MASTEROPPGAVE

Growth, muscle cellularity and proximate composition of juvenile Lumpfish (*Cyclopterus lumpus* L.); replacing fish meal in the diet with plant protein.

Nimalan Nadanasabesan

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N. Nimalan

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Abstract

Norway is the largest producer of Atlantic salmon (*Salmo salar*) in the world and there is a need for new tools to overcome the salmon lice challenge the industry is facing. The interest for using biological treatments like cleaner fishes is a new environmental friendly trend that supports the sustainability goal of the industry. Lumpfish (*Cyclopterus lumpus*) is a promising salmon lice grazer with better performances compared to wrasse fish species which are less temperature tolerant. However, the knowledge regarding the nutrition of the lumpfish is more or less none existing. The aim of the study was therefore to investigate the performance of the fish when replacing fish meal in the diet with soy and pea protein concentrate (SPC & PPC) at different inclusion levels (0%, 25%, 50% and 75%). Lumpfish (n = 2000 per tank), with mean weight of 4 g were purchased from a commercial lumpfish farmer (Mørkvedbukta AS) and allocated randomly in twelve tanks with triplicate treatments. Biometrical data such as body weight, standard length, width and height were measured at week 0, 2.5, 5, and 7.5. In addition, proximate composition and histological analysis to study the growth and development of the muscle were at each sample point during the experiment.

At the start of the experiment, weight of the fish (mean ± SEM) was 7.29 ± 0.13 g (post acclimatization) and there was no mortality during the experimental period, indicating that the fish were in good health. All biometric parameters measured throughout the experiment did not show any changes with diets ($P > 0.05$). Similarly, plasticity of skeletal fast white muscle fibre was not significantly affected with plant protein inclusion ($P > 0.05$), and hyperplasia was documented to be the dominant mechanism of muscle growth during the experimental period. Proximate composition of the fish did neither vary between diets ($P > 0.05$). It is therefore concluded that, fish meal in the diet of juvenile lumpfish can be replaced with 75% of SPC & PPC without compromising the growth performance, muscle cellularity and proximate composition.

Key words: *Cyclopterus lumpus,* Lumpfish, sea lice, growth, muscle cellularity, proximate composition, hyperplasia.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ANOVA	Analysis of variance
BL	Body length (Standard)
BW	Body weight
cm	Centimeter
CPSP 90	Pre-digested fish meal
CTRL	Control diet (0% plant protein)
DHA	Docosahexaenoic acid
dph	Days post hatch
EAA	Essential amino acids
EPA	Eicosapentaenoic acid
FAO	Food and Agriculture organization
FD	Fast muscle fibre density
FFD	Fast muscle fibre diameter
FM	Fish meal
FN	Fast muscle fibre number
g	Grams
H	Body height
hr	Hour
HSI	Hepatosomatic index
K	Condition factor
Kg	Kilo grams
l	Litre
LW	Liver weight
MCP	Monocalcium phosphate
mm	Millimeter
N	Number of counted fibre
NOK	Norwegian kroner (Currency)
P	Probability (α level)
PDFs	Probability density functions
PPC	Pea protein concentrate
SBM	Soybean meal
SEM	Standard error of mean
SGR	Specific growth rate
SPC	Soy protein concentrate
t	Time (days)
TCA	Total cross sectional area of fast muscle fibre
US$	Untied states of America currency
VSI	Viscera somatic index
VW	Viscera weight
W	Body width
µm	Micrometer

1. Introduction

The need for more food production is increasing as the world population is growing (FAO, 2017). Aquaculture is one of the promising industries which provided around 80 million tons sea food in 2014, and can sustainably contribute to food security while minimizing the ecosystem impacts (FAO, 2016). However aquatic animal disease is major constraint to the production (Murray and Peeler, 2005; Asche et al., 2009; Aunsmo et al., 2010). Therefore, significant portion of cost of production is spent for disease treatment. In 2006, the cost for sea lice treatments on 1.6 million tons global production of salmon was 305 million Euro (Costello, 2009a). In 2010, over 77 million US$ were spent in Norway on fish disease management, including the implementation of legislation and support to surveillance and control programs (Liu and Bjelland, 2014). Since then the numbers has increased further.

While the global aquaculture production is growing, the demand for sustainable supply of feed is also increasing. As a consequence, the price of the feed ingredients especially the fish protein became one of the more expensive macro nutrients. Over the decades in the aquaculture feed industries, finding the alternative fish protein especially the vegetable protein sources is a trend due to the global concerns on the over-exploited fishery industries. Therefore, seeking for sustainable protein sources for the fish feed ingredients is vital to the prevailing aquaculture industries to sustain.

1.1 Salmon farming and challenges

Atlantic salmon (*S. salar*) is one of the major aquaculture fish species in Norway (Torrissen et al., 2011; Taranger et al., 2015). In 2015, the production from Norway was 1.39 million tons with the first-hand value of NOK 46.7 billion. First sea lice, *Lepeophtheirus salmonis* (Krøyer) infestation outbreaks reported on Norwegian Atlantic salmon farm during the 1960's soon after cage culture began (Pike & Wadsworth, 1999). Commercial aquaculture in open net cages provide opportunity to increased number of susceptible hosts, and thus to elevated reproduction and spread of parasites leading a threat to the affected fish farms (Costello, 2009b). The outbreak of salmon louse is one of the main drawbacks of salmon production in Norway (Torrissen et al., 2013; Liu and Bjelland, 2014). In 2015, there were

129 salmon production sites in Norway using lumpfish as cleaner fish (Bornø et al., 2016), reported 83% of salmon mortality to specific diseases and 33% loss to bad handling and husbandry practice (Jonassen et al., 2017).

Salmon lice graze on the skin, muscle and mucosal tissue of the fish (Pike and Wadsworth, 1999; Boxaspen, 2006) causing secondary infections and osmotic stress which then leads to death (Wells et al., 2007; Johansen et al., 2011). Most severe tissue damage is caused by the mobile pre-adult and adult stage of sea lice (Wells et al., 2007). Physiological effects, reduced feeding were reported to be more severe for fish infested two weeks after transfer from fresh water to sea water (Dawson, 1998). Mortality range of 25 – 46% reported on hatchery-reared sea trout within 10 – 20 days exposure (Wells et al., 2007).

The Norwegian salmon aquaculture industry is facing increased difficulties with salmon lice (Nilsen et al., 2017) including resistant build up against most chemotherapeutants (Jansen et al., 2016). Abolofia et al., (2017) have estimated that sea lice parasitism cost US$ 436 million to the Norwegian industry in 2011. Jansen et al., (2016) have reported an average cost for the sea lice control treatment in Norwegian salmon farms was 2-5 NOK per kg produced salmon in 2014.

Fish welfare is one of the growing concerns for the sustainable aquaculture production (Brandal et al., 1976; Asche et al., 2005; Krkošek et al., 2006). By considering that, control and prevention of sea lice is important in salmon farming (Krkošek et al., 2006). In order to control sea lice, aquaculture industries heavily relied on chemotherapeutic treatments either bath with hydrogen peroxide or in-feed with avermactin benzoate or more recently synthetic pyrethroids (Denholm et al., 2002). However, Torrissen et al., (2013) suggested resistant accumulation to all these compounds in the sea lice population. Furthermore, such chemotherapeutic treatments are stressful to salmon (Burka et al., 1997), expensive (Costello, 2009a) and hazardous to the ecosystem (Burridge et al., 2010).

Therefore, emergence of biological control of sea lice (Treasurer, 2002) with the use of cleaner fish such as wrasse species (Bilal et al., 2016): ballan wrasse (*Labrus bergylta*), goldsinny wrasse (*Ctenolabrus rupestris*), rock cook (*Centrolabrus exoletus*), corkwing wrasse (*Symphodus melops*) and lumpfish (*C. lumpus*) has become feasible, effective and sustainable option in Atlantic salmon aquaculture (Denholm et al., 2002). Imsland et al.,

(2014) have suggested that lumpfish is the effective fish which control the sea lice infestation in cold-water, removing up to 97% of mature female sea lice from farmed salmon.

1.2 Biology of Lumpfish

The lumpfish is geographically distributed along the Norwegian coast (Holst, 1993) and in the arctic margins of the North Atlantic; in the east from 80^0 north Spitsbergen and Nova Zemlya in the north to Portugal in the south. In the west, along the coasts of America from Cape Cod to Canada and the coasts of Greenland 70^0 north in the north-west (Davenport, 1985; Nytrø et al., 2014; Kasper et al., 2014). Lumpfish (*C. lumpus*) belongs to Family: Cyclopteridae; Order: Scorpaeniformes; Class: Actinopterygii and Phylum: Chordata under Animalia kingdom (Wikipedia, 2017).

The lumpfish is a semi-pelagic species (Eriksen, Durif, & Prozorkevich, 2014); it can be easily distinguished by their high dorsal crest that covers the first dorsal fin entirely. It is a scale-less, short and thick fish. The pelvic fins of lumpfish are modified to constitute a ventral suction disc, allowing it to rest on substrate like rocks and algae. Juveniles spend one to two years in the pelagic intertidal zone (Moring & Moring, 1991).

Average lifespan of lumpfish is up to 6-7 years and maximum length of females can grow up to 60 cm and can weigh maximum 5 kg. Lumpfish spawn naturally from early spring until mid-summer. However, the spawning activities vary due to location and temperature of sea water. Female Lumpfish can lay around 60,000 (half liter) eggs in low tidal zones. Male Lumpfish protects the fertilized eggs until hatching (Garcia-Mayoral et al., 2016). Length of newly hatched larvae are around 4.5 – 5 mm. Larvae are born with suction discs, but lack swim bladder. The lumpfish larvae have special body shape like tadpoles and change colors during the first months of life from yellow-orange to dark brown, grey and green-blue (Vargas, 2016).

1.3 Lumpfish farming

Currently, wild-caught brood stocks are used to produce the lumpfish intensively. In Norway, currently 16 licenses were granted to produce lumpfish along the coast of Agder to Tromsø, with the estimated production of 12-14 million juveniles in 2015 (Vargas, 2016).

In aquaculture farms, lumpfish juveniles are produced by stripping the gametes from male and female, fertilized eggs are incubated around 270 – 300 degree-days depending on the temperature. Under optimum conditions, hatching rate of lumpfish is higher (98%). Larvae start to feed around 4 days post hatching (dph). During larval period, *Artemia* was used as live feed, since larvae can feed on microparticulate diet and many hatcheries dropped to use *Artemia* as start feed, as it is less work demanding and cuts production costs. Lumpfish is typically fed with 150 µm microparticle diets and shift to bigger particle size as they grow. At the size of approximately 4g the fish is ready for 1 - 1.5 mm pellets. Examples on commercial lumpfish diets are Gamma products (Skretting AS), Inicio Plus (Biomar AS) and Otohime (Marubeni Nisshin Feed CO., LTD). When fish reach approximately 0.2 g wet weight, first grading is often conducted. Lumpfish are graded every second or third week, before they reach market size of approximately 20 g. Size grading is important as lumpfish is very cannibalistic post hatching and up to approximately 4g. It is essential to keep high density and provide enough feed to suppress the cannibalistic behavior (Vargas, 2016). Juvenile grows faster in high temperature at 13 ^{0}C than low at 10 ^{0}C. However juveniles show good appetites to sea lice even at low temperature. Juvenile lumpfish is sensitive to water oxygen level and develops hypoxia below 80% in aquaculture (Jørgensen et al, 2017). It is also important to maintain a good water quality as to avoid bacterial infestation (Vargas, 2016). Lumpfish health and the development of efficient vaccines (Bilal et al., 2016) against some of the most common bacteria related problems is currently a hot developmental area (Haugland et al, 2017).

Imsland et al., (2015) has reported that lumpfish can easily adhere to artificial smooth, plastic surfaces than natural surfaces such as kelp; this characteristic behavior could make them be cultured in farming condition with reduction of stress and improving welfare.

1.4 Lumpfish as a cleaner fish

Among the potential cleaner fish, the lumpfish is a new and promising marine candidate, well suited for cultivation under cold northern climates (Imsland et al., 2014) while other wrasse species showed decrease in appetite (Lein et al., 2013) and temperature sensitive (Sundt & Jørstad, 1998). Groner et al., (2013) have reported that lumpfish efficiently graze on pre-adult and adult lice in salmon net pens. They are deployed in net pens when they reach the standard length of approximately 7 cm (Schaer & Vestvik, 2012).

Therefore, Lumpfish is better delousing agent in salmon farms, especially in northern Norway (Jørgensen et al., 2017) since it has greater temperature range tolerance (from 4 - 7 ^{o}C and lower), more robustness, relatively high survival during hatch and sea transfer at a size of 20 - 25 grams, fast growth, less susceptible to *Vibrio* infection and can be use in greater density than wrasse. Another aspect with the use of lumpfish is the increased sustainability of the salmon industry, making salmon production greener providing biological and environmental friendly solutions with the use of less traditional chemicals in the battle against salmon lice (Treasurer, 2002).

It is noted that small lumpfish (20 g) have a higher overall preference for natural food items, including sea lice, compared to larger ones (Imsland et al., 2016a). This makes slow to moderate and uniform growth of lumpfish more desirable than fast growth for its optimal use as cleaner fish in salmon aquaculture. Maintaining regular food source for lumpfish reared in salmon sea cages is vital especially in winter time to maintain healthy population (Imsland et al., 2015a), and feed blocks can be deployed in a proper way for that purpose without affecting their feeding behavior (Imsland et al., 2018). Potential studies have confirmed that juvenile lumpfish effectively graze on pre-adult and adult sages of sea lice attached to salmon (Willumsen, L. 2001; GIFAS, 2012).

1.5 Nutritional demands of lumpfish

Lumpfish can switch their preference towards food item that are most readily available to them within their environment (Imsland et al., 2015a). Although there are few published articles on the nutrition of lumpfish, Imsland et al., (2016b) has reported that feeding preference of lumpfish is linked to different family background.

Rincón-Cervera et al., (2009) have studied high levels of EPA and DHA accumulation in the roe of lumpfish, and the fat content between the sexes appeared to be different, as males having more lipid in the muscle than females. Davenport & Kjørsvik (1986) have suggested that the metabolism of lipid fractions may vary with gender. Imsland et al. (2015b) studied that nutritional problems may ensue if lumpfish only feed on salmon pellets after deployment. Novel formulated feeds are produced based on body composition and with a lower oil composition for rearing lumpfish in cages at rations of 4 – 6% body weight per day (Skretting, 2016). Anyhow, very little is known about complete nutritional demand for juvenile lumpfish. Attempt to investigate the whole body composition and muscle cellularity of juvenile lumpfish is even rare or almost no scientific publications up to date (2018) available to our knowledge.

1.6 Replacing fish meal with plant proteins

To replace fish protein with that of vegetable protein sources has been a trend since early 2000 and is linked to the global concerns on the over-exploited wild fish stocks and increased feed price (Gatlin et al., 2007; Hardy, 2010). Soybean (*Glycine max*) protein is the most available and economic solution with good amino acid profile (Peres and Lim, 2008; Jobling, 2012). Our study aims to replace fish meal with soy (*G. max*) and pea (*Pisum sativum*) protein concentrates (SPC & PPC). Therefore it is reasonable to overlook the quality of SPC and PPC. SPC has crude protein approximately equal to fish meal (FM) and considerably higher than soybean meal (SBM).

Soy protein contains higher crude fibre, minerals and much lower ash than those of fish meal. Soy protein has higher potassium, but lower calcium and phosphorous, than FM.

Anyhow, most phosphorous in soybean products is in the form of phytic acid which is relatively unavailable for fish. But, it can be mitigated with addition of exogenous enzyme (phytase). It is noted that, among plant protein sources, SPC protein has one of the best essential amino acid profile (EAA) including higher levels of arginine, similar to SBM, but lower in methionine and cystine (Peres & Lim, 2008). Heat treatment such as cooking and extrusion enhance the carbohydrate, protein and energy digestibility of soybean products. Furthermore, supplementation of deficient EAA, and minerals, addition of palatability enhancers improve the nutritional value of soybean products (Peres & Lim, 2008).

There are several studies suggested that partial or full replacement of fish meal is feasible without affecting the growth of fish species (Kissil et al., 2000; Chou et al., 2004; Hernández et al., 2007; Lim and Lee, 2008; Kader et al., 2012; Silva-Carrillo et al., 2012; Zhang et al., 2016). Anyhow conflict results have shown that replacement of fish meal with soy protein in fish species have adverse effects on growth performance (Lim et al., 2011; Ye et al., 2011; Song et al., 2014; Yaghoubi et al., 2016). However, the nutritional demand and published studies on lumpfish are rare.

The effects of replacing the feed fish meal protein by plant protein on the growth of fish have been studied for several species including Atlantic cod (Hansen et al., 2007; Colburn et al., 2012), Atlantic salmon (Carter and Hauler, 2000; Refstie et al., 2001; Øverland et al., 2009; Penn et al., 2011), European seabass (Gouveia and Davies, 2000; Kaushik et al., 2004), Gilthead Seabream (Kissil et al., 2000), Yellowtail (Watanabe et al., 1998), Milkfish (Borlongan, Eusebio, & Welsh, 2003), Rainbow Trout (Refstie et al., 2000; Thiessen et al., 2003; Zhang et al., 2012), freshwater Crayfish (Fuertes et al, 2013), and Spotted rose snapper (Silva-Carrillo et al., 2012), Red Seabream (Takagi et al, 2001) Red Drum (Davis, Jirsa, & Arnold, 1995), Turbot (Day & Gonzalez, 2000) and Atlantic Halibut (Grisdale-Helland et al., 2002). According to Colburn et al., (2012) juvenile Atlantic cod, *Gadus morhua* fed with the 50% fish meal replacement diets, grew as well or better than the control. Cod fed the 100% fish meal replacement diet exhibited the lowest growth and differed from the control with respect to final body weight, growth, specific growth rate, and thermal-unit growth coefficient. There were no enteritis was observed in histological sections. Colburn et al., (2012) indicated that 100% fish meal replacement is not recommended, but 50% replacement could be used without significant reductions in growth or condition indices. With the support of those tremendous studies on several fish species, our

study aims to focus on finding the effects of replacing fish meal with plant protein (SPC & PPC) at different inclusion levels on growth of juvenile lumpfish. Thus, it is vital to overlook how the skeletal muscle structure, growth and development of fish is affected.

1.7 Muscle structure, growth and development

The axial musculature of fish is the largest and fast growing organ (Alami-Durante et al., 1997) and constitutes up to 40% of the total body mass during early stages (Galloway, Kjørsvik, & Kryvi, 1999). Fish myotomes which have a W-shape in two dimensions and consist of overlapping cones in three dimensions (Van Leeuwen, 1999) are mostly composed of white muscle fibres (Luther et al., 1995; Koumans and Akster, 1995) that provide power during swimming (Rowlerson and Veggetti, 2001; Johnston, 2006). The rest is the superficial layer of aerobic slow fibres that powers sustained activity and intermediate muscle fibre type, if present, have aerobic and glycolytic capabilities and intermediate contractile properties to slow and fast muscle fibres (Johnston et al., 1977; Luther et al., 1995). Figure 1A illustrates the position of different muscle fibre types in a fish[1].

Figure 1B illustrate the microscopic diagram of different muscle fibre types. Muscle fibre is composed of a bundle of cylindrical cross-striated structures, the myofibrils which contain the contractile material. Muscle sarcomere which is composed of myosin and actin filaments, is the composing unit of myofibrils that gives rise to cross-striated appearance (Videler, 1993; Luther et al., 1995). Figure 1C illustrates the structure and components of the skeletal muscle fibre of vertebrate.

Muscle growth in fish differs from mammals because of muscle recruitment continues throughout the life in fish (Greer-Walker, 1970). Girth of fish increase mainly by hypertrophy while synthesis of contractile filaments, whereas large body size is attained in Atlantic salmon mainly by hyperplasia (Johnston, 1999). However, Hagen et al., (2008) have reported that myotube production in fast myotomal muscle is stopped at shorter body lengths in male than female Atlantic halibut as a consequence, lower final fibre number.

[1] Figure 1(A) and (B) were adapted from Koumans and Akster, (1995), and (C) from Videler, (1993).

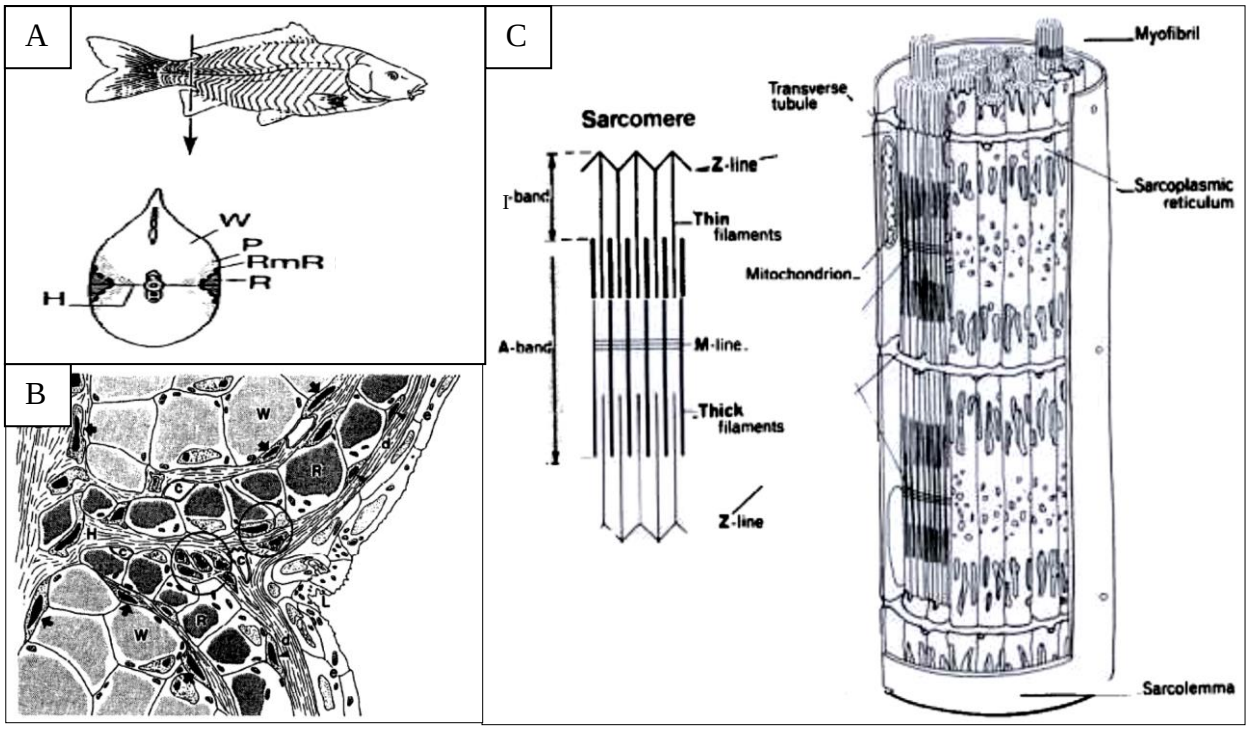

Figure 1: (A) Position of different muscle fibre types in the tail of a fish. W= fast white fibre, P= intermediate pink fibre, R= slow red fibre, RmR= remnant of superficial red zone, H= horizontal septum. (B) Diagram showing muscle fibre types of *Clarias* larva. C= capillary, d= dermis, arrows= myogenic cells, arrowheads= external cells, circles= precursor cells of red muscle zone. (C) Structure and nomenclature of a muscle fibre.

