

THE EFFECT OF DIETARY FISH OIL REPLACEMENT WITH  
SOYBEAN OIL ON GROWTH AND HEALTH OF DUSKY KOB,  
*ARGYRO SOMUS JAPONICUS* (PISCES: SCIAENIDAE)

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By

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“With Earth’s burgeoning population to feed  
we must turn to the sea with new understanding and new technology.  
We need to farm it as we farm the land”  
Jacques Cousteau, 1973

## ABSTRACT

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Lipids are essential components for fish because they contain fatty acids that are vital for regular growth and health. Fish oil is rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are essential fatty acids for carnivorous fish, and therefore this product has traditionally been used as the main source of lipids in fish feeds. However, with declining fisheries resources worldwide and the rapid expansion of the aquaculture industry pressuring this finite resource, such ingredients are becoming less available and more expensive. It is therefore necessary to explore the utilization of ingredients that are sustainable and competitive alternatives to fish oil in marine finfish feeds. This work investigated the effects of the substitution of fish oil with soybean oil on the growth performance, feed efficiency, fatty acid composition of the liver tissue and some health parameters in juvenile dusky kob, *Argyrosomus japonicus*; an increasingly popular sciaenid marine aquaculture species in South Africa. Six diets (18 % total lipid and 46 % protein) with increasing percentage substitution of fish oil with soybean oil (1, 14, 28, 42, 56 and 70 %) were fed to juvenile kob. After 84 days of feeding these diets to the fish, no significant differences in fish length and weight between treatments were observed. However, there was a significant trend of a decrease in specific growth rate, ranging from ( $\pm$  standard error)  $0.87 \pm 0.06$  to  $0.72 \pm 0.04$  % body weight day<sup>-1</sup>, and condition factor, ranging from  $1.59 \pm 0.03$  to  $1.54 \pm 0.02$ , with increasing vegetable oil replacement in the diets between days 56 and 84. There were no differences in red blood cell count, haematocrit and haemoglobin concentration after 206 days of feeding. However, visceral fat index (VFI) increased significantly from  $1.08 \pm 0.17$  % for fish fed diets with 28 % soybean oil, to  $2.24 \pm 0.15$  % for fish fed diets with 70 % soybean oil. Similarly, hepatosomatic index (HSI) increased significantly from  $0.84 \pm 0.08$  % to  $1.80 \pm 0.12$  % in the control diet and the 56 % soybean oil diet, respectively. After 206

days of feeding, fish fed diets with 42 to 70 % soybean oil showed greater number of lipid vacuoles in the liver, which were also larger in size, and hepatocytes nuclei were displaced to the cell periphery. The fatty acid composition of the liver tissue strongly corresponded to the fatty acid composition of the diets. Linoleic acid accumulated in the liver of the fish fed increasing soybean oil in the diets. In contrast, EPA and DHA decreased from 13.63 to 1.97 %, and 14.34 to 3.28 %, respectively, in the liver tissue of fish fed diets with increasing soybean oil content; consequently the n-3/n-6 ratio was also significantly reduced with inclusion of vegetable oil in the diets. The trend of decreasing growth rate with increasing oil replacement towards the end of the trial corresponds with increases in VFI, HSI, as well as the fatty acid accumulation and lipid vacuoles in the liver. This suggests that dusky kob is less able to metabolise soybean oil at increased substitution levels which would account for the poorer growth at higher levels. The dependence of fish on dietary marine oil decreased significantly with each inclusion of soybean oil in the diets. Nonetheless, the calculations based on the nutrient ratio presented positive outcomes for all treatments, that is, values of marine oil dependency ratio were below one for all treatments. It is concluded that soybean oil can replace fish oil in formulated diets for dusky kob up to a level of 28 % of total dietary lipids, as evidenced by the good growth and feed efficiency, and no apparent negative health effects observed up to this level.

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## CHAPTER 1

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### GENERAL INTRODUCTION

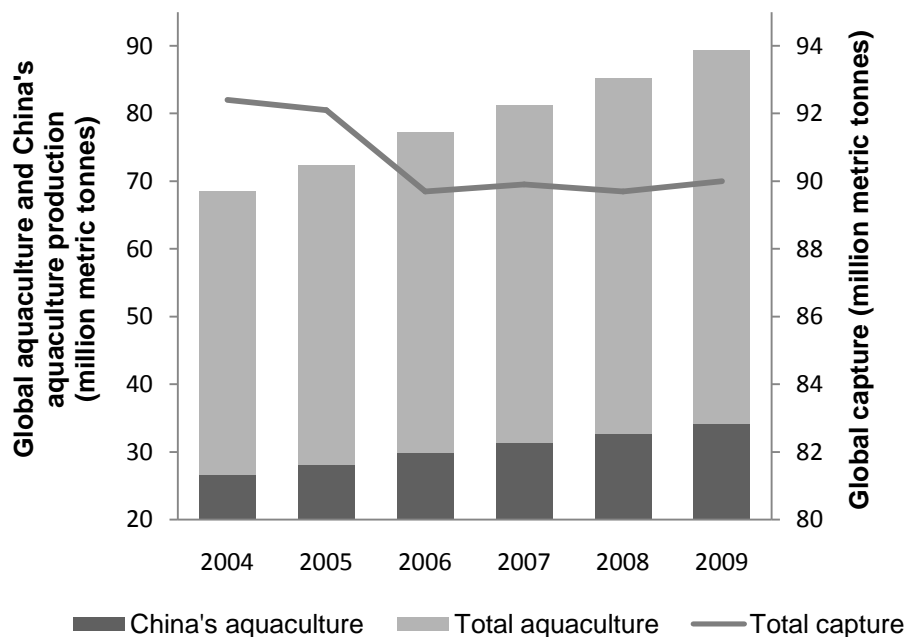
As the aquaculture industry continues to grow, so does the requirement for fish oil. Fish oil is used as the main lipid source in fish feeds (Turchini *et al.*, 2009), especially for marine carnivorous species due to the content of n-3 HUFA (highly unsaturated fatty acids), which is considered essential for these species (Izquierdo *et al.*, 2003). However, the supplies of this product are finite and the production of fish oil may not be enough to compensate the demand for animal feed (Tacon *et al.*, 2006). As a consequence, there has recently been an increasing interest in the investigation of sustainable alternative lipid sources. Oils of plant origin are considered a good option (Turchini *et al.*, 2009). The substantial increase in availability of plant oils combined with their traditionally lower prices makes them suitable alternatives to fish oil. Nonetheless, the challenge is to maintain a high-quality healthy fish when fatty acids are altered in their diets. This work assesses the effects of fish oil substitution by a vegetable oil on growth, feed efficiency and health of *Argyrosomus japonicus*, a promising species for culture in South Africa (Hecht *et al.*, 2006).

#### **Aquaculture in the world**

Fishing activities have been a primary source of protein for humanity since the very beginnings of civilization. However, at the present time, there are concerns about the sustainability of this activity. Twenty five percent of the global fisheries were overexploited or depleted and 52 % had been operated at maximum sustainable production limit in 2004 (FAO, 2005). The demand for seafood continues to grow worldwide due to increasing population and the requirement for high-quality protein

as well as health benefits attributed to fish. Global fish consumption per capita increased from five kg year<sup>-1</sup> in 1961 to 25 kg year<sup>-1</sup> in 2001 (Hashim, 2006), and a large part of this demand has to be supplied by aquaculture.

Aquaculture is currently the fastest growing food sector in the world, with more than 220 species of finfish and shellfish being farmed (Naylor *et al.*, 2000). Total worldwide aquaculture yield increased from 41.9 million tonnes in 2004, to 55.1 million tonnes in 2009 (Figure 1.1; FAO, 2010b). In 1970, the contribution of aquaculture to total fisheries worldwide, excluding aquatic plants, accounted for 3.9 % (FAO, 2002a). By 2009 that proportion had increased almost tenfold, to about 38 % (FAO, 2010b) and continues to grow. This rapid increase can be explained mainly by China's participation in world aquaculture (FAO, 2010b). Nonetheless, even excluding China's production, aquaculture is still increasing (Figure 1.1; FAO, 2010b).



**Figure 1.1.** Global aquaculture production, China's aquaculture production, and global capture (excluding aquatic plants), in million metric tonnes, from 2004 to 2009. Data obtained from FAO (2010b).

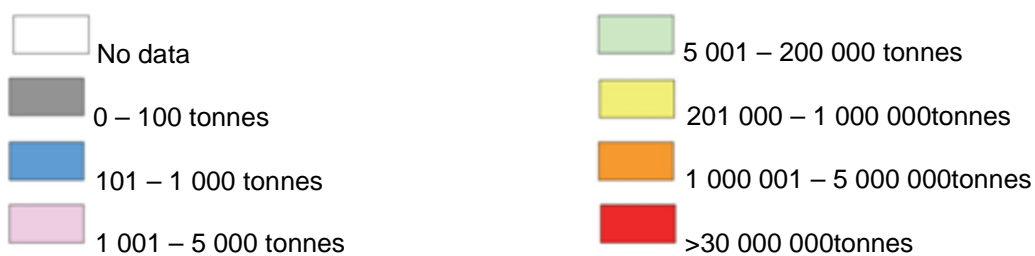
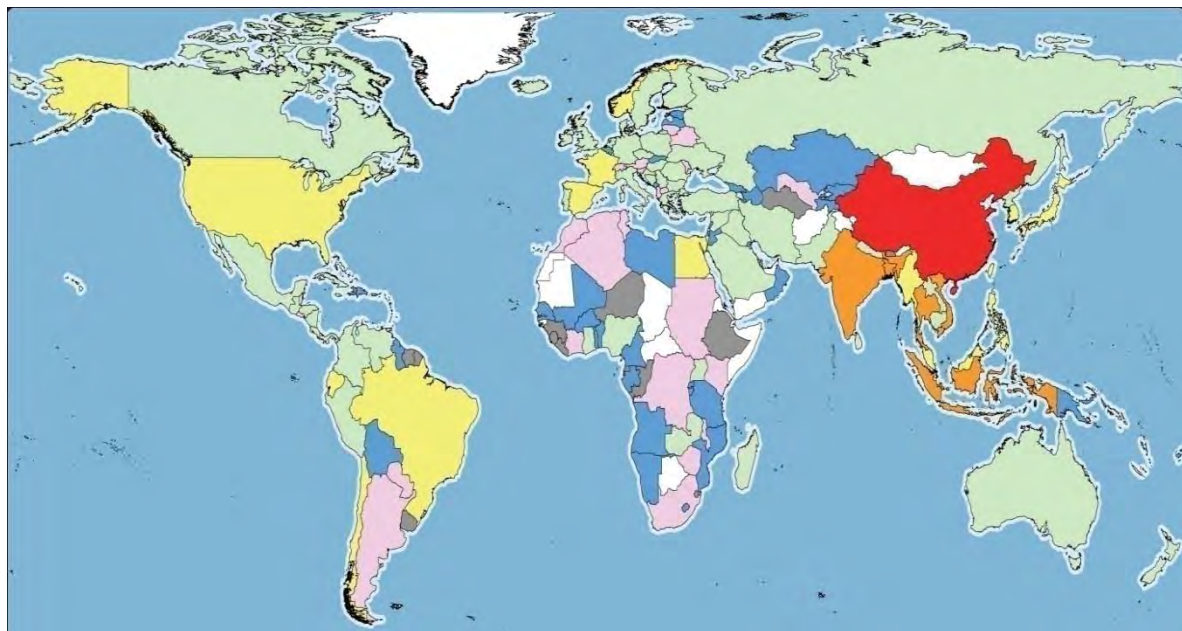
Fishmeal and fish oil are important feed ingredients in aquaculture. Of the 142 million tonnes from the total fisheries supply in 2008, around 20.8 million tonnes was used for fish meal and fish oil production (FAO, 2010b). Only 115 million tonnes was used for direct human consumption as food (FAO, 2010b), and the remaining was used for other non-food purposes, such as bait, pharmaceutical, as well as for direct feeding in aquaculture and terrestrial animals (Hertrampf & Piedad-Pascual, 2000; FAO, 2010b). Total worldwide compound aquafeed production in 2003 was approximately 19.5 million tonnes (Hardy, 2006). By 2006, global aquafeed production was between 20.2 and 22.7 million tonnes, from a mere 36 reporting countries (Tacon & Metian, 2008).

Aquafeeds for fish bred in captivity depends mainly on fish oil, which is extracted from the waste, or fish with low or no economic value for human consumption such as herring, sardine, anchovy, capelin, among others (Hertrampf & Piedad-Pascual, 2000; Péron *et al.*, 2010). The content of long chain n-3 fatty acids in these species can be up to 35 % of the total body fat (Pike & Jackson, 2010), and they generally store the oil in their bodies instead of the livers (Pike & Jackson, 2010). Between 20 and 30 % of the fish landings are pelagic fish (FCRR, 2006). As a result of the high demand for these species, the prices increase, consequently increasing the price of aquafeeds. Furthermore, these are important species on food-webs, hence, overfishing could cause great imbalance in ecosystems (Venegas-Calación *et al.*, 2010).

### **Aquaculture and aquafeeds in South Africa**

In South Africa aquaculture was implemented more than one hundred years ago with the introduction of trout for sport angling purposes (Safriel & Bruton, 1984). The only viable aquaculture industries in the mid 1980's were rainbow trout, *Salmo gairdneri*, oysters, *Crassostrea gigas*, waterblommetjies, *Aponogeton distachyos* and ornamental fish, mainly goldfish, *Canassius auratus* (Safriel & Bruton, 1984). Since then there has been an aquaculture explosion. The contribution to global aquaculture in Africa, especially in north Africa, has increased from 0.005 % in 1995 to 1.19 % in

2004, a growth of 334 % (FAO, 2006; Hecht & Jones, 2009). Moreover, it is expected that the sub-Saharan Africa will produce a total of approximately 200 000 to 380 000 tonnes per annum of fish by the year 2013 (Hecht *et al.*, 2006). Despite this, the current participation of South Africa in global aquaculture sector is small, producing about 3 433 tonnes (Figure 1.2; FAO, 2011). This industry is still fairly limited to few species of mussels, oysters, abalone, and prawns (Griffiths *et al.*, 2008). However, there are exciting prospects for the aquaculture development (DWAF, 2005). In particular, mariculture is expected to increase substantially with the introduction of finfish culture in South Africa (FAO, 2010a).



**Figure 1.2.** Aquaculture production of aquatic animals for human consumption (in tonnes) in 2009. Adapted from FAO (2011).

One potential problem with the increase of African aquaculture is a lack of locally produced high-quality feed (Gabriel *et al.*, 2007). South Africa was producing only 1 500 – 2 000 tonnes of compound aquafeed in 2006 (Tacon & Metian, 2008). Currently, finfish culture in South Africa relies on locally produced trout feed, or imported feeds of which prices fluctuate considerably due to currency exchange rates (Woolley *et al.*, 2010). However, as South African aquaculture is a promising activity, and the projections are that this industry will indeed grow, locally produced aquafeeds are crucial for the development and sustainability of this industry (Hecht *et al.*, 2006).

As previously mentioned, fishmeal and oil are the main ingredients in aquafeeds. This is no different in Africa, and the majority of these products come from pelagic fish species. The contribution of small pelagic fisheries in Africa and the Near East, even though fairly small, is mainly for human consumption (Hecht & Jones, 2009). Nonetheless, South Africa is the only country in this region that has a dedicated reduction fishery (Hecht & Jones, 2009). From 1990 to 2000 the average total landing of pelagic fish was 375 000 tonnes, which represented more than 60 % of total South African fish landings (Stefanus, 2002). From 2000 to 2004 this average (5-year average) increased to 541 568 tonnes (FAO, 2006). However, this latter data cannot be divided into reduction to fishmeal and oil, human consumption and other uses (Hecht & Jones, 2009). The average fish oil production in South Africa in the period 1968 to 1983 was approximately 64 400 tonnes (FAO, 1986). However, it decreased to an average of 2 500 tonnes between 2001 and 2006 (Péron *et al.*, 2010). Furthermore, some pelagic fish species are extremely dependant on environmental conditions which may cause fluctuations in the biomass from one year to the other (Stefanus, 2002). Thus, the challenge for the aquaculture industry is that environmental factors make it difficult to forecast the abundance of pelagic fish (anchovy, for example), and therefore the availability of fishmeal and fish oil cannot be anticipated with precision (Stefanus, 2002).

Some local industries have done pioneering work in developing technologies to produce indigenous species such as dusky kob. This species has been declared over exploited due to overfishing in South Africa (Griffiths, 1997b), but identified as a good candidate for aquaculture (Hecht, 2000). On the South African coast, dusky

kob is a major angling species in the inshore and estuarine environments (Griffiths & Heemstra, 1995). This interest in this species has led to a number of studies of its biology, ecology and stock status (Griffiths, 1996; Griffiths, 1997a; Taylor *et al.*, 2005; Silberschnneider & Gray, 2008; Silberschnneider *et al.*, 2009; Bernatzeder *et al.*, 2010)

### **Dusky kob, *Argyrosomus japonicus*, and a review of its culture**

Global culture of scianeids species is rapidly expanding, and there is potential for it to keep growing (Hong & Zhang, 2003; Duncan *et al.*, 2009). Many scianeids are farmed in the world, including *Sciaenops ocellatus*, *Umbrina cirrosa*, *Argyrosomus regius*, and *A. japonicus* (Jiménez *et al.*, 2005). They are produced not only for human consumption but also for restocking to rehabilitate wild fish stocks (Silberschnneider & Gray, 2008).

Dusky kob is a large carnivorous fish that can live to 30 years or more (PIRSA, 2001), and reach a maximum weight of about 75 kg (Griffiths & Heemstra, 1995). It is a cosmopolitan species, occurring along the African southeast coast from the Cape of Good Hope to southern Mozambique (Griffiths & Heemstra, 1995). In Australia it occurs along the entire southern seaboard (Starling, 1993) and along the Chinese coast to southern Korea and Japan (Griffiths, 1997b). It occurs in estuaries, in the surf zone and in the nearshore zone (Griffiths & Heemstra, 1995).

Due to its large size, palatability (Griffiths, 1996) and high market value, *A. japonicus* is an increasingly popular mariculture species (Silberschnneider & Gray, 2008). This species is an excellent candidate for commercial culture in sea cages, ponds and inland recirculating systems (PIRSA, 2001). It tolerates poor water and low oxygen levels, and wide range of salinities (Fitzgibbon *et al.*, 2007). It also easily adapts to intensive culture systems (Love & Langenkamp, 2003) and formulated feeds (Daniel 2004). It has a fast initial growth rate (Griffiths, 1996), good feed conversion ratios (PIRSA, 2001; Silberschnneider & Gray, 2008) and is highly fecund (Pirozzi *et al.*,

2010c). In addition, juvenile dusky kob can tolerate a high stocking density of up to 50 kg m<sup>-3</sup> (Collett *et al.*, 2001).

Farming dusky kob is becoming increasingly popular. In Australia *A. japonicus* (mulloway or Jewfish - as it is known there) is a highly prized sportfish and an important commercially farmed species (Taylor *et al.*, 2005; Partridge *et al.*, 2008). The species was bred for the first time in 1992, and since then large numbers of mulloway fingerlings have been successfully produced in intensive and extensive systems (Love & Langenkamp, 2003). Australia produced approximately 0.5 tonnes of dusky kob in 2001 and 2002 (O'Sullivan & Savage, 2004) and the production increased to 558 tonnes in 2004 and 2005 (O'Sullivan *et al.*, 2007). In recirculating systems in Australia *A. japonicus* reached above market size of 500 g in 10 months. In 16 months it reached one kg, and up to 1.2 kg average in about 2 years (PIRSA, 2003). This fast growth could be attributable to their "hovering" behaviour that possibly saves the energy for growth instead of for swimming (PIRSA, 2001).

Since this species is becoming more popular for culture, studies are progressively being done on its biology and culture practices. A rearing protocol for juvenile *A. japonicus* was developed, and the best rearing temperature for juveniles was at 25.3° C, within a light intensity range of 23-315 lux (Collett *et al.*, 2008a; Collett *et al.*, 2008b). In addition, a stocking density up to 50 kg m<sup>-3</sup> did not affect growth or feed conversion ratio (Collett *et al.*, 2001). An optimal protein and lipid levels in the diets of juvenile dusky kob were found to be at 46 and 18 %, respectively (Woolley *et al.*, 2010). Furthermore, some of the nutritional requirements, including the replacement of fishmeal with soybean meal in the diets were also studied (Daniel, 2004; Lee, 2009). These later studies proposed that, replacing up to 30 % of fishmeal with soybean meal did not affect growth, body composition and feed efficiency. However, feed intake was reduced due to the palatability of the diets containing soybean meal (Daniel, 2004). Nonetheless, even with this increasing interest on the culture of this species, little is known about lipid substitution on *A. japonicus* diet.

## Lipids in fish nutrition

Lipids are the primary source of energy for marine fish. If the requirement of lipids in the diets are not met, the protein, which is the most expensive ingredient used in aquafeeds, will be metabolised by the fish as the energy source (Higuera, 2001). As lipids are high-energy nutrients, they can be utilized by fish to partially spare protein (Corraze, 2001). In salmonids, for example, when the lipid level was increased from 15 to 20 %, the protein content could be lowered from 48 to 35 % without affecting growth performance (Corraze, 2001). Carbohydrates are a low-cost source of energy; however, carnivorous fish such as dusky kob have a limited capacity to utilize carbohydrates as energy source (Silva & Anderson, 1995), and therefore lipids are a better alternative. This has been reported for carnivorous species such as red drum, *Sciaenops ocellatus* (Serrano *et al.*, 1992), and yellowtail, *Seriola quinqueradiata* (Silva & Anderson, 1995). It is imperative, thus, to meet the energetic requirements of a species when formulating finfish diets.

Lipids are crucial for fish health and survival. They supply energy for growth from the egg to the adult fish, as well as for reproduction, swimming, and for heat production in fish that maintain thermal homeostasis (Wiegand, 1996; Sargent *et al.*, 2002). Furthermore, lipids are an important source of vital fatty acids, which play an important role on the maintenance of functional and structural integrity of cell membranes and as precursors of eicosanoids (Sargent *et al.*, 1999a).

Fatty acids are the key components of all lipids. The concentration of polyunsaturated fatty acids (PUFA) of the n-3 series in the liver or muscle of fish varies among species and depends on factors such as age, sex, seasonality, water quality and especially the diet consumed (Vlieg & Body, 1988; Regost *et al.*, 2003). The highly unsaturated fatty acids (HUFA) such as eicosapentaenoic acid (EPA 20:5 n-3) and the docosahexaenoic acid (DHA 22:6 n-3) are found in high levels in marine fish (Ribeiro *et al.*, 2007). This is because they are produced by marine algae that are consumed by zooplankton and finally by the fish (Pike & Jackson, 2010). Thus, the fatty acid composition of fish tissues generally reflects that of the diets (Guillou *et al.*, 1995; Izquierdo *et al.*, 2005; Mourente & Bell, 2006). In freshwater species,

however, EPA and DHA are usually found in lower levels in the fish tissues (Ribeiro *et al.*, 2007).

Marine fish have a dietary requirement for n-3 long chain PUFA. Plants are the only organisms that can produce series of n-3 and n-6 fatty acids. Consequently, animals require these fatty acids in their diets, and therefore they are called essential fatty acids (IFFO, 2008). The n-3 series is derived from the  $\alpha$ -linolenic acid (18:3 n-3), and the n-6 from the linoleic acid (18:2 n-6). From these fatty acids the arachidonic acid (AA 20:4 n-6), EPA, and DHA are synthesised (Figure 1.3; Tocher, 2003). Fresh water species have the ability to bioconvert C<sub>18</sub> PUFA into long chain PUFA (Turchini *et al.*, 2009). Albeit marine fish have the enzymes to bioconvert linoleic and  $\alpha$ -linolenic acids, they adapted their food habits to diets rich in HUFA; therefore it is believed that they lost their ability to perform *de novo* biosynthesis of C<sub>18</sub> PUFA to n-3 HUFA (Sargent *et al.*, 2002; Turchini *et al.*, 2009). Thus, marine fish require n-3 HUFA in their diets.

n-6	enzymes	n-3
18:2 n-6		13:3 n-3
↓	$\Delta^6$ -desaturase	↓
18:3 n-6		18:4 n-3
↓	Elongase	↓
20:3 n-6		20:4 n-3
↓	$\Delta^5$ -desaturase	↓
20:4 n-6		20:5 n-3
↓	Elongase	↓
22:4 n-6		22:5 n-3
↓	Elongase	↓
24:4 n-6		24:5 n-3
↓	$\Delta^6$ -desaturase	↓
24:5 n-6		24:6 n-3
↓	$\beta$ -oxidation peroxisomes	↓
22:5 n-6		22:6 n-3

**Figure 1.3.** Elongation and desaturation pathway of n-6 and n-3 fatty acids and the precursors of eicosanoids. Adapted from Tocher (2003).

