place to control the fishing of the species (Palmer, 2008). Juveniles, in particular, are a highly exploited group as a result of poor law enforcement and poor management control (Britz *et al.*, 2001; Mirimin *et al.*, 2014). The farming of dusky kob is thus being developed by the commercial aquaculture sector as a viable option to meet the market demand.

Dusky kob, also called jewfish, mulloway and dusky meagre elsewhere in the world, is one of 10 Sciaenids that occur in South African waters (Fennessy, 2000). The family Sciaenidae contains approximately 70 genera and up to 270 species worldwide with 28 species restricted to freshwater environments. The dusky kob is a migratory fish occurring both in the southern and northern hemispheres. It is an inshore species found in estuaries and the surf zone, but adults are also found offshore to a depth of 50-100 m from Cape Point to Mozambique. Within South Africa, the occurrence of the species is primarily from Cape Agulhas to northern KwaZulu-Natal. Dusky kob also occur along the southern seaboard of Australia and from Hong Kong northwards along the Chinese coast to South Korea and Japan (Griffiths, 1996; Griffiths & Heemstra, 1995). In Australia, dusky kob is distributed along the eastern, southern and western seaboards from the Burnett River in Queensland to North West Cape in Western Australia (Silberschneider & Gray, 2008). There is currently very little work done on genetic variation of the different populations of dusky kob throughout its distribution range. Although studies on otoliths suggested that the South African stocks of dusky kob are three or more strains (Griffiths & Hecht, 1995), Griffiths and Heemstra (1995) concluded, after examining the South African and Australian populations that the stocks should be recognised as single species pending the availability of genetic evidence (Griffiths & Heemstra, 1995). A recent genetics study by Mirimin *et al*., (2014) confirmed that dusky kob exist as a single species

throughout its distribution range within South African waters, which has greatly simplified the management of the aquaculture broodstock collected from the wild.

Dusky kob has aquaculture characteristics, due to its high fecundity, fast growth rates, high price, marketability and good food conversion ratios (Benjamin, 2006; Britz *et al.*, 2001; Ferguson & Ward, 2003; Griffiths & Hecht, 1995). During 2013, farm gate prices of R60-70/kg were obtained by local aquaculture operations. The demand from European and Asian markets makes a strong case for the production of dusky kob (Daniel, 2004). Dusky kob is closely related to meagre (*Argyrosomus regius*) which is being cultured and widely consumed in the Mediterranean region.

The biology of dusky kob makes it a suitable candidate for culture under a wide range of salinity and temperature environments (Fielder & Bardsley, 1999). Juveniles prefer hyposaline waters (Ferguson & Ward, 2003) and temperatures between 24 and 26 °C (Collett *et al.*, 2008) for maximum growth, however, optimal growth can still be attained at full strength seawater. Some other studies with aquaculture relevance on dusky kob include artificial spawning in ponds (Battaglene & Talbot, 1994), effects of salinity in egg development and hatching (Ginindza, 2008), effects of salinity on larval development (Fielder & Bardsley, 1999), effect of photoperiod and feeding interval (Ballagh *et al.*, 2008), culture in saline or groundwater (Doroudi *et al.*, 2003; Hutchinson & Gluis, 2008), estimating metabolic rates under different temperatures (Pirozzi & Booth, 2009) and oxygen levels (Fitzgibbon *et al.*, 2007), and some parasitology (Amin & Christison, 2005; Hayward *et al.*, 2007).

As intensive fish culture is practised in modified environments, farmed fish generally have no access to natural food. Consequently, such fish are highly dependent on artificial feed, making the need for nutritionally complete feeds critical (Lovell, 1998). Feeding costs make up a large percentage (30-60 %) of the total expenses of an intensive aquaculture operation and for those trying to culture new species, achieving a competitive economic performance is a high priority (Davies & Gouveia, 2008; Pirozzi, 2009). In aquaculture, it is generally desirable to minimize dietary protein concentration (that chiefly determine the feed cost) without compromising growth of the fish. Low dietary protein not only reduces feed costs, but also reduces undesirable nitrogenous waste production (McGoogan & Gatlin III, 1998). During the last decade, commercial feeds for fish farming have incorporated increasing lipid levels while reducing protein levels. The high lipid diets have been considered beneficial for growth, feed efficiency, protein-sparing and for reducing phosphorus and nitrogen losses. Feed costs have thus been reduced through the protein-sparing emanating from low protein-high fat diets (Kaushik & de Oliva Teles, 1985; Naylor *et al.*, 2000; Williams & Robinson, 1988).

Lipids include triglycerides, wax esters, sterols and phospholipids. Fatty acids, both the saturated and unsaturated groups, are key components of lipids. Lipids are important nutrients that facilitate the optimal utilization of dietary proteins. In fish, lipids are highly digestible and are a preferred nutrient source for energy as compared to carbohydrates (Mohanta *et al.*, 2008). The energy provided by lipids is almost double that of protein and even more than double that from carbohydrates. However, protein is the primary energy source in marine fish (Glencross, 2004). Lipids are required by fish as a source of available energy, as structural components of bio-membranes, carriers of fat-soluble vitamins, precursors to eicosanoids, hormones and vitamin D, and as enzyme co-factors (Lovell, 1998; NRC, 2011). In the lipid component, fish are unable to synthesize the unsaturated fatty acids C18, C20 and C22 *de novo* because of their very limited gene expression of delta 6 and 5 desaturases. Therefore, these fatty acids must be supplied in the diet in accordance with the fatty acid requirements of the species (Montero *et al.*, 2008; NRC, 2011).

There is no fixed percentage of dietary lipid that has been specified for fish diets. Dietary lipid content has to consider the type of lipid as well as the protein and energy content of the diet. Lipid concentrations of up to 20 % of dietary content typically yield optimum results in marine fish culture (NRC, 2011). Increasing the lipid content in feed is generally a more effective method to increase energy levels because lipids are more energy-dense than proteins or carbohydrates (Cho *et al.*, 2005). The use of high lipid content feeds has been shown to drastically increase growth rates and reduce feed conversion ratios, and also increase feed intake (Caballero *et al.*, 1999; Cho *et al.*, 2005; Vergara *et al.*, 1999). When excessive dietary lipid or energy, is fed, feed consumption decreases and results in growth reduction because of a lack of other nutrients necessary for optimal growth (Akpınar *et al.*, 2012; Olsen *et al.*, 2008).

In some fish species, protein digestibility tends to decrease with an increase in the levels of dietary lipid levels (Lee *et al.*, 2002a). This suggests that protein content in feeds is poorly utilised and results in an increase in the excretion of nitrogenous nutrients. The accumulation of nutrients in water bodies may result in localised eutrophication and possibly algal blooms (Muzaffar Bazaz & Keshavanath, 1993; Pitcher & Gilbert, 2005). It is therefore important to balance protein and lipid levels (by extension, energy levels) to optimise protein digestibility and absorption, and reduce nutrient loss into the environment. Loss of nitrogenous components into the water is essentially the loss of essential amino acids and value of the feeds (NRC, 2011).

Excess dietary lipid can have some undesirable effects on the product quality of muscle tissue of aquaculture species upon slaughter. Muscle tissue with excess lipids increase the amount of substrate for oxidative processes and the risk of rancidity during processing and storage (Jensen *et al.*, 1998; Torrissen, 1985). In fish, the tissue characteristics are generally linked to diet composition, although the effects seem to vary among species. For example, it is well known that body fat increases when fish are fed diets containing low protein/energy ratios (Akpınar *et al.*, 2012). Studies on yellowtail have described a decrease in the texture of fish muscle due to increased muscle fat (Suárez *et al.*, 2009; Thakur *et al.*, 2002).

Dietary lipid levels also affect product acceptability to consumers and the shelf life of the product (Coello *et al.*, 1999). During storage of fish, fat and fat-soluble molecules contribute towards the loss of the tissue's characteristic texture, flavour and aroma. When formulating feeds, dietary lipid levels need to be supplied adequately to minimise negative impact on storage life of fish (Huang *et al.*, 1998). As dusky kob is a relatively new candidate species, its nutritional requirements under culture conditions, in particular the lipid levels for best fillet, is not known and need to be determined to optimize shelf life. Anecdotal reports on the market acceptance of farmed kob suggest that consumers prefer the taste of the farmed product due to its higher lipid content (P. Britz, Rhodes University, 2014 pers. comm.)

Previous studies on the nutritional requirements of dusky kob have focussed on optimizing growth and feed utilization through protein and energy balance and pellet characteristics. Some of the key findings of these studies were that wild-caught dusky kob juveniles readily consume dry formulated feeds (Daniel, 2004); soybean meal can replace up to 30 % of fishmeal, while soybean oil can replace up to 28 % of fish oil without negatively affecting the growth performance of dusky kob (Daniel, 2004; Rossetti, 2011); the protein requirements of dusky kob fall within the 40 – 50 % range of sciaenids (Davis & Arnold, 1997; Pirozzi, 2009; Woolley *et al.*, 2010); the dietary protein to energy ratio of dusky kob decreases with increasing body size up to 2 kg (Daniel, 2004; Fernandes, 2013; Pirozzi, 2009); and pellet size (ranging between 3 and 8 mm) and texture does not affect growth performance (Benjamin, 2006; Woolley *et al.*, 2010).

As dusky kob is a relatively new aquaculture candidate species, comprehensive information on its nutritional requirements under culture conditions, in particular the lipid levels for best fillet, are not known and need to be determined to optimize consumer acceptability and shelf life. Furthermore, the impact of dietary lipid levels in the diet of dusky kob on fatty acid profile are not known. There is also a gap in our knowledge of the nutrient loss to the environmental as a result of leaching or poor digestibility. The post-prandial metabolic rates and waste

accumulation in the environmental as a result of dietary lipid levels have not been quantified. Being mindful of these knowledge gaps, the objectives of this research were to:

1. Determine the effects of different lipid levels on production performance and proximal and fatty acid composition of dusky kob.
2. Determine the effect of dietary lipid levels on chemical quality changes of refrigerated dusky kob fillets.
3. Quantify the effects of different lipid levels in aquafeeds on nitrogenous excretion and oxygen consumption in dusk kob.

CHAPTER 2

EFFECT OF DIETARY LIPID LEVEL ON GROWTH PERFORMANCE, CARCASS PROXIMAL AND FATTY ACID COMPOSITION

2.1 Introduction

Dietary lipids are generally digested and used more efficiently than carbohydrates as energy sources in marine fish as they lack carbohydrases (Mohanta *et al.*, 2008). Lipids typically provide almost double the energy from protein and more than double that from carbohydrates (Glencross, 2004). Along with carbohydrates, lipids are included in formulated feeds to fuel the metabolic energy requirements of aquaculture species. In turn, lipids increase the protein retention efficiency of nutrients and thus reduce nitrogen and phosphorus waste production to the environment (Olsen *et al.*, 2008). High-energy diets have been successfully used to save on feed costs due to the protein-sparing effect in common dentex and sea bream (Company *et al.*, 1999); some studies such as in shi drum have reported some negative effects of this approach (Akpınar *et al.*, 2012). The production of energy-dense diets requires supplementation with appropriate antioxidants to counter the harmful effects of auto-oxidation (NRC, 1993).

High lipid levels and part of protein supply in aquaculture feeds, often lead to lipogenesis (Alvarez *et al.*, 1998). Increased lipogenesis is also associated with negative effects such as increased tissue adiposity, reduced pigment visualization and reduced smoking performance, leading to processor and retailer rejection and consumer dissatisfaction (Tocher *et al.*, 2003). In addition, high lipid content can change the sensory characteristics (taste, flavour, colour and texture and also affect storage stability) of the fish product (Ng & Bahurmiz, 2009; Suárez, 2005; Waagbø *et al.*, 1993).

Dietary lipid requirements for maximum growth rate in aquaculture differ among various fish species and according to age and size classes. The type, percentage and source of dietary lipids have been shown to affect growth performance of fish differently. Lipid concentrations of up to 20 % have been reported to give optimum results with some species (NRC, 1993). Dietary lipid affects the nutrient digestibility, muscle RNA to DNA ratio and digestive enzyme activity of fish (Muzaffar Bazaz & Keshavanath, 1993). In certain species, protein digestibility tends to increase with the increase in the levels of dietary lipid. The increased protein digestibility indicates that protein content can be reduced and still achieve similar growth rates, which is indicative of a protein-sparing effect (Lee *et al.*, 2002b). Excretion of both phosphorus

and nitrogen are indicative of metabolised protein that is not converted into muscle growth (Olsen *et al.*, 2008).

Fish species have specific protein, lipid and carbohydrate requirements that are necessary for attaining their genetically disposed optima for growth, health and general condition. When a balance of nutrients in feeds is not achieved, undesirable effects such as poor growth, health deterioration and histopathology often manifest in fish (Hardy *et al.*, 2011). The negative effects of feeding an energy-excessive diet often manifests in the viscera and tissue composition. The body composition of fish is not only controlled by diet and environment, but also by endogenous factors such as fish size and hormones. Whereas the protein content of growing fish is solely dependent on endogenous factors (such as fish size), lipid level is affected by both endogenous and exogenous factors (Shearer, 1994). The value of fish is directly affected by its body composition which, in turn, is largely determined by diet composition. There needs to be a balance in diet formulations to ensure optimal growth and good nutrient composition of fish tissue (El-Sayed, 1998; Martínez-Llorens *et al.*, 2007).

The availability of locally manufactured aquaculture feeds that produce good performance is a constraint to the development of the South African aquaculture industry. Defining nutritional requirements, particularly balancing the quality, quantity and source of proteins and lipids (energy) is the main challenge that has to be addressed prior to be commercial production of fish. Research on the dietary requirements of cultured sciaenid species include studies on dusky kob (Benjamin, 2006; Daniel, 2004; Dobberstein, 2006; Pirozzi, 2009; Rossetti, 2011; Woolley *et al.*, 2010), red drum (Serrano *et al.*, 1992; Thoman *et al.*, 1999), meagre (Chatzifotis *et al.*, 2010) and Atlantic croaker (Davis & Arnold, 1997). Daniel (2004) demonstrated that dusky kob can readily be weaned onto artificial aquaculture diets.

Dietary protein and lipid levels have been shown to affect growth and tissue proximate composition in sciaenids. Protein utilisation in dusky kob is negatively affected by diets containing low protein and high lipid content, indicating that the species has a limited capacity to show protein-sparing effect (Pirozzi, 2009). Studies on dusky kob (Woolley, 2009), meagre (Chatzifotis *et al.*, 2010), and red drum (McGoogan & Gatlin III, 1999; Thoman *et al.*, 1999), have demonstrated that a 45 % protein diet generally performs best at dietary lipid levels ranging between 13 and 18 %. Woolley (2009) obtained better growth rates when dusky kob were fed diets containing 46 % protein and 18 % lipid compared to dietary combinations containing higher dietary protein and/or lower dietary lipids. In formulated feeds containing 46 % protein and 18 % lipid, inclusions of soybean oil of up to 28 % of dietary lipids in dusky kob feeds did not affect growth but caused negative health effects at high soybean oil levels

(Rossetti, 2011). By contrast, a diet containing 45 % dietary protein with 8-10 % dietary lipid content yielded best growth rates for dusky kob. Further increases in dietary lipid levels produced an inverse relationship between growth and nutrient utilisation (Daniel, 2004). In other sciaenid species including shi drum (Akpınar *et al.*, 2012), yellow croaker (Duan *et al.*, 2001) and red drum (McGoogan & Gatlin III, 2000), no improvement in growth performance and nutrient utilisation were found when dietary lipid levels were increased from 10-19 %. However, none of these studies focussed on the effect of dietary lipid levels on carcass composition and product quality which is important from a market perspective.

In view of the limited data available on the effect of lipid in dusky kob diets, an experiment was conducted to determine the effect of different dietary lipid levels in artificial feeds on the feed conversion efficiency, proximal carcass composition and fatty acid composition of dusky kob fed.

2.2 Materials and Methods

2.2.1 Experimental fish

A total of 288 dusky kob juveniles spawned from wild collected broodstock were sourced from Ocean Wise Pty (Ltd), East London for the experiment (Figure 2.1). All experimental fish were similar in body weight (35.12 ± 10.3 g, P = 0.718) and standard length (122.90 ± 11.9 mm, P = 0.062). Twenty-four fish were randomly distributed in each of the 12 tanks and acclimatised for 14 days prior to beginning the experiment. Before weight and length measurements were taken, the fish were starved for 24 hours then anaesthetized in 0.2 ml/l dose of 2-phenoxyethanol, and gently drained of excess water. Individual weights were determined on a MonoBloc Viper SW15 balance and determined to the nearest 0.1 g. Length was measured on a measuring board to the nearest 0.1 cm. To monitor growth, the first weight measurement was taken 30 days after the start of the experiment; thereafter the fish were weighed every 25 days for the duration of the experiment.

Figure 2.1. Dusky kob was used to test the effect of different dietary four lipid levels.