Muscle development through hyperplasia in fish can be categorized into three phases[2] as shown in Figure 2: embryonic, stratified and mosaic hyperplasia (Johnston, 2006). During embryonic myogenesis, the adaxial and posterior cells generate the superficial and deep muscle cells respectively (Devoto et al., 1996; Rescan, 2005 and 2008), components of the primary myotome (Stellabotte & Devoto, 2007). Even though both fibre types are aerobic, they will then differentiate into slow contraction speed red colour aerobic (red) and fast contraction speed white colour anaerobic (white) fibres around metamorphosis (Johnston, 1999).

[2] Figure 2 was adapted from Johnston, (2006) based on Johnston et al., (2004).

After that, growth of the primary myotome takes place by stratified hyperplasia. During stratified hyperplasic stage, fibres from the external cells shift from outer to the inner surface of slow fibres to position in discrete germinal zones located at the dorsal and ventral regions of the myotome (Stellabotte & Devoto, 2007). Lastly, mosaic hyperplasia forms new fast fibres between the existing fibres and give rise to an assortment of fibre sizes (Johnston, 2006).

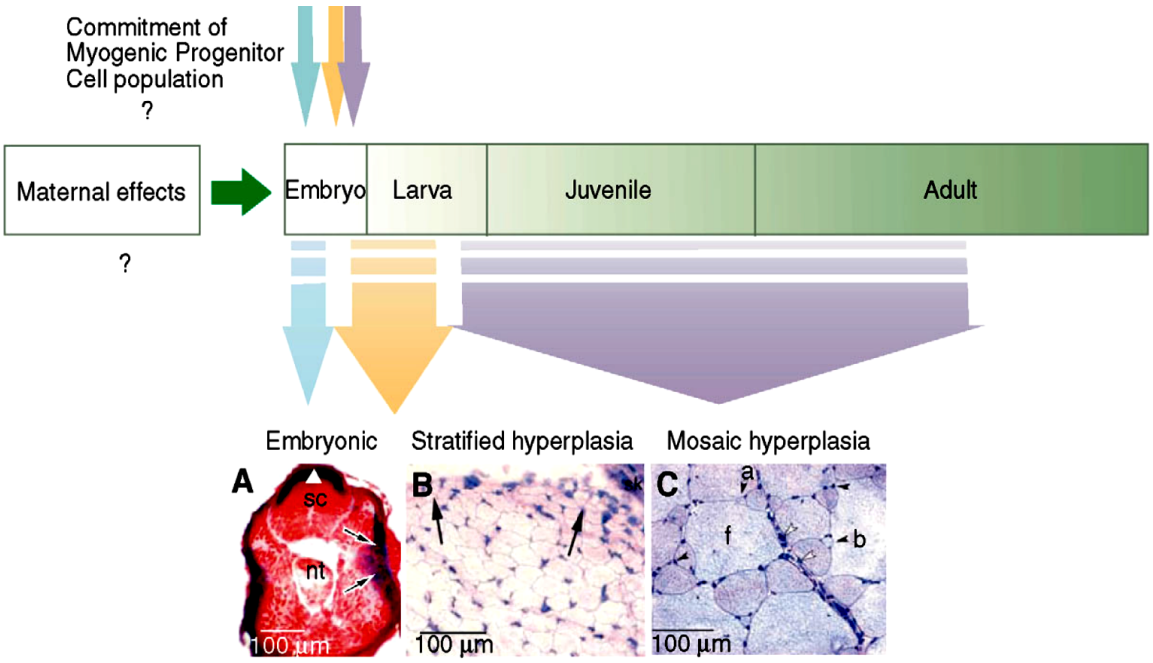

Figure 2: The three phases of myogenesis in fast myotomal muscle of the arctic charr *Salvelinus alpines*: embryonic (blue arrow), stratified hyperplasia (orange arrow) and mosaic hyperplasia (mauve arrow). (A) The rostral somites of an arctic charr embryo, arrows illustrate intense staining for Pax 7, arrowhead shows intense staining in the dorsal region of the spinal cord. nt= notochord; sc= spinal cord. (B) Stratified hyperplasia (arrows) in the apical regions of the fast muscle layer of the myotome in an arctic char juvenile. sk= skin. (C) Mosaic hyperplasia in the fast muscle of arctic charr, f= mature fibre, (a) and (b) are 14 and 18 µm daughter fibres respectively. Filled and unfilled arrow heads represent myonuclei and connective tissue nuclei.

Occurrence and duration of mosaic hyperplasia depends on the fish species, late but large size in fast-growing species and greatly reduced with small ultimate size in slow growing species (Johnston, 1999). Campos Vargas et al., (2015) have reported that the total number of fast muscle fibres showed a 10-fold increase, and the diameter of fast fibre also increased in Atlantic cod larvae regardless of ploidy.

Johnston, (1999, 2006) has summarized that skeletal muscle of fish shows high phenotypic plasticity to environmental factors like temperature, swimming activity and diets. Kiessling et al., (1991) have studied the number of muscle fibres recruited in rainbow trout was affected by diet ration. Similar studies conform it in several fish species (Alami-Durante et al., 2010; Matos et al., 2012). Total fish meal replacement resulted significant reduction in muscle cross sectional area due to reduced fibre size in Senegalese sole fish (Valente et al., 2016). In contrast, rice protein concentrate fed blunt snout bream did not show adverse effect on the fibre recruitment, but higher muscle fibre frequency in the 20 - 50 μm class but less >50 μm class was observed (Cai et al., 2018).

However, literature on the muscle growth dynamics for juvenile lumpfish none exists and more research is needed to increase the scarce knowledgebase for this upcoming aquaculture species.

1.8 Thesis Objectives

The present study was conducted to investigate how juvenile lumpfish respond to a diet in which fish meal was gradually replaced by commonly used plant based proteins (SPC & PPC). It is important to evaluate at what level fish meal can be replaced without affecting the performance of juvenile lumpfish. The performance of the fish in terms of 1) growth, 2) survival, 3) proximate composition and 4) white muscle fibre growth dynamics were therefore investigated.

2. Materials and methods

The experiment including all procedures and fish handling were conducted in accordance to the guidelines set by the National Animal Research Authority (Forsøksdyrutvalget, Norway). All students and staff involved in this project received training and certificate which was approved by the Forsøksdyrutvalget, prior to conducting the experiment.

2.1 Fish and fish rearing

The feed experiment was carried out at the Marine Research Station (Mørkvedbukta), and laboratory analyses were carried out at the laboratory of the faculty of biosciences and aquaculture at Nord University, Bodø, Norway. In order to conduct a 7.5 weeks experiment, juvenile lumpfish (*C. lumpus*) were obtained from Mørkvedbukta AS fish farm, Bodø, Norway. The initial weight of 4 g juveniles were transported to research station (Hall 4). 12 cylindrical, green colour tanks of 500-liter holding capacity were arranged. Around 2000 juveniles per tank were randomly distributed. Each tank was aided with a special automatic feeder and separate oxygen supply.

The rearing conditions including water temperature, oxygen, salinity, water flow, pH and light intensity, but except feed were kept identical in all experimental tanks. Juveniles were acclimatized for 2.5 weeks at the new experimental unit and they were fed with Skretting Gemma Silk. The light density was controlled by four florescent lamps (24 hr) mimicking the commercial rearing light set up (dim and upwards facing). Water flow was kept at 400 l/h. The average temperature was 8 ^{o}C, salinity was 34% from 250 m depth and the average oxygen level was 9.0 mg/l. Feed was supplied to all the tanks continuously to apparent satiation with an automatic feeder (automated with average body mass increase; at eight time intervals as following: 06.00 – 08.30, 08.30 – 11.00, 11.00 – 13.00, 13.00 - 15.00, 15.00 – 17.00, 17.00 – 19.00, 19.00 – 21.00 and 21.00 – 22.00).

Experimental unit was monitored daily with daily routines including cleaning and flushing of excess feed and faeces in all tanks. Oxygen level and temperature were measured with a hand held OxyGuard. Uneaten feed and feaces were cleaned with the tube-siphon in every cleaning schedule and after every sampling is done.

2.2 Experimental design

At the research station (Mørkvedbukta), 12 tanks were randomly marked with different color code in order to represent four different feeding regimes with triplicate tanks. The experiment was conducted at the research station from 08th May 2017 to 15th July 2017. Sampling was done at week 0 prior to feed the fish with experimental diets. After the introduction of experimental diets, three more sample points were selected at 2.5 weeks time interval. Figure 3 shows completely randomized experimental design.

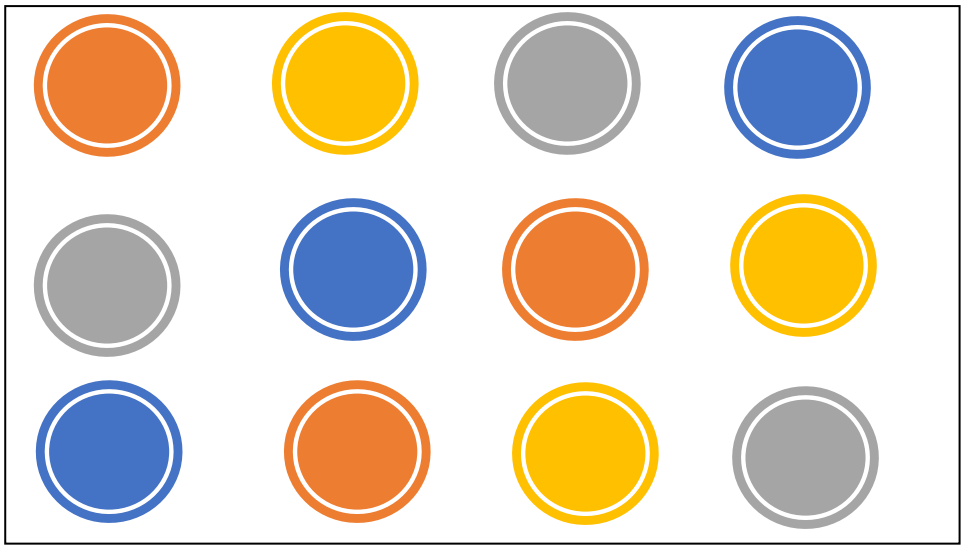

Figure 3: Experimental design – different feed allocation with colour codes: Blue – control (0%), Orange – 25%, Ash – 50%, Yellow – 75%. Each diet was in 3 replicate tanks and fish was randomly distributed in 12 tanks for the four diet groups. Each tank is experimental unit.

2.3 Feed formulation and proximate composition

As per the objective of the study, four isonitrogenous and isoenergetic experimental diets were formulated in which 0, 25, 50 and 75% of fish meal in diet were replaced by SPC & PPC as shown in Table 1. The inclusion level of krill meal, CPSP 90, wheat gluten, pea starch, fish oil, vitamin & mineral premix PV01, lutavitE50, antioxidant powder were kept constant in the four diets, whereas wheat meal, krill oil, MCP, L-tryptophan, DL-methionine inclusion level were used to adjust the total to 100% (Table 1). Commercial diet was kept as control (CTRL) and tested against the treatment diets (25%, 50% and 75%). Proximate composition of the experimental diets is shown in Table 2.

Table 1: Experimental diet and formulated feed ingredients for juvenile lumpfish.

	Experimental diet			
Diet code	**CTRL(0%)**	**25%**	**50%**	**75%**
Ingredients, %	%	%	%	%
Fishmeal 70 LT (NORVIK)	58.000	43.500	29.000	14.500
CPSP 90	2.500	2.500	2.500	2.500
Krill meal (Aker Biomarine)	5.000	5.000	5.000	5.000
Soy protein concentrate (Soycomil)	0.000	7.200	14.450	21.670
Pea protein concentrate	0.000	7.200	14.450	21.670
Wheat gluten	7.000	7.000	7.000	7.000
Wheat meal	10.000	9.160	6.950	4.650
Pea starch	5.330	5.330	5.330	5.330
Fish oil - SAVINOR	6.800	6.800	6.800	6.800
Krill oil	1.700	2.450	3.250	4.050
Rapeseed oil	0.000	0.000	0.000	0.000
Vit & Min Premix PV01	1.000	1.000	1.000	1.000
Lutavit E50	0.050	0.050	0.050	0.050
Soy lecithin - Powder	0.000	0.000	0.000	0.000
Antioxidant powder (Paramega)	0.200	0.200	0.200	0.200
Sodium propionate	0.100	0.100	0.100	0.100
MCP	0.000	0.000	0.980	2.100
Carophyll Pink 10% - astaxanthin	0.050	0.050	0.050	0.050
Nucleotides (Nucleoforce)	0.500	0.500	0.500	0.500
Garlic extract	0.500	0.500	0.500	0.500
L-Histidine	0.250	0.250	0.250	0.250
L-Tryptophan	0.000	0.090	0.170	0.260
DL-Methionine	0.000	0.000	0.350	0.700
L-Taurine	1.000	1.100	1.100	1.100
Yttrium oxide	0.020	0.020	0.020	0.020
Total	**100.000**	**100.000**	**100.000**	**100.000**

Table 2: Proximate composition, amino acid profile, vitamins & minerals and fatty acids of the experimental diets for the juvenile lumpfish.

	Experimental diet			
As fed basis	**CTRL (0%)**	**25%**	**50%**	**75%**
Crude protein	53.85	53.86	53.88	53.86
Crude fat	13.42	13.40	13.41	13.42
Fiber	0.34	0.69	1.02	1.34
Starch	9.24	9.45	8.83	8.15
Ash	11.30	9.49	8.26	7.10
Gross Energy	20.01	20.27	20.34	20.38
Arginine	3.46	3.72	3.98	4.23
Histidine	1.40	1.40	1.40	1.41
Isoleucine	2.01	2.13	2.25	2.36
Leucine	3.85	3.93	4.00	4.07
Lysine	3.93	3.94	3.94	3.94
Threonine	2.47	2.35	2.22	2.10
Trptophan	0.55	0.55	0.55	0.55
Valine	2.51	2.55	2.59	2.63
Methionine + Cysteine	2.27	1.96	1.98	2.00
Phenylalanine + Tyrosine	4.50	4.51	4.52	4.53
Taurine	1.18	1.24	1.21	1.17
Total Phosphorous	1.72	1.47	1.43	1.43
Vitamin C (mg/kg)	1000	1000	1000	1000
Vitamin E (mg/kg)	350	350	350	350
EPA	1.59	1.70	1.82	1.95
DHA	1.96	1.84	1.73	1.62
EPA+DHA	3.55	3.54	3.55	3.56
Total phospholipids	2.72	2.68	2.66	2.65

2.4 Fish sampling and data collection

At the start of the experiment, all individual weights, standard length, width and height of the fish were recorded. Similarly, all the measurements mentioned were recorded at the end of the experiment. Fish (n= 35/tank, 420/sampling point) for the various analysis were randomly collected with four sampling points at 2.5 weeks intervals, week 0 (reference sampling), 2.5, 5 and 7.5. Prior to collect the samples, fish were anaesthetized with Ms-222 (Tricaine methane sulphonate; Argent Chemical Laboratories, USA; 30g /l). At each sampling point 5 fish/tank were randomly sampled for the liver proteomics, digestive enzyme assays (results not present in the thesis) and muscle histology. In addition, 20 fish/ tank were sampled for whole body chemical composition analysis. The biometric data were recorded

for all sampled fish, i.e. weight, standard length, height, width, liver and visceral weight. After dissecting the fish for the liver and gastrointestinal tract, the same remaining carcass was used for the muscle histology.

2.5 Biometric measurements

The following formulas were used for the calculations of specific growth rate (SGR) (Houde & Schekter, 1981), condition factor (K), hepatosomatic index (HSI) and viscera somatic index (VSI):

1) SGR = (e^{b}−1) × 100, where b = (ln (W2) − ln (W1)) / (t2−t1) andW2 = final wet weight (g), W1 = initial wet weight (g), t2 = final day and t1 = initial day.
2) K = (Body wet weight / $Length^{3}$) × 100
3) HSI = (Liver wet weight / Body wet weight) × 100
4) VSI = (Gastrointestinal tract wet weight / Body wet weight) × 100

2.6 Muscle histology

Juveniles were sectioned transversely to the body axis at post-anal level, and the anterior muscle steaks (left – A, and right - B) were taken separately as shown in Figure 4.

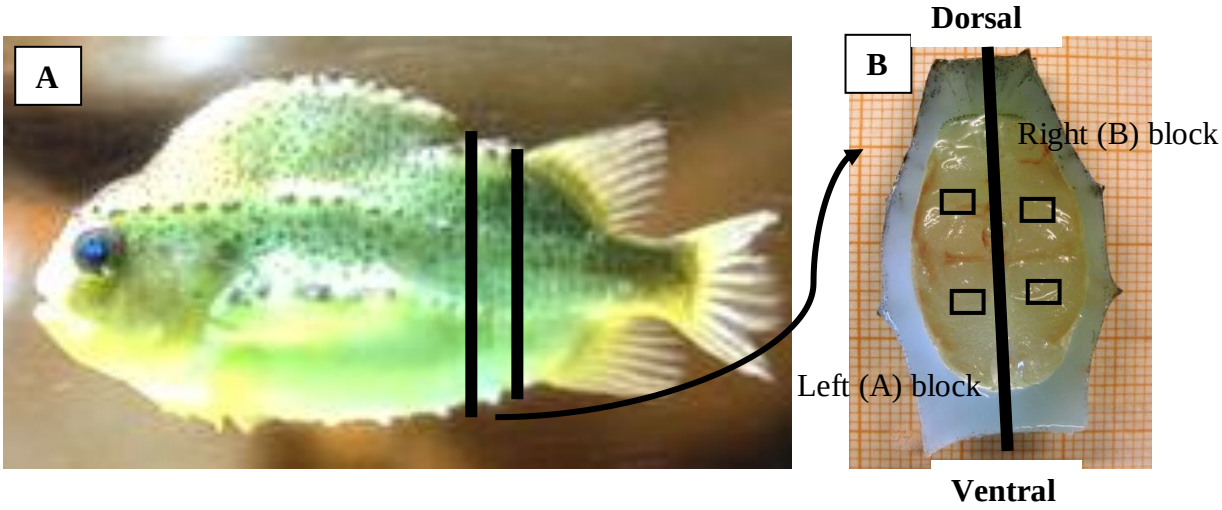

Figure 4: Location of muscle steak cut (0.5cm thick) at post-anal level (A) and the location of different blocks (B) used for muscle fibre count.

The blocks were mounted in pre-labeled cork pieces using cryomatrix (Anatomical pathology/Bergmann As, Oslo, Norway) and immediately frozen for 60 seconds in 2-methyl butane (Isopentane, C_6H_{12}). The frozen muscle blocks were wrapped with pre-labeled aluminum foil and temporarily stored in liquid nitrogen (at – 159 ^{o}C) container, after that they were placed in a -80 ^{0}C freezer.

The muscle blocks were acclimated for 15 minutes in the cryostat (Microm HM 550, MICROM International/Bergmann AS, Oslo, Norway) at -18 ^{0}C prior to sectioning. Before mounting, empty slides were treated with poly-L-lysine for 5 minutes and air dried overnight. The muscle block was trimmed with 20 µm and sectioned at 7 µm thick. The suitable cut muscle section was mounted on empty slide and air-dried for 45 seconds with a hair dryer. The slides were stained with Harris Haemotoxyline solution for 3.5 minutes and washed with clean tap water for 8 minutes. The air-dried slide was covered with a cover glass after adding a drop of Glyserol gelatin (Sigma Aldrich, Steinheim, Germany).

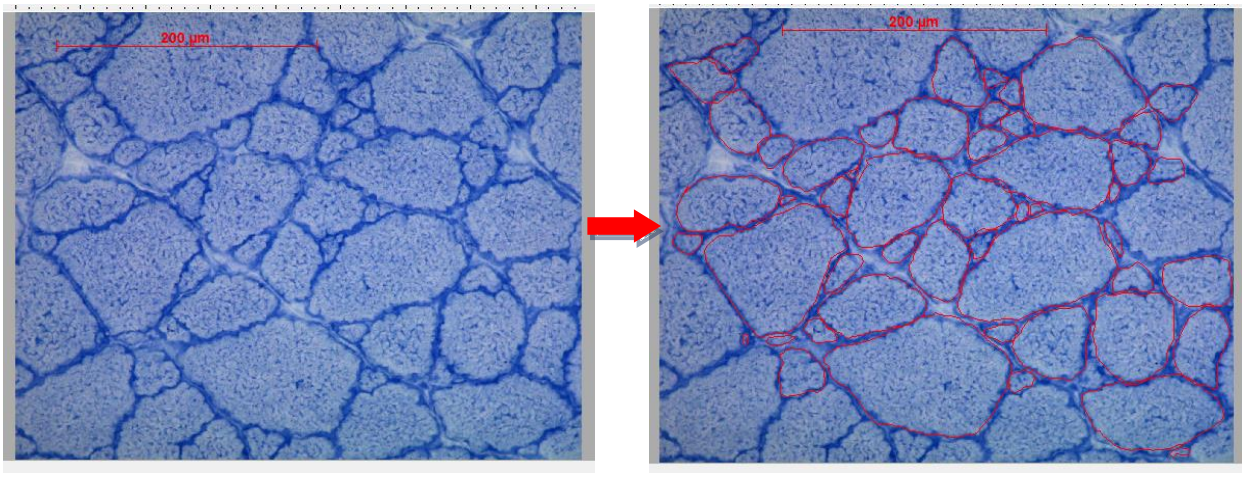

Figure 5: The fast white muscle fibre under light microscope (left), demarcated the same fibre area using the Axio vision software (right) for juvenile lumpfish at week 7.5. Weight= 49.48g, length= 9cm, height= 4.5cm and width= 3.5cm. Control group. Scale bar= 200 µm.