Lipids are also vital for human health. The most beneficial fatty acids for human health are those found in the fish flesh (Sahena *et al.*, 2009). Fish contains a large amount of unsaturated fatty acids, and the n-3 PUFA found in marine fishes are essential for human health (Sahena *et al.*, 2009). Long chain PUFA such as EPA and DHA are found in the human body and they are vital for growth and health. They are also important in human cardiovascular system (Din *et al.*, 2004). Epidemiological studies associate the low incidence of cardiovascular diseases in Eskimos with the consumption of fatty acids from marine oils (Kromann & Green, 1980). An additional reason why EPA and DHA are recommended in the human diet is because the deficiencies or imbalance of these fatty acids are associated with cancer, as well as inflammatory and autoimmune diseases (Simopoulos, 2002). Consequently, since the fatty acids in fish tissues generally reflect those of their diets, a change in the fish's dietary lipids can therefore compromise the final product, and lose the beneficial properties for humans.

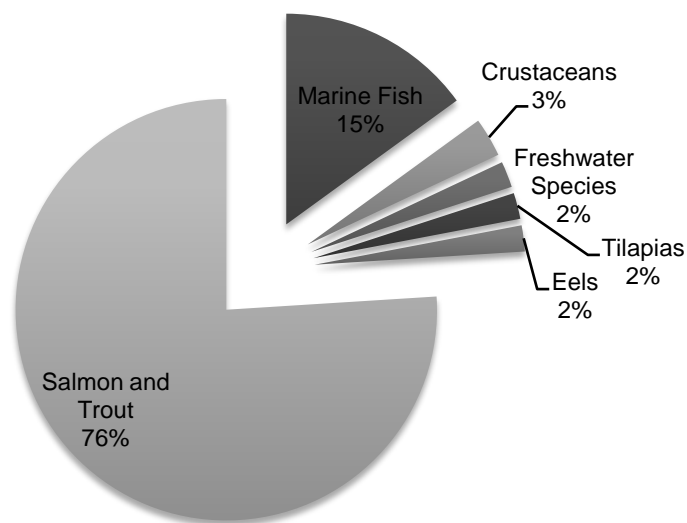
### **Fish nutrition, fish oil and alternative feed ingredients**

In this era of modern aquaculture systems the success of commercial aquaculture depends on factors such as rearing technologies, market, and the biology of the chosen species. A crucial aspect of the biology of the animal is its nutrition. Research into nutrition has thus become an important tool in fish production. The interest in nutrition increases simultaneously with the development of the aquaculture industry, as fish farmers spend about 35 to 50 % of production costs on feed (Piedecausa *et al.*, 2007).

Most of the fish oil produced in the world is used in the aquafeed industry. Although dietary requirements change among fish species, fishmeal and fish oil are the preferred ingredients used in aquafeeds, especially for carnivorous fish (Naylor *et al.*, 2000). The culture of carnivorous species is rapidly expanding (Naylor *et al.*, 2001). As a result, approximately 87 % of world fish oil production was used in aquaculture in 2003 (Hecht & Jones, 2009); and in 2006 it was estimated that the sector consumed 88.5 % of the total fish oil production (Tacon & Metian, 2008). However,

supplies of fishmeal and fish oil have remained relatively consistent for many years (FAO, 2002b). Except for the more severe El niño years (1992 and 1998), fish oil production have ranged between 1.0 to 1.7 million tonnes over the past 20 years (Shepherd *et al.*, 2005). Moreover, fish oil sources are finite and may not meet the increasing demand for animal feed in aquaculture and other animal cultivation.

The culture of salmonid species consumes most of the fish oil destined to the aquaculture industry, and salmon production is rapidly growing. In 1970 the global salmon production was around 500 tonnes and in 2005 it increased to 1.3 million tonnes (Liu & Sumalia, 2008). For salmon, “high-energy” feeds with lipid levels as high as 40 % have been used. The use of fish oil in aquafeeds for salmonid fish accounted for 76 % of the total fish oil used in aquaculture in 2008, and 15 % was used in feed for marine fish (Figure 1.4; Jackson & Shepherd, 2010).



**Figure 1.4.** The usage of fish oil in aquaculture feeds in 2008. Adapted from Jackson and Shepherd (2010).

For a successful aquaculture production, animals must grow in short periods and with low costs. The aquaculture industry needs to formulate diets that reach a fish’s nutritional requirements, and does not negatively affect growth, immune function, and disease resistance. The diets should also provide a high feed conversion ratio

and digestibility. Aquaculture uses up to five times more fish protein as fishmeal to feed farmed species than what is produced (Tacon, 1997). Nonetheless, in a long-term supply, this industry will be more limited by the availability of fish oil than of fishmeal (Jackson & Shepherd, 2010). It is necessary, thus, to assess alternative ingredients to supplement or replace conventional ones with higher prices, reducing the cost of the feed and the absolute dependence on fish oil (Izquierdo *et al.*, 2003). There are several alternative sources of lipids for aquafeeds, such as vegetable oils, animal fat, aquatic by-products (Turchini *et al.*, 2009), marine products from lower trophic levels (Olsen *et al.*, 2004), amongst others. A number of studies have been conducted to investigate the effects of partial or total replacement of fish oil by vegetable oils (one single oil or a blend of oils) in fish diets (Nematipour & Gatlin III, 1993; Guillou *et al.*, 1995; Bransden *et al.*, 2003; Yildiz & Sener, 2004; Montero *et al.*, 2005; Mourente *et al.*, 2005; Francis *et al.*, 2006; Francis *et al.*, 2007a; Lin & Shiau, 2007; Piedecausa *et al.*, 2007; Fountoulaki *et al.*, 2009; Pettersson *et al.*, 2009; Wassef *et al.*, 2009; Babalola *et al.*, 2011; Trushenski *et al.*, 2011). Amongst other factors, these studies analyze how vegetable oils affect growth, nutrient digestibility, muscle fatty acid composition, lipid metabolism, and fillet quality, in several different marine and freshwater fish species.

Oils of plant origin are increasingly being produced, and with greater availability are becoming cost competitive alternatives (Turchini *et al.*, 2009). The production of the major vegetable oils (i.e. crude palm oil, soybean oil and canola/rapeseed oil) increases yearly (Table 1.1). The prices of these three oils have historically been lower than the price of fish oil (Turchini *et al.*, 2009). Soybean oil is one of the most important vegetable oils for human consumption (Hertrampf & Piedad-Pascual, 2000) and is the vegetable oil with the second largest production globally, with 43.2 million metric tonnes in 2011 (USDA, 2011).

**Table 1.1.** World production (million metric tonnes) of the major vegetable oils from 2007 to 2011. Data obtained from the United States Department of Agriculture (2011).

	2007/08	2008/09	2009/10	2010/11	2011/12
<b>Vegetable oil</b>					
Palm	41.08	43.99	4.86	47.67	50.28
Soybean	37.83	35.91	3.87	41.26	43.11
Rapeseed	18.43	20.49	22.34	23.23	22.72
Sunflowerseed	10.03	12.00	11.70	11.46	12.83
Palm Kernel	4.88	5.17	5.50	5.65	5.66
Cottonseed	5.21	4.77	4.62	4.97	5.35
Peanut	4.86	5.02	4.68	5.16	4.97
Coconut	3.53	3.53	3.62	3.68	3.68
Olive	2.78	2.78	3.05	3.01	3.02

### **Aim and objectives**

The aim of this study was, thus, to investigate the effects of fish oil replacement with a vegetable oil in the diet for *A. japonicus*. The objectives were:

1. to assess the effects of dietary soybean oil on growth, feed efficiency, whole body proximate composition, and survival of dusky kob;
2. to investigate the effects of dietary soybean oil on the hepatosomatic and visceral fat indexes;
3. to analyze the fatty acid composition of the liver tissue of fish fed diets containing graded levels of soybean oil replacing fish oil; and
4. to assess the effects of feeding dusky kob diets containing soybean oil on the histology, as well as the glycogen content of the liver.

## CHAPTER 2

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### **GROWTH PERFORMANCE OF JUVENILE DUSKY KOB, *ARGYRO SOMUS JAPONICUS*, FED DIETS WITH FISH OIL REPLACEMENT BY SOYBEAN OIL**

#### **Introduction**

The rapid increase and development of aquaculture places pressure on the small pelagic fisheries worldwide, as the main ingredients for fish feeds are fishmeal and fish oil derived from these fish (Péron *et al.*, 2010). Fishmeal is obtained by cooking, pressing and drying these pelagic species, and the oils are obtained from the pressing process (Palos, 2010). The utilization of fish oil is one of the aspects that affect the costs of aquafeeds, and prices of fishmeal and oil are increasing substantially (Rana *et al.*, 2009).

Besides the high costs, the sustainability of aquaculture remains a concern due to the stagnation of fisheries (FAO, 2010b). Moreover, with the criticism by nongovernmental organizations (NGOs) that fishmeal and oil producers are wasting natural resources by feeding these products to fish, instead of using them for direct human consumption (IFFO, 2009), sustainable aquafeeds are becoming a prerequisite due to consumer demand. Hence, ways to evaluate the efficiency of fish farming and the overall impact of aquaculture practices on fish supply, based upon its use of nutrients obtained from fishmeal and fish oil are being developed for the salmon industry (Tacon & Metian, 2008; EWOS, 2009). These efficiency ratios consider various steps: the production of fishmeal and fish oil, the aquafeed production, the feeding practices, and also fish growth. The ratios that are generally used are the fish in-fish out ratio (FIFO; Jackson, 2009), the feed fish equivalency ratio (FFER; WWF, 2011), as well as the marine oil dependency ratio (MODR) and marine protein dependency ratio (MPDR; EWOS, 2009). The MODR and the MPDR developed for the salmon industry are nutrient based efficiency ratios that consider two important factors that the other ratios do not take into account (EWOS, 2009): 1)

it is assumed that the excess fishmeal is not wasted because it is used elsewhere, and 2) that salmon are of higher nutritional value than the average wild caught fish.

Although producing farmed fish requires a greater equivalent of wild fish biomass than can be harvested from farmed fish, considering a 10 % energy flow value between trophic levels, 10 units of food (small pelagic fish) is required to produce one unit of predatory wild fish, compared to 2-5 units to produce a unit of farmed fish (Naylor *et al.*, 2000). More recently, however, it has been stated that this situation is more complex. Firstly because fish meal and fish oil are not obtained exclusively by pelagic fishes, but also by trimmings of captured and cultured fish, as well as “trash” fish, i.e. fish with little or no economic value (Bendiksen *et al.*, 2011; WWF, 2011). Furthermore, the use of other ingredients such as plant meals and oils, by-products from terrestrial animals, and other ingredients are being used by the aquafeed industry to reduce the strong dependence on fish meal and fish oil originated from pelagic species (Bendiksen *et al.*, 2011). Thus, ratios of wild fish to farmed fish, calculated by the MODR, below one have recently been achieved by the salmon industry (Crampton *et al.*, 2010; Bendiksen *et al.*, 2011).

The production of vegetable oils has increased considerably from 1980 to 2006 (Turchini *et al.*, 2009), and these oils are likely to remain more available than limited fish oil resources (Turchini *et al.*, 2009). As such, it is expected that more vegetable sources will be included in aquafeeds in the future (Bendiksen *et al.*, 2011). Soybean oil is one of the largest sources of vegetable oils in the world (Turchini *et al.*, 2009). It is rich in vitamin E and is cholesterol-free (Figueiredo-Silva *et al.*, 2005). It contains high levels of polyunsaturated fatty acids (PUFA) such as linoleic acid (18:2 n-6); however, it is deficient in eicosapentaenoic acid (EPA 20:5 n-3) and docosahexaenoic acid (DHA 22:6 n-3). Soybean oil has been used to substitute fish oil in the diets without affecting fish growth in some freshwater species (Figueiredo-Silva *et al.*, 2005; Martins *et al.*, 2006), as well as marine fishes such as Atlantic salmon, *Salmo salar* (Grisdale-Helland *et al.*, 2002), cobia, *Rachycentron canadum* (Trushenski *et al.*, 2011), European sea bass, *Dicentrarchus labrax* (Figueiredo-Silva *et al.*, 2005; Martins *et al.*, 2006), red drum, *Sciaenops ocellatus* (Tucker *et al.*, 1997), and others. Unlike many fresh water fish, most marine species are not able to bioconvert fatty acids with 18 carbons (C<sub>18</sub>) to long chain PUFA (Lochmann & Gatlin

III, 1993). Therefore, EPA and DHA remain essential dietary ingredients for most marine fishes (Sargent *et al.*, 1997). Thus, it must be established whether and to what degree lipids from vegetable sources can fully satisfy the lipid nutritional requirements of a fish and, consequently, whether and to what extent fish oil can be replaced with vegetable oils in marine finfish feeds.

As marine finfish culture is still emerging in South Africa, with dusky kob (*A. japonicus*) the forerunner, studies on production technology (Collett, 2007; Ballagh *et al.*, 2008; Collett *et al.*, 2008a; Collett *et al.*, 2008b; Partridge & Lymbery, 2009; Pirozzi *et al.*, 2009) are still limited. Similarly, few nutritional studies have been undertaken. Although there has been some initial research into nutritional requirements of dusky kob (Daniel, 2004; Pirozzi *et al.*, 2010a; Pirozzi *et al.*, 2010c; Pirozzi *et al.*, 2010b; Woolley *et al.*, 2010), there are no studies on the replacement of fish oil by vegetable oils in the diets for this species. Hence, the aim of this trial was to evaluate the effects of the substitution of fish oil with soybean oil on the diet of *A. japonicus* on growth, feed efficiency and whole body proximate composition of fish.

## **Material and methods**

### Experimental system

The experiments were conducted at the Rhodes University, Marine Research Laboratory in Port Alfred, South Africa.

The experimental system consisted of eighteen circular tanks each with a maximum operating volume of 0.82 m<sup>3</sup>, and a maximum operating depth of 800 mm (Figure 2.1). These tanks were covered with a black 30 % shade cloth. Water was drained by gravity from each tank, via a central up-stand pipe, to a 1 000 L submerged sediment-settling tank. It was pumped (Speck Pumps SA Pty Ltd, Aquadrive 500, 1.1 kW, South Africa) through a 1 000 L oyster shell and plastic media biological filter (Figure 2.2). The same pump delivered water to the tanks at a flow rate of 200 L h<sup>-1</sup> tank<sup>-1</sup>.

The water of the system was passed under an ultraviolet light (55W Pro UV, Ultrazap, Johannesburg), and heated to 22-24 °C using a heat pump (SIRAC POOL, 3.7 kW, China). A protein skimmer filtered the water of the whole system. Ten percent of the volume of the system was replaced daily with water from the Kowie River Estuary. The water was aerated with airstones placed in each tank. Fish were maintained in natural photoperiod throughout the experiment.

Forty-two days after the start of the trial a biological trickle filter was installed (Figure 2.2). It consisted of a 1 000 L tank composed of a manifold for dispersing the water over Celpad wet wall trickle media.



**Figure 2.1.** Circular tanks at Rhodes University, Marine Research Laboratory in Port Alfred.



**Figure 2.2.** (a) The sedimentation settlement tank (1 000 L), (b) the biological filters including a submerged (1 000 L) and (c) a trickle filter (1 000 L).

### Experimental fish and acclimation

Captive bred *A. japonicus* were obtained from Espadon Marine Pty Ltd (East London, South Africa), with a mean weight of approximately 0.7 g. They were grown-out for two months before the experiment started. During the first month they were fed two different imported feeds: first a 1.8 mm pellet (52 % protein; 20 % lipid; Skretting Gemma, Italy), followed by a 2 and 3 mm pellet (50 % protein; 15 % lipid; BernAqua, Belgium). They were fed six times a day to apparent satiation for the first month. In the second month, they were fed a commercial trout diet (45 % protein; 14 % lipid; Aquanatro, Nutroscience Pty Ltd, South Africa) to apparent satiation, three times a day.

Two weeks before the start of the experiment fish were fed the same trout diet twice a day at 09h00 and 16h00 to apparent satiation, seven days a week, at a maximum

of 3.85 % of their body weight day<sup>-1</sup> (Collett, 2007), to acclimate them to the experimental feeding regime.

#### Starting weight and stocking density

After the acclimation period, fish were purged for 24 h, anesthetized with 2-phenoxyethanol at 0.2 mL L<sup>-1</sup>, weighed to the nearest gram and standard length was measured to the nearest millimetre. Fish with an average weight ( $\pm$  standard error) of 112.56  $\pm$  18.47 g fish<sup>-1</sup>, were randomly distributed among the 18 tanks (20 fish per tank). The volume in each tank was reduced by lowering the up-stand pipe to achieve a stocking density of five kg fish m<sup>-3</sup>. Every 28 days the water level in the tanks was adjusted to maintain this stocking density, based on the mean weight of all fish in that tank.

#### Experimental diets

Six isonitrogenous and isoenergetic diets were formulated according to the previous requirements established for dusky kob, with a total lipid content of 18% and a total protein content of 46% (Woolley *et al.*, 2010), with fishmeal and soybean meal as the main protein sources at 90 and 10 % of the total protein, respectively (Table 2.1).

All diets had the same basal composition, formulated on a dry weight-basis (Table 2.1). The lipid composition was replaced in each diet with a decrease in the contribution of fish oil (refined fish oil; Energy Oil Pty Ltd, Gauteng, South Africa), and a corresponding increase in soybean oil (crude soybean oil; Energy Oil Pty Ltd, Gauteng, South Africa), ranging from 0 to 68.8 % replacement. This resulted in the following total vegetable lipid in the diets: 1, 14, 28, 42, 56 and 70 % (Table 2.1).

The diets were manufactured at the Department of Ichthyology and Fisheries Science, feed manufacture laboratory, Rhodes University. The dry Ingredients were weighed and mixed with an industrial food mixer (Macadams baking systems, SM-

201, Cape Town, South Africa). The fish oil, soybean oil and water were subsequently introduced to the dry ingredients and mixed into homogenous dough. The dough was cold extruded, cut into pellets, placed on trays and dried at 38 °C for about 16 h. The feed was placed into sealed packets, which were stored in buckets at -20 °C.

**Table 2.1.** Ingredients (raw ingredient g 100 g diet<sup>-1</sup>) and formulated proximal composition of the experimental diets with different inclusion of fish oil / soybean oil.

	Vegetable oil 1 %	Vegetable oil 14 %	Vegetable oil 28 %	Vegetable oil 42 %	Vegetable oil 56 %	Vegetable oil 70 %
<b>Ingredients (g 100 g diet<sup>-1</sup>)</b>						
Fishmeal	60.45	60.45	60.45	60.45	60.45	60.45
Soybean meal	9.85	9.85	9.85	9.85	9.85	9.85
Fish oil	12.71	10.41	7.89	5.37	2.85	0.33
Soybean oil	0.00	2.30	4.82	7.34	9.86	12.38
Starch	16.77	16.77	16.77	16.77	16.77	16.77
Vitamix	0.22	0.22	0.22	0.22	0.22	0.22
<b>Formulated proximal composition (%)</b>						
Total protein	48	48	48	48	48	48
Total lipid	18	18	18	18	18	18
Portion of lipid from vegetable oil (%)	1	14	28	42	56	70
Portion of lipid from fishmeal (%)	0.08	0.08	0.08	0.08	0.08	0.08

Each of these diets was fed to the fish in three randomly selected tanks (i.e. three replicates per treatment) to apparent satiation, limited to a maximum of 3.85 % of their body weight day<sup>-1</sup> (Collett, 2007), split into two meals day<sup>-1</sup>.

### Data Collection

At the start of the trial, a composite sample of 10 randomly selected fish from the original population, from which the fish in the trial were selected, were sacrificed and immediately frozen for whole body proximate analysis.

Fish were purged for 24 h before handling at the start of the trial. They were then anesthetized with 2-phenoxyethanol at 0.2 mL L<sup>-1</sup>. Individual fish weight (0.01 g) and standard length (1 mm) were recorded using an electronic scale (Denver Instruments, MXX-612, New York, USA). The fish were then returned to their respective tanks. This procedure was repeated every 28 days for a total of 84 days.

Dead fish were removed from the tanks on a daily basis and mortality was recorded.

Condition factor (CF) was calculated using Equation 1 (Collet, 2007):

$$CF = W \times L^3 \times 106 \quad (1)$$

where  $W$  represents fish weight (g) and  $L$  stands for length (mm).

Weight gain ( $W$  gain, %) was calculated using Equation 2:

$$W \text{ gain} = (W_f - W_i) \times (W_i)^{-1} \times 100 \quad (2)$$

where  $W_f$  represents final weight of the fish (g) and  $W_i$  represents the initial fish weight (g).

Length gain ( $L$  gain, mm) was calculated using Equation 3:

$$L \text{ gain} = L_f \times L_i \quad (3)$$

where  $L_f$  represents final length of the fish (mm) and  $L_i$  represents the initial fish length (mm).

Specific growth rate (SGR, % day<sup>-1</sup>) was calculated using Equation 4:

$$SGR (\% \text{ day}^{-1}) = [\ln(W_f) - \ln(W_i)] \times D^{-1} \times 100 \quad (4)$$

where  $W_f$  represents final weight of the fish (g),  $W_i$  represents the initial fish weight (g), and  $D$  represents the number of days.

Food conversion ratio (FCR) was calculated using Equation 5:

$$FCR = WF \times W_{\text{gain}}^{-1} \times 100 \quad (5)$$

where  $WF$  represents the weight of the dry feed fed to the fish (g) and  $W_{gain}$  stands for weight gain of the fish (g).

Protein efficiency ratio (PER) was calculated using Equation 6 (Francis *et al.*, 2006):

$$PER = (W_f - W_i) \times MP^{-1} \quad (6)$$

where  $W_f$  represents final weight of the fish (g),  $W_i$  represents the initial fish weight (g), and  $MP$  represents the mass of protein fed to the fish (g).

Marine oil dependency ratio (MODR) was calculated using Equation 7 (EWOS, 2009):

$$MODR = [(FO_{feed}) + (FM_{feed}) \times (FO_{FM}) \times FCR] \times (FO_{fish})^{-1} \quad (7)$$

where  $FO_{feed}$  represents the concentration of fish oil in the feed (%),  $FM_{feed}$  represents the concentration of fishmeal in the feed (%),  $FO_{FM}$  stands for concentration of fish oil in the fishmeal used in the diets (as a proportion), and  $FO_{fish}$  represents the concentration of fish oil in the whole fish basis (%).

Marine protein dependency ratio (MPDR) was calculated using Equation 8 (EWOS, 2009):

$$MPDR = [(FM_{feed} \times P_{FM}) \times FCR] \times (P_{fish})^{-1} \quad (8)$$

where  $FM_{feed}$  represents the concentration of fishmeal in the feed (%),  $P_{FM}$  represents the concentration of protein in the fishmeal used in the diets (as a proportion), and  $P_{fish}$  stands for concentration of protein in the whole fish basis (%).

FCR and PER were recorded for the period starting on day 56 and ending on day 84. At the end of the experiment one fish from each tank was euthanized with a 2-phenoxyethanol at  $0.4 \text{ mg L}^{-1}$  (Brown, 2003) and immediately frozen for proximate analyses.

### Proximate analyses

Three composite samples from each experimental diet, a composite sample of fish at the start of the trial and a fish from each replicate sampled at the end of the growth experiment were sent for proximate analyses to the University of Kwazulu-Natal, Department of Animal and Poultry Science. These samples were analysed for crude protein, fat, ash, moisture, and the gross energy was analysed for the feed. Crude protein was determined using the nitrogen by combustion, according to Dumas combustion method (AOAC Official Method 990.03), in a LECO Truspec Nitrogen Analyser. Fat was extracted by the Soxhlett procedure (AOAC Official Method 920.39), using a Buchi 810 Soxhlett Fat extractor. For the determination of ash (AOAC Official Method 942.05), the samples were ashed in a furnace for four hours at 550 °C, and for moisture (AOAC Official Method 934.01), the samples were dried in an air circulated hot oven at 95 °C for 72 h. Gross energy of whole body was determined using a DDS isothermal CP500 bomb calorimeter (Digital Data Systems Pty Ltd, Johannesburg, South Africa).

### Water quality

Water temperature and pH were measured daily with a hand held electronic probe (Hanna Instruments HI 98128, Rhode Island, USA). Dissolved oxygen (DO) was also measured daily with a DO meter (YSI 85 DO/SCT Meter, Ohio, USA). Total ammonia, nitrite and nitrate were determined colourmetrically every week between 08h00 and 09h00 (NH<sub>3</sub> NH<sub>4</sub><sup>+</sup>, NO<sub>2</sub>, NO<sub>3</sub> Kits, Red Sea Fish Pharm, Israel). Salinity was also measured weekly with a refractometer (Atago S/Mill-E, Tokyo, Japan).

Mean pH values were calculated by converting all the pH values to H<sup>+</sup> ion concentrations. The average of this concentration was then used to calculate mean pH (EIFAC, 1986).

## Statistical analysis

Treatment means were compared using a one-way analysis of variance (ANOVA) and a Tukey's multiple range analysis at  $p < 0.05$ . Kruskal-Wallis ANOVA ( $p < 0.05$ ) was used if the data did not meet the assumptions of ANOVA (Shapiro-Wilk's test for normality of the residuals and Levene's test for homogeneity of variance). Regression models were calculated using the mean parameter of each tank as the unit of measure ( $p < 0.05$ ), whereas the combined mean and standard errors were plotted on the same axis as the regression models. All statistical analyses were performed using the software STATISTICA version 9 (Statsoft, Tulsa, OK, USA).