2.2.2 Experimental system

The experiment was conducted at the Aquaculture Research Facility, Sea Point (South Africa) in a semi-recirculation system (Figure 2.2). The experiment ran for 15 weeks from April to July 2011. The temperature of the system was maintained between 22 and 24 °C with immersion heaters. Aeration was provided with air-stones in each of 12 polyethylene tanks (500 l capacity). A constant supply of seawater (34.0 ± 2.4 ppt) was topped up at approximately 11 l/min to compensate for water lost in the filtration system and when clearing uneaten feed and faeces. The photoperiod was maintained at 10L:14D with light period between 7am and 5pm local time. Uneaten feed and faeces were flushed routinely. The protein skimmer and sump were scrubbed and siphoned weekly while the cartridge filter was changed monthly. On some days, adverse sea conditions increased turbidity of the intake water, reducing the clarity in the aquarium system. The water clarity, however, improved with time and any possible negative was temporary.

Figure 2.2 Arrangement of tanks in the experimental system

2.2.3 Experimental diets

2.2.3.1 Diet formulation

Experimental diets were formulated to contain lipid levels ranging from 8 to 20 % using practical feed ingredients used in locally manufactured commercial diets. The diets were formulated to be isonitrogenous and isoenergetic. The feed was manufactured at the Marifeed (Pty) Ltd factory from marine fish oil, fishmeal, starch, vitamin-mineral premix, and soya (Table 2.1). The fatty acid profile of the four experimental diets are shown in Table 2.2. The fishmeal and fish oil were sourced from Oceana (Pty) Ltd, Cape Town; soya was imported from Molinos Rio de la Plata, Argentina; starch from Gelatia, Krugersdorp, South Africa; and vitamin-mineral premix were sourced from ADVIT in Johannesburg, South Africa. The fishmeal (66.6 % crude protein, 8.4 % lipid) and soya (47.4 % crude protein, 1.6 % lipid) were the main protein sources.

Table 2.1. Formulation and proximate composition (% dry matter) of the four experimental diets containing between 8 % and 20 % dietary lipid levels.

	Experimental diets			
g/100g diet	8F	12F	16F	20F
Fishmeal	62.16	62.16	62.16	62.16
Soya	5.56	6.24	6.92	7.6
Maize	29.46	24.78	20.1	15.41
Vitamin-mineral mix	0.22	0.22	0.22	0.22
Marine fish oil	2.6	6.6	10.6	14.61
Calculated composition				
Crude protein	46.00	46.00	46.00	46.00
Crude lipid	8.01	12.00	16.00	19.99
Energy ($kJ.g^{-1}$)	18.97	19.67	20.37	21.08
Protein-Energy ratio (mg/kJ)	24.25	23.39	22.58	21.82

Table 2.2 Profiles of saturated, monounsaturated and polyunsaturated fatty acids (percentage of total fatty acids excluding *trans* fatty acids) in four diets that were fed to dusky kob juveniles.

Fatty acid composition	Experimental diets			
	8F	12F	16F	20F
Saturated fatty acids (% of total fatty acids)				
C14:0 (myristic)	5.95	6.38	6.56	6.66
C16:0 (palmitic)	18.97	18.92	18.91	18.90
C18:0 (stearidonic)	4.99	4.38	4.12	3.99
C20:0	0.48	0.43	0.41	0.40
C22:0	0.19	0.15	0.14	0.13
C24:0	0.26	0.18	0.14	0.12
Σ SFA	30.84	30.44	30.28	30.20
Monounsaturated fatty acids (% of total fatty acids)				
C16:1 (palmitoleic)	7.00	7.88	8.24	8.44
C18:1n-9c (oleic)	14.86	14.40	14.21	14.10
C18:1n-7	3.27	3.18	3.14	3.12
C20:1n-9	3.17	2.77	2.60	2.51
C24:1	1.35	1.13	1.04	0.99
Σ MUFA	29.65	29.36	29.23	29.16
Polyunsaturated fatty acids (% of total fatty acids)				
C18:2n-6 (linoleic)	1.59	1.55	1.53	1.52
C18:3n-6	0.19	0.23	0.25	0.26
C18:3n-3 (α-linolenic)	0.57	0.68	0.72	0.75
C20:2n-6	0.48	0.43	0.41	0.40
C20:3n-6	0.17	0.19	0.20	0.20
C20:3n-3	0.04	0.06	0.07	0.08
C20:4n-6 (arachidonic)	1.41	1.11	0.99	0.92
C20:5n-3 (eicosapentaenoic)	17.15	20.46	21.82	22.57
C22:4n-6	0.11	0.08	0.07	0.07
C22:5n-3 (docosapentaenoic)	2.82	2.49	2.36	2.29
C22:6n-3 (docosahexaenoic)	14.77	12.75	11.92	11.46
C22:5n-6	0.46	0.40	0.37	0.36
Σ PUFA	39.76	40.43	40.71	40.88
n-3/*n*-6	8.02	9.13	9.66	9.96

2.2.3.2 Fish feeding

The fish were fed to apparent satiation twice daily (09:00 and 16:00). After each feeding period, uneaten feed was collected from the settlement compartment (after 10-20 minutes), oven dried at 60 °C and weighed. Feed consumption was determined by deducting the weight of the uneaten feed from that of the pre-determined amount given.

2.2.4 Chemical analysis

2.2.4.1 Crude protein

Percentage crude protein was calculated from total nitrogen content from a sample which was determined in a LECO FP528 system following acid digestion and titration of sample distillate according to the Official Methods (AOAC, 2000).

2.2.4.2 Crude lipid

Percentage crude lipid was determined gravimetrically following chloroform-methanol extraction from a dried sample in a Soxhlet extractor (Lee *et al.*, 1996).

2.2.4.3 Ash

Percentage ash content was determined as total inorganic matter by incineration of sample at 550 °C overnight. The sample was weighed into a pre-weighed crucible (porcelain) and incinerated in a Muffle Furnace (LASEC EMF 072). The crucible was removed from the muffle furnace, cooled in a desiccator and weighed. The total ash content was calculated as:

$$Ash\ (\%) = \frac{ash\ weight \times 100}{sample\ weight}$$

2.2.4.4 Gross energy

Gross energy content in the dried sample was determined by combustion to ash in a CP 500 isothermal bomb calorimeter according to the Digital Data System CP 500 operating manual (Animal Science Laboratory, University of Stellenbosch, Stellenbosch).

2.2.4.5 Fatty acids

A portion of each sample was weighed and homogenized in chloroform: methanol solution containing butylated hydroxytoluene (BHT) to break down the bonds between lipids and other compounds. An aliquot with internal standard was evaporated and transmethylated before being analysed by gas chromatography to determine the fatty acid profile (Animal Science Laboratory, University of Stellenbosch, Stellenbosch).

2.2.5 Biological measurements

2.2.5.1 Specific growth rate (SGR)

SGR (%/d) expresses the logarithm of weight gained over the duration of the culture period in days and then converted into percent using the formula below (Albrektsen *et al.*, 2006):

$$SGR = 100 \times \left(\frac{\ln W_t - \ln W_0}{t}\right)$$

Where: W_0 is the initial weight of the fish, W_t is final weight after t days and t = time (days).

2.2.5.2 Feed intake (FI)

Feed intake (g feed/fish/day) as per the formula below (Albrektsen *et al.*, 2006), expresses the amount of feed consumed per unit mass of a fish in a day.

$$FI = \frac{consumed\ feed}{n \times t}$$

Where: *n* is the number of fish, and *t* is time in days.

2.2.5.3 Feed conversion ratio (FCR)

FCR (g feed/g fish produced) as per the formula below that expresses the amount of feed that is required to produce 1 gram of fish (Chatzifotis *et al.*, 2010).

$$FCR = \frac{consumed\ feed}{\mathrm{W_t} - \mathrm{W_0}}$$

Where: W_0 is the initial weight of the fish in grams and W_t is final weight after t days.

2.2.5.4 Lipid efficiency ratio (LER)

LER (g fish/g lipid) as per the formula below (Daniel, 2004), expresses the amount of consumed lipid (g) derived from proximate composition that is retained by deposition in the carcass.

$$LER = \frac{\mathrm{W_t} - \mathrm{W_0}}{\mathrm{consumed\ lipid}}$$

Where: W_0 is the initial weight of the fish in grams and W_t is final weight after t days.

2.2.5.5 Protein efficiency ratio (PER)

PER (g fish/g protein) as per the formula below (Akpınar *et al.*, 2012), expresses the amount of consumed protein (g) derived from proximate composition that is retained by deposition in the carcass.

$$PER = \frac{\mathrm{W_t} - \mathrm{W_0}}{\mathrm{consumed\ protein}}$$

Where: W_0 is the initial weight of the fish and W_t is final weight after t days.

2.2.5.6 Viscerosomatic index (VSI)

VSI (%) as per the formula below (Woolley, 2009), is an index generally used to assess the physiological status of fish based on the relationship of the visceral (internal organs such as heart, pyloric caeca, liver, spleen, intestine, swim bladder, kidney and stomach) and total body weight. The VSI was calculated using the formula of. The VSI was calculated as follows:

$$VSI = \frac{100 \times \text{viscera weight}}{\text{body weight}}$$

2.2.5.7 Headed and gutted fish (H&G)

H&G (%) as per formula below (Tejada & Huidobro, 2002), indicates fish weight that remains after a fish is beheaded and gutted. H&G was calculated as follows:

$$H\&G = \frac{100 \times H\&G \text{ tissue weight}}{\text{body weight}}$$

2.2.5.8 Condition factor (CF)

The condition factor (CF) as per formula below (Ballagh *et al.*, 2008), is an index generally used to describe the well-being of a fish based on the length and weight relationship. The CF was calculated as follows:

$$CF = \frac{100 \times W_t}{L^{2.88}}$$

Where: W_t is final weight after t days and L is length in cm. The constant of 2.88 is determined from the length–weight relationship ($W = aL^b$) of the sampled population.

2.2.6 Water quality

Water quality parameters (mean ± SD) were monitored daily with a Hanna multi-parameter instrument (Hanna HI 9828). Oxygen (5.6 ± 0.7 mg/l), temperature (23.4 ± 1.4 °C), salinity (34.0 ± 2.4 ‰) and pH (7.3 ± 0.2) remained around these reported values during the experiment. The instrument was calibrated once per week or when values seemed out of range.

2.2.7 Statistical analysis

The data was subjected to a normality test before being analysed for differences using a one-way ANOVA. This was followed by a post-hoc test where significant differences existed and a regression to determine the relationship of the measured variable to the dietary lipid level. Data that was in percentage was log-transformed prior statistical analyses. A confidence level of 95

% was applied and data considered to be statistically different at $P < 0.05$. Where there were significant differences, the Tukey's test was applied to determine differences between means. Means were assigned ascending alphabetical superscripts according to the Tukey's test results. All statistical analyses were done using STATISTICA® software, version 7.0. Results are presented as mean ± standard deviation (SD).

2.3 Results

The results for feed consumption, weight gain, feed conversion and nutrient efficiencies are summarised in Table 2.3. Feed consumption did not differ significantly between treatments. All fish more than tripled their weight indicating the experimental diets were effective. The final weight and specific growth rate was similar between all treatments except for the 20F diet which was slightly lower. Significant differences ($P = 0.001$) in percentage weight gain and growth rates between treatments were due to dietary treatment formulations. FCR was in the range expected for commercial diets, but was significantly poorer ($P = 0.034$) for the 16F and 20F diets. Lipid efficiency ratio (LER) was significantly different ($P < 0.001$) between the tested groups. Protein efficiency ratio (PER) was significantly better ($P = 0.042$) in the 8F and 12F treatments and showed no relationship ($r = –0.89$. $P = 0.112$) with the dietary lipid levels. The LER showed statistically significant relationship ($r = –0.96$. $P = 0.037$) with dietary lipid levels. Survival was generally high (93 to 100 %) and similar ($P = 0.227$) across all treatments during the experimental phase. Mortalities were generally associated with some outward pathological signs which could be related to the experimental environment rather than the diet. The most obvious pathological signs associated with mortalities was tail-rot and gas bubbles in the eyes.

The results for carcass proximate composition, saturated fatty acids (SFA) and monounsaturated fatty acids are summarised in Table 2.4. Carcass dry matter, lipid and energy were significantly ($P < 0.05$) lower in the 8F diet and increased with an increase in lipid (and corresponding protein levels) percentage. There was no significant positive linear correlation observed in both dry matter ($r = 0.95$, $P = 0.051$), lipid ($r = 0.94$, $P = 0.061$), and energy ($r = 0.75$, $P = 0.254$) with an increase in lipid percentages. On individual basis, detected SFA increased with increasing lipid levels in diet, while most monounsaturated fatty acids decreased with increasing lipid levels in diet. Palmitic acid accounted for more than 65 % of SFA, while oleic acid made up more than 53 % of monounsaturated fatty acids.

Table 2.3. Mean of body weight, specific growth rate, feed conversion ratio, feed intake, nutrient efficiency ratio and survival of dusky kob juveniles fed four experimental diets. Data are presented as mean ± standard deviation. Means with the same superscript letters within a row are not statistically different. The absence of superscripts for the FCR and PER values

indicate no significant difference in Tukey's test, although means of groups were statistically different $F_{(3, 8)} = 4.79$, $P = 0.034$ and : $F_{(3, 8)} = 4.40$, $P = 0.042$, respectively.

Growth indicators	Experimental diets			
	8F	12F	16F	20F
Initial weight (g)	33.48 ± 2.92[a]	36.16 ± 4.89 [a]	35.35 ± 0.15 [a]	35.49 ± 1.49 [a]
Final weight (g)	178.39 ± 23.69 [a]	176.27 ± 19.26 [a]	173.47 ± 5.33 [a]	158.64 ± 6.18 [a]
SGR (%/d)	1.58 ± 0.04[a]	1.50 ± 0.03[a]	1.51 ± 0.01[a]	1.41 ± 0.03[b]
FCR	1.24 ± 0.05	1.29 ± 0.05	1.40 ± 0.09	1.39 ± 0.04
Feed intake (g/fish/day)	1.70 ± 0.17 [a]	1.70 ± 0.12 [a]	1.85 ± 0.13 [a]	1.61 ± 0.07 [a]
Energy intake (kJ/fish/day)	32.16 ± 3.31 [a]	33.41 ± 2.26 [a]	37.77 ± 2.70 [a]	33.96 ± 1.56 [a]
PER (g fish/g protein)	1.73 ± 0.06	1.69 ± 0.07	1.53 ± 0.12	1.56 ± 0.06
LER (g fish/g lipid)	9.93 ± 0.34[a]	6.47 ± 0.26[b]	4.41 ± 0.34[c]	3.59 ± 0.14[d]
Survival (%)	95.83 ± 4.17 [a]	100.00 ± 0.00 [a]	93.06 ± 6.36 [a]	98.61 ± 2.41 [a]

Table 2.4. Proximate composition (g/100g of wet weight), saturated and short chain fatty acids (percentage of total fatty acids excluding *trans* fatty acids) of dusky kob juveniles fed the four experimental diets. Data are presented as mean ± standard deviation. Means (n = 15 per treatment) with the same superscript letters within a row are not statistically different.