The white muscle fibers were analyzed using a light microscope (Axioscop 2 mot plus; Carl Zeiss INC., Germany) equipped with a camera. The area of 800 – 850 fibres from the left and right epaxial (dorsal) and hypaxial (ventral) side of the steak of white muscle sections (Figure 5) were calculated for each fish using the software Axio Vision (Rel.4.2, Carl Zeiss INC., Germany). Calculations were done using following formulas:

1) The fast fibre diameter (FFD) = $2\times\sqrt{}$ (Area of fibre/π)

2) The total fibre number (FN) = [10^6 x TCA (mm^2) x N] / [Total area of fibres (μm)].

3) The fibre density (FD) = [10^6 x N] / [Total area of fibres (μm)].

Where, N = number of counted fibre, TCA = total cross-sectional area of fast muscle steak, calculated using the Sigma Scan pro software (v.5.0, Systat, Inc.).

2.7 Proximate composition

2.7.1 Sample preparation for the proximate composition analysis

The fish were pooled (10fish/pool, 2-pooled samples/tank) and minced into a homogeneous mass for 6 x 15 seconds using a conventional food processor (Bosch GmbH, CNCM11, Slovenia). Part of this homogenate used for determine the moisture and ash content in whole fish (as fed basis). Remained fish homogenate was freeze dried (96 hours at -70 ^{0}C) and dry matter was recorded. The dried samples were frozen at -80 ^{0}C before re-grind (3x15 sec) it into the fine powder, for the crude protein and fat analysis (dry basis).

2.7.2 Moisture and ash content

The empty weight of crucible was measured. From each pooled sample 2.0 g of minced fish was measured for moisture and ash content analysis. The dry matter was determined gravimetrically after drying in an oven at 104 ^{0}C for 24 hours. The dry weight was measured after placing it in the dessicator to cool down to room temperature. To determine the ash content of the whole fish, the remaining content from moisture removal was then placed in the muffle furnace at 540 ^{0}C for 8 hours. The final weight was recorded after placing it in the dessicator. The following formulas were used to calculate the moisture and ash content (wet basis).

1) Moisture (%) = $\frac{\text{(Initial wet weight of fish – Dried weight of fish) (g)}}{\text{Initial wet weight of fish (g)}} \times 100$

2) Ash content (%) = $\frac{\text{(Weight after muffle furnace) (g)}}{\text{Initial wet weight of fish (g)}} \times 100$

2.7.3 Crude protein

The crude protein was determined by the Kjeldahl method which includes digestion, neutralization and titration. Two pooled samples weighing 0.500g were taken from weeks 2.5, 5.0, and 7.5 sampling points, while 0.200g was taken from the initial sampling point as the fish were too small at week 0. The freeze-dried sample was weighed in a nitrogen free paper and put into a digestion tube and then digested by heating (240 ^{0}C, for 50 minutes) it in the presence of the concentrated sulfuric acid (98%, 20 ml) and catalyst (2 Kjeltac tablets). The result product (ammonia) is then cooled down to room temperature. The digestion flask was inserted into automatic Kjeltec machine for neutralization and titration after adding 50 ml of distilled water. The machine automatically calculated (Crude protein = extracted nitrogen × 6.25) and provided the protein value in dry basis. Following formula was used to convert it into wet basis:

1) Protein (%) (Wet basis) = (Protein % (dry basis) × dry matter content %) / 100

2.7.4 Crude fat

The crude fat was determined gravimetrically after ethyl acetate extraction. From each sampling point, 1.00g of freeze-dried, re-homogenized whole fish sample was weighed in a plastic container. The sample was transferred into a bottle container after adding 20 g of sodium sulfate. In order to extract the fat, the content was mixed and stirred well for an hour after pouring 50 ml of ethyl acetate in a fume hood. The weight of the empty petric dish was measured. The extract was drained into a measuring cylinder and transferred into a petric dish, and allowed to dry over the hot water-bath for 20 minutes. The petric dish was placed in an oven at 104 ^{0}C for 20 minutes. The final weight was measured using the digital balance after it was taken from the oven and placed into the desiccators for another 20 minutes. Following formula was used to calculate the crude fat % in dry basis:

1) Crude Fat (%) = $\frac{\text{(dry weight of fat and petric dish – empty petric dish) g}}{\text{(Dry sample weight) g}} \times 100$

2.8 Statistical analysis

Statistical analysis was conducted mainly using SPSS (version 24). When necessary, data were logarithmically (log 10) transformed. All raw and transformed data were tested for normality of distributions (Shapiro Wilk's test) and homogeneity of variance (Levene's F test) (Zar, 1984). Normally distributed and homogenous variance data were compared with a one-way analysis of variance (ANOVA). Observed significant differences ($P < 0.05$) among treatment diets were followed by paired comparisons (Tukey's HSD) for multiple comparisons. Welch's F test was performed if the homogeneity of variance is violated. Non-parametric Kruskal-wallis test was performed if assumptions of normality and homogeneity of variance were not met. A significance level (α) of 0.05 was used if not stated otherwise. Detailed information regarding variable mean ± SEM (graphs were created using Microsoft Excel 2010) statistics are given in the appendix as well.

The following linear model was used:

$A = X \beta + \varepsilon$ Where, A is the vector of parameter of juvenile lumpfish, X is a design matrix that accounts for the experimental diets (fixed effect), β is the unknown vector of parameter estimates for experimental diets. The ε is the vector of unknown random error which is no longer required to be independent or homogenous.

The muscle cellularity data especially the probability density function (PDFs) was analyzed with R software[3] and a developed programme called FibreA.prg[4]. Nonparametric statistical techniques were used to fit smoothed probability density functions (pdfs) to the 800 fast fibres diameter measured per individual using a kernel function (Johnston et al. 1999). A nonparametric Kruskal-Wallis rank sum test used to check for differences in the pdfs between groups. The basic R code for the smooth PDFs graph is represented in the appendix.

[3] Accessed: (http://cran.r-project.org/) on 11/12/2017.
[4] Developed by the staff of Nord University, Bodø, Norway.

3. Results

3.1 Growth performance

The results showed that body weight (BW), body length (BL), body width (W), body height (H), liver weight (LW), viscera weight (VW), specific growth rate (SGR), condition factor (K), hepatosomatic (HSI) and viscera somatic (VSI) indices were not significantly affected by the different dietary plant protein inclusion ($P > 0.05$).

Body weight

The initial body weight of juvenile lumpfish prior to the introduction of experimental diets (mean ± SEM) at week 0 was in the range of 7.05 ± 0.21 g and 7.38 ± 0.10 g for all diet groups and increased to 42.25 ± 1.93 g, 41.04 ± 1.08 g, 45.92 ± 1.37 g, and 41.63 ± 3.50 g for CTRL, 25%, 50% and 75% diets respectively, at week 7.5 (Figure 6, $P > 0.05$). Body weight showed increasing throughout the whole experiment. This represents a weight increase of approximately 6 fold in just 7.5 weeks. Even though the 50% diet group had tendency of higher end weight, this was not different from the other diets ($P > 0.05$).

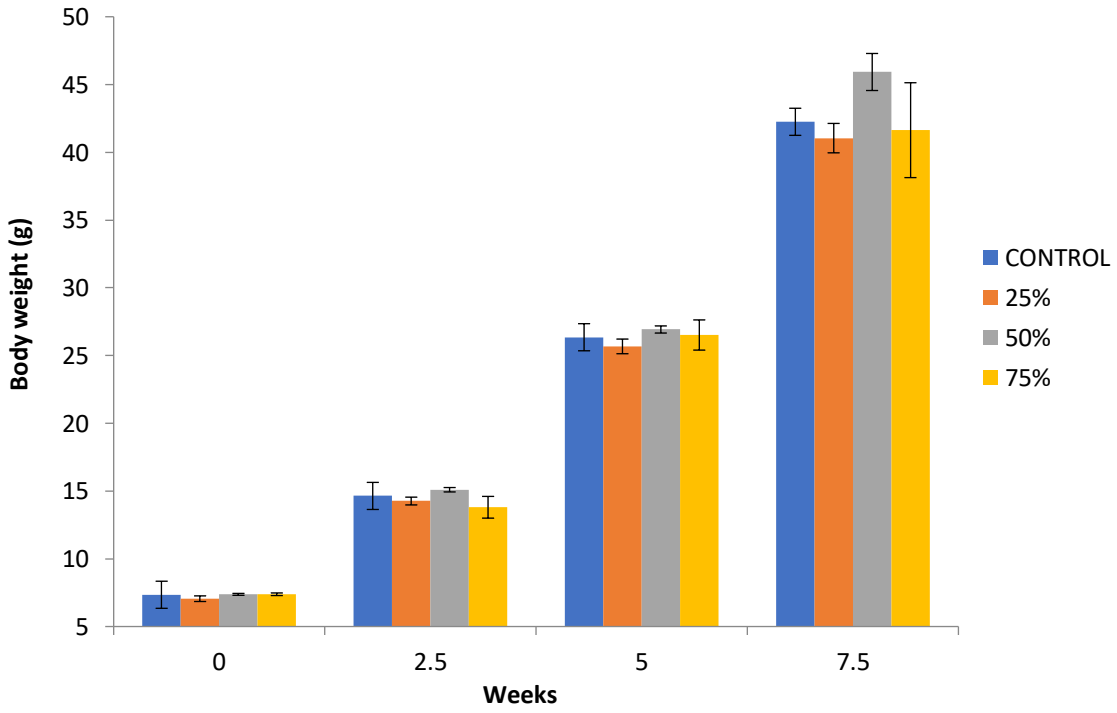

Figure 6: Whole body weight (mean ± SEM) of juvenile lumpfish fed diet with different levels of plant protein (Blue – Control, Orange - 25%, Ash - 50% and Yellow -75%). N=3. If any, different letters indicate significant difference, P<0.05.

Body length

The initial body length (mean ± SEM) at week 0, were between 4.87 ± 0.07 cm to 4.95 ± 0.03 cm for all groups with only minor differences (Figure 7, P > 0.05). The final body length (mean ± SEM) at week 7.5, were 9.40 ± 0.56 cm, 8.65 ± 0.13 cm, 8.84 ± 0.20 cm, and 8.39 ± 0.16 cm for CTRL, 25%, 50% and 75% diets respectively (Figure 7, P > 0.05). This represents an average increase in length for all groups of approximately 1.8 fold in 7.5 weeks.

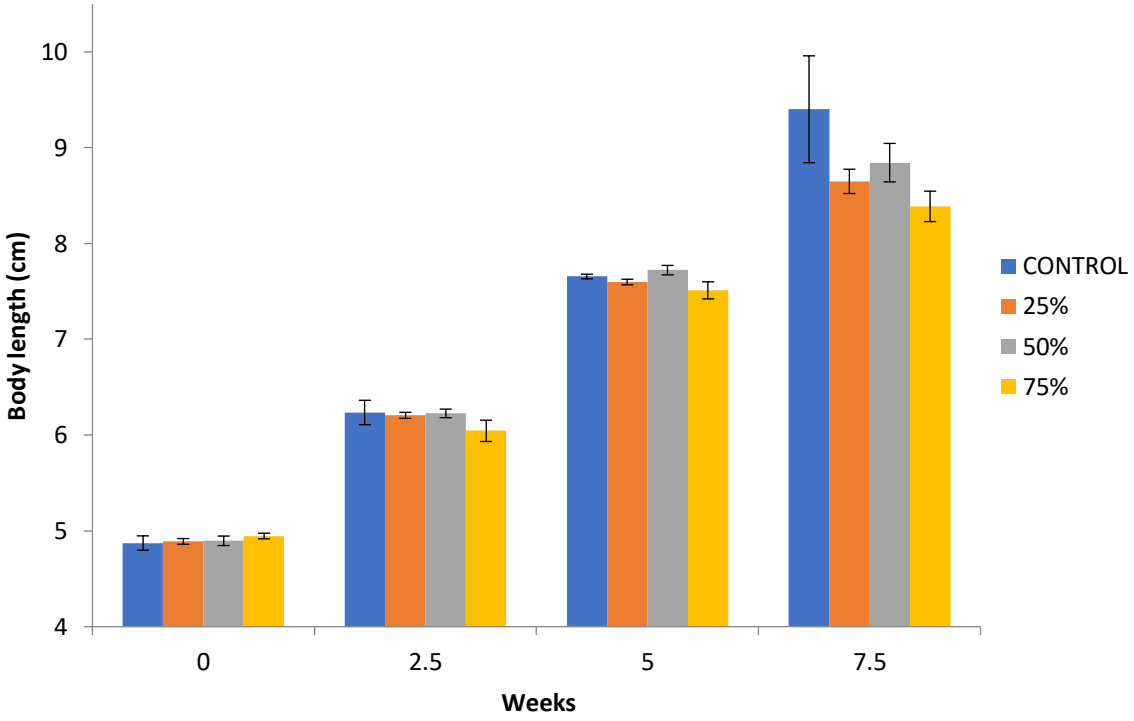

Figure 7: Body length (mean ± SEM) of juvenile lumpfish fed diet with different levels of plant protein (Blue – Control, Orange - 25%, Ash - 50% and Yellow -75%). N=3. If any, different letters indicate significant difference, P<0.05.

Body width

Width of juvenile lumpfish increased by approximately 2 folds over the experimental period, but with a declining rate over the experimental period (Figure 8). The largest increase in width (on average 1.6) took place between week 0 and week 2.5. The body width at week 0 were approximately 17 mm for all groups and this increased to 3.38 ± 0.23 cm, 3.42 ± 0.18 cm, 3.47 ± 0.20 cm, and 3.33 ± 0.06 cm at week 7.5 for diet groups CTRL, 25%, 50% and 75% respectively (Figure 8, $P > 0.05$).

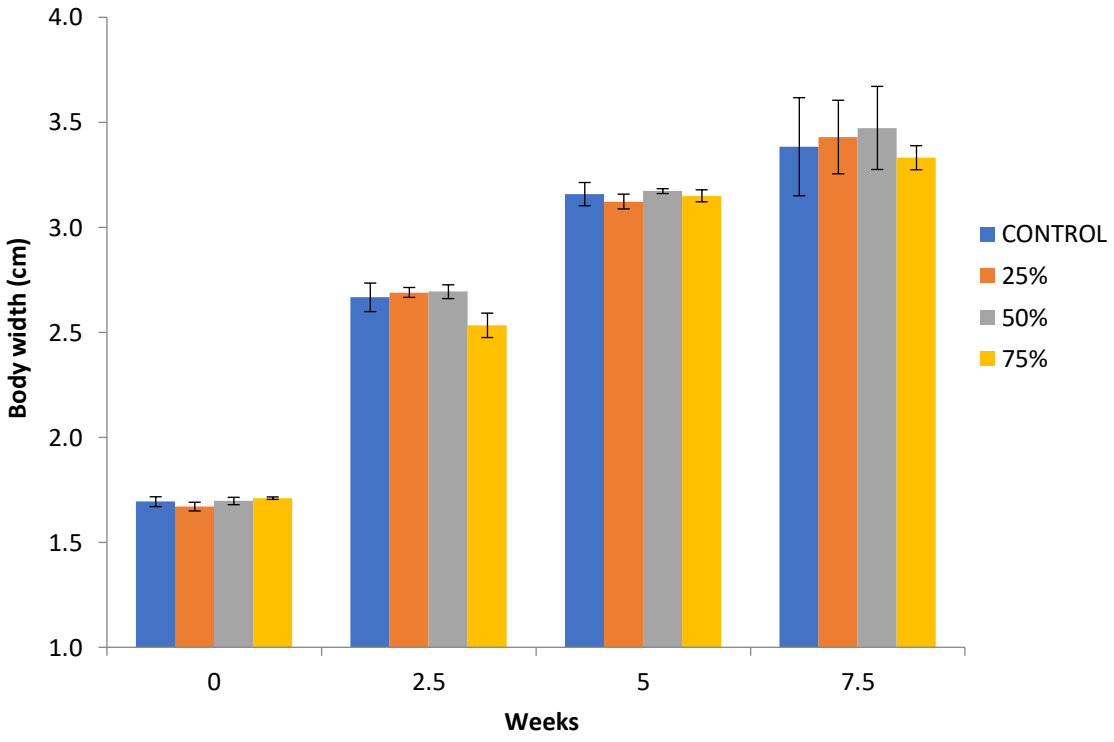

Figure 8: Body width (mean ± SEM) of juvenile lumpfish fed diet with different levels of plant protein (Blue – Control, Orange - 25%, Ash - 50% and Yellow -75%). N=3. If any, different letters indicate significant difference, P<0.05.

Body height

The initial body height (mean ± SEM) at week 0, were between 2.37 ± 0.03 cm and 2.44 ± 0.01cm for all groups with only minor differences (Figure 9, P > 0.05) and this increased to 4.32 ± 0.09 cm, 4.35 ± 0.04 cm, 4.50 ± 0.09 cm, and 4.33 ± 0.13 cm at week 7.5 for diet groups CTRL, 25%, 50% and 75% respectively (Figure 9, P > 0.05). This represents an average increase in height for all groups of approximately 1.8 fold in just 7.5 weeks.

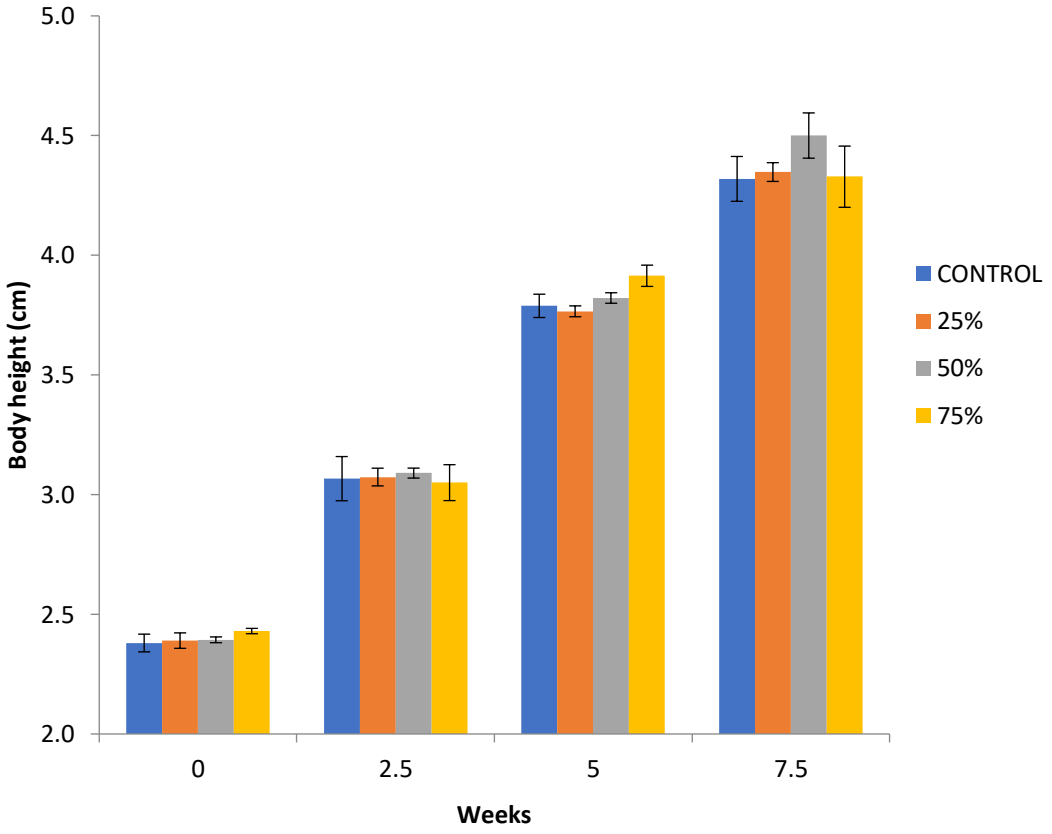

Figure 9: Body height (mean ± SEM) of juvenile lumpfish fed diet with different levels of plant protein (Blue – Control, Orange - 25%, Ash - 50% and Yellow -75%). N=3. If any, different letters indicate significant difference, P<0.05.

Liver weight

The initial liver weight of juvenile lumpfish prior to the introduction of experimental diets (mean ± SEM) at week 0 was in the range of 0.15 ± 0.03 g and 0.19 ± 0.01 g for all diet groups and increased to 1.04 ± 0.09 g, 0.96 ± 0.04 g, 1.09 ± 0.03 g, and 1.05 ± 0.07 g for CTRL, 25%, 50% and 75% diets respectively at the end of the experiment (Figure 10, $P > 0.05$). This represents a liver weight increase of approximately six fold during the experiment (7.5 weeks).

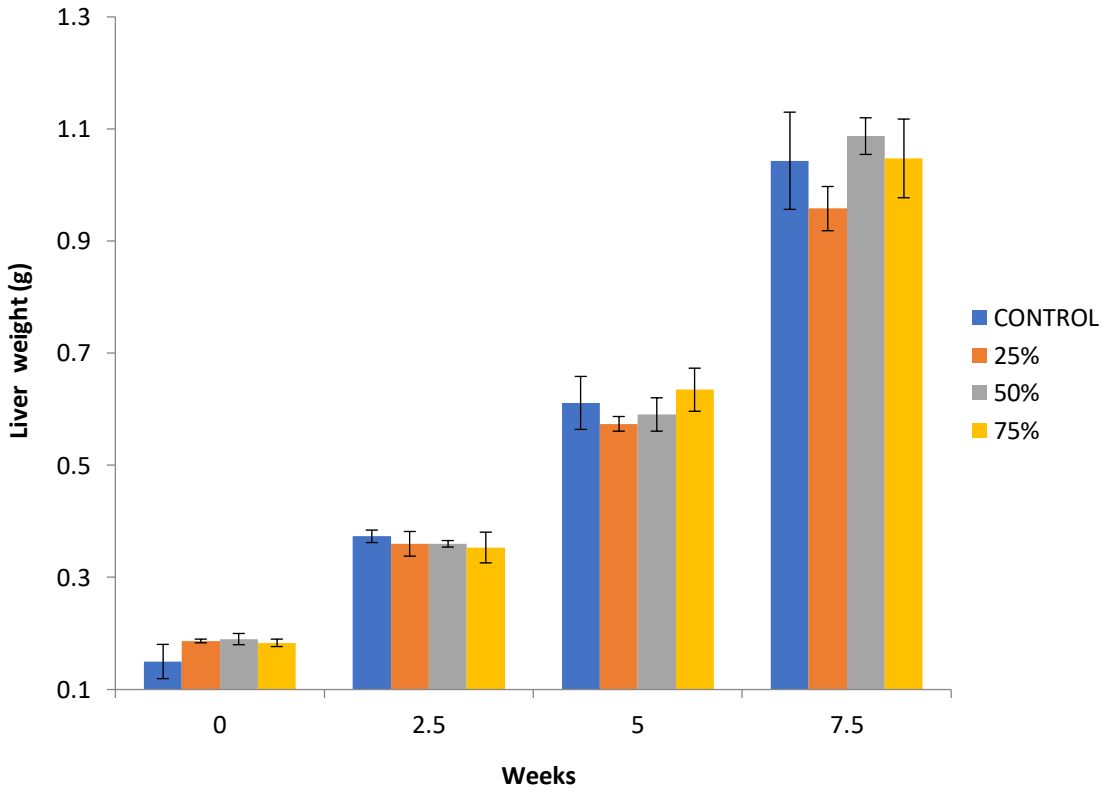

Figure 10: Liver weight (mean ± SEM) of juvenile lumpfish fed diet with different levels of plant protein (Blue – Control, Orange - 25%, Ash - 50% and Yellow -75%). N=3. If any, different letters indicate significant difference, P<0.05.

