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Phospholipids vs. neutral lipids: Effects on digestive enzymes in Atlantic cod (Gadus morhua) larvae

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Abstract:

The aim of this study was to evaluate the effect of dietary lipid classes (phospholipid vs. neutral lipid) and level of n-3 highly unsaturated fatty acids (HUFAs) on growth, survival and digestive enzymatic activity in Atlantic cod (Gadus morhua) larvae. Larvae were fed enriched rotifers from mouth opening and were weaned to formulated diets during a co-feeding period lasting from 17 days post hatch (dph) to 24 dph. Larvae were fed exclusively compound diets from 24 dph until the end of the experiment. Three isoproteic and isolipidic compound diets with gradually decreasing levels of DHA and EPA in the dietary phospholipid fraction (diet PL3 > PL1 > NL1) and increasing levels of total dietary neutral lipids (PL3 < PL1 < NL1) were used. Larvae fed the PL3 or PL1 had a significant higher dry weight than larvae fed the NL1 diet at the end of the experiment (45 dph). Survival did not differ significantly between treatments. A gut maturation index based on the relation between the amount of the brush border enzyme alkaline phosphatase and the cytosolic enzyme leucine-alanine aminopeptidase showed that the enterocyte maturation on 35 and 45 dph was better in larvae fed the PL3 and PL1 diet than larvae fed the NL1 diet. Dietary composition did not significantly affect the activity of α-amylase, but an age specific decrease in activity was observed. The dietary composition did affect the specific activity of trypsin, although showing variation in pattern between different larval ages at sampling. The activity of neutral lipase showed high variability between dietary treatments, but at the end of the experiment the specific activity of neutral lipase was positively connected to the dietary neutral lipid levels. The results from this study showed that cod larvae use DHA and EPA in the phospholipid fraction more efficiently compared to those in the neutral lipid fraction.

Keywords: Atlantic cod larvae; Early weaning; Phospholipids; Digestive enzymes; Gut maturation

1. Introduction

The commercial juvenile production of Atlantic cod (*Gadus morhua*) is still a challenge, much due to suboptimal nutrition during the first stages. Cod larval rearing is still largely

depending on a live feed period with rotifers (*Brachionus sp.*) and *Artemia* during the early larval phase. The nutritional value of these commonly used live feed organisms is variable, and in particular the *Artemia* lipid composition after enrichment is rather unstable (Olsen et al., 2004). Due to high costs in live feed production, one main objective in cod larval rearing is to formulate a compound diet that can substitute live prey, in particular *Artemia,* as early as possible during larval development. Cahu et al., (2003a) demonstrated that complete substitution of live feed by formulated diets 51 in marine fish larval rearing is possible when larval sea bass (*Dicentrarchus labrax*) were reared only with compound diets from mouth opening. So far this is not achieved with cod larvae and cod larvae fed formulated diets before development of the stomach have shown less growth than larvae fed rotifers and *Artemia* (MacQueen Leifson, 2003). A shift from rotifers to *Artemia* or to a formulated diet 20-30 dph is usual in cod aquaculture industry (Hamre, 2006). Baskerville-Bridges and Kling (2000) and MacQueen Leifsson (2003) demonstrated that cod larvae can be weaned directly from rotifers to a specialized microparticulate diet without incorporating an intermediate *Artemia* phase, although growth was poorer than in larvae fed *Artemia*. The present study is one of few early-weaning experiments performed with cod larvae where *Artemia* is entirely substituted by formulated diets as early as 17 days post hatching (dph) to investigate the effect of dietary lipid composition on larval growth and development. During the last two decades ontogeny of digestive enzymes and developmental features of the digestive tract have been well documented in several species (Zambonino Infante and Cahu, 2001; Kjørsvik et al., 2004). The development of pancreatic enzymes follows a genetically programmed pattern which is subtly modified by the diet composition (Cahu and Zambonino Infante, 2001; Hoehne-Reitan and Kjørsvik, 2004). All digestive enzymes, except the stomach enzymes, seem to be present in pelagic marine fish larvae during the first period of feeding (Kjørsvik et al. 2004). This indicates that marine fish larvae are capable of digesting formulated diets from the beginning of start feeding. Recent work has described the activity of several key enzymes throughout the ontogeny of larvae cod (Perez-Casanova et al., 2006) in order to investigate the development of cod larval digestive capacity. The authors concluded that cod larvae are capable of digesting lipids, as also reported by Hoehne (1999) and protein at the time of mouth opening and that they have a limited capacity to digest

carbohydrates. So far the dietary effects on digestive enzyme activity in cod larvae have not been shown.

