UNIVERSITY OF NORDLAND

MASTER THESIS

Nutritional programming in lumpsucker, *Cyclopterus lumpus* - a transcriptomic approach

Iuliia Belova

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PREFACE

This master's thesis is the final part of a two-year Master of Science program at the Faculty of Biological Sciences and Aquaculture, University of Nordland, Bodø, Norway. The thesis is a scientific work of 60 credits within a field of aquaculture.

The work "Nutritional programming in lumpsucker, *Cyclopterus lumpus* - a transcriptomic approach" is a part of MarLip (Marine Larvae Innovation Platform) research project: Development of a new functional and specialized feed for marine fish larvae.

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Iuliia Belova

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

ABSTRACT

The study of nutrient requirements of lumpsucker larvae during early development for aquaculture is still at a very early stage. To investigate the effects of different feeding diet on lumpsucker during early development, larvae and early juveniles were fed with four different diets: preserved feed, planktonic feed, dry commercial feed and control. *Artemia nauplii* were used as first-feeding in all groups, except the control fish, which were fed only dry commercial feed during all the experiment. Larvae reared at different diets were not morphometrically similar, however two groups (preserved feed and dry commercial feed) showed the best growth and survival rates in comparison to Control. RNA-Seq expression analysis was performed to compare the intestinal transcriptome between preserved feed and control feed groups, since they displayed significant differences in growth at 84th day. It revealed 97 differentially expressed genes that were affected by feeding diet. Among these transcripts, *ppp1r3c*, *capn10* and *stbp2* were significantly down-regulated and *ranbp2*, *utp20*, *samd9*, *cenpf*, *mll3*, *mdn1* were significantly up-regulated in preserved feed compared to control group. The differentially expressed genes are involved in a number of processes, including lipid metabolism, and they may improve, at least partly, the growth performance of fish fed preserved feed compared to their control counterparts. Taken together, the results from this study demonstrate that larval growth, survival and intestinal gene expression in lumpsucker are influenced by feeding regimes. Also, it seems that the use of live feed is more crucial on early stages of lumpsucker ontogenesis than artificial food. Importantly, this is the first report on the effects of diets on larvae during the weaning process in this species and this fundamental information will be most useful for successful production of lumpsucker larvae.

1. INTRODUCTION

1.1. Biological characteristic and general description of lumpsucker

Lumpsucker (*Cyclopterus lumpus* Linnaeus, 1758) or lumpfish is primarily a demersal fish and the only species of the genus *Cyclopterus*. They are widely distributed in the boreal region of both sides of the North Atlantic and are found in the Barents sea, White Sea, along the Norwegian, Danish, Dutch, Belgian, United Kingdom, French, and Spanish coasts and as far south as the northern coast of Portugal (Davenport, 1985; Stevenson and Baird, 1988; Nytrø, 2013).

In Europe they are commonly referred to as lumpsucker, but is also known as henfish, seasnail, lump, paddle-cock and poule de mer (Davenport, 1985; Stevenson and Baird, 1988).

The fish has been given the name "lumpfish" due to the short, thick and blunt body, but the name "lumpsucker" is also used due to the "sucker" under the side of the chest formed by a modification of the ventral fins (Basby, 1997) (Fig. 1).

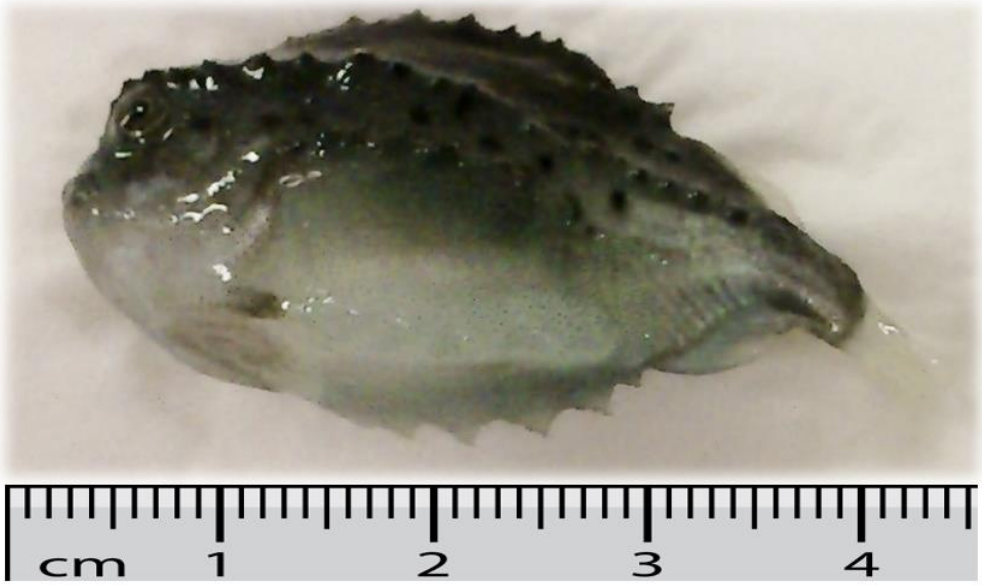

Figure 1. General view of lumpsucker (128 dph)

Coloration with a great variety of tints from blue, bluish-grey, to greenish and brownish, and dependent on spawning season. Furthermore, sexual dimorphism is typical for this species and females are generally bigger than males. Females may attain a total length of 61 cm and weigh up 9.1 kg, while male lumpsucker can grow to 35.6-38.1 cm length and weight 1.4-2.7 kg. (Davenport, 1985; Stevenson and Baird, 1988; DFO, 1999).

They are found in the cold waters of the Arctic, North Atlantic, and North Pacific oceans; from Portugal to the White Sea and are common along the Norwegian coast (Cox and Anderson, 1922; Moen and Svensen, 2003) (Fig. 2).

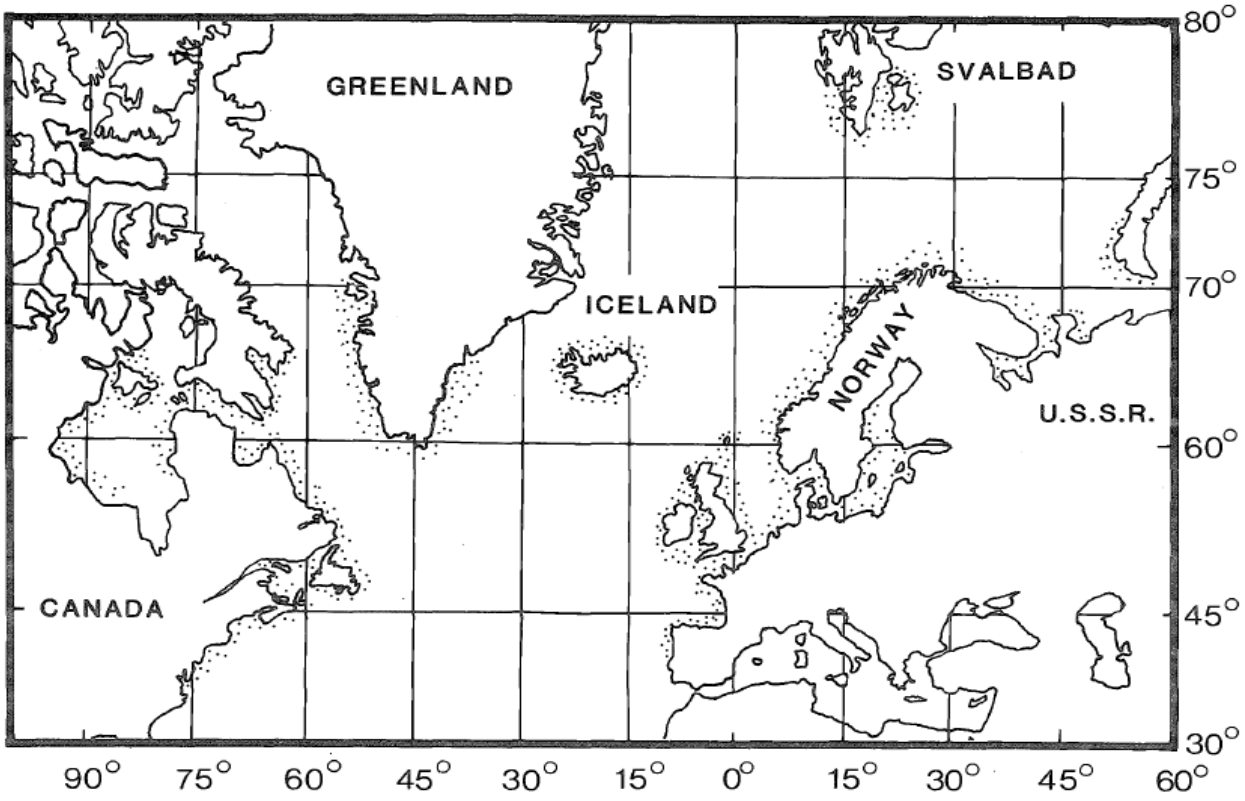

Figure 2. Distribution of lumpfish, where dotted areas indicate spawning grounds (Davenport, 1985)

The lumpsucker is primarily a semi-pelagic fish, because the adults, living in pelagic zones, becomes demersal during spawning season. Juveniles typically hide in algae (Davenport, 1985).

1.1.1. Reproduction and development

During spawning between late spring and early summer (April to May), *Cyclopterus lumpus* makes migration for very long distances towards shallow coastal waters and usually migrate back to deeper water (at depths greater than 300 m) during late summer and early autumn. Species should be at least five-six years old to spawn (Davenport, 1985). In the breeding season, males have a bright pink or red coloration of underside, while females remain in blue green. Females partially release of their eggs in a large spongy mass with intervals of 8-14 days two or three times. Usually, the number of eggs (approximately 2.3 mm in diameter) is 100.000; however, it can vary from 100.000 to 400.000 and higher, depending on the size and age of the females.

Males play a key role in rearing the eggs, aerating the eggs and guarding them against predation. (Davenport, 1985; Stevenson and Baird, 1988; DFO, 1999; Nytrø, 2013).

The fertilized eggs contain several oil droplets, which fuse into one single oil globule during embryo development. The incubation period lasts about 6-8 weeks, depending on water temperature. Usually, the outer layer eggs develop more rapidly than inner layers (Davenport, 1985; DFO, 1999; Hustad, 2008).

The newly hatched larvae average 6 mm in length and weight 2.4 mg. During the first year they grow rapidly and can reach 5 and 7.5 cm in length. Davenport (1985) found that newly hatched larvae have a continuous fin running along the back, round the tail and on the underside to the vent. Pectoral fins are rudimentary at this stage, and no ventral fins ever appear. Ventral suction disc is an active. The median fin breaks up into separate fins by the length of about 8-9 mm, and fin rays are visible in all fins. At this stage the lumpfish has a perfectly normal first dorsal fin, but this is gradually overgrown by the characteristic dorsal "hump", and a 32-mm specimen is essentially a miniature of the adult fish (Davenport, 1985; DFO, 1999).

1.1.2. Nutrition

The literature on the feed preferences for larvae of *Cyclopterus lumpus* is limited. In 1910 Apstein was the first to carry out the first seriously study of feeding in lumpfish from the North Sea and Baltic. As he reported, gut content lumpsuckers consists chiefly of mysids (*Mysis mixta*) or ctenophores (*Pleurobrachia spp.*). In addition, the amphipod, young sandeels (*Ammodytes*) and seagrass (*Zoostera*) were found in small quantity. Cox and Anderson (1922), working upon Canadian material, found the euphausiid shrimps (*Meganyctiphanes*), medusa *Aurelia flavidula*, amphipod crustaceans (*Hyperia*), large numbers of young clupeids and caprellid crustaceans with the remains of small fish in the stomachs of lumpsuckers. Myrseth (1971), investigating lumpfish caught at North Norway, reported about amphipods, isopods and *Nereis pelagica*, however, the euphausids were dominated. Therefore, it can be concluded that the planktonic organisms are main components in the diet of *Cyclopterus lumpus*, but benthic organisms are also present sometimes. According to Daborn and Gregory (1983), juveniles also fed upon near surface plankton until they are small, and shifting to the amphipods *Calliopus laeviusculus* and *Parathemisto gaudichaudi* as they grew (Davenport, 1985).

During spawning migrations lumpfish almost completely stops exogenous feeding, and all a big waste of energy during prolonged and further movement happens due to the endogenous supply, at the expense of a transformation of reserve substances in the body. However, fish

receives all materials are needed for the endogenous supply by exogenous feeding, which is one of main reason why exogenous feeding is so important (Stroganov, 1962; Davenport, 1985; Mitamura et al., 2007).

1.2. Lumpsucker and sea lice control

The lumpfish is poorly studied fish and the specific literature about their behavior and biology is limited. However, in recent years, many aquaculture companies and marine organizations around the world are researching this species as a biological solution of problems caused by sea lice.

The sea louse *Lepeophtheirus salmonis* (Krøyer, 1837) is small marine ectoparasitic (external parasites) copepod crustacean that affects both on wild and farm fish, especially salmonids. *Lepeophtheirus salmonis* and *Caligus elongatus* are the major genera of sea lice leading to serious economic damage on salmon aquaculture in Northern Europe (Costello, 2006).

L. salmonis has a direct life cycle, meaning a single host, and consists of ten stages. The life cycle of the salmon louse can be divided on non-parasitic larval stages (nauplii I, II) and parasitic stages (copepodid; chalimus I–IV; pre-adults I, II; adults) (see below Fig. 3). The adult female sea louse can produce 6–11 pairs of egg strings during life cycle (Whelan, 2010; Hamre et al., 2013). Both of nauplii stages are planktonic, non-feeding and they drift passively with ability to vertical migration in water column. The nauplii moult into infective free-living copepodids. The copepodid attaches to the host tissue (especially on the fins of the fish or the scales) by their antenna, and then undergoes a moult to chalimus. All four chalimus stages are sessile and parasitic stages, which attach by puncture the epidermis of the host with a special frontal filament (penetrative thread) to feed. Pre-adults I, II are two mobile stages, which able to move around on the surface of the fish to feed and can also swim in the water column. They finally moult to adult male or female. The lifespan of the louse is difficult to measure under natural conditions; however laboratory experiments are showed that females have lived for up to 210 days (Heuch et al., 2000; Whelan, 2010; Hamre et al., 2013).

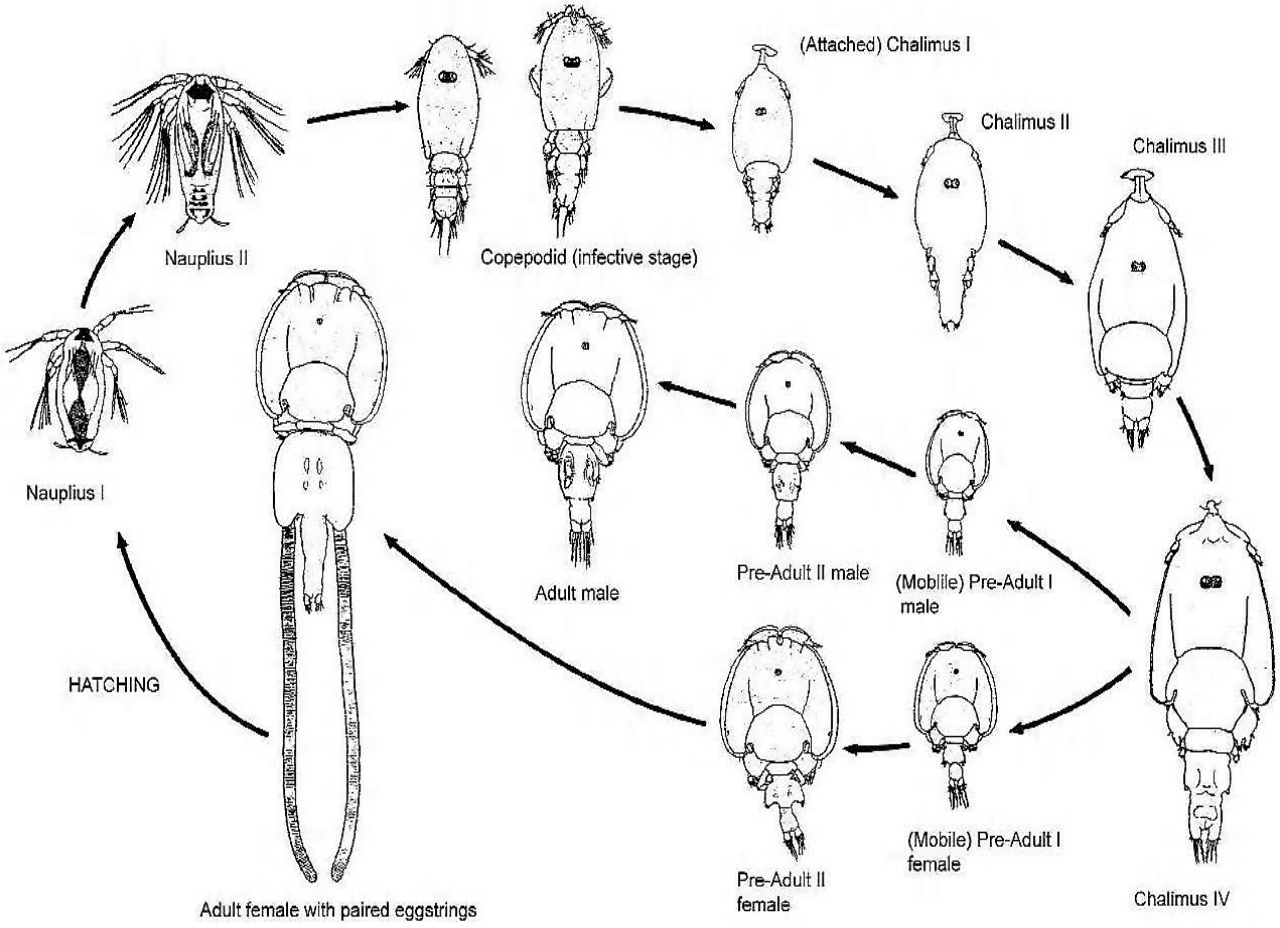

Figure 3. The life cycle of the salmon louse *Lepeophtheirus salmonis* (Whelan, 2010)

Sea lice abundance depends on many factors such as water temperature, salinity, host species, age and immune system of the fish. Sea lice cause physical and enzymatic disturbances, when attach to the fish skin and remove mucus, blood, skin and underlying tissue in general lead to the fish mortality (Costello, 2009; Whelan, 2010). Mobile stages of salmon lice represent the greatest threat to the fish due to their ability to swim quickly along the host's surface. As Whelan (2010) reports this causes various destructive process of the host's skin such as epithelium loss, bleeding, increased mucus discharge, tissue necrosis. In addition, host fish showed a poor growth performance due to loss of appetite. These changes and prolonged stress lead to reduce of lymphocyte level in blood, ionoregulatory disturbance and elevated cortisol, meaning reduction of osmoregulatory and respiratory ability, impaired in immune response, reduced swimming and cardiac performance, behavioral changes that increase predation risk. Therefore, if appropriate measures are not taken it will result in host death (Costello, 2009; Morton et al., 2004; Whelan, 2010; Price et al., 2011; Brauner et al., 2012).