Viscera weight

The initial viscera weight of juvenile lumpfish prior to the introduction of experimental diets (mean ± SEM) at week 0 was in the range of 0.86 ± 0.10 g and 0.96 ± 0.04 g at week 0 for all diet groups and increased to 6.07 ± 0.54 g, 5.84 ± 0.21 g, 6.61 ± 0.22 g, and 5.57 ± 0.43g at week 7.5 for diet groups CTRL, 25%, 50% and 75% respectively (Figure 11, $P > 0.05$) This represents a viscera weight increase of approximately seven fold over 7.5 weeks.

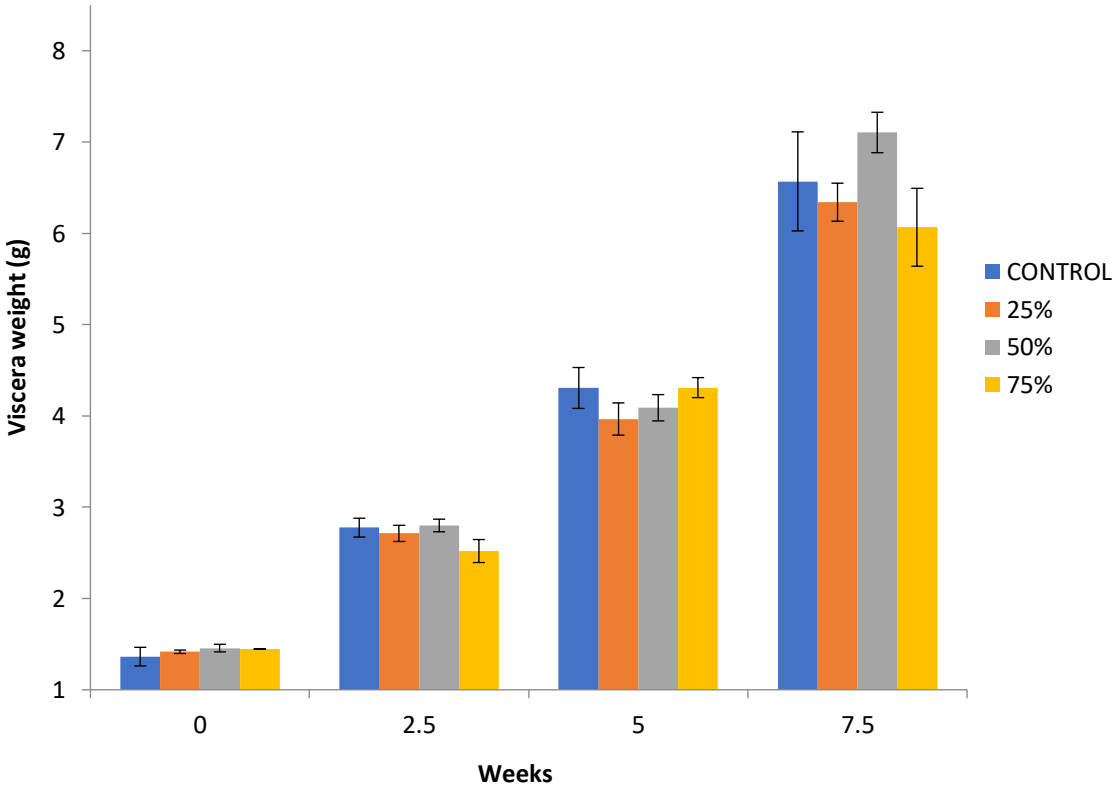

Figure 11: Viscera weight (mean ± SEM) of juvenile lumpfish fed diet with different levels of plant protein (Blue – Control, Orange - 25%, Ash - 50% and Yellow -75%). N=3. If any, different letters indicate significant difference, P<0.05.

Specific growth rate

Specific growth rate of juvenile lumpfish decreased over the experimental period (Figure 12). Specific growth rate (mean ± SEM) at week 0 was in the range of 3.46 ± 0.34% and 3.98 ± 0.11% for all diet groups with minor differences, but decreased to 3.30 ± 0.08%, 3.32 ± 0.10%, 3.34 ± 0.05%, and 3.25 ± 0.18% for CTRL, 25%, 50% and 75% diets respectively at the end of the experiment ($P > 0.05$).

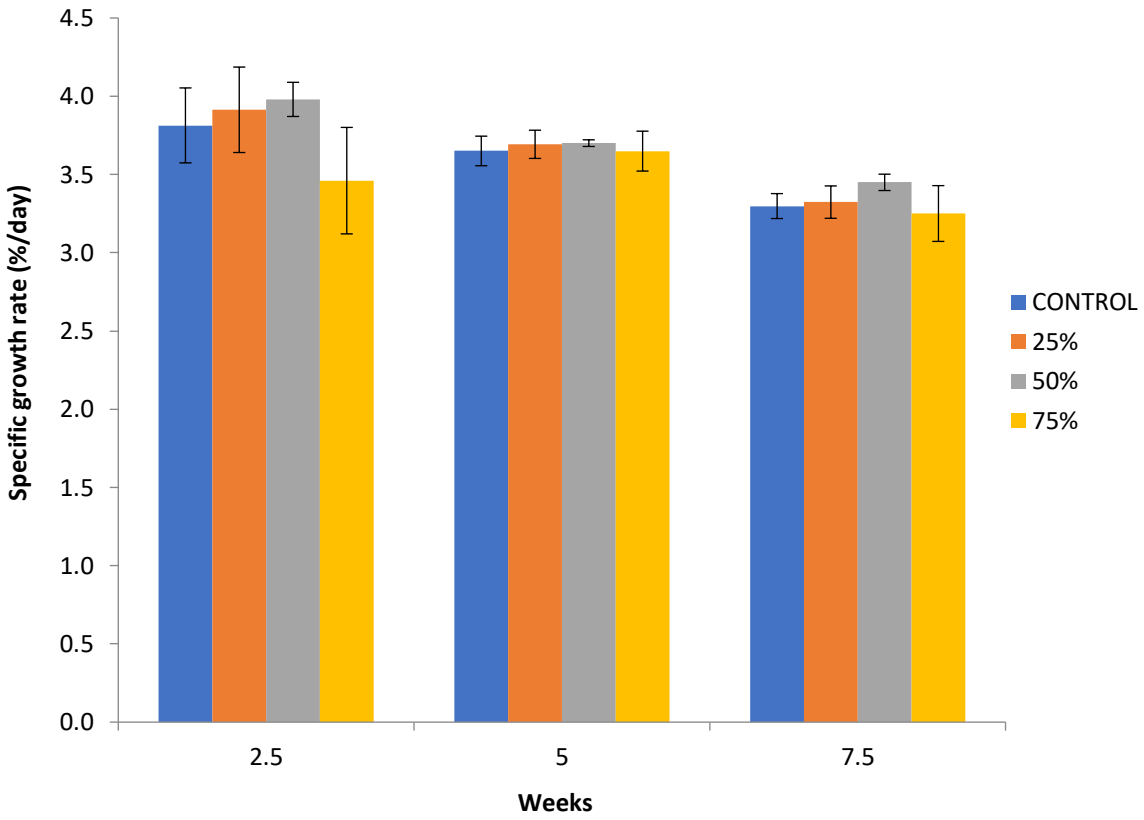

Figure 12: Specific growth rate (mean ± SEM) of juvenile lumpfish fed diet with different levels of plant protein (Blue – Control, Orange - 25%, Ash - 50% and Yellow -75%). N=3. If any, different letters indicate significant difference, P<0.05.

Condition factor

Condition factor of juvenile lumpfish ranges from 5.36 – 7% over the experimental period (Figure 13). Significant differences observed for condition factor at week 5 and multiple pair-wise comparisons showed that 75% diet group has higher condition factor value than 25% and 50% diet groups ($P < 0.05$). However there is no significant difference observed for mean condition factor among all dietary groups ($P > 0.05$) at week 0, 2.5 and 7.5.

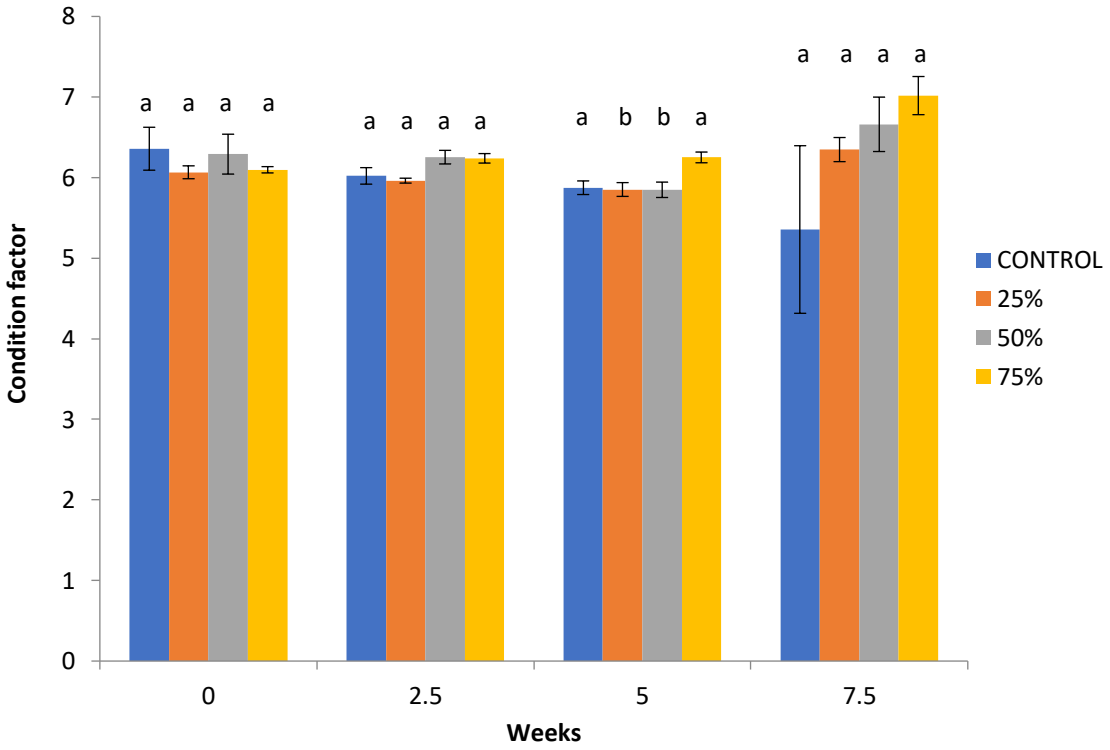

Figure 13: Condition factor (mean ± SEM) of juvenile lumpfish fed diet with different levels of plant protein (Blue – Control, Orange - 25%, Ash - 50% and Yellow -75%). N=3. If any, different letters indicate significant difference, P<0.05.

Hepatosomatic index

The hepatosomatic index (HSI) of the juvenile lumpfish showed an irregular pattern and it increases and decreases throughout the experimental period. Initial HSI percentage (mean ± SEM) in the range of 2.06 ± 0.44% and 2.65 ± 0.10% for all diet groups with minor differences (Figure 14, P >0.05). The final HSI% change to 2.46 ± 0.10%, 2.33 ± 0.06%, 2.37 ± 0.03%, and 2.53 ± 0.10% for CTRL, 25%, 50% and 75% diets respectively (P > 0.05). Slightly higher HSI% was observed in 75% diet groups at week 2.5, 5 and 7.5 (Figure 14), this is not significant compared to other diet groups (P > 0.05).

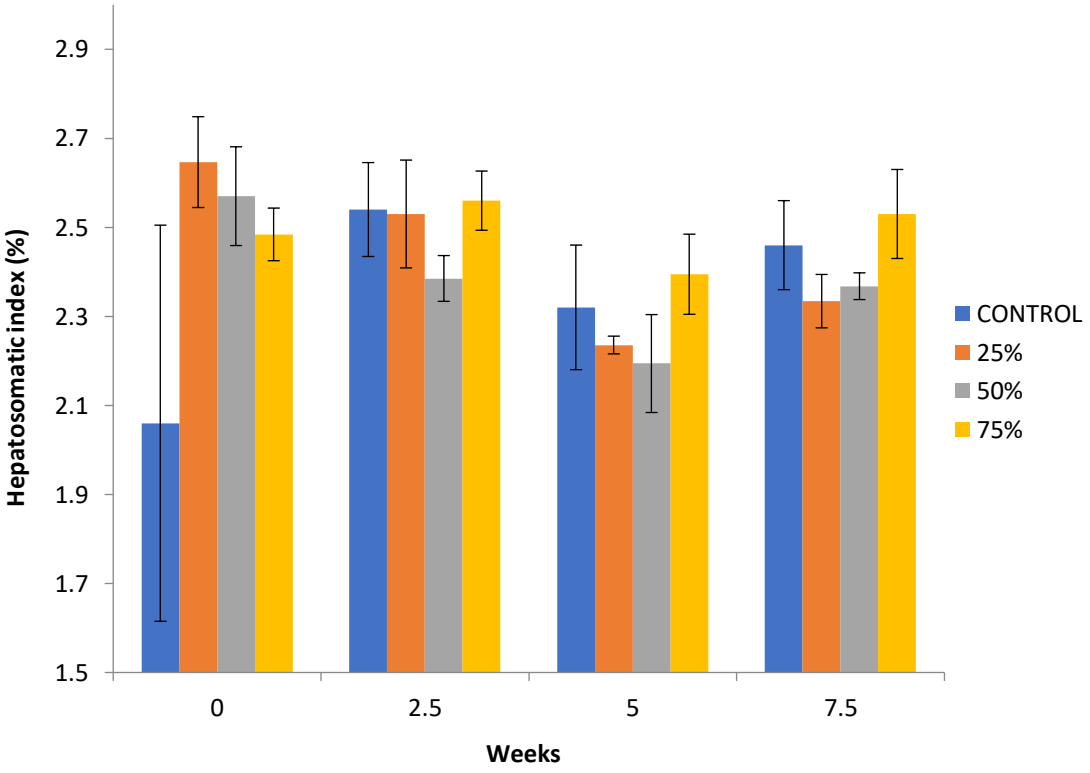

Figure 14: Hepatosomatic index (mean ± SEM) of juvenile lumpfish fed diet with different levels of plant protein (Blue – Control, Orange - 25%, Ash - 50% and Yellow -75%). N=3. If any, different letters indicate significant difference, P<0.05.

Viscera somatic index

Initial VSI percentage (mean ± SEM) at week 0, was in the range of 11.81 ± 1.56% and 13.00 ± 0.13% for all diet groups with minor differences (Figure 15, P > 0.05). VSI percentage showed an irregular pattern and it reached maximum at week 2.5 in the range of 14.62 ± 0.26% and 15.60 ± 0.65% for all diets groups (P > 0.05). Afterwards it decreases at week 5, and the final VSI% at week 7.5 were 14.31 ± 0.66%, 14.23 ± 0.33%, 14.38 ± 0.19%, and 13.39 ± 0.25% for CTRL, 25%, 50% and 75% diets respectively (P > 0.05).

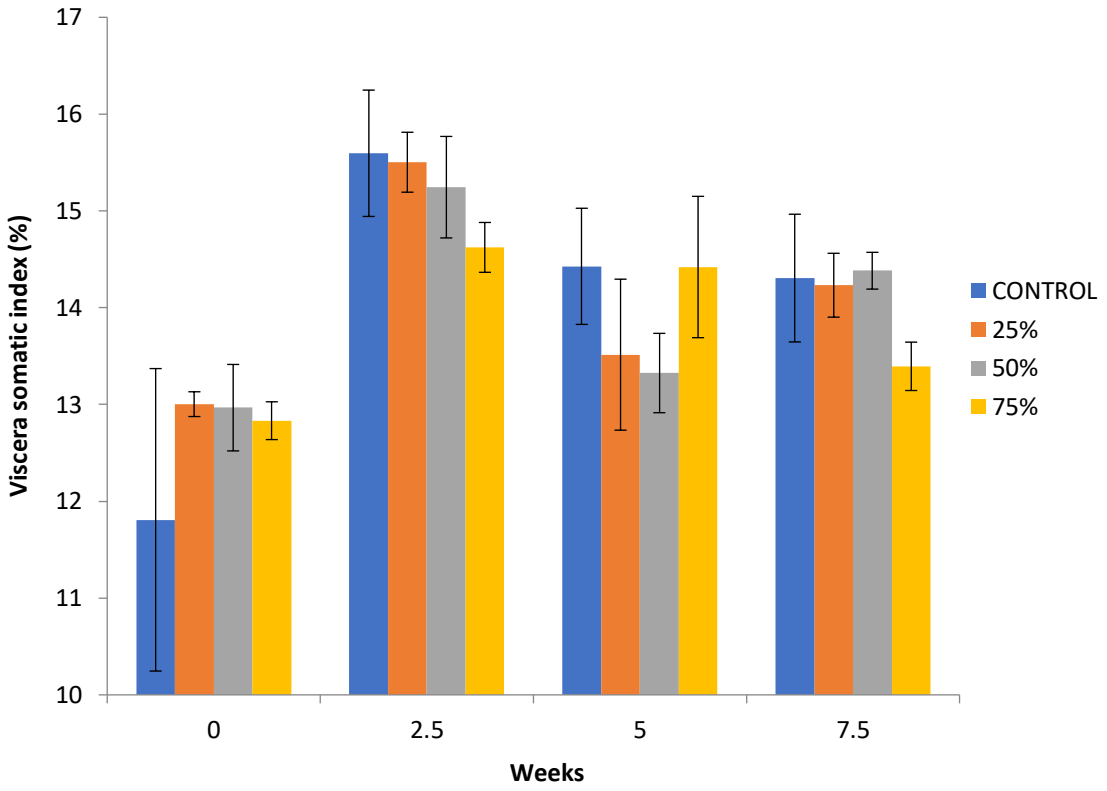

Figure 15: Viscera somatic index (mean ± SEM) of juvenile lumpfish fed diet with different levels of plant protein (Blue – Control, Orange - 25%, Ash - 50% and Yellow -75%). N=3. If any, different letters indicate significant difference, P<0.05.

3.2 Muscle cellularity

Total cross sectional area (TCA)

The total cross sectional area (mean ± SEM) of fast muscle fibre at week 0 was in the range of 76.37 ± 3.65 mm^2 and 81.93 ± 9.76 mm^2 for all diet groups (P > 0.05) and it increased to 409.68 ± 26.22 mm^2, 346.77 ± 12.01 mm^2, 399.30 ± 26.30 mm^2, and 346.95 ± 31.18 mm^2 at week 7.5 for diet groups CTRL, 25%, 50% and 75% respectively (Figure 16, P > 0.05). This represents a TCA increase of approximately 500% in 7.5 weeks.

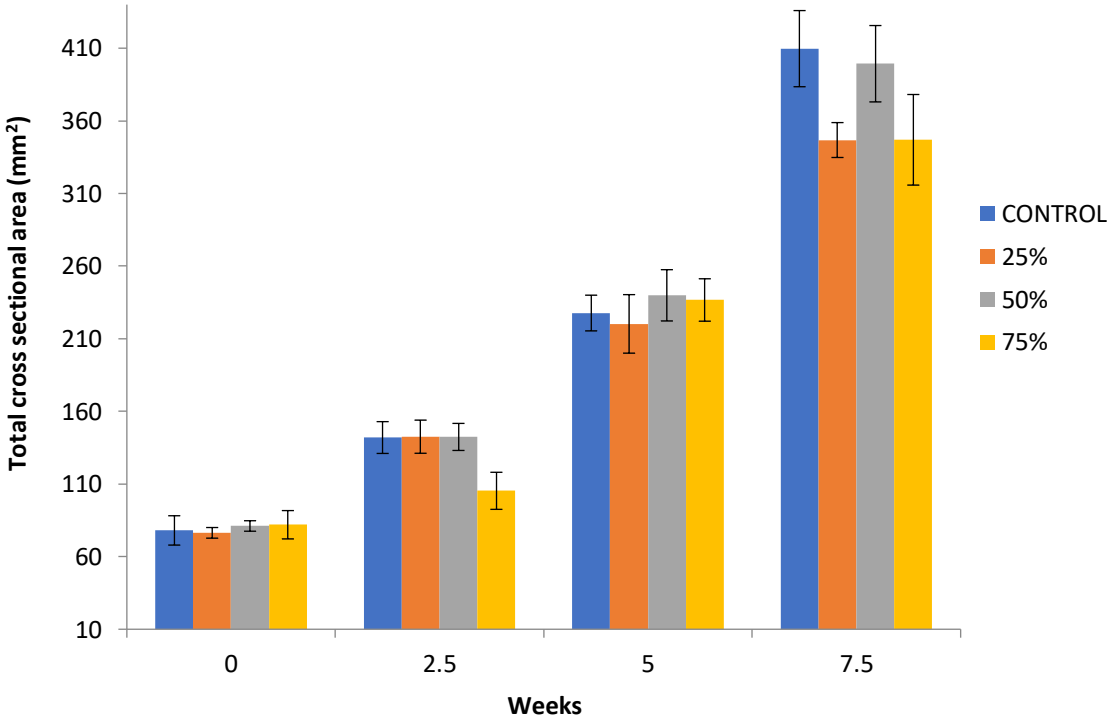

Figure 16: Total cross sectional area (mean ± SEM) of fast muscle fibre of juvenile lumpfish fed diet with different levels of plant protein (Blue – Control, n=12, Orange - 25%, n=24, Ash - 50%, n=24 and Yellow -75%, n=48). N=3. If any, different letters indicate significant difference, P<0.05.

n = number fish per sampling point (Control; n=1/tank, 25% & 50%; n=2/tank, 75%; n= 4/tank), N = triplicate.