Dietary lipids are the main source of energy for developing fish larvae (Sargent et al., 2002), and the n-3 highly unsaturated fatty acids (HUFA) have been identified as essential dietary components for marine fish since they cannot synthesize them de novo (Cahu and Zambonino Infante, 2001; Bell et al., 2003). The optimal fatty acid composition and lipid levels for cod larvae are not known. Generally, marine fish larvae require large amounts of docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) (Rainuzzo et al., 1997; Sargent et al., 2002; Izquierdo, 2004) and the optimal level of EPA+ DHA in marine finfish larvae seem to be about 3% of dietary dry matter (Cahu and Zambonino Infante, 2001; Sargent et al., 2002). The use of diets with a high DHA/EPA ratio (>1.5 - 2.0) generally shows better larval survival and growth than a lower DHA/EPA ratio (Kjørsvik et al., 2004). Marine phospholipids (PLs) are carriers of HUFAs and are considered as good lipid sources for starter feeds for marine fish larvae (Sargent et al., 2002). Marine fish larvae possess a high capacity to utilize phospholipids (Geurden et al., 1998; Salhi et al., 1999; Izquierdo et al., 2001) and micro diets containing more marine phospholipids than marine triacylglyceroles (TAG) resulted in better growth in larval sea bass (Cahu et al., 2003a; Gisbert et al., 2005). It is recommended that marine fish larvae are given 10% marine phospholipids of dietary dry matter (Sargent et al., 2002; Cahu et al., 2003b).

The aim of the present study was to evaluate the effects of dietary incorporation of n-3 HUFA in different lipid classes (phospholipids vs. neutral lipids) and the level of n-3 HUFA in formulated diets on growth, survival and activity of some digestive enzymes in early weaned cod larvae. Growth, survival and specific activity of pancreatic- and intestinal digestive

enzymes were followed. The experimental design was based on isolipidic and isoproteic microdiets from 17 until 45 dph.

2. Material and methods

2.1 Experimental design

Atlantic cod eggs were obtained from Troms Marine Yngel A/S (Tromsø, Norway) two days before hatching. Eggs were disinfected in glutaraldehyde in seawater (0.4g/l) for 10 min (Salvesen and Vadstein, 1995) and incubated at a stocking density of 150 eggs/l in nine 160 l cone bottomed black tanks in darkness at 7.5° C (salinity 34 ‰). Each dietary treatment was run in three replicate tanks.

After hatching, light was turned on (24h) and the temperature was gradually increased from 8 to 12°C between 1 and 6 dph, and then kept constant at 12°C ± 0.2°C. Water exchange was gradually increased from 0.1 l/min at hatching to 0.9 l/min from 30 dph. Larval rearing lasted up to 45 dph. Dead larvae were removed every second day from 1-17 dph and were removed and counted every day from 17 dph onwards.

2.2 Live feed enrichment and larval feeding

The larvae were fed rotifers (*Brachionus. "Nevada" within the* Brachionus *plicatilis* cryptic species complex, Gomez et al., 2002), long-term enriched with the marine emulsion Marol E provided by SINTEF Fisheries and Aquaculture (Trondheim, Norway). Cod larvae were fed rotifers three times every day from 3 dph. Feed density was 3000-5000 ind/l until 4 dph, and then increased to 5000-7000 ind/l from 5 dph. Algal paste (*Nannochloropsis*, Reed mariculture, USA*)* was added 1-3 times a day from 2 dph until the end of the rotifer phase in a