Proximate composition	Experimental diets			
(g/100g)	8F	12F	16F	20F
Dry matter	23.69 ± 0.93^{a}	24.25 ± 3.19^{ab}	25.63 ± 1.98^{b}	25.68 ± 1.44^{b}
Lipid	3.33 ± 1.11^{a}	4.44 ± 1.36^{ab}	4.72 ± 1.63^{b}	5.04 ± 1.18^{b}
Ash	1.66 ± 0.23^{a}	1.76 ± 0.66^{a}	2.07 ± 0.55^{a}	2.00 ± 0.48^{a}
Crude protein	18.09 ± 1.35^{a}	16.76 ± 2.42^{a}	17.95 ± 2.46^{a}	18.10 ± 2.20^{a}
Gross energy (MJ/kg)	21.80 ± 0.89^{a}	22.34 ± 1.22^{ab}	22.93 ± 0.62^{b}	22.50 ± 0.50^{ab}
Saturated fatty acids (% of total lipid)				
C14:0 (myristic)	2.51 ± 1.13^{a}	4.18 ± 0.80^{b}	4.92 ± 0.64^{bc}	5.64 ± 0.84^{c}
C15:0	0.39 ± 0.08^{a}	0.48 ± 0.07^{b}	0.49 ± 0.04^{b}	0.51 ± 0.06^{b}
C16:0 (palmitic)	25.75 ± 1.69^{a}	25.78 ± 3.60^{a}	24.22 ± 2.09^{a}	25.62 ± 7.02^{a}
C18:0 (stearidonic)	8.07 ± 0.75^{a}	6.71 ± 0.67^{b}	5.84 ± 0.51^{c}	5.52 ± 0.58^{c}
C20:0	0.30 ± 0.02^{a}	0.38 ± 0.03^{b}	0.45 ± 0.03^{c}	0.44 ± 0.04^{c}
C21:0	0.09 ± 0.02^{a}	0.07 ± 0.01^{ab}	0.07 ± 0.01^{b}	0.07 ± 0.01^{b}
C22:0	0.39 ± 0.03^{a}	0.41 ± 0.03^{a}	0.42 ± 0.08^{a}	0.41 ± 0.05^{a}
C24:0	0.04 ± 0.01^{a}	0.04 ± 0.01^{a}	0.05 ± 0.02^{a}	0.05 ± 0.02^{a}
Σ SFA	37.55 ± 2.57^{a}	38.06 ± 4.89^{a}	36.47 ± 3.01^{a}	38.26 ± 5.92^{a}
Monounsaturated fatty acids (% of total lipid)				
C14:1	0.08 ± 0.02^{a}	0.11 ± 0.02^{b}	0.11 ± 0.01^{b}	0.12 ± 0.01^{b}
C15:1	0.05 ± 0.02^{a}	0.04 ± 0.01^{a}	0.04 ± 0.01^{a}	0.04 ± 0.01^{a}
C16:1 (palmitoleic)	6.55 ± 0.95^{a}	7.37 ± 0.80^{b}	8.57 ± 0.55^{c}	8.76 ± 0.79^{c}
C18:1n-9c (oleic)	16.2 ± 0.77^{a}	12.9 ± 0.72^{b}	11.9 ± 0.43^{c}	11.2 ± 0.90^{d}
C18:1n-9t	0.18 ± 0.07^{a}	0.23 ± 0.19^{a}	0.15 ± 0.07^{a}	0.15 ± 0.10^{a}
C20:1	0.22 ± 0.02^{a}	0.25 ± 0.03^{b}	0.26 ± 0.02^{b}	0.25 ± 0.03^{b}
C22:1n-9	0.04 ± 0.00^{a}	0.03 ± 0.00^{b}	0.03 ± 0.00^{b}	0.03 ± 0.00^{b}
C24:1	1.14 ± 0.16^{a}	0.92 ± 0.10^{b}	0.80 ± 0.07^{c}	0.80 ± 0.10^{c}
Σ MUFA	24.23 ± 1.16^{a}	21.67 ± 1.40^{b}	21.77 ± 0.90^{b}	21.22 ± 1.78^{b}

A summary of the polyunsaturated fatty acid profile of experimental fish is presented in Table 2.4. Docosapentanoic and docosahexaenoic acids accounted for over 69 % of total polyunsaturated fatty acids. Docosapentanoic and docosahexaenoic acids accounted for over 69 % of total polyunsaturated fatty acids. On an individual basis, most polyunsaturated fatty acids (PUFA) were significantly different ($P < 0.05$) and decreased with an increase in dietary lipid levels. The ratios of n-3 to n-6 were significantly higher in the 20F treatment ($P < 0.05$)

and not significantly correlated (r = 0.94, *P* = 0.061) with dietary lipid levels. When fatty acids were grouped according to their classes (Figure 2.4), there was no significant difference (*P* > 0.05) in SFA across all treatments and did not correlate to lipid/protein levels in diet (r = 0.09, *P* = 0.913). The sum of monounsaturated fatty acids (MUFA) and PUFA were significantly different (*P* < 0.05) across all treatments. The correlation between diet and both MUFA (r = –0.85, *P* = 0.152) and PUFA (r = 0.75, *P* = 0.250) were not significant. The variations in SFA and PUFA within each treatment also increased with increasing lipid levels in diet.

Table 2.5. Comparison of polyunsaturated fatty acids (percentage of total fatty acids excluding *trans* fatty acids) in dusky kob fed the four experimental diets. Data are presented as mean ± standard deviation. Means (n = 15 per treatment) with the same superscript letters within a row are not statistically different.

Polyunsaturated fatty acids (%)	Experimental diets			
	8F	12F	16F	20F
C18:2n-6c (linoleic)	5.21 ± 0.27^{a}	4.21 ± 0.38^{b}	3.74 ± 0.26^{c}	3.56 ± 0.38^{c}
C18:2n-6t	0.04 ± 0.02	0.04 ± 0.02	0.03 ± 0.01	0.03 ± 0.01
C18:3n-6	0.57 ± 0.04^{a}	0.58 ± 0.06^{ab}	0.62 ± 0.04^{b}	0.61 ± 0.06^{b}
C18:3n-3 (α-linolenic)	2.41 ± 0.20^{a}	2.51 ± 0.19^{a}	1.91 ± 0.56^{b}	1.70 ± 0.22^{b}
C20:2	0.22 ± 0.02^{a}	0.18 ± 0.02^{ab}	0.19 ± 0.08^{ab}	0.16 ± 0.02^{b}
C20:3n-6	2.21 ± 0.20^{a}	2.04 ± 0.23^{ab}	1.98 ± 0.15^{b}	1.92 ± 0.24^{b}
C20:3n-3	0.20 ± 0.02^{a}	0.18 ± 0.03^{ab}	0.17 ± 0.02^{b}	0.16 ± 0.02^{b}
C20:4n-6 (arachidonic)	0.54 ± 0.14^{a}	0.69 ± 0.07^{b}	0.78 ± 0.06^{c}	0.75 ± 0.07b^{c}
C20:5n-3 (eicosapentaenoic)	0.15 ± 0.04^{a}	0.16 ± 0.02^{a}	0.22 ± 0.04^{b}	0.23 ± 0.03^{b}
C22:2	0.12 ± 0.03	0.11 ± 0.03	0.10 ± 0.03	0.09 ± 0.04
C22:5n-3 (docosapentaenoic)	11.02 ± 0.90^{a}	14.43 ± 1.68^{b}	17.27 ± 0.93^{c}	16.76 ± 1.69^{c}
C22:6n-3 (docosahexaenoic)	15.35 ± 1.37	14.89 ± 1.66	14.61 ± 1.07	14.39 ± 1.66
Σ PUFA	38.00 ± 2.73^{a}	40.00 ± 3.87^{ab}	41.57 ± 2.51^{b}	40.34 ± 4.22^{ab}
n-3/*n*-6	3.41 ± 0.12^{a}	4.27 ± 0.20^{b}	4.80 ± 0.14^{c}	4.86 ± 0.15^{c}

Omega 3 fatty acids (n-3) were significantly (P < 0.001) lower in the 8F treatment and correlation with increasing dietary lipid levels was not significant (r = 0.85, P = 0.154), while omega 6 fatty acids (n-6) were significantly (P < 0.001) higher in the 8F treatment only and negatively correlated to increasing lipid levels in diet (r = –0.96, P = 0.044) as shown in Figure 2.5. The effect of dietary lipid levels on tissue indices are summarised in Table 2.6. Viscerosomatic index (VSI) and headed and gutted fish (H&G) were significantly affected by dietary lipid and protein levels.

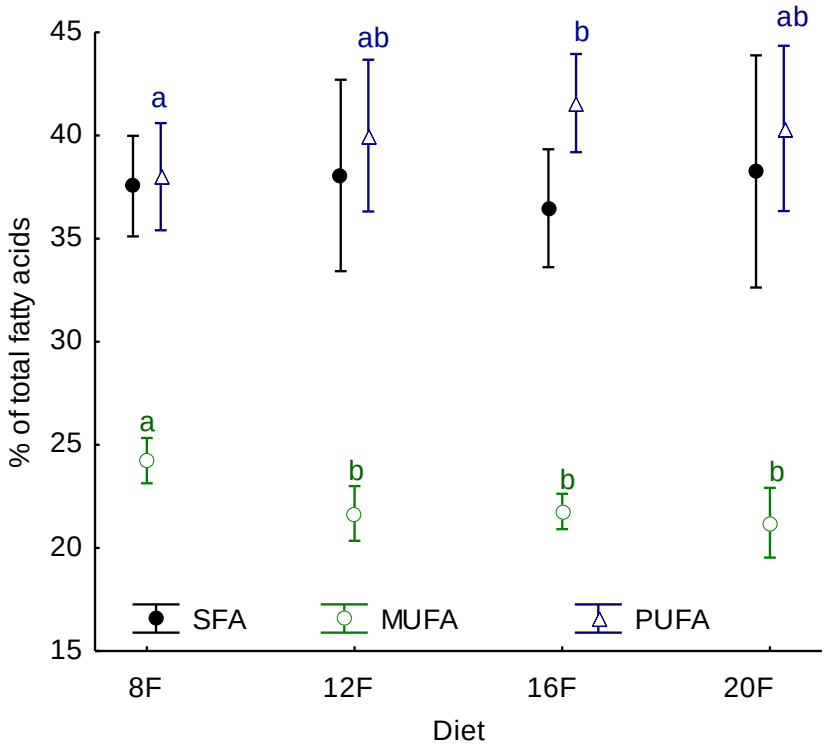

Figure 2.4. Saturated fatty acids (SFA, ●), monounsaturated fatty acids (MUFA, ○) and polyunsaturated fatty acids (PUFA, ∆) in dusky kob fed experimental diets with four dietary lipid and protein levels. Different letters above error bars denotes significant difference between dietary treatments.

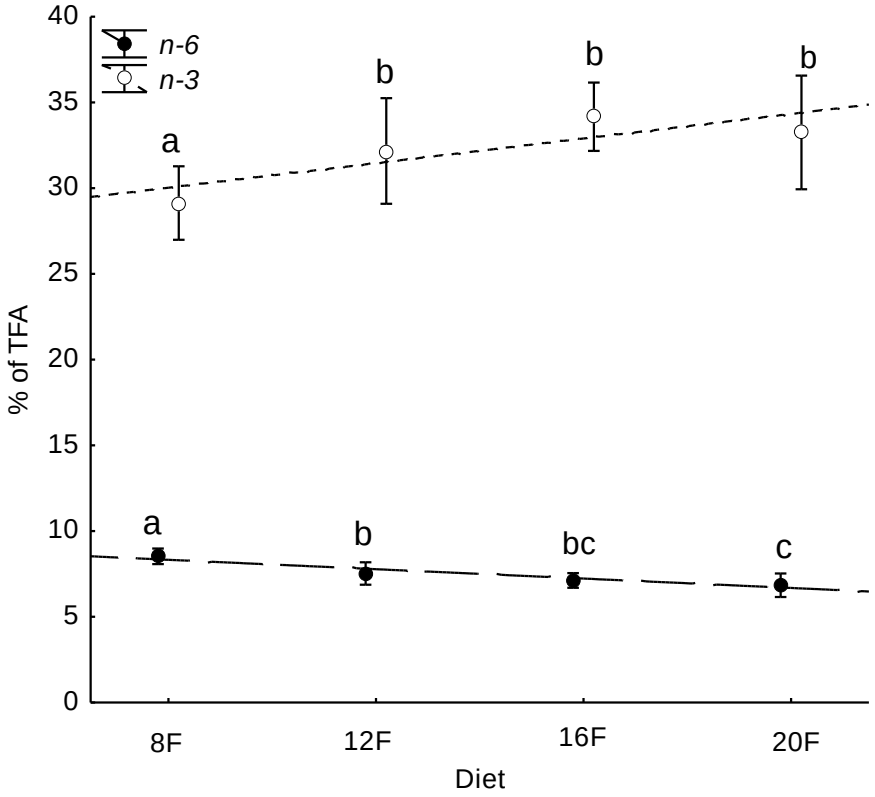

Figure 2.5. Omega 3 (○, n-3) and omega 6 (●, n-6) fatty acids in dusky kob fed the experimental diets with four dietary lipid levels. The dashed lines indicate a linear relationship between dietary lipid levels and n-3 ($y = 27.16 + 0.36x$; $r = 0.85$; $P = 0.154$) and n-6 ($y = 9.42 - 0.14x$; $r = -0.96$; $P = 0.044$). Different letters above error bars denotes significant difference.

Table 2.6. Viscerosomatic index (VSI), headed and gutted yield (H&G) and condition factor (CF) of dusky kob fed four experimental diets. Data are presented as means ± SD. Means (n = 25) with the same superscript letters within a row are not statistically different.

Tissue indices	Experimental diets			
	8F	12F	16F	20F
VSI (%)	6.92 ± 0.95^{a}	7.84 ± 1.04^{b}	8.57 ± 0.95^{b}	8.06 ± 1.29^{b}
H&G (%)	55.53 ± 2.84^{ab}	57.38 ± 5.16^{a}	56.79 ± 4.43^{a}	53.63 ± 4.10^{b}
CF (%)	2.57 ± 0.06^{a}	2.64 ± 0.06^{a}	2.60 ± 0.03^{a}	2.58 ± 0.04^{a}

2.4 Discussion

During the growth phase of the experiment, feed consumption was similar between dietary treatments. Fish will eat as much as they can until their energy requirements are met. If the energy levels of the consumed feed are low, feeding will only be restricted by the capacity of the fish's stomach. Conversely, if the energy levels are high, as is seen in high lipid diets, the feed consumption is reduced. This phenomenon has been demonstrated in malabar grouper (Tuan & Williams, 2007), sea bream (Lupatsch *et al.*, 2001), tiger puffer (Kikuchi *et al.*, 2009), meagre (Chatzifotis *et al.*, 2010) and Atlantic salmon (Paspatis & Boujard, 1996). The similar feed consumption recorded in the present study is consistent with the iso-energetic composition of the experimental diets.

Weight gain and growth rates were better at lower dietary lipid treatments and decreased slightly at the 20 % dietary lipid level. The decreased growth rate observed at the 20 % lipid may be due to a reduced ability to digest and assimilate feeds that have higher lipid content. Similar findings of reduced growth at high dietary lipid levels (>18 %) have been observed for meagre (Chatzifotis *et al.*, 2010) and red drum (Williams & Robinson, 1988). At the protein level of 42 % Woolley *et al.* (2009) found reduced growth at 18 % dietary lipid level. It has been reported that at comparatively high lipid levels, a feedback mechanism may be responsible for the depression of growth due to the accumulation of excessive lipid metabolites or satiety hormones in the blood (Kause *et al.*, 2009). Fish will only grow up to a point, optimizing their energy requirements, beyond which lipids tend to be deposited throughout the body, predominantly in the viscera (Chatzifotis *et al.*, 2010; Luo *et al.*, 2005). Fish fed diets high in lipids generally tend to spare the protein utilization for energy. Under such conditions, the dietary lipids are instead used for energy while the available protein is used for muscle growth (Hillestad & Johnsen, 1994; NRC, 2011).

Our data suggest that there was no protein-sparing effect with increasing dietary lipid level, as PER declined slightly with increasing dietary lipid level. Similarly, Woolley *et al.* (2010) observed that while growth performance in dusky kob increased with increasing lipid content (6-18 % lipid) in fish fed a 46 % protein diet, the PER was not significantly affected. As the 18 % lipid diet reported in Woolley *et al*. (2010) was of similar composition to our 20F diet, the better growth performance of our fish fed the comparatively low lipid but higher protein treatments was probably due to the higher protein to energy ratio used in the current study. This finding suggests that the optimal P:E ratio for dusky kob may be higher than 22.6 mg/kJ as recommended by Woolley *et al*. (2010). The determination of an optimal P:E ratio and

protein level for maximum growth in cultured dusky kob will, however, require further controlled investigations.

Feed conversion ratio was directly affected by dietary lipid level with feed conversion best at the lower lipid levels and worsening with increasing dietary lipid inclusion. A high lipid level in feed has been shown to negatively affect the digestive capacity of several fish species including Atlantic croaker (Davis & Arnold, 1997), red snapper (Miller, 2003), grouper (Lin & Shiau, 2003), meagre (Martínez-Llorens *et al.*, 2011) and juvenile ayu (Lee *et al.*, 2002b). Reduced digestive capacity reduces the bioavailability of nutrients, which in turn affects the amount of nutrients that can be metabolised (Cahu *et al.*, 2000; Lovell, 1989). Contrary to these findings, increased lipid levels have been shown to significantly improve feed conversion in meagre (Chatzifotis *et al.*, 2010), red drum (Thoman *et al.*, 1999), rainbow trout (Gèlineau *et al.*, 2001) and jade perch (Song *et al.*, 2009). Other studies found no significant differences on feed conversion ratios from different dietary lipid levels, e.g. meagre (McGoogan & Gatlin III, 2000), dusky kob (Woolley, 2009), and juvenile hybrid sturgeon (Guo *et al.*, 2011). In tiger puffer (Kikuchi *et al.*, 2009) and juvenile sea bass (Peres & Oliva-Teles, 1999) feed efficiency increased with increasing lipid levels to about 17 %, but then it decreased with further increases in dietary levels. In our study, the observed poor FCR probably reflects the decline in LER with the increasing dietary lipid content.