In the wild, such a phenomenon as migratory allopatry (the spatial separation of age classes) protects the juvenile salmon from infestation with the parasites until they are bigger and start spawning. However, the coastal marine salmon aquaculture involves the use of floating sea cages for all period of product rearing (about 18 months). Thus, salmon farming is carried out in high densities and the risk of infection by sea lice is growing. These living conditions are not typical for wild salmon, but escaped domestic fish provides the transmission of the disease and lead to the decline of some wild salmonid populations and ultimately to violation of the ecological balance in the environment (Costello, 2009; Price et al., 2011; Kristoffersen et al., 2014; Liu and Bjelland, 2014).

Sea lice are one of the most significant problems in such the salmon producing countries as Scotland, Chile, Canada and especially in Norway, where farmed salmon provides a major economic income for these countries (Nagasawa, 2004; FAO, 2005; Costello, 2009). Liu and Bjelland (2014) reported that the total production of Atlantic salmon decreased by almost two times from 2002 to 2012 (1.1 billion and 2.1 billion tons, respectively) that constitute a serious threat to these countries. In Norway the cost of sea lice infection (including consequences from abundance of lice and treatment) are estimated approximately 790 million NOK or 0.79 NOK per kg in 2011 and continues to increase. Sea lice monitoring of fish farms is allowed to estimate such important indicators as level of salmon lice infection and demography in the surrounding sites (Kristoffersen et al., 2014). The Norwegian government and responsible authorities are exercised a control and supervision of compliance with mandatory requirements for monitoring of salmon farming by a special laws and regulations. Accordingly to National Action Plan for sea lice on salmon farms (1997), all farms are not permitted to exceed an infection level of on average 0.5 mature female salmon lice (*Lepeophtheirus salmonis*) or two mobile male lice per fish. Salmon farms must count sea lice every week provide the corresponding monthly reports (Wilson et al., 2009; Kristoffersen et al., 2014).

Researchers believe that prevention, immediate treatment and management strategies are best interventions to limitation the influence of parasites. In Norwegian salmon farms, these methods can be divided on three main approaches:

— Medicine (in-feed pellet – oral treatment);

— Chemical (bath delousing);

— Biological (cleaner fish).

Ememectinbenxoate (SLICE) and Teflubenzuron (Ektobann/Skretting) are typically used as main ingredients in-feed treatment anti-parasitic medicines in Norway and other salmonid producing countries. The present procedure consists in covering feed pellets with drugs, which have high selective toxicity for the parasite (Lees et al., 2008; Liu and Bjelland, 2014).

Bath treatments against sea lice are more complex and usually are used at the end of the production cycle, when using of the in-feed treatments requires a significant investment. Deltamethrin (Alphamax), Azamethiphos (Salmosan) and H_2O_2 (hydrogen peroxide) are the main chemicals, used in bath treatments (Liu and Bjelland, 2014).

Usually, both medicine and chemical approaches are used at the early stage, when salmon is not so big (around 2 kg), because the using of them on later stages require large financial expenses for aquaculture industry due to its high costs. Despite medicine and chemical treatments are quite effective, many scientists found that drug resistant parasites occurs. For example, as S. Carrell (2012) reported, the overall use of different pesticides on salmon farming in Scotland was dramatically increased during 2008 to 2011 years: from 188 kg in 2008 to 395 kg last year with an increase in some of them to 110%, meaning that sea lice becomes resistant to treatment (Fig.4).

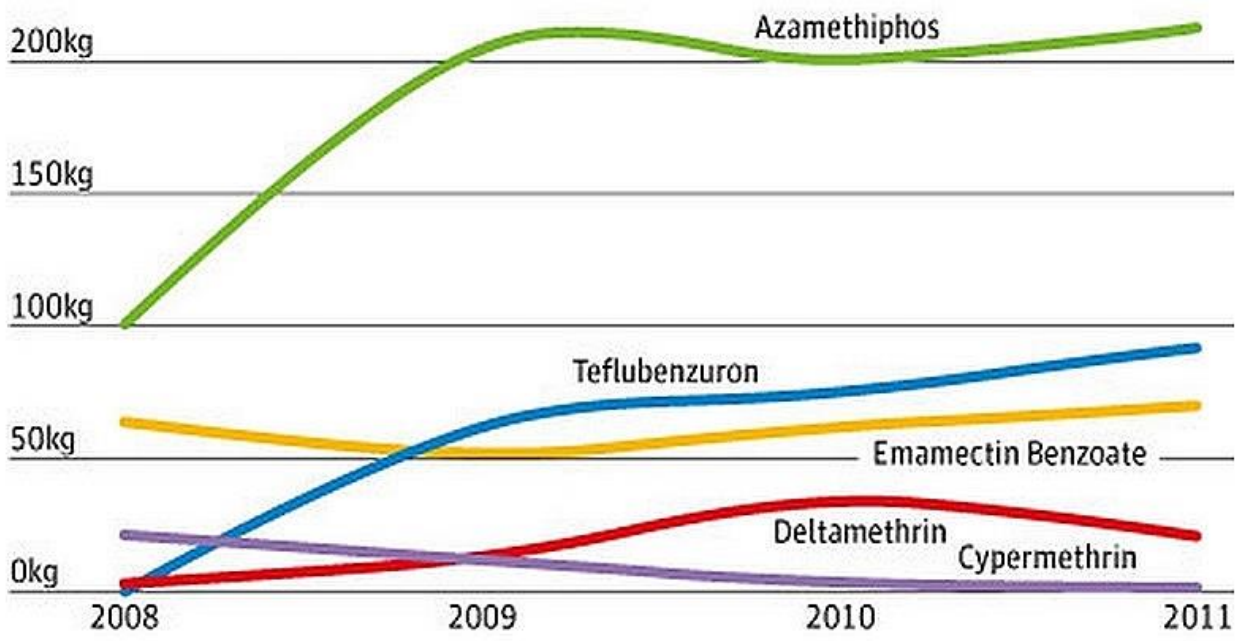

Figure 4. The using of different pesticides against salmon lice on Scottish fish farms during 2008 to 2011 years (Carrell, 2012)

Furthermore, medicine and chemical treatments can effect on health of fish and lead to environmental concerns (Grave et al., 2004; Lees et al., 2008; Carrell, 2012; Hamre et al., 2013; Torrissen et al., 2013; Aaen et al, 2015; Liu and Bjelland, 2014).

One of the main alternative to pharmaceutical methods and therefore ecological and cost-effective way of sea lice control is the use of cleaner fishes, which form a symbiotic relationship with salmon by removing the sea lice attached to salmon skin (Chilvers, 2013). Currently, goldsinny wrasse (*Ctenolabrus rupestris*), corkwing wrasse (*Symphodus melops*), rock cook (*Centrolabrus exoletus*) and juvenile ballan wrasse (*Labrus bergylta*) are four wrasse species used as cleaner fish in Norway (Skiftesvik et al., 2014). Among them, ballan wrasse (*Labrus bergylta*) is more popular and high effective on fish farms. This species is most suitable in biological delousing on larger salmon production due its largest size. However, the wrasses are very sensitive to the low temperatures (< 6 °C) that lead to inability of feeding for winter and ultimately to mortality. This a serious limitation for use in many farms of northern regions, especially the salmon farms of Northern Norway (Chilvers, 2013; Imsland et al., 2014).

Another species that is preferable as a cold-water alternative to wrasses is lumpfish for environmental and economical reasons is the lumpfish or lumpsucker (see section 1.1). As H. Chilvers (2013) reported, lumpsuckers have several main advantages over wrasses: they are more robust, hardy with faster growth rate (5-6 cm for 4-5 months compared to 1.5 years), greater temperature range tolerance (from 4-7°C and lower) with ability to feed all year round, easier to farm and less susceptible to vibrio infection. Furthermore, lumpfish can be used at a greater density than wrasse (10% compared to 4%) (Chilvers, 2013).

1.3. Effect of nutrition on larval growth and development in marine species

The larval stage is a key period in the ontogeny of the fish, when there are significant changes in the structure, physiology, size and morphology. Thus, over the last twenty years numerous studies are devoted to investigations of fish larvae development, namely ontogeny and functioning of the digestive tract (RCN, 2009; Zambonino-Infante and Cahu, 2010).

The body of fish is constantly spending energy and therefore it must constantly replenish it by exogenous feeding (Stroganov, 1962). From the opening of the mouth (day 5 post-hatching in sea bass, day 21 in halibut), fish progressively combines exogenous feeding with endogenous and completely converted to exogenous feeding when yolk and oil globule are totally depleted (day 15 in sea bass, day 27-30 in halibut). In terms of aquaculture focus, the weaning processes (switch from live preys to compound diet feeding sequence) is a bottleneck area in the first feeding of marine fish in Norway and abroad, and will be affected by the composition of the exogenous feeding (RCN, 2009; Zambonino-Infante and Cahu, 2010; Almli, 2012; Piccinetti et al., 2013).

It is well known that the nutritional requirements of larval fish are different from juveniles and adult fish (RCN, 2009; Zambonino-Infante and Cahu, 2010; Piccinetti et al., 2013). Many scientists, who investigated this question found that live prey motion (rotifers, artemia, copepods) and their biochemical characteristics are important for early stages of fish. However, rotifers and artemia due their inadequate fatty acid profile and size are largely inferior copepods. Accordingly to studies, marine fish larvae reared on natural copepods show the best survival and growth rate and lower prevalence of bone deformations than fish larvae grown on rotifers or artemia. Results of experiments demonstrated that preserved copepods retain precious fatty acid characteristics and lead to gene expression and improvement of crucial developmental processes, such as lipid metabolism and metamorphosis (Luizi et al., 1999; Imsland et al., 2006; RCN, 2009; Olivotto et al., 2010; Kortner et al., 2011; Almli, 2012; Demeny et al., 2012; Piccinetti et al., 2013).

The successful artificial larval feed or diet preparation must satisfy essential species-specific requirements for amino and fatty acids, vitamins, minerals and macronutrients (protein, lipid, carbohydrate) to ensure proper development and high survival rates during the early life stages (Leaver et al., 2008; RCN, 2009).

1.3.1. Effect of protein fraction supply

Larval period is generally characterized by the most intensive growth of fish and the requirement in protein increases. However fish larval requirements in protein is still poorly studied (Hatlen et al., 2005; Zambonino-Infante and Cahu, 2010; Kortner et al., 2011).

In 1996 Péres et al. studied sea bass (*Dicentrarchus labrax*) larvae fed four isoenergetic feeding diets with varied proportion of protein and carbohydrate. As they reported, larvae fed diet containing 50 to 60 % protein showed better growth in contrast diet with only 30 and 40 % protein. Furthermore, the extensive amylase synthesis was observed only on early stages of larvae development, whereas trypsin activity increased on later stages. Thus, it has been suggested the regulation of trypsin activity and other digestive enzymes in fish larvae is age dependent (Péres et al., 1996).

Hatlen at al. (2005), working upon two size groups (60 and 800 g) of Atlantic halibut (*Hippoglossus hippoglossus*) fed four diets with different of protein and carbohydrate composition, found that optimal dietary protein level for Atlantic halibut depends on body size. In addition, big fish will have a lower optimal dietary protein level and less prone to dietary carbohydrates than a smaller fish (Hatlen et al., 2005).

In 2011 Kortner et al. investigated the effects of dietary constituents on the ontogeny of digestive functions in Atlantic cod (*Gadus morhua*) larvae, using molecular approaches. As showed the experiment, genes involved in digestive system were differentially expressed after different first feeding regimes, meaning that the growth rate can be regulated by optimal diet during early developmental stages in marine fish larvae (Kortner et al., 2011).

Researchers also believe that a partial replacement of native protein by dietary peptides has a positive effect on the health status and total larvae development. The explanation of this is that fish larvae have the lowest trypsin activity at this stage of ontogenesis, but high levels of peptidases. According the results of experiments, marine fish larvae fed on diet with substitution of 10 % to 20 % of fish meal by hydrolysate showed the best survival and growth rates with early stimulation of the digestive enzymes and improved immune functions, compared larvae fed on diets without hydrolysate or at a replacement of 40 %. However, an incorporation of a high concentration of peptides (50 % and 75 %) led to skeletal (spinal or jaw) abnormalities and opposite results (Kolkovski and Tandler, 2000; Charu et al., 2003; Kotzamanis et al., 2007; Zambonino-Infante and Cahu, 2010).

1.3.2. Effect of lipid fraction supply

Fish lipid are extremely complex system and the predominant source of energy for fish (Stroganov, 1962; Leaver et al., 2008). As Zambonino-Infante and Cahu (2010) report, lipid constitutes with free amino acids the most important energy reserve in fish embryos. Larvae require high energy and show the best development with high dietary lipid level: 18 % of dry lipid in sea bream, 25 % for *Paralichthys olivaceus*, 25 – 30 % in sea bass. However, not all lipids are useful. According to studies in different marine fish species, the specific growth rate was significantly decreased and survival was lower in dietary group with 26 % or mainly lipids of neutral nature, whereas high growth and survival were observed in diet with 14 % neutral lipid and 12 % phospholipid. In addition, phospholipids have a specific role in the synthesis and secretion of chylomicrons and VLDL (Very Low Density Lipoproteins) from the intestinal mucosa into the circulatory system, and therefore affect lipid absorption and transport. Thus, the fatty acid composition of the lipids as important as high level of energy (Zambonino-Infante and Cahu, 2010).

Highly unsaturated fatty acids (HUFA), namely eicosapentaenoic acid (EPA) and docohexaenoic acid (DHA), are performed a key role in marine fish larval development and survival. Cellular membrane of marine fish consists from phospholipids that can be synthesized

only from EPA and DHA, explaining why these fatty acids so important constituents. Optimal level of total EPA and DHA brought as phospholipids around 3 % of dry weight of larvae diet. According to studies, when EPA+DHA level exceed or reduce this optimal value, it cause different skeletal and other deformities during larval development. As Zambonino-Infante and Cahu (2010) report, HUFA modulate the transcription of genes involved in their metabolism, through their nuclear receptors, the PPARs (Peroxisome Proliferator Activated Receptors). These receptors form heterodimers with retinoid X receptors to regulate the expression of more than 500 genes, involved in lipid metabolism, energy balance, morphogenesis and bone synthesis (Zambonino-Infante and Cahu, 2010).

Phospholipid content in copepod nauplii can provides about 50 % of total lipid in fish larvae, compared to rotefer or artemia (34-43%) (Zambonino-Infante and Cahu, 2010; Almli, 2012).

1.4. Aims of this study

The lumpsucker or lumpfish is very effective as a biological agent against sea lice, especially for northern Atlantic salmon farms. In spite of the growing interest from the aquaculture industry, we know very little about the dietary requirements of lumpsucker, especially during larval and juvenile stages.

The main aims of this study were to follow growth in lumpsucker groups fed different diets and compare the effect of different diets in whole gut transcriptomes to better understand the transcription profiles of genes involved in digestive system of *C. lumpus*.