126 concentration of 2 mg C/l. From 17 to 24 dph there was an overlap between rotifers and
127 formulated diets, and the amounts of rotifers were gradually reduced. From 24 dph cod larvae
128 were fed formulated diets exclusively.
129
130 Small amounts of the experimental formulated diets were fed manually to the larvae (0.15 g x
131 10) on day 17 to start weaning. On 18 dph, 3g/day of formulated diets were added using
132 continuous automatic belt feeders, gradually increasing the amount of feed added per day to
133 each tank to 10g from 31 dph. A pellet size of < 200μm was used from 17 to 30 dph with an
134 increase to 200-400μm from 30 dph. An overlap using a mixture of both pellets sizes was
135 conducted from 30 to 36 dph.
136
137 *2.3 Formulated diets*
138 Three isoproteinic and isolipidic diets which varied in their lipid class composition, PL vs. NL,
139 were used (Table 1). The lipid composition differed by the amount of cod liver oil, marine
140 lecithin and soybean lecithin added. PL3 and PL1 comprised a mixture of marine and soybean
141 lecithin as only source of lipids. The phospholipids were incorporated in two different levels
142 and in inverse relationship into the two different diets. NL1 contained only soybean lecithin
143 as the PL source and marine TAG (cod liver oil). These differences gave a proximate
144 composition with gradually decreasing levels of DHA and EPA in the dietary phospholipid
145 fraction (PL3>PL1>NL1) and increasing of total dietary neutral lipid levels (PL3<PL1<NL1)
146 (Table 1).
147 All diets contained 51% defatted fish meal and 14% hydrolyzed fish meal (CPSP), 8%
148 vitamin mixture, 4% mineral mixture and 2% of betaine. The total lipid content in all diets
149 was 21%. The diets were manufactured by UMR 1067 of Fish Nutrition, IFREMER (France)
150 according to Gisbert et al. (2005).

2.4 Sampling

All larvae were anaesthetised with Metacainum (Tamro 257675) and rinsed in distilled water before further treatment. Larvae collected for enzyme assays were immediately frozen in liquid nitrogen and stored at -80ºC. Larvae sampled to monitor growth were collected individually in tin capsules and dried for 48 hours at 60ºC for dry weight (DW).

2.5 Growth and survival

On 1 dph 15 larvae were sampled randomly from all tanks, and on 10, 17, 24, 35 and 45 dph, 15 larvae were sampled from each tank.

The larval specific growth rate (SGR, %/days) was calculated according to Kjørsvik et al. (2004):

$$SGR = \ln (W_t/W_0) /t \qquad \text{(equation 1)}$$

W_0 is the initial individual dry weight and W_t is the individual dry weight at time t.

The daily weight increase (DWI in %) was calculated from the specific growth rate according to Kjørsvik et al. (2004):

$$\% SGR = (e^{SGR} -1) \times 100 \qquad \text{(equation 2)}$$

Larval survival was calculated by daily counting of dead larvae from 17 dph, and by counting remaining larvae at the end of the experiment. Numbers were corrected for sampled larvae.

2.6 Spectrophotometric determination of trypsin, α-amylase, alkaline phosphatase (AP) and leucin-alanin aminopeptidase (leu-ala) specific activity

Before the introduction of the microdiets on 17 dph, 90 larvae were sampled and pooled from all tanks. Larvae were sampled separately from each tank on 24 dph (n = 50/tank), 35 dph (n

= 30/tank) and 45 dph (n = 30/tank). In order to determine enzymatic activities in specific segments, larvae older than 24 days were dissected after thawing as described by Cahu and Zambonino Infante (1994). The pancreatic segment, besides pancreas, comprised liver, heart muscle and spine. The intestinal segment contained the intestine, muscle and spine. Dissection was conducted under a binocular on a glass plate cooled on ice.

Seventeen and 24-days-old larvae were not dissected due to their smaller size. These larvae were homogenized with a Vortex mixer at maximum speed for 30 s and centrifuged at 2000 g (1 min and 4ºC) in order to tear up the larval abdomen and intestine. The supernatant was kept and treated as the intestinal segments of dissected larvae. The pellet was homogenized in 500 µl cold distilled water and treated as pancreatic segments in dissected larvae.
Dissected samples (pancreatic and intestinal, 35 and 45 dph) were homogenized in 500 µl cold distilled water using a homogenizer (Polytron, PT-MR 2100) at maximum speed for 30 s. The homogenate was centrifuged at 3300 g (3 min and 4°C) and the supernatant was collected.
Intestinal segments were homogenized to purify brush border membranes by the method of intestinal scrapping (Crane et al., 1979) and modified for intestinal segments of fish larvae (Cahu and Zambonino Infante, 1994). Before $CaCl_2$ was added in the procedure, 50-100µl of homogenate was removed for assays for total intestinal enzyme activity.
Due to the different procedures, larvae from 17 and 24 dph were not statistically compared with larvae from 35 and 45 dph.