Fast muscle fibre diameter (FFD)

The fast muscle fibre diameter (mean ± SEM) at week 0 was in a range of 32.04 ± 1.61 µm and 39.33 ± 2.74 µm for all diet groups with minor differences (P > 0.05) and it decreased to 33.48 ± 0.87 µm, 32.78 ± 0.73 µm, 32.14 ± 0.84 µm, and 31.48 ± 1.07 µm at week 7.5 for diets CTRL, 25%, 50% and 75% respectively (Figure 17, P > 0.05). On average, smallest fibre diameter such as 28.45 µm and 29.01 µm was observed at week 2.5 and 5 respectively for all diet groups (P > 0.05).

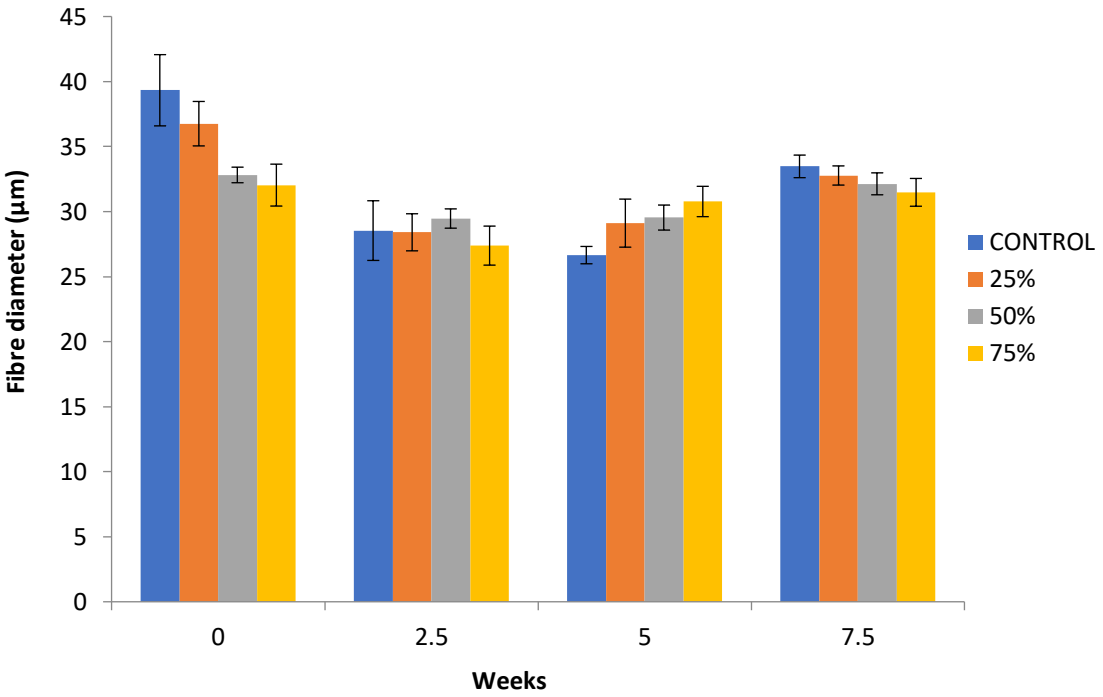

Figure 17: Fast muscle fibre diameter (mean ± SEM) of juvenile lumpfish fed diet with different levels of plant protein (Blue – Control, n=12, Orange - 25%, n=24, Ash - 50%, n=24 and Yellow - 75%, n=48). N=3. If any, different letters indicate significant difference, P<0.05.

Fibre number (FN)

At week 0 the total number of fibers was in the range of 48787 ± 8159 and 73730 ±11970 for all diet groups with minor differences (P > 0.05) and it increased to 310234 ± 32277, 284228 ± 16640, 331335 ± 26949, 298406 ± 24714 at week 7.5 for diet group CTRL, 25%, 50% and 75% respectively (Figure 18, P > 0.05). This represents approximately a 5 fold increase in just 7.5 weeks.

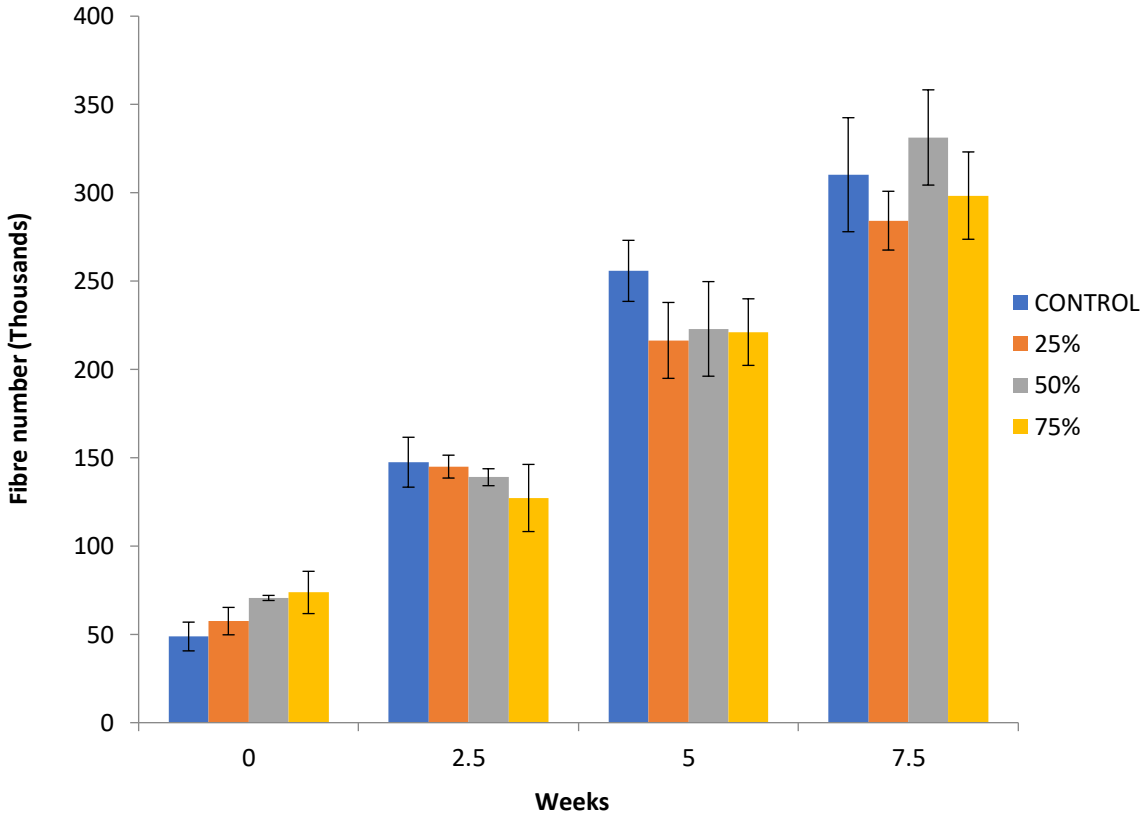

Figure 18: Total fibre number (mean ± SEM) of juvenile lumpfish fed diet with different levels of plant protein (Blue – Control, n=12, Orange - 25%, n=24, Ash - 50%, n=24 and Yellow -75%, n=48). N=3. If any, different letters indicate significant difference, P< 0.05.

Newly recruited fast muscle fibres

Table 3 shows the total number of newly recruited fast muscle fibres in between different sample points.

Table 3: Total number (mean) of recruited fast muscle fibres throughout the experimental period.

Sample point (week)	Plant protein inclusion levels			
	CTRL (0%)	25%	50%	75%
0 – 2.5	98691	87493	68351	53484
2.5 – 5	108333	71433	83978	93910
5 – 7.5	54423	67800	108386	77282

Hyperplasia and Hypertrophy

It is noted in this study that juvenile lumpfish grows mainly by hyperplastic growth compared to hypertrophy during the experimental period as shown in figure 19.

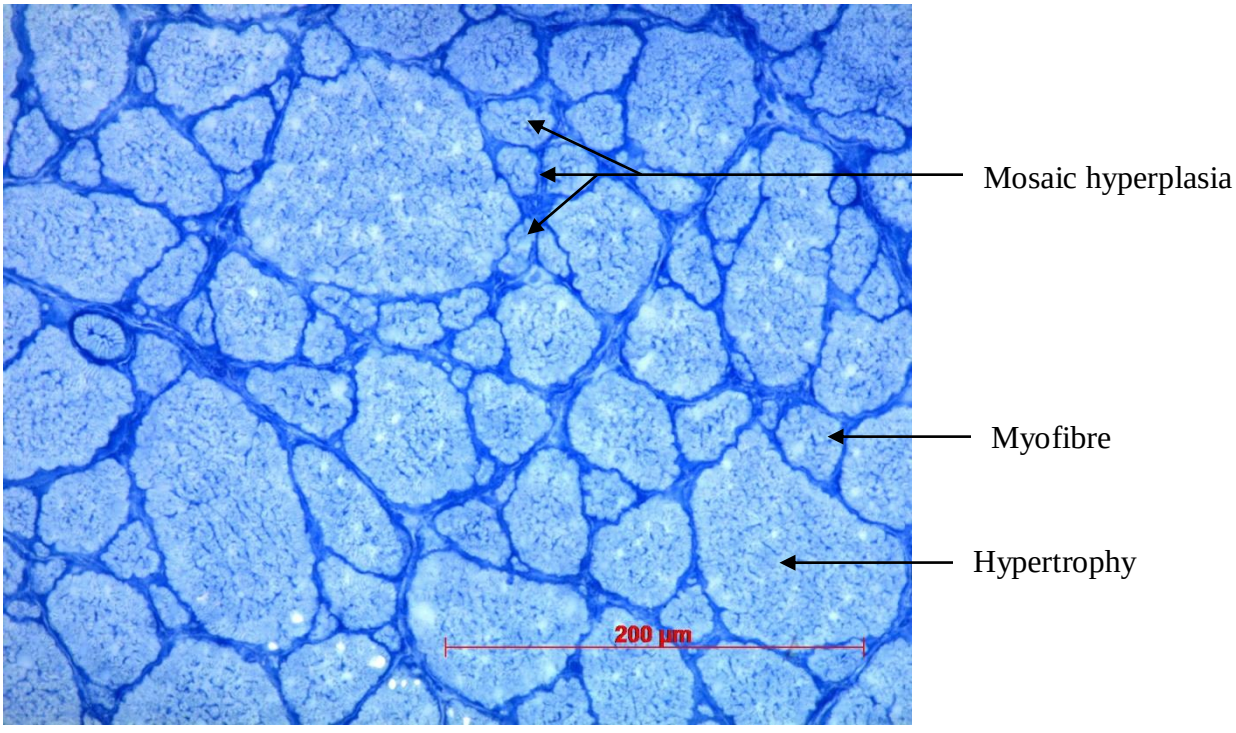

Figure 19: Growth process of juvenile lumpfish (mosaic hyperplasia and hypertrophy) at week 7.5 (Transverse section under light microscope. Diet group= 75%. Weight= 36.68 g. Length= 8cm. Scale bar 200µm).

Fast muscle fibre < 10 µm

At week 0, the number of fibers below 10 µm was in the range of 16 ± 6 and 60 ± 21 for all diet groups and it increased to 144 ± 28, 143 ± 15, 120 ± 11, and 112 ± 12 at week 2.5 for diet group CTRL, 25%, 50% and 75% respectively (Figure 20, P > 0.05). This represents approximately a three-fold increase. Afterwards it decreases throughout the experimental period and reached a range of 43 ± 3 and 59 ± 5 with minor differences at week 7.5 (P > 0.05).

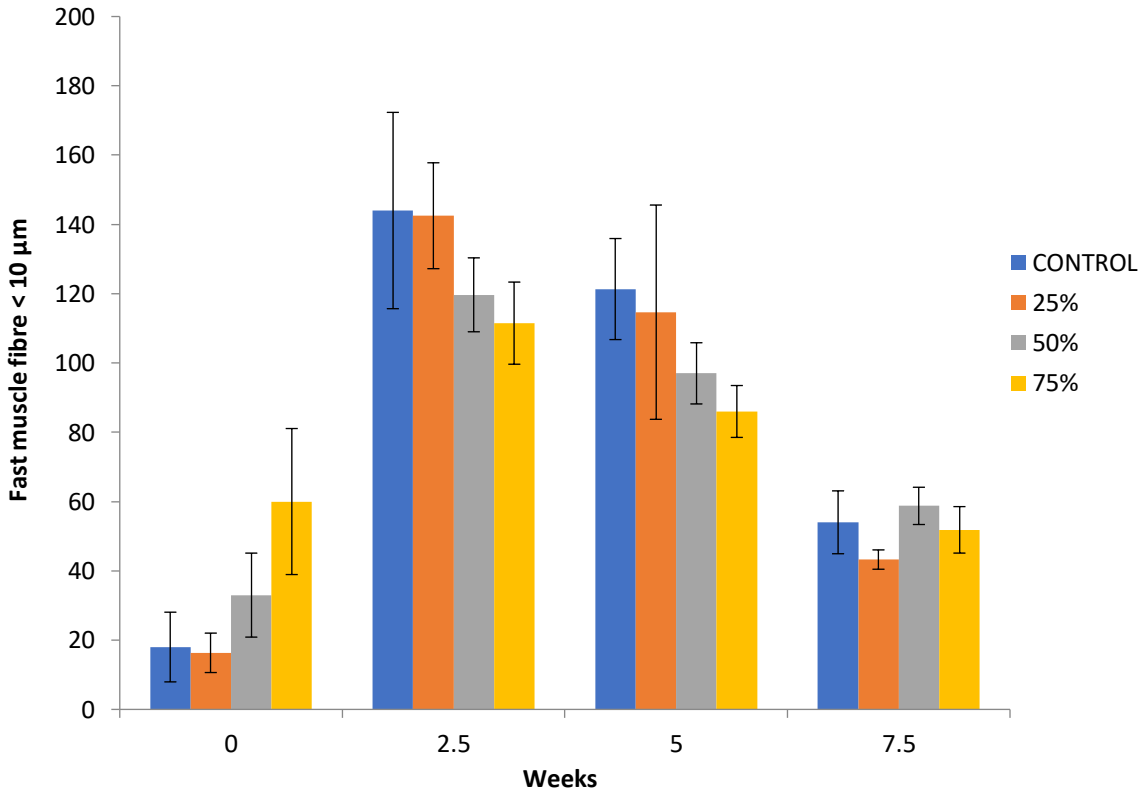

Figure 20: Number of fibers below 10 µm (mean ± SEM) of juvenile lumpfish fed diet with different levels of plant protein (Blue – Control, n=12, Orange - 25%, n=24, Ash - 50%, n=24 and Yellow -75%, n=48). N=3. If any, different letters indicate significant difference, P<0.05.

Fibre density (FD)

The fast muscle fibre density (mean ± SEM) at week 0 was in a range of 643 ± 131 and 890 ± 54 for all diet groups with minor differences (P > 0.05), and reached to maximum in a range of 969 ± 31 and 1200 ± 135 at week 2.5 for all diet groups. Afterwards FD decreased and at week 7.5, FD was 751 ± 35, 794 ± 42, 832 ± 30, and 869 ± 48 for diets CTRL, 25%, 50% and 75% respectively (Figure 21, P > 0.05).

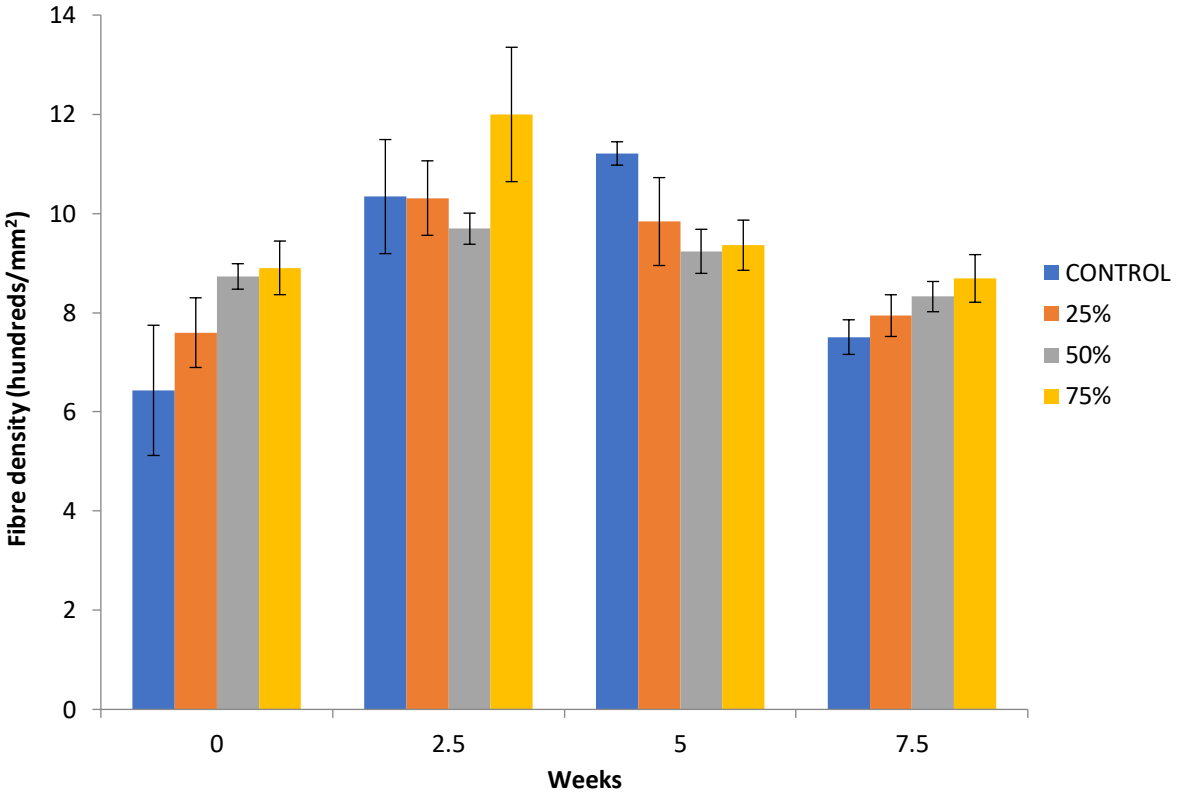

Figure 21: Fibre density (mean ± SEM) of juvenile lumpfish fed diet with different levels of plant protein (Blue – Control, n=12, Orange - 25%, n=24, Ash - 50%, n=24 and Yellow -75%, n=48). N=3. If any, different letters indicate significant difference, P<0.05.

Distribution of fast fibre diameter

Smooth distributions were fitted to 800 numbers of measurements of fast fibre diameter per fish using a kernel function and the corresponding probability density functions (pdfs) were plotted at each sample point. Comparisons of the distribution of fast fibre diameter showed that for week 0 and 7.5, the pdf peak corresponded to a muscle fibre diameter of approximately 30 µm for all diet groups. Obtained p values are 0.183, 0.904, 0.980 and 0.989 for week 0, 2.5, 5 and 7.5 respectively. At week 2.5 and 5, there was a tendency towards an increase in probability (above 0.03) of fibre diameter while the peak pdfs remains approximately same for all diet groups (Figure 22, 23, 24 & 25).

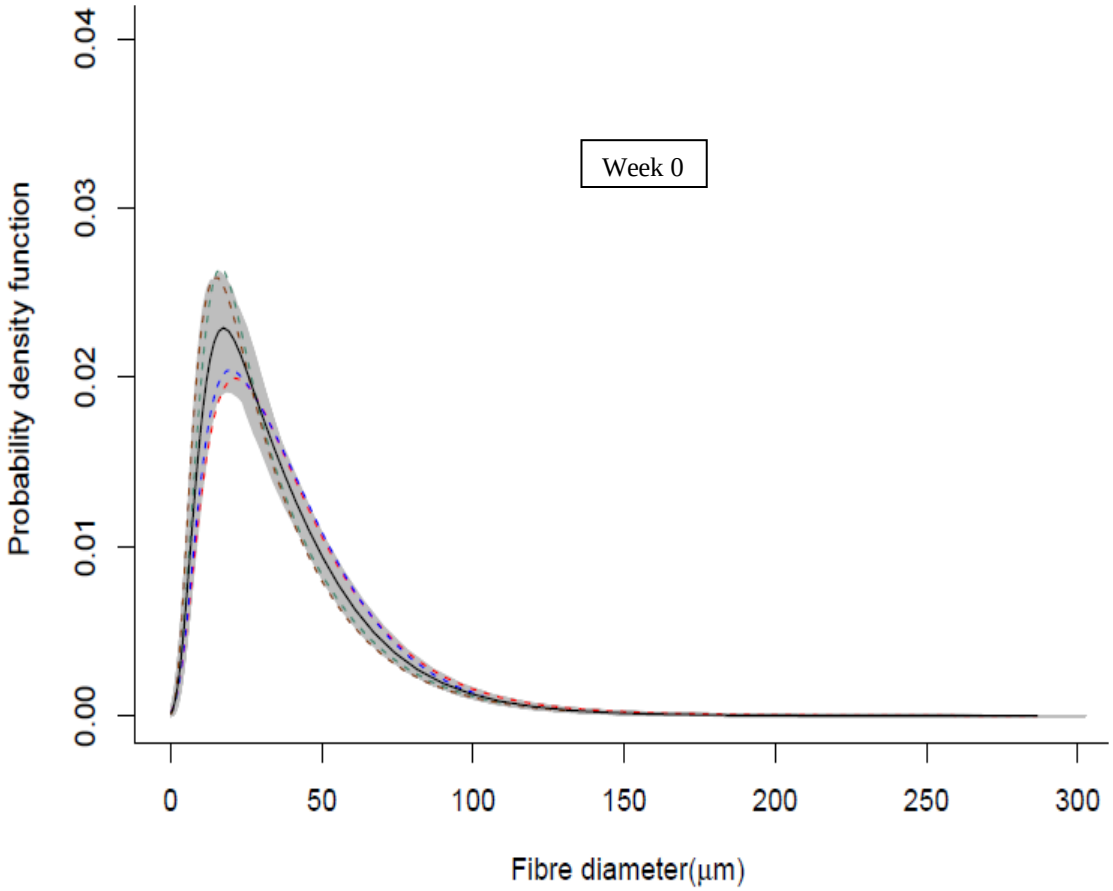

Figure 22: Probability density functions (PDFs) of fast muscle fibre diameter of juvenile lumpfish fed the experimental diets at week 0. Dashed lines represent the average PDFs for each dietary group and solid central line corresponds to the average PDF for combined groups. The shaded area shows 1000 bootstrap estimates from combined populations of fibre diameter.