The tissue lipid and fatty acid composition of the experimental fish mirrored that of the consumed feed. Carcass composition in fish is controlled by both endogenous and exogenous factors. Carcass dry matter, lipid and energy increased with increasing dietary lipid levels, while inorganic matter and proteins were not affected. A similar correlation between lipid levels in the diet and in the fish tissue were obtained in other studies, including research on dusky kob (Daniel, 2004), meagre (Chatzifotis *et al.*, 2010), pike perch (Kowalska *et al.*, 2011), juvenile ayu (Lee *et al.*, 2002b), southern flounder (Alam *et al.*, 2009), juvenile white sea bass (López *et al.*, 2009) and red drum (Williams & Robinson, 1988). Shearer (1994) postulated that while the protein content of growing fish is determined by fish size (endogenously controlled), lipid level is determined by both endogenous and exogenous factors. From the present study, an expected inverse relationship between moisture and lipid in the muscle tissue was also observed. In other fish, a significant negative correlation was observed between protein content in the muscle and lipid levels in the diets of juvenile rockfish (Lee *et al.*, 2002a), tiger puffer (Kikuchi *et al.*, 2009) and juvenile cobia (Wang *et al.*, 2005). Lipid levels in dusky kob muscle tissue increased with increasing dietary lipid levels, while inorganic matter and proteins in muscle tissue were not affected.

The carcass saturated fatty acid (SFA) content was not affected by dietary lipid levels. However, the values obtained in this study are higher than those obtained in meagre (Hernández *et al.*, 2009; Piccolo *et al.*, 2008) and other dusky kob (Guy & Nottingham, 2014; Rossetti, 2011), but, less than those obtained in brown meagre (Cakli *et al.*, 2006). The MUFA obtained in this study are in the range obtained in meagre (Piccolo *et al.*, 2008), despite decreasing with increasing dietary lipid levels. However, this study found lower MUFA concentrations than in meagre (Giogios *et al.*, 2013; Piccolo *et al.*, 2008) and other dusky kob (Guy & Nottingham, 2014; Rossetti, 2011), but higher than that of a separate study on meagre (Fernandes, 2013). The higher concentrations of SFA and MUFA can be attributed to high levels of palmitic and oleic acids, respectively. Plant-based ingredients such as soybean and maize are rich in palmitic and oleic acids (NRC, 2011). The lower lipid diets with greater dietary proportion of oleic acid had more oleic acid in muscle tissue. Dusky kob tissue composition reflects the accumulation of largely unchanged dietary saturated and monounsaturated fatty acids. The results could also mean dusky kob has a limited capacity for fatty chain elongation and desaturation, irrespective of dietary lipid levels.

The PUFA composition increased with an increase in dietary lipid levels of up to 16 %, particularly the n-3 fatty acids, EPA and DHA. These results are consistent with known trends that FA composition of fish muscle tissue is directly linked to the composition of the diet (Caballero *et al.*, 2002). The DHA values in this study were closer to the 15 – 17% range in meagre (Poli *et al.*, 2003), lower than the 18 % obtained in another meagre study (Giogios *et al.*, 2013), and higher than the 5.9 % in other dusky kob (Guy & Nottingham, 2014). It is not clear what may have caused the elevated levels of docosapentaenoic acid (C22:5n-3), as the values from this study are much higher than in several studies on sciaenids where docosapentaenoic acid values range between 0.4 – 2.3 % of total fatty acids (Fernandes, 2013; Giogios *et al.*, 2013; Piccolo *et al.*, 2008; Poli *et al.*, 2003). The *n*-3/*n*-6 ratios in this study increased with increasing dietary lipid levels.

Of the tissue indices, only VSI and H&G yield were affected by differences in dietary lipid levels. The increase in VSI agrees with other studies on juvenile grass carp (Du *et al.*, 2005), rainbow trout (Gèlineau *et al.*, 2001) and cobia (Wang *et al.*, 2005). In pikeperch (Kowalska *et al.*, 2011), meagre (Chatzifotis *et al.*, 2010) and juvenile hybrid sturgeon (Guo *et al.*, 2011), however, no corresponding differences between tissue indices and dietary lipid levels were observed. For those studies, an increase in VSI (including this study) was recorded. It appears that the consumed lipids end up stored in the visceral tissues and, which if not measured independently, may be mistaken as deposition in the edible parts of the fish. High dietary lipid levels depresses *de novo* fatty acid synthesis and encourages lipid storage. This depressed

synthesis may lower the nutritional value of fish. The increase in VSI and reduction of headed and gutted yield in dusky kob with increasing dietary lipid levels may well be interpreted as energy and value loss from the feed. Mathis *et al*. (2003) found a strong negative correlation between VSI and H&G. Such a relationship was, however, not apparent in the current study. The lowest H&G yield in the highest lipid level treatment agrees with those findings on rainbow trout (Jobling *et al.*, 1998), Eurasian perch (Mathis *et al.*, 2003) and Atlantic salmon (Einen & Roem, 1997). Although the relationship between H&G and dietary lipid levels was weak in this study, it can be said that lipid levels of feed has a direct effect on the value of cultured fish and can affect the yield of the headed and gutted fish.

2.5 Conclusion

Dusky kob fed 8 %, 12 % and 16 % dietary lipid yielded similar growth rates, feed conversation ratios, and lipid conversion efficiencies, while performance for these parameters was slightly poorer at 20 % dietary lipid inclusion. The results of this study suggest that increasing dietary lipid levels increases tissue lipid levels but does not affect tissue protein levels. The increase in dietary lipid levels does not affect the saturated fatty acid composition in tissue, but does increase the content of the polyunsaturated fatty acids, particularly *n*-3 fatty acids. Consequently, higher dietary lipid levels increased the *n*-3/*n*-6 ratios.

CHAPTER 3

EFFECT OF DIETARY LIPID LEVELS ON QUALITY CHANGES OF CHILLED DUSKY KOB FILLET

3.1 Introduction

Fresh and good quality fish are a highly beneficial source of nutrition as they contain all essential amino acids and fatty acids (Kandemir & Polat, 2007). The health benefits of fish have, over the years, increased the demand for fish and has resulted in increased fishing pressure and overexploitation of certain fish species. The decline in fish stocks has resulted in a scarcity of some fish species, particularly fresh fish as opposed to frozen fish (FAO, 2014b). Aquaculture guarantees constant supply of good quality fish because most parameters that affect fish quality can be controlled to a certain extent (Paterson *et al.*, 1997). Fish quality is affected by both intrinsic and extrinsic factors such as fish species, fish size, spawning period, stress level, food composition, body composition, handling during capture, holding temperature and preservation method (Haard, 1992; Huss, 1995; Köse & Erdem, 2001). Chilled and iced storage are common preservation methods used by most retailers of fresh fish who are aware that quality is of paramount importance to the consumer (Coello *et al.*, 1999). However, even at low temperatures, there is still a degree of sensory, chemical and microbiological changes that occur in the muscle tissues thus reducing freshness (Simeonidou *et al.*, 1998).

The freshness of fish can be determined through organoleptic and biochemical techniques that are universally accepted. In some instances microbiological techniques are employed in addition to organoleptic and biochemical tests (Alasalvar *et al.*, 2002; Huss, 1995). Autolytic degradation is the primary chemical reaction that takes place and is characterised by the loss of the original odour and taste. In contrast, the final stages of fish quality deterioration are characterised by softening or toughening of the flesh texture and production of volatile, unpleasant odours and flavours, mainly due to microbial activity (Fraser & Sumar, 1998; Widjaja *et al.*, 2009).

Fat content has a major effect on fish degradation during storage with high fat content species being more prone to rancidity than others (Jensen *et al.*, 1998). Fatty species generally have large variations in their lipid content between seasons, feeds and age (Liang *et al.*, 2002). Besides the obvious organoleptic consequences of lipid degradation there are also potential human health concerns. There is sufficient evidence to health risks associated with rancid or oxidised fats. The oxidation by-products are in part cytotoxic and genotoxic compounds. Reports on have shown that the health risks, such as induction and development of

atherosclerosis in humans that may be posed by lipid peroxidation (Godwin & Prabhu, 2006; Trevor, 2004; Wąsowicz *et al.*, 2004).

Preservation techniques such as freezing, canning, icing, chilling and others were developed long ago to preserve the quality of fresh meat including fish. These techniques, however, substantially change the character of the meat relative to that of freshly caught product (Pedrosa-Menabrito & Regenstein, 1990). The freezing process induces muscle tissue changes as a result of the formation and accumulation of ice crystals. The ice crystals disrupt the cells resulting in dehydration and an increase in solute concentration, eventually affecting the value of the fish (Huss, 1995). Chilled storage (temperatures around 5ºC) is very effective in preserving freshness in fish as the process retards bacterial growth and enzymatic activity resulting in minimal damage to the muscle tissue (Huidobro *et al.*, 2001).

Quality deterioration in stored fatty fish has been attributed to both changes in lipids and loss of protein functionality (Lauzon *et al.*, 2010). Marine fish products are composed of high levels of polyunsaturated fatty acids (PUFA). Various pro-oxidants such as hemi-proteins and free transition metals are in fish products, hence PUFA in fish and fish products decompose (oxidise) very rapidly (Bórquez, 2003). The high lipid content which is deposited in muscle tissues tend to increase the susceptibility of fish products to lipid oxidation, and are closely correlated to the lipid content in feed (Huang *et al.*, 1998). The produced lipid oxidation compounds interact with proteins leading to protein denaturation, nutritional losses, and modification of profiles of proteins (Aubourg *et al.*, 1998a). Endogenous enzymes such as lipases and phospholipases remain active even at frozen storage temperatures and yield free fatty acids (FFA) that are a more labile substrate for oxidation. Other factors that accelerate lipid oxidation include the presence of haem components, light, heat, microorganisms and metals (Shahidi & Zhong, 2005). Generally, measured oxidation by-products are the peroxide value (PV) and thiobarbituric acid reactive species (TBARS). The PV, which is a primary indicator of oxidation of lipids, is between 20-40 milliequivalents of oxygen per kg of sample at the beginning of spoilage (Daramola *et al.*, 2007). For the presence of secondary oxidation products from autoxidation, TBARS levels below 6 mg malonaldehyde/kg (83µmol/kg) in fish products are regarded as acceptable for human consumption (Owaga *et al.*, 2009).

Fresh seafood has a high commercial value (EUMOFA, 2014), and quality loss in conventionally frozen or chilled fish is a big concern for producers and consumers. The quality losses are not only from lipid degradation, but also from the breakdown of nitrogenous molecules. Total volatile basic nitrogen (TVBN, a measure of freshness) measures the total nitrogenous compounds (such as trimethylamine, dimethylamine, ammonia and other volatile nitrogenous compounds) that develop during muscle tissue deterioration (Huss, 1995). These

compounds are formed from autolysis, bacterial activities and deamination of proteins and are primarily responsible for the offensive fish odour during spoilage. Trimethylamine (TMA) is responsible for the fishy smell, while unionised NH_3 gives rise to the ammoniacal smell. TMA arises from the bacterial reduction of trimethylamine oxide, a naturally-occurring osmoregulatory substance found in most marine fish, or from the deamination of amino acids (Hernández *et al.*, 2009). Fish are generally considered to be fresh if levels of TVBN are less than 20 mg N/100 g sample; at level ≥35 mg N/100 g the fish is regarded as unfit for consumption (Fraser & Sumar, 1998; Melaku, 2007). Based on proximate composition, dusky kob can be classified in the same group as turbot in the EU guidelines for TVBN limits for fish which is 25 mg-N/100g tissue. The accumulation of the volatile bases during storage results in some degree of pH increase, while glycogenolysis during post mortem results in a drop of muscle pH. The limit in pH value of fish differs between species, although, muscle tissues with pH values above 7.0 are generally regarded as unfit for consumption (Köse & Erdem, 2001).

High-fat diets are known to produce fish with a higher fat content, thus increasing the amount of substrate for oxidation processes and the risk that fats will become rancid during processing and storage (Torrissen, 1985), and reducing shelf-life (Jensen *et al.*, 1998). However, consumer preference of the final product, particularly lipid content, differs. In most regions fish with a fishy odour are considered to be of lower quality because that is often linked to the onset of rancidity (Huss, 1995). In some regions (parts of Spain) these fish have higher levels of acceptability (Coello *et al.*, 1999). In light of this, some studies have investigated dietary manipulation to modify the susceptibility of fish to quality deterioration (Alvarez *et al.*, 1998).

Aquaculture research on dusky kob (*Argyrosomus japonicus*) has generally been conducted to define nutritional, environmental, reproductive, health requirements to optimise growth, feed and nutrient efficiency, and general husbandry. There are, however, no studies that have sought to quantify the quality changes in dusky kob edible tissue under refrigerated storage. Fish muscle tissue is an excellent source of protein and fatty acids, however, during post-mortem it becomes a suitable substrate for enzymatic activities and bacterial growth. Intrinsic and extrinsic factors directly influence fish quality and storage life. Studying lipid and protein hydrolysis is very important in fish quality studies because hydrolysed lipids and proteins have direct effect on fatty acid oxidation. Other quality losses directly linked to FFA accumulation include taste loss, loss of protein solubility and protein denaturation (Lauzon *et al.*, 2010). Aquaculture has the potential of supplying both refrigerated and frozen dusky kob products. This study sought to determine the effect of dietary lipid levels on chemical quality changes of refrigerated dusky kob fillets. The chemical quality changes refers to total volatile basic nitrogen, pH, moisture, free fatty acids and thiobarbituric acid-reactive species.

3.2 Materials and Methods

3.2.1 Experimental fish and diets

A total of 32 dusky kob fish from the growth experiment in Chapter 2 were used for this experiment. The fish were reared at the Aquaculture Research Facility (Sea Point) under experimental conditions and were fed the 8F, 12F, 16F and 20F as explained in Chapter 2. The diet names indicate percentage of dietary lipid (F) and protein (P). Eight fish (172.38 ± 15.71 g) from each treatment were randomly taken and euthanized by immersion in an ice-cold water bath, filleted and refrigerated (6.6 ± 1.5 °C). Each chilled fillet was finely-ground in a blender and stored in individual containers for the 18-days trial. The ground muscle tissue was mixed using a spatula before taking a sample at each sampling interval. The proximate and fatty acid composition (Table 2.4 and Table 2.5) of experimental muscle tissue was analysed at the beginning of the storage trial which was at the end of the growth experiment in Chapter 2.

3.2.2 Total volatile basic nitrogen (TVBN)

TVBN (mg/100g) was extracted from muscle tissue using perchloric acid and alkalinisation in sodium hydroxide. This solution then underwent steam distillation wherein the volatile bases were trapped in an acid and then determined by titration. A standard was run to determine recovery of the nitrogenous compounds from a known concentration of ammonium sulphate. Then the TVBNs were calculated according to the EU protocol (EU, 2005).

$$TVBN = \frac{(V_1 - V_0) \times 14 \times 0.05 \times 100}{m}$$

Where: V_1 is the volume of HCl in the sample, V_0 is the volume of HCl in the blank, m is the weight of the sample in grams.

3.2.3 pH

One gram of fish muscle was homogenized with 10 ml distilled water and then the pH of the homogenate was measured using a digital pH meter (Rawdkuen *et al.*, 2008).

3.2.4 Moisture

The moisture content was determined by drying a pre-weighed sample at 100 °C for 24 hours followed by cooling in desiccators before reweighing (AOAC, 2002).

3.2.5 Free fatty acids (FFA)

FFAs (g/100g) were analysed at the Cape Peninsula University of Technology. Two grams (2 g) of each sample was weighed and homogenized in chloroform: methanol (containing butylated hydroxytoluene). An aliquot with internal standard added, was evaporated and transmethylated at 70 °C. Samples were analysed as methyl esters by Focus Gas Chromatography with Flame Ionization as detector. Analyses were conducted under the following parameters and conditions: 60 m capillary column x 250 µm internal diameter BPX70 with 0.25 µm film thickness. The initial column temperature was 160 °C, then increased at 3 °C per minute to 220 °C and held for 1.5 minutes. Hydrogen at a constant flow rate was used as carrier gas (Functional Food Research Unit, Department of Agriculture and Food Science, Cape Peninsula University of Technology).

3.2.6 Thiobarbituric acid-reactive substances (TBARS)

The TBARS (nmol MDA/ g) were determined following a modification of the spectrophotometric method (Pegg, 2001). A 2.5 g muscle tissue was homogenized in 1.25 ml of an antioxidant solution and 25 ml trichloroacetic acid for 1 minute. This homogenate was homogenized for another minute after adding 25 ml ice-cold distilled water before filtering through a Whatman GF/F filter. A 5 ml of filtrate was transferred to a 15 ml centrifuge tube followed by addition of 5 ml thiobarbituric acid. A blank was treated in a similar way except that trichloroacetic acid-water replaced the filtrate. A standard was prepared from serial dilutions of tetraethoxypropane (TEP) solution (0.2 mM MDA, malondialdehyde) to range between 0 and 20µM in trichloroacetic acid-water and 5 ml thiobarbituric acid was added to the standards. All the tubes were capped and mixed thoroughly before incubation for 35 minutes in a boiling water bath. After incubation, all tubes were allowed to cool in water bath at room temperature before reading absorbance at 532 nm. TBARS were then calculated as:

$$TBARS = \frac{\text{nmol TEP (per tube)}}{\text{g wet weight eq in 5 ml extract (i.e per tube)}}$$

3.2.7 Statistical analysis

Data was analysed using a 2-way ANOVA followed by a post-hoc test where significant difference existed. Regression was done to determine the relationship of the measured variable to the dietary lipid level in diet. A confidence level of 95 % was applied and data considered to be statistically different at $P < 0.05$. Where there were significant differences, the Tukey's test was applied to determine differences between means. Means were assigned ascending alphabetical superscripts according to the Tukey's test results. All statistical

analyses were done using STATISTICA® software, version 7.0. Results in tables are presented as mean ± standard deviation (SD), while graphs are reported as mean ± 95 % confidence interval.