## **Results**

### Survival

After 42 days, all fish from one replicate of the treatments 42, 56 and 70% vegetable oil diets died. A further five fish died in one of the 70 % soybean oil replicate, and three fish from one replicate of the control treatment also died. Fish samples were immediately collected for necropsy, and gas bubble disease was diagnosed by a fish veterinarian. The disease was caused by nitrogen supersaturation in the water of the system. Apart from that, all fish survived the experiment.

### Growth performance, feed conversion ratio and protein efficiency ratio

There were no significant differences in weight, length and CF of fish among treatments at the start of the experiment, with overall means ( $\pm$  standard error) of  $112.56 \pm 1.36$  g (ANOVA:  $F_{(5, 12)} = 0.44$ ,  $p = 0.81$ ),  $200.9 \pm 0.09$  mm (ANOVA:  $F_{(5, 12)} = 0.62$ ;  $p = 0.69$ ), and  $1.46 \pm 0.007$  (ANOVA:  $F_{(5, 12)} = 1.05$ ,  $p = 0.43$ ), respectively (Table 2.2).

There were no apparent trends in SGR, weight gain, length gain and CF with an increase in vegetable oil portion in the diet for the first 56 days of the trails ( $p > 0.05$ ).

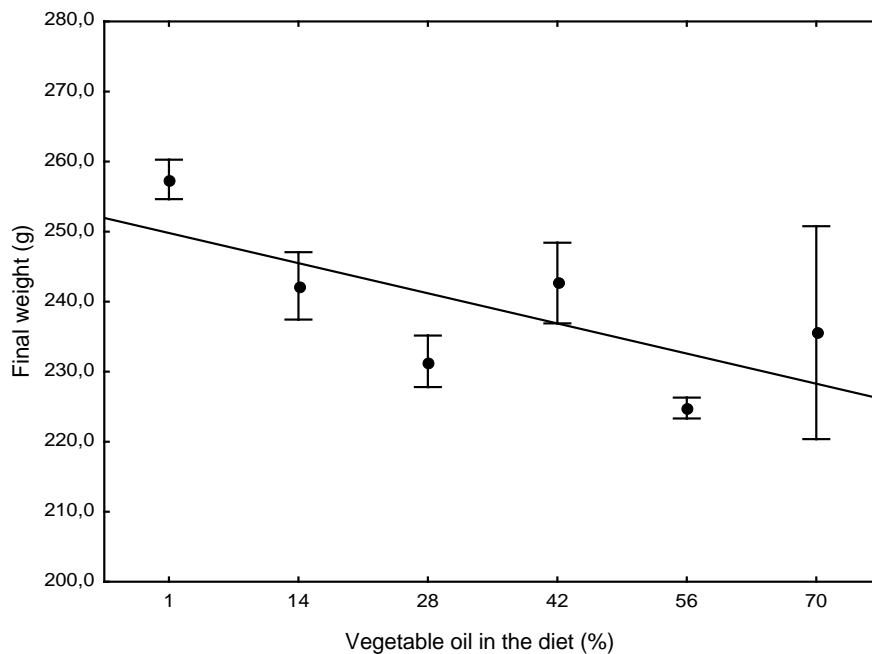
However, various significant trends became apparent after the fish had been exposed to the diets for about two months ( $p < 0.05$ ; Figures 2.3 to 2.8).

No significant differences nor trends were observed in final length of fish among treatments after 84 days with a combined mean of  $252.3 \pm 0.11$  mm (ANOVA:  $F_{(5, 9)} = 3.14$ ,  $p = 0.07$ ; Table 2.2) and similarly, there was no difference in length gain with a combined mean of  $50.9 \pm 0.10$  mm (Kruskal-Wallis:  $H_{(5, 15)} = 7.82$ ,  $p = 0.17$ , Table 2.2).

**Table 2.2.** Mean ( $\pm$  standard error) weight, length, and condition factor (CF) of *A. japonicus* at the start and end of the experiment, and feed conversion ratio (FCR) and protein efficiency ratio (PER) of fish fed diets where fish oil was replaced with graded levels of soybean oil for 84 days (ANOVA / Kruskal-Wallis;  $p > 0.05$ ).

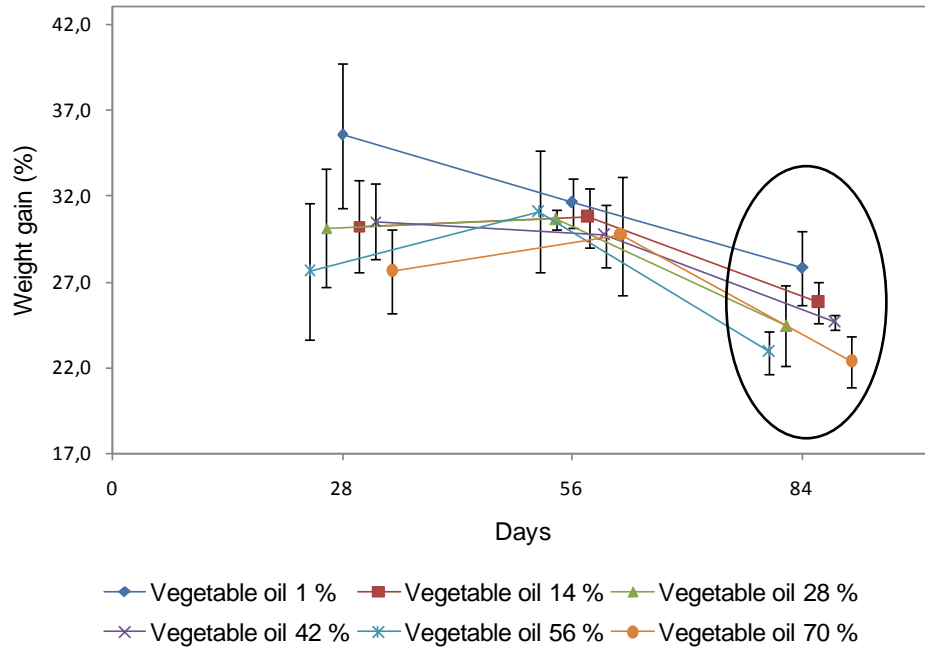
	Vegetable oil 1 %	Vegetable oil 14%	Vegetable oil 28 %	Vegetable oil 42 %	Vegetable oil 56 %	Vegetable oil 70 %	F / H value	p value
Initial weight (g)	113.4 $\pm$ 5.64	113.2 $\pm$ 2.73	109.6 $\pm$ 3.77	113.1 $\pm$ 3.70	110.0 $\pm$ 3.53	116.1 $\pm$ 0.58	$F_{(5, 12)} = 0.44$	0.81
Initial length (mm)	201.2 $\pm$ 0.40	202.6 $\pm$ 0.20	199.5 $\pm$ 0.15	201.1 $\pm$ 0.28	198.1 $\pm$ 0.21	203.2 $\pm$ 0.11	$F_{(5, 12)} = 0.62$	0.69
Initial CF	1.47 $\pm$ 0.01	1.44 $\pm$ 0.02	1.46 $\pm$ 0.02	1.47 $\pm$ 0.01	1.49 $\pm$ 0.01	1.46 $\pm$ 0.02	$F_{(5, 12)} = 1.05$	0.43
Final weight (g)	257.45 $\pm$ 2.82	242.25 $\pm$ 4.81	231.48 $\pm$ 3.68	242.65 $\pm$ 5.76	224.80 $\pm$ 1.50	235.57 $\pm$ 15.20	$H_{(5, 15)} = 9.90$	0.08
Weight gain (%)	128.19 $\pm$ 11.43	114.29 $\pm$ 7.11	111.76 $\pm$ 8.58	107.72 $\pm$ 4.70	111.13 $\pm$ 1.35	102.42 $\pm$ 14.45	$F_{(5, 9)} = 0.89$	0.53
Final length (mm)	25.72 $\pm$ 0.17	25.26 $\pm$ 0.22	24.84 $\pm$ 0.13	25.45 $\pm$ 0.12	24.80 $\pm$ 0.12	25.21 $\pm$ 0.42	$F_{(5, 9)} = 3.14$	0.07
Length gain (mm)	56.0 $\pm$ .023	50.0 $\pm$ 0.14	48.9 $\pm$ 0.09	50.7 $\pm$ 0.17	52.0 $\pm$ 0.13	47.9 $\pm$ 0.50	$H_{(5, 15)} = 7.82$	0.17
Final CF	1.59 $\pm$ 0.03	1.58 $\pm$ 0.02	1.58 $\pm$ 0.02	1.55 $\pm$ 0.01	1.55 $\pm$ 0.01	1.53 $\pm$ 0.01	$F_{(15, 27)} = 1.12$	0.38
SGR (% body weight per day)	0.98 $\pm$ 0.06	0.91 $\pm$ 0.04	0.89 $\pm$ 0.05	0.87 $\pm$ 0.03	0.89 $\pm$ 0.00	0.84 $\pm$ 0.09	$F_{(5, 9)} = 0.89$	0.53

After 84 days of feeding, no significant differences were observed in final weight of fish fed the different diets (Kruskal-Wallis:  $H_{(5, 15)} = 9.90$ ,  $p = 0.08$ ; Table 2.2). However, there was a significant trend of a decrease in fish weight ranging from  $257.45 \pm 2.82$  to  $224.80 \pm 1.50$  g with increasing soybean oil after 84 days of feeding ( $r^2 = 0.35$ ,  $p = 0.02$ ; Figure 2.3).

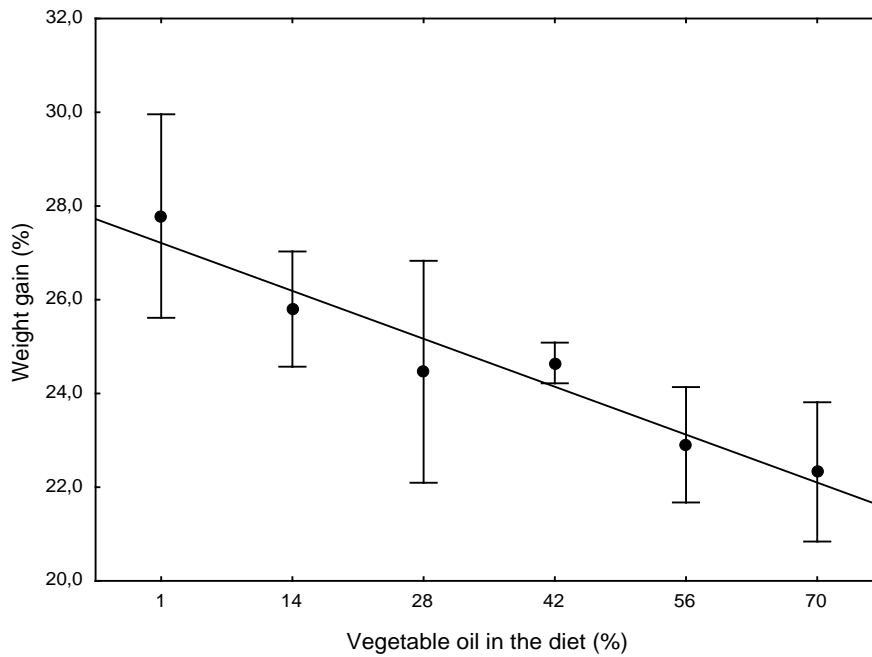


**Figure 2.3.** Mean ( $\pm$  standard error) weight of *A. japonicus* fed diets with graded levels of fish oil replaced by soybean oil for 84 days ( $y = 250.103 - 0.3267x$ ,  $r^2 = 0.35$ ,  $p = 0.02$ ).

The trend in weight gain was only apparent during the last month of the trial; that is, there was largely no difference in weight gain among treatments between days 28 and 56, with a drop in weight gain between days 56 and 84 (Figure 2.4). This drop was significant from  $27.79 \pm 2.17$  to  $22.33 \pm 1.49$  % with increasing soybean oil in the diets ( $r^2 = 0.35$ ,  $p = 0.019$ ; Figure 2.5). There were no significant differences in weight gain from the start of the experiment until day 84 ( $F_{(5,9)} = 0.89$ ,  $p = 0.53$ , Table 2.2).

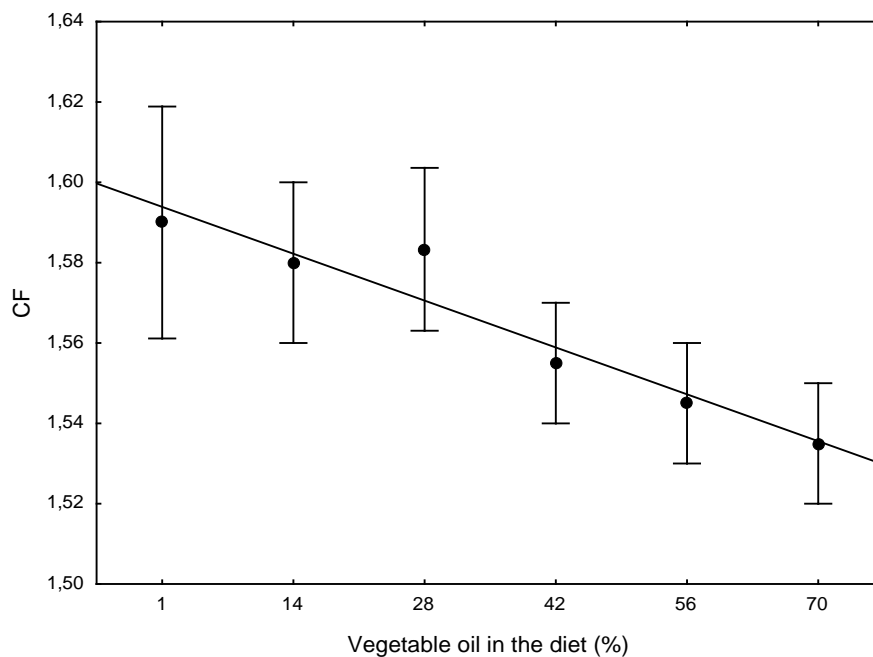


**Figure 2.4.** Mean ( $\pm$  standard error) weight gain (%) of *A. japonicus* fed diets with graded levels of fish oil replaced by soybean oil from the start of the experiment to day 28, between day 28 and 56 and between days 56 and 84. Data within the circle are represented in Figure 2.5.

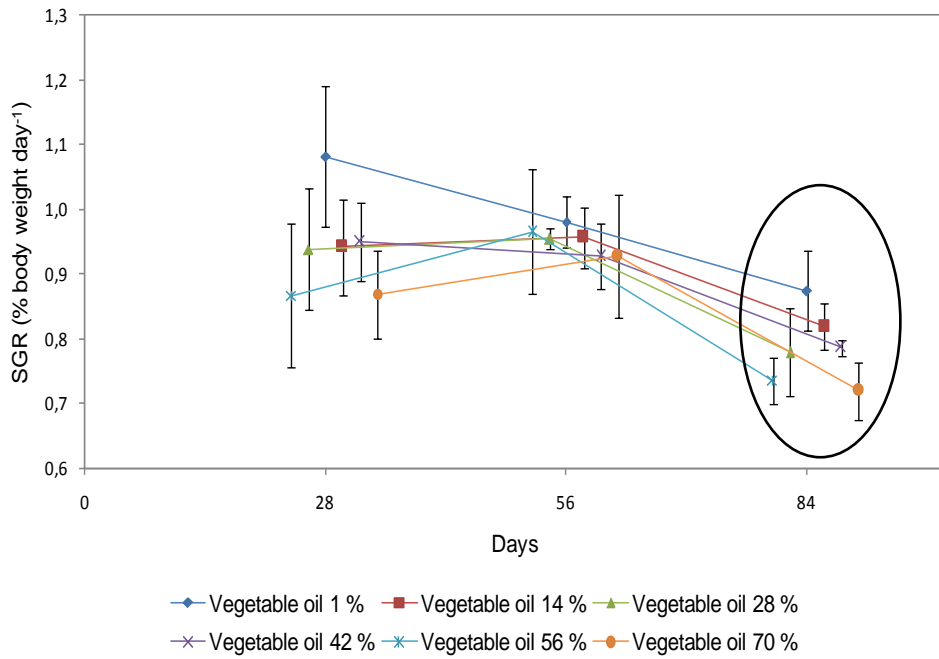


**Figure 2.5.** Mean ( $\pm$  standard error) weight gain of *A. japonicus* fed diets with graded levels of fish oil replaced by soybean oil between days 56 to 84 ( $y = 27.245 - 0.0748x$ ,  $r^2 = 0.35$ ,  $p = 0.019$ ).

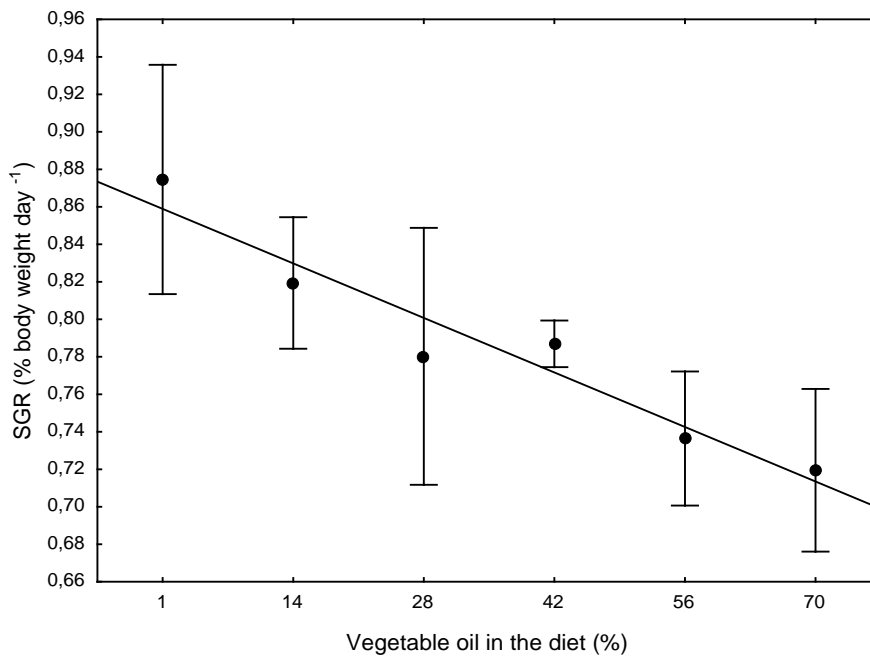
Over the feeding period of 84 days there was no significant difference in the mean CF of fish fed diets containing increasing level of soybean oil ( $F_{(15, 27)} = 1.12$ ,  $p = 0.38$ ; Table 2.2). Yet, there was a significant trend in CF of fish fed the different treatments from days 56 to 84, decreasing from  $1.59 \pm 0.03$  to  $1.54 \pm 0.02$  with an increase in dietary vegetable oil ( $r^2 = 0.32$ ,  $p = 0.028$ ; Figure 2.6). Again, this trend was not apparent among treatments during the first 56 days of the trial ( $p > 0.05$ ). Similarly, the SGR trends observed among treatments were significant only after 56 days of feeding (Figure 2.7); and it decreased significantly from  $0.87 \pm 0.06$  to  $0.72 \pm 0.04$  % body weight day<sup>-1</sup> with an increase in vegetable oil replacement between days 56 and 84 ( $r^2 = 0.35$ ,  $p = 0.019$ ; Figure 2.8).



**Figure 2.6.** Mean ( $\pm$  standard error) condition factor (CF) of *A. japonicus* fed diets with graded levels of fish oil replaced by soybean oil for 84 days ( $y = 1.594 - 0.0008x$ ,  $r^2 = 0.32$ ,  $p = 0.028$ ).



**Figure 2.7.** Mean ( $\pm$  standard error) specific growth rate (SGR; % body weight day<sup>-1</sup>) of *A. japonicus* fed diets with graded levels of fish oil replaced by soybean oil from the start of the experiment to day 28, between day 28 and 56 and between days 56 and 84. Data within the circle are represented in Figure 2.8.



**Figure 2.8.** Mean ( $\pm$  standard error) specific growth rate (SGR; % body weight day<sup>-1</sup>) of *A. japonicus* fed diets with graded levels of fish oil replaced by soybean oil between days 56 and 84 ( $y = 0.856 - 0.0021x$ ,  $r^2 = 0.35$ ,  $p = 0.019$ ).

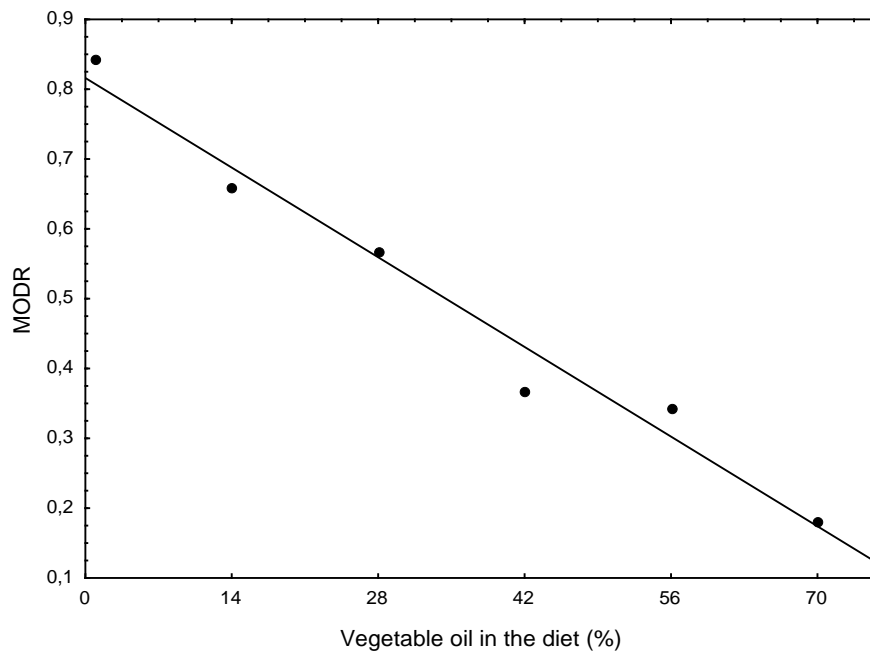
From days 56 to 84, the lowest FCR values were observed for fish fed the diets with 14, 42, 1, and 28 % of vegetable oil (0.99, 1.02, 1.09, and 1.11, respectively; Table 2.3). The highest values were observed, in the same period, for the fish fed diets with 56 and 70 % vegetable oil (with values of 1.36 and 1.25, respectively; Table 2.3). The exact same sequence was observed for PER. Fish fed the diets with 56 and 70 % vegetable oil inclusion had the lowest values (1.55 and 1.70, respectively; Table 2.3). Fish fed diets 14, 42, 1, and 28 % of vegetable oil had the highest PER values (2.13, 2.09, 1.96, and 1.90, respectively; Table 2.3).

**Table 2.3.** Feed conversion ratio (FCR) and protein efficiency ratio (PER), both as a composite of three replicates per treatment, of *A. japonicus* fed diets with graded levels of fish oil replaced by soybean oil between days 56 and 84.

	Vegetable oil 1 %	Vegetable oil 14 %	Vegetable oil 28 %	Vegetable oil 42 %	Vegetable oil 56 %	Vegetable oil 70 %
FCR	1.09	0.99	1.11	1.02	1.36	1.25
PER	1.96	2.13	1.90	2.09	1.55	1.70

#### Marine oil dependency ratio and marine protein dependency ratio

There was a significant trend of a decrease in MODR, ranging from 0.84 for fish fed the control diet to 0.18 for fish fed the 70 % vegetable oil diet ( $r^2 = 0.97$ ,  $p = 0.0003$ ; Figure 2.9). No trends were observed in MPDR ( $r^2 = 0.47$ ,  $p = 0.13$ ), with an overall mean of 0.48.



**Figure 2.9.** Marine oil dependency ratio (MODR) of *A. japonicus* fed diets with graded levels of fish oil replaced by soybean oil for 84 days ( $y = 0.82 - 0.009x$ ,  $r^2 = 0.97$ ,  $p = 0.0003$ ).

#### Proximate composition of the diets

The proximate composition of the experimental diets was similar among treatments with overall means ( $\pm$  standard error) of  $47.13 \pm 0.21$  % for crude protein,  $17.64 \pm 0.05$  % for crude fat,  $11.36 \pm 0.16$  % for ash,  $5.34 \pm 0.10$  % for moisture, and  $21.05 \pm 0.03$  MJ kg<sup>-1</sup> for gross energy (ANOVA;  $p > 0.05$ ; Table 2.4).

**Table 2.4.** Means ( $\pm$  standard error) proximate composition of the experimental diets fed to *A. japonicus* wherein fish oil was replaced with graded levels of soybean oil. (ANOVA / Kruskal-Wallis;  $p > 0.05$ ).