Trypsine activity was assayed according to Holm et al. (1988) and amylase activity was assayed according to Métais and Bieth (1968) in both pancreatic and intestinal isolates.
Intestinal and pancreatic trypsine activity was determined using Nα-Benzoyl-DL-arginine-p-

nitroanilide (Sigma B4875) as substrate (pH 7, 25 ºC). Amylase activity was determined using starch as substrate (pH 7, 30 min and 37 ºC).

Alkaline phosphatase (AP) activity was assayed in intestinal homogenate and isolated brush border according to Bessey et al. (1946) using p-nitrophenylphosphate (Merck, 6850) as substrate (pH 7, 2 min and 37 ºC).

Assays for leucine-alanine peptidase (leu-ala) activity were performed on intestinal segments for larvae according to the method of Nicholson et al. (1974). Leucine-p-nitroanilide (Sigma, L9125) was used as substrate (pH 7, 2 min and 37 ºC). Absorbance was read at 407 nm (trypsine, amylase, AP) and 410 nm (leu-ala) in a spectrophotometer (Unicom Heλlios α).

Larval protein content was determined by the method of Bradford (1976) and enzyme activity was expressed as specific activity being μmol of substrate hydrolysed per min per mg protein (U/mg protein).

2.7 Spectrofluorometric determination of neutral lipase (n-lipase)

Ten larvae were randomly sampled from all treatments on 35 and 45 dph. Larvae were dissected as described above. Samples were homogenized on ice in 600 μl potassium phosphate buffer (50 mM, pH 7.8) by an ultra-turrax homogenizer (T8, IKA Labortechnik). After centrifugation at 3300 g (4 min and 4 ºC), the supernatant was collected. Larval samples from each treatment were denatured at 80 ºC for 1 h to inhibit any enzyme activity and thus were used as blanks.

N-lipase activities were determined by the method of Roberts (1985) as described by Izquierdo and Henderson (1998) using 4-methylumbelliferyl heptanoate (4-MUH, Sigma, M2514) as substrate. The substrate was added as liposomes to the enzymatic reaction (pH 7.8, 15 min and 35 ºC). The product of the hydrolysis was highly fluorescent and was quantified in

a spectrofluorometer (Perkin Elmer LS 50 B) at an excitation setting of 365 nm and an emission setting of 450 nm. Enzyme activity was calculated by using a standard curve based on 4-methylumbelliferone (4-MU) concentrations.

Sample protein in the samples for lipolytic activities was determined by a BioRad microassay kit based on the method of Bradford (1976) and enzyme activity was expressed as μmol of substrate hydrolysed per min per mg protein (U/mg protein).

2.8 Statistics

Data were tested for homogeneity of variances using a Levene test. To compare means, the group data were statistically tested using one-way ANOVA followed by a Student-Newman-Keuls-Test for more than two means. When variances were not homogenate; a non parametric Kruskal-Wallis test was accomplished. Two means were compared by a Student`s t-test. A 5 % level of confidence was used throughout. All statistical analysis was performed using the software SPSS 14.0 for Windows.

3. Results

At the end of the rearing period, final dry weight was significantly higher in larvae fed the PL3 (2.67 ± 0.39 mg) and PL1 (2.75 ± 0.52 mg) diets than the NL1 (2.23 ± 0.16 mg) diet (Fig. 1). Dry weight increased from 0.1 to 0.4 mg from hatching to 24 (dph) (Fig. 1). Between 24 and 45 dph larvae fed the PL3 and PL1 diets increased their dry weight nearly 7 folds while larvae fed the NL1 diet increased their dry weight 5.5 times. Growth in terms of daily weight increase (DWI) was reduced in all treatments during the first 11 days (24 to 35 dph) when larvae started to be fed dry feed only, compared to the co-feeding period (significant only in the PL3 treatment). Thereafter the DWI increased significantly in the PL3 and PL1 treatment

during the last ten days of the experiment (35 to 45 dph, Table 2). When comparing different dietary treatments at equal time intervals there were no significant differences in DWI. Larval survival was not significantly affected by the different experimental diets (Fig. 2). At the end of the experiment the average survival was 12.2 ± 0.5 % for larvae fed the PL3 diet, 12.8 ± 3.5 % for PL1 and 15.7 ± 4.5 and for NL1-diet.