The specific objectives were:

1. Increase the molecular data available for lumpsucker;

2. Characterize the gut transcriptome;

3. Determine how diet influences expression of key genes in gut.

2. MATERIALS AND METHODS

2.1. Fish stock and rearing conditions

The present study was carried out at Mørkvedbukta (Marine Research Station) and Campus of University of Nordland, Bodø, Norway. Larvae (*C. lumpus*) were obtained from hatching at Artic Cleaner Fish AS, Lofoten, Norway. The larvae (2 dph) were transported to Mørkvedbukta (Hall 6) on 30 May 2014. There were in total 12 cylindrical black plastic tanks with black bottoms (80 L) and stocking density ca. 4000 larvae per tank randomly distributed. Each tank was equipped with special automatic feeders and feeding tubes (1.5-2 m length) (Fig. 5).

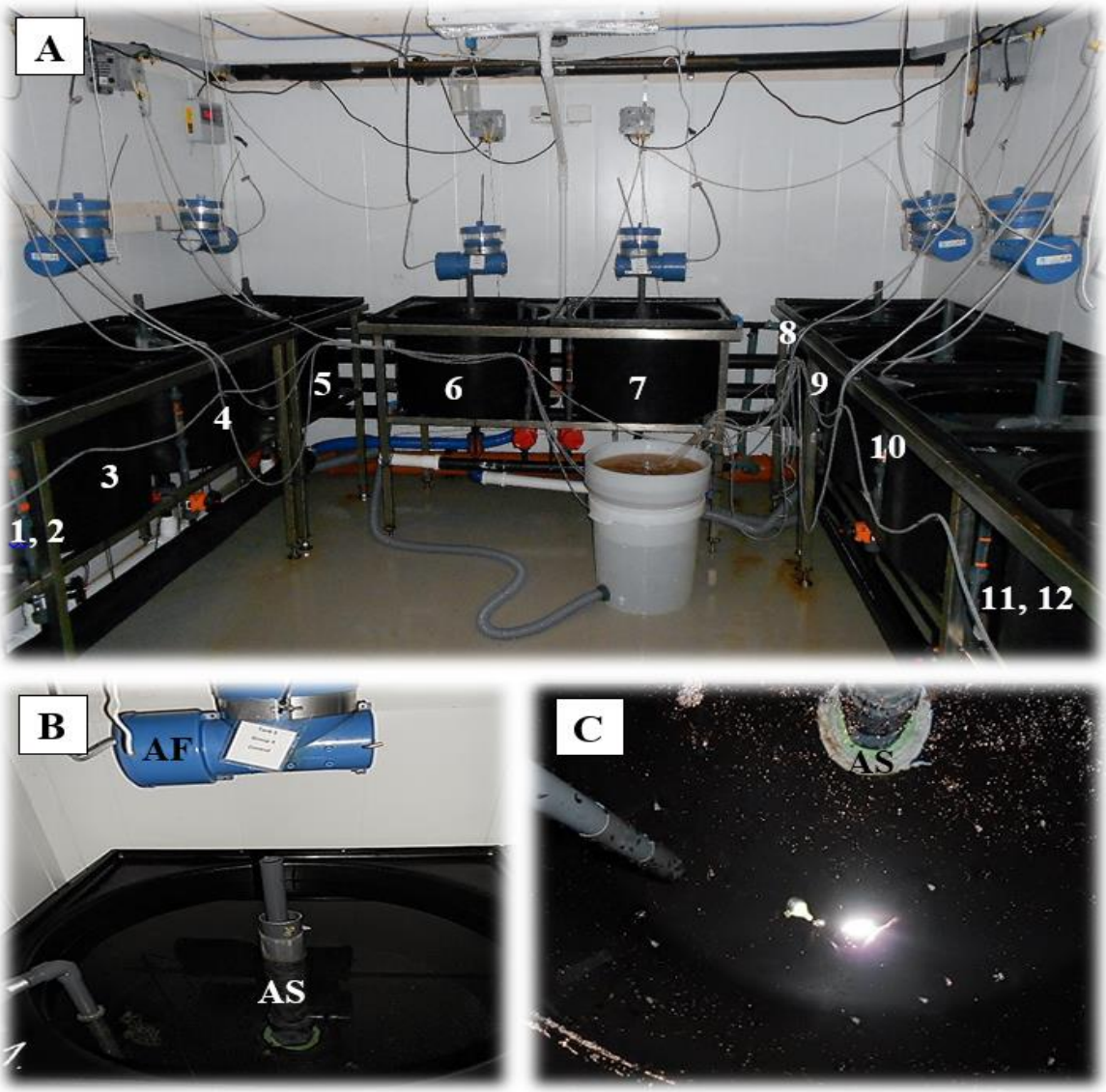

Figure 5. Experimental design. Photo A: general view; the 12 experimental unit with individually automatic feeder and feeding tube. Photo B: automatic feeder (AF) was marked with treatment information (number of tank, number of feeding group, name of feeding group). Photo B, C: aeration system (AS) and larvae distribution

The rearing conditions and sampling treatments were identical in all experimental units. Larvae and juveniles were reared at continuous a temperature controlled room with white limited light (one fluorescent lamp, LD 24:0) throughout the experimental period. Initial seawater temperature was 7.5 ^{o}C and was gradually increased to 9 ^{o}C and to 10 ^{o}C after 3 and 5 days, respectively. Salinity was 33 – 34 ‰, oxygen level approximately 9.8 mg l^{-1}.

Daily routines included monitoring of fish behavior, cleaning and flushing excess feed and faeces in all tanks. Water temperature and oxygen saturation were measured daily with a hand-held OxyGuard® Handy Polaris (Point Four Systems Inc., Canada) in each unit.

Cleaning of fish tanks (bottom and walls) and water exchange (running seawater 2.5 l min^{-1}) were performed daily after the last feeding. Small glass-siphon was used to take out dirt from the bottoms and walls of the tanks and surface skimmer was used to clean the water surface. Tending-equipment (bucket and tub fitted with inlet water) was used to collect dead larvae. Dead larvae were counted and removed from each tank daily during the scheduled cleanings.

2.2. Experiment

2.2.1. Experimental design

The experiment was conducted at Mørkvedbukta during the period from 03 June 2014 until and including 03 September 2014.

The fish larvae were divided in four groups of different feeding regimes with triplicate tanks (see Table 1 and Fig. 6).

The four feeding diets were Diet #1, Diet #2, Diet #3 and control:

— Diet #1: artemia for one week, then mixture of preserved feed and artemia for one week, then only preserved feed (Preserved feed);

— Diet #2: artemia for one week, then mixture of artemia and planktonic feed for one week, then only planktonic feed (Planktonic feed);

— Diet #3: artemia for one week, then mixture of artemia and dry commercial feed for one week, then only dry commercial feed (Dry feed);

— Control: only dry commercial feed.

Figure 6. General distribution of tanks with different types of diets

Table 1. Feeding regime layout

Weeks from starting of the experiment	1	2	3	4	5	6	7	8	9	10
DPH	5-11	12-18	19-25	26-32	33-39	40-46	47-53	54-60	61-67	67-73
Temperature (°C)	10.0	10.1	10.6	10.3	10.5	10.5	10.5	10.5	10.4	10.6
Oxygen (mg l⁻)	9.8	9.4	9.8	9.8	9.9	9.4	9.2	9.5	9.4	9.2
Photoperiod	24LD									
Feeding frequency	8 times day ¹									
Mortality	x	x	x	x	x	x	x	x	x	x
Diet #1	*Artemia* (enriched with Ori-Green)		*Preserved feed*							
Diet #2	*Artemia* (enriched with Ori-Green)		*Planktonic feed*							
Diet #3	*Artemia* (enriched with Ori-Green)		*Dry commercial feed*							
Control	*Dry commercial feed*									
Sampling	x	x	x	x	x		x			x

Thus, during first week all groups of larvae had the same feeding regime and were fed exclusively on *Artemia sp. nauplii* enriched with Ori-Green (Skretting, USA). From second week there were a co-feeding period with *Artemia sp. nauplii* cultivated on Ori-Green enrichment and formulated diet accordingly to number of group. From the third to end of experiment the larvae were fed only formulated feed: preserved feed- Diet #1; planktonic feed- Diet #2 and dry commercial feed- Diet #3. Larvae of control group were fed only dry commercial feed during all experiment.

Larvae were fed 8 times per day with different diets accordingly to group. In the daytime (at 09:00, 12:00, 15:00) and at evening (at 20:00) manually.

2.2.2. Fish feed

Artemia production

There were used two 150 L conical tanks (for hatching and feeding of artemia), which were equipped with heaters, air- and oxygen-stones, filters and lights.

There were measured up 6g of 120 g of artemia-cysts, and putted them into the hatching tank (60 L of seawater). Ca. 22 g of enrichment (Ori-Green, Skretting, USA), and putted it into 1 L of water in the mixer. Mixed for 2 min on medium speed and putted into enrichment tank (45 L of seawater). For more detailed description of *Artemia* production see Appendix 1.

The cysts hatch into *nauplii* about 12 to 24 hrs, depending on the strain of artemia, quality of cysts and water temperature. The optimum environmental condition required for proper hatching are: temperature - 27 to 30 °C, salinity - 25 to 30 ‰, pH-7.5 to 8.5, light intensity-1000 lux and dissolved oxygen - up to saturation point (Das et al., 2012).

Dry commercial feed (GEMMA Micro, Skretting, USA)

Approximately 2 g of dry feed was added in each tank of the Diet #3 and Control with gently spreading it around of water surface in tank.

Preserved feed (raw material and copepods Acartia tonsa)

Approximately 0.5 g of preserved feed was weighed in the small beaker and added to each tank of the Diet #1.

Planktonic feed (copepods Acartia tonsa)

There was measured up 6 g of planktonic feed into a measuring-jug and filled it up with 600 ml of seawater. Stirred it until the feed was dissolved in the water, and then gave each tank of the Diet #2 about 200 ml of this.

2.3. Sampling procedures

Samples of larvae were taken on the day of first feeding (D0). In the subsequent month, sampling was conducted on a weekly basis (D7, D14, D21 and D28), one sampling point after two weeks thereafter (D42), one sampling point after three weeks (D56) and one sampling point after four weeks (D84). Sampling for morphometric and transcriptomic analyses were conducted on the same days for all four experimental groups.

2.3.1. Sampling for transcriptomic analyses

During each sampling day, two pool of three larvae were sampled from every tank (N=12). Larvae were caught at random, using a plastic 3ml pipette. Each plastic pipette used has the tip cut off in order to accommodate the largest larvae without damaging them. Net and forceps were used for juveniles. Each larvae pool was anesthetized in glass beaker containing a MS 222 solution (Tricaine Methane Sulfonate; 300 mg/L). The dead larvae were transferred onto a lint-free tissue using a plastic 3ml pipette with a cut off tip, and then transferred to a 2 ml screw-cap tube, using a pair of tweezers. Care was taken not to damage the larvae in the process. Tubes were sealed well and submerged in liquid nitrogen for 30 sec. After submerging, the tube was wiped dry and adhesive stickers with information is needed attached. Each tube was labeled outside with day of sampling, experimental group, tank number and pool (A or B). In addition, a piece of paper with replicate of all necessary information was written in pencil was included in each tube for extra check. To prevent defrosting, labels were attached as quickly as possible, without touching the bottom of the tube too much. During sampling procedure, tubes were stored in dry ice. Juveniles of D42, D56 and D84 were wrapped in an aluminum foil and instantly frozen using liquid nitrogen.

During sampling procedure, they were kept on dry ice in pre labeled plastic bags. To prevent mixing tubes/pools, each larval/juvenile pool was processed individually. Sampling of the next pool was commenced after the previous tube was stored on dry ice.

After sampling was completed, tubes/bags were stored at -80°C until the RNA extraction.

2.3.2. Sampling for morphometric analyses

During each sampling day, one pool of five larvae were sampled from every tank (N=12). Larvae were caught at random, using a plastic 3ml pipette. Each plastic pipette used has the tip cut off in order to accommodate the largest larvae without damaging them. Net and forceps were used for juveniles. Each larvae pool was anesthetized in glass beaker containing a MS 222 solution (Tricaine Methane Sulfonate; 300 mg/L). The dead larvae were transferred to a 50 ml screw-cap pre-labeled tube, using plastic 3ml pipette with a cut off tip and a pair of tweezers. Excess solution was carefully removed with a pipette. Care was taken not to damage the larvae in the process. All samples were fixed in 4% PFA in PBS (Paraformaldehyde solution in Phosphate-Buffered Saline). Fixing of samples was taken place in a fume hood, using all necessary security measures. Each tube was labeled outside with species, solution, day of sampling, experimental group, tank number and number of fish.

After sampling was completed, tubes were stored at 4°C until the measuring.

2.4. Estimated parameters

Mortality

The number of dead larvae was counted during all experiment. The mortality at a particular sampling day was expressed in percentage of the number of stocked fish using the following formula:

$$\text{Mortality rate [\%]} = \frac{\text{initial number of larvae in each tank} - \text{number of larvae survived to the next sampling day}}{\text{initial number of larvae in each tank}} \times 100$$

Morphometric measures of larvae

Standard length (SL) and weight of fixed larvae were examined to estimate growth rate.

SL was measured in mm along the midline of the body from the tip of the snout to the end of the notochord. Every five larvae from the same feeding group were taken out of the tube with 4 % PFA in PBS solution and dried a little on a tissue. Then they were rinsed in a Petri dish filled with 4 % PFA in PBS for a couple of minutes. 4 % PFA in PBS solution was changed between feed groups. After it larvae were dried again on a clean tissue and measured. After measuring of one pool was completed, larvae were putted back in the original tubes. SL analysis was conducted using the graph paper (also known as grid paper) with squares of 10 x 10 mm sizes. Measuring of samples was carried out in a fume hood, using all necessary security measures. Weight was measured in g using scales with 0.0001 g accuracy, which were closely spaced to the fume hood.

2.5. RNA extraction

RNA extraction was performed on gut of juvenile fish from 84th day for Diet #1 (preserved feed) and control group. Total RNA was individually extracted from each sample.

For RNA extraction, the guts were dissected out carefully avoiding contamination and washed in sterile PBS (Sigma, Missouri, USA). Then, they were transferred to Petri dish (with aluminum foil on bottom) that was placed on top of dry ice and were cut into the required size (no more 0.1 g tissue).

Total RNA was extracted using the *mir*Vana™ miRNA Isolation Kit (Life technologies, California, USA, 2011); sections *II.E. Organic Extraction* on page 11 and *II.F.I. Total RNA Isolation Procedure* on pages 12-13 were used with some changes. The kit employs an organic extraction followed by immobilization of RNA on glass-fibers to purify total RNA from tissue samples.

The work area and other materials are needed for RNA extraction were disinfected with RNase*Zap* solution (Sigma, Missouri, USA) to avoid contamination. The tissue samples stored at -80°C were taken out and placed on dry ice to prevent thawing of samples and degradation of RNA.

The organic extraction was performed in three steps. At first, 700 µl of tissue samples were homogenized using 70 µl of miRNA Homogenate Additive in 2 ml microtubes, containing approximately 100 – 200 mg zirconium beads (1.4 mm). Samples were handled on ice for 10 min while processing to avoid degradation of RNA and centrifuged after it at 12,000 rpm at 4°C for 1 min. At the second step, the resultant homogenate without any tissue particles was then transferred into a 2 ml Eppendorf tube, containing 700 µl of Acid-Phenol: Chloroform, to remove most of the

other cellular components, leaving a semi-pure RNA sample. The resultant mixture was then vortexed for 1 – 2 min and centrifuged at 12,000 rpm at 4 °C for 10 min. This led to the formation of three distinct phases with the uppermost clear aqueous phase comprising of RNA. The interphase was compact. At the third step, the aqueous (upper) phase was carefully removed without disturbing the lower phase and transferred to a fresh 1.5 ml Eppendorf tube and centrifuged at 12,000 rpm at 4°C for 1 – 2 min to be sure that interphase was not touched.

For total RNA isolation procedure, 712 µl of room temperature 100 % ethanol was added to the resultant upper aqueous phase. This lysate/ethanol mixture was then pipetted into 1.5 ml Eppendorf tube with filter cartridge and centrifuged at 10,000 rpm at 4 °C for 15 sec to allow the passing of mixture through a filter cartridge without destruction of the filter. The second centrifugation of 10 – 15 sec was also carried out to remove the remaining alcohol from the tubes. After three washing steps with miRNA Wash Solution 1 and Wash Solution 2/3 (provided with the kit), the filter cartridge was transferred into 1.5 final collection tube without cup. The pre-heated (95°C) nuclear-free water (50 µl) was used for the dissolution of the RNA.

The dissolved RNA was stored in 1.5 ml Eppendorf tubes at -80°C for further quantity and quality evaluation.

2.6. RNA quantity and quality measurements

2.6.1. RNA quantity measurement

The quantity of RNA extracted from the samples was measured using Quant-iT™ RNA Broad Range Assay Kit and Qubit® fluorometer (Invitrogen, California, USA).

The kit reagents comprised of a Quant-iT™ RNA Broad Range buffer, Quant-iT™ Broad Range reagent, Quant-iT™ RNA Broad Range standard-1 (0 ng/µl) and Quant-iT™ Broad Range standard-2 (100 ng/µl).). The kit components are stored at room temperature except for the standards.