	Vegetable oil 1 %	Vegetable oil 14 %	Vegetable oil 28 %	Vegetable oil 42 %	Vegetable oil 56 %	Vegetable oil 70 %	F / H value	p value
Crude Protein (%)	46.86 $\pm$ 0.41	47.25 $\pm$ 0.43	47.21 $\pm$ 0.26	47.06 $\pm$ 0.44	47.21 $\pm$ 0.20	47.19 $\pm$ 0.24	$F_{(5,12)} = 0.06$	1.00
Crude Fat (%)	17.54 $\pm$ 0.05	17.84 $\pm$ 0.08	17.61 $\pm$ 0.01	17.73 $\pm$ 0.08	17.65 $\pm$ 0.08	17.46 $\pm$ 0.04	$F_{(5,12)} = 1.03$	0.44
Ash (%)	11.34 $\pm$ 0.25	11.28 $\pm$ 0.29	11.55 $\pm$ 0.25	11.27 $\pm$ 0.33	11.36 $\pm$ 0.27	11.37 $\pm$ 0.23	$H_{(5,N=10)} = 2.48$	0.78
Moisture (%)	5.47 $\pm$ 0.07	5.20 $\pm$ 0.12	5.33 $\pm$ 0.08	5.40 $\pm$ 0.15	5.21 $\pm$ 0.26	5.43 $\pm$ 0.18	$F_{(5,12)} = 1.60$	0.23
Gross energy (MJ kg <sup>-1</sup> )	21.02 $\pm$ 0.06	20.96 $\pm$ 0.05	21.09 $\pm$ 0.05	20.97 $\pm$ 0.04	21.15 $\pm$ 0.02	21.10 $\pm$ 0.02	$F_{(5,12)} = 0.17$	0.97

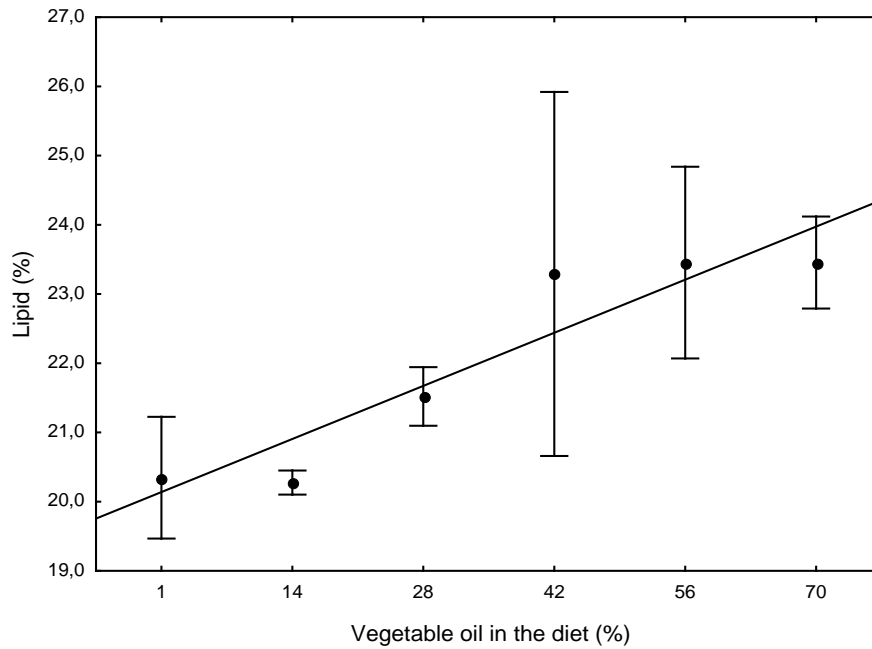
### Proximate composition of the fish

After 84 days of feeding, the inclusion of soybean oil in the diets did not affect protein (mean: 57.64  $\pm$  0.61 %; ANOVA:  $F_{(5,9)} = 0.69$ ,  $p = 0.64$ ), ash (mean: 15.31  $\pm$  0.18 %; Kruskal-Wallis:  $H_{(5,15)} = 5.38$ ,  $p = 0.37$ ) or moisture (mean: 68.51  $\pm$  0.69 %; Kruskal-Wallis:  $H_{(5,15)} = 1.68$ ,  $p = 0.89$ ) content of whole body of fish (Table 2.5).

**Table 2.5.** Mean ( $\pm$  standard error) whole body proximate composition of *A. japonicus* at the start of the trial (i.e. initial pop) and of those fed diets where fish oil was replaced with graded levels of soybean oil for 84 days (ANOVA / Kruskal-Wallis;  $p > 0.05$ ).

	Initial pop	Vegetable oil 1 %	Vegetable oil 14 %	Vegetable oil 28 %	Vegetable oil 42 %	Vegetable oil 56 %	Vegetable oil 70 %	F / H value	p value
Crude protein (%)	58.72	56.58 $\pm$ 1.20	59.02 $\pm$ 0.05	58.95 $\pm$ 0.40	57.85 $\pm$ 1.65	56.55 $\pm$ 2.31	56.02 $\pm$ 2.86	$F_{(5,9)} = 0.69$	0.64
Ash (%)	15.62	15.8 $\pm$ 0.48	15.80 $\pm$ 6.00	14.80 $\pm$ 0.37	14.98 $\pm$ 1.01	14.93 $\pm$ 0.23	15.33 $\pm$ 0.13	$H_{(5,15)} = 5.38$	0.37
Moisture (%)	70.91	65.93 $\pm$ 2.65	69.29 $\pm$ 1.48	69.83 $\pm$ 0.33	68.47 $\pm$ 2.63	69.10 $\pm$ 0.16	68.64 $\pm$ 1.31	$H_{(5,15)} = 1.68$	0.89

Body fat content increased significantly from 20.28  $\pm$  0.17 to 23.46  $\pm$  0.67 %, with the inclusion of soybean oil in the diets ( $r^2 = 0.48$ ,  $p = 0.004$ ; Figure 2.12).



**Figure 2.10.** Mean percentage ( $\pm$  standard error) of the body lipid content of *A. japonicus* fed diets with graded levels of fish oil replaced by soybean oil for 84 days ( $y = 20.051 - 0.0561x$ ,  $r^2 = 0.48$ ,  $p = 0.004$ ).

### Water quality

The mean water temperature and pH were  $22.3 \pm 0.03$  °C, and 7.86, respectively, and did not differ among treatments ( $H_{(5, 156)} = 1.81$ ,  $p = 0.87$ , and  $F_{(5, 150)} = 0.50$ ,  $p = 0.77$ , respectively). Similarly, there was no significant difference in DO between treatments with an overall mean of  $7.2 \text{ mg L}^{-1}$  ( $H_{(5, 1071)} = 8.35$ ,  $p = 0.14$ ). There were also no significant differences in ammonia (ranging from 0 to  $0.25 \pm 0.01 \text{ mg L}^{-1}$ ;  $H_{(5, 24)} = 3.55$ ,  $p = 0.62$ ), nitrite (ranging from 0.10 to  $0.20 \pm 0.01 \text{ mg L}^{-1}$ ;  $F_{(5, 18)} = 0.75$ ,  $p = 0.60$ ), and nitrate (ranging from 10.0 to  $20.0 \pm 1.50 \text{ mg L}^{-1}$ ;  $F_{(5, 6)} = 0.34$ ,  $p = 0.90$ ) concentrations between treatments. Salinity remained constant at  $35 \text{ mg L}^{-1}$  during the experimental period.

## Discussion

The substitution of fish oil appeared not to affect the final weight and length of dusky kob after 84 days of feeding; however, if the experiment had been continued for a longer period, the observed decline in weight gain and SGR with increasing dietary soybean oil content between day 56 and 84 would probably have produced significant differences in weight and length. Similar to the present work, there was no significant difference in final weight of European sea bass (*Dicentrarchus labrax* L.) when up to 60 % of fish oil was replaced with a blend of two vegetable oils in their diet. The initial weight of fish was about five grams and they were fed for 64 weeks (Richard *et al.*, 2006). In another experiment with *D. labrax*, fish fed a diet with a five percent soybean oil inclusion showed no significant difference in final weight compared to others fed a five percent fish oil or sunflower oil inclusion rate in the diet (Yildiz & Segner, 1996). Conversely, final weight and length of red drum, *Sciaenops ocellatus*, were negatively affected when fed a diet with a 12.7 % addition of soybean oil (Tucker *et al.*, 1997). The weight of gilthead sea bream, *Sparus aurata*, and turbot, *Psetta maxima*, were also affected by the inclusion of soybean oil in the diets (Kalogeropoulos *et al.*, 1992; Alexis, 1996; Regost *et al.*, 2003; Izquierdo *et al.*, 2005; Montero *et al.*, 2008). This negative effect on weight was also evident in some freshwater species such as Eurasian perch, *Perca fluviatilis*, (Xu & Kestemont, 2002), and Murray cod, *Maccullochella peelii peelii* (Francis *et al.*, 2007b). The studies on the effect of lipid substitution on fish growth appear to be contradictory. The reasons for that might be due to the differences in experimental design, such as the initial size of fish (Francis *et al.*, 2006; Francis *et al.*, 2007b), length of experiment (Francis *et al.*, 2007b; Turchini *et al.*, 2009) and the statistical analysis used (Shearer, 2000; Turchini *et al.*, 2009), for example.

The majority of the studies on the substitution of fish oil with vegetable oils in finfish diets are carried out over short feeding periods with only few replicates (Turchini *et al.*, 2009). Longer feeding periods, larger replicate numbers and possibly different statistical tests besides ANOVA, should be used to avoid misinterpretation of the ANOVA test (Shearer, 2000). Regression analysis

would be useful since it is not restricted by the limited power of ANOVA and the type II error that is common in this kind of research (Turchini *et al.*, 2009). As an example, there were no significant effects on growth and feed efficiency of Murray cod, *M. Peelii peelii*, when fed a diet with 100 % canola oil inclusion (Francis *et al.*, 2006). These fish had an initial body weight of 6.5 g and were fed the canola oil based diets for 84 days (Francis *et al.*, 2006). In a later study, Murray cod with an initial body weight of 21 g were fed similar diets for 112 days (Francis *et al.*, 2007b). A significant difference followed by a decreasing trend in growth was observed (Francis *et al.*, 2007b). The authors point out the discrepancies between the results, suggesting that it might be due to the differences in the initial weight of the fish, and due to the length of the experiment. The use of linseed oil to replace fish oil was also analysed in this latter study. No significant differences were found in final weight, weight gain, and SGR among fish in the different treatments. However, when regression analysis was used, they found a decrease in growth with an increase in dietary linseed oil (Francis *et al.*, 2007b). The authors also consider the different results obtained in the same study, using different statistical tools. For example, it was concluded that 75 % of the fish oil could be substituted by canola oil, based on SGR data analysed using ANOVA. In addition, this test suggests that up to 100 % of fish oil can be replaced by linseed oil without affecting growth of Murray cod. However, it was only with the use of regression that the differences became apparent, showing a significant growth reduction when canola and linseed oils were used to replace fish oil (Francis *et al.*, 2007b). Similarly, the use of different statistics in this study resulted in different conclusions. Although an ANOVA showed no significant difference in final weight between treatments, the use of regression showed a significant decreasing trend in final weight with increasing level of soybean oil substitution. These results confirm the suggestion by Francis *et al.* (2007b) and Turchini *et al.* (2009); that regression analysis is likely to be more appropriate than ANOVA, since regression analysis appears to eliminate the type II error associated with ANOVA.

The growth reduction resulting from soybean oil substitution in the present study was not immediately apparent, as the declines in percentage weight gain, CF

and SGR with increased oil substitution only started to show after two months of feeding (i.e. after 56 days). Furthermore, the higher the inclusion of vegetable oil, the more apparent the decrease in growth rate became. Similarly, when *S. aurata* were fed an essential fatty acid deficient diet, fish did not start to show inferior weight gain compared to fish fed diets with higher contents of n-3 fatty acids until six weeks into the feeding trial (Ibeas *et al.*, 1994). The delay on fish growth indicates the importance of conducting longer studies reproducing farming conditions that can better demonstrate the growth effects of substitution of fish oil by vegetable oils in aquafeeds. Weight gain was also affected by dietary lipids when sunshine bass, *Morone chrysops* ♀ x *M. Saxatilis* ♂, were fed a diet in which fish oil was partially or totally replaced by vegetable oils (Nematipour & Gatlin III, 1993). Similarly, weight gain was significantly different when Eurasian perch, *P. fluviatilis*, were fed diets supplemented with olive, safflower and linseed oils, compared to fish fed a cod liver oil based diet (Xu & Kestemont, 2002). The latter authors suggest that this effect, in Eurasian perch, was due to the palatability of the linseed oil diet compared to the one containing cod liver oil. SGR was also affected with the increase in soybean oil in the diets only from days 56 to 84. The mean SGR of all treatments were lower than those found for other marine species fed diets with total or partial replacement of fish oil by vegetable oils (Izquierdo *et al.*, 2003; Xue *et al.*, 2006; Montero *et al.*, 2008). Fish size and a number of other factors may influence growth rate (Jobling, 1993). Hence, as fish grow faster in early stages, the lower SGR in this study is possibly due to the larger initial size of the fish compared to the fish used in these studies mentioned above. Similar to this study, Murray cod (*M. peelii peelii*), European sea bass (*D. labrax*), and gilthead sea bream (*S. aurata*) had a significant lower SGR when fed diets with partial or total replacement of fish oil with vegetable oils (Menoyo *et al.*, 2004; Yildiz & Sener, 2004; Francis *et al.*, 2006). The reduced growth performance of fish fed the diets with vegetable oil replacing fish oil in this study is consistent with such studies. This suggests that diets containing lower levels of vegetable oil are more effective, resulting in better growth. Furthermore, growth suppression is a sign of essential fatty acid deficiency (Watanabe, 1982). Hence, the decrease in final weight, weight gain, and SGR demonstrate that dusky kob fed diets with fish oil replacement

displayed increasingly poor growth. This may be related to essential fatty acid deficiency of the vegetable oil used in the diets (Castell *et al.*, 1972). This is discussed further in Chapter 3.

The inclusion of soybean oil in the diets also affected the CF of *A. japonicus* from days 56 to 84. CF is an indicator of how heavy a fish is in relation to its weight and is used to assess different feeding conditions, as well as how temperature, stocking densities and other environmental conditions affect the length-weight relationship of fish (Satake *et al.*, 2009). CF decreases when fish are subjected to stress (Goede & Barton, 1990). It might also fluctuate as a reflection of nutrient availability in the feed (Goede & Barton, 1990). In this study, the availability of nutrients, and the fatty acids were altered with the decrease in fish oil and the inclusion of soybean oil in the diets (Chapter 3), and consequently the fish might have been nutritionally challenged. This may explain the decreasing trend in CF of dusky kob fed diets with increasing levels of soybean oil.

The efficiency of feed conversion was negatively affected in dusky kob fed diets with high levels of soybean oil. From days 56 to 84, FCR values were best for fish fed diets with up to 42 % soybean oil inclusion. These values (0.99, 1.02, 1.09 and 1.11) are similar to the 1.05 FCR value observed by Woolley *et al.* (2010) for dusky kob fed a 46% protein, 18% lipid diet, in which fish oil was the only source of lipid. Consistent with the present work, FCR increased from 1.35 to 1.70 when fish oil was totally replaced by soybean oil, and other vegetable oils in the diets for *S. aurata* (Montero *et al.*, 2008). The higher FCR values found here suggest that diets with high levels of soybean oil were not efficiently utilized by the fish.

Protein was used more efficiently by fish fed diets with low levels of soybean oil. The PER was highest (2.13, 2.09, 1.96 and 1.90) for fish fed diets with lower vegetable oil levels. Similarly, the PER decreased in flounder (*Paralichthys olivaceus*) when they were fed diets deficient in essential fatty acids (Kim & Lee, 2004). This ratio is often applied to express how well a protein supply is used to support growth. Thus, the low PER values (1.55 and 1.70) of fish fed diets with

higher levels of vegetable oil (i.e. 56 and 70 % soybean oil) suggest that protein was not being well utilized. This might be explained by the limited essential fatty acids in the diets with fish oil replaced by soybean oil (Chapter 3).

Increasing levels of dietary soybean oil resulted in higher levels of body fat in the experimental fish. The whole body fat content of fish fed the control diet in the present study (19.55 %) was however similar to that reported in previous work where a diet of similar composition was fed to dusky kob (Woolley *et al.*, 2010). The level of lipid in *A. japonicus* diets has been shown to be correlated with the carcass lipid level for fish fed fishmeal/fish oil based diets (Riddin, 2009). However, even though the amount of lipid in the diets was constant in this study (i.e. 18 % for all diets), the whole body content of lipid increased with increasing soybean oil substitution.

Some specific fatty acids, which are not typical in the natural diets of marine fish, can accumulate in the carcass, in the visceral cavity or in some organs, e.g. liver. This may influence the concentration of lipids in the whole body of the fish. Besides the growth suppression, fatty acid deficient diets may also cause the accumulation of fat in some of the fish organs (Ibeas *et al.*, 1994). This is discussed further in Chapter 3. The results of lipid content in whole body of dusky kob fed diets with increasing soybean oil suggest that the lipid originated from this oil was possibly not being used for metabolism by the fish, but was being deposited in the body.

Survival of fish appeared to be indirectly affected by dietary lipids. Fish fed diets containing higher levels of soybean oil (i.e. 42, 56 and 70 %) seemed to be more susceptible to other environmental stressors. During the experiment, after 42 days of feeding, fish were diagnosed with gas bubble disease. This led to the mortality of all fish in three tanks of the treatments 42, 56 and 70 % vegetable oil diets, as well as five and two fish from two other tanks (control and the 70 % vegetable oil diet, respectively). All fish in the 1, 14 and 28 % vegetable oil treatments survived the gas bubble incident. In a similar manner, sharpsnout seabream, *Diplodus puntazzo*, fed diets containing linseed oil had a significantly higher mortality rate compared to fish fed a fish oil based diet (Piedecausa *et*

*al.*, 2007). The authors suggest that the mortality occurred due to opportunistic bacterial infection related to handling the animals in high temperatures. Lower survival rates were recorded for rainbow trout, *Oncorhynchus mykiss*, fed diets deficient in essential fatty acids for eight weeks (Rinchard *et al.*, 2007). Conversely, essential fatty acid deficient diets had no impact on survival rate of juvenile gilthead seabream (*S. aurata*); however, the combination of these diets and high stocking densities did cause high mortality rates (Montero *et al.*, 2001). Terrestrial vegetable oils contain different fatty acids than those present in marine fishes natural feed. Hence feeding fish for a long period with unusual fatty acids could alter the blood parameters (Babalola *et al.*, 2009), and, consequently, lead to implications to their immune systems (Mourente *et al.*, 2005). Therefore the link involving nutrition, immunology, and disease resistance in fish is of great interest (Bruslé, 1990).

Fish fed diets wherein soybean oil replaced fish oil were less dependent on wild caught fish. The dependency on marine oil was highest in fish fed the control diet, in which only fish oil was added as a lipid source. This dependence decreased with each inclusion of soybean oil. In the present study all MODR values were lower than one. These results are similar to the MODR values for Atlantic salmon, *Salmo salar* (Bendiksen *et al.*, 2011). Fish presented values below one when fed diets with pristine fish oil or silage fish oil (Bendiksen *et al.*, 2011). Values below one indicate that fish are depositing more fat and protein in their bodies than what is supplied to them in the form of fish oil and fishmeal (Bendiksen *et al.*, 2011). However, with the replacement of fish oil by vegetable oils, and consequently a lower dependence on marine oils, the essential fatty acids are likely to be lacking in the finfish for human consumption (Crampton *et al.*, 2010). Thus, it would be interesting to evaluate the quantity of essential fatty acids in the fillet of fish, in order to verify if they have an adequate amount of these fatty acids available for humans.

## **Conclusion**

Growth was negatively affected when vegetable oil contribution to total dietary lipid was increased in the diet, shown by the decreasing trends in final weight, weight gain and SGR. However, these effects only became apparent after the fish had been fed the experimental diets for an extended period of about two months. As the growth trial was only conducted over a period of about three months, the effects of oil replacement might have been greater had the trial been continued. Fish fed the control, 14 and 28 % vegetable oil diets displayed similar CF. There was, however, a transition from the 28 to the 42 % vegetable oil diets. A similar break point between these two diets was observed in the body fat of dusky kob, wherein fish fed diets with higher levels of soybean oil (i.e. 42, 56 and 70 %) exhibited higher levels of body fat. The FCR and PER were best in fish fed diets with up to 42 % vegetable oil inclusion. Moreover, mortality rate was higher in the experimental fish fed diets with higher levels of soybean oil (i.e. 42, 56 and 70 %). It would be interesting, thus, to investigate the effects of fish oil substitution on the health of fish, to obtain a better understanding of the reasons for the differences in growth and condition of the fish observed here. This is further assessed on Chapter 3.

## CHAPTER 3

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### THE EFFECT OF DIETARY FISH OIL REPLACEMENT BY SOYBEAN OIL ON THE HEALTH OF DUSKY KOB, *ARGYRO SOMUS JAPONICUS*

#### Introduction

Increasing aquaculture production has stimulated a rise in the global demand for fish oil (FAO, 2010b). Because fish oil production varies with catches, which have reached a ceiling, prices have risen, and vegetable oils are being progressively investigated as substitutes for fish oil in aquafeeds (Turchini *et al.*, 2009). The effect of the inclusion of vegetable oils on growth parameters of fish has been extensively evaluated. Besides direct effects on growth rate, the effect on the health of fish is also an important factor to be elucidated, as the inclusion of these lipid sources in the diets may cause imbalances in the fatty acids, therefore affecting the tissues (Alexis, 1996; Wassef *et al.*, 2007).

Polyunsaturated fatty acids (PUFA) are essential for fish to maintain normal cellular structure and function (Bell *et al.*, 1999). These fatty acids must be obtained through their diets since they cannot be synthesised *de novo* by the fish (Bell *et al.*, 1999). Marine fish require long-chain PUFA, also known as highly unsaturated fatty acids (HUFA), especially eicosapentaenoic acid (EPA 20:5 n-3) and docosahexaenoic acid (DHA 22:6 n-3; Turchini *et al.*, 2009). Dietary lipid changes are, however, not reflected in the same way in the fish tissues for the different classes of lipids; neutral lipids are greatly influenced by dietary fatty acid composition when compared to phospholipids, whose concentration remains almost constant (Corraze, 2001).

In some fish species, the liver stores energy in the form of triacylglycerols (Lie *et al.*, 1986; Corraze, 2001). Hence, when dietary lipid or energy is in excess and the hepatic cells are incapable of oxidising the fatty acids, or when protein

synthesis decreases, a large synthesis and deposition of triacylglycerols occurs (Caballero *et al.*, 2004). This lipid deposition might lead to fatty liver disease as there is no pathway for the excretion of excess lipid (Caballero *et al.*, 2004). Moreover, imbalance in dietary fatty acids might result in deficiency syndromes, which may also cause fatty infiltration in the liver (Cowey & Roberts, 1978; Watanabe, 1982). The many enzymes that regulate lipid metabolism in fish liver, including synthesis and degradation of fatty acids, have affinities for different fatty acids (Caballero *et al.*, 2004). Therefore an imbalance in dietary fatty acids may also modify the function and morphology of the liver (Caballero *et al.*, 2004).

Fish are sensitive to changes in their environment, and the structures in the tissue cells are often affected by these changes. Histological investigation of cellular disturbance in target organs can be used as indication that fish have been subjected to environmental stress, including nutritional stress (Harper & Wolf, 2009). Histology can be an important tool in determining if fish were nutritionally challenged in feeding trials (Mosconi-bac, 1990; Ostaszewska & Sawosz, 2004; Bolla *et al.*, 2011).

Histopathological analysis of fish liver is a good health indicator (Richardson *et al.*, 2010). This organ is an efficient model when studying interactions between environmental factors and its structures and functions (Bruslé & Anadon, 1996). Fish liver structure can vary in relation to gender, age, temperature, and food, especially regarding to glycogen and lipid content (Genten *et al.*, 2009). The teleosts liver can be used to indicate the nutritional status of the fish, the nutritional balance of the diet, as well as evaluate the sanitary conditions of the organism's environment (Bruslé, 1990). Furthermore, research on fish liver is becoming progressively important in the field of stress induced by aquaculture (Verreth *et al.*, 1994; Robaina *et al.*, 1995; Morais *et al.*, 2001).

The fish liver is a relatively large digestive gland crucial in many aspects of intermediary metabolism associated with digestion. It controls the metabolism of proteins, lipids and carbohydrates, as well as detoxification, glycogenolysis, and catabolism of nitrogen (Bruslé & Anadon, 1996). In some teleosts, the liver is a

compound organ in the form of a hepatopancreas, while in others the pancreas is found as a separate organ (Roberts & Ellis, 2001). It is usually reddish brown in carnivores and lighter brown in herbivores; however, in cultured fish it tends to be lighter in colour when compared to the wild species, due to the higher level of lipids in their diets (Bruslé & Anadon, 1996; Roberts & Ellis, 2001).

