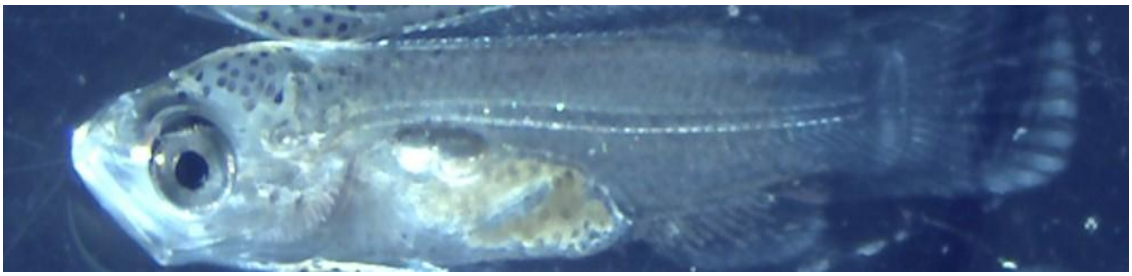
Genetic ontogeny of pancreatic enzymes in *Labrus bergylta* larvae and the effect of feed type on enzyme activity and gene regulation



Masters degree in Aquaculture biology

Truls Wergeland Hansen

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Front page illustration: *L. bergylta* larvae (34 dph) produced at MH Labrus in 2011

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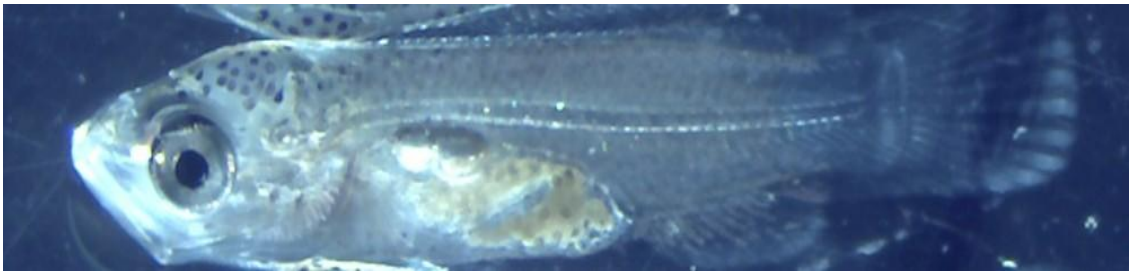

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NIFES

NATIONAL INSTITUTE OF NUTRITION AND SEAFOOD RESEARCH

Alternative front page illustration: L. bergylta larvae (34 dph) produced at MH Labrus in 2011

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Finally, I would like to thank the larvae for sacrificing their lifes in the name of science.

Abstract

In the later years increased interest in the use of *Labrus bergylta* (ballan wrasse) as a cleaner fish on *Salmo salar* (Atlantic salmon) has caused the emergence of commercial farming of *L. bergylta*. In order to cultivate such a species, knowledge about its digestive physiology and its nutritional requirements is important, especially in larvae. It has therefore been suggested that a thorough investigation of digestive enzymes related to the pancreas in larvae could be valuable.

There were two separate aims of the study. A descriptive part regarding the ontogeny of genes coding for pancreatic enzymes in larvae prior to metamorphosis were performed. Here, the aim was to investigate the effect of age on the relative expression of genes which are related to pancreatic digestion of proteins (trypsin), lipids (Cyp7 A1, BAL, $sPLA_2$ 1B) and carbohydrates (amylase, chitinase). Larvae were sampled from the age of 2–55 dph in weekly intervals. In addition to this, an experimental part was performed where the aim was to investigate the effect of different diets on pancreatic enzyme activity in the larvae. We also wanted to investigate the possibility of exogenous enzyme contribution by rotifers (*B. plicatilis*). Samples of successfully weaned larvae were compared to rotifer fed larvae, including larvae with no gut content. A sample of enriched rotifers was analyzed for comparison. Enzyme activity assays were performed on trypsin, neutral lipase, $sPLA_2$, amylase and chitinase.

Our results suggest a consistent feed- dependency in larval *L. bergylta* at a transcriptional level, where rotifers somehow stimulate upregulation and the formulated feed has the opposite effect. The regulation of BAL was the only exception, where an upregulation was observed as a result of weaning both in the ontogeny series and the experimental part.

We did not observe an effect of diet on enzyme activities of neutral lipase, chitinase and $sPLA_2$ in the experimental part. Trypsin and amylase activity assays showed a trend towards a higher activity in rotifer fed larvae, but these values were below the manufacturers LOD (limit of detection).

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Abbreviations

ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
BAL	Neutral bile activated lipase
cDNA	Complementary DNA
Cyp7 A1	Cholesterol 7- alpha- monooxygenase
ddH_2O	Double distilled, RNase- free H_2O
DEPC	Diethyl pyrocarbonate
DNTB	2.4 dinitro-1-thiocyanobenzene
DMSO	Dimethyl sulfoxide
EtOH	Ethanol (C_2H_6O)
GM 150	Gemma Micro 150 µm
LOD	Limit of detection
MH	Myotome height
MH Labrus	Marine Harvest Labrus
MNE	Mean normalized expression
mRNA	Messenger RNA
MS 222	Tricaine methanesulfonate
NaAc	Sodium acetate
NIFES	National Institute of Nutrition and Seafood Research
PBS	Phosphate buffered saline
PUFA	Poly- unsaturated fatty acid
R	Rotifer group
ROT	Rotifer sample
RU	Unfed larvae in the rotifer group

SEM	Standard error of means
SD	Standard deviation
SL	Standard length
$sPLA_2$	Secretory phospholipase group A_2
TE buffer	Buffer consisting of Tris and EDTA
W	Weaned group
WU	Unfed larvae in the weaned group

1 Introduction

1.1 Salmon farming and sea lice

The Norwegian coastline is home to one of the largest industries of *Salmo salar* (Atlantic salmon) farming in the world, an industry which arose in the 1970s. Since then, there have been numerous improvements in production techniques and technology which has led to a significant increase in the number of farms and produced salmon. Norwegian farms produced ~1 million tonnes of *S. salar* at a value of ~5 billion USD in 2010 alone (FAO, 2012). Despite the apparent success of salmon farming, there have been several challenges throughout the years. One of the most severe issues has been infections of the ectoparasitic sea louse *Lepeoptheirus salmonis* (from here on termed sea lice). Sea lice have plagued farmers since the inception of the industry and in later years (Brandal and Egidius, 1977, Heuch et al., 2005). The parasite feeds off the skin, mucous and blood of the host, causing secondary infections and osmoregulatory difficulties leading to illness and death. Infections have been reported on farmed salmonid species such as *S. salar*, *Salmo trutta* (sea trout) and *Onchorynchus mykiss* (rainbow trout) in Norway and other countries such as Scotland, Ireland, the Faroe Islands, Canada, USA and Japan throughout the years (Grant, 2002). Costs due to sea lice infections exceeded 500 million NOK in Norway in 1997 and £15-30 million in Scotland in 1998 (Pike and Wadsworth, 1999, Rae, 2002). A significant increase in the sea lice population caused by the increase in quantity and density of hosts and an all- year-round availability in net pens could also represent a serious threat to wild salmonid populations, and has been blamed for the collapse of *S. trutta* stocks in several countries (Heuch et al., 2005).

Throughout the years a variety of chemical components have been used to treat lice infections. These are avermectins, pyrethroids, hydrogen peroxide (H_2O_2), chitin synthesis inhibitors and organophosphates and they have been given by bath treatment or as feed additives. They lack specificity and can therefore be harmful to the environment (Egidius and Moster, 1987, Haya et al., 2001). The chemical treatments can also be stressful to the salmon (Salte et al., 1987), and cause disease among farmers handling the pesticide (Cantor et al., 1992). More importantly, resistant sea lice have been reported, compromising several of the treatments used and making it more difficult to reduce lice infections (Fallang et al., 2004, Treasurer et al., 2000, Jones et al., 1992). Government regulations and supervision of aquaculture and sea lice status have been on the agenda in many countries in the later years, i.e. *Lakselusforskriften* in Norway (2009),

including implementation of new methods that are less harmful to the environment (i.e. vaccines, land based facilities, selective breeding). At present there has not been any fully successful method towards a significant reduction of sea lice infections in aquaculture. The use of specialized cleaner fish could possibly pose a solution to the problem. There are however challenges related to available quantities in the wild and the stocking of two species together in a net pen (disease, aggressive behavior etc.).

1.2 Cleaner fish

Cleaning activity by labrid species (Teleostei: Labridae), commonly known as wrasse, in temperate waters have been documented several times throughout the years (Hobson, 1976, Bjordal, 1990). By investigating stomach content, Potts (1973) recorded cleaning behavior by three different labrid species; *Ctenolabrus rupestris* (goldsinny), *Ctenolabrus melops* (corkwing) and *Ctenolabrus exoletus* (rockcook) in their natural environment. This behavior is based on a 3-party relationship between a cleaner fish, a parasite and a host (Losey, 1987). Labrid fish possess a highly developed pharyngeal jaw apparatus and a protrudable mouth which is related to their ability to feed on louse that are attached to a host (Liem, 1973).

The use of cleaner fish in a commercial scale represents a biological control method which is considered advantageous compared to chemicals because it reduces the possibility of resistance, and is less harmful to the environment. Specialized cleaner fish have been reported to successfully reduce lice infections on commercial salmon farms in Norway (Ottesen et al., 2008), Shetland, Scotland and Ireland (Treasurer, 1994, Deady et al., 1995, Tully et al., 1996). The formerly mentioned labrid species, including *Labrus mixtus* (cuckoo) and *L. bergylta* have all shown a potential for use in commercial aquaculture as cleaner fish (Bjordal, 1990, Deady et al., 1995, Tully et al., 1996). *L. bergylta* have shown a greater potential compared to the other species of wrasse due to its tolerance of winter sea temperatures and its ability to delouse larger salmon (3-5 kg) (Ottesen et al., 2008). Figure 1.1 illustrates the reduction of lice on a salmon farm by *C. rupestris* compared to a control group, where no cleaners were added.

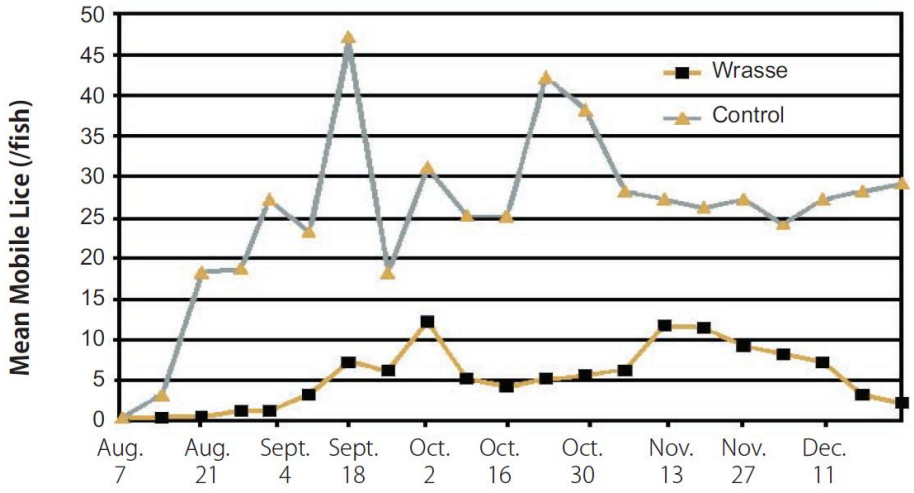

Figure 1.1: Lice reduction achieved on farmed *S. salar* by *C. rupestris*. From Ottesen et al. (2008).

1.3 *Labrus bergylta*

The *L. bergylta* is the largest of the European labridae species, with a recorded maximum length of 60 cm and spawn in early summer. It is distributed along the coastline of the eastern Atlantic, from Norway in the north to Morocco in the south. The coloration is variable and specimens can be found with dark shades of green, red, brown and grey, depending on age and reproductive state (fig. 1.2). *L. bergylta* is a protogynous hermaphrodite (changes sex from female to male) and live on rocky and algal substratum in the littoral zone (10- 20 m). Juvenile fish often live in intertidal areas. It is a relatively long- lived and slow- growing species, and the time of sexual inversion varies widely, from 5 to 14 years (Dipper et al., 1977). The oldest specimen recorded was a 29 year old male.

Figure 1.2: Adult *L. bergylta*. From: Ottesen et al. (2008).

Dipper et al. (1977) suggested an omnivorous diet of *L. bergylta* with crustaceans, isopods and molluscs as dominant food items, based on measurements of gut, teeth and mouth to body length ratios. Artüz (2005) found that the diet consisted mainly of mussels, echinoderms and crustaceans. Algae, sediment and occasional small fish were also found in their gut, suggesting an opportunistic feeding strategy. However, algae and sediment could have been ingested involuntarily together with i.e. a mussel or other food items (Sæle, Ø., pers. comm., NIFES, 2012). Results that were similar in relation to diet was found by Figueiredo et al. (2005), who investigated the feeding ecology of *L. bergylta* in the Azores. These authors also suggested that the *L. bergylta* fed on harder prey as they grew.

Approximately one- sixth of the fish species that belong to the genus Labridae are agastric (Wilson and Castro, 2011), which mean that they lack a functional stomach. A functional stomach is defined by the secretion of gastric juice (HCl and pepsinogen) (Smit, 1968). No such digestive components are secreted and the forgut leads directly into the intestine where mucus is secreted. Hence, no stomach grinding, denaturation (acidic pH by HCl) or enzyme degradation by pepsinogen is possible. Figure 1.3 illustrate the intestine of an adult male *L. bergylta*. The absence of a functional stomach in wrasse was reported by Chao (1973) and later confirmed by Yasugi (1987), who found that pepsinogen activity was not present. The pyloric cecae are also absent in wrasse, so the absorbtive surface to volume ratio is relatively small compared to other species.

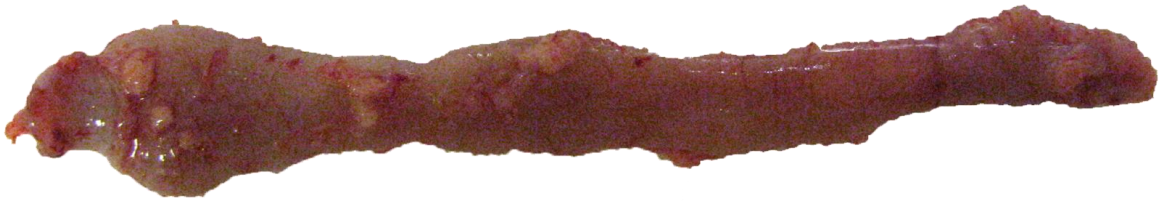

Figure 1.3: Alimentary tract of an adult male L. bergylta, with no stomach or pyloric caeca. Photo by Øystein Sæle, NIFES, 2011.

At hatching, most marine larvae are very immature, *L. bergylta* is no exception. Newly hatched larvae have been measured to be around 3.6 mm SL (Dunaevskaya, 2010). Little is known in regards to the natural feeding habits of larval *L. bergylta*, but it is assumed that the diet mainly

consists of various panktonic copepods, a diet which is similar to that of larval *Gadus morhua* (Atlantic cod) (Fossum and Ellertsen, 1994).

1.4 Cultivation of *Labrus bergylta*

The salmon aquaculture industry has increased its demand for wrasse significantly in the later years. The total amount of wrasse species fished in Norway in 2011 was ~109 metric tonnes (whole fish) at a value of 36.6 mill NOK (Råfisklaget, 2012). Increased selective fishing could alter the population structure and cause overfishing on wild stocks along the coastlines, a situation that should be avoided. In order to meet these demands from the industry, and based on commercial interests, Marine Harvest Labrus (Øygarden, Norway, from here on termed MH Labrus) initiated large- scale production of *L. bergylta* in 2009. This facility was formerly used to produce *G. morhua* fry until financial problems in the industry caused the company to phase out the production in 2008 (Grøtan, E., pers. comm., 2011). Two other Norwegian companies, Profunda (Barstadvik, Norway) and Nordland leppefisk (Lovund, Norway), have recently begun farming this species as well. A stable production of *L. bergylta* juveniles could contribute with sufficient numbers of lice eaters at the right size all year round to facilities on several locations along the Norwegian coast and on other similar locations. However, the fact that this species have never been farmed before makes cultivating it more demanding. The feed need optimized macronutrient content and digestibility to give the best growth in the fish. This is especially important for the larvae. In order to acquire knowledge on the farming of this species, research activity have been initiated on *L. bergylta* produced at MH Labrus in collaboration with scientists at NIFES (the National Institute of Nutrition and Seafood Research, Bergen, Norway) and IMR (the Institute of Marine Research, Bergen, Norway). The primary objectives of MH Labrus are to further improve a current production protocol and to optimize the use of the farmed *L. bergylta* in salmon production facilities. Specific objectives include optimization of nutrition, both for broodstock and juveniles, temperature, and light conditions.

1.5 Larval digestive system development and requirements

The main objective of the digestive system is to supply the body with the nutrients required for growth and survival. Larvae are growing rapidly during their first stages of life, and most of the energy available is used for morphological and physiological changes. At the time of transition from endogenous to exogenous feeding, there is a major change in the digestive physiology and morphology of the larvae (Ferraris et al., 1987, Beccaria et al., 1991). Incidents of poor quality and survival in first- feeding larvae and early juveniles have been relatively common throughout the years for many different farmed species (Shields et al., 1999, Evjemo et al., 2003, Hamre, 2006). One of the reasons to this is the performance of the digestive system in relation to the rapid growth and relative vulnerability of the larvae compared to adults. When cultivating a fish species, it is therefore crucial to acquire knowledge about species- specific nutrient requirements and the larval digestive system in general. Such knowledge can be used to synchronize feeding practices and rearing protocols according to the nutritional needs of the larvae.