To prepare a working solution the Quant-iT™ RNA broad range reagent was mixed with Quant-iT™ RNA broad range buffer at a ratio of 1:200 respectively. The resultant mixture was used further for the quantification of the RNA. One µl of the total RNA was mixed with 199 µl of working solution followed by vortexing for 3 sec. After it the samples were allowed to stand at room temperature for at least 2 min. The two standard solutions were prepared by mixing 10 µl of standard-1 and standard-2 separately with 190 µl of working solution of RNA reagent, followed by vortexing for 3 sec and incubation for 2 min.

The Qubit® fluorometer was calibrated using the Standard-1 and Standard-2 with following measurement of the RNA concentration in the presented samples. The concentrations were recorded in ng/µl.

2.6.2. RNA quality evaluation

Approximately 0.6 g agarose was weighed, transferred to a conical flask and dissolved in 65 ml of 0.5 x TBE buffer by heating in a microwave oven for 2 – 3 min (until the complete dissolution of all the agarose particles) to obtain a clear solution. When the mixture was cooled down to 60 degrees, 2 µl of Sybr Safe (Invitrogen, California, USA) was added and allowed to polymerise on the gel-casting tray in room temperature for 10 – 20 minutes. The 1 µl dissolved RNA was made up to 10 µl using RNAse free water and subjected to heat denaturation at 65°C for 3 min. The resultant RNA was then mixed with 2 µl of 6 x DNA Loading Dye (Invitrogen, California, USA) and loaded into wells of the agarose gel, which was run at 100 V for 30 min. After 30 min the gel was analyzed under UV light using Kodak™ Gel Logic 200 Imaging system (Eastman Kodak Company, USA).

According to results of the gel electrophoresis, there was the presence of two clear bands, corresponding to 28S and 18S rRNA, that was considered as the indication of good quality RNA (Appendix 2).

2.7. cDNA library preparation

Double-stranded cDNA libraries were constructed using the TruSeq® RNA Sample Preparation Kit v2 (Illumina, USA), following the manufacturer's instruction, with some changes.

During Purification and Fragmentation process the polyA, containing RNA molecules, was purified with using oligo-dT attached magnetic beads, using two rounds of purification; during the second elution of the polyA RNA, the RNA was also fragmented and primed for cDNA synthesis. For this the total RNA was diluted to a final volume of 50 µl using RNase-free water and three procedures were carried out:

— RNA Denaturation (65 °C for 5 min, 4 °C hold) to denature the total RNA and facilitate binding of the polyA RNA to the beads;

— RNA Elution 1 (80 °C for 2 min, 25 °C hold) to elute the total RNA from the beads;

— RNA Elution 2- Fag- Prime (80 °C for 2 min, without hold) to elute, fragment and prime the RNA.

The following components were used:

Table 2. Reaction mixture for the Purification and Fragmentation process

Component	Amount
Bead Binding Buffer	50 µl
Bead Washing Buffer	200 µl
Elution Buffer	50 µl
Elute, Prime, Fragment Mix	19.5 µl

During First Strand cDNA synthesis the cleaved RNA fragments primed with random hexamers were reverse transcribed into first strand cDNA, using reverse transcriptase and random primers. The first strand cDNA was synthesized from 17 µl of the supernatant (fragment and primed total RNA) with adding the 8 µl reverse transcription master mix. The reverse transcription master mix was prepared by mixing 1 µl SuperScript II Reverse Transcriptase with the 9.5 µl First Strand Master Mix. A final volume of 25 µl cDNA was obtained and incubated according the 1st Strand program.

The Synthesis Second Strand cDNA was performed using 25 µl Second Strand Master Mix, 90 µl AMPure XP beads were used to separate the ds cDNA from the Second Strand Reaction Mix, and 53 µl Resuspension Buffer. A final volume of 50 µl ds cDNA was obtained and then stored at -20°C.

For Performing End Repair procedure the 10 µl diluted End Repair Control was added to 50 µl ds cDNA. Diluted End Repair Control was prepared by mixing 99 µl Resuspension Buffer and 1 µl End Repair Control. The 40 µl End Repair Mix was added to convert of the overhangs resulting from fragmentation into blunt ends. After incubation and cleaning the 15 µl of supernatant was stored at -20°C before the next step.

For Adenilation 3’ Ends the 2.5 µl diluted A-Tailing Control to the 15 µl of supernatant. Diluted A-Tailing Control was prepared by mixing 99 µl Resuspension Buffer and 1 µl A-Tailing Control. The 12.5 µl A-Tailing Mix was used to prevent 3’ ends of the blunt fragments from

ligating to one another during the adapter ligation reaction. The final mixure was incubated according the ATAIL70 program.

During Ligation Adapters stage, multiple indexing adapters was ligated to the ends of the ds cDNA, prepared them for hybridization onto a flow cell. The following components were used:

Table 3. Reaction mixture for the Ligation Adapters process

Component	Amount
Ligation Mix	2.5 µl
Ligation Control	1 µl
Resuspension Buffer	175 µl
RNA Adapter Index	2.5 µl
Stop Ligation Buffer	5 µl
AMPure XP beads	92 µl per sample
Freshly prepared 80% ethanol	800 µl per sample

The final supernatant volume was 20 µl.

PCR was used to selectively enrich DNA fragments that had adapter molecules on both ends and to amplify the amount of DNA in the library. PCR conditions were as follows:

Table 4. Reaction mixture for PCR

Component	Amount
PCR Master Mix	25 µl
PCR Primer Cocktail	5 µl
Resuspension Buffer	32.5 µl
AMPure XP beads	50 µl per sample
Freshly prepared 80% ethanol	400 µl per sample

Thermocycling conditions:

— 98 ºC for 30 sec

— 15 cycles of:

- 98 ºC for 10 sec
- 60 ºC for 30 sec
- 72 ºC for 30 sec

— 72 ºC for 5 min

— Hold at 10 ºC

Thus, the total volume of 30 µl of clear supernatant was obtained and then stored at -20 °C.

Real-time PCR was performed to quantify the resulting libraries according to the Illumina Sequencing Library qPCR Quantification Guide (Part # 11322363) (Illumina, USA). The titration curve of serial 800-fold dilution (1:0800, 1:16000, 1:32000) was prepared for each template cDNA (6 in total) and run on plates. Libraries for quantification were diluted to approximately 4 nM in Qiagen EB Buffer. The following reaction mix was prepared for each library:

Table 5. Reaction mixture for the library preparation

Component	Amount/well
Illumina library (approx 10 nM)	10 µl
KAPA SYBR FAST Master Mix Universal (2x)	15 µl
qPCR Primer 1.1	0.2 µl
qPCR Primer 2.1	0.2 µl
Nuclease-free Water	7.6 µl

Thermocycling conditions:

— 95 ºC for 10 min

— 40 cycles of:

- 95 ºC for 10 sec
- 60 ºC for 30 sec

Quality and purity of the libraries were measured using a Bioanalyzer 2100 (Agilent Technologies). One µl of resuspended construct was loaded on Bioanalyzer 2100, using a DNA-specific chip such as the Agilent DNA 1000 and, thus, the size and purity of sample were checked.

Samples were multiplexed (three samples per lane) and sequenced by the MiSeq® (Illumina, USA) platform using sequenced runs of 2 × 300 paired-end reads.

All sequencing data was transferred to BaseSpace® (Illumina, USA) cloud platform for automatic analysis and storage, with the option of retaining data for local hosting.

2.8. Bioinformatics

The raw data for each pool of samples were separately trimmed with the CLC Genomics Workbench software (Version 8.0, CLC Bio, Denmark) to remove ambiguous reads, low quality (ambiguous limit: 2, quality limit: 0.03) and short reads (50 bp). The combined high quality reads (pools/samples) were assembled in a unique file using the CLC Genomics Workbench software.

The overlap settings for *de novo* assembly were a mismatch cost of 2, an insertion cost of 3, a deletion cost of 3, a minimum contig length of 200 bp, a length fraction of 0.5, a similarity of 0.8 and a trimming quality score of 0.05.

RNA-Seq was performed using the CLC Genomics Workbench software with following parametres: a minimum read length fraction = 0.8, minimum read similarity fraction = 0.8, and unspecific read match limit = 10 in relation to the reference values.

The sequencing reads of selected contigs were converted into FASTA format by CLC Genomics Workbench and were annotated using Blastx against the NCBI non-redundant protein sequences (NR) database (ncbi.nlm.nih.gov).

Figures and tables were made using Microsoft Office Excel 2013. Morphological data were expressed as means ±SD.

3. RESULTS

General Observations

Experiment showed that during the first week of lumpfish exogenous feeding, larval growth, development and survival were better on *Artemia nauplii* than on dry commercial feed that was also observed by other researches (Cañavate and Fernández-Díaz, 1999; Drossou et al., 2006; Demeny et al., 2012). However, results of analysis also revealed an interesting feature: during the experimental period was found that larvae fed planktonic feed from their natural habitat had lowest developmental and survival rates, compared with other feeding groups. These observations were significantly different from that usually described for most marine fish larvae, where results were the opposite (Luizi et al., 1999; Shields et al., 1999; Rajkumar and Kumaraguru vasagam, 2006; Imsland et al., 2006; Kortner et al., 2011; Almli, 2012; Das et al., 2012).

3.1. Analysis of morphometric characteristics

The larval-early juvenile period for most marine fish is affected by feeding diet (Luizi et al., 1999; Drossou et al., 2006; Imsland et al., 2006; Leaver et al., 2008; RCN, 2009; Olivotto et al., 2010; Kortner et al., 2011; Almli, 2012; Demeny et al., 2012; Piccinetti et al., 2013; Pradhan et al., 2014). Therefore, we used morphometric characteristics to determine if there was effects of feed on larvae and early juveniles of *C. lumpus*.

3.1.1. Standard length

To D7 larvae fed with *Artemia nauplii* (Diet #1, Diet #2 and Diet #3) grew to 0.75±0.005 mm (Diet #1), 0.76±0.008 mm (Diet #2) and 0.77±0.006 mm (Diet #3) standard length (SL) in comparison to D0 before experimental period (0.69 mm), while larvae fed with dry commercial feed (Control) grew to 0.7±0.004 mm (see Fig. 7). Thus, Diet #1, Diet #2 and Diet #3 had a slight advantage over Control group.

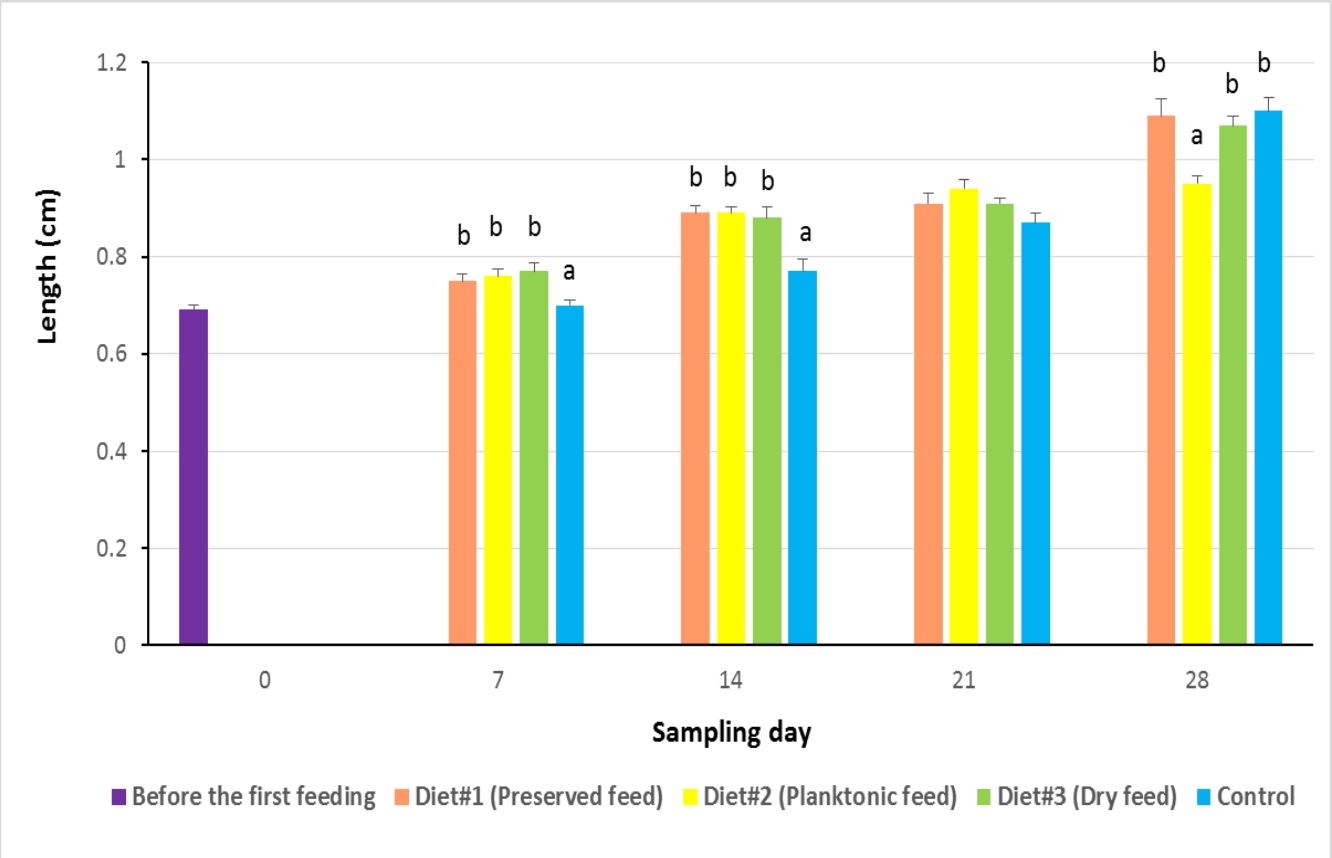

Figure 7. Average length (SL) of *C. lumpus* (larvae and early juveniles) at 0-28th days for all experimental groups. Error bars ± SE. Different letters above bar diagram indicate statistically significant differences between the feeding groups

On D21 there was started shifting to formulated diets: preserved feed (Diet #1), planktonic feed (Diet #2) and dry commercial feed (Diet #3). Among the groups, which are fed artemia before D21, Diet #2 was a bit longer (0.94±0.004 mm), than Diet #1 (0.91±0.013 mm) and Diet #3 (0.91±0.005 mm). Control group had the smallest size: 0.87±0.01 mm. However, from D28 to D56 Diet #2 showed the significantly slow growth, compared with other groups, and eventually died to the D84 (Fig. 7 and Fig. 8).

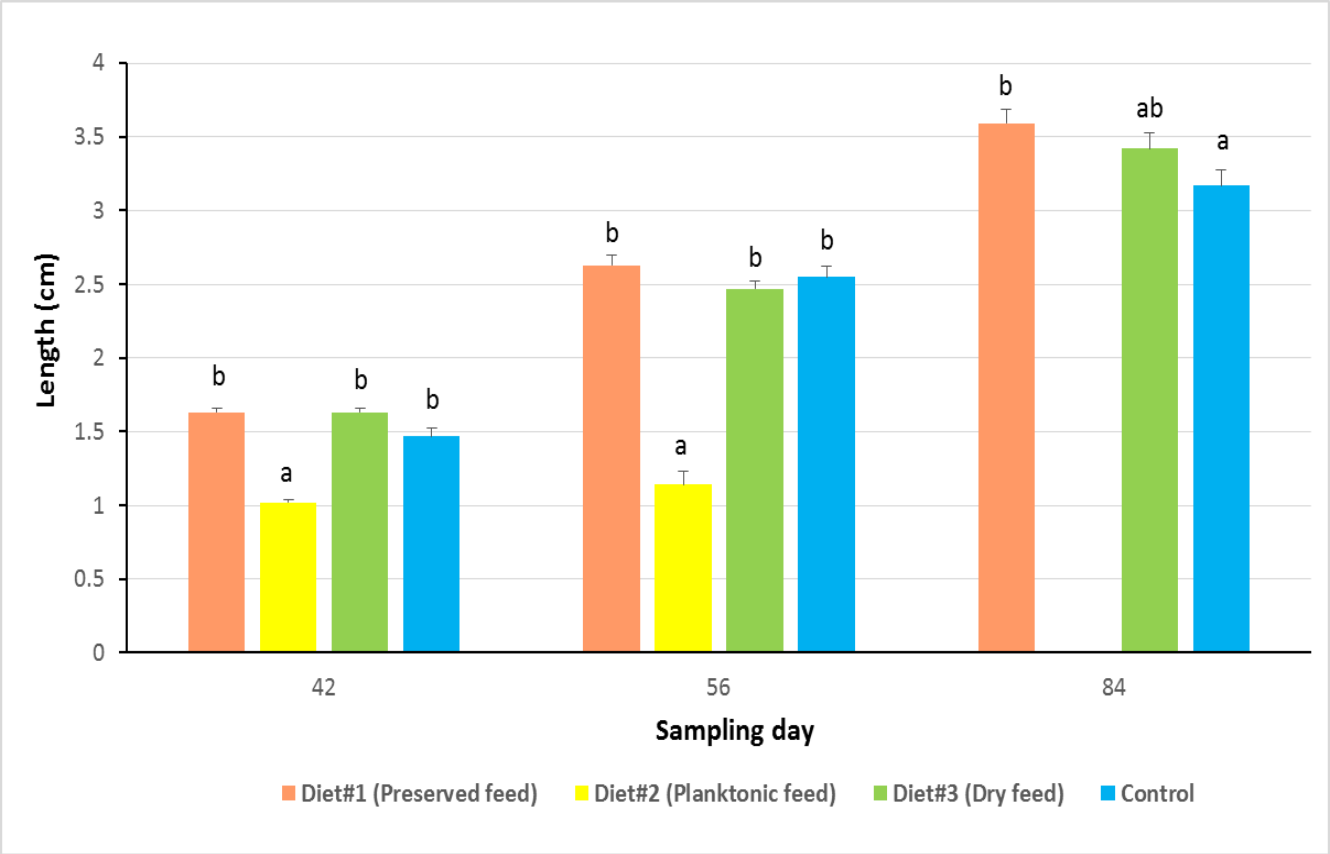

Figure 8. Average length (SL) of *C. lumpus* (larvae and early juveniles) at 42th-84th for all experimental groups. Error bars ± SE. Different letters above bar diagram indicate statistically significant differences between the feeding groups

At the last sampling day, D84, the biggest differences were found between Diet #1 (Preserved feed) and Control: 3.59±0.09 mm and 3.17±0.11 mm, respectively.