Activity of the brush border enzyme alkaline phosphatase (AP) was low in larvae on 17 and 24 dph. On 17 dph the average activity was 29.1 ± 6.7 mU/ mg protein, and no significant alterations in AP activity were observed in larvae on 24 dph (Fig. 3A). AP activity increased strongly between 24 and 35 dph in all treatments, and on 45 dph activity of AP was significantly higher in the PL3 treatment (4227 ± 290 mU/mg protein) than in the PL1 (3310 ± 325 mU/mg protein) and NL1 (2625 ± 496 mU/mg protein) treatment (Fig. 3B).

The activity of the cytosolic peptidase leu-ala in larval homogenates increased significantly from17 dph to 24 dph in all treatments (Fig. 3C). As for alkaline phosphatase a strong increase in leu-ala activity was seen from 24 to 35 dph, while the activity decreased in all treatments thereafter, being significantly lower in PL1 and NL1 treatment. On 45 dph significantly higher leu-ala activity was apparent in PL3-larvae (1498 ± 76 U/mg protein) than in the PL1- (1175 ± 96 U/mg protein) and NL1-larvae (1252 ± 106 U/mg protein) larvae (Fig. 3D).

The ratio of the amount of AP in brush border membrane to the amount of leu-ala on 35 and 45 dph (Table 3) showed a tendency towards higher values in larvae fed DHA and EPA in the PL fraction of the diet. The ratio was significantly higher in larvae fed the PL3 diet on 35 dph and in larvae fed the PL1 diet on 45 dph, being lowest in NL1-larvae on both days.

Pancreatic activity of amylase decreased significantly in all treatments between 17 and 24 dph (Fig. 4A), whereas a slight increase in intestinal activity was observed (Fig. 4B). The activity was higher in the pancreatic segment than in the intestinal segment on 35 dph (Fig. 4C and 4D). Between 35 and 45 dph amylase activity decreased in the pancreatic segments, while it was fairly stable in the intestinal segment. No significant differences in activity between dietary treatments were evident during the experimental period.

The trypsin activity in the intestinal part was approximately one third of the activity in the pancreatic part on 17 dph (Fig. 5A and 5B). Between 17 and 24 dph, activity in pancreatic segments decreased in all dietary treatments, being significantly lower in the PL3 and PL1 treatment. Simultaneously an increase in intestinal trypsin activity was observed. The intestinal trypsin activity on 24 dph was not significantly different between treatments, while NL1-larvae had a significantly higher activity in the pancreatic segment on that day than the other treatments.

On 35 dph significant higher levels of trypsin activity in pancreatic segments were observed in larvae fed the PL1 diet (Fig. 5C). Intestinal activity of trypsin was also higher in PL1-larvae, although not significantly (Fig. 5D). On 45 dph intestinal trypsin activity was significantly higher in larvae fed the PL1 or the PL3-diet than the NL1 diet. The same tendency was seen for the pancreatic trypsin activity, although not significant.

Specific activity of neutral lipase (n-lipase) in the pancreatic segments of 35- and 45-days-old -larvae showed no differences between dietary treatments (Fig. 6A). However, the specific activity in intestinal segments showed significant differences between treatments on both days

(Fig. 6B). On 35 dph the activity was higher in the PL3 treatment compared to the NL1 treatment, with PL1 being intermediate.

Between 35 and 45 dph the specific activity decreased significantly in the PL3 and PL1 treatment, being significantly higher in the NL1-treatment. On 45 dph the n-lipase pancreatic activity was positively connected to the dietary level of triglycerides.