1.5.1 The alimentary tract

In most species, including wrasse, the alimentary tract of first- feeding larvae is a straight undifferentiated tube with a sphincter in the anal opening (Kamisaka et al., 2003). With a few exceptions, a functional stomach is lacking and does not develop until the transition from larvae to juvenile, also known as metamorphosis (Govoni et al., 1986). Later in life the alimentary tract differentiates and grows, and becomes increasingly adapted to the adult way of acquiring nutrients.

1.5.2 Pancreas

The pancreas is an important digestive organ, both hormonally and enzymatically. In the early larval phase, the pancreas is one distinct organ (*pancreas compactum*). The adult form is developed in the late larval and early juvenile stage and is termed the *pancreas diffusum*, due to its more diffuse location around the alimentary tract (Kurokawa and Suzuki, 1996). It is the main source of release of many key digestive enzymes involved in the digestion of proteins, lipids and carbohydrates. It consists of a hormone releasing endocrine part and an enzyme secreting exocrine part. The endocrine pancreas mainly release glucagon and insulin, hormones that act antagonistically on each other in relation to appetite and growth (Murashita et al., 2006, Kurokawa and Suzuki, 1995). The exocrine pancreas contains acinar cells. Within these cells are zymogen granules, where enzymes are being produced and stored (Kurokawa and Suzuki, 1995).

From there, enzymes are released into the lumen when stimulated hormonally. Alkaline fluid is also secreted by the acinar cells, which in turn increases the pH in the intestinal lumen. Lipase and amylase are secreted in active forms, while proteolytic enzymes are secreted as pro- enzymes and activated in the lumen of the intestine.

1.5.3 Liver and gall bladder

The liver is an important organ in relation to metabolism. Hepatocytes within the liver produce and secrete bile acids, which are stored in the gall bladder and have powerful detergent properties. Bile acids serve an important role in lipid digestion, by emulsificating dietary lipids and fat- soluble vitamins (vitamin A, D, E and K), easing the following digestion by lipases. For several fish species, authors have concluded that the liver and gall bladder is functional prior to first- feeding (Govoni, 1980, O' Connell, 1981). An important part of digestion is the conversion of cholesterol to bile acids in the liver of most mammals. This is done by the enzyme CYP7 A1 (cholesterol 7α- hydroxylase) (Myant and Mitropoulos, 1977). Few studies have been performed on this enzyme in fish larvae despite its important role.

1.6 Digestion

Digestion is the process of modifying the ingested feed into molecules and elements that can be absorbed across the intestinal wall. When feed is digested, nutrient polymers (proteins, fats and carbohydrates) are broken down by hydrolysis and solubilization. This is done by a large number of digestive enzymes in the lumen of the alimentary tract. From here, smaller fragments are further digested in the epithelium in the gut wall and released into the bloodstream. The fragments released include peptides and amino acids, monosaccarides and fatty acids.

Several studies of different larval species and their digestion, especially on enzyme activity, have been carried out throughout the years (Infante and Cahu, 1994b, Gawlicka et al., 2000, Uscanga-Martinez et al., 2011). Most of these studies have focused on lipid and protein digestion, whilst little is known in regards to carbohydrates. In the recent years, new methods yielding more accurate information about the molecular basis of digestion have been applied (Perez-Casanova et al., 2006, Sæle et al., 2010, 2011). Such information can be used as an indicator of digestive system performance and larval acceptance to the feed given (Nolting et al., 1999). Important enzymes in regards to larval digestion will be presented in the following sections. Knowledge on

the molecular basis of these enzymes in *L. bergylta* larvae, including their activity in regards to feed type will provide essential knowledge on how to cultivate this species more efficiently.

1.6.1 Protein

Fast- growing fish larvae need considerable amounts of digestive proteins for muscle growth and energy. When digested, proteins are broken down to peptides and amino acids (AA) by different proteases such as trypsin and pepsin. As mentioned earlier, agastric species do not have the ability to initiate the digestion of proteins by enzymatic cleavage and denaturation. However, Infante and Cahu (1994b) suggested that a stomachless fish, in this case *D. labrax*, is not dependent on pepsin for sufficient protein digestion. In such a case, trypsin is left as the main proteolytic enzyme. Because a functional stomach is absent in most marine fish larvae until metamorphosis is initiated (Govoni et al., 1986), trypsin is considered the main proteolytic enzyme in the larval stage. Trypsin is a serine protease that hydrolyzes peptide bonds on the carboxyl side of the basic AA. This enzyme is secreted from the pancreas as trypsinogen, an inactive form which is activated by enterokinase. In turn, this enzyme activates other pancreatic proenzymes.

1.6.2 Lipid

Lipids are considered particularly important during the larval stages, as the larvae require high amounts of energy and structural components for growth and development. The enzymatic actions of bile salt- activated lipase (BAL) play an important role in the hydrolyzation and digestion of lipids in fish. According to Sæle et al. (2010), BAL is the only neutral digestive lipase found in fish. This enzyme is a glycoprotein with broad substrate specificity towards triacylglycerols, phospholipids, cholesterol esters and lipid soluble vitamins. Marine fish prey organisms have shown to contribute significantly with dietary wax esters and triacylglycerols, which are high in PUFA (Cowey and Sargent, 1977). The PUFAs include eicosapentaneoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3). Murray et al. (2003) observed that specific activities of BAL in larval *Pseudopleuronectes americanus* (winter flounder) increased with age. Similar results have been found in larval *Scaeniops ocellatus* (red drum), where specific activity increased after 10 dph and onwards. However, the activity decreased after a peak on 18 dph (Lazo et al., 2007). The specific activity of BAL in larval *G. morhua* seems to be stable with a slight decrease during the first 60 dph. However, molecular analysis of BAL mRNA in the same trial showed a significant upregulation of the gene at around 60 dph (Sæle et al., 2010). Another

trial on BAL mRNA in larval *G. morhua* showed opposite trends, with a peak at 20 dph followed by a significant decrease to low expression values from 30 dph and onwards (Kortner et al., 2011).

A second lipolytic enzyme which is responsible for the digestion of dietary PL (phospholipids) in the small intestine of adult fish, is a group of enzymes called $sPLA_2$ (secretory phospholipase A_2). These enzymes belong to the phospholiase A_2 superfamily, in which it is the secreted group ($sPLA_2$) along with four other distinct groups; the cytosolic ($cPLA_2$), the Ca^{2+} independent (iPLA2), the platelet- activating factor acetylhydrolases (PAF-AH) and the lysosomal PLA_2s (Schaloske and Dennis, 2006). The $sPLA_2$ enzyme is an anionic zymogen that is activated by tryptic cleavage in the presence of calcium ions. This group of enzymes hydrolyzes fatty acids from the sn-2 position of glycerophospholipids, yielding a lysophospholipid and a fatty acid (Schaloske and Dennis, 2006). Activity of these enzymes has been detected in several fish species (Ozkizilcik et al., 1996, Iijima et al., 1997, Izquierdo and Henderson, 1998). Hama (2009) found that intestinal phospholipase activity is not dependent on pancreas function in *Danio reiro* (zebrafish), indicating a requirement of calcium ions for catalysis. The $sPLA_2$ enzymes consist of ten identified isozymes (Murakami and Kudo, 2002), one of which is the $sPLA_2$ group 1B with a distinct feature called the pancreatic loop. This group was cloned from the hepatopancreas of *Pagus major* (red sea bream) and consists of three isoforms (Iijima et al., 2001). A characterization of activity in $sPLA_2$ 1B, both on a genetic and protein level, was recently performed on *G. morhua* by Sæle et al. (2011), showing positive correlation between the two. The total enzyme activity remained stable and relatively high during the larval stages, until a sudden decrease at the onset of weaning occurred. Kortner et al. (2011) measured a different mRNA expression profile of $sPLA_2$ 1B in the same species, where a significant upregulation occurred at 20 dph and onwards to 60 dph. This discrepancy could be due to different feeding regimes in the two trials, enabling the possibility that $sPLA_2$ 1B mRNA expression is regulated according to the diet (Kortner et al., 2011).

1.6.3 Carbohydrate

The availability of carbohydrates for energy is generally low in the marine environment. Omnivorous and herbivorous species are capable of digesting a greater portion of carbohydrate-containing feeds. This is reflected in anatomical and physiological differences of the gastrointestinal tract (GI- tract) (Krogdahl et al., 2005). Carbohydrates are applied in commercial

feeds primarily as a source of energy and as binding components in the pellets. Economically, the use of carbohydrates is advantageous compared to proteins and lipids, because it is available in great quantities at low prices (Krogdahl et al., 2005).

Amylase activities in newly- hatched larvae have been detected in species such as *D. labrax*, *Solea senegalesis* (sole) and *S. ocellatus* (Lazo et al., 2000, Ribeiro et al., 1999, Infante and Cahu, 1994a). Péres et al. (1998) observed a difference in the expression of amylase mRNA during ontogeny of larval *D. labrax*, where a sudden decrease was observed after 29 dph. A recent study have shown high activities of amylase in larval *P. splendida* until 15 dph and onwards, when a sudden decrease was observed (Uscanga-Martinez et al., 2011).

Chitin (1.4β-*N*-acetyl glucosamine) is the second most abundant carbohydrate after cellulose, as it occurs in bacterial cell walls and plants, in the exoskeleton of arthropods and cuticles of annelids and mollusks (Krogdahl et al., 2005). It is considered likely that fish feeding on crustaceans, such as *L. bergylta* will encounter chitin in their diet. Evidence of endogenous production of intestinal chitinase has been observed in *G. morhua* (Danulat, 1986). Chitinolytic enzyme activities have been detected in *Coryphaenoides rupestris* (roundnose grenadier) (Fange et al., 1979) and in the stomach of *Pennahia argentatus* (silver croaker) (Ikeda et al., 2009). Three different chitinase genes were indentified in *Paralichthys olivaceus* (japanese flounder), where it was suggested that two of the genes encoded dietary chitinases (Kurokawa et al., 2004).

Studies on larval digestion and absorption of carbohydrates are relatively scarce. This is mostly due to the importance of lipids and proteins in the larval diet. However, larvae feeding on copepods might require a chitinase- like enzyme that aid in the digestion of these species, since they are covered by a chitinous exoskeleton (Klusemann et al., 1990). The identification of a chitin degrading enzyme in marine fish larvae could be helpful in many ways, i.e. when selecting live prey for cultivation containing more or less chitin. This enzyme would also be important to adult *L. bergylta* when feeding on sea lice attached to *S. salar* in commercial farms.

1.7 Exogenous contribution by live prey

Exogenous contribution of digestive enzymes from prey animals, especially in the larval phase, has been a topic of discussion in the later years, but no conclusive results have been made. Fish larvae may have low proteolytic and lipolytic capacities, which make them more dependent on an exogenous source of such enzymes. Several authors have suggested that this contribution takes place in many species (Dabrowski and Glogowski, 1977, Lauff and Hofer, 1984), but other authors have opposed this suggestion, stating that the contribution is negligible (Cahu and Infante, 1997, Kurokawa et al., 1998, Gawlicka et al., 2000, Perez-Casanova et al., 2006). A trial that solely focuses on the question of exogenous contribution from live prey is thus clearly needed, where enzymatic activities are compared between larvae fed live prey and larvae fed a compound diet. Also, a comparison of fed and unfed larvae would provide more information, as the unfed larvae has no gut content or possible exogenous enzymes present. Such knowledge would also be beneficial in relation to the digestive system of the species in question and its adaption to the live prey.

1.8 Aims of the study

In this study we wished to elaborate the ontogeny of genes coding for key digestive enzymes related to the pancreas in larval *L. bergylta*. We also wanted to compare enzyme activities and gene expression in larvae fed live prey (*B. plicatilis*) with larvae fed a compound diet, as well as contrasting fed and unfed larvae.

To describe the larval ontogeny we:

- Sampled larvae from four similar production tanks at MH Labrus, ranging from 2-55 dph in age, and examined the mRNA- expression of enzymes related to the pancreas mediated digestion of proteins, lipids and carbohydrates. The expression profiles of the following genes were analyzed by qPCR; amylase, pancreatic chitinase, trypsin, $sPLA_2$ 1B, BAL and Cyp7 A1.

To investigate the effect of live prey and formulated feed we:

- Compared enzyme activities in successfully weaned larvae, rotifer- fed larvae, and unfed larvae of both groups. Activities in enriched rotifers were also measured for comparison. Enzyme activity measurements were done spectrophotometrically of the following enzymes; amylase, chitinase, trypsin, $sPLA_2$ and neutral lipase.
- Analyzed mRNA- expression of the same genes as in the ontogeny series and compared these to the corresponding enzyme activities.

2 Materials & methods

2.1 Background

The sampled larvae were produced in Marine Harvest Labrus' facilities during the first weeks of June 2011, as a part of the second 2011 larval production cycle. The broodstock fish were caught from the wild (in the waters around Hellesøy, Øygarden) using specialized gill nets a few weeks prior to spawning and transferred to the facility. At the predicted time of spawning, specialized mats of Astroturf (approx. 0.5 m^2) were placed on the bottom of eight spawning tanks late afternoon (after feeding and cleaning) and left overnight. The spawning tanks each contained approximately 50 adult fish and ~ 4 males per female. The following day, any mats containing ≥ 10.000 eggs were removed and incubated in a separate room. Mats collected on the same day, from any of the spawning tanks, were placed in an incubation tank and disinfected every second day prior to hatching and once more upon transfer to the larval rearing room. The larval rearing room contains eight production tanks (9 m^3) and each tank was stocked with approx. 1 million larvae. Each day of spawning did not give a net production of this many larvae, therefore 1-3 days of age difference were common in each rearing tank. The number of possible parent fish for each tank was thus ~400, although the actual number of fish most likely was significantly lower.

2.2 Ontogeny series

2.2.1 Set- up and sampling

The sampling took place once a week from 16th of June to the 5th of August 2011, on larvae aged 2- 55 dph. Initially, samples were collected from a total of eight production tanks within the facility. Throughout the sampling period mass mortality was observed in some of the tanks, and trials with different treatments (light regime, feeding regime/amount) were performed on others. Four tanks were therefore selected for further analyses due to similar treatment conditions. One of these tanks (tank 6) had no clay added from 29 dph and onwards, making it a pseudo replicate compared to the three other tanks. The other tanks had clay added until 48 dph. The larva in another tank (tank 3) was batch fed six times a day with three hour intervals at 38 dph, as opposed to continuous feeding. The amount of total feed/day was similar to the other tanks. The total number of proper replicates was considered to be 2, whilst 4 if the pseudo replicates were included (see discussion). The age of the larvae varied with 3 days between the tanks, hence the age interval at the sampling points (fig. 2.1).

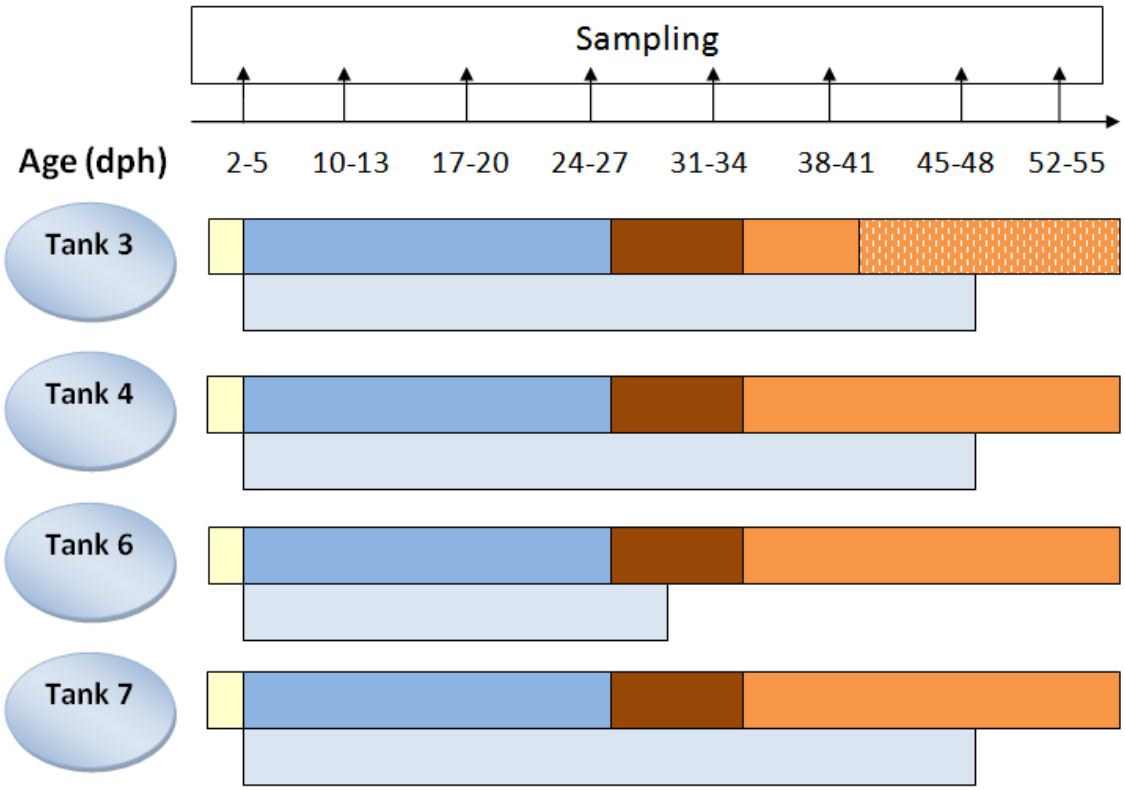

Figure 2.1: Sampling overview for the ontogeny series. Samples were taken from tank 3, 4, 6 and 7. Grey bars indicate the period when clay was suspended in the individual tanks. In tank 3, 4 and 7 the clay was added between 4- 48 dph, and in tank 6 between 4- 29 dph. The connected bars with different colours indicate feeding regimes. Yellow bar: endogenous yolk- sac stage (0-4 dph). Blue bar: rotifer feeding stage (4- 27 dph). Dark red bar: gradual weaning from rotifers to formulated feed (GM 150). Orange bar: formulated feed stage (34 dph and onwards). A different feeding regime was applied on tank 3 at 38 dph, hence the scattered white dots. Vertical arrows with connected age intervals indicate sampling points, as larvae were of different age at a given sampling date.