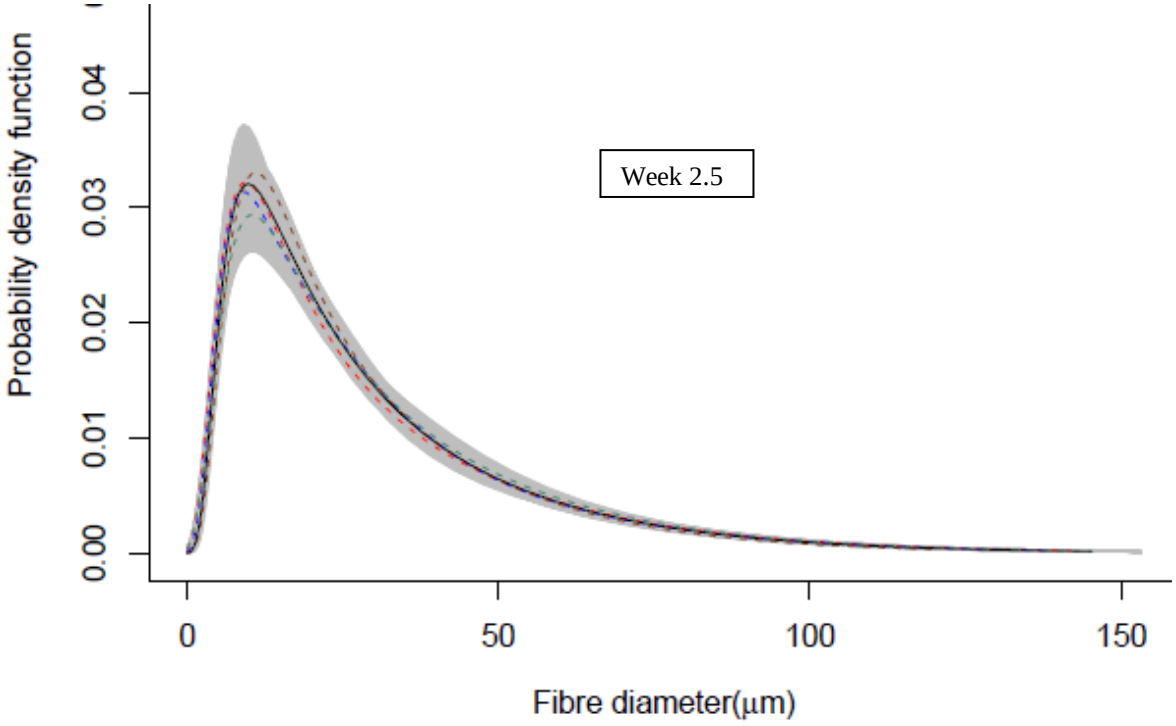

Figure 23: Probability density functions (PDFs) of fast muscle fibre diameter of juvenile lumpfish fed the experimental diets at week 2.5. Dashed lines represent the average PDFs for each dietary group and solid central line corresponds to the average PDF for combined groups. The shaded area shows 1000 bootstrap estimates from combined populations of fibre diameter.

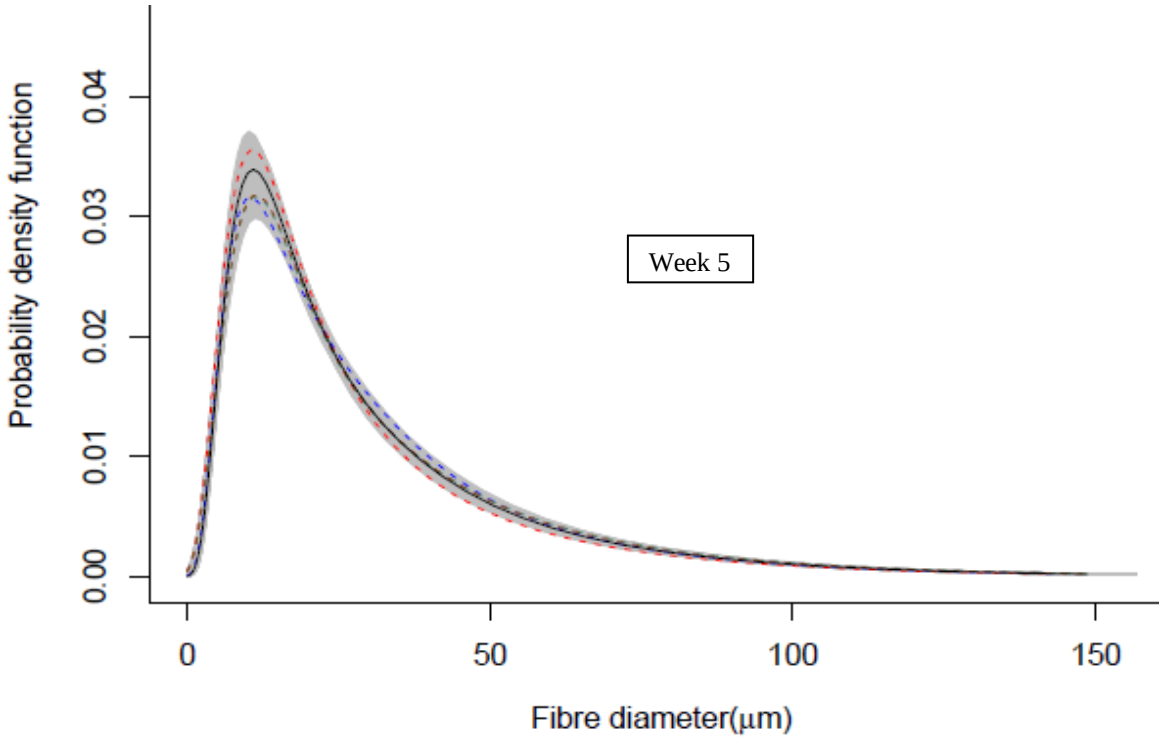

Figure 24: Probability density functions (PDFs) of fast muscle fibre diameter of juvenile lumpfish fed the experimental diets at week 5. Dashed lines represent the average PDFs for each dietary group and solid central line corresponds to the average PDF for combined groups. The shaded area shows 1000 bootstrap estimates from combined populations of fibre diameter.

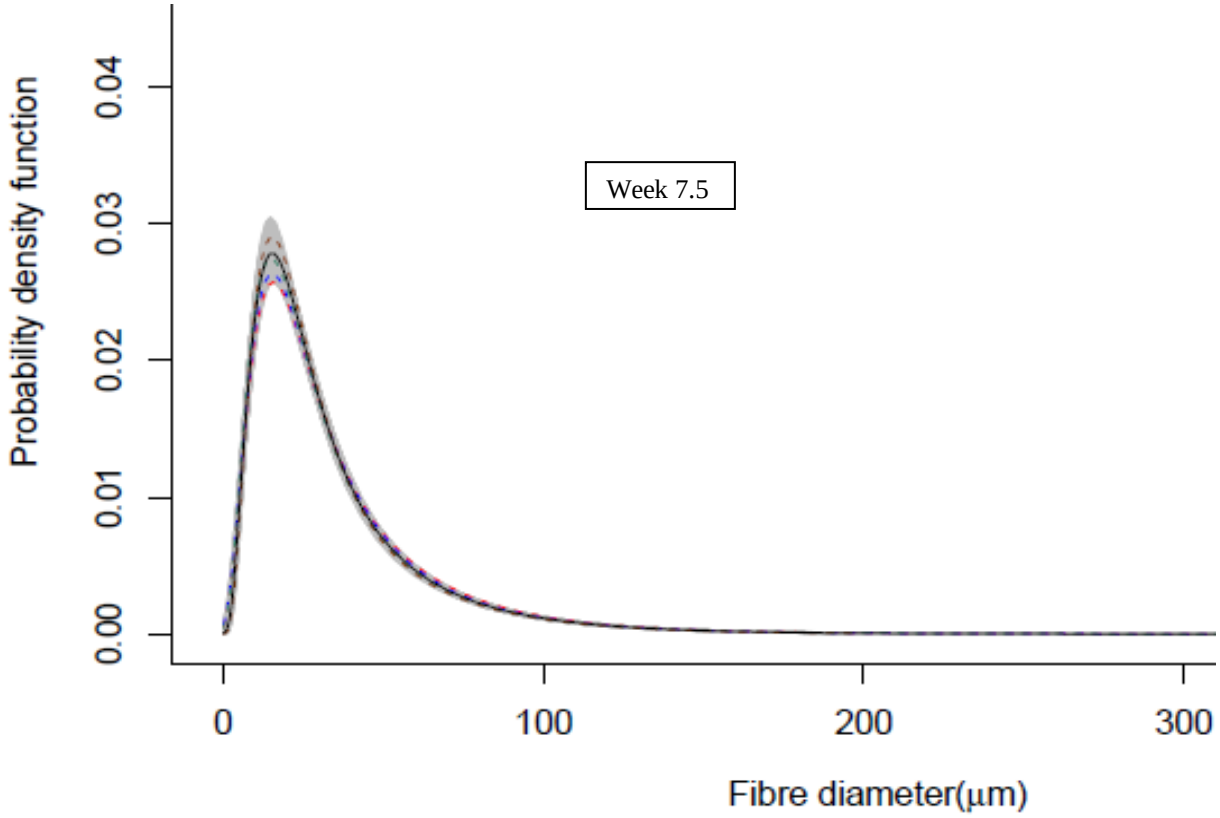

Figure 25: Probability density functions (PDFs) of fast muscle fibre diameter of juvenile lumpfish fed the experimental diets at week 7.5. Dashed lines represent the average PDFs for each dietary group and solid central line corresponds to the average PDF for combined groups. The shaded area shows 1000 bootstrap estimates from combined populations of fibre diameter.

3.3 Proximate composition

Table 4 shows the whole body's mean (SEM) dry matter, moisture, crude protein, crude fat and ash content of juvenile lumpfish at each sample point.

The dry matter (mean ± SEM) content was in a range of 12.89 ± 0.03 and 13.33 ± 0.16 at week 0 and it showed an increasing rate except for diet 25% and 50% at week 5. At week 2.5, the mean whole body dry matter was not significantly different for all diet groups, but diet group 75% showed a tendency of lower value than other three diet groups ($P = 0.072$). Dry matter increased to 14.51 ± 0.16, 13.79 ± 0.23, 14.04 ± 0.07 and 13.94 ± 0.18 for diet groups CTRL, 25%, 50% and 75% respectively at week 7.5 (Table 4, $P > 0.05$).

The moisture content of lumpfish decreased over the experimental period especially after week 2.5. The final moisture value was lesser than the initial moisture value for all diet groups. At each sample point the whole body moisture percentage of juvenile lumpfish showed no significant difference (Table 4, $P > 0.05$) for all diet groups.

At the beginning of the experiment crude protein (mean ± SEM) was in a range of 7.80 ± 0.01 and 8.06 ± 0.11. Crude protein showed increasing for all diets throughout the experiment except for diet 25% at week 5 which was slight reduced amount but this was not significant. The crude protein increased to 8.81 ± 0.13, 8.42 ± 0.20, 8.73 ± 0.06, 8.52 ± 0.10 for diet groups CTRL, 25%, 50% and 75% respectively at week 7.5 (Table 4, $P > 0.05$).

Crude fat (mean ± SEM) was in a range of 0.91 ± 0.03 and 0.95 ± 0.04 at week 0 ($P > 0.05$). After the introduction of the experimental diets (from week 0 to week 2.5) the fat content for all the diet groups decreased below the initial amount. After week 2.5, the fat content increased for all diets. At week 7.5, crude fat reached to 1.18%, 1.02%, 1.02%, and 1.08% for CTRL, 25%, 50% and 75% diets respectively (Table 4, $P < 0.05$). Diet containing 25% and 50% plant protein showed lower fat content compared to other two diet groups. The Tukey HSD yielded the adjusted p value for the paired wise treatment combination as follows: 0.0220 (25%-Ctrl), 0.0222 (50%-Ctrl), 0.1469 (75%-Ctrl), 0.9999 (50%-25%), 0.5563 (75%-25%) and 0.5594 (75%-50%).

The ash content (mean ± SEM) was in a range of 1.31 ± 0.05 and 1.43 ± 0.18 at week 0 for all diet groups with minor differences (P > 0.05) and increased to 1.62 ± 0.01, 1.63 ± 0.04, 1.65 ± 0.03, and 1.62 ± 0.02 for diet groups CTRL, 25%, 50% and 75% respectively. At each sample point the ash content of juvenile lumpfish showed no differences (Table 4, P > 0.05) for all the diet groups.

Table 4: Proximate composition of juvenile lumpfish (mean ± SEM) fed diets with different plant protein levels. N=3. If stated, different superscript letters indicate significant difference, P < 0.05.

Parameters	Duration (week)	Plant protein inclusion levels				
		CTRL (0%)	25%	50%	75%	P value
Dry matter (%)	0 (W)	13.33 ± 0.16	13.05 ± 0.04	13.19 ± 0.21	12.89 ± 0.03	0.0940
	2.5 (K)	13.41 ± 0.05	13.29 ± 0.12	13.32 ± 0.01	12.90 ± 0.10	0.0720
	5	13.69 ± 0.04	13.14 ± 0.19	13.31 ± 0.19	13.29 ± 0.12	0.1251
	7.5 (K)	14.51 ± 0.16	13.79 ± 0.23	14.04 ± 0.07	13.94 ± 0.18	0.0980
Moisture content (%)	0	87.35 ± 0.62	87.66 ± 0.64	86.69 ± 0.18	87.50 ± 0.47	0.5866
	2.5 (K)	87.49 ± 0.38	87.25 ± 0.42	87.78 ± 0.53	87.72 ± 0.53	0.6910
	5	86.24 ± 0.10	86.61 ± 0.16	86.58 ± 0.17	86.55 ± 0.17	0.3419
	7.5	85.59 ± 0.14	86.21 ± 0.17	85.99 ± 0.10	85.98 ± 0.13	0.0655
Crude protein (%) wet basis	0	8.06 ± 0.11	7.93 ± 0.08	7.88 ± 0.11	7.80 ± 0.01	0.2893
	2.5 (K)	8.11 ± 0.08	8.12 ± 0.13	8.16 ± 0.01	7.94 ± 0.09	0.2820
	5	8.38 ± 0.10	8.07 ± 0.05	8.22 ± 0.07	8.33 ± 0.12	0.1607
	7.5	8.81 ± 0.13	8.42 ± 0.20	8.73 ± 0.06	8.52 ± 0.10	0.2241
Crude fat (%) wet basis	0	0.95 ± 0.01	0.91 ± 0.03	0.95 ± 0.04	0.91 ± 0.01	0.5408
	2.5 (W)	0.91 ± 0.03	0.83 ± 0.01	0.89 ± 0.01	0.80 ± 0.05	0.0520
	5 (K)	1.01 ± 0.02	0.91 ± 0.02	0.94 ± 0.02	0.88 ± 0.07	0.1290
	7.5 (HSD)	1.18^{a} ± 0.02	1.02^{b} ± 0.03	1.02^{b} ± 0.02	1.08^{a} ± 0.03	0.0162
Ash content (%) wet basis	0 (K)	1.42 ± 0.13	1.31 ± 0.05	1.43 ± 0.18	1.37 ± 0.11	0.9720
	2.5	1.64 ± 0.21	1.65 ± 0.19	1.60 ± 0.16	1.53 ± 0.11	0.9745
	5 (K)	1.62 ± 0.04	1.59 ± 0.02	1.58 ± 0.01	1.60 ± 0.03	0.6120
	7.5	1.62 ± 0.01	1.63 ± 0.04	1.65 ± 0.03	1.62 ± 0.02	0.7972

If not stated, P values are from one way ANOVA. If stated in second column, K = Kruskal-wallis test, W = Welch F test and HSD = ANOVA followed by Tukey HSD.

4. Discussion

Replacing fish feed ingredients is not a new trend, but rather an innovative and sustainable solution for the aquaculture sector. Over the decade numerous studies have been conducted to investigate the possible impacts of replacing fish meal protein with plant protein on growth of various fish species. Some of these studies have also included muscle cellularity analysis, but these are fewer. However, the studies regarding lumpfish nutrition or feed is limited or none existing and the results in this thesis will therefore be discussed in the light of other relevant species.

4.1 Growth performance

In the present study, the growth of juvenile lumpfish fed diets that replaced fish meal with all inclusion levels of SPC & PPC performed just as well as the fish receiving the fish meal based control diet. No pathological signs or deformities were observed during the experiment and the palatability of the experimental diets and the fish acceptance seemed to be similar between diets, based on visual inspections. Also, based on the feed formulations there was no reason to believe that, the test diet had any nutritional deficiencies compared to the fish meal based control diet. The present study has shown that mean body weight of juvenile lumpfish increased from 7.29 g to 42.71 g, or approximately six-fold in 53 days for all diet groups. This is quite a remarkable increase in body weight. In comparison, Morken et al., (2016) have reported that the body weight of juvenile lumpfish increased from 1.7 g to 18.9 g in 56 days and they found highest body weight in higher and medium gross energy diets. In a recent feed study, lumpfish with mean weight of 125.4 g showed higher final body weight for pellet feed (230g) than block feed (180g) in 41 days (Imsland et al., 2018). However, the initial body weight (size class) and duration reported in the previous studies differ from our study.

In our study, overall mean specific growth rate of juvenile lumpfish ranged from 3.25 to 3.98% day^{-1} for all diet groups. Almost similar results were reported in juvenile lumpfish fed diets with different gross energy where the specific growth rate ranged from 3.9 to 4.6% and was inversely related to the biological feed conversion ratio (Morken et al., 2016). In our

study, we observed a slight decreased specific growth rate during the experimental period. When juveniles grow, their body size increase, as a consequence the growth rate declines which is explained by Nytrø et al., (2014) that "growth rate of lumpfish declines with increasing size". In contrast, Imsland et al., (2018) have reported an increased SGR for adult lumpfish fed with pellet and block feeds. In our study, condition factor (K) of juvenile lumpfish ranged between 5.36% and 7% during the experimental period. At week 5, the 75% diet group showed higher K value than 25% and 50%. It could be explained by the tendency of reduced standard length since the body weight remained same. Whereas Imsland et al., (2018) have reported K of approximately in a range of 3.6 to 4.2 % in their study.

Our findings were similar to those reported for several other species, such as for example the Atlantic cod, growth was not impaired with significant inclusion of plant-based proteins (Hansen et al., 2006; Karalazos et al., 2007; Hansen et al., 2007; Walker et al., 2009; Árnason et al., 2010). However conflicting results on cod have been reported in previous studies that replaced fish meal with soybean meal reduced growth with 30% replacement (Decken and Lied, 1993) and has no effect on growth (Refstie et al., 2006; Karalazos et al., 2007). Hansen et al., (2007) stated that there is a great potential for using quite high inclusions of plant proteins in cod diets. They found that high growth and feed utilization were obtained up to 50% plant protein inclusion level, thus above that level growth is reduced. Colburn et al., (2012) investigated that Atlantic cod fed with diet replaced by 50% SPC & SBM grew well or better than the control for all variables such as final body weight, specific growth rate and thermal-unit growth coefficient. The fish did not suffer from any signs of enteritis. They indicated that 100% fish meal replacement is not recommended, but 50% replacement can be used without significant reductions in growth or condition indices. In this context, Fuertes et al., (2013) concluded that a 28.2% pea protein concentrate can be included in formulated diets for juvenile *Pacifastacus* without impairing growth. Whereas some other findings reported partial replacement of fish meal by soybean meal is feasible and positively affects the fish growth performance in Atlantic salmon (Refstie et al., 2001), Asian sea bass (Boonyaratpalin, Suraneiranat, & Tunpibal, 1998), milkfish (Shiau et al., 1988), and coho salmon (Fowler, 1980) and by soy protein concentrate in turbot (Peng et al., 2013), Atlantic halibut (Berge et al., 1999) and juvenile cobia (Salze et al, 2010) and by soybean peptide in yellow catfish (Zhao et al., 2016). Increased performance could be explained by various antioxidant activities of soybean such as flavor potentiator, antitumor, water solubility and higher digestibility (Lan et al., 2010; Peña-Ramos & Xiong, 2001; Zhao et al.,

2016). Feeding PPC has been reported to produce acceptable weight gain in Atlantic salmon (Carter & Hauler, 2000) and rainbow trout (Thiessen et al., 2003). In this context, Øverland et al., (2009) has found that PPC can replace 20% of fish meal in Atlantic salmon without any adverse effect on growth performance. In contrast, growth performance decreased by increasing soy protein levels in diet of silvery-black porgy juveniles (Yaghoubi et al., 2016), by soy protein isolates in yellowtail (Nguyen et al., 2011), by soybean peptide in starry flounder (Song et al., 2014). Decreased performance could be explained by reduced feed intake and protein synthesis (Mambrini et al., 1999; Xu et al., 2012), poor amino acid profile and amino acid oxidation and endogenous excretion (Zhang et al., 2002) leading to poor cell growth (Zhao et al., 2016). Nevertheless, lumpfish seems to be very robust and can tolerate a very high level of SPC & PPC compared to many other marine species. Natural prey of lumpfish includes crustacean larvae, halacarid mites and planktons (Ingólfsson & Kristjánsson, 2002); moreover whatever available in the environment (Imsland et al., 2015a) including plant based materials. This behavior is probably linked to that the lumpfish is omnivorous with a relatively long gut compared to more carnivorous species, probably make them better suited to digest a wider range of feeds compared to more carnivorous species.

In our study, approximately six-fold increase in liver weight was observed at the end of this experiment. There was a tendency of higher final liver weight for 50% diet group.. Rosenlund et al., (2004) have reported increased liver size of juvenile cod fed with imbalanced protein. However, the present study maintained a balanced protein content, therefore it contradict with the previous study on cod which stores all of the lipids in the liver. Weight of viscera increased to approximately 7 fold for all diet groups. In previous studies on cod, gastrointestinal growth was reported when replacing fish meal with high levels of plant proteins (Refstie et al., 2006). In present study, HSI ranged from 2.33 to 2.53% at final sampling and slightly higher HSI% was observed in 75% diet groups at week 2.5, 5 and 7.5. Similarly, HSI ranged from 12.7 to 14.3% in cod fish (Hansen et al., 2007). In this context, highest HSI was observed in silvery-black porgy juveniles fed the SP75 diet (Yaghoubi et al., 2016). Similar results were observed in Atlantic salmon (Espe et al., 2010), European sea bass (Kaushik et al., 2004), Gilthead sea bream (Sitjà-Bobadilla et al., 2005), Atlantic cod (Hansen et al., 2007) and Japanese flounder (Pham et al., 2007) fed with high plant protein diet. This might be due to stimulated lipogenesis in the liver and adipose tissues through transcriptional mechanisms linked to enhanced glucose metabolism (Kamalam et al., 2013). In our study, VSI percentage showed an irregular pattern and it reached maximum at week

2.5 in the range of 14.62% and 15.60% for all diets groups. Whereas, previous study on cod showed that higher VSIs and lower HSIs in fish receiving the 100% fish meal replacement diets (Albrektsen et al., 2006; Refstie et al., 2006a).