3.1.2. Weight

To D7 larvae fed with *Artemia nauplii* grew to 0.007± 0.0002 g (Diet #1), 0.0066±0.0001g (Diet #2) and 0.007 g (Diet #3) weight in comparison to D0 before experimental period (0.0036±0.0001 g), while larvae fed with dry commercial feed (Control) grew to 0.0055± 0.0002g (Fig. 9).

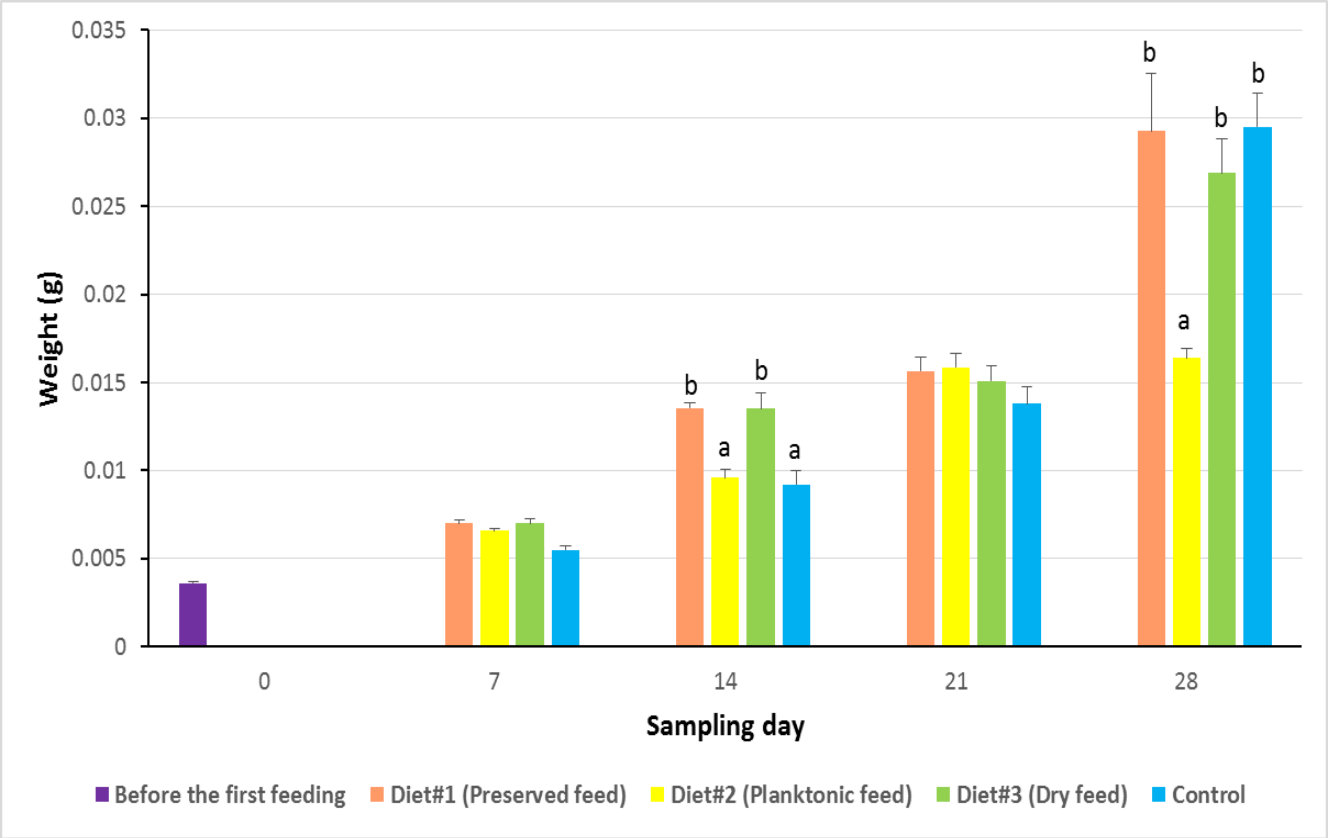

Figure 9. Average weight of *C. lumpus* (larvae and early juveniles) at 0-28th days for all experimental groups. Error bars ± SE. Different letters above bar diagram indicate statistically significant differences between the feeding groups

On D14 among the group fed artemia in beginning of experiment, Diet #2 (Planktonic feed) had the significantly slow growth (0.0096±0.0001 g), than Diet #1 (Preserved feed, 0.0135±0.0002 g) and Diet #3 (Dry feed, 0.0135±0.0003 g), but was a bit bigger than Control group (0.0092±0.0002 g).

However, from D28 to D56 Diet #2 showed the smallest size, compared with other groups, and eventually died to the D84 (Fig. 9 and Fig. 10).

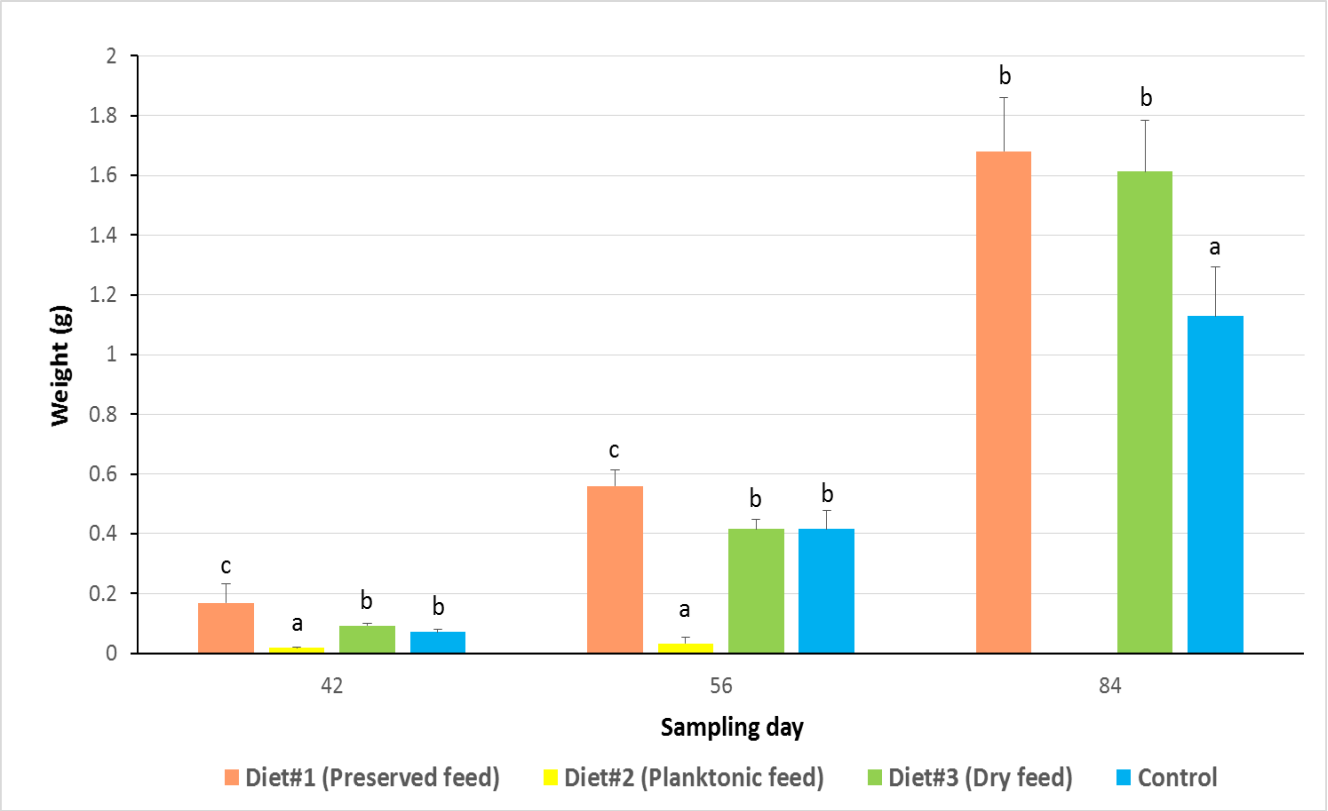

Figure 10. Average weight of *C. lumpus* (larvae and early juveniles) at 42th-84th for all experimental groups. Error bars ± SE. Different letters above bar diagram indicate statistically significant differences between the feeding groups

At the last sampling day, D84, the significant differences were found between Diet #1 (preserved feed) and Control: 1.6807±0.1791 g and 1.1280±0.1669 g, respectively.

3.2. Mortality

Dead larvae were counted from each tank daily and mortality was expressed in percentage of the number of stocked fish in each feeding group. Mortality rates were monitored from first until tenth week of the experiment for all feeding group (see Appendix 3 for this data).

The results demonstrated that low mortality rates were registered in all experimental groups during the first week (Fig. 11).

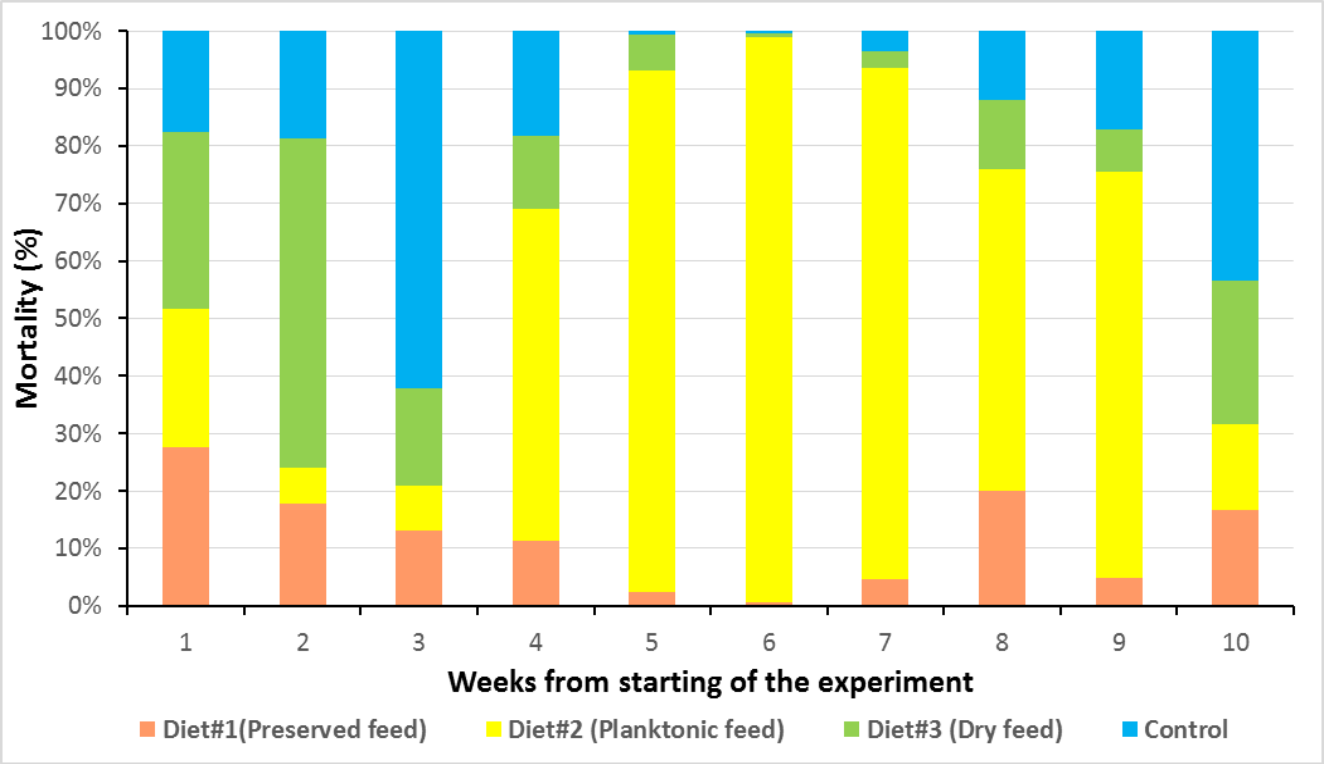

Figure 11. Mortality rates of *C. lumpus* (larvae and early juveniles) by experimental diets (feeding groups) during the 1st-10th weeks from starting of the experiment

After shifting larvae on formulated diets (3th week) control group fed only dry commercial feed had the highest mortality rates, compared with all the other diet groups. Diet #2 fed only planktonic feed started to die at a higher rate from the 4 – 9th week of the experiment.

In contrary, the mortality of the rest feeding groups was high only during the third week and showed in general an opposite results with a lower mortality rate from 4th until and including 10th week.

Total mortality rate was lower for larvae fed with *Artemia nauplii* during the first week and then formulated diets (Diet #1 – 3.4 %, Diet #3 – 5.3 %), than for Control group (6.4 %) fed only dry commercial feed during all experiment. However, mortality of larvae was not significantly high in these groups in contrast with Diet #2 (51.7 %).

3.3. Transcriptomic analysis

Preserved feed (Diet#1) and control group were chosen for this analysis due to significant differences between them compared with other groups, according to results of morphometric analysis and mortality.

3.3.1. Sequence assembly

The final sequence assembly results using CLC Genomics Workbench software was 31,632 contigs with a minimum length of 500 bp, a maximum length of 16,343 bp, and an average length of 1,612 bp with GC-content of 48.1 % and an N75 of 1,165 bp, N50 of 2,114 bp and N25 of 3,594 bp (see Appendix 4 for each library data).

3.3.2. RNA-Seq expression analysis

RNA-Seq analysis was conducted using CLC Genomics Workbench by mapping each sequencing read against the final sequence assembly and counting the mapped reads to generate expression values. The expression level of each transcript was quantified in reads per kilobase of the transcript per million mapped reads (RPKM).

3.3.2.1. Distributions of expression values

To check the genes similarly under the conditions considered and avaluate an overall normalazed expression level in samples, a box plot was created for a visual presentation of the distributions of normalized expression values in samples (Fig. 12).

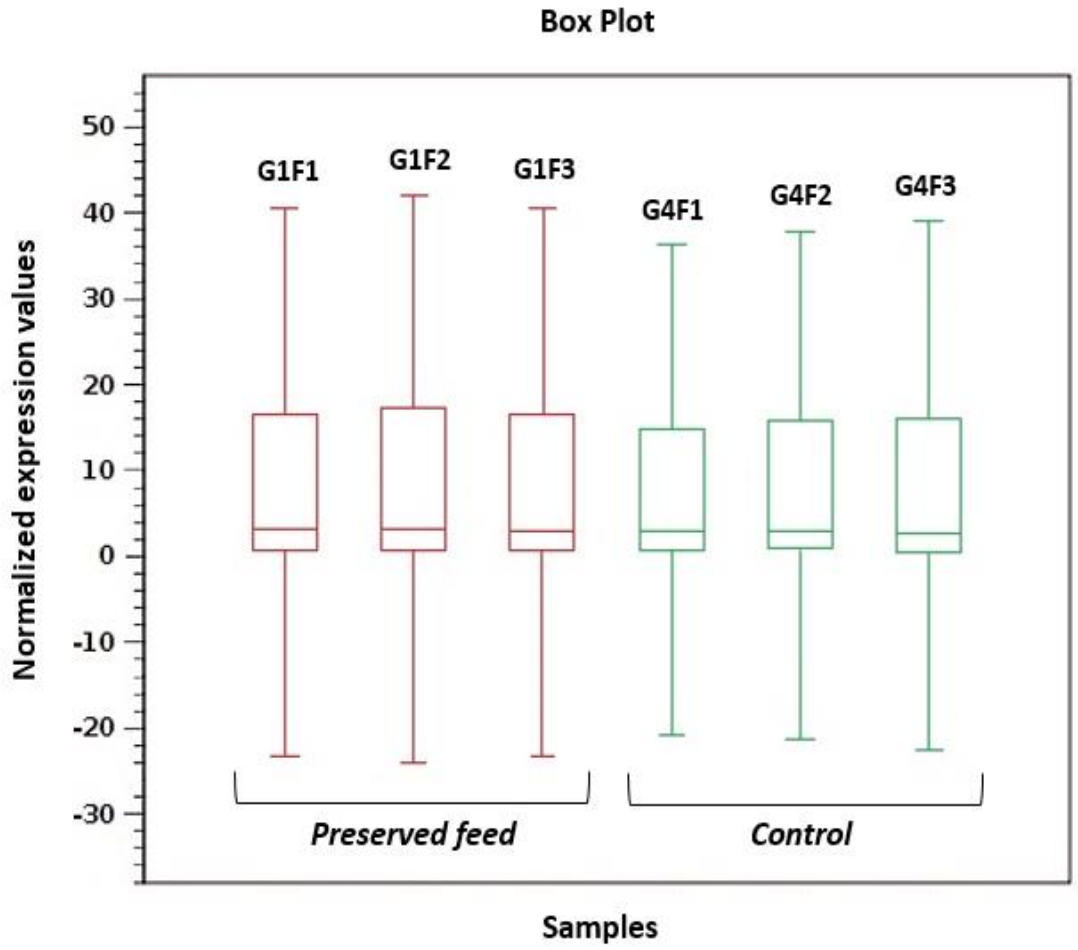

Figure 12. A box plot of six samples in a two-group experiment (preserved feed and control) after normalization by totals and colored by group

As one can see the distribution of values in the first sample from the control group is a bit differ from those of other samples, but it not deviates from the norm due to small differences. Thus, none of the samples in both groups stands out from the rest as having distributions that are atypical: the boxes, scales and whiskers ranges are about equally sized. Therefore, different libraries have the same or at least a comparable median expression level.