In order to get a representative sample of a tank, several locations were reached by using a cylindrical glass tube (~1.5 m length) and a small rake (5-10 cm^2). Larvae and water were sucked into the glass tube by lowering the pole into the water whilst covering the upper end with the thumb. The small rake was used in order to collect surface and near- surface dwelling larvae. Most of the sampled larvae were in aggregations because that was where the healthiest fish with the highest growth and survival rates were swimming (Grøtan E., pers. comm., 2011). The collected larvae were mixed gently and sedated with MS 222 (Western Chemical Inc.,

Washington, USA). Larvae for RNA extraction and subsequent quantitative real- time PCR analysis (n=10-30, depending on size) were collected and transferred to RNase/DNase free 1.5 ml eppendorf tubes (with holes in the lid) and flash frozen in liquid nitrogen. Later, the samples were stored at -80 °C awaiting further analysis. Samples for measurements of standard length (SL) and myotome height (MH) (n=10-20, depending on size) were collected in batches of five, photographed under a stereomicroscope (Motic Images Plus, ver. 2.0, Motic®, Xiamen, China) and flash frozen and stored in the same way as the RNA- samples. All sampling were performed late afternoon (16.00-17.00), right after the 16.00 rotifer feeding. ImageJ (NIH, Maryland, USA) was used to measure SL (distance from snout to posterior end of last vertebra) and MH (vertical distance from anus ventrally to the end of myotome segment dorsally) of larvae in the pictures.

2.2.2 Larval rearing and live feed

The temperature of each larval rearing tank was 16.0 ± 0.2 °C with a flow of 40-70 l/min (increasing flow with larval age). Continuous light (24 hours/day) was applied during the whole larval period. The temperature in all tanks was lowered to 15.0 °C at the 12th of July (28-37 dph). Enriched rotifers (*Brachionus plicatilis*) were fed to the larvae from 4 – 33 dph. The rotifers were cultivated and enriched within the facility. They were continuously fed *Chlorella sp*. (1.8 ml/million rotifers/day) and enriched with Multigain® (BioMar, Myre, Norway) (2.0 g/ million rotifers or 300-500 g/m^3). Before initiation of weaning, four batches of enriched rotifers were fed to the larvae per day at specific times (08.00, 16.00, 20.00 and 02.00). A weaning period or co-feeding onto a formulated diet (Gemma Micro, Skretting, Norway) overlapped with rotifer feeding from 27 – 34 dph, by gradually increasing the formulated feed: rotifer ratio. At the time of first feeding, suspended inorganic clay (K148, Alt for Keramikk AS, Nøtterøy, Norway) was added to each tank as a way of lowering bacterial numbers compared to the use of algae (Björnsdottir et al., 2011) whilst sustaining normal grazing behavior in the tanks (Muller-Feuga, 2000). The larvae were kept at the larval room until after metamorphosis.

Daily routines included cleaning the tank bottom, siphoning out and estimating mortality, removing waste material from the skimmer, careful flushing (especially when larval size is small), hand- feeding three times a day (30-50 g x 3) and general cleaning. Of the tanks which it was sampled from, antibiotic treatments were performed twice on tank 4 (9, 19 dph) and three times on tank 6 and 7 (6, 11/12, and 21/22 dph). Formaldehyde (CH_2O) treatment was performed on tank 4 at 52 dph, due to high mortality rates.

2.3 The feeding trial

The feeding trial was performed at Marine Harvest Labrus´ facilities from 11th- 18th of July 2011, in a specialized experimental room. The trial lasted for a total of 8 days. Weaning *L. bergylta* larvae at MH Labrus had not previously been performed successfully, the weaning strategy (rotifer amount, dry feed/day, hand feed) was therefore based on the strategy in the production tanks (see table A.1 in appendix). The amount of feed given was based on the volume of the tanks, and was not regulated according to the number of larvae in a given tank. This was done to assure that the larvae were fed in excess during the whole feeding trial. The different environmental parameters (flow, temperature, larval density) were set according to previous similar experiments at the facility of MH Labrus.

Enzymatic activities of the following five digestive enzymes were measured in the larvae; amylase, chitinase, neutral lipase, $sPLA_2$ and trypsin. Gene expression analysis of amylase, pancreatic chitinase, BAL, $sPLA_2$ 1B, trypsin and Cyp7 A1 was also performed.

2.3.1 Experiment

Randomly selected larvae (27 dph) were sampled from rearing tank 4 and transferred to six individual tanks (120 l, 1000 larvae/tank, 8.33 larvae/l). These were the same group of larvae as those sampled for the ontogeny series. Hence, the larvae in all six experimental tanks were from the same batch of eggs.

Larvae in three replicate tanks were fed only rotifers (6600 rotifers/larvae/day), and larvae in the three other replicate tanks were gradually weaned by co- feeding onto a formulated diet (Gemma micro® 150, Skretting, Stavanger, Norway) by means of a belt feeder, which was completed by day 7. The temperature of the tanks was 15.2-16.0 °C (105 % sat., 10.0 mg O_2 (measured day 8)), and the flow in all tanks were 4 l/min. No algae or clay were suspended in the water during the experiment, as opposed to the clay used in the production tanks. Dead larvae were siphoned out and counted each day, along with biological waste (faeces and sedimented feed) (fig. 2.2).

2.3.2 Sampling

Fig. 2.2 illustrates the experimental set- up and sampling. After completion of weaning at day 7, samples were taken (W, R) from all tanks and a period of 24 hours without feed was initiated, following a second sampling of unfed larvae (WU, RU). The samples were of individual larvae and representative of the larval population in each tank, except for the weaned fish (W). Here,

only about 10 % of the larvae had full stomach content. In order to fully investigate enzyme activity in the gut of these fish, their gut needed to be filled with feed. Fed and unfed larvae were easily distinguishable by eye, and only fed larvae were selected for the samples. Therefore these samples were not representative for each tank, and the sample sizes were also reduced due to the lack of fed larvae. The second sample was of the starved larvae and was assumed to be representative of each individual tank. A reference sample of enriched rotifers was taken simultaneously with sampling E. The rotifers enzyme activity was later compared with enzyme activities in the sampled larvae.

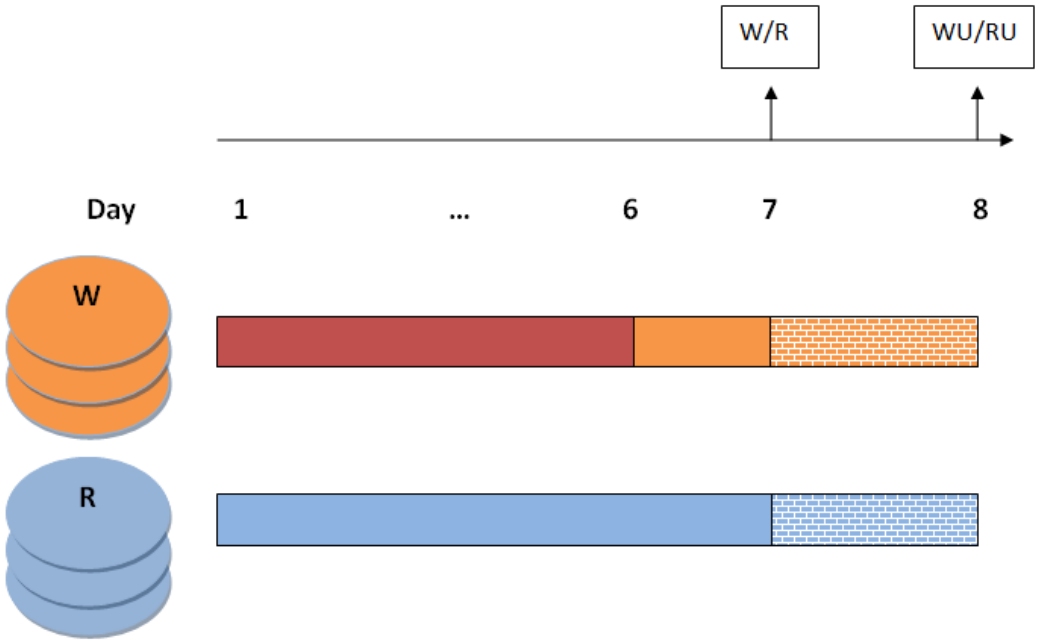

Figure 2.2: Set- up of the feeding trial. Orange and blue circles indicate three replicate tanks, which was gradually weaned (W) and fed only rotifers (R), respectively. Vertical arrows indicate sampling points from both groups, where fed larvae was sampled at day 7 (W/R) and unfed larvae sampled at day 8 (WU/RU). Dark red bar: gradual weaning onto GM 150 from day 1- 6. Orange bar: only GM 150 feeding. Blue bar: rotifer feeding from day 1-7. Bars with pattern (both colors): no feeding from day 7-8 (approx. 24 hours).

2.4 Gene expression analyses

2.4.1 RNA extraction and purification

Every step of every procedure was performed under sterile conditions to avoid contamination of the samples. This involved the use of plastic gloves (Sempermed, Wien, Germany) and a lab coat within the molecular lab. The working area was treated with RNase Zap® (Sigma- Aldrich, Missouri, USA) to avoid contamination from ribonuclease (RNase), a RNA degrading enzyme.

To extract mRNA from the samples, several steps were necessary. First, 1.0 ml of Qiazol Lysis Reagent® (Qiagen, Hilden, Germany) and 5 ceramic beads were added to each tube. The samples were then homogenized as quickly as possible, to avoid RNA degradation. This was done by the "precellys program" (AH Diagnostics, Aarhus, Denmark); 6000 rpm, 3 x 15 seconds, 10 second pauses between each shaking.

After 5 minutes in room temperature, 200 µl of chloroform were added to separate RNA from proteins and DNA. Then the tubes were shaken by hand for 15 seconds, following another 2-3 minutes in room temperature. In order to completely separate the RNA from the rest of the sample, 15 minutes of centrifuging (12 000 x g, 4 °C) was necessary. The upper transparent layer (supernatant) containing the RNA was transferred to 1.5 RNase free tubes, and 500 µl of isopropanol were added to each tube in order to separate the RNA from the watery phase. The solutions were mixed by turning the tubes up and down a couple of times. Further incubations included 10 minutes in room temperature, followed by 10 minutes at 4 °C. Thirty minutes of centrifuging (12 000 x g, 4 °C) was necessary in order to produce an RNA pellet.

The supernatant was removed from the samples by means of a vacuum suction pipette. To wash the remaining pellet, 1 ml of pre-cooled 75 % EtOH with DEPC was added, and vortexing caused the pellet to detach from the bottom of the tube, followed by 10 minutes of centrifuging (7 500 x g, 4 °C). A thorough second removal of the supernatant with the vacuum suction pipette was then performed, focusing on removing as many drops of supernatant as possible. At last, 50- 250 µl ddH_2O were added, following homogenization by vortexing. The samples were stored in a -80 °C freezer for later analysis.

2.4.2 DNA- free kit

In order to remove remaining DNA, the samples were treated with DNase I enzymes. Firstly, 10 x DNase buffer I (0.1 x sample volume) and 1-2 µl DNase I (depending on amount of RNA) were added to each sample, mixed carefully and incubated in 37 °C for 30 minutes in a heating cabinet. After incubation, 0.1 x sample volume of homogenized DNase inactivation reagent (pre-vortexed) was added. The samples were then stored in room temperature for 1 minute, before the supernatant was transferred into new RNase free 1.5 ml tubes.

2.4.3 RNA precipitation

All samples were precipitated as a part of the process of purification. Various amounts of 3M NaAc (pH 5.2) and 75 % EtOH were added according to initial sample volume. For NaAc, 0.1 x initial volume was added, and for EtOH 2.5 x total volume (sample + NaAc) following careful vortex of the samples. All samples were then incubated at – 80 °C overnight. On the following day, the samples were washed using the same procedure as for the washing of the initial RNA pellets.

2.4.4 RNA concentration and purity

The concentrations and purity of the RNA in the samples were measured with a Nanodrop 1000 (Saveen Werner, Limhamn, Sweden) full spectrum spectrophotometer. It measures the absorbance of 1 µl of solution at 260 nm, with a detection range from 1.5- 3000 ng/µl RNA.

Concentration:

- [RNA] (ng/ µl) = A_{260} x 40 ng/ µl

Quality/purity:

- $A_{260/280}$ ratio: This indicates the purity of the sample and should be around 2.0, but can range from 1.8- 2.2. If the numbers are not within this range, the sample could contain leftover DNA, proteins or phenol.
- $A_{260/230}$ ratio: The value should be >1.8. A lower number could imply that the sample contain leftover salt or phenol.

If the numbers were not satisfactory, it was necessary to precipitate the samples as described in section 2.4.3.

2.4.5 RNA quality

In order to allow further analyses in Real Time PCR, the quality of the RNA samples need to be adequate. By using the RNA 6000 Nano LabChip Kit (Agilent Technologies, California, USA, art. nr. 5065-4476) the integrity (RIN – RNA Integrity Number) of the RNA, ranging from 1-10, can be determined. A sample with a RIN value of >8.0 is considered adequate for use in RT-PCR reactions (Imbeaud et al., 2005). A sample with a poor RIN value will indicate degradation of the total RNA in the sample. In addition to the RIN value, the Agilent 2100 BioAnalyzer gives a gel photo and an electropherogram (graph) (see appendix fig. A.2). The RNA labchip contains micro channels used for separation of nucleic acid fragments by electrophoresis, based on fragment size. The RNA samples used were randomly selected. The concentration of the RNA needed to be in the range of 25-500 ng/µl, it was therefore necessary to know the concentrations in the initial samples beforehand. Knowing the initial concentrations, one can calculate how much ddH_2O is needed in order to dilute a 1-2 µl volume of RNA to the preferred concentration in 1.5 ml RNase free tubes.

Prior to the analysis a few preparing steps were required. Stock solutions of 550 µl RNA 6000 Nano Gel matrix were prepared beforehand by centrifuge (15 000 x g in 10 min), and stored in aliquots of 32.5 µl in RNase free tubes in 4 °C. The Gel- Dye mix consisted of the 32.5 µl, pre-equilibrated (30 min in 25° C) gel matrix and an added 0.5 µl dye concentrate. This mix was centrifuged for 10 minutes in 13 000 x g. The electrodes in the bioanalyzer machine were decontaminated for 1 minute using a specialized cleaner- chip containing 350 µl RNase Zap, and thereafter by a different cleaner-chip containing 350 µl ddH_2O for 10 seconds. After the removal of the last chip, the lid was left open for 10 seconds so that the remains of the ddH_2O on the electrodes evaporated.

The Nano Chip was placed in a specialized Chip Priming station, and 9.0 µl of the gel- dye mix were pipetted into the bottom of the well marked G (fourth column, third row). The well was then pressurized for 30 seconds using a plunger in the priming station, so that the gel dye- mix was spread along the micro channels in the chip. Then, another 9.0 µl were pipetted into each of the two wells marked G (fourth column, first and second row). Exactly 5.0 µl Nano Marker were pipetted into the well market with a ladder (fourth column, fourth row) and 5.0 µl in each of the 12 sample wells. Subsequently, 1.0 µl of pre- denatured (3 minutes at 70 °C) RNA molecular

weight marker (Invitrogen, Carlsbad, USA) into the well marked with a ladder. The RNA samples were also heat denatured before loaded into the chip, and 1 µl of each sample were loaded into each well (marked 1- 12). The chip was then vortexed for 1 minute at 2400 rpm. This was done in order to mix the reagents. The chip was then ready to be put into the bioanalyzer. The electrodes in the bioanalyzer were decontaminated using ddH_2O water by means of the specialized cleaner chip.

2.4.6 Primer preparations

Genes were identified in a contig assembly that has been based on transcriptome sequences (454 GS- FLX, Roche Applied Sciences, Basel, Switzerland) of a pooled sample of three fish homogenates. Gene identification was based on best BLAST® (NCBI, Maryland, USA) hit.

Gene specific primers were designed using Primer 3 (Rozen and Skaletsky, 2000). Primers were designed for the mRNA sequences for amylase, Cyp7 A1, pancreatic chitinase, BAL, trypsin, and sPLA2 1B. RPL37 and ubiquitin were chosen as reference genes based on (Sæle et al., 2008) (table 2.1).

Table 2.1: Genes used in qPCR: their amplicon size (bp) primer sequences and efficiencies. Ubiquitin and RPL37 were used as reference genes.

Gene	Amplicon size (bp)	Forward sequence	Reverse sequence	Mean efficiency from qPCR
Amylase	114	GCGTGGATAGCGACGAAGGGG	TGGCCAGAAGGACGGAGGCA	1.821
BAL	171	TAGCTGGAGATGCTGTGGTG	AACCGGCCGTACTTACTCCT	1.842
Cyp7 A1	160	GTGTGGCGCTTTCCCCTTGGT	CCGTTCTCCACGGCAGGCTC	1.986
pChitinase	186	CTTGCAGGTTTCTGGCTTTC	GGTGACCAACTCGTTTGCTT	1.941
sPLA2 1B	170	CTGCTTCTGTGGGAAAGGAG	TCTTGTTTGCCTCGTCACAG	1.825
Trypsin	162	CGATCAGGGTAGTTGCTTCC	CCTGGACAATGACATCATGC	1.848
Ubiquitin	183	GGCCAGCTGTCTGAGAGAAG	GTCAAGGCCAAGATCCAAGA	1.961
RPL37	140	CCTCCGTGTTTCACTGGGCAGAC	ACCCAGACGTGCTGCAGTGG	2.010

2.4.6.1 Primer specificity

Specificity of primers was tested by means of the OneStep RT-PCR kit (Qiagen, Hilden, Germany), a kit that enables the user to perform both reverse transcription and PCR amplification in one step. Aliquots of forward and reverse gene specific primers all had a concentration of 50 µM, and RNA- samples and reagents were thawed on ice and mixed prior to use. Every step of the procedure was performed in an RNase free environment.