This study has utilized SPC & PPC instead of SBM which contains relatively high levels of heat stable anti-nutritional and antigenic factors including protease inhibitors, oligosaccharides, saponins, isoflavones, phytate and tannins (Refstie et al., 2005; Knudsen et al., 2007; Iwashita et al., 2009) that can cause enteritis in salmonids, whereas those factors are relatively low in PPC (Francis, Makkar, & Becker, 2001) and absent in SPC (Peres & Lim, 2008). In combination with DL-methionine, SPC can be replaced 100% fish meal in rainbow trout (Kaushik et al., 1995). Normally, inclusion of SPC results reduced feed efficiency due to the fibre content in SPC. The crude fibre in SPC is 3.5% as fed basis and SPC lacks DL-methionine (Peres & Lim, 2008) and PPC also have low content of methionine (Øverland et al., 2009). The content of crude fibre in the SPC & PPC used in the experimental diets was in a range of 0.69 – 1.34% and plant protein diets have almost less portion of carbohydrates and minerals (ash) compared to fish meal diet. Thus, juvenile lumpfish might have a higher feed intake to obtain the same amount of available energy from the SPC diet compared to control diet. Otherwise inclusion of krill meal might have increased the palatability of our treatment diets. Notably, our experimental diets contain almost similar macro and micro nutrient profile with inclusion of similar amount of minerals and vitamins compared to control diet. Therefore, in our study similar nutrient profile (with similar palatability, digestibility, availability and metabolism) might be the reason for the similar growth performance observation for all diets. Anyhow previous study has investigated that wild juvenile lumpfish in floating seaweed fed mainly on prey organisms found on the seaweed but also consumed organisms from the plankton as well (Ingólfsson & Kristjánsson, 2002), it was later suggested that lumpfish seem to switch natural food choice to whatever available to them within their environment (Imsland et al., 2015b). Therefore replacing fish meal with up to 75% plant protein (SPC & PPC) in lumpfish diet did not show any adverse effects on growth performance. Anyhow significant ontogenetic variation in optimum temperature for growth in juvenile lumpfish was observed and growth of the juvenile lumpfish heavily depends on the rearing temperature (Nytrø et al., 2014) and water oxygen saturations (Jørgensen et al., 2017). Our study was conducted at 8 ^{0}C and oxygen level was kept at 90% which is considered to be in the optimum range for the species.

4.2 Muscle cellularity

Fast muscle fibre, which comprises approximately 70% of the bulk of myotomes, is the predominant components of the skeletal muscle in fish (Zhang, Swank, & Rome, 1996). The present study has investigated the parameters such as; total cross sectional area (TCA), fibre diameter, total fibre number, number of fibre < 10 µm and probability density function of fast fibre. The present study confirmed that inclusion of SPC and PPC in juvenile lumpfish diets have no significant impact on muscle cellularity (for all parameters) conforming earlier observations in other fish species (Kiessling et al., 1991; Akster et al., 1995).

Present study showed that TCA of white muscle increased approximately five-fold throughout the experiment as a consequence of new fibre recruitment. Dietary plant protein seems to provide essential amino acids for the new protein synthesis similar to the fish meal. This is supported by a study in herring, larvae growth from 8 to 16 mm length involved a three-fold increase in TCA largely due to the hypertrophy of the embryonic red and white muscle fibre (Johnston, 1998). In contrast Valente et al., (2016) have reported that plant protein replacement reduced the TCA and led to poor growth.

In our study, fast white muscle fibre diameter on average remained within the range of 29.01 – 39.33 µm throughout the experimental period due to the recruitment of new fibres. Even though average fibre diameter is a relatively insensitive and unreliable indicator of hypertrophic growth because of the recruitment of new muscle fibres. Similar results have been shown for rainbow trout in which the average fibre diameter of the white myotomal muscle remained similar relatively unchanged (90 – 95 mm) between 34 and 52 cm body length due to the addition of new fibres, but increased to 135–140 mm at 62 cm body length once recruitment had ceased (Stickland, 1983). In contrast, fish meal replacement leads to adverse effect on the fibre diameter, for example, Cai et al., (2018) have reported a decrease in the large diameter of white muscle fibre in blunt snout bream. Similarly, pea protein inclusion had adverse effect on fibre diameter in Sea bream (Matos et al., 2012), Rainbow trout (Alami et al., 2010) and Senegalese (Valente et al., 2016), it conforms that the muscle plasticity to dietary manipulation (Johnston, 2006; Silva et al., 2009).

The present study confirms the occurrence of hyperplastic growth process by evaluating the number of newly recruited white muscle fibres < 10 µm in juvenile lumpfish. Three fold increases in number of fibres < 10 µm was observed in between week 0 and week 2.5 which is a critical stage for lumpfish, as a consequence it is noted that two fold increase in width occurred at same stage. Fibre density of juvenile lumpfish showed similar trend to the number of fibres < 10 µm, it indicated that when new fibres are recruited, fibre density increases and vice versa. It is also noted that fibre density reached to maximum at week 2.5 as seen for number of fibres < 10 µm. This was supported by previous studies that fibres < 20 µm in diameter could represent the fibres recruited by hyperplasia (Zimmerman and Lowery, 1999; Michelato et al., 2016). Similarly, in Senegalese hyperplasia was observed in fish fed 100PP (Valente et al., 2016). These results suggest a prevalence of hyperplastic growth in lumpfish fed all diets, rather than by hypertrophy throughout the experimental period.

The present study has shown that white muscle fibre number is also not adversely affected by any inclusion levels of plant protein at each sample point. Changes in muscle fibre number during growth have been determined in other fish species including rainbow trout (Stickland, 1983), common carp (Koumans et al., 1994), Atlantic herring (Johnston et al., 1998) and Atlantic salmon (Johnston, 2003). In present study, the recruitment of additional white muscle fibres increased throughout the experiment. In this context , Valente et al., (2016) have reported that the total number of fibre in Senegalese did not vary significantly among treatment. Anyhow Johnston, (2003) has reported that photoperiod and feeding have impacts on the number of proliferating myogenic progenitor cells. However, present study documents that plant protein inclusion seems to have similar impact on the proliferation of myogenic progenitor cells, resulting similar growth of fast muscle fibre for all diet groups.

In this study, fish fed with experimental diets showed similar muscle cellularity with fish meal diet at each sample point, and it can be linked to similar observation for growth performance. This may be explained by involvement of lysine in metabolic pathway for muscle growth by hyperplasia and hypertrophy (Valente et al., 2013). Muscle cellularity is expected to be plastic with respect to different feeding regime (Johnston, 1999; Johnston, 2006). Relatively few studies elaborated the effects of replacing fish meal with plant protein diet on muscle cellularity. Some other feed experiments, for example; *Artemia* and dry food

showed similar effects on average diameter of white muscle fibre in *Clarias* (Akster et al., 1995) whereas similar results were obtained in rainbow trout that the distribution of white muscle fibre size was independent of diet (Kiessling et al., 1991). These findings as well as our study contradict with plasticity of skeletal white muscle growth dynamics which was previously shown to be affected by ration level (Kiessling et al., 1991) or by changes in nutrient supply (Fauconneau et al., 1997). In support of muscle plasticity theory by Johnston (2006), Alami et al., (2010) have reported high substitution levels of plant protein significantly reduced the median diameter of white muscle fibres. Muscle plasticity has been reported in Atlantic cod, when growth rate was manipulated by altering feed intake or diet quality (Ostaszewska et al., 2008; Valente et al., 2013; Salze et al., 2014). For example, Galloway et al., (1999) reported that higher DHA: EPA diet was responsible for higher growth rate in cod solely the result of hyperplasia. However, our study maintained similar dietary DHA: EPA in the experimental diets, and it was found that hyperplasia was the main muscle growth mechanism during that time, therefore it might have resulted similar muscle cellularity compared to the control diet.

4.3 Proximate composition

Proximate whole body composition is used to determine the moisture, protein, lipid and ash content of the fish and it is essential in order to maximize their utilization (Silva and Chamul, 2000), moreover it is considered as a good physiological condition and health indicator of fish (Saliu, Joy, & Catherine, 2007). Therefore, fish meal (eg: NORVIK) in the commercial diet of juvenile lumpfish can be reduced from 58% to 14.5% as fed basis, while substituting SPC (Soycomil) and PPC up to 21.67 % each, without affecting the whole body proximate composition of juvenile lumpfish. In present study, proximate composition was not significantly different for all diet groups at each sample point except for crude fat at the end of the experiment. The dry matter, crude protein and ash showed an increasing trend while moisture was decreasing throughout the experiment. However, crude fat decreased to lowest level at week 2.5, and then it increased throughout the experiment. Our results are consistent with previous study on silvery-black porgy juveniles in which whole body moisture, protein and ash were not significantly different among dietary treatments ($P > 0.05$), but fish fed with the SP75 had highest whole body lipid content (Yaghoubi et al., 2016).

Protein is a main constituent of tissue and organs; it is the precursors of enzymes, hormones, neurotransmitters, cofactors etc, and play as an important energy source. Fish build its new proteins when they intake protein or EAA consistently. Inadequate inclusion of protein in the diet results in reduced growth. If excess protein is supplied, fish either use it for more protein synthesis or transform into energy. Fish digest the protein and absorb the free amino acids by intestinal tract (Robaina & Izquierdo, 1995). In our study, all diets contain almost same level of crude protein (53.8%) and amino acid profile. Even though the protein source of diets is altered by SPC & PPC, it is seemed that the digestibility and availability was not affected by different ration. Otherwise, lumpfish might have capability to digest the plant protein and absorb the amino acids regardless of the sources, similar to fish meal protein. Similar result was reported in previous study (Kissinger, García-Ortega, & Trushenski, 2016). Unbalanced amino acid profile in the feed of juvenile lumpfish lead to cataract development as a consequence of osmotic imbalance (Mattilsynet, 2016). Therefore, alleviating malnutrition especially undisturbed protein metabolism is important to maintain the health of juveniles.

In our study, the moisture content of lumpfish decreased over the experimental period especially after week 2.5. The final moisture value was lower than the initial moisture value

for all diet groups. In contrast, moisture reduction was documented when the fish meal was replaced by SPC, for example, a study has reported a lower moisture value for longfin yellowtail fed the FM100 diet than FM40 treatment (Kissinger, García-Ortega, & Trushenski, 2016). Lipid plays a role as dietary energy source and necessary for fish physiological functions such as cellular membrane fluidity regulation, prostaglandin precursors, fat-soluble vitamins and carotenoid pigments carriers and hormones (Robaina & Izquierdo, 1995). In our study, all the diets contained 13.4% of crude fat (as fed basis) with almost similar levels of essential fatty acids including EPA & DHA. In present study, crude fat of juvenile lumpfish was in a range of 0.91% and 0.95% at week 0. After the introduction of the experimental diets the fat content for all the diet groups decreased below the initial amount. It is because of water content increase (Cowey & Sargent, 1977) during that time period which phenomena (" moisture and lipid content of fish are inversely related") is clearly evidenced in the current study (FAO, 1999). Moreover, lumpfish might have catalyzed the body lipid to attain gross energy since fat is less expensive energy source than protein. Lumpfish might have partitioned its gross energy from the fat reserve to energy needed for synthesis of new myofibrils and development of viscera. Therefore it is noted that lumpfish seems to optimize fat metabolism and save protein for the muscle growth. After week 2.5, the fat content increased for all diets due to the anabolism of fat. At week 7.5, average crude fat reached to 1.07% for all diets. Diet containing 25% and 50% plant protein showed significantly lower fat content compared to other two diet groups at week 7.5. It can be due to the tendency of higher moisture content in juvenile lumpfish at that sample point.

Similar results to our study has been achieved for several other experiments, for example no effects on whole fat contents compared to control diet was reported on rainbow trout (Luo et al., 2006; Harlíoğlu, (2011) and gilthead sea bream (Francesco et al., 2007), moreover Luo et al., (2006) also reported no significant differences on the body composition of rainbow trout. Whereas, Kissinger, García-Ortega, & Trushenski, (2016) have reported higher lipid content in fish fed the soybean concentrate diets. On the other hand, Harlíoğlu, (2011) revealed that body lipid content decreased as the amount of plant protein increased which could be explained by different lipid metabolism in fish due to different protein sources (Francesco et al., 2007; Harlíoğlu, 2011). In spite of, lipid metabolism pathway of juvenile lumpfish was not altered by the SPC & PPC inclusion in our study.

Regarding the use of SPC & PPC in our study, lumpfish may be tolerant to high (43.34%) dietary inclusion levels. The concentration of amino acids including; methionine, tryptophan, and fatty acids including; EPA and DHA, and essential nutrient taurine were similar among all experimental diets and comparable to the fish meal. Amino acids especially DL-methionine and L-tryptophan supplementation in the experimental diets likely contributed to their success in supporting juvenile lumpfish growth. Whereas, specific essential amino acids or fatty acids requirements for juvenile lumpfish have not been published, concentration of arginine, isoleucine, leucine, valine, phenylalanine, taurine and EPA in all experimental diets were higher than in control diet. It has been expressed that taurine is an essential nutrient in diets for some fish species (Salze et al., 2011; Jobling, 2016). Given the adequate dietary supplementation of essential nutrients especially essential amino acids; DL-methionine and L-tryptophan, and an essential nutrient called taurine, juvenile lumpfish seems to have tolerance for high SPC & PPC inclusion in the diet, without affecting the growth performance, muscle cellularity and proximate composition. Therefore, the balanced / similar nutrients contained feed (proteins, lipids, carbohydrates, vitamins, & minerals) is digested and absorbed by juvenile lumpfish; the absorbed similar nutrients (amino acids, fatty acids, sugars, vitamins & minerals) might have similarly influenced the gene activation and transcription, enzyme activities and metabolism. Thus, similar gene expression might have profiled (Transcriptomics) similar protein expression (proteomics) and metabolites (metabolomics) yielding (Jobling, 2016) similar growth for all the diet groups.

5. Conclusion

This thesis has documented that fish meal in the diet for juvenile lumpfish can be replaced with up to 75% SPC & PPC without compromising the growth performance of the fish. Moreover, fast skeletal muscle growth in juvenile lumpfish occurs mainly through hyperplasia during the experimental period and did not show any differences in proximate composition. Nevertheless, effort should be put into establishing the nutritional requirements of this species since it is a rapid growing commercial species in aquaculture.

6. References

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Appendix

Appendix 1: Biometrics of juvenile lumpfish fed diets with different plant protein levels.

Table 5: Growth performance – whole body weight, body length, body width, body height, liver weight, viscera weight, specific growth rate, condition factor, HSI and VSI of juvenile lumpfish (mean ± SEM) fed diets with different plant protein levels (0%, 25%, 50% and 75%). Different superscript letters indicate significant difference, P<0.05.

Parameter	Duration	Plant protein inclusion levels				
	(week)	CTRL (0%)	25%	50%	75%	P value
Body weight (g)	0	7.34 ± 0.12	7.05 ± 0.21	7.37 ± 0.07	7.38 ± 0.10	0.321
	2.5	14.64 ± 0.89	14.34 ± 0.26	15.09 ± 0.16	13.80 ± 0.80	0.545
	5	26.35 ± 0.46	25.67 ± 0.54	26.92 ± 0.26	26.51 ± 1.11	0.635
	7.5	42.25 ± 1.93	41.04 ± 1.08	45.92 ± 1.37	41.63 ± 3.50	0.434
Body length (cm)	0	4.87 ± 0.07	4.89 ± 0.03	4.90 ± 0.05	4.95 ± 0.03	0.763
	2.5	6.24 ± 0.13	6.21 ± 0.03	6.23 ± 0.04	6.04 ± 0.11	0.429
	5	7.66 ± 0.02	7.60 ± 0.03	7.72 ± 0.05	7.51 ± 0.09	0.116
	7.5	9.40 ± 0.56	8.65 ± 0.13	8.84 ± 0.20	8.39 ± 0.16	0.211
Body width (cm)	0	1.69 ± 0.02	1.67 ± 0.02	1.70 ± 0.02	1.71 ± 0.01	0.510
	2.5	2.67 ± 0.07	2.69 ± 0.02	2.69 ± 0.03	2.54 ± 0.06	0.173
	5	3.16 ± 0.06	3.12 ± 0.04	3.17 ± 0.01	3.15 ± 0.03	0.796
	7.5	3.38 ± 0.23	3.43 ± 0.18	3.47 ± 0.20	3.33 ± 0.06	0.948
Body height (cm)	0	2.38 ± 0.04	2.39 ± 0.03	2.39 ± 0.01	2.43 ± 0.01	0.602
	2.5	3.07 ± 0.09	3.07 ± 0.04	3.09 ± 0.02	3.06 ± 0.07	0.988
	5	3.79 ± 0.05	3.77 ± 0.02	3.82 ± 0.02	3.91 ± 0.04	0.084
	7.5	4.32 ± 0.09	4.35 ± 0.04	4.50 ±0.09	4.33 ± 0.13	0.522
Liver weight (g)	0 (K)	0.15 ± 0.03	0.19 ± 0.00	0.19 ± 0.01	0.18 ± 0.01	0.588
	2.5	0.37 ± 0.01	0.36 ± 0.02	0.36 ± 0.01	0.35 ± 0.01	0.933
	5	0.61 ± 0.05	0.57 ± 0.01	0.59 ± 0.03	0.63 ± 0.04	0.643
	7.5	1.04 ± 0.09	0.96 ± 0.04	1.09 ± 0.03	1.05 ± 0.07	0.535
Viscera weight (g)	0 (K)	0.86 ± 0.10	0.92 ± 0.02	0.96 ± 0.04	0.95 ± 0.00	0.646
	2.5	2.28 ± 0.10	2.21 ± 0.09	2.30 ± 0.07	2.02 ± 0.13	0.259
	5	3.81 ± 0.22	3.47 ± 0.18	3.59 ± 0.14	3.81 ± 0.11	0.438
	7.5	6.07 ± 0.54	5.84 ± 0.21	6.61 ± 0.22	5.57 ± 0.43	0.322

Table 4 continues…

Parameter	Duration (week)	Plant protein inclusion levels				
		CTRL (0%)	25%	50%	75%	P value
Specific growth rate (%/day)	0 -2.5 (K)	3.81 ± 0.24	3.91 ± 0.27	3.98 ± 0.11	3.46 ± 0.34	0.557
	2.5 - 5	3.65 ± 0.09	3.69 ± 0.09	3.70 ± 0.02	3.65 ± 0.13	0.965
	5 – 7.5 (K)	3.30 ± 0.08	3.32 ± 0.01	3.45 ± 0.05	3.25 ± 0.18	0.655
Condition factor	0	6.36 ± 0.27	6.07 ± 0.08	6.29 ± 0.25	6.10 ± 0.04	0.639
	2.5	6.02 ± 0.10	5.96 ± 0.03	6.25 ± 0.08	6.24 ±0.06	0.051
	5.0 (HSD)	5.87 ± 0.08[a]	5.85 ± 0.09[b]	5.85 ± 0.10[b]	6.25 ± 0.07[a]	0.024
	7.5 (K)	5.36 ± 1.04	6.35 ± 0.15	6.66 ± 0.34	7.02 ± 0.24	0.200
Hepato somatic index (%)	0 K	2.06 ± 0.44	2.65 ± 0.10	2.57 ± 0.11	2.48 ± 0.06	0.459
	2.5 (K)	2.54 ± 0.11	2.53 ± 0.12	2.39 ± 0.05	2.56 ± 0.07	0.589
	5.0	2.32 ± 0.14	2.24 ± 0.02	2.19 ± 0.11	2.39 ± 0.09	0.521
	7.5	2.46 ± 0.10	2.33 ± 0.06	2.37 ± 0.03	2.53 ± 0.10	0.369
Viscera somatic index (%)	0 (K)	11.81 ± 1.56	13.00 ± 0.13	12.97 ± 0.45	12.83 ± 0.20	0.910
	2.5	15.60 ± 0.65	15.50 ± 0.31	15.24 ± 0.52	14.62 ± 0.26	0.487
	5.0	14.43 ± 0.60	13.51 ± 0.78	13.32 ± 0.41	14.42 ± 0.73	0.515
	7.5	14.31 ± 0.66	14.23 ± 0.33	14.38 ± 0.19	13.39 ± 0.25	0.330

If not stated, P values are from one way ANOVA. If stated in second column, K= Kruskal-wallis test, W= Welch F test and HSD= ANOVA followed by Tukey HSD.

Appendix 2: Muscle cellularity of juvenile lumpfish fed diets with different levels of plant protein.

Table 6: Muscle cellularity – Total cross sectional area, Fast fibre diameter, Fibre number, Fibre diameter below 10 µm, Fibre density and of juvenile lumpfish (mean ± SEM) fed diets with different plant protein levels (0%, 25%, 50% and 75%). If stated, different superscript letters indicate significant difference, P < 0.05.