3.3 Results

There was significant interaction between dietary lipid levels and storage period on total volatile basic nitrogen (TVBN) – (Factorial ANOVA: $F_{(12, 140)} = 4.50$, $P < 0.001$). TVBN increased in all treatments and showed positive correlation with increased lipid levels over the storage period (Table 3.1). The 20F was the first treatment to reach critical limit for human consumption on day 8, followed by the 16F on day 12. On day 18, the 16F and 20F treatments had the highest concentrations of volatile bases. The changes in pH of dusky kob fillets from different treatments during storage are summarised in Table 3.2. There was a significant interaction (Factorial ANOVA: $F_{(12, 140)} = 11.161$, $P < 0.001$) between dietary lipid levels and storage period on pH. A general increase in pH across all treatments was evident, except for the 12F treatment. The 8F treatment was the first to reach the critical pH limit for human consumption on day 12. The moisture content in the fillets was not affected by dietary lipid levels during refrigerated storage period (Table 3.3) and displayed no significant correlation with dietary lipid level ($r = –0.95$; $P = 0.051$). On day 18, moisture content was similar across all treatments, and an insignificant positive correlation ($r = 0.87$; $P = 0.131$) between moisture content and dietary lipid levels was observed. Free fatty acids (FFA) increased significantly ($P < 0.05$) in all treatments with time of storage and showed a general positive correlation with lipid levels in tissue, although there were fluctuations in FFA levels between sampling intervals (Table 3.4).

Table 3.1. The means (n = 8) of TVBN (mg-N/100g) in dusky kob fillets during an 18-day period of refrigerated storage. Data are presented as mean ± standard deviation. Means with the same superscript letters within a row are not statistically different.

Storage (days)	Dietary lipid levels			
	8F	12F	16F	20F
0	9.05 ± 1.25^{a}	9.79 ± 0.73^{a}	8.68 ± 1.43^{a}	10.86 ± 0.88^{b}
4	11.13 ± 1.26^{a}	14.25 ± 2.34^{ab}	14.75 ± 4.69^{ab}	16.60 ± 1.91^{b}
8	18.42 ± 2.27^{a}	19.64 ± 0.94^{a}	23.83 ± 1.56^{b}	25.96 ± 1.77^{b}
12	22.54 ± 1.02^{a}	21.83 ± 0.46^{a}	26.07 ± 2.05^{b}	26.81 ± 1.94^{b}
18	26.74 ± 1.61^{ab}	24.01 ± 0.78^{a}	28.55 ± 3.59^{b}	27.91 ± 3.37^{b}

Attempts to measure thiobarbituric acid-reactive substances (TBARS) on days 0, 4 and 8 were unsuccessful due to ineffective filtration using the applied method. The Whatman No. 1 and 3 filters allowed substantial amounts of suspended solids to pass through into the filtrate which made reading on a spectrophotometer impossible (centrifuging was ineffective as well). It was only after filtering through Whatman GF/F filters (nominal pore 0.7 µm) where the filtrate was clear enough to be read on a spectrophotometer. On day 12, TBARS were significantly different ($P < 0.05$) and showed a not significant positive correlation ($r = 0.94$; $P = 0.057$) with dietary lipid levels. TBARS differences were statistically significant ($P < 0.05$) on day 18 and the highest value was recorded in the 16F treatment (Table 3.5).

Table 3.2. Changes in means (n = 8) of pH in dusky kob muscle tissue from fish fed four experimental diets with varying lipid levels during 18 days of refrigerated storage. Data are presented as mean ± standard deviation. Means with the same superscript letters within a row are not statistically different.

Storage (days)	Dietary lipid levels			
	8F	12F	16F	20F
0	6.65 ± 0.02^{a}	6.77 ± 0.08^{b}	6.68 ± 0.03^{a}	6.72 ± 0.04^{ab}
4	6.71 ± 0.05^{a}	6.72 ± 0.08^{a}	6.74 ± 0.10^{a}	6.67 ± 0.13^{a}
8	6.85 ± 0.05^{a}	6.75 ± 0.04^{bc}	6.78 ± 0.03^{b}	6.73 ± 0.02^{c}
12	7.12 ± 0.02^{a}	6.70 ± 0.10^{b}	6.97 ± 0.10^{c}	6.90 ± 0.06^{c}
18	7.12 ± 0.20^{a}	6.72 ± 0.11^{b}	7.16 ± 0.20^{a}	7.06 ± 0.14^{a}

Table 3.3. Changes in the means (n = 8) of moisture content (% of tissue) in dusky kob muscle tissue from fish fed four experimental diets with varying lipid levels during 18 days of refrigerated storage. Data are presented as mean ± standard deviation. Means with the same superscript letters within a row are not statistically different.

Storage (days)	Dietary lipid levels			
	8F	12F	16F	20F
0	76.31 ± 0.93^{a}	75.75 ± 3.19^{ab}	74.37 ± 1.98^{b}	74.32 ± 1.44^{b}
18	73.61 ± 1.21^{a}	73.46 ± 1.48^{a}	73.98 ± 0.88^{a}	75.14 ± 1.62^{a}

Table 3.4. Changes in means (n = 8) of FFA (g/100g of lipid) in dusky kob muscle tissue from fish fed four experimental diets with varying lipid levels during 18 days of refrigerated storage. Data are presented as mean ± standard deviation. Means with the same superscript letters within a row are not statistically different.

Storage (days)	Dietary lipid levels			
	8F	12F	16F	20F
0	3.11 ± 0.12^{a}	2.10 ± 0.03^{b}	2.81 ± 0.00^{c}	4.46 ± 0.07^{d}
4	2.24 ± 0.07^{a}	2.74 ± 0.01^{b}	3.97 ± 0.07^{c}	2.63 ± 0.04^{b}
8	3.82 ± 0.04^{a}	4.25 ± 0.04^{b}	7.01 ± 0.02^{c}	5.16 ± 0.07^{d}
12	3.44 ± 0.02^{a}	4.05 ± 0.02^{b}	3.44 ± 0.02^{a}	6.04 ± 0.05^{c}
18	5.43 ± 0.01^{a}	3.96 ± 0.00^{b}	6.91 ± 0.00^{c}	4.94 ± 0.02^{d}

Table 3.5 Changes in means (n = 8) of TBARS (µmolMDA/kg of tissue) in dusky kob muscle tissue from fish fed four experimental diets with varying lipid levels during 18 days of refrigerated storage. Data are presented as mean ± standard deviation. Means with the same superscript letters within a row are not statistically different.

Storage (days)	Dietary lipid levels			
	8F	12F	16F	20F
12	3.66 ± 0.09^{a}	3.58 ± 0.08^{a}	5.95 ± 0.14^{b}	7.33 ± 0.66^{c}
18	96.06 ± 4.24^{a}	93.23 ± 6.96^{a}	107.18 ± 10.09^{b}	99.07 ± 6.93^{ab}

3.4 Discussion

The quality deterioration of dusky kob fillets was mainly driven by enzymatic activities on tissue protein and lipid. The tissue from higher dietary lipid levels had shorter storage life and reached critical limits much earlier than tissue from lower dietary lipid levels.

The initial cause of change in muscle tissue properties are constitutive enzymes that maintain their activities post mortem (Sikorski & Kotakowski, 2000). Although TVBN is a highly recognised indicator of fish spoilage, it cannot be solely used to determine freshness (Olafsdóttir *et al.*, 1997). In this study, the TVBN concentrations between treatments were not expected to vary much because the starting nitrogenous content reported as protein content was similar across all treatments. The tissue concentration of volatile bases at the end of the

experiment was however positively correlated with dietary lipid level, suggesting that diet significantly influences the formation of volatile bases in stored dusky kob fillets. No comparative aquaculture studies that investigated the impact of dietary lipid levels on storage life of fish could be found. However, the observed increase in TVBN with storage in this study agrees with other studies that monitored its formation during storage such as in Atlantic cod (Duun & Rustad, 2007), Mediterranean horse mackerel and blue jack mackerel (Tzikas *et al.*, 2007). Contrary results are given in Huidobro *et al*. (2001) and Hernández *et al.* (2009) who observed a decrease in TVBN during chilled storage of gilthead sea bream and meagre, respectively. It was around 25 mg-N/100g where the obvious fishy and putrid odour was noticed in the present study. The TVBN values suggested that dusky kob can have up to 12 days of acceptable storage if fed lipid levels ranging between 6 and 12 %. Dietary lipid levels above 12 % reduced the storage life to around 8 days (16F) or less (20F). TVBN therefore accumulates much quicker in muscle tissue of dusky kob fed high lipid diets.

Tissue pH generally drops during *rigor mortis* and then increases again. This is caused by the lactic acid accumulation in tissues from glycogenolysis and to a lesser extent from fatty acids after lipid hydrolysis (Hernández *et al.*, 2009). Higher lactic acid formation is generally linked to stress before slaughter of fish (Özogul *et al.*, 2005). The pH of fresh fish is between 6.0 and 6.5 with an acceptable upper pH limit of 6.8. Once the pH reaches 7.0, the fish is declared unfit for human consumption (Belitz *et al.*, 2009; Köse & Erdem, 2001). The initial pH (6.65 – 6.77) of the fillets was suitable for most bacterial growth, particularly the spoilage bacteria as indicated by the increase in TVBN production across all treatments. The 8F treatment was the first treatment to be unfit for human consumption between 8 and 12 days of storage, while other treatments could only be declared unfit after 12 days of storage. The general increase in pH for all treatments observed in this study is normal in fish during storage and is primarily contributed by the production of alkaline compounds such as ammonia. Similar increases in pH have been reported for Mediterranean horse mackerel and blue jack mackerel (Tzikas *et al.*, 2007), whiting (Köse & Erdem, 2001), Nile tilapia (Liu *et al.*, 2010), Mediterranean hake and striped mullet (Simeonidou *et al.*, 1998) and European eel (Özogul *et al.*, 2005). pH values above 7.0 are directly linked to texture loss in stored fillets and favours the growth of spoilage bacteria. pH can, however, not be a reliable indicator in fish with higher ratio of dark to white muscles because they have higher glycogen levels in tissues. Therefore pH should be used in combination with other quality indicators to determine quality loss and storage life of fish tissue. These results suggest that the experimental dusky kob remained suitable for human consumption for at least 12 days in chilled storage in all treatments except in the 8F treatment.

Higher moisture content in fish provides a medium rich in both organic and inorganic compounds for bacterial growth. Increased water activity speeds up quality deterioration in stored fish (Huss, 1995). On the other hand consumers prefer a succulent fillet to a drier one. Hydrated fillets fetch a higher price due to the texture and general appearance, than fillets that have lost their water holding capacity. The results of this study clearly indicate that dietary lipid level did not affect the moisture content of muscle tissues during storage. More importantly, the moisture content of muscle tissue remained within the range of semi-fatty fish, though it decreased slightly in all treatments during storage. The decrease in moisture content of chilled muscle tissue is consistent with findings in bagrid catfish (Gandotra *et al.*, 2012) and African catfish (Ahmed *et al.*, 2013). It is still unclear what may have caused the increase in moisture content in the high lipid (20F) treatment, but it is speculated that it can have something to do with the higher muscle drip from proteolysis. An increase in moisture content was similarly observed in sea bream (Zaragozá *et al.*, 2013) and gilthead sea bream (Kyrana *et al.*, 1997) stored under similar conditions. The rate of proteolysis and protein denaturation has an effect on drip loss as these factors directly affect the water holding capacity of tissues (Huff-Lonergan & Lonergan, 2005). In the present study, higher development of volatile bases may be linked to loss of protein integrity, thus resulting in lower water holding capacity. A drop in pH during *rigor mortis* also decreases the tissue's water holding capacity because it brings the proteins closer to an iso-electric point (Huff-Lonergan & Lonergan, 2005). Although pH was not measured during *rigor mortis,* tissues from the highest lipid treatments may have reduced pH more than the other treatments, which together with the effect of proteolysis may have increased the amount of water loss from tissue.

During chilled storage, glycerol-fatty acid esters in muscle tissue undergo some degree of hydrolysis thus releasing fatty acids and glycerol molecules, which indicates quality loss in stored fish (Huss, 1995; Refsgaard *et al.*, 2000). The results of this study, show that there was a steady increase in FFA levels in stored dusky kob fillets that was positively correlated to storage time. It was also interesting to see the positive correlation between FFA and dietary lipid levels. The positive correlations probably reflects the differential rate of lipid hydrolysis in the treatments. There are few studies that have considered lipid hydrolysis during refrigerated and iced storage. However, the slow FFA development obtained in this study is consistent with results for farmed turbot (Aubourg *et al.*, 2005), horse mackerel (Aubourg, 2001), farmed giant catfish (Rawdkuen *et al.*, 2008) and blue whiting (Aubourg *et al.*, 1998b). The results suggest that lipid hydrolysis (FFA accumulation) in dusky kob tissue increases with increasing dietary lipid levels during storage. Quality deterioration is thus faster in stored dusky kob tissue with high lipid contents.

Lipid oxidation in chilled storage gives rise to a broad odour spectrum and sometimes a yellow discolouration of fish muscle tissue mainly due to accumulation of aldehydes that can be determined as thiobarbituric acid reactive substances (Decker *et al.*, 1988; Halamíčková & Malota, 2010). The results indicate that diet composition directly influenced TBARS formation during storage of dusky kob fillets, and the oxidation rate was higher in tissues with higher lipid contents. Despite these apparent differences, deterioration rates were considerably more elevated after day 12 in all treatments. The marked increase in TBARS was probably due to the destruction of hydroperoxides into the secondary oxidation products, particularly aldehydes. This is often due to the increased degradation of muscle proteins (indicated by the TVBN accumulation), which released pro-oxidants as well as the non-heme iron from the degraded muscles with increasing storage time (Medina *et al.*, 1999; Rawdkuen *et al.*, 2008). The results on day 18 show that all the treatments had exceeded the TBARS limit of 83 µmol/kg, and were thus considered unfit for human consumption. The lipid oxidation levels were directly linked to the amount of polyunsaturated fatty acids in muscle tissue. The relationship between lipid oxidation levels and PUFA in muscle tissue was also observed in other fish species such as hybrid tilapia (Huang *et al.*, 1998), rainbow trout (Chaiyapechara *et al.*, 2003), Arctic charr (Olsen & Henderson, 1997), and some Mediterranean fish species (Simeonidou *et al.*, 1998). Huang *et al*., (1998) further implied that high levels of PUFA may either inhibit or destroy natural antioxidants such as vitamin A, C and E thus promoting lipid oxidation. Off-flavours are primary indicators of lipid oxidation in stored fish, and in this study the strong rancid odour was more obvious in the higher lipid contents (16F and 20F). This perhaps explains why the FFA levels did not increase sharply; rather there was a steady increase throughout storage. Based on the various fish species studied by Decker *et al*., (1988), the optimal pH for lipid oxidation occurred over a range of 6.5–6.9, and this fits well with the observations made in this study. The development of TBARS in dusky kob tissue during refrigerated storage are directly influenced by dietary lipid levels.

3.5 Conclusion

High dietary lipid levels shortened the shelf-life of refrigerated dusky kob fillets through the accumulation of volatile bases and lipid oxidation. Lipolysis increased steadily during storage, thus availing FFA to oxidation. Dusky kob fillets of lower lipid contents were generally of an acceptable quality for longer, but only to a limited period of time. Fillets of higher lipid contents had shorter shelf-lives. The results of this study showed that volatile bases contribute more towards quality deterioration initially. Thereafter, there was a rapid increase in lipid oxidation that is positively correlated with lipid levels in the diet.

CHAPTER 4

EFFECT OF DIETARY LIPID LEVELS ON NUTRIENT EXCRETION RATES

4.1 Introduction

In addition to optimising production and minimising input costs, aquaculture nutrition research seeks to reduce environmental pollution by minimising the excretion of waste nutrients. It is of great importance to reduce nutrient loss both into water from leaching, and from urine and faeces (Boyd, 2012; NRC, 2011). The protein component of a diet is the most expensive and some studies have used lipid inclusion to reduce the dietary protein content. Dietary protein reduction reduces formulation costs, and nitrogen and phosphorus loss into the environment (Kaushik & de Oliva Teles, 1985; Williams & Robinson, 1988). The discovery of protein-sparing mechanisms in some fish species have encouraged the increase in lipid inclusion in aquafeeds without hampering feed intake, growth and nutrient digestibility and absorption (Lee *et al.*, 2002a).