The primers were diluted with TE buffer to a concentration of 0.05 nmol/µl. They were then vortexed for 15 seconds and stored in 50 µl aliquots (50 µM) at – 20 °C. This was done to avoid unnecessary thawing and freezing that might cause degradation of the primer DNA. An RNA pool from 25 randomly selected samples (2 µl from each) was prepared and the concentration measured in Nanodrop 1000.

A master mix was prepared in a volume according to the number of primers being tested, plus a 10 % surplus. The master mix contained all reagents and the pool of RNA. RNase- free tubes (0.2 ml) for the RT and PCR reaction were prepared and 24 µl of master mix were pipetted into each tube. The forward (0.5 µl) and reverse (0.5 µl) primers of each gene being tested were then added to each of the 0.2 ml tubes (for overview of reagents, see appendix table A.2).

The thermal cycler was programmed according to the set- up listed in appendix, table A.3. The samples were then either stored at -20 °C or prepared for electrophoresis in agarose gel.

2.4.6.2 Gel electrophoresis

The electrophoresis enables a separation of cDNA fragments by size in an agarose gel. The cDNA fragments are negatively charged and will therefore be drawn towards a positively charged pole. The PCR product (cDNA) was run on a 1 % agarose gel, using Gel Red Nucleic Acid Stain (Biotium, California, USA) as a dye. The running buffer consisted of 1x TAE buffer. One control well was filled with 6 µl 50 bp DNA ladder (Biolabs, London, UK), and the remaining wells were each filled with 8 µl of the product being tested and 2 µl loading buffer (Blue juice®, Invitrogen, Carlsbad, USA). The gel was run at 80 V for approximately one hour. After the run, the gel was visualized and photographed in UV- light (fig. 2.3). All primers were tested.

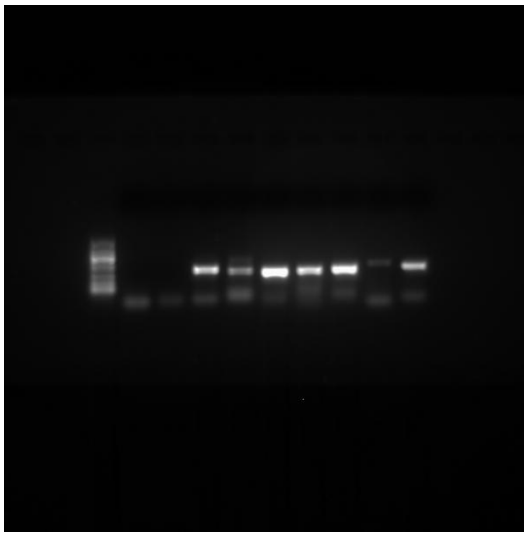

Figure 2.3: Separation of primer cDNA fragments by size, viewed in a gel photo. From left; lane 1: control well, lane 2: amylase, lane 3: Cyp7 A1, lane 4: pancreatic chitinase, lane 5: $sPLA_2$ 1B, lane 6: trypsin, lane 7: BAL, lane 8: ubiquitin, lane 9: RPL37, lane 10: ELF A1. Primers in lanes 4, 5, 6, 7, 8 and 10 were chosen for qPCR based on this separation.

2.2.7 Reverse Transcription (RT) PCR reaction – from RNA to cDNA

By means of the enzyme reverse transcriptase, the sample RNA is converted into complementary DNA. This is necessary in order to run the RT- qPCR reaction. TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA) were used for the RT reaction mix.

Aliquots of forward and reverse gene specific primers all had a concentration of 0.5 µM, and RNA- samples and reagents were thawed on ice and mixed prior to use. Every step of the procedure was performed in an RNase free environment.

The standard curve was made out of a pool 2 µl of each of the RNA samples (except from two samples with low concentrations). By means of Nanodrop 1000, the pool was then used to make a standard curve with six serial dilutions in triplicates, with concentrations ranging from 3.1- 100 ng/ µl/ well (± 1 %). The 45 individual samples were set up in triplicates on two 96- well plates (Roche Applied Sciences, Basel, Switzerland) with a concentration of 50 ng/ µl/ well (± 2 %). Two negative controls on each plate were prepared; the non- amplification control (nac) (no enzymes) and the non- template control (ntc) (ddH_2O instead of RNA). Table A.4 in appendix shows volumes and reagents required for the reaction mix (50 µl/ well reaction). A master mix comprising of the non- enzymatic reagents were firstly made and vortexed. Secondly, the

enzymes were added and carefully mixed. The 40 µl reaction mix was then added to each well following the addition of 10 µl RNA sample.

Newly cleaned 96- well plate covers (washed with soap, ddH_2O and 70 % EtOH and dried - respectively) were placed on top of the cDNA- plates. The plates were then centrifuged for 1 minute at 50 x g. The plates were then ready for the PCR- reaction which was performed with the GeneAmp PCR 9700 (Applied Biosystems, Foster City, UA, USA). Table A.5 in appendix shows the program used for the reaction. The duration of the program was 75 minutes, and the RT-product (cDNA plate) could be stored in the machine for up-to 24 hours (hence the ∞). The finished cDNA plates were covered with tape pads (ABgene, Surrey, UK) and stored at -20 °C until used for real- time quantitative PCR.

2.2.8 Real Time quantitative PCR (qPCR)

The cDNA plates from the RT-PCR functioned as a template for the qPCR reaction. The following method was used to give a relative quantification of the target gene by measuring cDNA amplification and fluorescence. The reaction mix used was a product from Roche Applied Sciences (Basel, Switzerland) which included CYBR green master®. This dye is fluorescent when conjugated to the cDNA. The quantity of cDNA is expressed as the cycle threshold value (C_t) – the number of heating/cooling cycles it takes before the increase in fluorescence is linear.

The cDNA plates were thawed on ice before adding 50 µl ddH_2O to each well, bringing the total volume per well up to 100 µl. Each plate was then centrifuged at 1000 x g for 1 minute and vortexed at 1100 rpm for 3 minutes. Table A.6 shows the amounts of reagents needed for each well for a 10 µl reaction in Light Cycler 480® RT- qPCR system (Roche Applied Sciences, Basel, Switzerland).

A pipetting robot (Biomek® 3000, Beckman Coulter, California, USA) was programmed to transfer 8 µl reaction mix and 2 µl cDNA from a single well to each well on a 384- well qPCR plate. Two target genes and two cDNA plates were pipetted onto one qPCR plate at a time, a process lasting for approx. 20 minutes. After transfer, the plate was covered by an optical adhesive cover and spun down (1500 rpm, 2 minutes). The plate was then put into the Light Cycler® 480 Real Time PCR System (Roche Applied Sciences, Basel, Switzerland). Table A.7 shows the set- up of the qPCR reaction and figure A.1 illustrates the temperature shifts of the

reaction. Results and calculations of standard curves and efficiencies were performed using the software Light Cycler® 480, version 1.5.039.

2.5 Enzyme activity assays

2.5.1 Sample homogenization

Samples from the feeding trial were homogenized in PBS- buffer in order to later determine larval enzyme activity spectrophotometry. The samples were kept on dry ice before homogenization and on regular ice during the remains of the procedure (see fig. 2.2 for overview of the trial).

The larval wet weight in milligram (mg) was determined using an ultra- sensitive weight (Mettler Toledo, Greifensee, Switzerland). The larvae were then transferred to 2.0 ml RNase/DNase- free tubes with round bottoms. Secondly, PBS- buffer comprising of four times the wet weight of each sample was added to get a 5x dilution, i.e.; 88.0 mg wet weight in a sample, 352 µl PBS- buffer added. Two steel beads were then added to each tube, and the tubes were homogenized in a Retch MM310® shaking device (Retch GmbH, Haan, Germany) (15 seconds x 3, 20 rotations per second). In order to separate the supernatant, a centrifugal step was required (12 x g, 4 °C, 15 minutes) before the supernatant was removed and put in six similar aliquots (volume is the same).

2.5.2 Amylase activity

The assay was performed according to the kit and protocol provided by abcam® (ab102523, Cambridge, UK, 2011) on a 96- well flat bottom plate specialized for colorimetric measurements.

Prior to the run, the amylase positive control bottle was dissolved in 50 µl assay buffer. Two positive control wells were filled with 5 µl amylase positive controls and 45 µl ddH_2O, and duplicate sample wells of 10 µl and 40 µl ddH_2O followed. A nitrophenol standard curve in duplicates contained various amounts of 2 mM nitrophenol standard mix to create 0, 4, 8, 12, 16 and 20 nmol/well. The wells were adjusted to a total volume of 50 µl with ddH_2O. For each well, a 100 µl reaction mix was prepared, containing 50 µl assay buffer and 50 µl substrate mix. Immediately (< 1 min) after the addition of reaction mix to the wells, the plate was placed in a Labsystems iEMS reader MF spectrophotometer (Labsystems Ltd, Ramat- Gan, Israel). The machine was programmed to shake the plate for 15 seconds (720 rotations min^{-1}) and thereby measure the absorbance at 405 nm each minute for 30 minutes in 25 °C.

The reaction linear range of the measurements was used to calculate the change in absorbance ($\Delta A_{405} = T_1 - T_0$). These values were applied to the plotted nitrophenol standard curve to get B nmol of nitrophenol generated by amylase between T_1 and T_0. According to abcam® (Cambridge, UK), one unit of amylase is the amount of amylase that cleaves ethylidene-pNP-G7 to generate 1 µmol of nitrophenol per min at pH 7.2 at 25 °C.

The following equation was used for the calculations of amylase activity (nmol/min/ml):

$$Amylase\ activity = \left[\frac{B}{T}\ x\ sample\ volume\ (ml)\right]\ x\ sample\ dilution$$

B: amount of nitrophenol calculated from standard curve (nmol)

T: time in minutes between T_1 and T_0

2.5.3 Chitinase activity

The assay was performed according to the assay kit and manual provided by Sigma- Aldrich® (St. Louis, Missouri, USA, catalog number CS0980). Three different substrates for the detection of chitinolytic activity were provided in this kit, and based on Gutowska et al. (2004), 4-Nitrophenyl N-acetyl-b-D-glucosaminide which detect exochitinase activity was chosen. The assay was performed on a 96- well flat bottom plate specialized for colorimetric assays.

The following reagents were prepared prior to the enzyme assay; stop solution (sodium carbonate dissolved in ddH_2O), substrate solution (1 mg substrate in 1 ml assay buffer), chitinase control enzyme (0.2 mg/ml, dissolved in PBS) and standard solution (5 µl 10 mM p-nitrophenol solution diluted with 995 µl stop solution). Blank reactions (100 µl substrate solution/well), standard reactions (300 µl standard solution/well) and positive controls (90 µl substrate solution and 10 µl 20- fold diluted chitinase control enzyme/well) were run in duplicates. The enzymatic samples were run in triplicates (90 µl substrate solution and 10 µl sample/well).

The substrate solution and standard solution were equilibrated by incubating for 5 minutes in a 37 °C water bath. Substrate solution and standard solution were added to their designated wells following the addition of the chitinase control enzyme and the enzymatic samples. The plate was shaken (15 seconds at 720 rotations min^{-1}) and incubated in 37 °C for 30 minutes in a Labsystems

iEMS reader MF micro reader (Labsystems ltd., Ramat- Gan, Israel). Stop solution (200 µl) was then added to each well except for the standard reaction wells, and the plate was placed back into the micro reader. The plate was shaken (15 seconds at 720 rotations $min^{-}1$) and the absorbance measured immediately at 405 nm. According to Sigma-Aldrich®, one unit chitinase will release 1.0 µmole of *p*-nitrophenol from the appropriate substrate at pH 4.8 at 37 °C. To calculate the enzymatic activity, the following equation was used:

$$Chitinase\ activity\ (units/ml) = \frac{(A_{405}sample - A_{405}blank)\ x\ 0.05\ x\ 0.3\ x\ DF}{A_{405}standard\ x\ time\ x\ V_{enz}}$$

A_{405} sample: absorbance of the sample at 405 nm

A_{405} blank: absorbance of the blank at 405 nm

0.05: µmole/ml of *p*- nitrophenol in the standard solution

0.3: final volume/well after all reagents are added

DF (Dilution factor): fold dilution of the original chitinase enzyme

A_{405} standard: absorbance of standard solution at 405 nm

Time: minutes into the reaction

V_{enz}: volume of the sample (ml)

2.5.4 Neutral bile- salt independent lipase activity

The method used by Murray et al. (2003), modified from Iijima et al. (1998) was used to measure the activity of neutral bile- salt independent lipase. The substrate solution consisted of 0.4 mM 4-nitrophenyl myrisate (Sigma- Aldrich, St. Louis, Missouri, USA). Other reagents included 25 mM ammonium bicarbonate, 0.125 M sodium cholate and 37.5 mM NaCl, giving the solution in each well pH 7.8 at 25 °C. The total assay volume in each well was 0.2 ml of which the enzymatic samples were 0.01 ml. The test samples were run in triplicates on a 96- well colorimetric assay plate on a micro reader (Labsystems iEMS Reader MF, Ramat- Gan, Israel) and the absorbance values read at 404 nm once every minute for 30 minutes at 25 °C.

The change in absorbance (A_{404}/min) was calculated in the reaction linear range. The following equation was used to calculate lipase activity:

$$Lipase\ activity\ (nmol/ml/min) = \frac{A_{404}/min}{2.2\ mM^{-1}}\ x\ \frac{0.20\ ml}{0.01\ ml}\ x\ sample\ dilution$$

A_{404}/min: the change in absorbance per minute at 404 nm

2.2 mM^{-1}: extinction coefficient for 4- nitrophenol under the assay conditions

0.20 ml: total assay volume/well

0.01 ml: volume of enzymatic sample/well

2.5.5 Phospholipase A_2 activity

The assay kit was provided by Cayman Chemical Company® (Michigan, USA, assay kit no. 765001), and the assay preparation, protocol and analysis was performed according to the manual provided together with the kit. The assay was performed using a 96- well plate specialized for colorimetric assay and a micro reader (Labsystems iEMS Reader MF, Ramat- Gan, Israel).

The blank wells were non- enzymatic controls. The absorbance rate measured in these wells was subtracted from the absorbance rate measured in the sample wells. Each blank well contained 10 µl 10 mM $sPLA_2$ DNTB and 15 µl assay buffer (pre- diluted with ddH_2O). Two positive control wells each contained 10 µl pre- diluted bee venom PLA_2 (1 µg/ml) and 10 µl 10 mM $sPLA_2$ DNTB. The sample wells contained 3 x10 µl (triplicates) of each sample, 10 µl DNTB and 5 µl assay buffer.

The substrate solution (1.66 mM $sPLA_2$ Diheptanoyl Thio- PC, pre- diluted in the diluted assay buffer, 200 µl/well) were added simultaneously to each well by using a multi- pipette after all other reagents were added, and initiated the enzymatic reactions. The plate was then placed in a plate reader. The plate reader was programmed to shake the plate (15 seconds at 720 rotations min^{-1}) and read the absorbance at 414 nm once every minute for 30 minutes at 25 °C.

The change in absorbance (ΔA_{414}) per minute was calculated from the linear portion of the curve, and the rate of ΔA_{414}/min in the non- enzymatic controls (blank wells) was subtracted from this rate in order to get a final value. According to Cayman Chemical Company® (Michigan, USA), one unit of enzyme hydrolyzes one µmol of diheptanol Thio- PC per minute at 25 °C.

A final calculation of $sPLA_2$ enzyme activity was done using the following equation:

$$sPLA_2\ activity\ (\mu mol/min/ml) = \frac{\Delta A_{414}/min}{10.66\ mM^{-1}}\ x\ \frac{0.225}{0.01}\ x\ Sample\ Dilution$$

A_{414}/min: the change in absorbance per minute at 414 nm

10.66 mM^{-1}: the extinction coefficient for DNTB at 414 nm under the assay conditions

0.225: total assay volume/well

0.01: volume of enzymatic sample/well

2.5.6 Trypsin activity

The trypsin activity assay kit and protocol was provided by abcam® (Cambridge, UK), and the assay performed on a 96- well colorimetric plate with flat bottoms.

Some of the reagents were in DMSO solution and was therefore warmed up to room temperature before use. These reagents included trypsin substrate, ρ-NA standard and chymotrypsin inhibitor. The positive control was dissolved in 100 µl assay buffer.

A standard curve was prepared in duplicates with various dilutions of 2 mM ρ-NA standard to create 0, 4, 8, 12, 16 and 20 nmol/well. The volume was adjusted to 50 µl/well with trypsin assay buffer. Duplicate sample wells were filled with 50 µl sample/well. The sample wells were treated with 1 µl 50X chymotrypsin inhibitor (TPCK) solution each and incubated for 10 minutes in room temperature. Two positive control wells were filled with 5 µl positive control solution and

adjusted to 50 µl with assay buffer. A 50 µl/well reaction mix containing 48 µl assay buffer and 2 µl trypsin substrate was prepared and pipetted into each of the wells on the 96- well plate. The plate was then placed in a Labsystems iEMS reader MF micro reader (Labsystems ltd., Ramat-Gan, Israel) which was programmed to shake the plate for 15 seconds (720 rotations min^{-1}) and then measure the absorbance at 405 nm for 30 minutes in 25 °C. The plate was then incubated in the dark (covered in aluminum foil) for 1 hour in room temperature, following a second shake and measurement of absorbance similar to the first.