4. Discussion

In the present study DHA and EPA seemed to be more beneficial to larval growth and development when incorporated in the polar lipid fraction (PL) than in the neutral lipid (NL) fraction, as indicated by increased dry weight at the end of the experiment. NL1-larvae grew slower than the PL3- and PL1-larvae, although they reached the exponential growth phase during the experiment. The diets containing high and moderate levels of DHA and EPA in the PL fraction (PL3 and PL1 respectively) induced the best growth and intestinal maturation as indicated by the gut maturation index. In previous studies the relation between the amounts of brush border enzymes and cytosolic enzymes have been used to express intestinal maturation in sea bass, Senegal sole (*Solea senegalesis*) and yellow croacker larvae (Zambonino Infante and Cahu, 1999 ; Buchet et al., 2000; Ribeiro et al., 2002; Ma et al., 2005). Higher values correspond to a faster maturational process of enterocytes.

This might be caused by the levels and chemical location of DHA and EPA in the diet, since the PL1 diet contained equal levels of DHA and EPA in the PL fraction as the NL1 diet contained in the NL fraction. Cod larval ability to improve utilization of dietary PL compared to NL was also reflected by a faster skeletal development of larvae fed the PL3 and PL1 diets compared to those fed the NL1 diet (Kjørsvik et al., unpublished). Increased levels of marine phospholipids have also resulted in better larval growth in larval European sea bass when

weaned to formulated diets from mouth opening (Cahu et al., 2003a; Gisbert et al., 2005) using the same dietary composition as in the present experiment. This is further supported by findings of MacQueen Leifson et al. (2003) showing that marine phospholipids had a beneficial effect on growth and enterocyte mitochondrial structures in larval turbot. Marine fish larvae possess a high capacity to utilize dietary phospholipids (Salhi et al., 1999; Izquierdo et al., 2001) and it has been shown for sea bass larvae that it is beneficial to incorporate at least some of the dietary DHA/EPA content into the PL fraction (Cahu et al., 2003a; Gisbert et al., 2005). The more efficient use of DHA and EPA supplied in the PL class of the diet might be related to the ability of young larvae to better modulate phospholipase A_2 expression than that of lipase, suggesting a more efficient capacity to assimilate PL than NL as showed in sea bass larvae (Cahu et al., 2003a).

However, differences in growth were not associated to cod larval survival in this experiment. This is in accordance with weaning experiments with sea bass larvae (Cahu and Zambonino Infante, 1994; Morais et al., 2004), when there are sufficient essential nutrients available in the formulated diets to keep up larval survival.

The differences in growth between the PL3 and PL1 diets and the NL1 diet between 35 and 45 dph were reflected by the specific activity of the intestinal digestive enzymes. The specific activity of AP remained low in whole larvae homogenates from 17 to 24 dph and increased thereafter, which may suggest an increase in cod larval digestive capacity. An increase in AP activity generally occurs during larval and post larval development (Cahu and Zambonino Infante, 1994; Ribeiro et al., 2002; Ma et al., 2005; Perez-Casanova et al., 2006). This increase corresponds to the maturation process of enterocytes and the settlement of an efficient digestion on the brush border level.

In early larval stages, the enterocyte microvilli layer is poorly developed and the activity of brush border membrane enzymes is low. During the development of larval cod in the present study, the specific activity of AP increased in all treatments between 24 and 45 dph. This increase in AP activity demonstrates the intestinal maturation and is supported by other experiments with larval cod using live feed (O`Brien-MacDonald et al., 2006; Perez-Casanova et al., 2006) and other marine species such as sea bass and yellow croaker (*Pseudosciaena crocea*) (Cahu and Zambonino Infante, 1995; Ma et al., 2005). While AP activity increased in both the PL3- (significantly) and PL1-treatment (not significantly) it did not increase at all in the NL1 treatment between 35 and 45 dph, indicating a slower maturation and pointing to the diet-dependent process as previously suggested in other studies with cod and other marine fish species (Cahu and Zambonino Infante, 1995; Zambonino Infante and Cahu, 1999; Ribeiro et al., 2002; O`Brien-MacDonald et al., 2006).

The lower activity is probably not caused by reduced larval growth due to an inadequate dietary composition of the NL1 diet. A decrease in AP activity usually accompanies feeding with inadequate diets (Gawlicka et. al., 1996) or starvation (Cousin et al., 1987). In the present study the relation between amounts of AP and leu-ala was higher in larvae fed the PL3 and PL1 treatments indicating a faster maturation of the intestine of these larvae than in the NL1 treatment.