3.3.2.2. Principal Component Analysis

The principal component analysis (PCA) was used for two-dimensional visualization of the samples to identify and quantify the directions of variability in the data set. Thus, PCA separated groups along both Projection 1 and Projection 2 (Fig. 13).

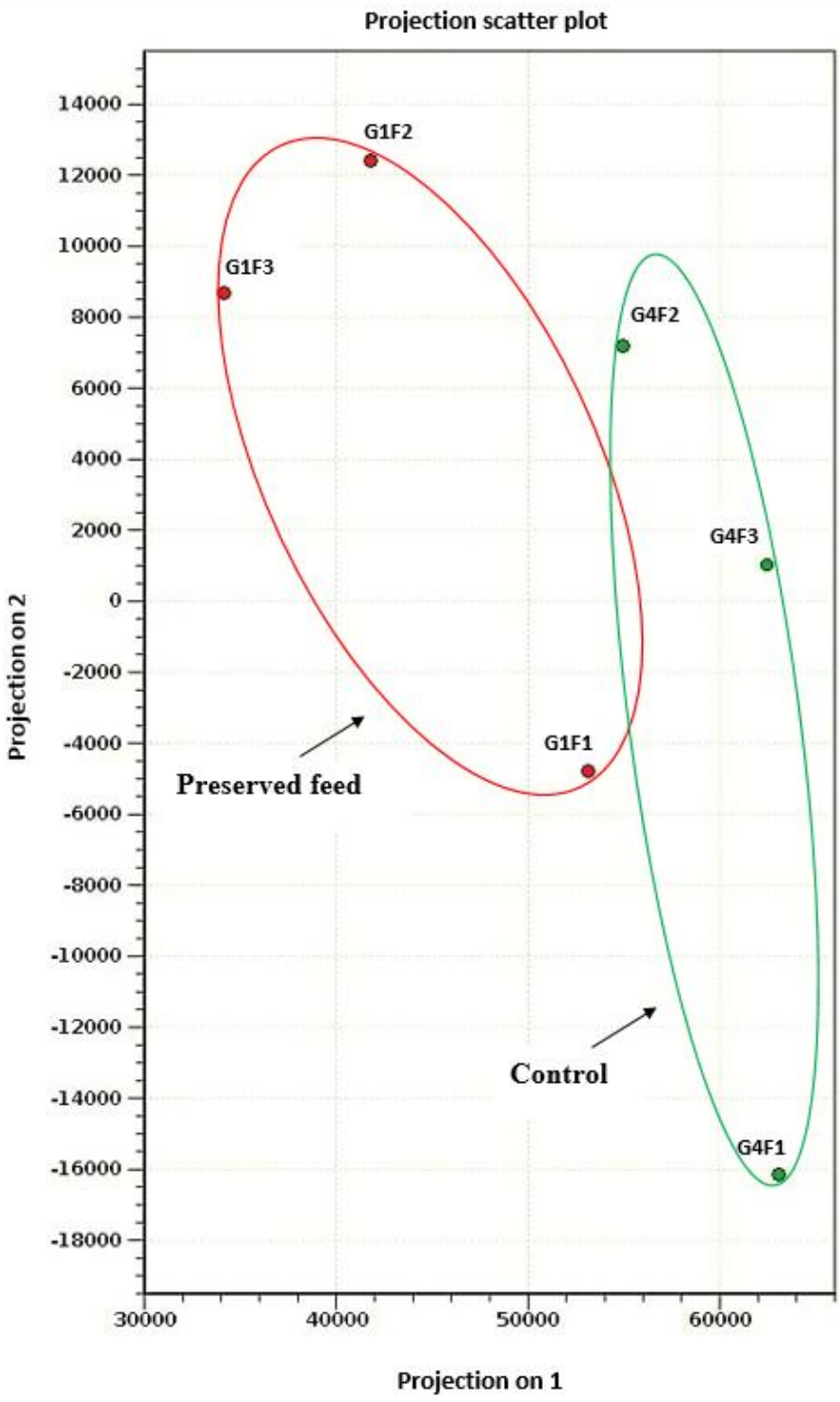

Figure 13. PCA score plot of six samples in a two-group experiment (preserved feed and control) and colored by group

As analysis showed, Control group (green dots) had a higher Projection 1 score, than the Preserved feed group (red dots). In the Projection 2 direction the Preserved feed group had a higher value, than the Control group, fed dry feed. Thus, samples are clustered according to groups with clear separation between them, meaning that genes were differently expressed.

3.3.2.3. Volcano plot

A volcano plot was performed to select the differentially expressed genes during filtering (total count filter cutoff=50, fold change abs. value >|4| and FDR p-value < 0.05) (Fig. 14).

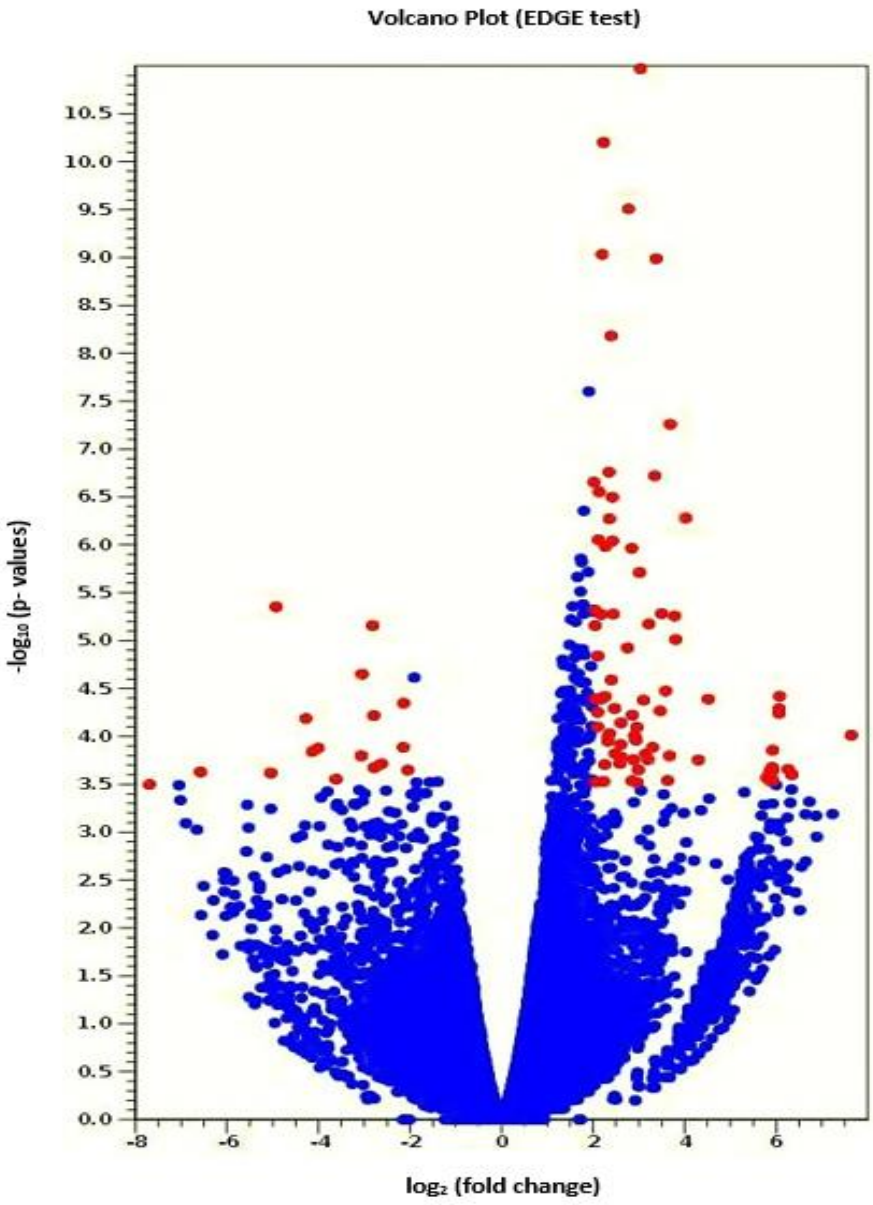

Figure 14. Volcano plot of 97 differently expressed genes found in both groups (common genes). Color dots are used to indicate up- and down-regulated genes (red and blue, respectively)

According to results of filtering, among 97 genes, which were differently expressed, 17 were found as down- regulated (negative fold changes) and 80 were up-regulated (positive fold changes). The complete list of up- and down- regulated genes in Preserved feed group and Control group are available in Appendix 5.

3.3.2.4. Hierarchical clustering of features

The contigs selected through a volcano plot were clustered according to their normalized RPKM values and grouped into a heat map (Fig. 15).

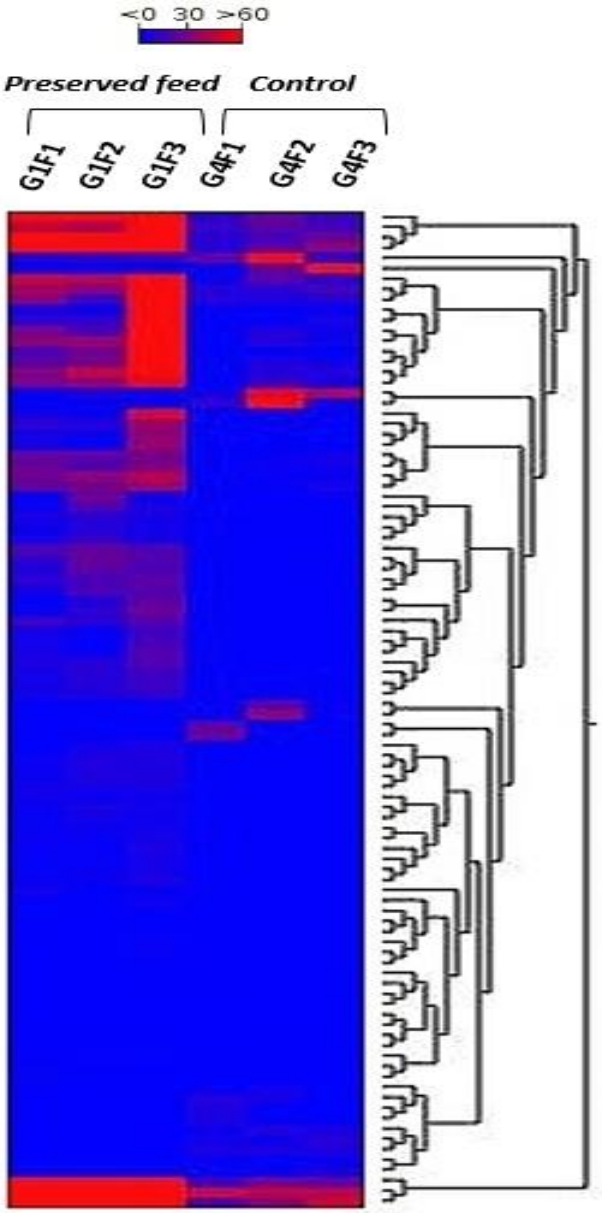

Figure 15. Heat map of differentially expressed genes found in both groups (common genes). Color code is shown to indicate up- and down-regulated genes (red and blue, respectively)

For this purpose, the Euclidian distance was estimated and a complete linkage was selected as a clustering strategy. The contig_26228 was not included in heat map due to its large expression level compared to the rest contigs. Thus, it was possible to visualize transcriptional differences between each group.

3.3.2.5. Blastx searching

All contigs of interest were annotated using sequence-based similarity search. The sequencing reads were converted into FASTA format by CLC Genomics Workbench and Blastx (search protein databases using a translated nucleotide query) was performed against the NCBI non-redundant protein sequences (NR) database (Tables 6 and 7).

According to the Blastx searching, the contig_2790, contig_4986, contig_3990 and contig_9314, contig_19385, represented down- and up-regulated genes respectively, had a failure in finding significant hits to the NR database. Similar results were observed in rainbow trout (Salem et al., 2015) and catfish (Wang et al., 2010).

The largest number of database matches were from large yellow croaker *Larimichthys crocea* (Teleostei, Sciaenidae). Other fish species in the BLASTx top-hit list were Japanese rice fish (*Oryzias latipes*), Nile tilapia (*Oreochromis niloticus*), black cod (*Notothenia coriiceps*), Amazon molly (*Poecilia formosa*), bicolor damselfish (*Stegastes partitus*), sablefish (*Anoplopoma fimbria*) and tongue sole (*Cynoglossus semilaevis*).

Table 6. Subset of down-regulated genes in preserved feed group (Diet#1) compared to control group

Feature ID	E-value	Gene name and abbreviations	Organism	Fold Change	FDR p-value
contig_23234	9E-57	Secretory phospholipase A2 receptor-like (*pla2r1*)	*Notothenia coriiceps*	-32.97	0.04
contig_20112	9E-5	Pyrrolidone-carboxylate peptidase (*pcp*)	*Streptomyces griseorubens*	-30.40	4.57E-3
contig_2790	—	No significant similarity found	—	-19.36	0.02
contig_1410	0.001	Serine/threonine-protein phosphatase PP1-beta catalytic subunit-like (*ppp1cb*)	*Poecilia formosa*	-17.53	0.03
contig_23393	2E-162	NAD(P)H dehydrogenase [quinone] 1-like (*nqo1*)	*Larimichthys crocea*	-16.09	0.03
contig_23103	0.25	N-acyl-D-amino-acid deacylase (*baci_c44860*)	*Bordetella bronchiseptica A1-7*	-12.27	0.05
contig_17416	2E-115	Alanine--glyoxylate aminotransferase 2, mitochondrial-like (*loc103368456*)	*Stegastes partitus*	-8.35	0.03
contig_9563	4E-72	Nucleoside diphosphate kinase B (*ndkb*)	*Anoplopoma fimbria*	-8.27	0.01
contig_4986	—	No significant similarity found	—	-7.04	5.12E-3
contig_19530	4E-56	T-cell surface glycoprotein CD3 zeta chain-like (*cd3e*)	*Larimichthys crocea*	-6.93	0.02
contig_14218	0	Heat shock protein HSP 90-alpha 1 (*hsp90aa1*)	*Cynoglossus semilaevis*	-6.88	0.04
contig_9329	4E-46	Saxitoxin and tetrodotoxin-binding protein 2-like (*stbp2*)	*Larimichthys crocea*	-6.17	0.04
contig_4666	0	Calpain-10 *(capn10*)	*Larimichthys crocea*	-4.42	0.03
contig_3990	—	No significant similarity found	—	-4.40	0.02
contig_15904	0	Protein phosphatase 1 regulatory subunit 3C (*ppp1r3c*)	*Larimichthys crocea*	-4.14	0.04

Table 7. Subset of up-regulated genes in preserved feed group (Diet#1) compared to control group

Feature ID	E-value	Gene name and abbreviations	Organism	Fold Change	FDR p-value
contig_19259	0	Fatty acid synthase (*fasn*)	*Larimichthys crocea*	12.85	2.19E-4
contig_10853	0	MAM and LDL-receptor class A domain-containing protein 1-like (*loc105358565*)	*Oryzias latipes*	13.73	4.60E-3
contig_18515	4E-153	RNA-directed DNA polymerase from mobile element jockey-like (*loc101169532*)	*Oryzias latipes*	13.96	7.00E-3
contig_14640	0	Metal transporter CNNM 2 isoform X1 (*cnnm2*)	*Oreochromis niloticus*	16.28	1.06E-3
contig_21674	9E-66	Acetyl-CoA carboxylase 2 isoform X2 (*acc2*)	*Larimichthys crocea*	19.69	0.04
contig_12172	1E-98	Phospholipase DDHD1-like (*ddhd1*)	*Stegastes partitus*	22.93	0.02
contig_17806	4E-154	Midasin (*mdn1*)	*Larimichthys crocea*	55.53	0.05
contig_16269	0	Histone-lysine N-methyltransferase MLL3 (*mll3*)	*Larimichthys crocea*	58.37	0.04
contig_26220	0	Centromere protein F (cenpf)	*Larimichthys crocea*	58.38	0.05
contig_18514	0	Sterile alpha motif domain-containing protein 9-like (*samd9l*)	*Larimichthys crocea*	60.17	0.04
contig_21532	0	Small subunit processome component 20 (*utp20*)	*Larimichthys crocea*	60.34	0.05
contig_22871	8.6	Hypothetical protein (*oeoe_0490*)	*Oenococcus oeni*	60.57	0.05
contig_3298	0.051	Sigma-54 modulation protein (*synpcc7942_2352*)	*Pseudomonas fluorescens BRIP34879*	60.60	0.03
contig_9314	—	No significant similarity found	—	60.89	0.04
contig_21711	0	SCO-spondin-like (*sspo*)	*Oreochromis niloticus*	66.79	0.02
contig_20949	0	E3 SUMO-protein ligase RanBP2 (*ranbp2*)	*Larimichthys crocea*	66.80	0.02
contig_19385	—	No significant similarity found	—	67.30	0.02
contig_25999	1E-07	Hypothetical protein (*lmg9581*)	*Bacillus azotoformans*	76.69	0.04
contig_20644	3E-07	Hypothetical protein (*so_4719*)	*Shewanella decolorationis*	81.12	0.04
contig_28082	3E-05	Transposase IS1016 (*n872_01860*)	*Neisseria meningitidis LNP27256*	199.67	0.03

4. DISCUSSION

4.1. Growth and survival

Information concerning the effects of nutrition on development and survival of larvae and early juvenile of lumpsucker is limited. However, it has been suggested that during early developmental stages in lumpsucker larvae, the growth performance could be regulated by optimal diet and *Artemia nauplii* are desired to best growth, development and survival during weaning process (Cañavate and Fernández-Díaz, 1999; Drossou et al., 2006; Demeny et al., 2012).