The change in absorbance ($\Delta A_{405} = A_2 - A_1$) generated by cleavage of the substrate was measured in the linear range of the reaction. After subtracting the 0 nmol/well standard well absorbance from all readings, a standard curve was made from the values of the ρ-NA standard wells. The change in absorbance was then applied to the standard curve to calculate the amount of ρ-NA created between T_1 and T_2 in the sample wells. According to the abcam® kit (ab102531, Cambridge, UK, 2011), one unit is defined as the amount of trypsin that cleaves the substrate, yielding 1.0 nmol of ρ-NA per minute at 25 °C.

The following equation was used to calculate trypsin activity in the samples:

$$Trypsin\ activity = \left[\left(\frac{B}{T_2 - T_1}\right)\ x\ sample\ volume\ (ml)\right]\ x\ sample\ dilution$$

B: ρ-NA calculated from standard curve (nmol)

$T_2 - T_1$: Time of second reading – time of first reading

2.6 Statistics

Microsoft Excel was used to treat raw data both from the ontogeny series and the feeding trial. Ct- values from the qPCR were imported into Excel where relative quantities and standard deviations were calculated.

The values of the reference genes were compared in the program geNorm version 3.5 in order to find the genes with the most stable expression patterns in all the samples from the ontogeny series and the feeding trial (Vandesompele et al., 2002). Genes were chosen based on an expression stability value given by this program (M- value). The two reference genes Ubiquitin and RPL had an M- value of 0.218, which is considered satisfactory. From this, a gene expression normalization factor was made for each sample. The expression values were re- calculated by dividing each value by its respective normalization factor, giving the MNE- value (mean normalized expression).

Statistica 10.0 (Statsoft®, Oklahoma, USA) was used for statistical analysis of the results. Larval growth in the ontogeny series was estimated by means of a regression analysis, and differences were tested by means of analysis of covariance (ANCOVA) (SL as dependent variable, age as continuous predictor and tank as categorical independent predictor). One- way analysis of variance (ANOVA) were performed on the MNE- values from the ontogeny series. A two- way ANOVA was used to test for significant differences between treatments in the feeding trial (feed type and status as factors). Differences in final SL between weaned- and rotifer fed larvae in the feeding trial were tested by a one- way ANOVA. For all the tests, level of significance was set at $p \leq 0.05$. In case of significance, a Tukey- Kramer HSD multiple comparisons test was performed to investigate which specific means differed from each other. Analyses of homogeneity of variance were done by means of a Hartley F_{max}- test.

If the values from the enzyme activity measurements were below the manufacturers LOD, no statistical analyses were performed. This was the case both for the amylase and trypsin activity assays. Negative activity values from the trypsin assay were set to zero (0) as a simplification.

3 Results

3.1 The ontogeny series: growth

L.bergylta larvae showed an exponential growth pattern with a sharp increase in standard length after 35 dph, when the larvae had started ingesting formulated feed. First- feeding larvae (2- 5 dph) had a mean SL of 3.97 ± 0.25 mm (n=57) and at a mean SL of 14.43 ± 2.08 mm (n=40) at 52- 55 dph (fig. 3.1). The MH was first measured at 10- 13 dph. The larvae then had a mean MH of 0.36 ± 0.11 mm (n=28). At 38- 40 dph, the larvae had a mean MH of 1.41 ± 0.39 mm (n=40), and 2.09 ± 0.42 mm (n=10, only tank 3) at 47 dph (fig. 3.2). There were no significant differences between the mean SL of the tanks (ANCOVA, log SL, p=0.46) while the effect of age on SL was highly significant (ANCOVA, log SL, p<0.00) (fig. 3.1).

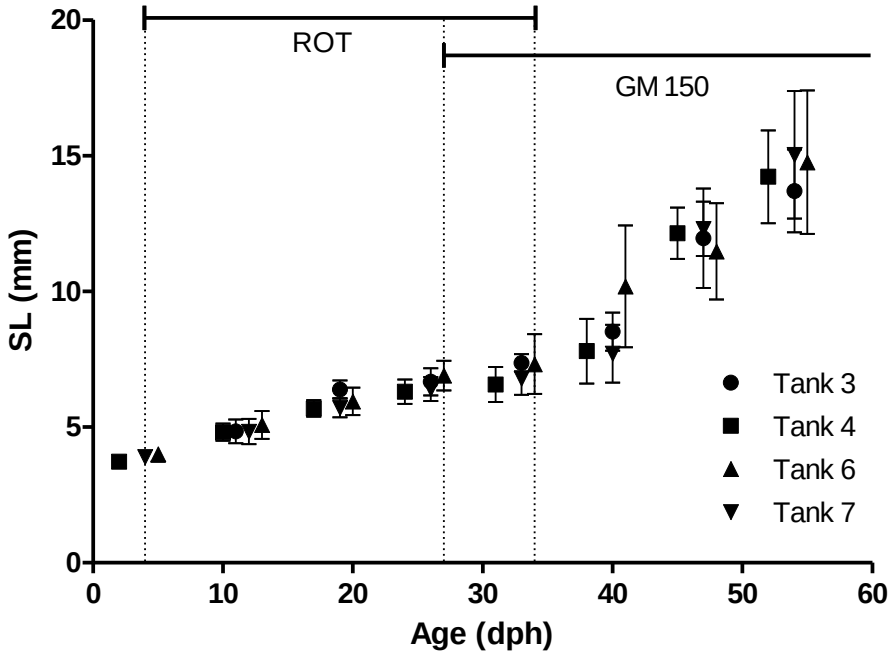

Figure 3.1: Mean SL ± SD during the first 60 dph in *L. bergylta* larvae (n=10-20, depending on size). The exogenous feeding regimes are shown, where rotifer feeding was initiated at 4 dph. Weaning was initiated at 27 dph. The larvae were then co- fed rotifers and GM 150 until 34 dph. From 34 dph and onwards the larvae were fed only GM 150. An exponential fit based on these values yielded the following formula for predicted SL at age: $y = 3.56 * \exp(0.0244*x)$, $r^2 = 0.89$.

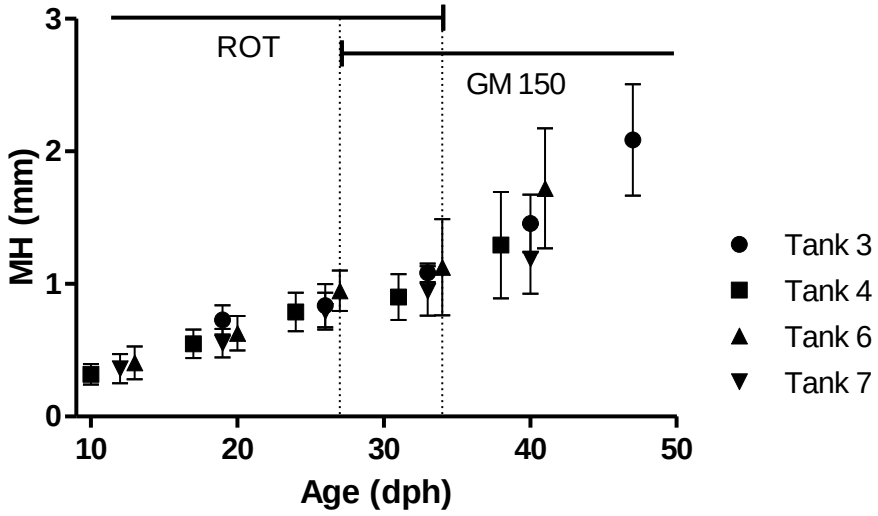

Figure 3.2: Mean MH ± SD of L. bergylta larvae from 10- 50 dph (n=10-20 depending on size). The exogenous feeding regimes are shown, where rotifer feeding was initiated at 4 dph, prior to MH measurements. Weaning was initiated at 27 dph. The larvae were then co- fed rotifers and GM 150 until 34 dph. From 34 dph and onwards the larvae were fed only GM 150.

3.2 The ontogeny series: gene transcriptions

3.2.1 Trypsin

The youngest larvae showed low genetic expression of trypsin but the expression increased up to a peak at 24-27 dph (0.21 ± 0.13 MNE). From there on, reduced expression values were observed on weaned larvae older than 30 dph, to the lowest levels at 45- 48 dph (0.02 ± 0.01 MNE) (fig. 3.3). The effect of age on gene expression was non- significant (one- way ANOVA, p=0.14).

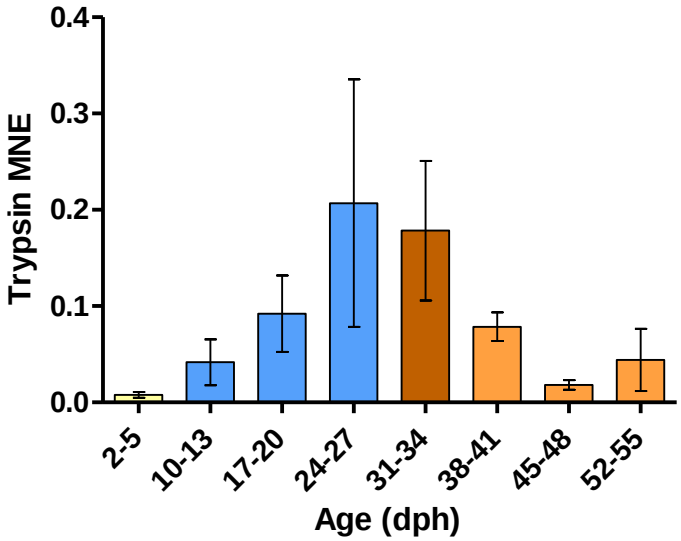

Figure 3.3: Mean MNE of trypsin ± SEM (n=4). Different colors indicate different feeding regimes. Yellow bar: yolk- sac larvae age 2-5 dph. Blue bars: rotifer fed larvae age 10-27 dph. Dark orange: larvae being co- fed rotifers and GM 150, age 31-34 dph. Orange: weaned larvae (100 % GM 150) age 38-55 dph.

3.2.2 Cholesterol 7- alpha- monooxygenase (Cyp7 A1)

Starting out with a low expression in yolk- sac larvae (0.2 ± 0.01 MNE), an increase in the relative expression to a stable ~0.2 MNE throughout the rotifer feeding stage was observed. A downregulation occurred just after initiation of weaning and shifting over to formulated feed, where the relative expression stabilized from 38- 41 dph at about 0.1 MNE (fig. 3.4).

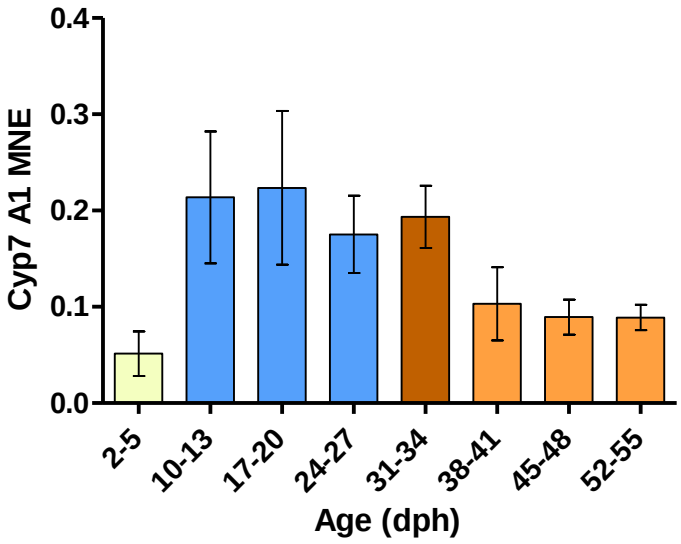

Figure 3.4: Mean MNE of Cyp7 A1 ± SEM (n=4). Different colors indicate different feeding regimes. Yellow bar: yolk- sac larvae age 2-5 dph. Blue bars: rotifer fed larvae age 10-27 dph. Dark orange: larvae being co- fed rotifers and GM 150, age 31-34 dph. Orange: weaned larvae (100 % GM 150) age 38-55 dph.

3.2.3 Neutral bile activated lipase (BAL)

The relative expression of this gene was close to zero until a sudden upregulation occurred at 31-34 dph (0.10 ± 0.06 MNE). From here on the expression values increased markedly with age to a peak at 45- 48 dph (0.96 ± 0.10 MNE) (fig. 3.5). The effect of age on relative expression was highly significant (one- way ANOVA, $p < 0.001$).

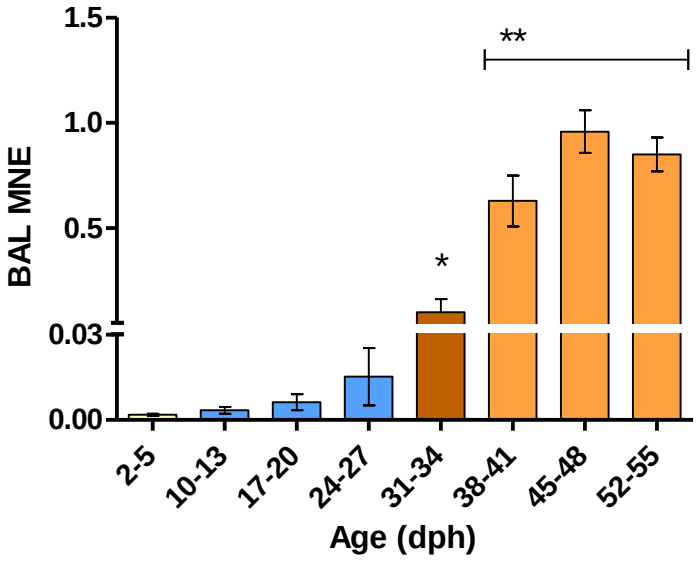

Figure 3.5: Mean MNE of BAL ± SEM (n=4). Different colors indicate different feeding regimes. Yellow bar: yolk-sac larvae age 2-5 dph. Blue bars: rotifer fed larvae age 10-27 dph. Dark orange: larvae being co- fed rotifers and GM 150, age 31-34 dph. Orange: weaned larvae (100 % GM 150) age 38-55 dph. A scale break was applied to the y- axis for a better overview. The lower scale ends at 0.03, and the upper scale starts at 0.05 MNE. Stars (*, **) denote significant differences between groups (ANOVA, Tukey HSD test, p<0.05).

3.2.4 Secretory phospholipase A_2 group 1B ($sPLA_2$ 1B)

An expression pattern similar to that of trypsin can be seen for $sPLA_2$ 1B in figure 3.6. An upregulation to a peak of ~0.2 MNE with high SEM at 24-27 (0.21 ± 0.13) and 31-34 dph (0.21 ± 0.11) was observed, followed by a downregulation of the gene by older larvae that were weaned onto formulated feed (fig. 3.6).

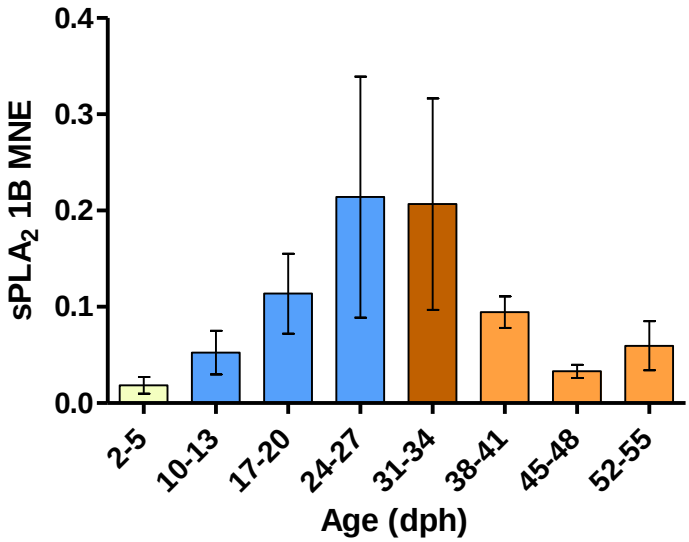

Figure 3.6: Mean MNE of $sPLA_2$ 1B ± SEM (n=4). Different colors indicate different feeding regimes. Yellow bar: yolk- sac larvae age 2-5 dph. Blue bars: rotifer fed larvae age 10-27 dph. Dark orange: larvae being co- fed rotifers and GM 150, age 31-34 dph. Orange: weaned larvae (100 % GM 150) age 38-55 dph.

3.2.5 Amylase

Amylase was detected prior to first- feeding (0.20 ± 0.10 MNE). A general increase in the relative gene expression of amylase towards 27 dph indicated upregulation of the gene by the larvae to a peak of 0.31 ± 0.05 MNE at 24- 27 dph. As the larvae grew older, especially from the moment the fish started eating formulated feed, there was a general downregulation of the gene expression, in which the lowest expression levels was observed at 45- 48 dph (0.05 ± 0.01 MNE). An upregulation was observed at 52- 55 dph (0.11 ± 0.06 MNE) (fig. 3.7). The effect of age on amylase MNE was not significant (one- way ANOVA, p=0.10).

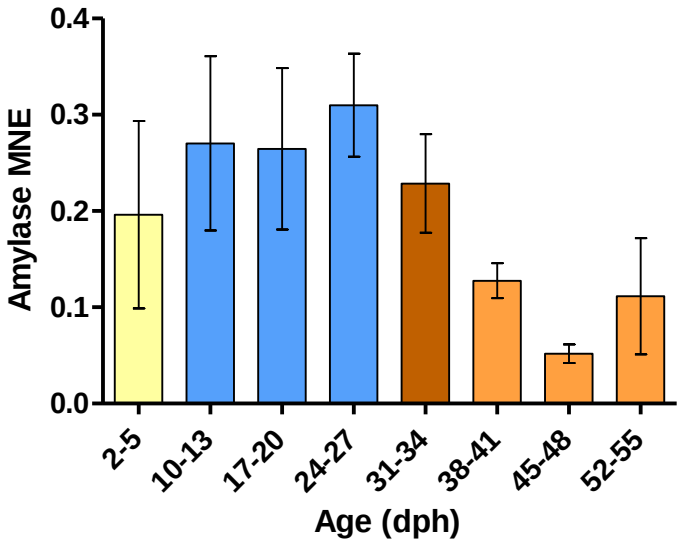

Figure 3.7: Mean MNE of amylase ± SEM (n=4). Different colors indicate different feeding regimes. Yellow bar: yolk- sac larvae age 2-5 dph. Blue bars: rotifer fed larvae age 10-27 dph. Dark orange: larvae being co- fed rotifers and GM 150, age 31-34 dph. Orange: weaned larvae (100 % GM 150) age 38-55 dph.