Parameter	Duration (week)	Plant protein inclusion levels				
		CTRL (0%)	25%	50%	75%	P value
TCA (mm^2)	0	78.04 ± 10.09	76.37 ± 3.65	81.09 ± 3.60	81.93 ± 9.76	0.9451
	2.5 (K)	141.97 ± 10.95	142.56 ± 11.41	142.39 ± 9.31	105.29 ± 12.77	0.2820
	5.0 (K)	227.68 ± 12.31	220.21 ± 20.14	239.86 ± 17.66	236.67 ± 14.60	0.6910
	7.5	409.68 ± 26.22	346.77 ± 12.01	399.30 ± 26.30	346.95 ± 31.18	0.2244
FFD (µm)	0	39.33 ± 2.74	36.76 ± 1.71	32.82 ± 0.60	32.04 ± 1.61	0.0695
	2.5	28.54 ± 2.30	28.41 ± 1.43	29.47 ± 0.74	27.39 ± 1.50	0.8336
	5.0	26.65 ± 0.67	29.12 ± 1.85	29.5 ± 0.96	30.78 ± 1.17	0.2009
	7.5	33.48 ± 0.87	32.78 ± 0.73	32.14 ± 0.84	31.48 ± 1.07	0.9328
FN	0	48787 ± 8159	57502 ± 7765	70620 ± 1444	73730 ±11970	0.1951
	2.5 (K)	147478 ± 14125	144995 ± 6476	138971 ± 4794	127214 ± 19013	0.7640
	5.0	255811 ± 17288	216428 ± 21488	222949 ± 26777	221124 ±18839	0.5729
	7.5	310234 ± 32277	284228 ± 16640	331335 ± 26949	298406 ± 24714	0.6339
Number of fibres < 10µm	0	18 ± 10.07	16± 5.70	33 ± 12.12	60 ± 21.08	0.1592
	2.5	144 ± 28.33	143 ± 15.28	120 ± 10.67	112 ± 11.86	0.5155
	5.0 (W)	121 ± 14.58	115 ± 30.93	97 ± 8.84	86 ± 7.49	0.3840
	7.5	54 ± 9.07	43 ± 2.78	59 ± 5.38	52 ± 6.71	0.4311
Fibre density	0	643 ± 131	760 ± 70	873 ± 26	890 ± 54	0.1871
	2.5	1034 ± 115	1031 ± 75	969 ± 31	1200 ± 135	0.4332
	5.0	1121 ± 24	984 ± 89	924 ± 44	936 ± 51	0.1311
	7.5	751 ± 35	794 ± 42	832 ± 30	869 ± 48	0.2515

If not stated, P values are from one way ANOVA. If stated in second column, K= Kruskal-wallis test, and W= Welch F test.

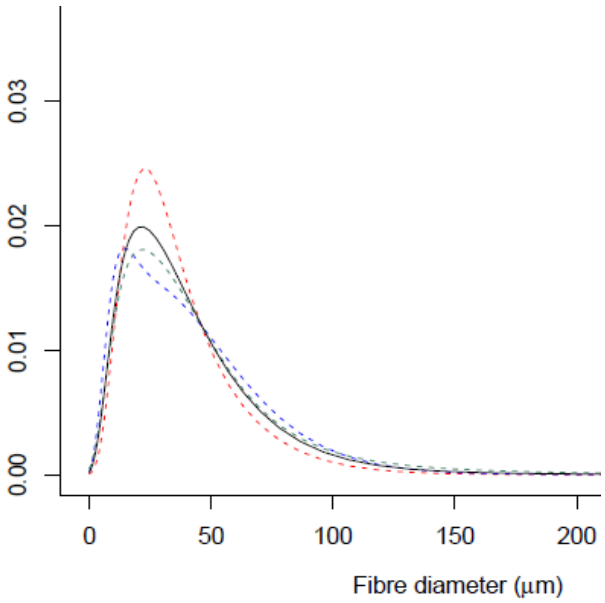
Appendix 3: R code for the probability density functions (PDFs) of juvenile lumpfish.

```
> install.packages("sm")
> load("FibreA.prg")
> library(sm)
> smsetup()##
> sm.options(rugplot=F)
> sm.options(xlab=expression(paste("Fibre diameter(",mu,"m)")))
> group1.mat <- matrix(scan("wk0ctrl.txt"),ncol=3,byrow=T)
> group2.mat <- matrix(scan("wk025%.txt"),ncol=3,byrow=T)
> group3.mat <- matrix(scan("wk050%.txt"),ncol=3,byrow=T)
> group4.mat <- matrix(scan("wk075%.txt"),ncol=3,byrow=T)
> random1.mat <- drawfish.prg(800, group1.mat)
> random2.mat <- drawfish.prg(800, group2.mat)
> random3.mat <- drawfish.prg(800, group3.mat)
> random4.mat <- drawfish.prg(800, group4.mat)
> H1 <- findh.prg(random1.mat)
> H2 <- findh.prg(random2.mat)
> H3 <- findh.prg(random3.mat)
> H4 <- findh.prg(random4.mat)
> View(findh.prg)
> fix(findh.prg)
> H1 <- findh.prg(random1.mat)
> H2 <- findh.prg(random2.mat)
> H3 <- findh.prg(random3.mat)
> H4 <- findh.prg(random4.mat)
> samlet <- list(random1.mat, random2.mat, random3.mat, random4.mat)
> hchecker.prg(samlet)
> View(hset.prg)
> fix(hset.prg)
> pdf_group1 <- hset.prg(random1.mat,H1$hmean, title="Group1")
> pdf_group2 <- hset.prg(random2.mat,H2$hmean, title="Group2")
> pdf_group3 <- hset.prg(random3.mat,H3$hmean, title="Group3")
> pdf_group4 <- hset.prg(random4.mat,H4$hmean, title="Group4")
> tiff(filename = "example.tif", pointsize = 50, width = 20, height = 20,
units="in", res = 300)
> pdf_group1 <- hset.prg(random1.mat,H1$hmean, title="Group1")
> dev.off()
> tiff(filename = "example.tif", pointsize = 50, width = 20, height = 20,
units="in", res = 300)
> pdf_group2 <- hset.prg(random2.mat,H2$hmean, title="Group2")
> dev.off()
> tiff(filename = "example.tif", pointsize = 50, width = 20, height = 20,
units="in", res = 300)
> pdf_group3 <- hset.prg(random3.mat,H3$hmean, title="Group3")
> dev.off()
> tiff(filename = "example.tif", pointsize = 50, width = 20, height = 20,
units="in", res = 300)
> pdf_group4 <- hset.prg(random4.mat,H4$hmean, title="Group4")
> dev.off()
> fix(hset.fixx.prg)
> fixx.pdf_group1 <- hset.fixx.prg(random1.mat, H1$hmean, col1=1,
xmax=300, title="Group1")
> fixx.pdf_group2 <- hset.fixx.prg(random2.mat, H2$hmean, col1=1,
xmax=300, title="Group2")
> fixx.pdf_group3 <- hset.fixx.prg(random3.mat, H3$hmean, col1=1,
xmax=300, title="Group3")
> fixx.pdf_group4 <- hset.fixx.prg(random4.mat, H4$hmean, col1=1,
xmax=300, title="Group4")
> fix(testband.prg)
> samlet <- list(random1.mat, random2.mat, random3.mat, random4.mat)
> hmean_all <- mean(H1$hmean, H2$hmean, H3$hmean, H4$hmean)
> test_all <- testband.prg(list1 = samlet, hmean = hmean_all, nboot =
1000)
```

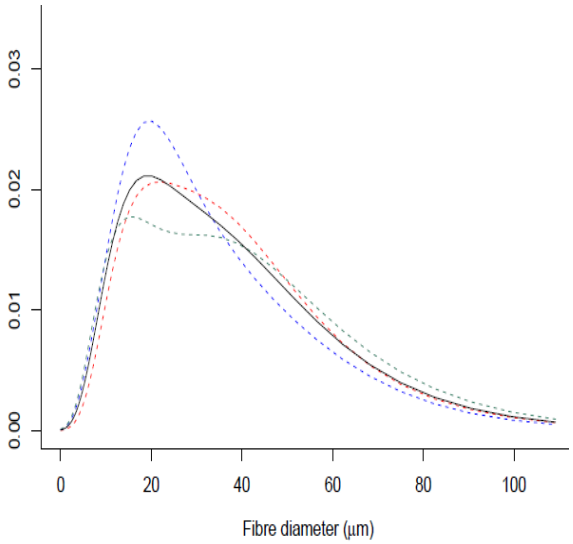
Appendix 3 cont..

Probability density functions for all diet groups at each sample point.

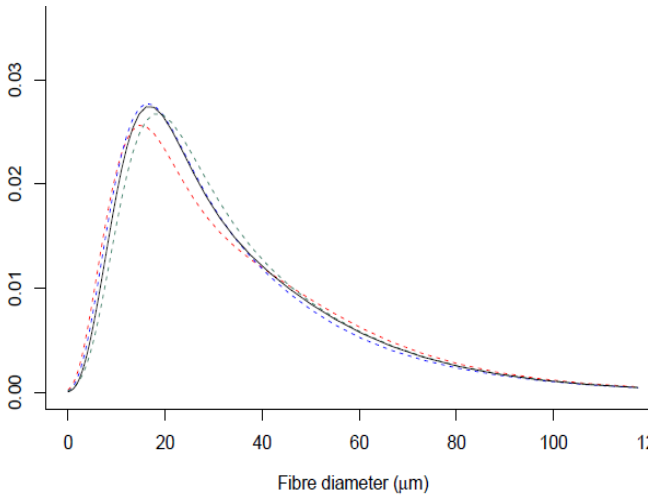
A-Week 0 - Control diet



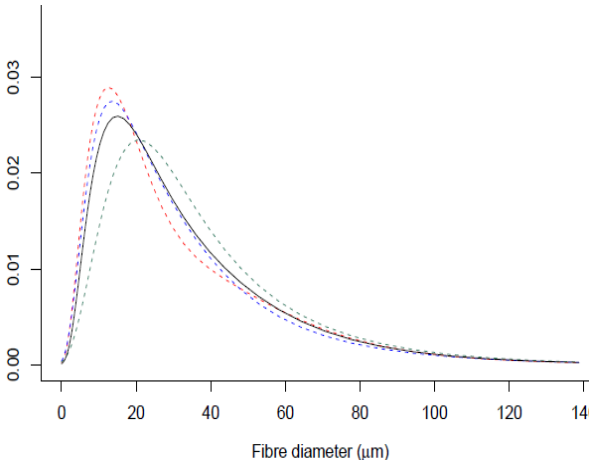
B- Week 0 – 25% diet



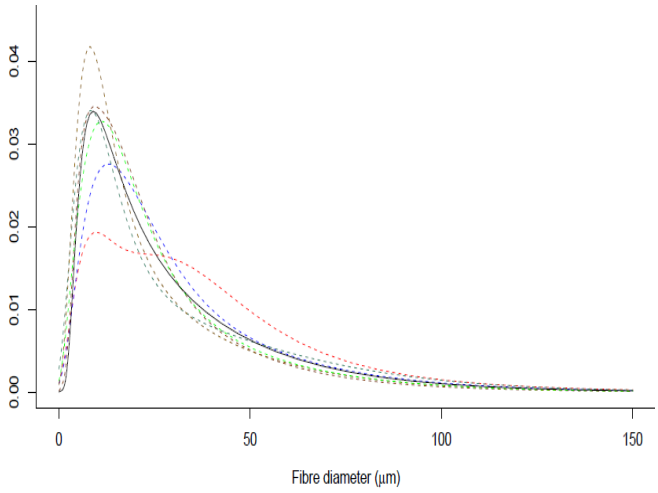
C-Week 0 - 50% diet



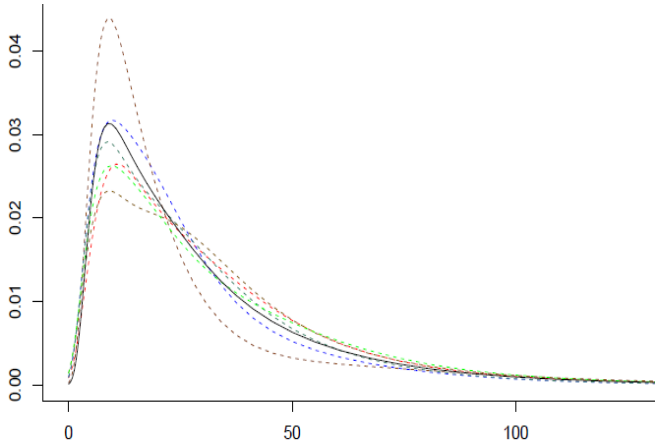
D-Week 0 – 75%



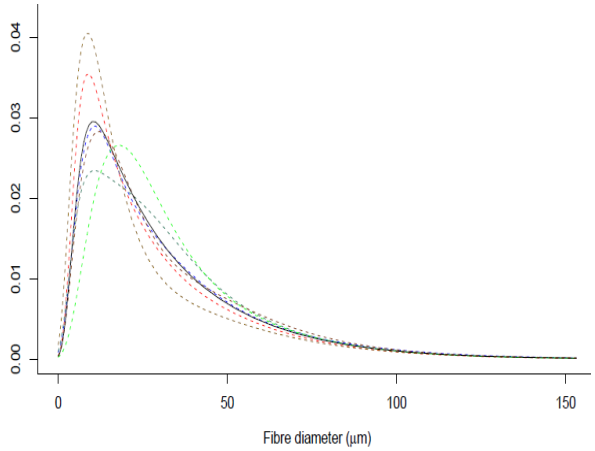
E-Week 2.5 – Control diet



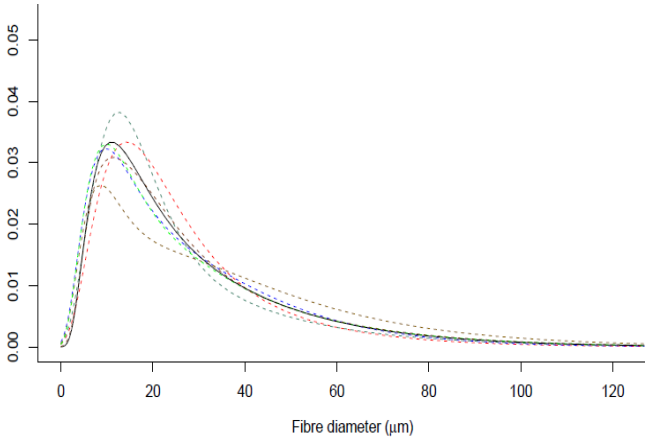
F- Week 2.5 – 25% diet



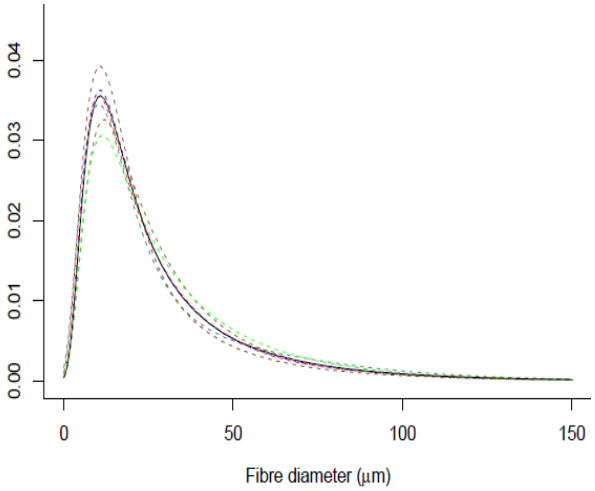
G- Week 2.5 – 50% diet



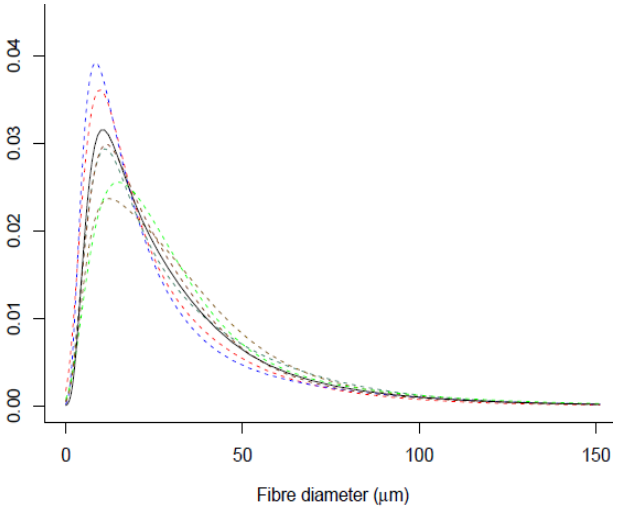
H- Week 2.5 – 75% diet



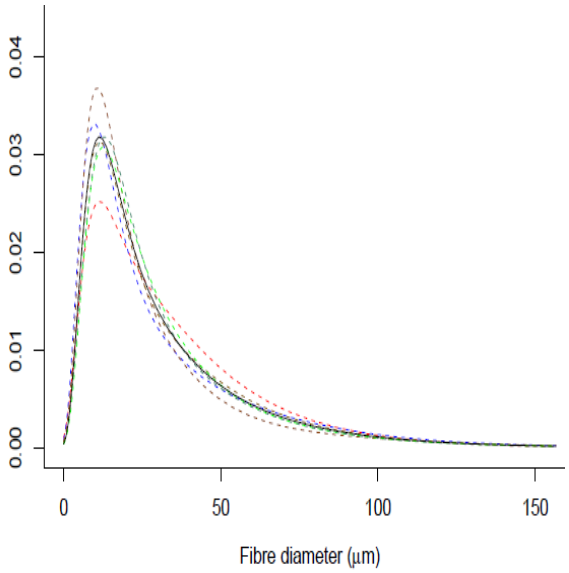
I - Week 5 – Control diet



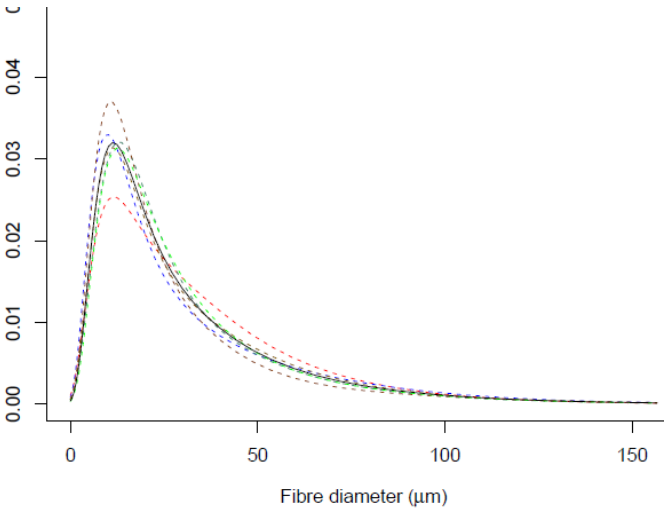
J- Week 5 – 25% diet



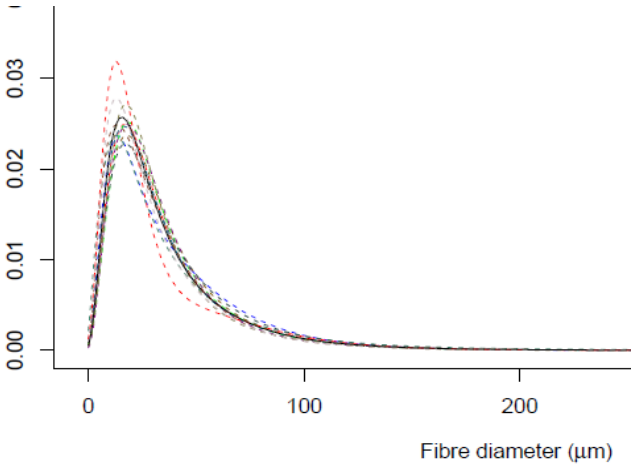
K - Week 5 – 50% diet



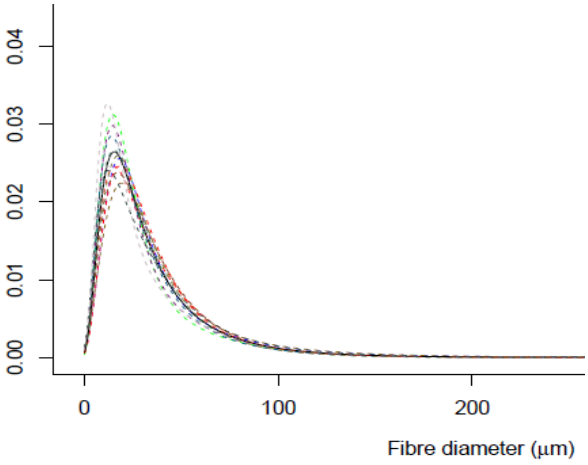
L- Week 5 – 75% diet



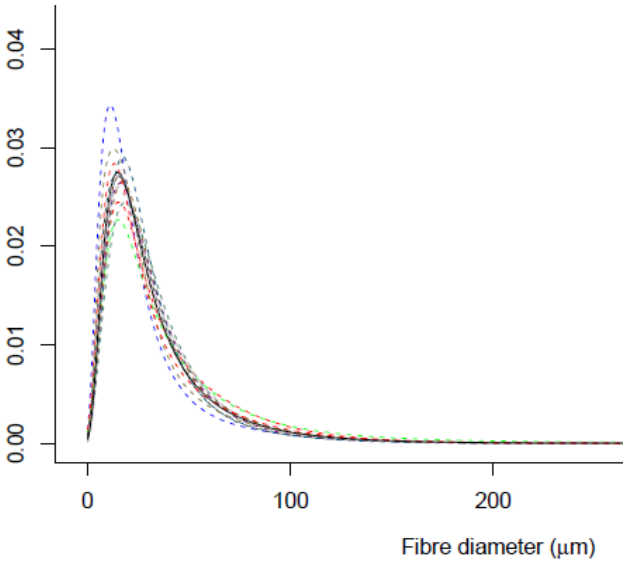
M- Week 7.5 – Control diet



N - Week 7.5 – 25% diet



0 - Week 7.5 – 50% diet



P- Week 7.5 – 75% diet

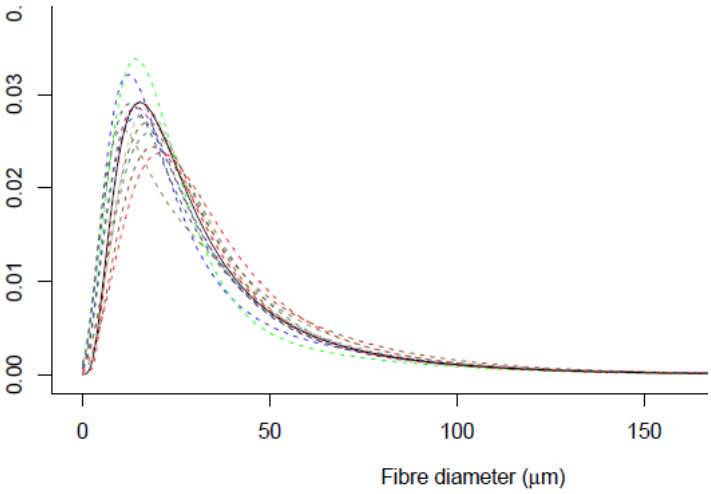


Figure 266: Probability density functions (PDFs) of fast muscle fibre diameter of juvenile lumpfish fed the experimental diets at week 0 (A=CTRL, B=25%, C=50% D=75%), 2.5 (E=CTRL, F=25%, G=50% H=75%), 5 (I=CTRL, J=25%, K=50% L=75%), & 7.5 (M=CTRL, N=25%, O=50% P=75%). Dashed lines represent the average PDFs for each fish and solid central line corresponds to the average PDF for combined fish.