Fish will consume feed until their energy requirements are met. Thus energy utilization for growth, maintenance, reproduction, and other activities needs to be understood when formulating aquafeeds. Maintenance energy requirements in fish are not as high as for land animals because fish do not need to regulate their temperature; also minimal energy is required to maintain position in water (De Silva *et al.*, 2012; NRC, 2011). Fish void more than 85 % of their nitrogenous wastes through an energy-free mechanism across the gills; some fish, however, do expend energy when clearing organic nitrogenous compounds such as urea and uric acid. This is because the synthesis of urea and uric acid is energy-dependant together with its excretion in urine (Dosdat *et al.*, 1996; Lovell, 1989). Energy consuming processes in fish include voluntary activities, reproduction, feeding, digestion, absorption, formation and excretion of metabolic waste, and assimilation of nutrients into tissues. In order to define metabolic energy budgets and nutrient losses, there is a need to quantify all by-products from metabolism of nutrients in fish (Hardy *et al.*, 2011).

Environmental parameters such as temperature, oxygen and ambient ammonia concentration are key in regulating metabolic rates in fish. Other parameters that has an impact, yet to a lesser extent, are salinity, photoperiod and water currents (Jobling, 1981a; Lovell, 1989). Not only is metabolic rate affected by the environment, it is also affected by dietary composition, feeding rate, reproduction stage, physiological condition, fish species, size, sex and age (Ai & Xie, 2005; Hillebrand, 2009; Jobling, 1981a; Jobling & Davies, 1980; Sundström, 2012). Some

human-induced factors have a direct impact on the metabolism of a fish such as the physical environment or confinement and anaesthesia (Belal, 2005).

Predatory marine fish such as dusky kob primarily utilize protein and lipid to satisfy their energy demands (NRC, 2011). The deamination process that forms part of protein catabolism is reflected in their metabolic wastes as ammonia (ionised and unionised). A major water quality management concern in an aquaculture operation is ammonia excretion, which accounts for more than 80 % of the total nitrogen excretion derived from proteins; urea and uric acid accounts for the balance (Boyd, 2012). Unionised ammonia is toxic to fish while urea and uric acid are not. About 80 % of ammonia is excreted through the gills, while roughly 10 % is excreted in faeces and the remaining 10 % is excreted as urine (De Silva & Anderson, 1995; Hardy *et al.*, 2011).

The production of ammonia has been proven to be directly linked to protein utilization and is regarded as an indicator of protein utilisation efficiency in cultured fish (Tantikitti *et al.*, 2005; Yang *et al.*, 2002). A fish's ammonia excretion rate is dependent on both the quality and the quantity of the ingested protein. Feed intake rapidly increases ammonia excretion to the water body, primarily due to deamination of amino acids in the diet (Engin & Carter, 2001). The excretion of ammonia reaches maximum concentrations several hours after ingestion of a meal depending on various factors such as feed composition, duration of fasting, feeding rate, meal time and water quality parameters (Agradi *et al.*, 1995; Ballestrazzi *et al.*, 1994). It is, however, worth mentioning that the nitrogen excreted into the water is not always directly linked to the nitrogen levels in diet because it can also be of endogenous origin (Boyd, 2012; NRC, 2011). The problem with ammonia build-up in the environment is that as ammonia concentration in water increases, ammonia excretion from the body decreases leading to increased ammonia levels in the blood. This then leads to the blood becoming alkaline which then affects normal physiological reactions and processes in the body (Boyd, 2012; Ip *et al.*, 2001).

In some fish species, lipid metabolism has a major influence on the overall metabolism of a fish, affecting the excretion of nitrogenous wastes (Chatzifotis *et al.*, 2010). In several fish species, elevated dietary lipid levels have been shown to redirect the use of protein from energy production to growth, thus reducing nitrogenous excretion (Company *et al.*, 1999; Morais *et al.*, 2001; Muzaffar Bazaz & Keshavanath, 1993; Skalli *et al.*, 2004; Vergara *et al.*, 1999). The use of protein for growth instead of energy generation not only reduces feed costs, which is chiefly determined by the protein content, but also reduces undesirable nitrogenous and phosphoric losses (Kaushik & de Oliva Teles, 1985; McGoogan & Gatlin III, 1998; Naylor *et al.*, 2000; Williams & Robinson, 1988). Studying the relationship between nutrient excretion

and dietary lipid level is crucial in order to reduce or minimise nitrogen loss into the environment. Dusky kob (*Argyrosomus japonicus*) have high aquaculture potential; there is, however, little information available on the species' waste excretion and general metabolism as a result of varying dietary lipid levels. This component of the study was aimed at quantifying the combined effect of dietary lipid and protein levels on nitrogenous excretions and oxygen consumption in dusk kob at constant temperature.

4.2 Materials and Methods

4.2.1 Experimental fish

Dusky kob juveniles were sourced from Irvin and Johnson (Pty) Ltd hatchery in Gansbaai for this experiment and maintained at the Sea Point Marine Research Aquarium for 2 months prior to the experiment. Triplicate of 31 fish (58.0 ± 13.2 g, 15.3 ± 1.6 cm) maintained on the experimental diets (Table 2.1) for 6 days, were stocked in a 400 l respirometer. The fish were fed to apparent satiety during the 6 day acclimation period. On day 7 (prior to the experimental run), the fish were starved between 24 and 30 hours, then anaesthetized (0.2 ml/l 2-phenoxyethanol), gently drained of excess water, and individually weighed on a MonoBloc Viper SW15 balance to the nearest 0.1 g. Length was measured on a measuring board to the nearest 1 mm.

4.2.2 Experimental system

The respirometer (Figure 4.1) was linked to a recirculation system and was kept in a recirculation mode during acclimation; the system was switched to a stagnant mode during sampling periods. The water volume was reduced to ~284 l during sampling to ensure an air tight environment. Oxygen was supplied by aeration during acclimation, and pure oxygen during the sampling phase. During acclimation, water temperature, dissolved oxygen, salinity and pH were maintained at 22.3 ± 0.3 °C, 6.6 ± 0.9 mg O_2/l, 33.9 ± 0.5 g/l and 7.80 ± 0.1, respectively. The tank was scrubbed, and all debris siphoned out, a day before measurements were taken. The photoperiod was maintained at 10L:14D, with light period between 7 am and 5 pm local time during acclimation, and 24L:0D during sampling days. Three submersible water pumps were fixed to the walls of the respirometer for adequate mixing of water. Water was replaced at approximately every 12-hour interval to reduce ammonia build-up.

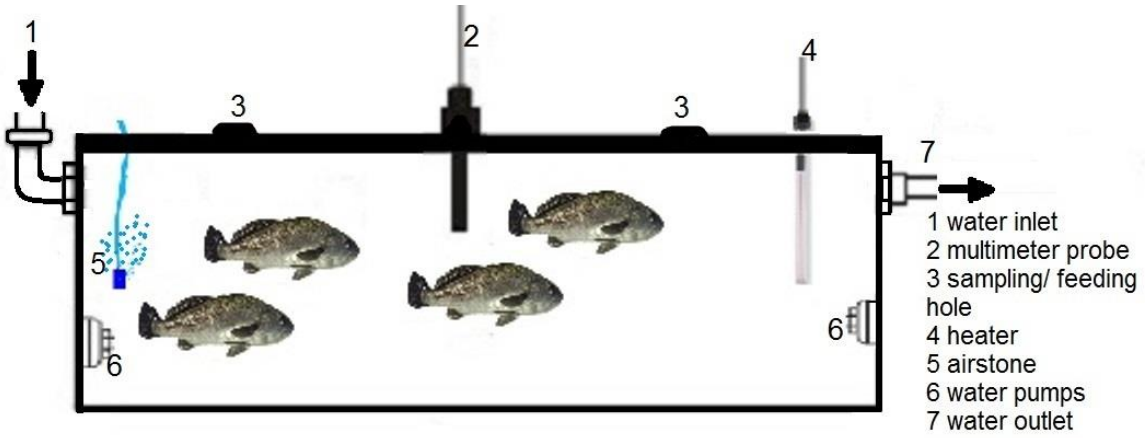

Figure 4.1 An illustration of the sealed respirometer where excretion and metabolic rate experiments were conducted on dusky kob juveniles fed 4 different dietary lipid and protein levels.

4.2.3 Feeds and feeding

Four experimental feeds (4 mm pellet diameter) were formulated to contain between 8 % and 20 % lipid and 45 % protein on a dry matter basis as described in Chapter 2 (Table 2.1). During acclimation (day 1-6), fish were fed three times per day to apparent satiety at 8:30, 12:00 and 16:30, while only one meal fed to satiety was provided at 10:00 during the sampling day (day 8). During the experiment, fish were given an additional 2 hours to settle after switching the respirometer into a stagnant mode before feed was supplied. The fish were fed and allowed to eat for 20 minutes. Thereafter, uneaten feed and faeces were removed by siphoning.

4.2.4 Sampling

Triplicate 20 ml water samples were collected from three different areas in the respirometer - the two feeding holes and the hole where the probe was fitted. The samples were filtered through a Whatman GF/F filter and combined for ammonium and urea measurements at 30-minute intervals for 2 hours prior to feeding. Water was sampled at 15-minute interval during the first hour post feeding, then at 30 minute intervals for the next 2 hours, and thereafter every hour until the end of the sampling duration. At each sampling interval, pH, salinity and temperature was measured with a calibrated Multi 350i multimeter. Oxygen levels were monitored very closely to avoid falling below 4 mg/l. Sampling continued until the fish returned to basal metabolic rate, some 25 to 30 hours after feeding.

4.2.5 Chemical analysis

4.2.5.1 Ammonium and ammonia

Ammonia-nitrogen was determined in triplicate following a spectrophotometric method according to Koroleff (1983). In the spectrophotometric method, ammonia reacts with hypochlorite to form monochloramine in the presence of catalytic amounts of nitroprusside ions and excess hypochlorite to give an indophenol blue colour (Koroleff, 1983).

4.2.5.2 Urea

Urea was determined in triplicate following a spectrophotometric method according to Koroleff (1983). In the spectrophotometric method, urea forms a condensation product with diacetyl monoxine in strongly acidic solutions in the presence of a weak oxidant. This product interacts with semicarbazide and manganous ions to produce a magenta complex (Koroleff, 1983).

4.2.6 Calculations

4.2.6.1 Metabolic rate (MR)

MR (mgO_2/kg/hr) was determined from oxygen consumption in the tank and calculated according to Ai & Xie (2006).

$$MR = \frac{\Delta O_2 \times v}{m \times t}$$

Where: ΔO_2 is the measured change in oxygen concentration (mg O_2/l) between the beginning and end of each test period, v is the volume (l) of water in the respirometer, t is the duration in hours of test period, and m is body mass (kg) of the test fish.

The first 12 hours after feeding were used to calculate the total ammonium and urea excretion emanating from a meal as that is generally the point where most excretion of the two metabolites occurs (Lovell, 1998; NRC, 2011). Additionally, using the first 12 hours avoided the complication of excretion of metabolites from catabolism of endogenous sources as though they were derived from the consumed feed. The percentage of excreted nitrogen was calculated from the excreted metabolites.

4.2.6.2 Nitrogen content in consumed feed (N_f)

The N_f (mg N) was calculated from the analysed content of the feed sample before feeding using basic mathematical calculations as follows:

① $$N_f = \frac{\%N_f \times m_f}{100}$$

Where: $\%N_f$ is the percentage of nitrogen in the whole feed sample, and m_f is the mass of the feed.

② Consumed nitrogen (mg N/kg fish) $= \frac{①}{\text{wet biomass}}$

4.2.6.3 Excreted nitrogen (E_N)

The total excreted nitrogen (mg N/kg fish) was calculated as the integral of the means of excretion rates during the 12-hour period using a mid-point, rectangular integration method (Mahaffy, 2004).

③ $$\int_a^b f(x)dx \approx \sum_{i=1}^{n} f(C_i)\Delta x$$

Where: a and b are the integrated concentration intervals, *n* is the number of concentration sub-intervals, Δx is the concentration of each sub-interval and C_i is the midpoint of each of the intervals.

④ % N excreted $= 100 \times \frac{③}{②}$

4.2.7 Statistical analysis

The data was subjected to a normality test before being analysed for differences using a one-way ANOVA. This was followed by a post-hoc test where significant differences existed and a regression to determine the relationship of the measured variable to the lipid level in diet. A confidence level of 95 % was applied and data considered to be statistically different at $P < 0.05$. Where there were significant differences, the Tukey's test was applied to determine differences between means. Means were sorted according to ascending dietary lipid levels and assigned ascending alphabetical superscripts according to the Tukey's test results. Distance-Weighted Least Squares Fitting was used to plot the excretion rates data from the various treatments during the entire sampling period to eliminate noise between sampling intervals. The relationship between nitrogenous excretion rates and dietary lipid levels, as dietary percentage, was described by a linear regression. All statistical analyses were done using STATISTICA® software, version 7.0. Results are presented as mean ± standard deviation (SD).

4.3 Results

The metabolic rates, measured as oxygen consumption rates, in all treatments increased sharply following feeding, peaking 4-10 hours later and returning to basal levels by 25-30 hours (Figure 4.2). The average metabolic rate of the 8F, 12F and 16F were very similar, but the 20F was consistently lower (Figure 4.3). Post-prandial metabolic rates among the experimental treatments rose by 47.39 – 81.12 % of the basal metabolic rate (Table 4.1). Dietary lipid level did not affect (P > 0.05) basal and maximum metabolic rate. There was no statistically significant trend between dietary lipid level and maximum metabolic rate.

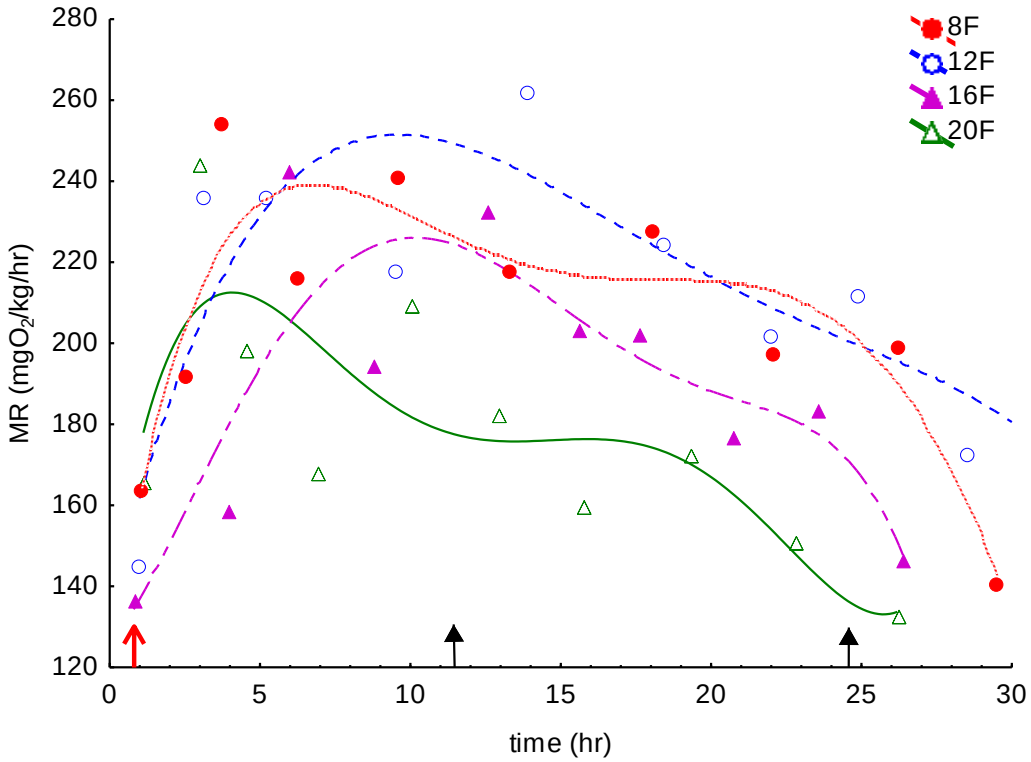

Figure 4.2 Metabolic rates (mgO_2/kg/hr) from dusky kob juveniles fed experimental feeds with various lipid inclusion levels over the sampling period. The lines are Distance-Weighted Least Squares Fitting of metabolic rates from each treatment. The red arrow (↑) indicates feeding time while the black arrows (↑) indicate the point of water exchange. Values are represented as means of three replicates.