3.2.6 Pancreatic chitinase

Pancreatic chitinase was detected prior to first feeding (0.22 ± 0.03 MNE). The relative expression increased gradually towards 17-20 dph (0.30 ± 0.05 MNE) with a sharp, but not significant increase towards 24-27 dph (0.60 ± 0.03 MNE). However, from 31-34 dph and onwards downregulation was observed corresponding with changing diet onto formulated feed, to 0.34 ± 0.05 MNE at 52- 55 dph. The levels were higher in weaned larvae compared to larvae aged 2- 20 dph (fig. 3.8).

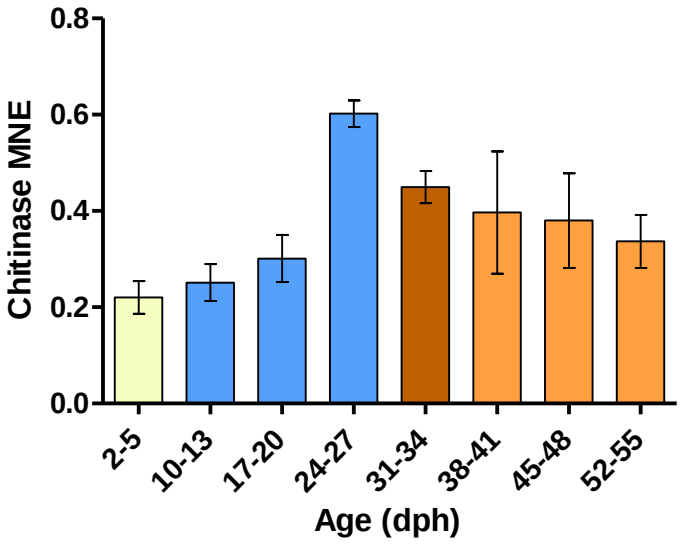

Figure 3.8: Mean MNE of pancreatic chitinase ± SEM (n=4). Different colors indicate different feeding regimes. Yellow bar: yolk- sac larvae age 2-5 dph. Blue bars: rotifer fed larvae age 10-27 dph. Dark orange: larvae being co-fed rotifers and GM 150, age 31-34 dph. Orange: weaned larvae (100 % GM 150) age 38-55 dph.

3.3 The feeding trial

3.3.1 Growth and survival

At day 6 of the weaning trial, the sampled rotifer fed larvae (R) had a mean SL of 7.06 ± 0.40 mm and mean MH of 1.10 ± 0.13 mm (n=45). The sampled larvae with gut content (approx 10 % of the larvae) from the weaning tanks (W) had a mean SL of 7.36 ± 0.42 mm (fig. 3.9) and mean MH of 1.19 ± 0.15 mm (n=40) (fig. 3.10).

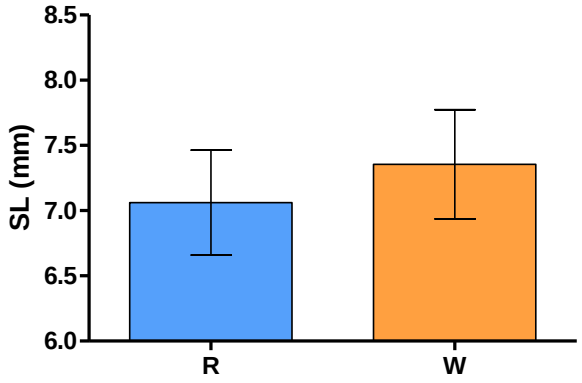

Figure 3.9: Mean SL ± SD at day 6 of the feeding trial. Blue bar: larvae in the rotifer group (R, n=45). Orange bar: larvae in the weaned group (W, n=40).

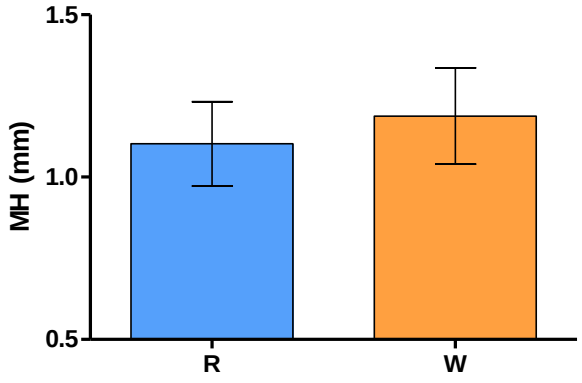

Figure 3.10: Mean MH ± SD at day 6 of the feeding trial. Blue bar: larvae in the rotifer group (R, n=45). Orange bar: larvae in the weaned group (W, n=40).

Dead larvae were removed and counted from the second day and onwards. At the second day of the trial, high mortality numbers were observed in both weaned and rotifer- fed larvae (208 ± 46 and 167 ± 38, respectively) and the survival rates decreased to 79.1 ± 4.6 % in weaned and 83.3 ± 3.8 % in the rotifer group. At day 6 the survival rates were 56.8 ± 4.7 % in the weaned group and 63.0 ± 2.2 % in the rotifer group, corresponding to daily average mortality rates of approx. 7.7 and 6.7 % per day, respectively (fig. 3.11).

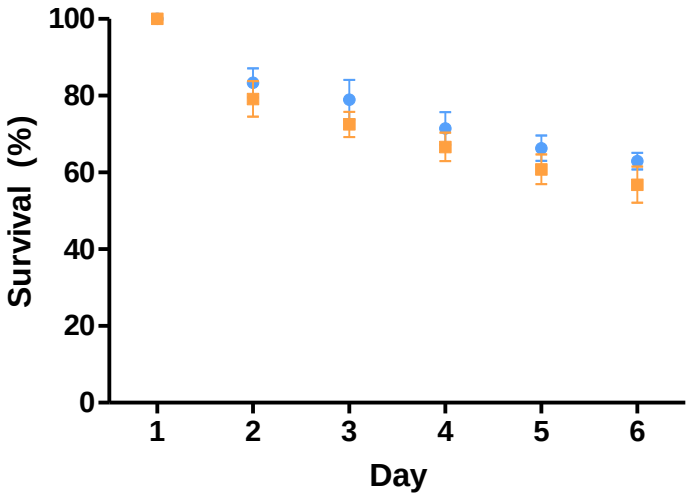

Figure 3.11: Survival ± SD (%) throughout the feeding trial. Blue dots: survival in the rotifer group (R). Orange dots: survival in the weaned group (W).

3.3.2 Enzyme activities and gene transcription

3.3.2.1 Trypsin

Larvae in the R and RU groups (3.67 ± 2.93 and 5.03 ± 3.01 pmol/min/ml, respectively) showed higher enzyme activities than in the W and WU groups, where negative values were measured in all samples except from one (WU- sample: 1.77 pmol/min/ml). The activity in the rotifer reference sample was higher, with 12.3 pmol/min/ml (fig. 3.12, A). All enzyme activities were below the manufacturer's LOD at 100 pmol/min/ml (abcam®, Cambridge, UK). Values of gene expression never exceeded 0.15 MNE, although the expression was higher in R and RU larvae (fig. 3.12, B).

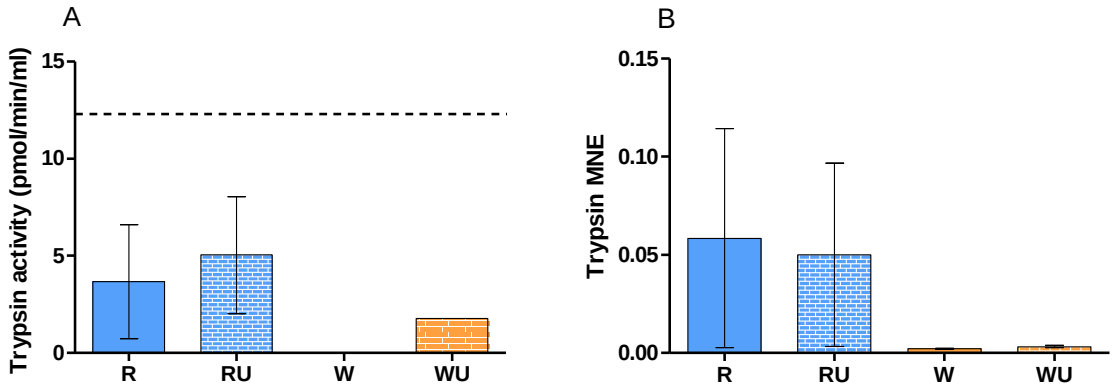

Figure 3.12: A: Mean enzyme activities of trypsin ± SD (n=3). Horizontal line represent rotifer activity (n=1). B: Mean trypsin MNE ± SEM (n=3). Blue bar: rotifer group (R). Blue bar with pattern: unfed rotifer group (RU). Orange bar: weaned group (W). Orange bar with pattern: unfed weaned group (WU).

3.3.2.2 Cholesterol 7- alpha- monooxygenase (Cyp7 A1)

A trend towards a higher gene expression in rotifer fed fish was observed, with 0.14 ± 0.04 MNE in the R- group and 0.10 ± 0.07 MNE in the RU group, compared to 0.07 ± 0.04 MNE in the W group and 0.08 ± 0.02 MNE in the WU group (fig. 3.13).

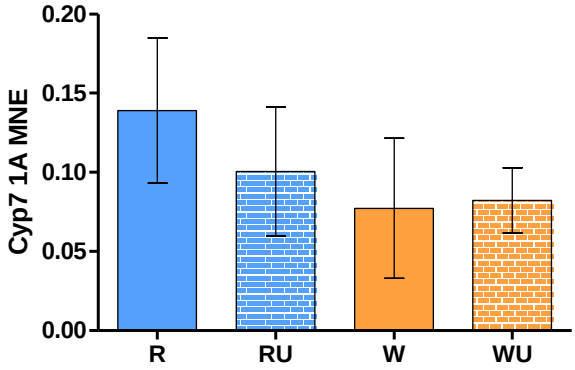

Figure 3.13: Mean Cyp7 A1 MNE ± SEM (n=3). Blue bar: rotifer group (R). Blue bar with pattern: unfed rotifer group (RU). Orange bar: weaned group (W). Orange bar with pattern: unfed weaned group (WU).

3.2.2.3 Neutral lipase and bile activated lipase (BAL)

The neutral lipase activity was similar in all four groups including the rotifer sample and no effects were significant (fig. 3.14, A). There was a trend towards higher gene expression of BAL in the W- (0.45 ± 0.08 MNE) and WU- group (0.28 ± 0.05 MNE) compared to the R- (0.20 ± 0.10 MNE) and RU- group (0.15 ± 0.05 MNE) (fig. 3.14, B).

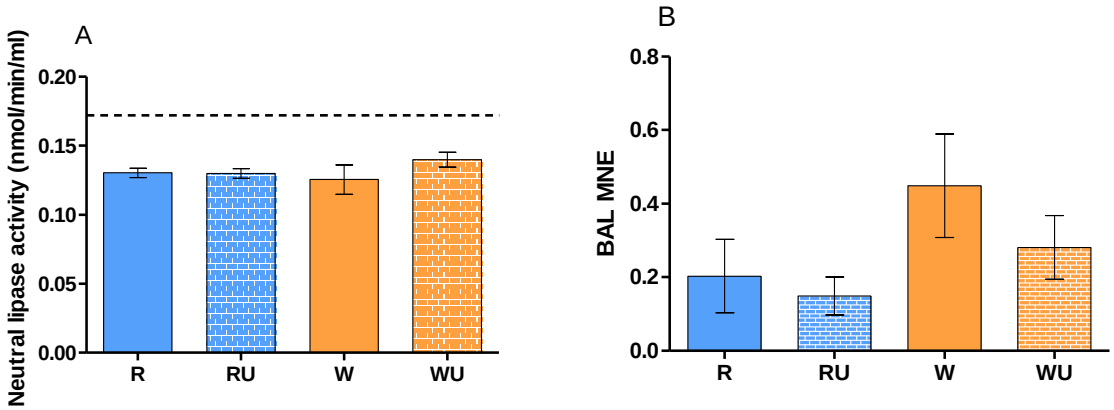

Figure 3.14: A: Mean neutral lipase activity ± SD (n=3). Horizontal line represents rotifer activity (n=1). B: Mean BAL MNE ± SEM (n=3). Blue bar: rotifer group (R). Blue bar with pattern: unfed rotifer group (RU). Orange bar: weaned group (W). Orange bar with pattern: unfed weaned group (WU).

3.2.2.4 Secretory phospholipase A_2 ($sPLA_2$ and $sPLA_2$ 1B)

The effect of diet was barely significant in the case of enzyme activities of $sPLA_2$ (two- way ANOVA, p=0.043), while none of the groups differed from the other (Tukey HSD- test) (fig. 3.15, A). The gene expression of $sPLA_2$ 1B was not significantly different between treatments. However, there is a trend towards a higher expression in rotifer fed larvae, with 0.06 ± 0.03 MNE in the R- group and 0.05 ± 0.02 MNE in the RU- group (fig. 3.15, B).

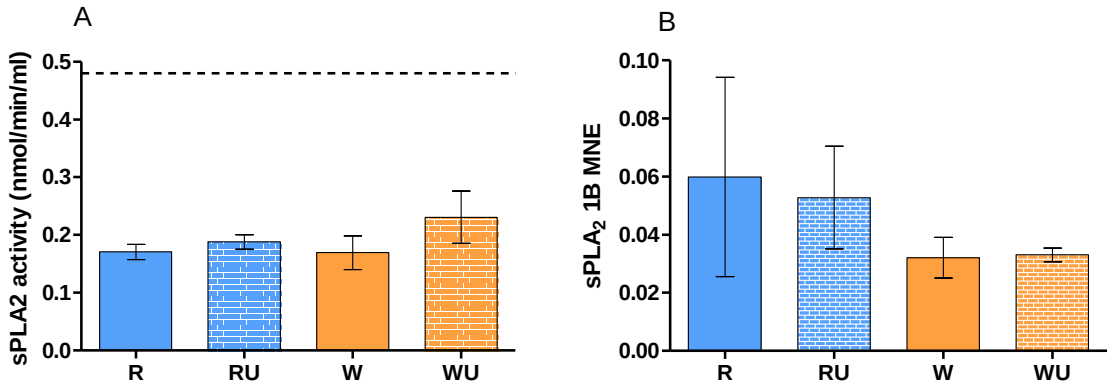

Figure 3.15: A: Mean $sPLA_2$ enzyme activity ± SD (n=3). Horizontal line represents rotifer activity (n=1). B: Mean $sPLA_2$ 1B MNE ± SEM (n=3). Blue bar: rotifer group (R). Blue bar with pattern: unfed rotifer group (RU). Orange bar: weaned group (W). Orange bar with pattern: unfed weaned group (WU).

3.2.2.3 Amylase

The larval enzyme activities were generally low (0-10 pmol/min/ml) compared to the rotifer sample (73.6 pmol/min/ml). The enzyme activities in the R group (8.2 ± 1.9 pmol/min/ml) were higher than in the W group (0.48 ± 0.28 pmol/min/ml) (fig. 3.16, A). The enzyme activity values were below the manufacturers LOD at 200 pmol/min/ml (abcam®, Cambridge, UK). The expression of amylase was similar in all groups except from the W group (0.006 ± 0.001 MNE) (fig. 3.16, B).

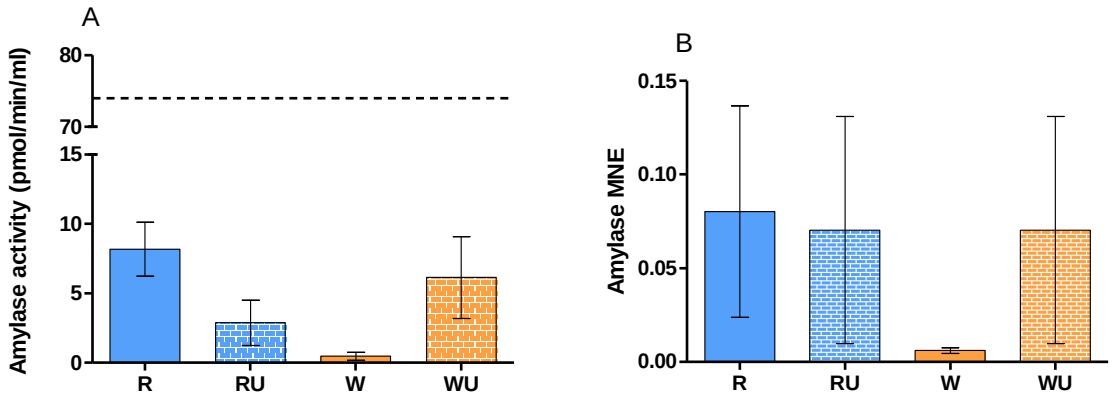

Figure 3.16: A: Mean amylase enzyme activities ± SD (n=3). Horizontal line represents rotifer activity (n=1). B: Mean amylase MNE ± SEM (n=3). Blue bar: rotifer group (R). Blue bar with pattern: unfed rotifer group (RU). Orange bar: weaned group (W). Orange bar with pattern: unfed weaned group (WU).