The histology of fish liver differs from mammalian since the hepatocytes are less likely to be displayed in cords or lobules (Ashley, 1975; Roberts & Ellis, 2001). The lack of such organization has real implications for pathologists because this valuable tool for the morphological categorization of liver disease is not applicable to fish (Wolf & Wolfe, 2005). In fish liver the intracellular bile canaliculi join together to form the bile ducts (Roberts & Ellis, 2001). These ducts are merged and eventually meet the gallbladder that stores the bile (Roberts & Ellis, 2001). Sinusoids are irregularly distributed between hepatocytes (Roberts & Ellis, 2001). They are fewer in number compared to mammals and lined by endothelial cells (Ellis *et al.*, 1978). Hepatocytes of the hepatic parenchyma of fish are polygonal-shaped cells with spherical nuclei, that typically contains a single central nucleolus (Ellis *et al.*, 1978; Bruslé & Anadon, 1996). They are separated from the exocrine pancreatic cells by a thin septum of connective tissue (Bruslé & Anadon, 1996).

Mammalian livers include Von Kupffer cells, which are part of the macrophage system, with the function of phagocytosis (Naito *et al.*, 2004). In most teleosts there is an absence of efficient Von Kupffer cells in the liver (Wolf & Wolfe, 2005; Genten *et al.*, 2009). However, pigment-containing macrophages are commonly observed in higher teleosts. They are concentrated in the melanomacrophage aggregates which are located in the stroma of hemopoietic tissue, around blood vessels and lymphatics, in the peritoneum and within healing wounds (Roberts, 1975). In some species melanomacrophage aggregates form discrete structures, but in others such as salmonids they are randomly distributed throughout the tissues that contain them and have a high proportion of dark pigments (Agius, 1985 cited by Agius & Roberts, 2003). Generally, macrophage cells increase in number and/or size (surface area) when fish are exposed to environmental changes and stress (Blazer *et al.*,

1987; Vogelbein *et al.*, 1987; Rios *et al.*, 2007). Moreover, pale staining pigment in melanomacrophage aggregates and degeneration in haemopoietic tissue occur when fish are fed diets with high contents of fat (George, 1987). Amongst all functions of the melanomacrophage aggregates, they process antigens in the immune response, and remove particulate and soluble material from the bloodstream. In addition, they store iron as hemosiderin, perform the catabolism of tissues and storage of waste products such as ceroid and lipofuscin as well as melanin (Roberts, 1975; Roberts, 1989).

Ceroid is one of the major components of melanomacrophage aggregates, and it accumulates with the age of the fish and damage of the tissue (Wolke *et al.*, 1985; Montero *et al.*, 1999a). This pigment is formed by the oxidative polymerization of PUFA (Agius & Roberts, 2003). The presence of ceroid pigment in fish has been associated to dietary deficiencies, especially vitamin E, which is a natural antioxidant, as well as stress conditions (Montero *et al.*, 1999a). Moreover, excessive fat in fish diets can also lead to an increase in ceroid pigment in fish liver (George, 1987).

Growing animals constantly form tissues in their bodies, and some of the dietary energy that is stored in these tissues is stored as protein, lipids or glycogen. Glycogen is the stored form of carbohydrates in fish muscle and liver (Harmon *et al.*, 2011), and its quantity in the fish varies between species (Wolf & Wolfe, 2005; Enes *et al.*, 2009). Fish liver generally contains considerable amounts of glycogen in the cytoplasm (Hibiya, 1982), especially captive fishes which are intensively fed (Wolf & Wolfe, 2005).

Glycogen levels in fish liver are a good indicator of stress (Barton, 2002). When liver glycogen is required it is broken down by enzymes and transported to the extrahepatic tissues as glucose (Enes *et al.*, 2009; Barcellos *et al.*, 2010). Hepatic glycogen is usually the primary energy source utilized by fish at the first moments of critical stress situations (Christiansen & Klungsoyr, 1987; Enes *et al.*, 2009); for example, glycogen content decreased when fish were exposed to toxicants, anoxia, and when they were deprived of food (Nilsson, 1990; Boeck *et al.*, 2010; Peres *et al.*, 2011; Saravanan *et al.*, 2011). In addition, glycogen

levels in fish liver also decreased due to the inclusion of vegetable ingredients in the diets (Robaina *et al.*, 1995).

Fish response to stress can be featured into primary, secondary and tertiary (Barton & Iwama, 1991). At first, fish mainly release cortisol and adrenaline into the bloodstream (Barton, 2002). The second responses, caused by the neuroendocrine stimulation of the primary responses, are the metabolic, haematological, hydromineral and structural (Barton & Iwama, 1991; Frisch & Anderson, 2000). The tertiary responses to stress are the “whole animal” changes, including growth and condition indices (Barton & Iwama, 1991; Barton, 2002). Thus, in addition to the histological evaluation of the liver, haematological assessment is also a good indicator of fish health and stress conditions (Celik & Aydin, 2006). Given that the aquaculture industry is rapidly developing, information on the blood parameters of cultured fish will increasingly gain attention (Akinrotimi *et al.*, 2010).

Blood is one of the most dynamic tissues of an organism and its characteristics may alter due to environmental changes (Ochang *et al.*, 2007; Araujo *et al.*, 2011). Haemoglobin is an important blood parameter to consider since its main function is the transport of oxygen to the tissues. The haemoglobin values of fish blood may decrease when they are fed essential fatty acid deficient diets (Tacon, 1995). Similarly, it may also reduce when fish are fed diets with oxidized lipids or deficient in some vitamins (Sargent *et al.*, 2002). Haematocrit is the packed red cell volume of the blood expressed as a percentage of the total column, and it is generally measured in micro-haematocrit tubes (Gallaughier & Farrell, 1998). An example of a stress situation that influenced haematocrit is for Atlantic cod, *Gadus morhua*, which had the percentage haematocrit decreased after two days of induced anaemia (Burke, 2009). The red cells in the blood are a major site of production of active oxygen species, hydrogen peroxide, hydroxyl radical and singlet oxygen (Roche & Bogé, 1996). The number of these cells are also influenced by environmental stressors; for instance, *Sparus aurata* had an increase in red blood cell counts when fed essential fatty acid deficient diets (Montero *et al.*, 2004). Similarly, when African catfish (*Clarias gariepinus*) were fed diets in which cod liver oil was replaced by

palm oil, fish displayed increasing levels of red blood cells with increasing palm oil in the diets (Ochang *et al.*, 2007). Hence, these parameters are susceptible to changes when fish are exposed to stress situations such as being fed diets with altered lipid sources.

The objective of this trial was to describe, using health indexes, as well as fatty acid analyses and macroscopic and microscopic examinations, the changes in the body and liver of *A. japonicus* fed experimental diets containing graded levels of soybean oil replacing fish oil as an alternative source of lipid. The aim was to gain some insight into the pathologic mechanism of the fatty acid deficient diets.

## **Material and methods**

### Experimental design

The data presented in this chapter were collected from the fish in the experiment described in Chapter 2. As such, these data were collected from fish subjected to the experimental conditions described in Chapter 2, from the same fish that were fed diets with 46 % protein and 18 % lipid, in which fish oil was increasingly replaced by soybean oil (Chapter 2, Table 2.1). Data were collected from fish that had been fed these diets for 206 days.

### Macroscopic and morphologic evaluation of liver, gills and heart

Three fish per tank were randomly sampled (i.e. nine fish per treatment) after 206 days of feeding the different diets. They were sacrificed with a 2-phenoxyethanol bath at a concentration of 0.4 mg L<sup>-1</sup> as recommended by Brown (2003), weighed to the nearest gram and measured to the nearest millimetre. Gills, heart and liver colour and condition were recorded.

### Visceral fat index and hepatosomatic index

The same fish that were sacrificed for the evaluation of the liver colour had their perivisceral fat and livers removed and weighed.

Visceral fat index (VFI, %) was calculated using Equation 1:

$$VFI \% = (W_{VF}) \times (W_{Fd})^{-1} \times 100 \quad (1)$$

where  $W_{VF}$  represents visceral fat weight (g) and  $W_{Fd}$  stands for eviscerated fish weight (g).

Hepatosomatic index (HSI, %) was calculated using Equation 2:

$$HSI \% = (W_{liver}) \times (W_{Fd})^{-1} \times 100 \quad (2)$$

where  $W_{liver}$  represents liver weight (g) and  $W_{Fd}$  stands for eviscerated fish weight (g).

### Haematological parameters

Three fish per tank (i.e. nine per treatment) were randomly captured with a net and anesthetized with 2-phenoxyethanol at 0.2 mL L<sup>-1</sup> after 206 days of feeding the different diets. Blood was then collected by an acute cardiac puncture with heparinised syringes (Saravanan *et al.*, 2011). Samples were stored in dipotassium-ethylenediamine-tetra-acetic acid (EDTA) vacuum tubes and immediately taken to the laboratory for analysis. They were analysed for haematocrit, haemoglobin and count of red blood cells in a haematology analyzer (Beckman Coulter® Ac.T 5diff).

### Lipid extraction and fatty acid analysis

Dietary total lipid was determined by a chloroform/methanol total lipid extraction (Phillips *et al.*, 1997). The fatty acids of the diets were assessed according to Jaarsveld (*et al.*, 2000).

After weighing the livers for HSI calculation, a small fraction of each sampled liver (i.e. nine per treatment) was freeze dried in liquid nitrogen and pulverized. Approximately 50 mg was weighed and the weight was recorded. A Chloroform/Methanol extraction method using gas chromatography (Folch *et al.*, 1957; Tichelaar *et al.*, 1989) was used. Heptadecanoic acid (17:0) was used as internal standard. In the case of the triglycerides, a similar method used by Hon *et al.* (2009) was used, but the triglyceride band was extracted.

### Histological preparation and analysis

The remaining fractions of each sampled liver were fixed in 10 % buffered formalin for histological examination. They were dehydrated in a graded ethanol series and then embedded in paraffin wax. Sections of four µm were cut and stained with haematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and periodic acid-Schiff diastase (PASD) for light microscopy examination (Genten *et al.*, 2009). The PASD stain was necessary to discriminate the PAS positive reaction due to glycogen from other PAS positivity, such as ceroid pigment (Page *et al.*, 2005).

Examination of histological slides of each fish was performed using a Nikon Eclipse E400 biological microscope (Nikon, Tokyo, Japan) at magnifications of 10x, 20x, 40x, and 100x. A semi-quantitative evaluation (Table 3.1) was made adapted from Wood *et al.* (1957), where:

- Liver architecture, bile ducts, hepatopancreas, and hepatocyte nuclei were rated as: 1 – abnormal, to 5 – normal. In the case of scoring hepatocyte nuclei, a normal nucleus was considered to be spherical, with

central nucleoli and an abnormal nucleus was shrunken and piknotic, with condensed chromatin content inside the nucleoli.

- Nuclei displacement was rated as: 1 – nuclei completely shifted to hepatocyte edges, to 5 – nuclei in the middle of the cells.
- Lipid and glycogen contents were rated as: 1 – no lipid or glycogen deposit, to 5 – entire cell filled with lipid or glycogen. After histological processing of the slides, the areas occupied by lipid droplets appeared as clear circular or polygonal vacuoles with H&E staining (Hibiya, 1982).
- Fat zones were rated as: 1 – prominent zones, to 5 – no zones.
- Melanin surrounding bile ducts and ceroid pigment in hepatocytes were rated as: 1 – large amount of melanin or ceroid pigment, to 5 – no melanin or ceroid pigment.
- Melanomacrophage aggregates were counted in two randomly chosen different areas of the slide in a magnification of 10x.

**Table 3.1.** Semi-quantitative evaluation of dusky kob histological features, with scores ranging from 1 to 5.

	1	—————>	5
General architecture	abnormal		normal
Bile ducts	abnormal		normal
Hepatopancreas	abnormal		normal
Hepatocyte nuclei	spherical with central nucleoli		shrunken and pycnotic
Hepatocyte nuclei displacement	nuclei shifted to hepatocyte edges		nuclei located in the middle of the hepatocytes
Lipid content	no lipid deposit		entire cell filled with lipid
Glycogen content	no glycogen deposit		entire cell filled with glycogen
Lipid vacuolated zones	prominent zones		no zones
Melanine surrounding bile ducts	large amount of melanine		no melanine
Ceroid pigment content	large amount of ceroid pigment		no ceroid pigment

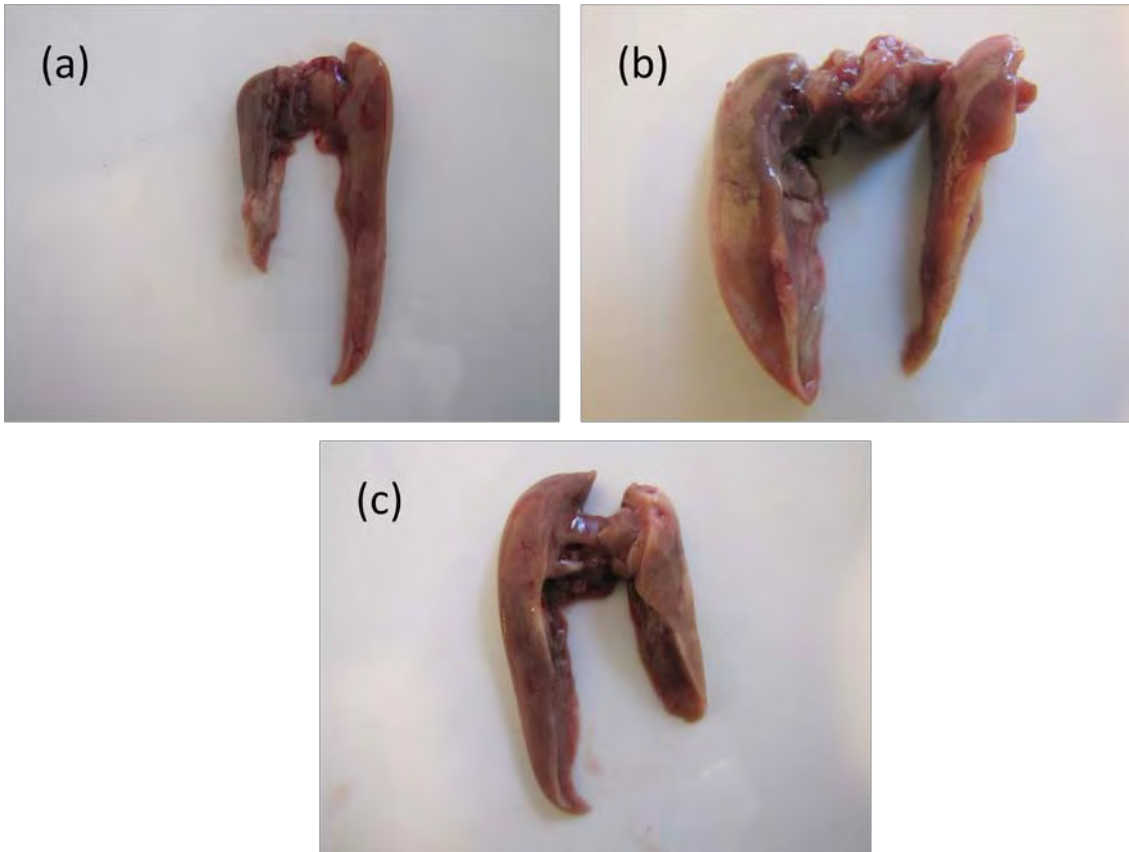
## Statistical analysis

Treatment means were compared with one-way analysis of variance (ANOVA). Tukey's test was performed if any significant differences were detected ( $p < 0.05$ ). If the data did not meet the assumptions of an ANOVA (Shapiro-Wilk's test for normality of the residuals and Levene's test for homogeneity of variance), treatment means were compared using a non-parametric Kruskal-Wallis test and a multiple comparison of  $z'$  values ( $p < 0.05$ ). Regression models were calculated using the mean parameter of each tank as the unit of measure ( $p < 0.05$ ), whereas the combined mean and standard errors were plotted on the same axis as the regression models. These analyses were carried out using STATISTICA version 9 (Statsoft, Tulsa, OK, USA). Chi square was used to determine differences in frequency distribution.

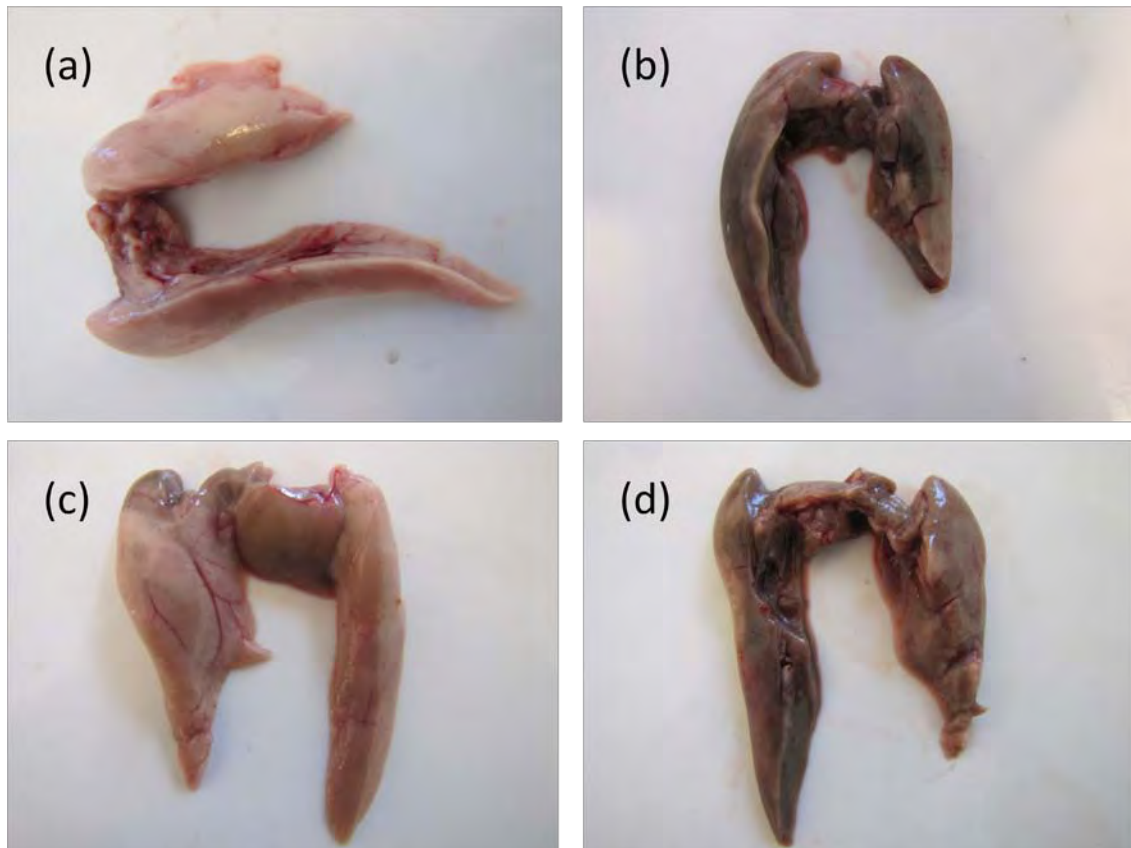
## **Results**

### Macroscopic and morphologic evaluation of the liver

No macroscopic and morphological changes were observed in liver from fish fed the control diet and diets with up to and including a 28 % vegetable oil diets. These livers showed a healthy, light reddish-brown colour (Figure 3.1). Moreover, liver tissue from fish fed these diets was "firmer" compared to the others. Livers from fish fed diets where 42 to 70 % of the lipids were originated from soybean oil were either noticeably pale or dark green, appeared swollen and were easily broken (Figure 3.2).



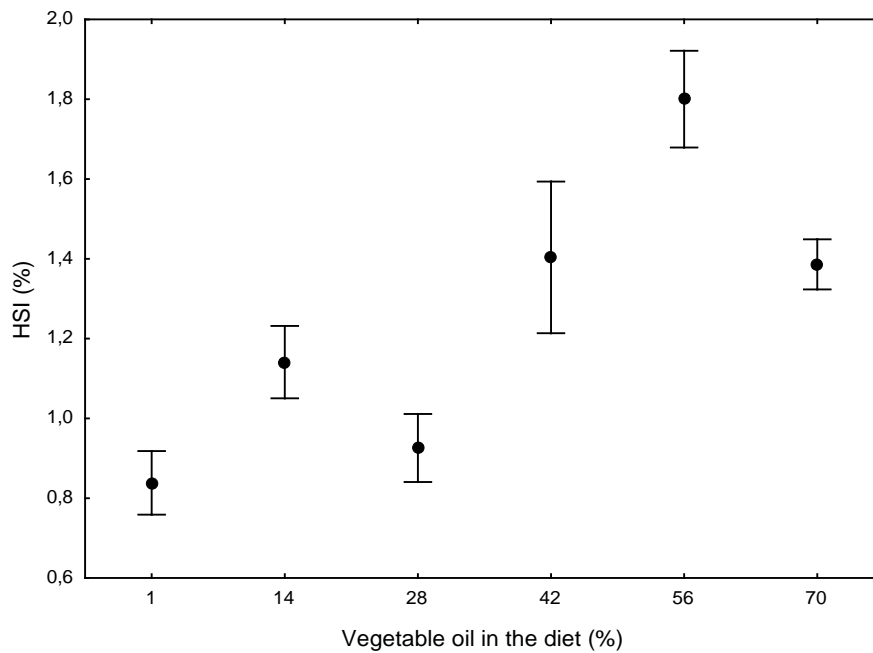
**Figure 3.1.** Healthy, reddish-brown livers from fish fed a diet with (a) no vegetable oil substitution (i.e. control), (b) 14 % vegetable oil substitution and (c) 28 % substitution, for 206 days.



**Figure 3.2.** (a) The pale liver and (b) dark green liver from fish fed a diet where 42 % of the total lipids originated from vegetable oil. (c) The pale liver of a fish fed a 56 % substitution diet and (d) dark green liver of a fish fed the 70 % substitution diet.

### Hepatosomatic index

There was a significant difference in HSI of dusky kob fed the different diets ( $F_{(5, 12)} = 9.90$ ,  $p = 0.0006$ ) after 206 days. Fish fed diets with higher inclusion of soybean oil (i.e. 42, 56 and 70 %) showed higher HSI values, compared to those fed diets with lower inclusion (i.e. control diet and diets with 14 and 28 % soybean oil), ranging from  $0.84 \pm 0.08$  to  $1.80 \pm 0.12$  % in the control diet and the 56 % soybean oil diet, respectively. Moreover, there was a significant increasing trend in HSI with the inclusion of soybean oil in the diets ( $r^2 = 0.49$ ,  $p = 0.01$ ; Figure 3.3).



**Figure 3.3.** Mean percent ( $\pm$  standard error) of hepatosomatic index (HSI) of *A. japonicus* fed diets with different levels of replacement of fish oil by soybean oil for 206 days ( $y = 0.873 + 0.0107x$ ,  $r^2 = 0.49$ ,  $p = 0.001$ ).

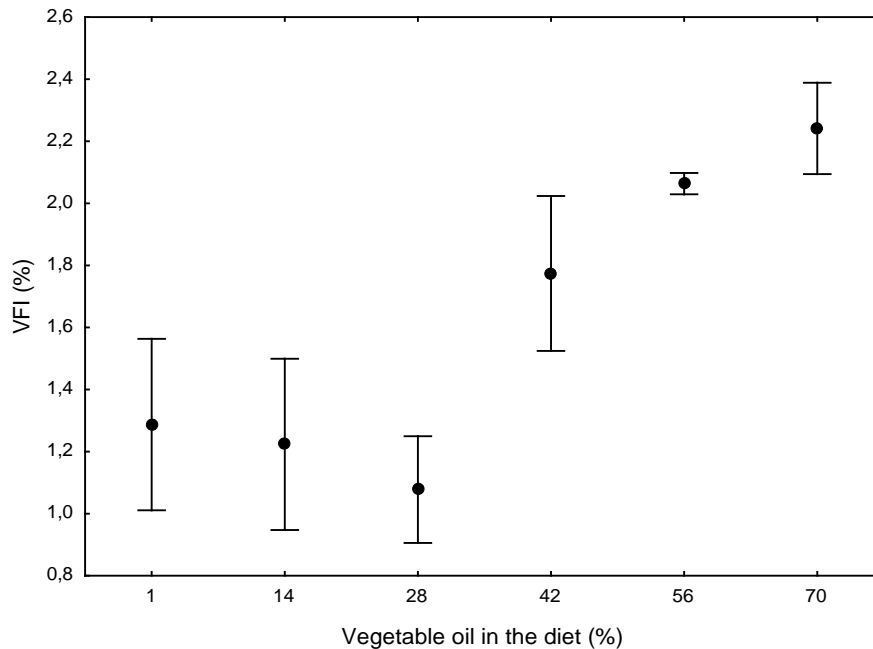
### Macroscopic evaluation of gills and heart

No evidence of anaemia was observed in the gills (Hardy, 2001), which showed red colouration. The heart of fish from all treatments exhibited a vivid-red colour and normal shape.