To test our suggestion, we used *Artemia nauplii* as initial feed during the first 7 days and on 21 day there was started weaning to formulated diets (Preserved feed, Planktonic feed and Dry commercial feed). Measurements done at 28 day of our experiment showed that the Diet#2 fed planktonic feed from their natural habitat group was having a lower increase in the length and weight compared to the other feeding group.

At first week was started the registering survival. The rate of larval death for the third week was very high in all feeding group. Control group fed only dry commercial feed had the highest mortality rates, compared with all the other diet groups fed artemia during the first feeding stage of weaning process. The reason why the feeding groups did not continue to grow at the same rate was probably because at 21 day was start the shifting from live feed (artemia) to formulated diets. The high mortality at the start of the experiment could be caused by starting of weaning process or sub-optimal environmental conditions in the tank (Almli, 2012).

Our results demonstrated that the groups fed artemia had a slight advantage over Control group fed only dry commercial feed. However, survival were better on artemia during the first week than on dry commercial feed that was also observed by other researches. Cañavate and Fernández-Díaz (1999) showed on the example of Senegal sole larvae (*Solea senegalensis*) that mixed diet are stimulated the normal development and survival during larval metamorphosis. Despite of that during the first week larvae had the similar growth performance, survival rate was higher in diet groups fed artemia than dry commercial feed. Otherwise, no growth or survival were observed in fish after metamorphosis without previous larval co-feeding (Cañavate and Fernández-Díaz, 1999). According to Demeny et al. (2012), the crucian carp (*Carassius carassius* L.) larvae fed with *Artemia nauplii* had a high survival and growth performance and larvae fed only with dry food had opposite results. Mixed feeding protocols (with low proportion of live food) were tested as well and larvae had satisfactory survival, but no so good growth rate and fitness as group with

artemia diet. Thus, results of our experiment also confirmed the benefiting of artemia during early larval life.

Indeed, the live food organisms are commonly known as "living capsules of nutrition", because they have all the necessary nutrients for the larvae. Artificial larval feeds can not successfully replace the protein rich live food organisms in terms of acceptance, nutritional and other factors. Consequently the most of marine larvae prefer live food than commercial food for their better development and survival (Ademola and Folagbade, 2005; Das et al., 2012).

The results of our experiment also revealed the interesting feature. From 4th until and including 9th week all feeding group in general had a lower mortality rate, except the group fed planktonic feed. The planktonic group (Diet#2) started to die at a higher rate from the 4 – 9th week of the experiment and completely died to 84 day. These findings are inconsistent with previous results that usually described for most marine fish larvae (Luizi et al., 1999; Shields et al., 1999; Rajkumar and Kumaraguru vasagam, 2006; Imsland et al., 2006; Olivotto et al., 2010; Kortner et al., 2011; Almli, 2012; Das et al., 2012; Pradhan et al., 2014).

In the natural habitat of marine fish larvae, zooplankton is a major component of food web and as it is suggested all nutritional requirements can be found in copepods (Das et al., 2012).

Kortner et al. (2011) observed that Atlantic cod larvae (*Gadus morhua*) showed high growth rates when fed natural zooplankton. Similar results have been found in Atlantic halibut (*Hippoglossus hippoglossus*), where larvae fed wild planktonic invertebrates (*Eurytemora velox*) during exogenous feeding period showed higher growth performance with the higher intestinal lipid digestion. As showed the results, rate of development and of differentiation of the digestive tract during organogenesis of the larvae were higher in copepod-fed groups in comparison to *Artemia*. In addition, there was a difference in the feed conversion in favor of copepods (Luizi et al., 1999). The same trend could be seen also in butter catfish (*Ompok bimaculatus*) larvae. After 7 days post hatch (dph) of weaning, larvae fed *Artemia nauplii* and zooplankton had the best growth and survival rates, compared with larvae fed microdiet. At the end of the trial at 17 dph, larvae were adapted to the microdiet. As scientists report, early weaning delayed the development of the digestive system and complete development of the digestive system will led to the adaptation of larvae (Pradhan et al., 2014). Thus, we can see that the including copepods in feed diet has a positive influence on the larval development of marine fish.

The reason why larvae could not adapt to the Planktonic feed (copepods *Acartia tonsa*) and the mass death of larvae occurred to the end of our experiment is unknown and required further

investigation. We can predict that it can be related to stress or inappropriate environmental conditions (Almli, 2012).

Later the similar results were found in R. Eckmann (1985) work. This study was focused on the investigation of the histological changes observed in the intestine of Whitefish (*Coregonus lavaretus*) larvae during their first days of feeding. According to the results of experiment, larvae fed live zooplankton from their natural habitat (mainly *Cyclops vicinus* and *C. abyssorum*) had very low survival rates and non-normal histological differentiation of the intestinal epithelium, compared with normal development of larvae fed *Artemia nauplii*. The histopathological alterations included intestinal hyperplasia and dysplasia. At the first 15 day, about 50 % of the larvae fed live zooplankton were found died. At 26 day cumulative mortality for this group was 90 %. Author concluded that these observations is related to reaction of the larvae's organism against a stressor present in the zooplankton and therefore eventually lead to the death of larvae (Eckmann, 1985). These findings are similar to ours, where lumpsucker larvae started died from 14 day until 56 day with total mortality rate of 51.7 %, following the mass death of larvae to 84 day. Therefore, the Eckmann's explanation could be applicable to our case.

4.2. Transcriptomic approach

Very little information is currently known about molecular mechanisms that involved in digestive system of lumpsucker, especially during the weaning process when the larvae undergo many developmental changes in response to change in feeding regime.

To test investigate level and nature of transcriptional activity and its role in controlling gene expression under the influence of different diets (preserved feed and control), transcriptome analysis was carried out. Total RNA was isolated and sequenced with Illumina from six different gut tissues of lumpsucker larvae. Thus, the RNA-Seq analysis was performed, using *de novo* transcriptome assembly.

Differential expression of the genes were found between preserved feed and control groups that not only points to the genes participating in general response to different diets, but also demonstrates contrasting behavior of the same gene products under the diet background. In total, we have found 97 common discordantly expressed genes. The majority of them (80) were up-regulated and 17 were down-regulated.

Results of Blastx analysis revealed interesting features. Some of the contigs represented down- and up-regulated genes respectively, had a failure in finding significant hits to the NR database. Similar results were observed in rainbow trout (Salem et al., 2015) and catfish (Wang et al., 2010). As Salem et al. (2015) report, the main explanations of these findings could be a sequence errors or lacking of protein sequences of related fish in the database.

According to the Blastx similarity searching of the selected transcripts, the largest number of database matched was from Large yellow croaker *Larimichthys crocea* (Teleostei, Sciaenidae). Thus, we can suggest a high degree of sequence conservation and homology of *Cyclopterus lumpus* to *Larimichthys crocea* genes. In addition, most of the species on the Blastx top-hit list were fish species such as Japanese rice fish (*Oryzias latipes*), Nile tilapia (*Oreochromis niloticus*), Black cod (*Notothenia coriiceps*), Amazon molly (*Poecilia formosa*), Bicolor damselfish (*Stegastes partitus*), Sablefish (*Anoplopoma fimbria*) and Tongue sole (*Cynoglossus semilaevis*). These findings could specify on a high quality of the assembled genes and a high level of phylogenetic conservation of genes between lumpsucker and other teleost species (Salem et al., 2015).

C. lumpus genes orthologous to the NAD(P)H dehydrogenase [quinone] 1-like (*nqo1*), T-cell surface glycoprotein CD3 zeta chain-like (*cd3e*), saxitoxin and tetrodotoxin-binding protein 2-like (*stbp2*), calpain-10 (*capn10*) and protein phosphatase 1 regulatory subunit 3C (*ppp1r3c*) were down-regulated in Preserved group compared to Control group:

— *nqo1* gene functions primarily to protect normal cells from oxidant stress or electrophilic attack and the higher level of *nqo1* expression may contribute to tumorigenesis (Lienhart et al., 2014);

— *cd3e* complex plays a crucial role for T-cell function, coupling antigen recognition to several intracellular signal-transduction pathways (Bettini et al., 2014);

— *stbp2* is involved in the transport and tetrodotoxin (TTX) accumulation of many marine fish (Arakawa O. et al, 2010; Hashiguchi et al., 2015);

— *capn10* is expressed primarily in tissues important in glucose metabolism and associated with type 2 or non-insulin-dependent diabetes mellitus (NIDDM) (Picos-Cárdenas et al., 2015);

— *ppp1r3c* catalyzes reversible protein phosphorylation and hence have diverse functions, including protein synthesis, cell death and glycogen metabolism (NCBI, 2015).

Among the genes, *ppp1r3c*, *capn10* and *stbp2* were more expressed with -4.14, -4.42, -6.17- fold down-regulation respectively, than *cd3e* (-6.93) and *nqo1* (-16.9). This finding can specify on that these genes were more closely involved in down-regulated molecular mechanisms of larvae fed Preserved feed. The protein synthesis and glucose metabolism are major energy production pathways that could impair other critical physiological processes by limiting available energy resources (Metzger, 2012). The transfer and accumulation of TTX lead to greatly negative effect on the maturation process of fish. Organisms with a good developed biological defense mechanism are showed a high resistance to TTX (Arakawa O. et al, 2010). Therefore, activity reduction of genes associated with these metabolic pathways will decrease the impact of undesirable process on larval growth and survival.

On the other hand, *C. lumpus* genes orthologous to the fatty acid synthase (*fasn*), acetyl-CoA carboxylase 2 isoform X2 (*acc2*), midasin (*mdn1*), histone-lysine N-methyltransferase MLL3 (*mll3*), centromere protein F (*cenpf*), sterile alpha motif domain-containing protein 9-like (*samd9l*), small subunit processome component 20 (*utp20*) and E3 SUMO-protein ligase RanBP2 (*ranbp2*) were found as up-regulated in Preserved group compared to Control group:

— *fasn* gene acts as catalyzer of the synthesis of palmitate from acetyl-CoA and malonyl-CoA in the presence of NADPH into long-chain saturated fatty acids (Bian, 2015);

— *acc2* or *accb* catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA and involved in the regulation of fatty acid oxidation (Abu-Elheiga et al., 2010);

— *mdn1* acts as nuclear chaperone for maturation and may be involved in the assembly/disassembly of macromolecular complexes in the nucleus (Garbarino and Gibbons, 2002);

— *mll3* gene plays important role in histone methylation activity and in initiation of transcription (NCBI, 2015);

— *cenpf* gene has a potential role in regulating skeletal myogenesis and in cell differentiation in embryogenesis, involved in cell cycle progression and immune response (NCBI, 2015);

— *samd9l* involved in regulating cell proliferation and apoptosis, inflammatory response to tissue injury and the control of extra-osseous calcification (Hershkovitz et al., 2011);

— *utp20* gene associated with biogenesis of ribonucleoproteins due to its role in pre-rRNA processing and ribosome assembly (Peng et al., 2003; NCBI, 2015);

— *ranbp2* have diverse cellular functions, including immune response and transport, due to its interactions with the nuclear membrane (NCBI, 2015).

The value of positive change were very higher for *ranbp2* (66.80), *utp20* (60.34), *samd9* (60.17), *cenpf* (58.38), *mll3* (58.37) and *mdn1* (55.33) genes in comparison to the rest: *acc2* (19.69), *fasn* (12.85). The molecular functions of highly expressed genes mainly was associated with cell differentiation and immune response.

Therefore, we can predict that the amplified activity of up-regulated genes in lumpfish larvae could be linked to the effect of Preserved feed diet on lumpsucker larvae and early juveniles that correlated to morphometric analysis.

In Olivotto et al. (2010) work was found that the biochemical characteristics of preserved copepods are important for early stages of fish. The preserved feed as a new technology is able to save of precious fatty acid characteristics and lead to improving of gene expression in crucial developmental processes, such as lipid metabolism and metamorphosis (Olivotto et al., 2010).

Indeed, during all experimental period the Diet#1 (Preserved feed) had the best growth and survival results than Control group. At 84 day, Diet #1 were 3.59±0.09 mm, 1.6807±0.1791 g, the length and weight of Control group: 3.17±0.11 m, 1.1280±0.1669, respectively. Total mortality rate was lower for larvae fed with *Artemia nauplii* during the first week and then formulated diets (Diet #1 – 3.4 %), than for Control group (6.4 %) fed only dry commercial feed during all experiment.

Eventually, the present study can indicate on that nutritional values and composition of copepods make them the most suitable live feed in the early rearing of lumpsucker larvae compared with artificial larval feed.

5. CONCLUSION

This study investigated the effects of different feeding diet on lumpsucker larvae and early juvenile.

The using of the *Artemia nauplii* in start-feeding of lumpfish resulted in an increased growth and survival during weaning process compared to larvae fed dry feed.

The results of morphometrical analysis have reflected that development and survival during the experimental period were diet-specific. Larvae fed diets containing artemia had higher length and weight in comparison to dry-fed larvae. The mortality was high only at the third week during larvae adaptation to formulated diet. The mass death of planktonic group (Diet#2) to the end of experiment did not correlated with other marine fish studies and could be related to reaction of the larvae's organism against a stressor present in feed.

RNA-seq analysis identified 97 differently expressed genes, where 80 of them were found as up-regulated and 17 were down-regulated in preserved feed group (Diet#1) compared to control fed dry commercial feed. The top-hit of Blastx searching revealed that the selected transcripts were matched mainly with teleost species and with a higher similarity to large yellow croaker *Larimichthys crocea* (Teleostei, Sciaenidae). These findings could specify on a high quality of the assembled genes and a high level of phylogenetic conservation of genes between lumpsucker and other teleost species.

Among the genes found, such as *ppp1r3c*, *capn10* and *stbp2* were significantly down-regulated and *ranbp2*, *utp20*, *samd9*, *cenpf*, *mll3*, *mdn1* were significantly up-regulated in preserved feed compared to control group. It was suggested that the amplification of the some genes and reduced activity of other tended to improve the biological defense mechanism and growth of lumpsucker larvae and early juvenile. These results correlated to morphometrical analysis.

According to the data, I can conclude that the effects of nutrition on larval development and survival of *C. lumpus* is likely to be different under various feeding regimes and live feed is more preferable on early stages of lumpsucker ontogenesis than commercial food.

The results of the present study could be useful in improving the lumpsucker culture due to the high influence of feeding diets on larval development and survival. However, to enrich the knowledge about this species all areas of the given research require further investigations.

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7. APPENDICES

Appendix 1

Protocol Artemia-production, feeding and tending Lumpsuckers, Hall 6

Artemia-production

1). Feed the tanks as described under "Feeding". Wait AT LEAST 30 minutes after feeding, before you disturb the tanks again (by cleaning them).

2). Shut off the big light in the artemia hatching-tank, and take out the aeration-stone and glass-rod.