3.2.2.4 Chitinase

The enzyme activities of chitinase were almost three times higher in the larvae than in the rotifer sample (R, RU, W, WU: ~1.0 units/ml, rotifer sample: 0.31 units/ml) (fig. 3.17, A). The expression values of pancreatic chitinase were highest in the R- group (0.31 ± 0.15 MNE), although the SEM was relatively high (fig. 3.17, B).

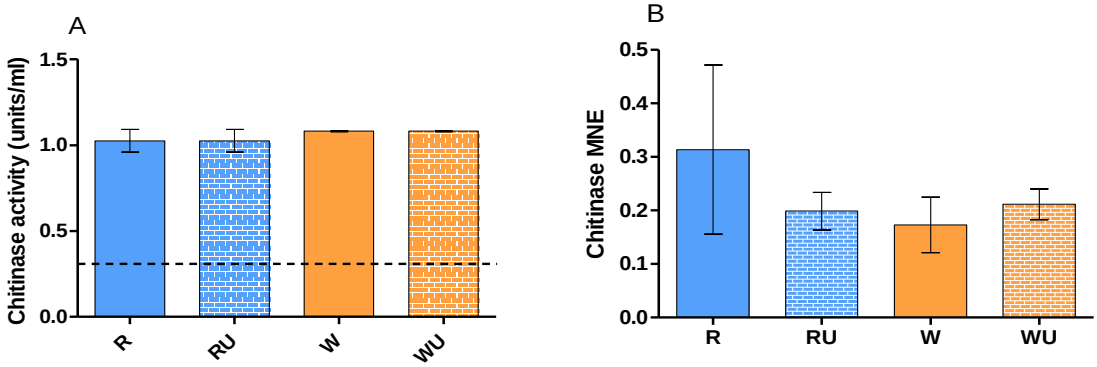

Figure 3.17: A: Mean chitinase enzyme activity ± SD (n=3). Horizontal line represents rotifer activity (n=1). B: Mean pancreatic chitinase MNE ± SEM (n=3). Blue bar: rotifer group (R). Blue bar with pattern: unfed rotifer group (RU). Orange bar: weaned group (W). Orange bar with pattern: unfed weaned group (WU).

4 Discussion

4.1 Methodological considerations

4.1.1 The feeding trial

Samples of weaned larvae were not representative of the population in the tanks, because only about 10 % of the larvae had visible gut content. Because the aim of this study was to investigate differences in enzyme activity between rotifer fed and weaned larvae, samples were only taken of fed larvae with visible gut content at the first sampling point (W/R). When sampling unfed larvae, the possibility of sampling larvae that initially did not ingest formulated feeds was high, since they were not distinguishable from pre- fed larvae. This could be weaker larvae with enzyme activities and mRNA- expressions that differ from healthy larvae.

4.1.2 Ontogeny sampling and measurements

The MH was not calculated in samples of larvae younger than 10 dph and older than 47 dph. This was because the youngest larvae were dorso- laterally placed on the surface of the microscope, making MH- measurements impossible. The larvae older than 47 dph were too large to be taken pictures of under the stereo microscope; we therefore measured SL manually by means of a ruler. Since there were no pictures of these larvae, MH measurements were not possible.

4.1.3 Enzyme activity measurements

Due to their small size and fragility, analyses of fish larvae have proven to be relatively difficult compared to larger specimens. This is especially the case for analyses of certain tissues which need to be isolated manually, a process that can be extremely demanding. In order to reduce workload and possible handling errors, samples were of whole larvae in this study. This is not ideal in from an analytical point of view. When quantifying enzyme activity in whole larvae homogenates, the resulting total activity could include activities of different origins (intracellular, from various tissues, exogenous prey or microbial enzymes) due to the unspecific nature of enzyme assays. The $sPLA_2$ assay does not differentiate between groups of $sPLA_2$ enzymes, which consist of ten secretory enzymes altogether (Murakami and Kudo, 2002). Sæle et al. (2011) compared $sPLA_2$ activities in dissected tissues (liver, brain, kidney tissue, gill and muscle) in *G. morhua* larvae, and reported similar activities in several tissues. Neutral lipase activity is also considered unspecific in that it measures all neutral lipases in a sample (Iijima et al., 1998). Any correlation analysis of BAL mRNA and neutral lipase activity should therefore not be performed.

The trypsin assay measures only trypsin and not its inactive precursor trypsinogen in whole larvae homogenates (Lazo et al., 2000). It has also been shown that trypsin inhibitors in the tissue can react with trypsin, following a lower estimation of the activity (Hjelmeland, 1983). Despite the apparent low specificity of these assays, Ueberschär et al. (1992) concluded that the fluorescence method that were used in this study is equally well suited as a highly sensitive radioimmunoassay method, based on a correlation analysis of the two. They did however demonstrate the need for individual measurements, as high individual variations were observed. As we pooled several individuals in a single sample, such variations were not detectable.

Despite the apparent disadvantages of enzyme activity measurements, it was decided to use the method in the feeding trial. Similar sized larvae that had only experienced differences in feeding regimes were compared. The resulting differences would therefore be connected to feed intake (enzyme secretion in the alimentary tract), in which the aim of the trial was to investigate the effect of different feeding strategies and the possibility of exogenous enzyme contribution by rotifers. It was decided not to measure enzyme activity in the ontogeny series. This was due to the differences in size of the larvae and relative size of the digestive system and enzymatic contribution during larval development. A comparison of enzymatic activities would thus be inappropriate.

The LOD of enzyme activity is set by the manufacturer. Values were below the LOD in the amylase and trypsin assays. The LOD were not defined in the chitinase assay or in the neutral lipase assay, the former because the reagents were homemade in accordance to Murray et al. (2003). The activities in the $sPLA_2$- assay were all within the LOD. The LOD are not always accurate, meaning that the manufacturer may have set the lower limit higher than it actually is. A possible way to set an own LOD would be to make a standard curve based on a series of dilutions of a positive control reagent, and from this calculate the lowest concentration of which activity is detected. If this was performed on the amylase and trypsin assays, lower LOD values might have been calculated that is closer to or below the measured contents. However, when the values are consistently below the LOD, any rigorous statistical testing is not warranted.

4.1.4 Enzyme activity and mRNA correlation

In this trial, I have analyzed mRNA encoding the enzyme and not the enzyme itself. It does not necessarily have to be a connection between the two. However, Sæle et al. (2011) investigated

the connection between mRNA expression and protein expression of $sPLA_2$ in *G. morhua* larvae by means of a linear correlation analysis, and found that 51 % of the protein expression could be explained by the gene expression (r^2= 0.51). This was considered sufficient by the authors, who suggested that the remaining 49 % was explained by post- transcriptional regulations and methodological variations. We therefore assumed a certain degree of connection between mRNA expression and target enzyme activity, although no analysis of correlation was performed.

4.1.5 Statistical considerations

In order to use the term replicate, one should be aware of potential causes of variation between tanks. This is important in order to have reasonable control of the statistical analysis of the material. For the ontogeny series, larvae from four tanks with somewhat similar treatments were analyzed. One tank had no clay suspended in the water after 29 dph, and another were subjected to a different feeding regime at 38 dph. These differences could potentially cause variation, although there was no significant difference in SL between tanks. The term pseudo replicates was therefore applied to the tanks, although they were treated as true replicates when doing the statistical analysis. An outlier was observed in the gene expression of BAL at 2-5 dph. This value deviated more than two- times the average predicted SD and was therefore removed.

4.2 Growth of *L. bergylta* larvae

Since larval growth is a good indicator for overall performance later in life, it is an important parameter to measure (McCormick and Molony, 1993). The growth of *L. bergylta* was initially relatively slow, until an exponential pattern occurred at around 35 dph, when the larvae had just recently been weaned onto GM 150. The growth of intensively reared *G. morhua* juveniles showed a similar exponential pattern with a significant increase from 10 dph, as measured by Sæle et al. (2010). These juveniles performed well compared to wild *G. morhua* juveniles fed zooplankton (Folkvord, 2005). The growth of larval *P. splendida* shows a similar pattern, accelerating from 45 to 60 dph (Uscanga-Martinez et al., 2011). Although the rapid increase in growth occurred later for *L. bergylta*, the SL was similar to *G. morhua* at around 50 dph. The growth in MH was similar to the growth in SL in the ontogeny series, with an exponential pattern occurring at around 35 dph. Hence, the larvae of *L. bergylta* in this study performed well in comparison to other trials, suggesting an efficient utilization of nutrients.

The apparent growth promoting effect of GM 150 on SL in *L. bergylta* can also be seen in the feeding trial. After only 7 days of weaning, both SL and MH of the weaned larvae were higher than that of the rotifer- fed larvae. It is however important to remember that only ~10 % of the larvae in the weaning group had been successfully weaned onto GM 150. These were the larvae that were sampled for SL- measurements, not the remaining 90 % (see section 4.1.2). In regards to these observations, one could expect that there is more energy available for growth in the formulated feed, as the protein content is higher than in rotifers (59 % vs. 42 %, respectively, see table 4.1 below). However, observations on cultivated *G. morhua* populations, which are not fed formulated feeds but cultivated plankton in excess (Folkvord, 2005), have shown a similar exponential growth pattern at a similar age compared to farmed *L. bergylta* and weaned *G. morhua* (Sæle et al., 2010). This age- dependent similarity might suggest that it is also the maturation and increased digestive efficiency of the digestive system that has caused the increasing growth rate. Dunaevskaya (2010) reported that the pancreatic tissue increased markedly in size and surrounded different structures in *L. bergylta* after 17 dph. Increased pancreas size and a concomitant improvement of digestive functions, as well as a growth promoting food source, is therefore a probable cause of the exponential growth pattern.

4.3 The effect of age and feed on pancreatic digestion

Knowledge about the effect of age/growth and diet on enzyme production, on a genetic and enzyme level is important in order to better understand the internal processes governing the growth and survival of the larvae. The digestive system of newly hatched *L. bergylta* larvae is a straight undifferentiated tube (Kamisaka et al., 2003) and a functional stomach is not developed later in life (Chao, 1973, Yasugi, 1987) (fig. 1.3). The natural diet of most marine larvae is high in lipids and proteins. Carbohydrates are also present in the diet of most species, although in relatively low amounts. Sufficient digestive enzyme activity is required at an early stage in order for the larvae to digest these nutrients efficiently. Several authors have suggested an influence of digestive nutrient content on enzyme activity in marine larvae (Cahu and Infante, 1994, Izquierdo et al., 2000, Infante and Cahu, 2007). Other authors have suggested otherwise (Lazo et al., 2000). An indication of influence by nutrients has also been suggested on a genetic level, where opposing ontogenetic expression profiles of BAL and PLA_2 was observed in *G. morhua* larvae with different feeding regimes (Sæle et al., 2010, 2011, Kortner et al., 2011). In our study, a general age or nutrient dependency was observed, with a trend of upregulation from hatching to

the onset of weaning, following a sudden decline in several genes towards metamorphosis. Interestingly, the gene coding for the lipolytic enzyme BAL showed a completely different trend with a significant increase from weaning (30 dph) towards metamorphosis.

The variable trends and patterns will be compared with age and feeding regimes and discussed in the following sections. It is important to remember that the capacity of marine larvae to digest formulated feed may be reduced compared to live feeds, due to their natural developed ability to feed on live prey (Cahu and Infante, 1997). Table 4.1 shows a comparison of the macronutrient content in formulated feed (GM 150) and rotifers enriched at MH Labrus.

Macronutrients	GM 150 (%)	Enriched rotifers (% of tot. DW)
Proteins	59.0	42.1
Oils and fats	14.0	9.1
Fiber/cellulose fiber	0.2	-
Ash/cellulose	10.0	-
Phosphorus	2.0	1.0
Calcium	1.5	0.2
Sodium	0.7	5.3
Nitrogen	-	9.4

Table 4.1: Macronutrient composition in GM 150 (%, Skretting, Stavanger, Norway) and rotifers enriched at MH Labrus (% of tot. DW, Hamre, K., pers. comm., 2012).

4.3.1 Protein digestion

In general, proteolytic enzymes in the intestine of marine larvae exhibit relatively high activities in the early stages, and trypsin is considered the main proteolytic enzyme in agastric fish species (Infante and Cahu, 1994b). It was therefore expected that we detected a regulation of the trypsin mRNA in the larval ontogeny of *L. bergylta*, as well as enzymatic activities in the feeding trial. Studies on proteolytic ontogeny have shown low specific enzyme activities in first- feeding *H. hippoglossus* larvae with an increase towards metamorphosis (Gawlicka et al., 2000). These results suggest that the regulation of trypsin is age- dependent in marine larvae. Perez- Casanova et al. (2006) found significant differences in proteolytic activity between *Melanogrammus aeglefinus* (haddock) and *G. morhua* when subjected to similar treatments, meaning that enzyme activity can differ between species.

Trypsin mRNA was barely present in yolk- sac larvae (2-5 dph) and increased up to a maximum at the time of weaning, following a decrease on older larvae. A recent study on the ontogenetic mRNA expression of trypsin in larval *G. morhua* has been performed by Kortner et al. (2011), in which they observed a similar pattern. Age- dependent enzyme activity patterns have been observed in *P. splendida* (Uscanga-Martinez et al., 2011) and *S. lalandi* (Chen et al., 2006). These species are however not agastric. The authors suggested that the decline in trypsin activity was corresponding with the development of gastric glands and a more prominent role of pepsin – factors which are not present in *L. bergylta* (Chao, 1973, Yasugi, 1987), as their alimentary tract consists of a relatively short intestinal tract with pancreatic tissue surrounding it (see fig. 1.3). A slight upregulation of trypsin mRNA was observed in the oldest larvae, an indication of further upregulation towards metamorphosis.

Protease activities in larvae have been assumed to be affected by dietary protein content by several authors (Grendell and Rothman, 1981, Tseng et al., 1982). However, Péres et al. (1998) concluded that this did not have an impact on neither mRNA levels nor specific activity of trypsin. The protein content in GM 150 is almost 30 % higher than in enriched *B. plicatilis* (table 4.1), so an effect could be possible in this study. In the feeding trial, it seemed as if larvae fed rotifers had upregulated trypsin mRNA and increased enzyme activity. All the values were however below the LOD, so it is possible that the variations are due to random noise measured in individual samples.

One could assume that formulated feeds, in this case GM 150, does not supply *L. bergylta* with the required amount and/or quality of digestive proteins that are needed to induce secretion of enzymes into the intestine (Cahu and Infante, 1997). This was however not reflected in their growth pattern. Since trypsin is considered the main proteolytic enzyme in fish (Infante and Cahu, 1994b), and based on evidence of trypsin activity in adult agastric fish species (Cohen et al., 1981), it is natural to assume a digestive role of trypsin later in life. Based on results from the feeding trial and the ontogeny series that are similar, a natural shift towards a more prominent lipid and carbohydrate digestion may be expected towards metamorphosis and later in life.

4.3.2 Lipid digestion

Dietary lipids are essential sources of energy for growth and development of marine larvae. The genetic expression of BAL, the only neutral lipase found in marine fish larvae (Sæle et al., 2010), has been analyzed, along with $sPLA_2$ 1B. The former enzyme is important in regards to the digestion of phospholipids in the small intestine and BAL catalyzes the hydrolysis of a wide range of neutral lipids. The genetic expression of Cyp7 A1 were included in the study due to its importance in the conversion of cholesterol to bile acids in the liver (Myant and Mitropoulos, 1977). Kortner et al. (2011) suggested that different BAL and PLA_2 expression patterns between their trial and the one by Sæle et al. (2010) might be caused by different feeding regimes, and that the lipid metabolism may be depending on the diet, as demonstrated by Lazo et al. (2000). The feeding regime in our trial was similar to Sæle et al. (2010, 2011), so was the expression pattern of BAL. Since BAL is activated by the secretion of bile acids, which in terms is depending on Cyp7 A1 mRNA levels, one could expect a similar pattern in the ontogeny of these genes. The lipid fractions are somewhat similar in GM 150 and enriched rotifers (table 4.1).

The mRNA levels of Cyp7 A1 were low in yolk- sac larvae, but quadrupled just after first-feeding and stabilized before a downregulation was observed in larvae older than 31 dph. In the feeding trial we observed a trend towards a downregulation of Cyp7 A1 in weaned larvae. The upregulation in first- feeding larvae might suggest activation of the gene in response to the presence of live feed. Jelinek et al. (1990) reported an upregulation of Cyp7 A1 in rats fed a diet high in cholesterol. A regulatory response to feeding could be the case in fish larvae as well, but few trials have been performed regarding this gene.

The pattern of expression BAL differed from Cyp7 A1. The mRNA levels remained at a constant near- zero until around 30 dph, when a significant upregulation occurred. This difference was in contrast to our expectations of similar patterns. Specific enzyme activities of BAL have been observed in larval *S. maximus* prior to first- feeding (Hoehne-Reitan et al., 2001), and other trials have detected BAL mRNA from hatch in *D. labrax* (Perez-Casanova et al., 2004) and from mouth opening in *P. americanus* (Murray et al., 2003). Kortner et al. (2011) registered an early upregulation of the gene in *G. morhua*, with a peak at around 20 dph, following a downregulation to low levels from 30 – 60 dph. However, opposing observations were made on the same species by Sæle et al. (2010). Low expression levels were observed during the first 60 dph until an

upregulation occurred at 62 dph. The authors suggested that this upregulation occurred in relation to the differentiation and development of the diffuse pancreas and pyloric caeca.

In the feeding trial, an upregulation of BAL mRNA was observed in weaned larvae, similar to the ontogeny series. Based on the ontogeny series alone, one could expect an age- dependent regulation, which was suggested in larval *G. morhua* by Sæle et al. (2010). But since the age of the rotifer fed and weaned larvae were similar in the feeding trial, it is natural to assume a more prominent effect of nutrient composition on the genetic regulation of BAL in larval *L. bergylta*. The enzymatic activities of neutral lipase did not differ between treatments in the feeding trial, but this similarity might be related to the unspecific nature of this assay (Iijima et al., 1998).