### Visceral fat index

The VFI of fish fed the different diets were significantly different after 206 days of feeding ( $F_{(5, 15)} = 5.25$ ,  $p = 0.009$ ). Although there was no significant difference between the VFI of fish fed the control diet with overall means ( $\pm$  standard error) of  $1.29 \pm 0.28$  %, and the diet with the highest inclusion of soybean oil ( $2.24 \pm 0.15$  %), fish fed the diet 70 % vegetable oil diet had significantly higher VFI compared to fish fed diets with 14 % ( $1.22 \pm 0.28$  %) and 28 % ( $1.08 \pm 0.17$  %) vegetable oil. In addition, there was a significant trend

of an increase in VFI ( $r^2 = 0.54$ ,  $p = 0.0005$ , Figure 3.4) with increasing soybean oil in the diets.



**Figure 3.4.** Mean percentage ( $\pm$  standard error) of visceral fat index (VFI) of *A. japonicus* fed diets with different levels of replacement of fish oil by soybean oil for 206 days ( $y = 1.029 + 0.0165x$ ,  $r^2 = 0.54$ ,  $p = 0.0005$ ).

### Haematological parameters

After 206 days of feeding, the percentage haematocrit and haemoglobin concentration in the blood were not affected by the substitution of fish oil with soybean oil in the diets with overall means ( $\pm$  standard error) of  $27.41 \pm 0.84$  % ( $H_{(5, 17)} = 3.76$ ,  $p = 0.59$ ), and  $6.92 \pm 0.20$  g dL<sup>-1</sup> ( $F_{(5, 11)} = 0.41$ ,  $p = 0.83$ ), respectively (Table 3.2). Similarly, there was no significant difference in red blood cell count with increasing replacement of vegetable oil in the diets with overall mean ( $\pm$  standard error) of  $2.76 \pm 0.08 \times 10^{12}$  L<sup>-1</sup> ( $X^2 = 0.03$ ,  $p = 0.99$ ; Table 3.2).

**Table 3.2.** Mean ( $\pm$  standard error) haematocrit, haemoglobin, and red blood cell count of *A. japonicus* fed diets with different levels of replacement of fish oil by soybean oil for 206 days.

	Vegetable oil 1 %	Vegetable oil 14 %	Vegetable oil 28 %	Vegetable oil 42 %	Vegetable oil 56 %	Vegetable oil 70 %	F / H value	p value	$\chi^2$
Haematocrit %	29.33 $\pm$ 2.19	28.67 $\pm$ 0.33	25.50 $\pm$ 0,50	26.00 $\pm$ 2.08	25.33 $\pm$ 3.67	29.00 $\pm$ 1.00	$F_{(5, 11)} = 3.76$	0.59	-
Haemoglobin g dL <sup>-1</sup>	7.35 $\pm$ 0.53	7.31 $\pm$ 0.90	6.62 $\pm$ 0,29	6.74 $\pm$ 0.56	6.59 $\pm$ 0.78	6.84 $\pm$ 0.47	$H_{(5, 11)} = 0.41$	0.83	-
Red blood cell count $\times 10^{12}$ L <sup>-1</sup>	2.93 $\pm$ 0.25	2.86 $\pm$ 0.04	2.63 $\pm$ 0,21	2.65 $\pm$ 0.15	2.62 $\pm$ 0.37	2.83 $\pm$ 0.09	-	0.99	0.03

### Fatty acid composition of the diets

The fatty acid composition of the experimental diets is presented in Table 3.3.

The control diet was characterized by high concentrations of EPA (20:5 n-3) and DHA (22:6 n-3). Both fatty acids decrease with increasing soybean oil content in the diets. The amount of linoleic acid (18:2 n-6) and  $\alpha$ -linolenic acid (18:3 n-3) in the diets increased with increasing fish oil substitution by vegetable oil. Consequently, the n-3/n-6 ratio was significantly reduced with every incremental increase of soybean oil inclusion, ranging from 5.8 in the control diet to 1.20 % in the diet with the higher substitution level. The content of oleic acid (18:1 n-9) slightly increased with increasing vegetable oil in the diets.

The control diet had higher saturated fatty acids and total n-3 fatty acids content. The EPA/DHA ratio was higher in the control diet, and it decreased with increasing soybean oil inclusion in the diets. Arachidonic acid (AA 20:4 n-6) was higher in the control diet and it decreased with soybean oil inclusion.

**Table 3.3.** Fatty acid profile (% total fatty acids) in the experimental diets, with graded levels of vegetable oil that were fed to *A. japonicus*.

Fatty acids	Vegetable oil 1 %	Vegetable oil 14 %	Vegetable oil 28 %	Vegetableoil 42 %	Vegetable oil 56 %	Vegetable oil 70 %
14:0	5.37	4.98	4.57	4.18	3.79	3.42
16:0	18.90	18.40	17.87	17.34	16.84	16.35
18:0	5.48	5.51	5.55	5.58	5.61	5.64
20:0	0.51	0.51	0.51	0.52	0.52	0.52
22:0	0.23	0.25	0.27	0.29	0.31	0.33
24:0	0.34	0.34	0.34	0.34	0.34	0.35
Saturated	30.83	30.00	29.11	28.25	27.41	26.59
16:1 n-7	6.01	5.53	5.00	4.50	4.01	3.53
18:1 n-9	15.18	15.49	15.82	16.14	16.45	16.75
18:1 n-7	3.28	3.15	3.02	2.90	2.77	2.66
20:1 n-9	3.41	3.27	3.13	2.99	2.85	2.72
24:1 n-9	1.49	1.43	1.37	1.31	1.26	1.20
Monounsaturated	29.36	28.87	28.35	27.84	27.34	26.86
18:2 n-6 (linoleic)	3.57	6.59	9.82	12.95	16.00	18.97
18:3 n-6	0.15	0.14	0.12	0.10	0.09	0.07
20:2 n-6	0.23	0.22	0.22	0.21	0.20	0.19
20:3 n-6	0.15	0.14	0.13	0.12	0.10	0.09
20:4 n-6 (AA)	1.61	1.56	1.50	1.45	1.40	1.35
22:4 n-6	0.13	0.13	0.13	0.12	0.12	0.11
22:5 n-6	0.49	0.47	0.45	0.43	0.41	0.39
Total n-6	6.34	9.26	12.36	15.38	18.32	21.18
18:3 n-3 ( $\alpha$ -linolenic)	0.75	1.28	1.85	2.40	2.93	3.45
20:3 n-3	0.02	0.02	0.01	0.01	0.00	0.00
20:5 n-3 (EPA)	13.75	12.43	11.02	9.65	8.31	7.01
22:5 n-3	2.99	2.86	2.72	2.58	2.45	2.32
22:6 n-3 (DHA)	15.94	15.28	14.58	13.90	13.23	12.59
Total n-3	33.46	31.87	30.18	28.53	26.93	25.37
AA/EPA	0.12	0.13	0.14	0.15	0.17	0.19
EPA/DHA	0.86	0.81	0.76	0.69	0.63	0.56
n-3/n-6	5.28	3.44	2.44	1.85	1.47	1.20

### Fatty acid composition of the liver

The fatty acid composition of the liver of dusky kob is presented in Table 3.4.

Fish fed the control diet showed the highest levels of total saturated fatty acids, which ranged from 28.63 % in the liver of fish fed the control diet, to 20.55 % in fish fed the 70 % vegetable oil diet. Similarly, monounsaturated fatty acids decreased with increasing vegetable oil in the diets. Percentage of oleic acid increased with increasing soybean oil in the diets, and, moreover, there was a significant difference (ANOVA  $F_{(5, 48)} = 68.42$ ,  $p < 0.01$ ) in the amount of oleic acid among treatments.

Due to the large content of linoleic acid in soybean oil and meal, the total n-6 fatty acids in fish liver increased substantially from 8.92 % in fish fed the control diet to 43.62 % in fish fed the diets with the highest substitution of fish oil. Furthermore, there was a significant difference in linoleic acid (Kruskal-Wallis  $H_{(5, 54)} = 51.55$ ,  $p < 0.01$ ), increasing from 5.48 to 42.05 % in liver of fish fed the control diet and those fed the diet with 70 % soybean oil, respectively. There was also a significant difference in  $\alpha$ -linolenic acid (ANOVA  $F_{(5, 48)} = 285.01$ ,  $p < 0.01$ ).

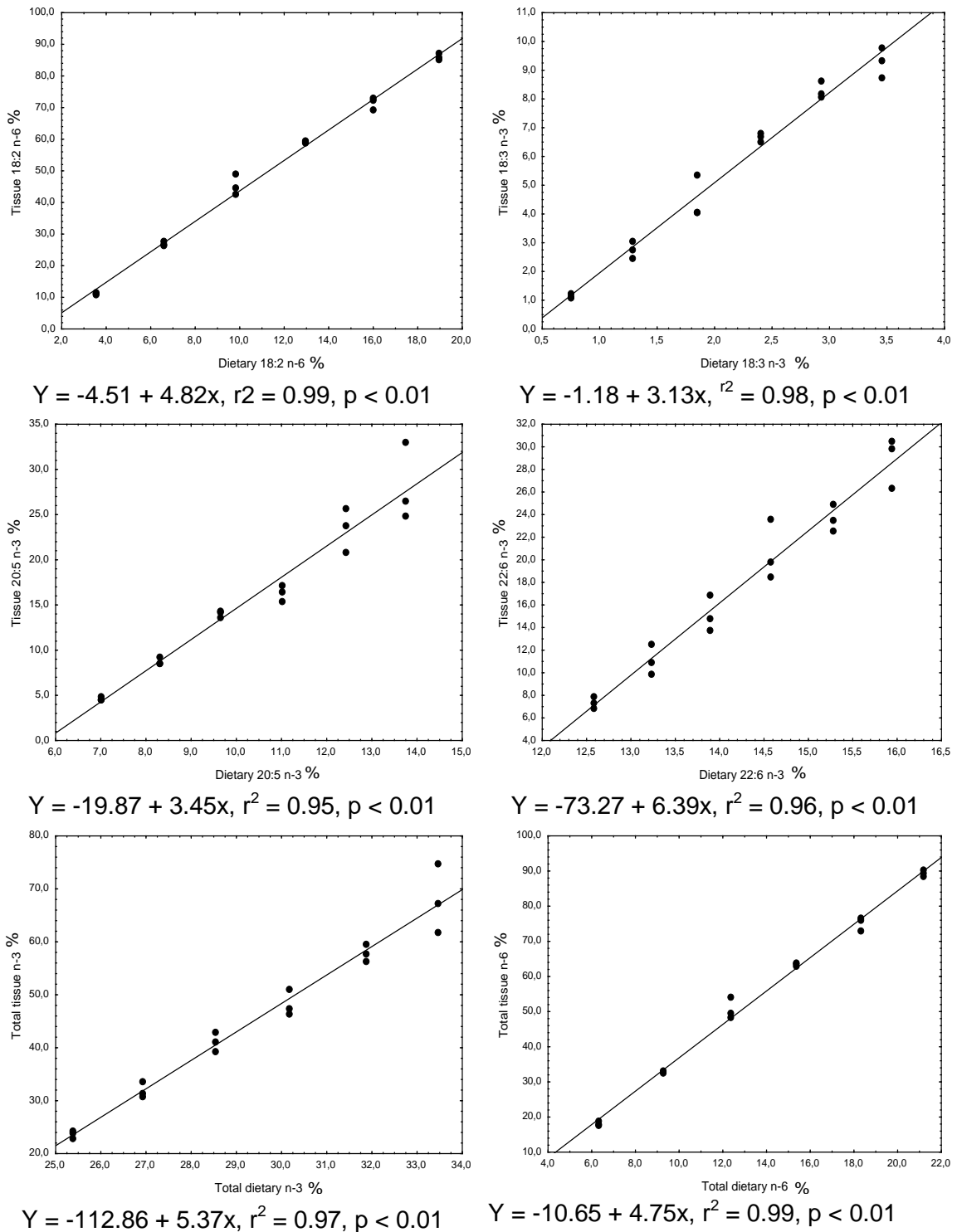
The percentage of DHA in dusky kob livers was significantly different, ranging from 14.34 % in fish fed the control diet, to 3.28 % in fish fed the 70 % soybean oil diet (ANOVA  $F_{(5, 48)} = 97.37$ ,  $p < 0.01$ ). A significant difference was also observed in the EPA content of the liver, ranging from 13.63 to 1.97 % in fish fed the control diet and fish fed the diet with the highest replacement level, respectively (Kruskal-Wallis  $H_{(5, 54)} = 50.33$ ,  $p < 0.01$ ). There was also a significant difference in EPA/DHA ratio among treatments (ANOVA  $F_{(5, 48)} = 10.30$ ,  $p < 0.01$ ). The n-3/n-6 ratio was significantly reduced (Kruskal-Wallis  $H_{(5, 54)} = 51.55$ ,  $p < 0.01$ ) with the inclusion of soybean oil in the diets. AA/EPA ratio was significantly different among treatments (ANOVA  $F_{(5, 48)} = 17.17$ ,  $p < 0.01$ ).

The concentrations of 18:2 n-6, 18:3 n-3, 20:5 n-6 and 22:6 n-3, as well as total n-3 and total n-6 in the diets were linearly correlated to their concentrations in the fish liver tissue (Figure 3.5).

**Table 3.4.** Fatty acid profile (% total free fatty acids) of liver from *A. japonicus* fed diets with different levels of soybean oil for 206 days.

Fatty acids	Vegetable oil 1 %	Vegetable oil 14 %	Vegetable oil 28 %	Vegetable oil 42 %	Vegetable oil 56 %	Vegetable oil 70 %
14:0	4.08	3.34	2.67	1.99	1.25	0.84
16:0	20.42	19.55	18.11	17.31	15.68	15.28
18:0	3.87	3.97	3.64	3.85	4.51	4.11
20:0	0.21	0.22	0.17	0.18	0.19	0.16
22:0	0.02	0.04	0.05	0.07	0.09	0.09
24:0	0.04	0.05	0.05	0.06	0.07	0.07
Saturated	28.63	27.18	24.69	23.47	21.78	20.55
16:1 n-7	11.54	9.87	7.84	6.10	4.15	3.16
18:1 n-9*	11.44 <sup>a</sup>	12.92 <sup>b</sup>	14.32 <sup>c</sup>	15.23 <sup>c</sup>	17.13 <sup>d</sup>	18.81 <sup>e</sup>
18:1 n-7	3.87	3.52	2.99	2.65	2.38	1.98
20:1 n-9	1.63	1.44	1.22	1.14	1.03	0.93
24:1 n-9	0.48	0.41	0.35	0.27	0.19	0.15
Monounsaturated	28.96	28.15	26.72	25.39	24.88	25.03
18:2 n-6* (linoleic)	5.48 <sup>a</sup>	13.45 <sup>ab</sup>	22.54 <sup>abc</sup>	29.25 <sup>bcd</sup>	35.91 <sup>cd</sup>	42.05 <sup>d</sup>
18:3 n-6	0.25	0.21	0.15	0.12	0.07	0.06
20:2 n-6	0.25	0.31	0.37	0.45	0.57	0.65
20:3 n-6	0.24	0.20	0.16	0.11	0.08	0.04
20:4 n-6* (AA)	2.14 <sup>a</sup>	1.70 <sup>b</sup>	1.49 <sup>bc</sup>	1.12 <sup>cd</sup>	0.80 <sup>de</sup>	0.62 <sup>e</sup>
22:4 n-6	0.17	0.16	0.13	0.10	0.08	0.06
22:5 n-6	0.40	0.34	0.28	0.22	0.15	0.13
Total n-6	8.92	16.37	25.12	31.35	37.67	43.62
18:3 n-3* (α-linolenic)	0.56 <sup>a</sup>	1.35 <sup>b</sup>	2.20 <sup>c</sup>	3.25 <sup>d</sup>	4.08 <sup>e</sup>	4.37 <sup>e</sup>
20:3 n-3	0.01	0.01	0.01	0.04	0.06	0.10
20:5 n-3* (EPA)	13.63 <sup>a</sup>	11.23 <sup>ab</sup>	7.77 <sup>ab</sup>	6.56 <sup>bc</sup>	4.19 <sup>c</sup>	1.97 <sup>c</sup>
22:5 n-3	4.94	4.03	3.42	2.59	1.84	1.08
22:6 n-3* (DHA)	14.34 <sup>a</sup>	11.68 <sup>b</sup>	10.05 <sup>b</sup>	7.35 <sup>c</sup>	5.50 <sup>d</sup>	3.28 <sup>e</sup>
Total n-3	33.49	28.30	23.46	19.79	15.67	10.80
AA/EPA*	0.16 <sup>a</sup>	0.15 <sup>a</sup>	0.19 <sup>a</sup>	0.17 <sup>a</sup>	0.19 <sup>a</sup>	0.31 <sup>b</sup>
EPA/DHA*	0.96 <sup>ab</sup>	0.97 <sup>a</sup>	0.79 <sup>bcd</sup>	0.91 <sup>abc</sup>	0.77 <sup>cd</sup>	0.61 <sup>d</sup>
n-3/n-6*	3.79 <sup>a</sup>	1.73 <sup>ab</sup>	0.94 <sup>abc</sup>	0.63 <sup>bcd</sup>	0.42 <sup>cd</sup>	0.25 <sup>d</sup>

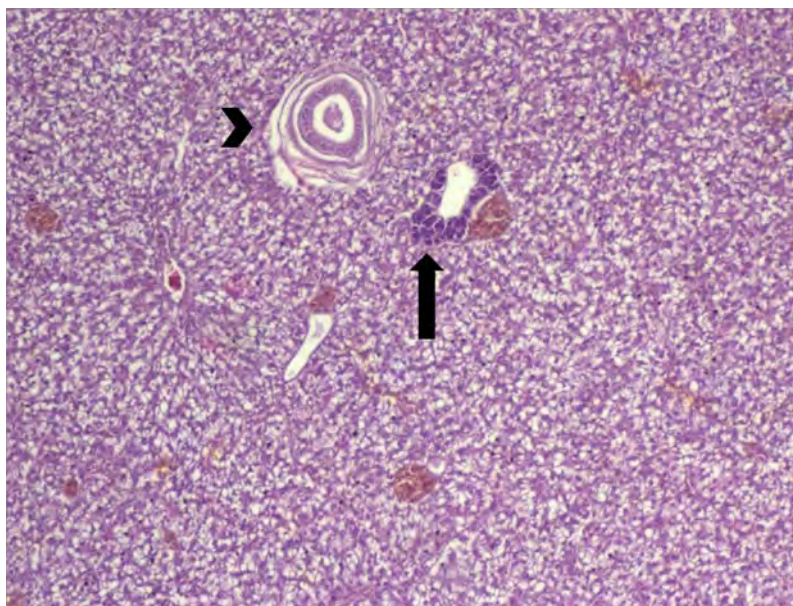
\* Fatty acids and ratios analysed for significant differences (ANOVA,  $p < 0.05$ )



**Figure 3.5.** Positive relationships between percentage dietary fatty acid concentrations and percentage liver tissue fatty acid concentrations of 18:2 n-6, 18:3 n-3, 20:5 n-3, 22:6 n-3, total n-3 and total n-6 in total lipids of *A. japonicus* fed experimental diets with graded levels of vegetable oil for 206 days.

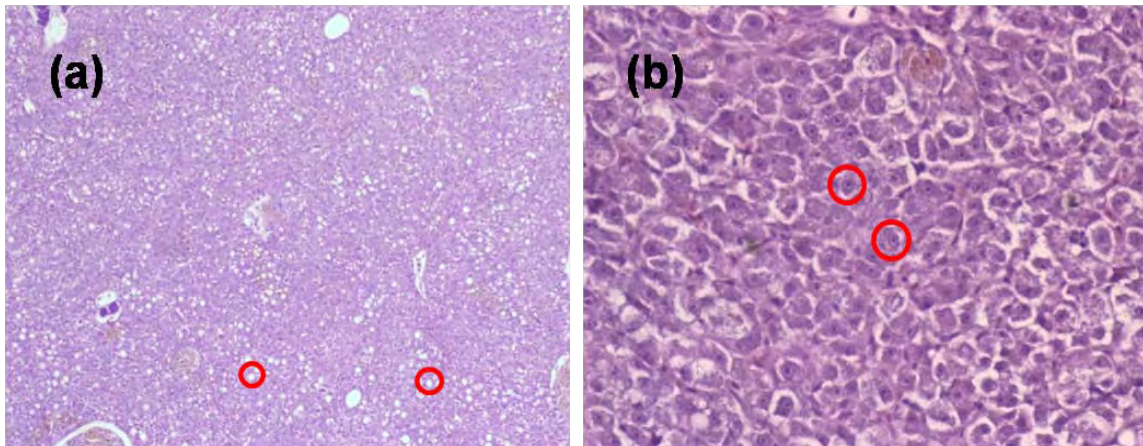
### Microscopic evaluation of the liver

The general architecture of the livers, the bile ducts and hepatopancreas showed no major abnormalities, with similar histological characteristics among all treatments (Figure 3.6).

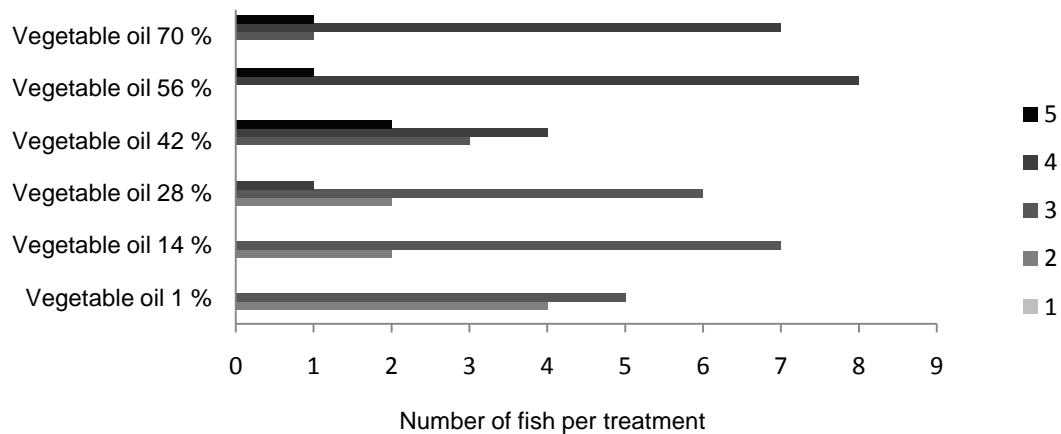


**Figure 3.6.** Section of liver (hematoxylin and eosin, 10x) from a fish fed a diet where 28 % of total lipids originated from vegetable oil, for 206 days. This photograph shows a bile duct surrounded by connective tissue (arrow head) and a hepatopancreas structure (arrow).

The hepatocytes of fish fed the control diet and diets with 14 and 28 % vegetable oil showed moderate accumulation of lipid with small vacuoles in the cytoplasm (Figure 3.7 and 3.8). Spherical nuclei, located in the centre of the cells and homogeneous in shape were observed in these treatments (Figures 3.7 and 3.9).



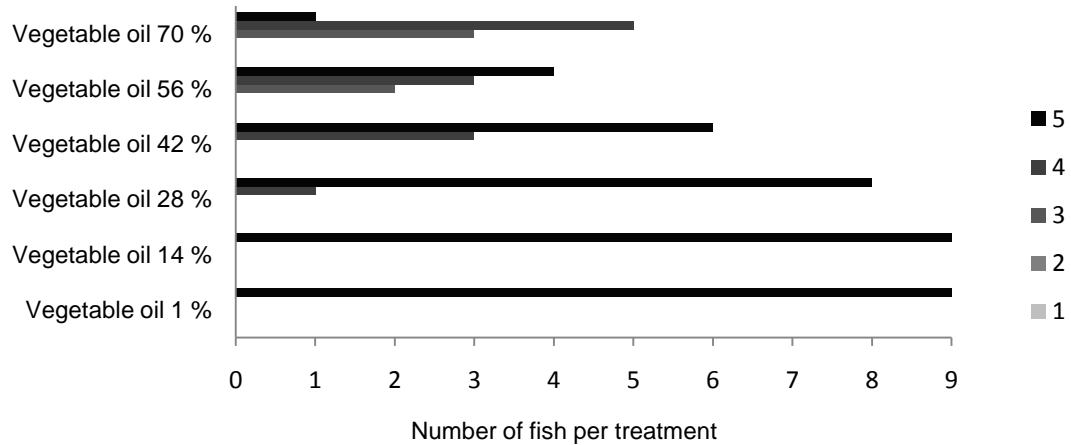
**Figure 3.7.** (a) Liver section (hematoxylin and eosin, 10x) of fish fed the control diet for 206 days, showing small and uniform lipid vacuoles in hepatocytes (red circles). (b) Liver section (hematoxylin and eosin, 20x) of fish fed a 28 % vegetable oil diet. The nuclei are spherical and located in the centre of the hepatocytes (red circles).