3). Place black plastic bags around the tank upper part and on top. Leave it for quite some time to settle.

4). Empty the feeding-cone inside Cell 1, and clean it with soap and water. Rinse thoroughly. Place back into the cooler.

5). Fill a 10L bucket with hot freshwater, and place the feeding glass-rods into the bucket.

NB! Make sure the feeding-tubes are placed on the outside of the tanks before doing this.

Run the timers until the bucket is empty. Wipe and clean off the ends of the glass-rods with paper and rinse then with hot water. Place them back into the feeding-cone.

6). Place the feeding tubes back into the tanks.

7). Shut off all heaters (enrichment, hatching and washwater-tanks)

8). Ready the washing equipment for the enriched artemia.

9). Take out the airstone and oxygen-stone, and shut off the oxygen on the valve in the roof (turn valve all the way to the right).

10). Wash the enriched artemia using water from the holding-tank.

11). The hose from the holding-tank should be inside the net. Fill up the tube with water before adding enriched artemia. Valve 1/3 open.

12). Put a hose on the enrichment-tank valve, put the first few liters on the floor (brown color), and then place hose into the washing-net. Valve no more than half open.

13). When waterlevel is getting down to under the beginning of the cone-part of the tank, trash the rest on the floor.

14). Wash enriched artemia until clean water outside net. Then use a measuring-jug, and transfer the clean artemia to a 10L bucket. Put the washed artemia in the feeding cone in Cell 1, and aerate it with the glass-rod.

15). Fill up the cone so there is 20L in total (with seawater from the white holding-tank outside).

16). Clean the enrichment tank, heater and air-stone properly (tank and heater with soap, air-stone with hot water). Rinse oxygen-stone properly with hot water.

17). Fill 45 L of seawater into the clean enrichment-tank from the white holding-tank, using the hose.

18). Put the oxygen-stone and the cleaned airstone into the enrichment-tank.

19). Clean, and ready the washing equipment (tub and net) for adding the hatched artemia.

20). Wash the hatched artemia using the same procedure as in point 10, 11 and 12.

21). When the water gets clear, empty the rest of the contend in the hatching-tank on the floor.

22). During this washing, we don't need to wash them for very long. The only purpose of adding them to the net is to concentrate them before adding ca 10L concentrated artemia to the enrichment-tank (same procedure as in point 14, except for the first sentence).

23). Should be ca 55 L in the enrichment-tank when you are done transferring.

24). Adjust the oxygen-stone as follows: turn all the way ON (left) and then turn the valve 10 rounds to the right (OFF).

25). Clean the hatching-tank, heater and air-stone/glassrod properly (tank and heater with soap, air-stone/glassrod with hot water).

26). After washing, and thorough rinsing, fill up the hatching tank with 60 L of water from holding-tank. Put the airstone and the glass-rod into the tank.

27). Measure up 120 grams of artemia-cysts (big bag in fridge), and put them into the hatching tank.

28). Make sure both lights are turned on.

29) Measure up 22 grams of enrichment (green stuff, small bag in fridge), and put it into 1 L of water (in the mixer). Mix for 2 minutes on medium speed. Put into enrichment tank.

30) Fill up the holding-tank with seawater using the grey aquarium-pump and the yellow hose.

Cleaning tanks

31). Use the small glass-siphone to take out as much dirt as you can from the bottoms of the tanks. Use tending-equipment (bucket and tub fitted with inlet water), to collect dead larvae into.

32). Live once has to be put back into the correct tank.

Count dead larvae from each tank. Put numbers of dead larvae in sheet.

33). Measure temperature and oxygen in tank number 5, and put in sheet.

34). Check the level of oxygen in enrichment-tank. Check whether feeding tubes back into the tanks.

35). Clean all equipment you have been using, and rinse/clean the floor.

Feeding

At approximately 09.00, check to see if there is still artemia in the ffeding-cone, and that the air-tube and glass-rods are still in.

If yes, turn the “pump-switch” from “TIMER” to “ON”, and leave it there for 1 minute before turning it back to “TIMER”. Check that all tubes and pumps are working properly, and that the tanks are receiving artemia (9 tanks, but NOT Control group).

If tubes are not working, check if the glass-rods are in the cone, that the tubes are not deplugged somewhere or the ends are bended, etc.

Collect the tree other feed-types from the fridge, marked “Dry-feed”, “Planktonic” and “Preserved feed”.

Dry feed (green cup): Use the spoon in the cup (ca. 2g) and give each tank in group 3 and 4 one spoon. Try to put it in gently and spread it around.

Preserved feed: Use a spoon and measure up ca. 0,5g in the small beaker. Give 0,5g of this to each tank in group 1.

Planktonic feed: Measure up 6g of this into a measuring-jug, and fill it up with 600 mL of seawater from buffertank 2 (black tank). Stir it until the feed is dissolved in the water, and then give each tank in group 2 200 mL of this.

At approximately 12.00 (after tending of all tanks), feed all 9 tanks (NOT CONTROL GROUP) with 0,2L of artemia from the cone.

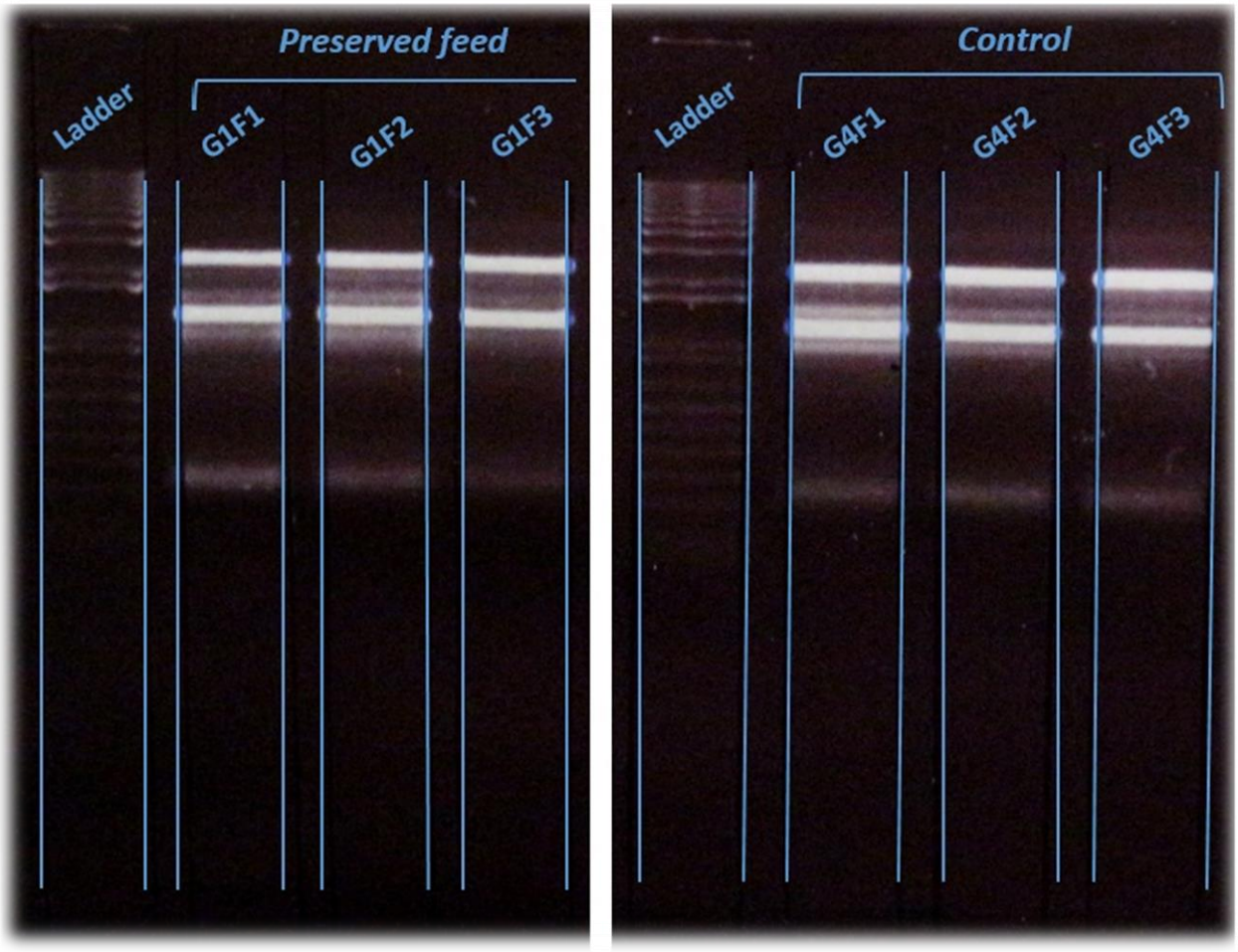
Fill the cone up again till 20L (add 2L of clean seawater).

Also repeat point 31-35.

Make sure all heaters (enrichment, hatching and holding-tank) are on, and put on 28 °C. Check whether all lights on. Check the level of oxygen in enrichment-tank and make sure airation are turned on.

Appendix 2

Agarose gel analysis of total RNA isolated from *C. lumpus* of two different feeding diet - the preserved feed (Diet#1) and control group, where G1F1- preserved feed, fish#1, G1F2- preserved feed, fish#2, G1F3- preserved feed, fish#3; G4F1- control, fish#1; G4F2- control, fish#2; G4F3- control, fish#3.



Appendix 3

Mortality rate in every feeding group during 1st -10th weeks of the experiment

Weeks from starting of the experiment	Feed	No. of dead larvae	Mortality rate
1	Diet#1	83	0,69 %
	Diet#2	73	0,61 %
	Diet#3	93	0,78 %
	Control	53	0,44 %
2	Diet#1	23	0,19 %
	Diet#2	8	0,07 %
	Diet#3	74	0,62 %
	Control	24	0,20 %
3	Diet#1	101	0,84 %
	Diet#2	59	0,49 %
	Diet#3	130	1,08 %
	Control	478	3,98 %
4	Diet#1	84	0,70 %
	Diet#2	432	3,60 %
	Diet#3	94	0,78 %
	Control	137	1,14 %
5	Diet#1	75	0,63 %
	Diet#2	2977	24,81 %
	Diet#3	202	1,68 %
	Control	23	0,19 %
6	Diet#1	13	0,11 %
	Diet#2	2420	20,17 %
	Diet#3	17	0,14 %
	Control	10	0,08 %
7	Diet#1	9	0,08 %
	Diet#2	177	1,48 %
	Diet#3	6	0,05 %
	Control	7	0,06 %
8	Diet#1	5	0,04 %
	Diet#2	14	0,12 %
	Diet#3	3	0,03 %
	Control	3	0,03 %
9	Diet#1	2	0,02 %
	Diet#2	29	0,24 %
	Diet#3	3	0,03 %
	Control	7	0,06 %
10	Diet#1	10	0,08 %
	Diet#2	9	0,08 %
	Diet#3	15	0,13 %
	Control	26	0,22 %

Appendix 4

Sequencing data of Illumina reads from *C. lumpus* gut (total RNA libraries; 2 pools with 3 fish in each).

Pools	Samples ID	Sample description	Total Reads	Average length	Trimmed reads	Percentage trimmed	Length after trim
1	G1F1	Group#1: preserved feed; fish#1	8,514,678	301.0	8,441,211	99.14%	250.4
1	G1F2	Group#1: preserved feed; fish#2	6,936,416	301.0	6,870,851	99.05%	254.0
1	G1F3	Group#1: preserved feed; fish#3	8,223,082	301.0	8,115,390	98.69%	250.5
2	G4F1	Control group; fish#1	8,259,126	301.0	8,192,616	99.19%	249.4
2	G4F2	Control group; fish#2	6,019,232	301.0	5,942,576	98.73%	249.6
2	G4F3	Control group; fish#3	5,138,974	301.0	5,060,303	98.47%	250.3

Appendix 5

The complete list of up- regulated (positive fold changes) and down- regulated (negative fold changes) genes in preserved feed compared to control group.

Feature ID	P-value	Fold Change	FDR p-value
contig_26228	3.17E-4	-207.25	0.05
contig_25446	2.35E-4	-95.47	0.04
contig_23234	2.42E-4	-32.97	0.04
contig_20112	4.43E-6	-30.40	4.57E-3
contig_2790	6.51E-5	-19.36	0.02
contig_1410	1.43E-4	-17.53	0.03
contig_23393	1.32E-4	-16.09	0.03
contig_23103	2.80E-4	-12.27	0.05
contig_17416	1.59E-4	-8.35	0.03
contig_9563	2.24E-5	-8.27	0.01
contig_4986	6.96E-6	-7.04	5.12E-3
contig_19530	6.05E-5	-6.93	0.02
contig_14218	2.12E-4	-6.88	0.04
contig_9329	1.93E-4	-6.17	0.04
contig_4666	1.30E-4	-4.42	0.03
contig_3990	4.50E-5	-4.40	0.02
contig_15904	2.25E-4	-4.14	0.04
contig_10214	2.22E-7	4.05	6.38E-4
contig_8142	4.79E-6	4.10	4.57E-3
contig_1471	4.06E-5	4.10	0.02
contig_15876	6.95E-6	4.12	5.12E-3
contig_16557	2.99E-4	4.14	0.05
contig_8089	7.94E-5	4.28	0.02
contig_4829	5.60E-5	4.28	0.02
contig_941	1.45E-5	4.29	9.18E-3
contig_754	8.89E-7	4.34	1.60E-3
contig_727	2.80E-7	4.38	7.38E-4
contig_8313	4.07E-5	4.51	0.02
contig_4410	5.34E-6	4.53	4.57E-3
contig_17725	3.95E-5	4.58	0.02

contig_6013	9.27E-10	4.59	6.51E-6
contig_1235	6.28E-11	4.69	9.93E-7
contig_13694	2.96E-4	4.70	0.05
contig_5863	1.97E-4	4.77	0.04
contig_1071	3.83E-5	4.80	0.02
contig_1064	1.04E-6	4.82	1.72E-3
contig_8658	1.12E-4	5.00	0.03
contig_12147	1.03E-4	5.02	0.03
contig_4742	1.74E-7	5.08	6.01E-4
contig_16911	5.35E-7	5.12	1.06E-3
contig_20655	9.36E-5	5.13	0.03
contig_7829	6.60E-9	5.26	3.48E-5
contig_5561	2.58E-5	5.27	0.01
contig_1559	3.19E-7	5.34	7.75E-4
contig_5630	9.11E-7	5.35	1.60E-3
contig_1072	5.29E-6	5.41	4.57E-3
contig_6749	5.11E-5	5.54	0.02
contig_16523	1.52E-4	5.59	0.03
contig_8599	1.92E-4	5.99	0.04
contig_13526	1.22E-4	6.07	0.03
contig_8386	7.22E-5	6.10	0.02
contig_12868	1.65E-4	6.15	0.04
contig_4266	1.19E-5	6.74	8.10E-3
contig_8537	3.11E-10	6.84	3.28E-6
contig_4741	1.09E-6	7.18	1.72E-3
contig_18741	2.90E-4	7.19	0.05
contig_18815	6.00E-5	7.24	0.02
contig_10775	1.75E-4	7.38	0.04
contig_14051	9.80E-5	7.51	0.03
contig_12366	1.10E-4	7.66	0.03
contig_18922	8.03E-5	7.77	0.02
contig_12319	3.03E-4	7.78	0.05
contig_18280	2.23E-4	7.93	0.04
contig_4608	1.94E-6	8.07	2.56E-3

contig_3711	1.07E-11	8.19	3.38E-7
contig_11470	4.18E-5	8.60	0.02
contig_18719	1.53E-4	8.88	0.03
contig_7929	1.75E-4	9.18	0.04
contig_7781	6.70E-6	9.29	5.12E-3
contig_21030	1.30E-4	9.83	0.03
contig_8538	1.90E-7	10.18	6.01E-4
contig_153	1.03E-9	10.40	6.51E-6
contig_13570	5.41E-5	11.05	0.02
contig_19641	5.22E-6	11.29	4.57E-3
contig_11202	3.36E-5	11.98	0.02
contig_13960	2.89E-4	12.40	0.05
contig_23745	1.59E-4	12.73	0.03
contig_19259	5.53E-8	12.85	2.19E-4
contig_10853	5.53E-6	13.73	4.60E-3
contig_18515	9.74E-6	13.96	7.00E-3
contig_14640	5.26E-7	16.28	1.06E-3
contig_21674	1.76E-4	19.69	0.04
contig_12172	4.08E-5	22.93	0.02
contig_17806	2.71E-4	55.53	0.05
contig_16269	2.31E-4	58.37	0.04
contig_26220	2.92E-4	58.38	0.05
contig_18514	2.11E-4	60.17	0.04
contig_21532	2.92E-4	60.34	0.05
contig_22871	2.88E-4	60.57	0.05
contig_3298	1.40E-4	60.60	0.03
contig_9314	2.27E-4	60.89	0.04
contig_21711	5.74E-5	66.79	0.02
contig_20949	5.13E-5	66.80	0.02
contig_19385	3.81E-5	67.30	0.02
contig_25999	2.21E-4	76.69	0.04
ontig_20644	2.50E-4	81.12	0.04
contig_28082	9.72E-5	199.67	0.03