The expression pattern of $sPLA_2$ 1B indicated an upregulation from yolk- sac larvae to weaning, and a downregulation after about 30 dph. A slight indication of upregulation in older larvae was observed at around 50 dph. A low expression of this gene was observed in *G. morhua* larvae, until a significant increase occurred at 62 dph, in relation to the expansion of the pancreas and development of the pyloric caeca (Sæle et al., 2011). A strong and consistent upregulation of PLA_2 was observed in *G. morhua* larvae after 20 dph by Kortner et al. (2011). Analyses of *L. bergylta* larvae older than 55 dph could give a better picture of the development towards metamorphosis, especially for $sPLA_2$ 1B where an upregulation could be expected.

A barely significant effect of diet on $sPLA_2$ enzyme activities was observed in the feeding trial. However, none of the groups differed statistically from each other, and higher activities in unfed larvae could be a result of the unspecific nature of the assay (Sæle et al., 2011) (see section 4.1.3). Interestingly, elevated mRNA expression values were observed in larvae fed enriched rotifers. This could suggest an upregulation of $sPLA_2$ 1B as a response to the rotifer diet.

4.3.3 Carbohydrate digestion

The formulated feed contains 10 % ash/cellulose (see table 4.1), which are mainly structural components with low nutritional value for the larvae. Live feeds that are used in cultivation have generally low contents of carbohydrates (Hamre, 2006). However, rotifers contain some glycogen (0.8 %) according to Litton et al. (1976) and chitin in their exoskeleton (Klusemann et al., 1990). We therefore expected some sort of regulation of chitinase and amylase in *L. bergylta* in relation to their diet. Enzyme activity of amylase has been detected in larvae prior to first- feeding in several species (Lazo et al., 2000, Ribeiro et al., 1999, Infante and Cahu, 1994a), including the

expression of the amylase gene in *G. morhua* (Kortner et al., 2011). The mRNA of amylase and pancreatic chitinase were both present in yolk- sac larvae in *L. bergylta*. This suggests that the genetic regulation of these enzymes is mediated internally, and not induced by the diet. These findings also indicate the importance of carbohydrates in the diets of first feeding larvae, possibly originating from algal material that is commonly encountered in their guts (van der Meeren, 1991).

During the rotifer feeding period, the amylase gene maintained a relatively high level, until a downregulation occurred at around 30 dph. A decrease in amylase expression has been observed from 29 dph in *D. labrax* larvae, where a change in diet did not affect the enzyme at a transcriptional level, suggesting a regulation independent of feed (Péres et al., 1998). In the ontogeny series, the downregulation in *L. bergylta* occurred at the time of weaning onto GM 150, a feed with low levels of digestive carbohydrates. All the measured values of amylase enzyme activities in the feeding trial were far below the manufacturers LOD, and the amylase mRNA levels were relatively low with high variance. Despite the low levels, we did measure lower enzyme activities and gene expression in weaned larvae, a result that is similar in relation to feed compared to the mRNA of the ontogeny series. Perhaps the 0.8 % glycogen in rotifers is not sufficient to stimulate amylase transcription. Some authors have found a positive effect of dietary carbohydrate ration (starch) on specific enzyme activities of amylase in *D. labrax* larvae (Péres et al., 1998, Yu et al., 2012). Incorporating higher levels of digestive carbohydrates (i.e. starch), both in live feed and in formulated feed could therefore stimulate amylase production in *L. bergylta* larvae, as well as in other marine larvae such as *D. labrax*.

Limited information is available regarding chitin digestion in marine larvae. However, chitinolytic activities have been detected in several fish species (Fange et al., 1979, Ikeda et al., 2009) although these had low pH optimums, an indication of gastric digestion. The genetic expression of pancreatic chitinase in *L. bergylta* was relatively high during larval ontogeny, with a peak at around 27 dph. Similar to amylase, the expression decreased in weaned larvae. It was however higher in larvae aged 30 + dph compared to younger rotifer- fed larvae. This could indicate the importance of the enzyme in degrading the chitinous exoskeleton of larger prey items (arthropods, mollusks, annelids) and the digestion of carbohydrates in general towards metamorphosis and later in life (Ikeda et al., 2009). The enzymatic activities of chitinase were

similar in all groups of larvae in the feeding trial, and the activity in the rotifer sample was less than one- third of these, a clear indication of endogenous enzyme production. The mRNA expressions were somewhat similar between the groups, except from the rotifer group which seemingly upregulated the gene. However, high variance in this group was observed. The upregulation in rotifer- fed larvae could be due to a transcriptional stimulus by rotifers on chitinase production. As few similar trials have been performed on chitinase, any conclusive remarks were difficult, but it seems as if chitinase is an important enzyme both in larval and adult *L. bergylta*.

4.4 Exogenous enzyme contribution

Some authors have suggested that live feed contribute with exogenous digestive enzymes (Dabrowski and Glogowski, 1977, Lauff and Hofer, 1984, Sæle et al., 2010, 2011), while others have suggested that the contribution is negligible (Cahu and Infante, 1997, Kurokawa et al., 1998, Gawlicka et al., 2000, Perez-Casanova et al., 2006). Enzyme activities in unfed larvae were compared with fed larvae of both groups of the feeding trial in order to answer this question. The possible exogenous contributions of trypsin and amylase from rotifers were not clearly apparent and will not be discussed further, as these enzyme activities were below the manufacturers LOD.

The activities of the lipolytic enzymes $sPLA_2$ and neutral lipase did not seem to be affected by rotifers. Similar activities of neutral lipase were observed when comparing fed and unfed larvae of the rotifer group, and the activity in the rotifer sample was only slightly higher. The activities were also similar in weaned larvae compared to rotifer fed larvae. The $sPLA_2$ activities varied more between groups. However, as mentioned earlier, the increased activities in unfed larvae is possibly due to the unspecific nature of the assay, where activities originating from other tissues have been measured as well (Sæle et al., 2011). These findings are in accordance to other studies on lipolytic enzyme contribution, stating that it is negligible.

The activities of chitinase were similar between groups as well, with a slight increase in weaned larvae. The activity in the rotifer sample was only about 30 % of the larval activities, suggesting that the chitinase enzyme is regulated independently of rotifers. These findings clearly indicate that the exogenous contribution by rotifers to chitinase activity is negligible, and that the larvae depend on endogenous production. Recent molecular evidence supports this assertion (Altschul et al., 1997, Oshima et al., 2002).

4.5 Conclusions

The farmed *L. bergylta* larvae at MH Labrus performed well in relation to growth. We conclude that the exponential growth pattern observed in the ontogeny series at around 30 dph was due to improved digestive functions and a growth promoting effect of the formulated feed (GM 150). The growth promoting effect could be related to a high amount of digestible proteins in the diet.

The detection of genes related to carbohydrate digestion (amylase and pancreatic chitinase) in yolk- sac larvae suggests an internal genetic regulation of these enzymes which is unaffected by the diet. Our findings also suggest an essential role of carbohydrates in first- feeding larvae, as well as juvenile/adult *L. bergylta*.

A consistent feed- dependency was observed on a transcriptional level in the ontogeny series. A genetic upregulation in rotifer fed larvae suggests that rotifers stimulate the transcription and consequent production of pancreatic enzymes. Exactly how, and to which degree the stimulation occurs is uncertain and beyond the scope of this study. Our results also suggest that the formulated feed given limits the transcription of several genes related to pancreatic digestion, with BAL being the only exception. We consider it unlikely that BAL is the only dominant pancreatic enzyme in weaned *L. bergylta* larvae, since a combination of nutrients is necessary for proper growth and survival.

Based on our observations of BAL mRNA both in the feeding trial and in the ontogeny series, we suggest that the regulation of this gene is more affected by nutrient type and/or composition than age. The genetic upregulation also indicate the importance of dietary lipids in the diet of late larval and juvenile *L. bergylta*.

Based on a thorough examination of enzyme activities in the feeding trial, it is concluded that the exogenous contribution of digestive enzymes by rotifers is negligible.

5 Future perspectives

It is clear that proper larval cultivation is needed for *L. bergylta* to become an efficient lice eater later in life. Based on our observations of downregulation in several genes related to weaning, an intermediate *Artemia* feeding step could stimulate further upregulation of these genes and perhaps also cause the larvae to be more strengthened at the initiation of weaning, given that the larvae respond positively to this type of feed. This strengthening could induce better growth and survival later in life, and also improve its skills in regards to lice removal on *S. salar*.

In regards to commercial feed production, there might be an element in the live feed that needs to be detected, in which it could be incorporated into a formulated feed. Such an element could induce a more natural production of pancreatic enzymes in marine larvae, and lead to better growth and survival in general.

Studies of molecular mechanisms of digestion in marine larvae are still in its infancy, and relatively expensive and time consuming. A new method of visualizing metabolism *in vivo* has recently been developed, where digestive metabolism in *D. reiro* is investigated by means of fluorescent reporters (Carten and Farber, 2009). These reporters are able to serve as readouts on lipase and protease activities *in vivo*, without hurting the fish. The method is considered less time consuming, and individual differences can be detected. Also, as *D. reiro* is agastric, parallels can be drawn between such trials on this species and *L. bergylta*. Such a method, combined with present molecular methods (qPCR) could provide enhanced knowledge in regards to the interplay between mRNA expression and enzyme activity.

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Appendix

Table A.1: Weaning strategy in the feeding trial

Day	Weaned tanks (rotifers/larva/day)	GM 150 (g/tank/day)	GM 150 handfed (g/tank/day)		Rotifer tanks (rotifers/larva/day)
1	5000	2.0	-		6600
2	5000	2.5	1.0		6600
3	5000	2.5	1.0		6600
4	3300	2.5	1.5		6600
5	3000	3.0	1.5		6600
6	1700	3.0	2.0		6600
7	-	3.0 (stop at 16.00)	2.0 (stop at 16.00)		6600 (stop at 16.00)
8	-	-	-		-

Table A.2: Reagents used in Qiagen One Step RT PCR

Component	**Volume (µl)**
ddH2O	11.25
10xdNTP	1
5x QIAGEN buffer	5
Q- solution	5
RNase inhibitor	0.25
QIAGEN enzyme mix	1
RNA (500 ng/µl)	0.5
Primer Forward (50 µM)	0.5
Primer Reverse (50 µM)	0.5
Total volume:	25

Table A.3: Thermal cycler program in Qiagen One Step RT PCR

Step	Duration	Temperature	Comment
Reverse transcription	30 min	50 °C	Can be increased to 60°C
PCR activation	15 min	95 °C	
3- step cycling:			
Denaturation	45 sec. (30-60)	94 °C	
Annealing	45 sec. (30-60)	55 °C (50-68 °C)	Approx. 5°C below primer T_m
Extension	1 min	72 °C	
Cycles	33 (25-40)		
Final extension	10 min	72 °C	

Table A.4: Reagents and concentrations for RT PCR

Reagents	Volume (µl)	Concentration
ddH_2O	8.9	
10x RT buffer	5.0	1X
10x $MgCl_2$	11.0	5.5 mM
10 mM dNTP mix	10.0	500 µM/dNTP
50 µM oligo $d(T)_{16}$	2.5	2.5 µM
RNase inhibitor	1.0	0.4 U/µl
Reverse transcriptase (50 U/µl)	1.67	1.67 U/µl
RNA sample (50 ng/µl)	10.0	500 ng
Total/well	50.0	

Table A.5: RT PCR reaction program

Step	Temperature (°C)	Time (minutes)
Incubation	25	10
Reverse transcription	48	60
Reverse transcriptase inactivation	95	5
End	4	∞

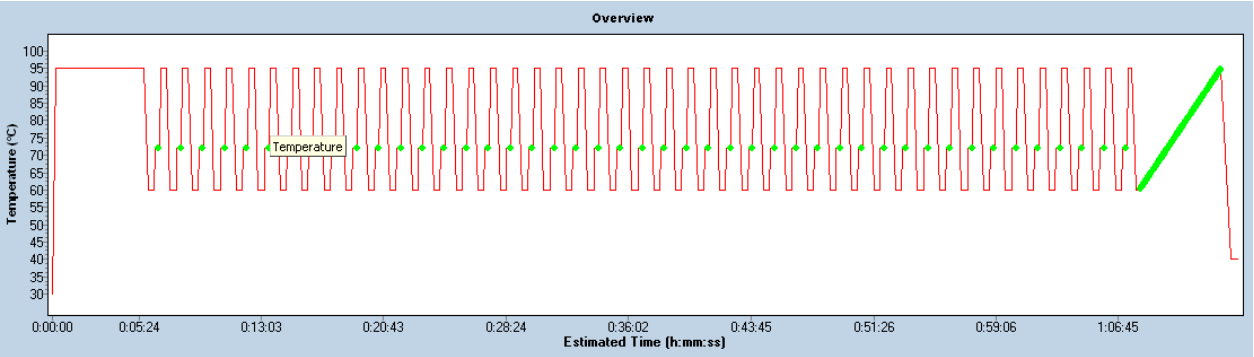
Table A.6: RT qPCR reaction mix

Reagents	Volume (µl)
ddH_2O	2,8
Forward primer (50 µm)	0,1
Reverse primer (50 µm)	0,1
CYBR Green mix	5

Table A.7: RT qPCR reaction program

Steps	Temperature (°C)	Time (min: sec)	Comment
Pre- incubation	95	05:00	Denaturation and activation of FastStart Taq DNA polymerase
Amplification			45 cycles, 3 steps
Denaturation	95	00:10	Separating DNA strands
Annealing	60	00:12	Primer binds to DNA strand
Elongation	72	00:05	Synthesis of double stranded DNA
Melting point analysis			
Denaturation	95	00:10	
Annealing	60	00:05	
Melting	95		
Cooling	40	00:30	

Figure A.1: Light Cycler® 480 program for RT qPCR reaction, illustrating temperature shifts during the reaction, where the x-axis represents temperature and the y-axis represents time (hour:min:sec). Fluorescence is measured at the green marks, during the elongation step.



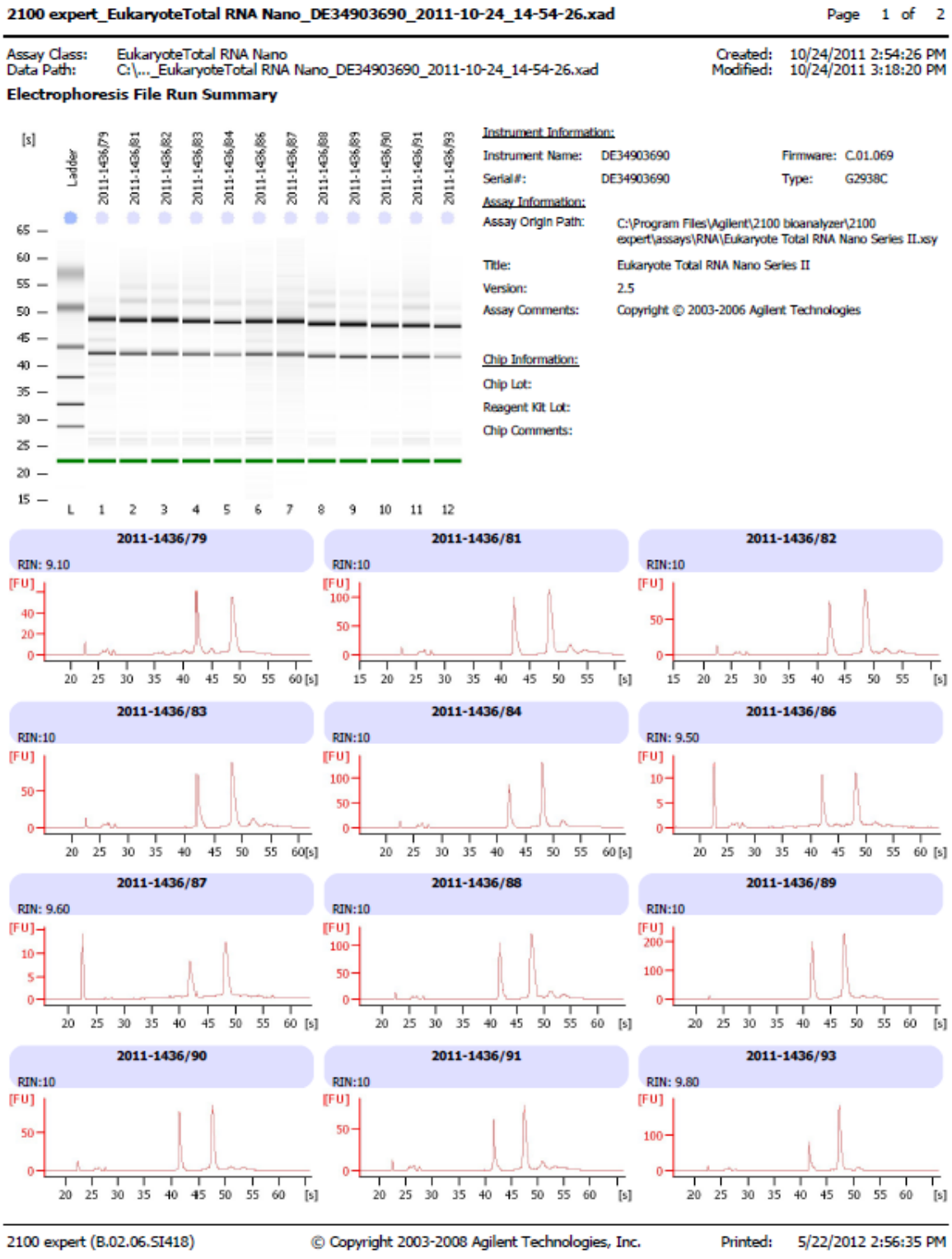


Figure A.2: RNA quality assessment with gel photo and electropherogram with connected RIN values on different larval samples.