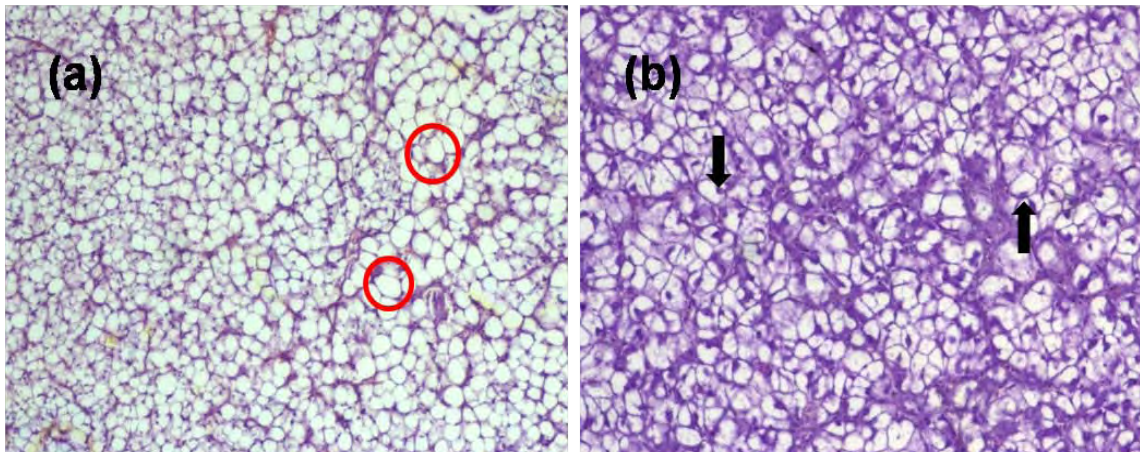
**Figure 3.8.** Scores for the livers of *A. japonicus* fed diets with graded levels of soybean oil for 206 days (n = 9 per treatment). Fat storage ranging from 1 (no lipid deposit) to 5 (entire cell filled with lipid)

The size of lipid hepatocellular vacuolization increased with higher levels of soybean oil in the diets (Figure 3.8 and 3.10). Fish fed diets with higher inclusion of soybean oil (42, 56 and 70 %) showed shrunken and pyknotic nuclei shifted to the periphery of the cells (Figures 3.9 and 3.10).

No signs of fatty degeneration of hepatocytes or necrosis were observed in any of the treatments.

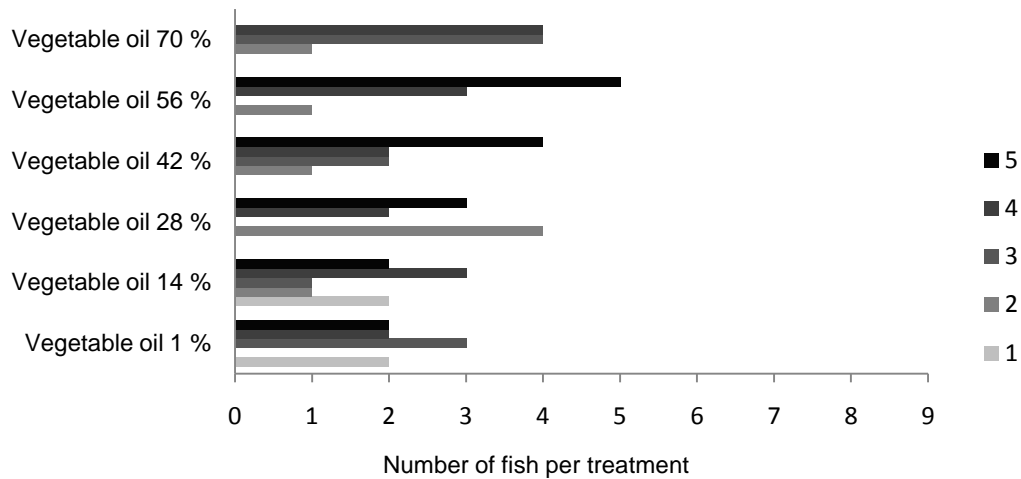


**Figure 3.9.** Scores for the livers of *A. japonicus* fed diets with graded levels of soybean oil for 206 days (n = 9 per treatment). Hepatocyte nuclei displacement ranging from 1 (nuclei completely shifted to hepatocyte edges) to 5 (nuclei in the middle of the cells).

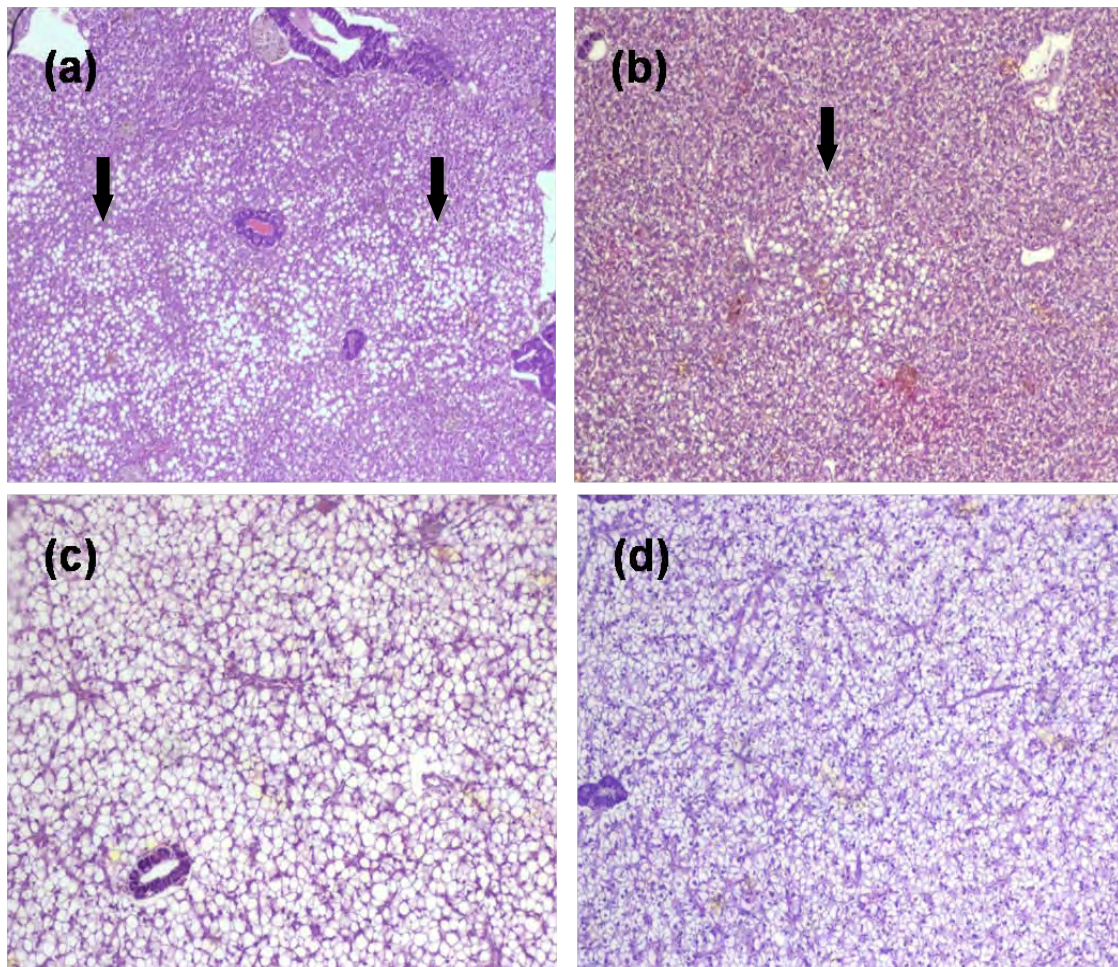


**Figure 3.10.** (a) Liver section (hematoxylin and eosin, 10x) of fish fed a 70 % vegetable oil diet. Red circles show large lipid vacuoles in hepatocytes with irregular sizes. (b) Liver section (hematoxylin and eosin, 20x) of fish fed a 56 % vegetable oil diet showing shrunken pycnotic nuclei located in hepatocyte peripheries (arrows).

Fish fed diets with higher levels of substitution of fish oil (i.e. 42, 56, and 70 %) showed less prominent lipid vacuolated zones compared to fish fed diets with lower substitution levels (Figures 3.11 and 3.12). The lack of evident lipid zones is because the fish fed the higher levels of substitution had vacuoles encompassing the whole liver sample (Figure 3.12).

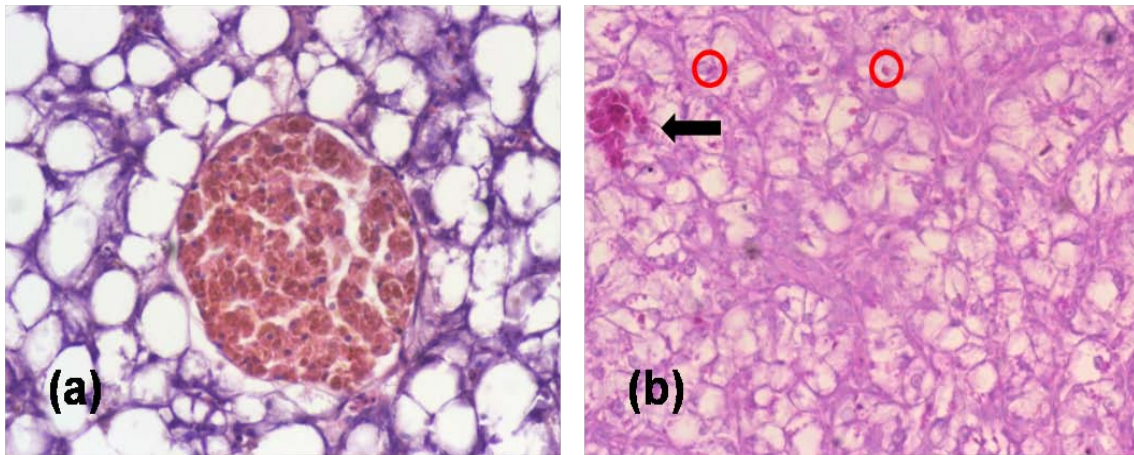


**Figure 3.11.** Lipid vacuoles zones scores for the livers of *A. japonicus* fed diets with graded levels of soybean oil for 206 days (n = 9 per treatment), ranging from 1 (prominent zones) to 5 (no zones).

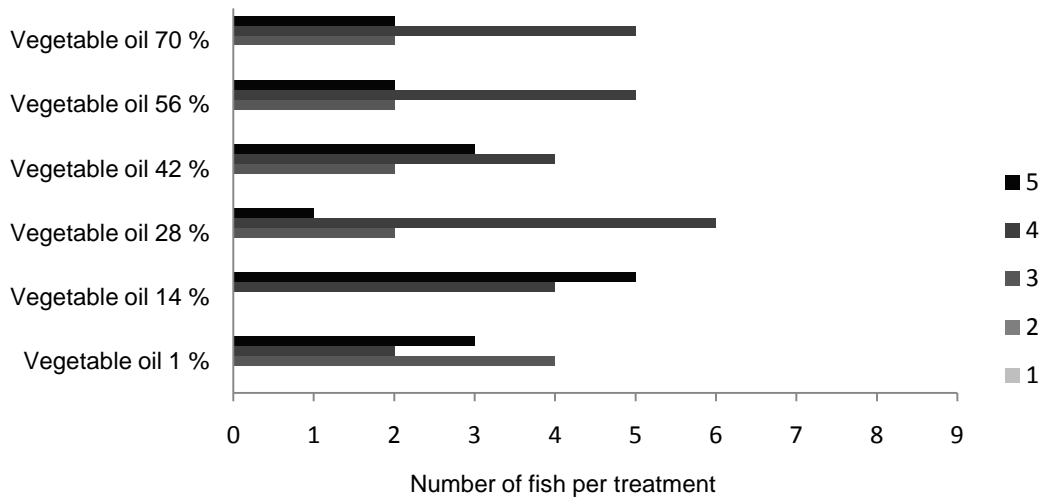


**Figure 3.12.** (a and b) Liver sections (hematoxylin and eosin, 10x) of fish fed the control diet showing prominent lipid vacuolated zones. (c) Liver section (hematoxylin and eosin, 10x) of fish fed a 70 % vegetable oil diet, with no lipid vacuolated zones. (d) Liver section (hematoxylin and eosin, 10x) of fish fed a 42 % vegetable oil diet showing no lipid vacuolated zones.

Melanomacrophage aggregates were observed in all livers samples (Figure 3.13). They were randomly distributed in the parenchyma, but mostly close to hepatopancreas and portal veins; however there appeared to be no differences in size and number among treatments. Ceroid pigment was found randomly distributed in hepatocytes of some sampled livers (Figure 3.13); but this seemed to be independent of diet (Figure 3.14).

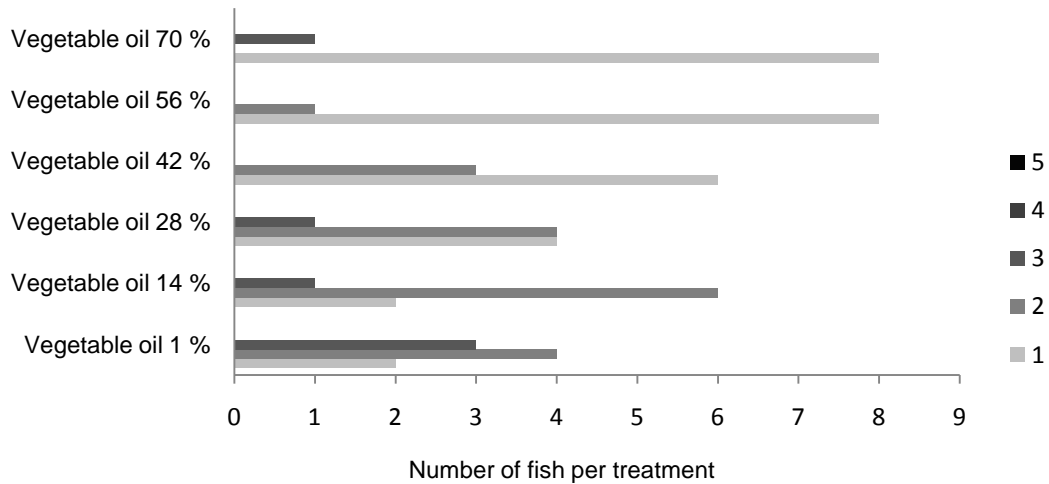


**Figure 3.13.** (a) Liver section (hematoxylin and eosin, x 40) of fish fed a 70 % vegetable oil diet for 206 days showing a melanomacrophage aggregate. (b) Liver section of fish fed a diet where 28 % of the total lipids originated from vegetable oil for 206 days. Red circles showing ceroid pigment and arrow showing melanomacrophage aggregate (periodic acid-Schiff diastase, 40x).

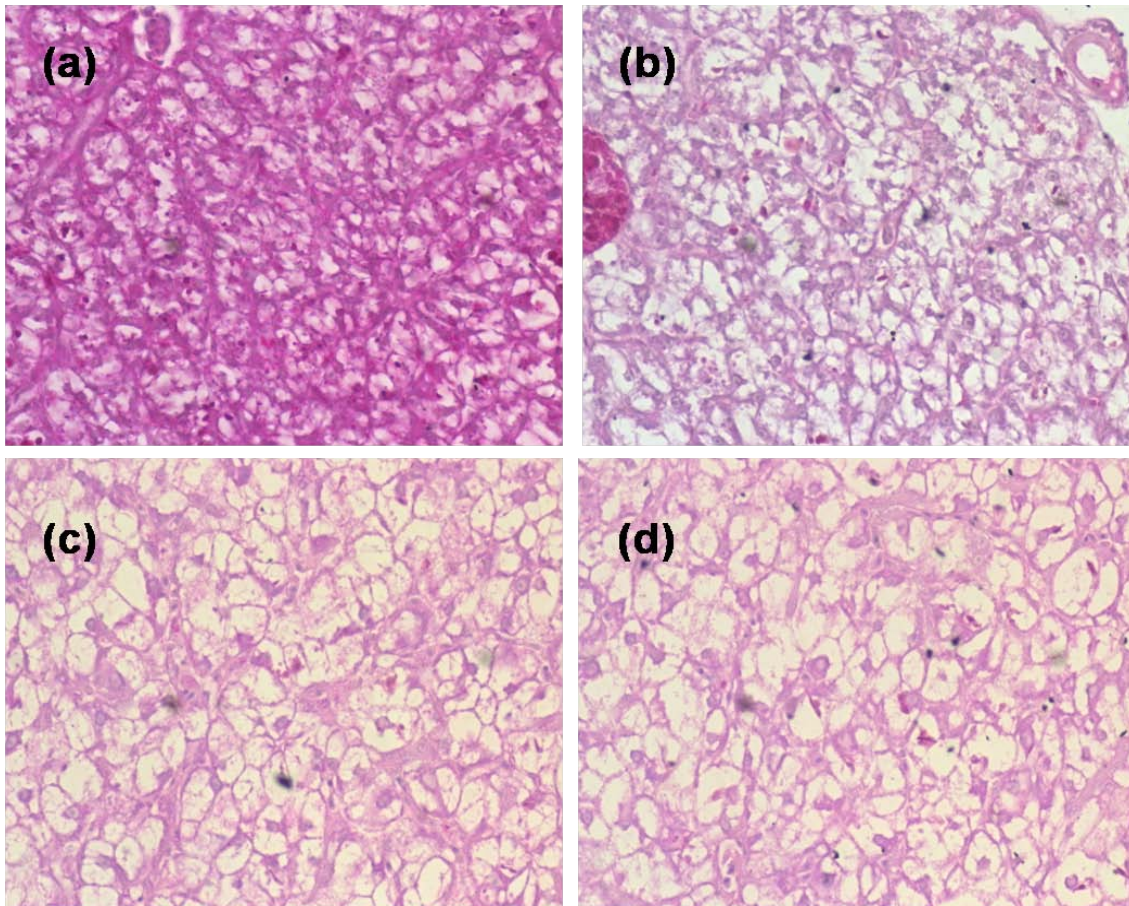


**Figure 3.14.** Ceroid pigment scores for the livers of *A. japonicus* fed diets with graded levels of soybean oil for 206 days (n = 9 per treatment), ranging from 1 (large amount of ceroid pigment) to 5 (no ceroid pigment).

All fish showed fairly low or no glycogen deposits in their livers; however fish fed diets with higher inclusion of soybean oil (i.e. 42, 56, and 70 %) had lower glycogen content compared to livers from fish fed diets with lower levels of soybean oil (i.e. control, 14, and 28 %; Figures 3.15 and 3.16).



**Figure 3.15.** Glycogen storage scores for the livers of *A. japonicus* fed diets with graded levels of soybean oil for 206 days (n = 9 per treatment), ranging from 1 (no glycogen deposit) to 5 (entire cell filled with glycogen).



**Figure 3.16.** (a) Liver section (periodic acid-Schiff, 40x) of fish fed the control diet showing darkly stained areas within hepatocytes, and (b) liver section of same fish (periodic acid-Schiff diastase, 40x) showing weak staining after diastase enzyme confirming glycogen content in liver. (c) Liver section (periodic acid-Schiff, 40x) of fish fed a 56 % vegetable oil diet. Weak staining before the diastase enzyme, and after diastase enzyme was used (d) the staining is still weak, confirming no glycogen content in the liver.

## Discussion

### Macroscopic examination of liver, gills and heart

Lipid liver disease might have been in the early stages of development in fish fed diets containing higher levels of soybean oil. From the macroscopic examination, there was evidence that fish fed diets that contained more than 28 % of the lipids originating from vegetable oil source displayed signs of lipid liver disease. However, this hypothesis was not supported by macroscopic

examination of other organs such as heart and gills. The pale colour and swollen livers from fish fed diets with 42, 56, and 70 % vegetable oil were also observed in other species fed diets with essential fatty acid deficiency (Takeuchi & Watanabe, 1982; Watanabe *et al.*, 1989). The colour of a healthy liver is generally reddish-brown, but may be more yellow when the liver concentrates high amounts of fat (Bruslé & Anadon, 1996). Fish with swollen liver, as well as extreme anaemia, and a bronzed, rounded heart could be suffering from lipoid liver disease, which is the accumulation of lipids in the liver (Hardy, 2001). The “firmer” consistency of the livers from fish fed the fish oil diet compared to those fed diets with high inclusion of soybean oil (i.e. 42, 56 and 70 %) was also observed in sharpsnout seabream, *Diplodus puntazzo* (Piedecausa *et al.*, 2007). Some of the sampled livers from fish fed 42 and 70 % vegetable oil diets exhibited a dark green colour, which is consistent to a condition called bile stagnation (Hibiya, 1982). In this study, livers from fish fed diets containing higher levels of vegetable oil presented a pale colouration and seem to be fairly swollen; however, hearts of all sampled fish appeared normal in shape and had a vivid-red colour. Furthermore, gills showed no signs of anaemia in any sampled fish. It is possible that lipoid liver disease was in its early stages of development and the other organs might have shown symptoms over a longer period. This should be investigated in future work.

#### Haematological parameters

Examination of the blood showed no evidence of stress among fish fed the different diets. There were no significant differences in haematocrit percentage, haemoglobin concentration and the number of red blood cells in the blood of *A. japonicus* fed the different diets for 206 days. Haematological parameters of fish have been increasingly used for diagnosing environmental stress, diseases and dietary deficiency in studies of the biology of fish or aquaculture conditions (Montero *et al.*, 1999b; Mourente *et al.*, 2005; Balfry *et al.*, 2006; Celik & Aydin, 2006; Gbore *et al.*, 2006; Dobšíková *et al.*, 2009; Araujo *et al.*, 2011). However, there is a lack in standardized methods and nomenclatures and therefore the

use of these measurements have been questioned (Klinger *et al.*, 1996). Blood parameters may vary between species (Larsson *et al.*, 1976), and within the same species it can also fluctuate depending on factors such as water quality (Klinger *et al.*, 1983) and sampling methodology (Oikari & Soivio, 1975; Gallagher & Farrell, 1998). These influences make it difficult to interpret the results and to make comparisons between studies. Nonetheless, the results of haematocrit percentage, haemoglobin concentration and the number of red blood cells found in this study concur with other studies that evaluated partial or total substitution of fish oil with different vegetable oils in different species, and found no significant differences (Thompson *et al.*, 1996; Mourente *et al.*, 2005; Wassef *et al.*, 2007).

The parameters analysed in this study are indicators of oxygen-carrying capacity (Lamas *et al.*, 1994). They establish the relationships between oxygen concentration available in the environment and the health of the fish (Lamas *et al.*, 1994). Haemoglobin and haematocrit were not affected when gilthead seabream, *S. aurata*, were fed diets containing different dietary lipids; however, the number of red blood cells was higher in fish fed a diet with fish oil as the only lipid source (Montero *et al.*, 2003). The authors suggest that this could be caused by a higher oxygen requirement from fish fed the diets containing fish oil, due to a higher peroxisomal  $\beta$ -oxidation. This was not observed in the present study. Haematocrit values tend to increase when fish are stressed (Gallagher & Farrell, 1998). The percentage of haematocrit for teleosts is generally greater than 20 % (Gallagher & Farrell, 1998). Furthermore, a decrease in haemoglobin value is a sign of reduced capacity of transport of gases in the body (Araujo *et al.*, 2011). As an example, when the temperature is lower than the one required for the species, the metabolism of the fish will be reduced, and thus the haemoglobin concentration is also reduced (Araujo *et al.*, 2011). This is an example of a stressful situation where the blood parameters were altered. A reduction on haemoglobin was not observed in the present study. Moreover, these data provide evidence that the fish were not stressed, since an increase in haematocrit value was not observed in dusky kob fed diets with vegetable oil inclusion compared to fish fed a fish oil diet, and the values

were greater than 20 %. Consequently, there were no abnormal effects in the blood chemistry of dusky kob fed diets where fish oil was replaced by vegetable oil.

#### Fatty acid composition of the liver

Approximately 98 % of the lipids in the dietary ingredients, and 90 % in the fish body occur as triglycerides (Webster & Lim, 2002). Since phospholipids were not analysed in this study, the summation of the triglycerides and free fatty acids were thus considered as the total lipids in the fish liver. Moreover, the effects of changing dietary lipids are more pronounced in the triglyceride fraction of the liver, when compared to the phospholipid fraction. This is due to the function of triglycerides which is the storage of lipids, serving as a long-term source of energy for when the energy required by the fish exceeds the energy available from the diet (Tocher, 2003; Ruyter *et al.*, 2006; Pettersson, 2010). Phospholipids, however, are essential components of biological membranes (Halver, 1980; Tocher, 2003).

Fatty acids of the liver were clearly influenced by dietary treatment. This is confirmed by the linear correlations between the percentage of individual fatty acids in the diets and their corresponding percentage in the liver. A number of studies have reported the influence of different dietary lipids and the respective concentration of these lipids on the body of many fish species (Izquierdo *et al.*, 2003; Regost *et al.*, 2003; Caballero *et al.*, 2004; Fountoulaki *et al.*, 2009). This effect is more obvious in marine fish due to their limited capacity of converting C<sub>18</sub> fatty acids into long chain PUFA (Lochmann & Gatlin III, 1993; Tocher, 2003). In this study this can be seen particularly with the incorporation of high levels of linoleic and  $\alpha$ -linolenic acids in the diets. These fatty acids accumulated in the livers of dusky kob, and were not absorbed or converted into long chain PUFA. This demonstrates that *A. japonicus* have a typical “marine” fish pattern (Turchini *et al.*, 2009). This inability to bioconvert 18:2 n-6 and 18:3

n-6 into HUFA is due to very low levels of  $\Delta^5$ -desaturase activity (Tocher & Sargent, 1990).