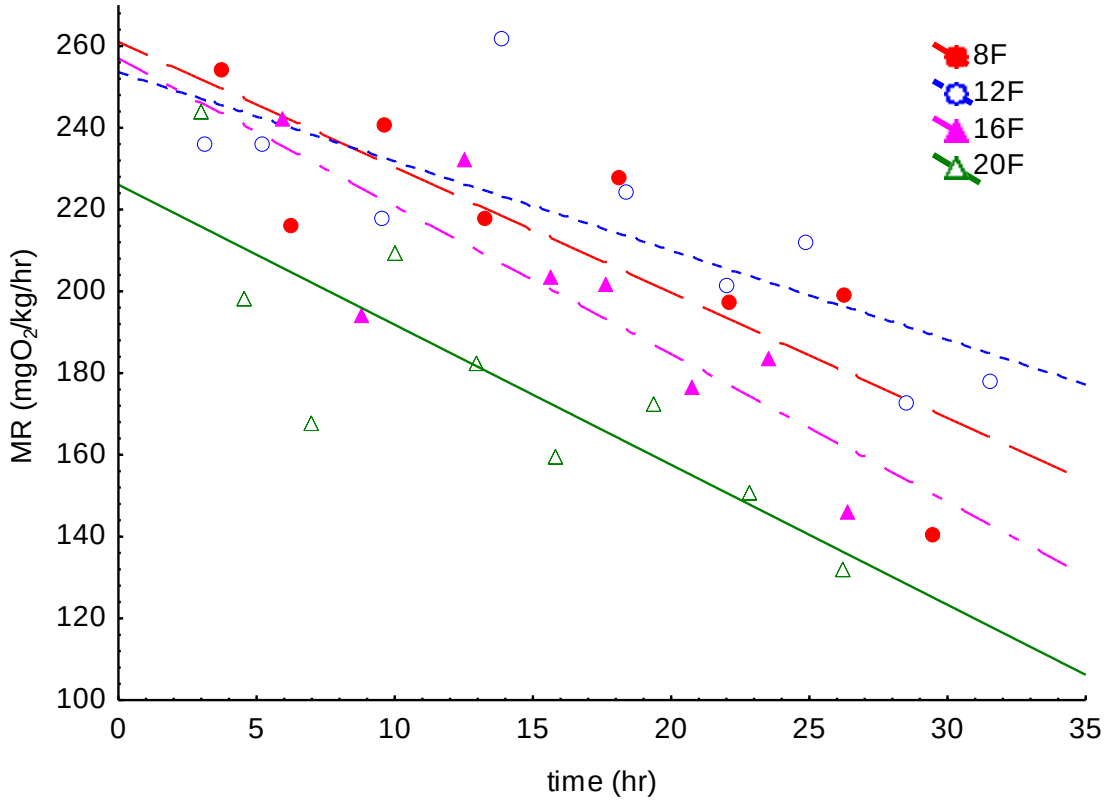

Figure 4.3. The decrease of metabolic rates (mgO_2/kg/hr) from dusky kob juveniles fed experimental feeds with various lipid inclusion levels after reaching the peak. The lines are linear regression of the excretion rates from the 8F ($r = -0.79,\ p < 0.001;\ y = 17.46 - 0.55x$), 12F ($r = -0.90,\ p < 0.001;\ y = 17.59 - 0.46x$), 16F ($r = -0.31,\ p = 0.156;\ y = 8.8 - 0.17x$), and 20F ($r = -0.72, p < 0.001;\ y = 5.17 - 0.16x$) lipid treatments.

Table 4.1. The means (n = 3) of basal and maximum metabolic rates and metabolic increase from dusky kob juveniles fed diets with varying lipid levels. Data are presented as mean ± standard deviation. Means with the same superscript letters within a row are not statistically different.

Rate	8F	12F	16F	20F
Basal metabolic rate (mgO_2/kg/hr)	163.68 ± 12.03[a]	144.74 ± 3.58[a]	158.40 ± 14.56[a]	165.7 ± 12.46[a]
Max metabolic rate (mgO_2/kg/hr)	254.04 ± 5.81[a]	262.16 ± 0.62[a]	242.72 ± 11.72[a]	244.22 ± 29.42[a]
Metabolic increase (%)	55.21 ± 15.05[a]	81.12 ± 4.05[b]	53.26 ± 6.75[a]	47.39 ± 6.72[a]

The NH_4-N concentration increased in all treatments, the peak being reached 5 hours after the start of the experiment or 3 hours after feeding (Figure 4.4). As with oxygen consumption, the peak of NH_4-N excretion rates were higher in the 8F and 12F treatments and decreased with increasing lipid levels, with the 20F diet registering the lowest overall excretion rates. The reduction pattern of NH_4-N excretion rates was comparable among the different treatments excluding the 16F treatment. The linear decrease in the NH_4-N excretion rates in the four treatments was comparable (Figure 4.5). The pattern of the 8F and 12F treatments were similar based on their slopes.

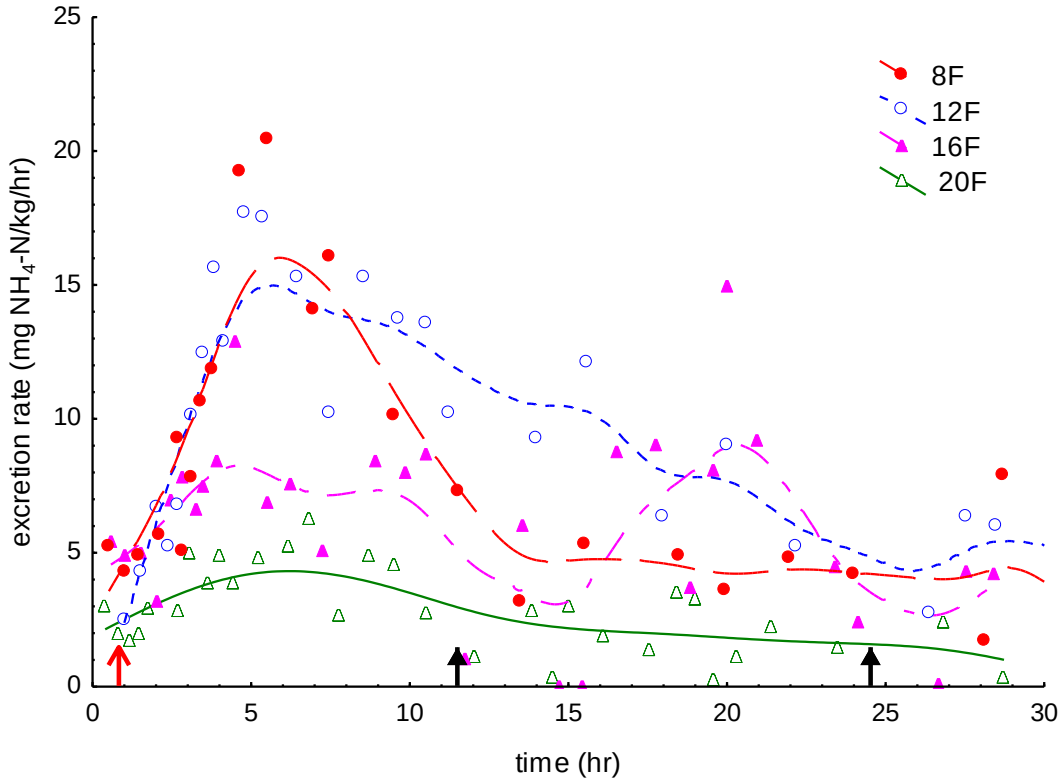

Figure 4.4 Ammonium-nitrogen excretion rates (mg NH_4-N/kg/hr) from dusky kob juveniles fed experimental feeds with various lipid inclusion levels over the sampling period. The lines are Distance-Weighted Least Squares Fitting of ammonium-nitrogen excretion rates from each treatment. The red arrow (↑) indicates feeding time while the black arrows (↟) indicate the point of water exchange. Values are represented as means of three replicates.

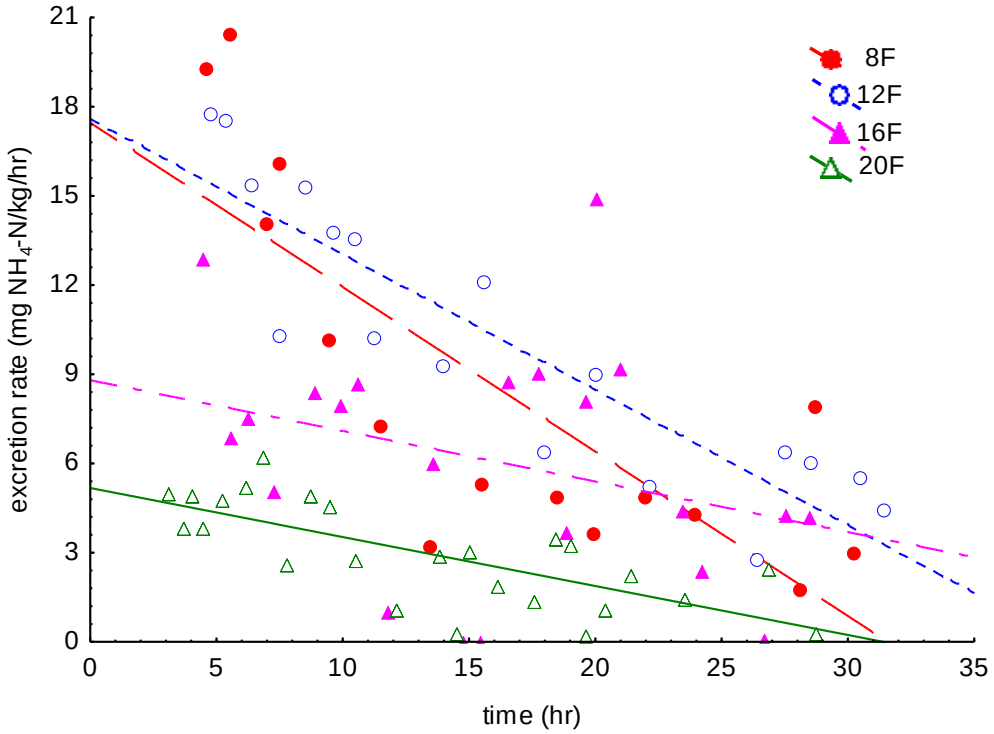

Figure 4.5 The decrease of ammonium-nitrogen excretion rates (mg NH_4-N/kg/hr) from dusky kob juveniles fed experimental feeds with various lipid inclusion levels after reaching the peak. The lines are linear regression of the excretion rates from the 8F ($r = -0.79,\ p < 0.001;\ y = 17.46 - 0.55x$), 12F ($r = -0.90,\ p < 0.001;\ y = 17.59 - 0.46x$), 16F ($r = -0.31,\ p = 0.156;\ y = 8.8 - 0.17x$), and 20F ($r = -0.72, p < 0.001;\ y = 5.17 - 0.16x$) lipid treatments.

There was a comparable pattern in the increase of urea excretion rates among treatments (Figure 4.6). The urea excretion rates showed marked variation throughout the sampling intervals and between treatments. The 8F and 20F treatments reached their peak of urea excretion rates between 3 and 6 hours after feeding, and decreased gradually. The data from the 12F and 16F treatments was too variable to discern clear peaks, however, the square fitting shows two urea excretion peaks in the 12F and 16F treatments. In general, the urea excretion data was too variable to discern differences and trends. The change in pH within the respirometer followed a variable pattern, increasing shortly after feeding then decreasing until stabilising 8 hours after feeding (Figure 4.7). In all treatments the pattern for pH change was similar.

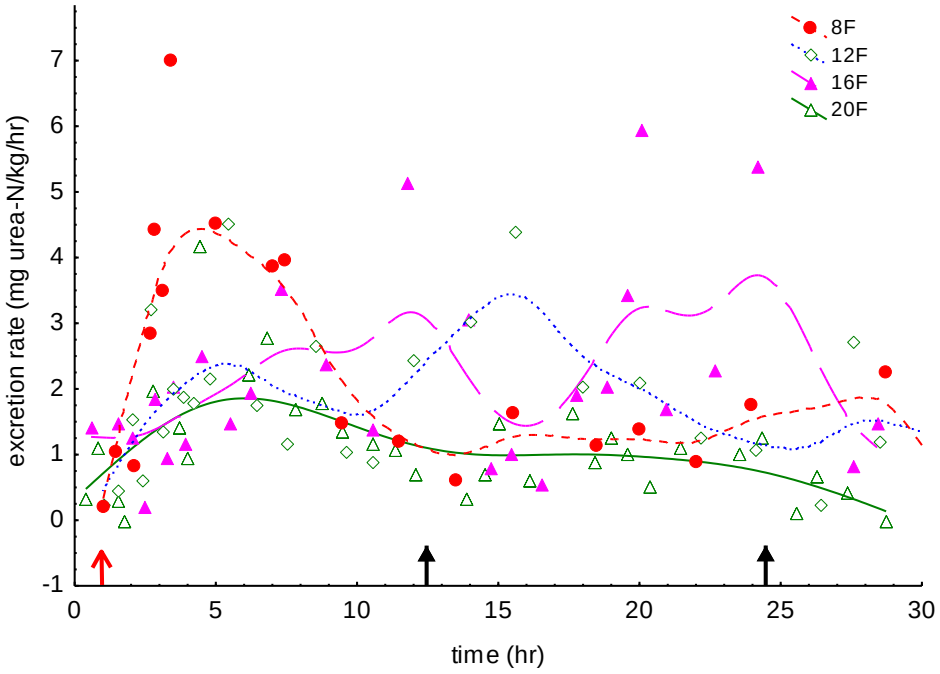

Figure 4.6 Urea excretion rates from dusky kob juveniles fed diets with varying lipid levels over the sampling period in a respirometer. The lines are Distance-Weighted Least Squares Fitting of urea-nitrogen excretion rates from each treatment. The red arrow (↑) indicates feeding time while the black arrows (↑) indicate the point of water exchange. Each point represents a mean of 3 replicates.

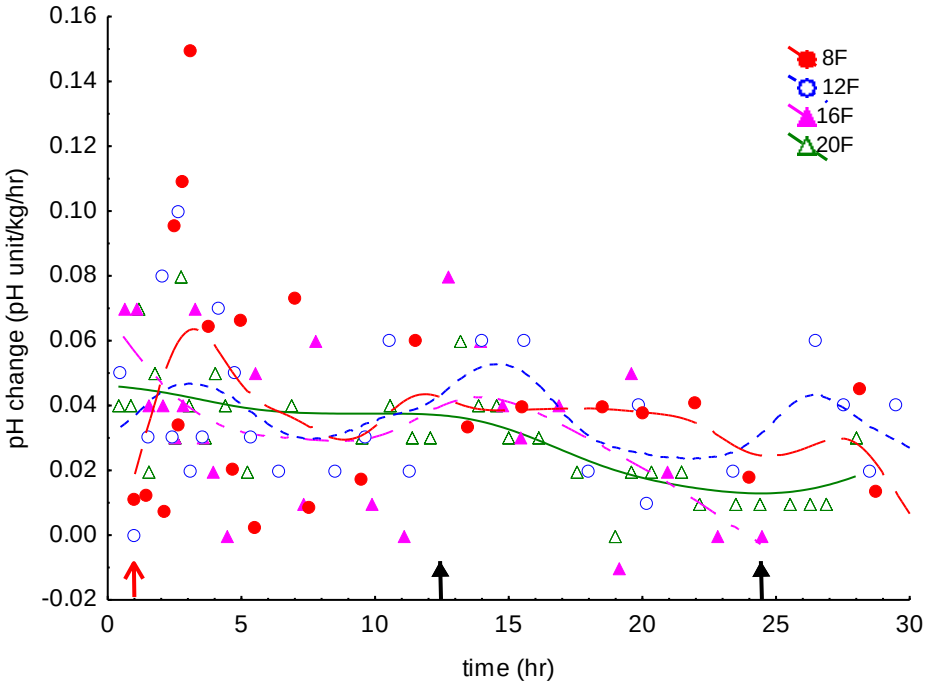

Figure 4.7 Rate of pH change from dusky kob juveniles fed diets with varying lipid levels over the sampling period in a respirometer. The lines are Distance-Weighted Least Squares Fitting of pH change rate from each treatment. The red arrow (↑) indicates feeding time while the black arrows (↑) indicate the point of water exchange.

Feed consumption among the treatments was significantly higher (P = 0.001) in the 12F and 16F (Table 4.2). There was no correlation between the consumed nitrogen and dietary lipid levels (r = –0.27, P = 0.072). Fish in the 12F treatment consumed a significantly higher (P < 0.001) amount of nitrogen while the 20F treatment consumed the lowest. Ammonium-nitrogen accounted for between 69.1–86.4 % of the measured nitrogenous excretion rates among the treatments, while urea-nitrogen excretion accounted for between 13.6–31.1 %. Dietary lipid levels affected the total percentage of excreted ammonium (P = 0.043) and urea (P = 0.009). The excreted nitrogen as a percentage of consumed nitrogen was high in the 8F treatment.