Accumulation of certain fatty acids in the liver of dusky kob may have altered whole body composition of lipid. There was a significant increase in lipid levels in whole body composition of dusky kob with increasing dietary vegetable oils described in Chapter 2 (Figure 2.10). This is possibly explained by the increasing percentages of 18:2 n-6 and 18:1 n-9 in the liver triglycerides (Ruyter *et al.*, 2006), particularly because marine fish generally store fat primarily in the liver (Figueiredo-Silva *et al.*, 2005). The accumulation of these fatty acids may be related to the low levels of n-3 HUFA available for fish fed diets containing higher quantities of soybean oil (Buzzi *et al.*, 1996). In addition to the accumulation of these two fatty acids, 18:3 n-3 was also accumulated in the liver of dusky kob. The increase in percentage of 18:2 n-6, 18:3 n-3 and 18:1 n-9 might have occurred because these fatty acids compete amongst each other for the  $\Delta^6$ -desaturase, which has a low substrate specificity (Mourente & Tocher, 1994). Consequently, this competition limits the conversion of C<sub>18</sub> to C<sub>20</sub> and C<sub>22</sub> HUFA (Mourente & Tocher, 1994). Moreover, high levels of oleic acid are a sign of essential fatty acid deficiency (Watanabe, 1982). This sign of deficiency have also been reported in other species (Ibeas *et al.*, 1994; Buzzi *et al.*, 1996; Seilliez *et al.*, 2003).

The increase in lipid deposition in the liver of dusky kob caused an imbalance in the n-3 to n-6 ratio. Because of the high content of linoleic acid in the diets with the inclusion of soybean oil, the n-3/n-6 ratio was significantly reduced. In general, the ratio of n-3/n-6 for wild marine fish species is relatively high, varying between 4 to more than 15 (Steffens, 1997; Bechtel & Oliveira, 2006; Guil-Guerrero *et al.*, 2011). This high values occur due to the high contents of long chain PUFA from the n-3 series in some phytoplankton species that make up part of the fish's diet (Fraser & Sargent, 1989). There is evidence that lipid metabolism is regulated by the dietary n-3/n-6 ratio (Turchini *et al.*, 2007). Excess dietary fatty acids from the n-3 or n-6 series present in an unnatural or atypical condition forces the fish to respond metabolically and to adapt to this abnormal situation (Yu & Sinhuber, 1976). Thus, fish might develop an

unbalanced n-3/n-6 ratio (Yu & Sinhuber, 1976). For example, when gilthead seabream, *S. aurata*, were fed a diet in which fish oil was replaced with soybean oil, this ratio was disturbed. As a result, the lipid metabolism was affected by energy depletion, which led to a mobilization of fatty acids for  $\beta$ -oxidation (Menoyo *et al.*, 2004).

One of the main signs of n-3 and n-6 fatty acid imbalances is the increase in lipid deposition in the liver (Takeuchi *et al.*, 1990). In this study, this imbalance on the n-3/n-6 ratio possibly account for the larger and higher number of lipid vacuoles in the histology of the liver of fish fed diets with higher fish oil substitution. This was confirmed by the histological examination discussed further in this chapter. The recommendation of an optimum dietary ratio of n-3/n-6 for marine species, e.g. juvenile sea bass (*Lates calcarifer*), is approximately 1.5-1.7/1 (Williams & Barlow, 1999). In the present study this ratio was achieved in diets containing 1 (control diet) to 42 % of soybean oil.

The experimental dusky kob showed signs of essential fatty acid deficiency. Besides the increase in 18:1 n-9 mentioned earlier, a decrease in total n-3 fatty acids in the liver of fish is also a sign of essential fatty acid deficiency (Watanabe, 1982). The reduction in EPA and DHA found on the present study is in agreement with other studies on fatty acid deficient diets, as well as on vegetable oils as alternatives to fish oil in finfish diets (Bell *et al.*, 1999; Montero *et al.*, 2001; Mourente *et al.*, 2005). The n-3 fatty acids, especially EPA and DHA, are essential for optimal health and development of marine fish species (Rimmer *et al.*, 1994; Izquierdo, 1996; Sargent *et al.*, 1997). Thus, this deficiency could explain the decreasing trend in final weight, weight gain and specific growth rate (SGR) of fish fed diets containing increasing levels of soybean oil (Chapter 2). Moreover, it implies that 20:5 n-3 and 22:6 n-3 are essential for superior growth of dusky kob.

In some fish species the n-3 HUFA, particularly EPA and DHA accumulate in the flesh of the fish, which makes the final product very nutritious and healthy for humans (Lands, 1990; Hunter & Roberts, 2000). Thus, reductions on these fatty acids are unsatisfactory. The flesh of *A. japonicus* was not analysed in the

present study; nevertheless it would be interesting for further research to investigate the changes in fish flesh especially concerning the final product for human consumption.

A change in the n-3/n-6 ratio directly modifies the ratio of AA/EPA (Turchini *et al.*, 2009). The AA/EPA ratio was elevated with the increase in fish oil replacement in the diets for *A. japonicus*. This occurred due to the marked decrease in dietary EPA. Similar results were found for *S. aurata* fed diets with soybean or linseed oils replacing fish oil (Menoyo *et al.*, 2004). The AA/EPA ratio was significantly higher in liver of fish fed the vegetable oil diets (Menoyo *et al.*, 2004). This ratio has been shown to be essential in the fatty acids desaturation and elongation processes in salmon (Bell *et al.*, 1997). However, high ratios of AA/EPA in the tissues of the fish result in enhanced eicosanoid activities related to immune response, blood clotting and inflammatory responses (Sargent *et al.*, 1999a; Tocher, 2003). In contrast, high EPA/AA ratios are likely to damp down eicosanoid actions (Sargent *et al.*, 1999a). This happens because AA-derived eicosanoids have a higher activity than EPA-derived ones (Turchini *et al.*, 2009). In this study, AA/EPA ratio increased in fish liver with the increase in vegetable oil in the diets. Nonetheless, higher mortalities were observed in fish fed diets with levels from 42 to 70 % vegetable oil. The optimal dietary ratio remains uncertain, and it is recognized that an unbalanced ratio can be damaging to fish (Sargent *et al.*, 1999a). Moreover, results of the effects of the AA/EPA ratio are often contradictory (Montero *et al.*, 2003). The unbalanced ratio of AA/EPA might explain why the survival of fish fed diets with high levels of soybean oil (i.e. 42, 56 and 70 %) was lower than the survival of fish fed diets with lower levels of soybean oil, when gas bubble disease was prevalent, as described in the Chapter 2. The production of eicosanoids and their effects on the immune response in dusky kob fed diets with vegetable oils should be further elucidated.

Changes in dietary EPA and DHA may cause an imbalance in the EPA/DHA ratio in the liver of the fish. A balanced dietary ratio of EPA/DHA is necessary for best growth of fish (Ibeas *et al.*, 1997). In this study, the EPA/DHA ratio decreased in dusky kob livers, with the increasing levels of soybean oil in the

diets. The same was observed for *S. aurata* when fed diets where vegetable oils replaced fish oil (Menoyo *et al.*, 2004). The influence of the EPA/DHA ratio is particularly evident in the larval stages of fish (Mourente *et al.*, 1993; Reitan *et al.*, 1994; Rodríguez *et al.*, 1998). However, the effects of this ratio on juvenile fish cannot be disregarded (Ibeas *et al.*, 1997). Increasing DHA contents will consequently decrease the EPA levels in the diets, which will alter the EPA/DHA ratio (Sargent *et al.*, 1999b). This alteration affects competitive interactions in eicosanoid production between EPA and DHA (Sargent *et al.*, 1999b). This imbalance might have caused, amongst other factors mentioned earlier, the decrease in final weight, weight gain and SGR of fish fed diets with increasing fish oil substitution (Chapter 2).

#### Visceral fat index and hepatosomatic index

VFI and HSI both remained relatively constant in fish fed up to 28% soybean oil diets, and then sharply increased at the 42 to 70 % vegetable oil levels. The fat in the visceral cavity, besides being considered an indication of poor health status related to the nutrition of the fish (Craig *et al.*, 1999; Mathis *et al.*, 2003), is undesirable to consumers when in excess. It affects the visual sense and the odour of the final product, since this fat usually has an unpleasant odour (Grigorakis, 2007). Additionally, the excess visceral fat decreases carcass yield (Mathis *et al.*, 2003). It is likely that *A. japonicus* was not able to metabolise the dietary vegetable fat and thus stored it.

The high HSI of fish fed diets with 42 to 70 % soybean oil suggests that these diets were deficient in essential fatty acids (Castell *et al.*, 1972). The HSI results of this study are in agreement with those found for gilthead seabream juveniles fed diets deficient in essential fatty acids (Montero *et al.*, 2001). In a similar manner HSI of gilthead seabream fed diets with higher levels of replacement of fish oil by soybean oil was significantly higher when compared to HSI of fish fed diets with lower levels of substitution (Kalogeropoulos *et al.*, 1992; Alexis, 1996). Higher HSI were also found for sharpsnout seabream (*Diplodus*

*puntazzo*) fed a soybean oil diet (Piedecausa *et al.*, 2007). The HSI is associated with the amount of fat in the liver and is sensitive to the nutritional condition of the fish (Bruslé & Anadon, 1996). The enlarged livers of the experimental fish fed the 42 to 70 % soybean oil diets implies a reduction of the utilization of the ingested soybean oil by dusky kob, leading to an accumulation of fat in the liver.

#### Histological examination of the liver

The histological examinations of the livers of fish fed diets with high levels of soybean oil (i.e. 42, 56 and 70%) support the results of the HSI and fatty acid composition of the liver. Histological examination of livers in this study demonstrated that all fish had lipid accumulation to some extent; however, there was an increase in number and size of lipid vacuoles in liver of dusky kob fed diets with 42 to 70 % soybean oil inclusion. This suggests that fish were less able to metabolise the vegetable oil, which therefore resulted in an accumulation of fat in the liver. The results of the present study are consistent with a similar study on Atlantic salmon, *Salmo salar*, fed diets supplemented with increasing levels of soybean oil at different temperatures (Ruyter *et al.*, 2006). At 5 °C, fish fed 100 % soybean oil diet had higher accumulation of fat in the liver compared to fish fed 100 % fish oil diet (Ruyter *et al.*, 2006). Furthermore, *S. aurata* fed a 60 % soybean oil diet showed intense steatosis and abundant lipid vacuoles in the liver in both, short-term and long-term trials (Caballero *et al.*, 2004). As a consequence of an n-3 deficient diet, *S. aurata* also showed accumulation of lipids in the liver (Ibeas *et al.*, 1994; Montero *et al.*, 2001). This accumulation could be caused by a deficient lipoprotein synthesis in the liver (Ibeas *et al.*, 1994). In line with this, the present study confirms that a reduction of essential fatty acids in the diets due to the inclusion of vegetable oil promotes accumulation of fat in dusky kob livers.

The presence of shrunken and pycnotic nuclei in fish fed diets with high vegetable oil contents is an indication of the possible onset of fatty liver disease.

Atrophy of the hepatocyte nuclei is a sign of fatty liver disease (Hibiya, 1982). The size of the hepatocyte nucleus is an indicator of hepatic metabolic activity (Segner *et al.*, 1988), and the higher cellular activity may lead to death of cells (Silva, 2008). This nucleus condition was observed for *S. aurata* growers fed a diet in which 60 % of the fish oil content was substituted by a mixture of cottonseed, sunflower, and soybean oils (Wassef *et al.*, 2007). Pycnotic nucleus has also been reported in many toxicological studies (Santos *et al.*, 2004; Jiraungkoorskul *et al.*, 2007; Rodrigues *et al.*, 2010). Pycnosis is a pathological change in the cell nucleus where the chromatin content becomes extremely condensed. Pycnosis are generally the changes that precede necrosis of hepatic cells (Hibiya, 1982). This structural alteration in the liver cells demonstrates that high levels of soybean oil in the diets are not suitable for dusky kob. Along with increased lipid storage in the liver of fish fed diets containing 42, 56 and 70% of soybean oil, the shrunken and pycnotic nuclei observed in fish fed these same diets suggests that fish may be developing fatty liver disease.

Melanomacrophage aggregates were found in livers of all sampled specimens, hence there is no conclusive evidence that this was a symptom of a nutritional imbalance or deficiency. The number, size and pigmentation of the melanomacrophage aggregates are known to vary between species and organs (Roberts, 1975; Montero *et al.*, 1999a). Their presence has been linked to fishes nutritional status, including starvation (Agius, 1981; Montero *et al.*, 1999a), age (Roberts, 1975; Woodhead *et al.*, 1983), pathological and inflammatory conditions (Roberts, 1975; Vogelbein *et al.*, 1987), heat stress (Blazer *et al.*, 1987), as well as environmental changes (Macchi *et al.*, 1992), and others. Aquaculture stress conditions have also been reported to influence the melanomacrophage aggregates (Montero *et al.*, 1999a). Since these structures were observed in all livers in the present study, it is not possible to conclude that they occurred due to stress caused by inclusion of the plant oil source in the diets.

Similar to melanomacrophage aggregates, there is no indication that ceroid pigment occurred in the liver as a result of the dietary treatments. While the

presence of ceroid pigment is one of the features associated with fatty liver disease (Roberts, 2002), in this study ceroid pigments were observed in some of the liver samples from all dietary treatments. Moreover, fish with high contents of unsaturated fatty acids and low vitamin E content in their tissues are prone to contain ceroid pigment (Agius & Roberts, 2003). In this study, vitamin levels were the same for all diets (Chapter 2, Table 2.1). Besides that, even though the unsaturated fatty acids increased in fish liver with the increase in soybean oil levels in the diets, the amount of ceroid pigment did not seem to be particularly affected by the increasing unsaturated fatty acids in the diets. No ceroid pigment was observed in the livers of red drum, *Sciaenops ocellatus*, juveniles after being fed diets with added soybean oil in different levels (Tucker *et al.*, 1997). Additionally, ceroid pigments were not found in liver of rainbow trout fingerlings fed a nutritionally adequate diet consisting of fresh beef, fresh beef liver and dry feeds (Wood & Yasutake, 1956). However, when rainbow trout were fed the same diet but the fresh beef liver was substituted by fishmeal, ceroid pigment was spotted throughout the spleen (Wood & Yasutake, 1956). According to the latter authors, fatty changes in liver and ceroid are not directly associated, as they observed livers with no noticeable fatty changes containing ceroid, and livers with dense fat infiltration without the presence of this pigment. Thus, the presence of ceroid pigments in liver of dusky kob might be a normal feature. It would be of interest to conduct a study comparing livers of fish fed diets with vegetable oils and wild fish.

The low glycogen contents in livers of dusky kob fed diets with higher levels of soybean oil are consistent with the results of a study on Eurasian perch (*Perca fluviatilis* L.) which recorded a decrease in glycogen when fish were fed vegetable oil diets, compared to fish fed a fish oil diet (Blanchard *et al.*, 2008). Liver glycogen is considered an emergency energy resource. It is generally used in the first critical moments of stress (Christiansen & Klungsoyr, 1987). Fish in good biological condition have adequate reserves of glycogen and lipid in the liver (Haard, 1992). Some fish species show a significant depletion of liver glycogen during the initial phase of fast (Metón *et al.*, 2003; Soengas *et al.*, 2006); however, other species tend to use their lipid deposits in this condition,

and therefore glycogen reserves change little (Moon, 1983). In the present study, liver lipid accumulated and glycogen decreased, suggesting that the accumulated oil in the high vegetable oil replacement diets was increasingly unavailable as a source of energy and that the fish could not build up glycogen reserves.

## **Conclusion**

The results indicate that dusky kob, like other marine fish, have a requirement for dietary EPA and DHA for maximum growth, and these fatty acids are not available in the required ratios in diets containing high levels of substituted terrestrial vegetable oils such as soybean oil. Furthermore, dusky kob appeared to be unable to metabolize 18:2 n-6, which was disproportionately accumulated in the liver. The low glycogen levels in the liver of fish fed diets with 42 to 70 % soybean oil suggest that the accumulated fat was unavailable as an energy source. Further research should be undertaken on the immune response of kob fed diets with vegetable oil inclusion. Moreover, the results of the macroscopic and the histological observations confirm that the replacement of high levels of fish oil by soybean oil (i.e. 42, 56 and 70 %) in the diets of dusky kob promotes fat accumulation in the liver. In addition, the highest vegetable oil inclusion in the diet caused hepatic damage. This damage indicates that fish oil should not be replaced by soybean oil at levels higher than 28 % in *A. japonicus* diets. It would be interesting, nevertheless, to test in future research, in a long-term trial, if the changes in the liver of dusky kob are reversible, to confirm if they are actual pathological features. It is also relevant to determine the length of time that dusky kob can be maintained on vegetable oil diets before the histological changes become irreversible.

## CHAPTER 4

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### GENERAL DISCUSSION

The aquaculture industry is rapidly expanding, and currently it is extremely reliant on the use of fish oil. Production of fish oil has not increased globally over the past few decades (Shepherd *et al.*, 2005). Therefore, this work aimed to investigate a sustainable and economically viable non-marine alternative lipid source in diets for juvenile *Argyrosomus japonicus*. This was achieved by assessing the effects of a graded increase in dietary soybean oil (i.e. from 1 to 70 % total soybean oil in the diets), and consequently decreasing fish oil, on growth, feed efficiency, proximate composition, as well as various health parameters in dusky kob.

The results of this work show that fish oil can be partially substituted by soybean oil in diets for dusky kob without significantly affecting fish growth. Nonetheless, to establish the optimum level of fish oil replacement, there are other factors that should be considered. For example, fish must remain healthy to maintain optimum growth and feed conversion in the long-term. As such, it was recommended here that a maximum concentration of 28 % of soybean oil can be introduced in the diet, replacing fish oil, with no apparent negative health effects.

When analysing the results of the growth of the fish with regression analyses, fish growth significantly decreased with the replacement of fish oil by soybean oil (Chapter 2). This decrease in growth could be due to differences in the fatty acid composition of the diets. The fatty acid profiles of the diets containing different levels of fish oil and soybean oil were markedly different. Dietary fatty acid composition has been found to be important and affects fish growth, but this effect varies considerably among species (Watanabe, 1982; Turchini *et al.*,

2009). Furthermore, inhibition of growth is a sign of essential fatty acid deficiency (Watanabe, 1982). In the present study there was evidence that the diets containing higher levels of vegetable oil were deficient in essential fatty acids. Besides growth suppression, fish fed the diets with 42, 56 and 70 % soybean oil exhibited increased HSI, as well as swollen and pale livers (Chapter 3, Figures 3.1 and 3.2). The same occurrence was observed in other species, such as rainbow trout *Salmo gairdneri*, coho salmon, *Onchorhynchus kisutch*, chum salmon, *Onchorhynchus keta*, white fish, *Coregonus lavaretus maraena*, and others, fed essential fatty acid deficient diets (Takeuchi & Watanabe, 1982; Watanabe *et al.*, 1989; FAO, 1992). EPA and DHA are essential fatty acids in fish for regular growth, health, reproduction and other bodily functions (Rimmer *et al.*, 1994; Sargent *et al.*, 1997). Hence, the decreasing trends found on growth probably occurred because the diets containing soybean oil did not provide the necessary amount of essential fatty acids required for regular growth of dusky kob. Furthermore, this confirms that *A. japonicus* have a reasonably high dietary requirement for n-3 highly unsaturated fatty acids (HUFA) for optimal growth. The alterations in dietary fatty acids also had an effect on the FCR and PER. Both of these observed parameters were superior in fish fed the control diet and diets with up to 42 % soybean oil. However, the feed efficiency of dusky kob fed diets with fish oil replaced by soybean oil should be further elucidated.

Changes in dietary fatty acids were responsible for the excessive accumulation of lipids in the body and liver of dusky kob. The fatty acid analysis of the diets described in Chapter 3 shows that certain fatty acids were significantly increased with the inclusion of soybean oil, especially the linoleic acid (18:2 n-6). The degree of lipids in *A. japonicus* body is strongly influenced by the dietary lipid level (Riddin, 2009; Woolley *et al.*, 2010). However, the significant increase in dietary soybean oil led to an increase in whole body lipids, even though the total lipid content of the diets did not change (Chapter 2, Figure 2.10). This suggests that these fatty acids were not metabolized by the fish, but accumulated in the body as fat. This accumulation of fatty acids, particularly linoleic acid, which accumulated disproportionately in the liver of fish fed diets

containing soybean oil, was probably due to the limited capacity of marine fish to bioconvert fatty acids with 18 carbons into longer polyunsaturated fatty acids (PUFA; Turchini *et al.* 2009).

In addition to lipid content of the whole body of the fish, lipid accumulation was confirmed by the liver histological analyses. Fish fed diets with higher contents of soybean oil, i.e. from 42 to 70 % soybean oil, exhibited a greater number of lipid vacuoles, which were also larger in size, than the liver from fish fed the other diets with lower contents of soybean oil (Chapter 3, Figures 3.7, 3.8 and 3.10). In addition, the nuclei of the hepatocytes of the fish fed these diets were shrunken and shifted to the edges of the cells due to the large size of the lipid vacuoles. These conditions have been observed in other fish species fed diets with vegetable oils (Alexis, 1996; Caballero *et al.*, 2004; Ruyter *et al.*, 2006; Wassef *et al.*, 2007). This accumulation of lipids in the liver suggests low utilization of these fatty acids by the fish (Izquierdo *et al.*, 2003).

Liver fatty acid profiles reflecting dietary fatty acid profiles have been observed in several fish species (Wu *et al.*, 2002; Regost *et al.*, 2003; Caballero *et al.*, 2004; Francis *et al.*, 2007b; Lin & Shiau, 2007). Differences in dietary formulation affected the composition of fatty acids in the fish liver tissue in this study, as demonstrated by the linear correlations of fatty acids in the diets and in the liver of the fish (Chapter 3, Figure 3.5). In general, EPA and DHA decreased, as well as the total n-3; but  $\alpha$ -linolenic acid, oleic acid, and particularly linoleic acid increased in the liver tissue of the fish fed diets with increasing soybean oil. These results confirm the differences in the metabolism of the different dietary fatty acids. The modification of fatty acids in the diets also caused an imbalance in the n-3/n-6 ratio in the fish liver tissue, which might have contributed to the increase in HSI (Menoyo *et al.*, 2004).

Changes in dietary fatty acids may have an effect on disease resistance mechanisms in fish (Blazer, 1992). The decrease in dietary EPA, due to the dietary vegetable oil inclusion, elevated the AA/EPA ratio in the fish liver (Chapter 3). Although an increase in AA/EPA ratio is considered to enhance the eicosanoid actions, and therefore enhance the immune response of fish

(Sargent *et al.*, 1999a), in this study immune response seemed to be compromised with the increase of this ratio because fish in the 42, 56 and 70 % vegetable oil treatments appeared more susceptible to the stress imposed by gas bubble disease (Chapter 2). The optimal dietary ratio of AA to EPA remains uncertain for fish (Sargent *et al.*, 1999a); however, an imbalance in this ratio in the fish can modify physiological responses as a result of eicosanoid production from the AA and EPA series (Bell *et al.*, 1997; Tocher, 2003). Nevertheless, further investigation should be made on the immunosuppression of dusky kob fed diets with fish oil replacement by vegetable oils.

Fatty acid profile changes in fish muscle, caused by the dietary vegetable oil inclusion, may influence the nutritional and quality parameters of the fish as food product for human consumption (Guillou *et al.*, 1995; Izquierdo *et al.*, 2003; Izquierdo *et al.*, 2005). One of the main disadvantages of the fish oil substitution in fish feeds is the loss of some fatty acids that provide benefits for human health (Hunter & Roberts, 2000; Turchini *et al.*, 2009). These fatty acids are from the n-3 series, especially EPA and DHA (Hunter & Roberts, 2000). These changes can, however, be reverted. Finishing diets, also called “wash-out” diets have been proven to be a good resource to reverse the changes in fatty acids caused by dietary vegetable oils. Moreover, fish previously fed vegetable oil diets with reduced n-3 HUFA might have their growth enhanced when reverted to a fish oil diet (Turchini *et al.*, 2007). Finishing diets are 100 % fish oil diets, fed to the fish on the final period of the production cycle. Since the alterations in muscle fatty acids in dusky kob fed diets with vegetable oil inclusion is a topic of concern, it would be interesting to assess the effects of a finishing diet on the growth, health and fillet fatty acid composition of dusky kob.

In conclusion, fish oil can be partially replaced by soybean oil in the diets for *A. japonicus*. The results of this study indicate an optimum concentration of 28 % of the total dietary lipids originated from soybean oil; this concentration provided good growth and feed efficiency with no apparent negative health effects. Substitution above 28 % is not recommended based on the increase in HSI and VFI, as well as the accumulation of lipids in the liver tissue of the fish. Further work is required to confirm the long-term effect of fish oil substitution on the

growth and health of dusky kob, and to better understand the optimization of aquafeeds and the metabolism of lipids in this fish.

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