Table 4.2 The means (n = 3) of consumed feed and energy, total nitrogenous consumption, total excretion of ammonium, urea and nitrogen over 12 hours for dusky kob juveniles fed diets with varying lipid and protein levels. Data are presented as mean ± standard deviation. Means with the same superscript letters within a row are not statistically different. The absence of superscripts for the total excreted urea values indicate no significant difference in Tukey's test, although means of groups were statistically different $F_{(3, 7)}$ = 5.21, P = 0.033.

	8F	12F	16F	20F
Gross Consumed Feed (g)	15.74 ±1.76^{ab}	27.75 ± 2.86^{c}	23.49 ± 2.75^{bc}	12.22 ± 2.98^{a}
Feed consumption (g/kg fish)	8.86 ± 0.99^{a}	12.92±1.33^{b}	8.82±1.03^{a}	4.49 ±1.10^{c}
Energy consumed (kJ/kg fish)	168.08 ± 18.75^{a}	254.11 ± 26.19^{b}	179.73 ± 21.04^{a}	94.72 ± 23.10^{c}
Energy (kJ/g)	18.97	19.67	20.37	21.08
Consumed nitrogen C_N (mg N/kg fish)	1157.36 ± 129.17^{ab}	2042.40 ± 210.50^{c}	1729.11 ± 202.38^{bc}	899.39 ± 219.33^{a}
Total excreted ammonium (mg N/kg fish)	129.47 ± 3.24^{a}	127.02 ± 4.74^{a}	88.04 ± 6.24^{b}	43.15 ± 7.24^{c}
Total excreted urea (mg N/kg fish)	32.90 ± 4.67	20.04 ± 3.77	28.99 ± 4.27	19.27 ± 4.77
Total excreted nitrogen, E_N (mg N/kg fish)	162.35 ± 10.32^{a}	147.06 ± 12.38^{a}	117.03 ± 9.71^{b}	62.43 ± 7.93^{c}
Ammonium N (%E_N)	79.80 ± 1.89^{ab}	86.37 ± 1.78^{b}	75.23 ± 4.07^{ab}	69.13 ± 8.88^{a}
Urea N (%E_N)	20.20 ± 1.89^{ab}	13.56 ±1.78^{b}	24.82 ±4.07^{ab}	31.11 ± 8.88^{a}
Excreted nitrogen (% C_N)	12.66 ± 1.31^{a}	6.90 ± 1.31^{a}	6.86 ± 1.42^{a}	7.03 ± 2.82^{a}

4.4 Discussion

The postprandial metabolic rates in dusky kob were not affected by the consumption of different dietary lipid and protein levels. The excreted ammonium-nitrogen peaked between 3-5 hours after feeding and was affected by the dietary lipid and protein levels. The urea excretion rates and pH change were too variable to discern clear trends and showed no relationship with dietary lipid levels. Ammonium-nitrogen accounted for 69 and 86 % of excreted nitrogen, while urea contributed between 14 and 31 % of measured nitrogenous excretions.

In most reported studies on fish, the maximum postprandial metabolic rate under optimum environmental conditions reached almost double the basal metabolic rate before gradually returning to basal rates (Jobling, 1981a; Lovell, 1998). The results of this study showed a comparable increase in post-prandial metabolic rate to that observed in other species such as southern catfish (Fu *et al.*, 2005) and sockeye salmon (Brett & Zala, 1975). All diets produced comparable metabolic rate peaks in the experimental fish ranging between 243 and 262 mgO_2/kg/hr, with higher lipid levels yielding the lowest maximum metabolic rates. This oxygen consumption can be primarily attributed to differences in digestion and absorption of nutrients, formation and excretion of metabolic wastes and assimilation of absorbed nutrients. A similar trend was reported in sea bass (Peres & Oliva-Teles, 2001) where an increase in lipid levels resulted in reduced oxygen consumption. When high dietary fat is not properly assimilated, it can trigger a negative feedback mechanism that can lead to suppressed metabolism (Kause *et al.*, 2009; NRC, 2011). Contrasting studies on rainbow trout and plaice, however, found the metabolic rate to be independent of the dietary lipid level (LeGrow & Beamish, 1986), and fully dependent on the ration size in rainbow trout (Jobling, 1981a) and plaice (Jobling & Davies, 1980). The ration argument may partially explain the low metabolic rate observed in the 20F treatment where food consumption was markedly lower than the other diets. A higher ration will generally require a longer digestion period, thus resulting in extended elevated metabolic rates (Beamish, 1974; Jobling & Davies, 1980). This was probably why, for the treatments where food intake was higher, the metabolic rates took longer to return to basal metabolism. This confirmed the finding that feeding rate has an impact on the duration it take for fish to return to basal excretion rates (James *et al.*, 1989; Jobling, 1981b).

In dusky kob, as in most fish, the rate of excretion of nitrogenous wastes such as ammonia is determined by the rate of catabolism of nitrogenous nutrients and the concentration gradient

between the water and the blood (NRC, 2011). The ammonium excretion rate observed in this study was similar to other studies as there was a rapid increase in nitrogenous excretion rates, which slowly returned to basal excretion levels over several hours. Ammonium excretion peaked in all treatments between 3 and 5 hours after feeding, which is in agreement with the findings for Japanese flounder (Kikuchi, 1995), sea bass (Almendras, 1994), Cape anchovy (James *et al.*, 1989), greenback flounder (Verbeeten *et al.*, 1999) and juvenile sea bream (Dosdat *et al.*, 1996). The 8F, 12F and 16F treatments peaked 3 hours after feeding, which is slightly before the 4–8 hours which is reported in species such as plaice (Jobling, 1981b), marble goby (Lam *et al.*, 2008), sea bass (Ballestrazzi *et al.*, 1994), sockeye salmon (Brett & Zala, 1975) and Australian short-finned eel (Engin & Carter, 2001). These differences may have arisen from the size and species tested, water temperature, feeding times, feed quality, environmental and physiological parameters. The highest excretion rate observed in the 8F and 12F diets is likely to have been influenced by the protein-energy levels in the two test feeds rather than just the dietary lipid level. This argument is consistent with other studies that have shown a positive correlation between protein content in diet and excreted nitrogen content in the water (Boyd, 2012; Engin & Carter, 2001). Despite the variation in the magnitude of peak ammonium excretion rate, the patterns were similar among the different treatments.

The ammonium production from this study, as measured by total ammonium excretion in the respirometer, was greatly reduced in fish fed the higher lipid levels. Similar results where a comparable increase in dietary lipid content resulted in reduced nitrogenous excretion were obtained in red drum (McGoogan & Gatlin III, 2000), European eels (Agradi *et al.*, 1995), lake trout (Jayaram & Beamish, 1992) and European sea bass (Boujard *et al.*, 2004). Such findings may have been the result of a protein-sparing mechanism whereby metabolic energy is supplied more from fat than protein catabolism (Company *et al.*, 1999). Another factor is that feed consumption was suppressed at the 20F lipid diet; hence a lower nitrogenous excretion in line with reduced nitrogen consumption. A study in tilapia asserted that, during physical activity, the polyunsaturated fat *n*-3 acts on the processes that regulate ammonia excretion by inhibiting the clearance of ammonia rather than regulating ammonia production – thus reducing production of nitrogenous waste (Agradi *et al.*, 1995). The results of this study suggest that higher dietary lipid levels reduce nitrogenous loss into the water. Minimising nitrogenous pollution is among the most important factors that feed producers seek to achieve, however, it does need to be balanced with good nutrient utilisation to maintain a good welfare and production status of the cultured fish.

The peak of urea synthesis and excretion in most teleost is regulated by environmental and hormonal factors. Urea excretion increased across all treatments in a variable manner and

peaked between 3 and 5 hours after feeding, but the 16F treatment peaked 10 hours after feeding, then decreased gradually. Similarly, urea excretion rates peaked between 4 and 8 hours after feeding in lake trout (Jayaram & Beamish, 1992), dusky kob (Dobberstein, 2006), Australian short-finned eel (Engin & Carter, 2001) sea bream, turbot, brown trout and rainbow trout (Dosdat *et al.*, 1996). In sockeye salmon (Brett & Zala, 1975) and red drum (McGoogan & Gatlin III, 1999), urea excretion showed no diurnal pattern, feeding or dietary lipid response. Plasma urea levels in Atlantic salmon were similarly found to be too variable after feeding and it was concluded that urea levels may not be confidently used for diagnostic or nutritional studies (Knoph & Måsøval, 1996). In goldfish, increased urea production and excretion is a coping mechanism to elevated ambient ammonia after feeding and is dependent on the environmental ammonia levels (Olson & Fromm, 1971). The variable urea excretion data of this study makes it difficult to compare urea excretion rates with ambient ammonia concentration. In general, urea excretion in the present study peaked in a similar pattern between 3-5 hours after feeding in all dietary lipid and protein treatments, suggesting that the peak of urea excretion in dusky kob is not affected by dietary lipid or protein levels.

After 10 hours in this study, urea excretion rates showed no pattern and lacked any relationship to consumed nitrogen or lipid levels, suggesting that urea excretion in dusky kob is independent of feed composition. Similar results were obtained in studies on juvenile dusky kob (Dobberstein, 2006) and Cape anchovy (James *et al.*, 1989), where diet composition and feeding rate showed minimal or negligible relation to urea excretion. Other studies have shown that urea excretion was similarly not attributed to diet composition, but was affected by feeding rates (Harris & Probyn, 1996; Kikuchi, 1995; Verbeeten *et al.*, 1999). The urea excretion in this study contrasts with studies where the excretion of urea-nitrogen was high in fish fed a high-lipid diet (Jayaram & Beamish, 1992) and high protein diet (Engin & Carter, 2001). Cortisol was reported to regulate urea production in sea raven because plasma cortisol levels were positively correlated with plasma urea, but not ammonia (Vijayan *et al.*, 1996). As cortisol levels are triggered by environmental stressors in some teleost such as in oyster toadfish, Lake Magadi tilapia and rainbow trout (Walsh, 1997), cortisol may be involved in the variable urea excretion rates in dusky kob after 10 hours of the experiment.

The contribution of urea-nitrogen in the total excreted nitrogen in water accounted for between 13.6–31.1 % of total nitrogen excretion among the treatments, which falls within the values that have been obtained for numerous teleost species. Although the ranges vary among the different studies, comparable values have been recorded such as 15–28 % in dusky kob (Dobberstein, 2006), 13–21 % in marble goby (Lam *et al.*, 2008), 2–28 % in plunderfish (Boyce, 1999), 18–22 % in turbot (Dosdat *et al.*, 1995), 14 % in rainbow trout (Dosdat *et al.*, 1996) and

10–20 % in sea bass (Dosdat *et al.*, 1996). In these studies, the relative contribution of urea to total nitrogen excretion was found to be linked to temperature and food intake. The relative contribution of urea to total nitrogen excretion was mostly due to the changes in ammonia excretion, resulting directly from feed intake, rather than to changes in urea excretion. The excretion rates recorded during the initial 12-hours can be attributed to feed intake, thereafter, there is no explanation for the variable excretion rates. Although the present results agree well with the majority of the published data on urea excretion, some studies have found a larger contribution of urea-nitrogen to total excreted nitrogen in species such as in short-finned eel where values ranged between 30 and 50 % (Engin & Carter, 2001) and in greenback flounder 58–63 % (Verbeeten *et al.*, 1999).

4.5 Conclusions

Dietary lipid levels in this study did not affect the post-prandial metabolic rates of dusky kob. The findings suggest that higher dietary lipid levels (20F) reduced the amount of nitrogenous metabolites excreted to the environment. The pattern of nitrogenous excretion was comparable to those observed in other studies, although, excretion in the dusky kob from this study peaked between 3–5 hours postprandial. Ammonium contributed the major (69-86 %) share of total excreted nitrogen with urea making up a smaller (14-31 %) but significant portion. Although increasing lipid levels in dusky kob diets have shown beneficial attributes of limiting nutrient loss into the environment, there is a need to balance growth performance and environmental pollution when selecting a specific feed composition.

CHAPTER 5

CONCLUDING DISCUSSION

The culture of dusky kob in South Africa is fairly new, and the availability of literature on formulated feeds for this species is limited. As a consequence, this study assessed the impact of lipid levels in dusky kob aquafeeds on feed conversion efficiency, carcass proximal and fatty acid composition, meat quality changes during storage and nutrient excretion rates. The aim was to contribute to the information on the nutritional requirements of dusky kob, particularly in light of the limited available knowledge. The relevance of this study, particularly, in contributing towards defining best feeds for rearing dusky kob, is highlighted. This section highlights the extent to which the mentioned objectives were met, significance of results, limitations of the study and proposes future research directions that were identified during this study. Furthermore, it lays a methodological foundation for future work.

Effect of lipids on feed conversion efficiency, carcass composition and fatty acid composition

Chapter 2 investigated the effect of varying dietary lipid and protein levels in aquafeeds fed to dusky kob on feed conversion efficiency, proximal carcass composition and fatty acid composition under experimental conditions. Growth rates were similar for the 8F, 12F and 16F diets, but was significantly lower for the 20F diet. Feed conversion ratios were within the ranges reported for other sciaenids, however, statistically poorer in the higher lipid treatments (16F and 20F). PER was significantly better for the low lipid diet indicating the absence of a protein-sparing effect. LER was significantly better in the low lipid diet indicating better use of dietary lipids. The decline in PER and LER was reflected in poorer feed conversion ratios in the higher lipid diets.

The lipid levels in tissue closely reflected the dietary lipid levels. Increasing dietary lipid levels improved the tissue concentration of polyunsaturated fatty acids, particularly, eicosapentaenoic acid and docosahexaenoic acid but decreased the concentration of monounsaturated fatty acids. There is no clear explanation for the high levels of docosapentaenoic acid values in this study as lower figures have been reported in other sciaenids (Fernandes, 2013; Giogios *et al*., 2013; Piccolo *et al*., 2008; Poli *et al*., 2003). The overall implication was that increasing dietary lipid levels improved the n-3/n-6 ratio in muscle tissue, which is desirable (healthier) from a product characteristic point of view as consumers perceive that a higher n3:n6 ratio is healthier.

Biochemical changes during storage

The available information indicates that the quality changes of chilled dusky kob, particularly in relation to feeds, have never been quantified even though being an aquaculture species. This study presents the first investigation into quality changes in refrigerated dusky kob. Despite the identified gaps in this study, there are some useful preliminary conclusions that can be drawn from the results. This study has demonstrated that increasing the lipid levels in dusky kob diets had a negative impact on storage life of fish fillets. Refrigerated dusky kob fillets can have up to 12 days of storage if fed dietary lipids. Further studies need to be done to assess the impact of various storage temperatures, preferably lower than those used in this study, on the biochemical and sensory changes of stored dusky kob fillets. It will also be worth quantifying the impact of different handling and slaughter methods on quality changes as it is well known that handling and storage conditions influence the storage life of a fish. A larger scale study may need to be done to determine how the results of this study can apply to commercial conditions wherein slaughter and handling of fish will invariably influence the quality.

Nutrient excretion

This study presents the first investigation to quantify the effect of dietary lipid and protein levels on metabolic and nitrogenous excretion rates in dusky kob. This study showed that the metabolic rates of the juvenile dusky kob peaked between 47 and 81 % of basal metabolic rate. The peak was lowest in the higher dietary lipid treatments. The post-prandial metabolic rates and NH_4-N excretion were highest in the lower dietary lipid treatments. These results are consistent with other studies suggesting that higher dietary protein-energy ratio demand more energy for metabolism. Similarly, the findings demonstrated that higher lipid levels reduced feed consumption, resulting in lower metabolic rate. There was no clear relationship between dietary lipid levels and urea. Both NH_4-N and urea excretion rates peaked between 3-5 hours after the first meal and started returning to basal excretion rates 13 hours after feeding. The results from this study lay a foundation for future studies into nitrogenous excretion in partial recirculation aquaculture systems used in South Africa. Further research needs to be conducted with controlled diets to establish an optimal P:E ratio where diets are formulated to minimise nitrogenous excretions but maximum growth.

Overall, the results of this study show that an increase in lipid levels in the diet of dusky kob of up to 16 % presents benefits such as lower nitrogen excretion and higher tissue concentration

of polyunsaturated fatty acids. However, there are drawbacks to higher dietary lipid levels (16-19 %). These include lower growth rates and shorter storage life. More research is needed within the 8-16 % dietary lipid range. While the three experiments provide preliminary data and insights into the effect of dietary lipid levels on proximate and fatty acid composition, nutrient assimilation efficiency, meat quality changes during storage and nutrient excretion, the methodologies proved to be viable tools that lay a foundation for future studies. The results from this study are useful for practical feed formulations for use in partial recirculation aquaculture systems, and pave the way for further studies into storage life for dusky kob and the effect of different storage temperatures on quality change.

CHAPTER 6

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