Leu-ala specific activity increased between 17 and 35 dph; which indicates that the cod larvae increased their capacity of intracellular digestion. A decrease in specific activity in intestinal segments of leu-ala between 35 and 45 dph, suggested a decrease in the intracellular digestion during development as also reported in sea bass (Zambonino Infante and Cahu,1997; Cahu et al., 2003a) and yellow croacker (Ma et al., 2005). The marked decrease with age of cytosolic enzymes and the concurrent and abrupt increase in brush border enzymes characterize the

normal maturation of the enterocytes in the developing animals (Henning, 1987), and this was observed in cod larvae between 35 and 45 dph in the present study.

With the exception on 24 dph, young cod larvae exhibited higher amylase activities in the pancreas than older larvae. The decrease in specific activity in pancreatic segments in cod larvae between 35 and 45 dph and the low activity in intestinal segments at the same age indicated a pancreatic development during the period. The amylase pattern in fish larvae may be compared with the decline of lactase expression observed during early development in mammals (Freund et al., 1990), and has previously been described in larval sea bass (Zambonino Infante and Cahu, 1994; Pères et al., 1996), walleye pollock (*Theraga chalcogramma,* Oozeki and Bailey, 1995), Solea *senegalesis* (Ribeiro et al., 1999) and yellow croacker (Ma et al., 2005). The higher amylase activities in younger larvae may express a predisposition of marine fish larvae to use carbohydrates during the early stages of life.

Generally, no clear differences in patterns of trypsin or amylase activity were observed between dietary treatments. This might be explained by the dietary compositions comprising comparable amounts of protein and starch in each of the diets. However, a significant higher trypsin activity was measured in pancreatic segments on 35 dph in larvae fed the PL1 diet. The same tendency was observed in intestinal segments. This might be explained due to higher ingestion rates of the PL1 diet, and thereby ingestion of a higher amount of protein. This assumption may also be supported by the tendency of PL1 larvae to possess higher daily weight increase values between 35 and 45 dph. Previous experiments have shown that trypsin activity is directly related to dietary protein content from 35 dph in sea bass larvae, whereas this regulatory process may not be functional in younger larvae (Pères, et al., 1996). Hjelmeland et al. (1988) showed that ingestion of inert polystyrene spheres induced higher

levels of trypsin in pancreas and intestine of larval herring than in starved larvae. Experiments with sea bass and turbot larvae, using live feed, also indicated better growth with high food levels due to higher larval ingestion rates when diets had the same composition (Zambonino Infante et al., 1996; Hoehne-Reitan et al., 2001). Pedersen et al. (1990) observed that trypsin and trypsinogen contents depended on food supply in *Clupea harengus* larvae fed copepods. Lower ingestion rates might also be the explanation to decreased pancreatic activity of amylase and trypsin in pancreatic segments during co-feeding. Cod larvae preferably seem to ingest live prey instead of formulated diets when live prey is available (Baskerville-Bridges and Kling, 2000).

The specific activity of n-lipase in intestinal segments was affected by the different diets. On 45 dph the lipase activity was 10 times higher in larvae fed the high NL levels (NL1) compared with low NL levels (PL3).

The activity of n-lipase showed high variability between dietary treatments on both 35 and 45 dph. On 45 dph there was a clear relation between enzyme activity and dietary lipid composition, suggesting that the mechanism of lipase regulation due to dietary composition in cod may be activated at the end of the larval phase. Lipase activity in cod larvae did not seem to be affected by the dietary lipid content in a comparable experiment during the first feeding phase (5 to 17 dph) (Hoehne, 1999) and turbot from 7 dph (Hoehne, 1999). However, lipase and PLA_2 activities were stimulated at the end of the larval phase in sea bass according to the increase in the respective dietary substrates triglycerides and phospholipids (formulated diets with 10-30% lipid) (Zambonino Infante and Cahu, 1999).

Overall the activity of lipase, trypsin and AP was lower in the present study than reported in other studies with larval cod (O`Brien-MacDonald et al., 2006; Perez-Casanova et al., 2006).

It is difficult to make precise comparisons between these studies due to different rearing protocols, diets, sampling procedures and analytical methods. Lower enzyme activity in larvae fed formulated diets have been reported by Hoehne-Reitan et al. (2003) who described that the specific activity of neutral lipase in turbot larvae (13 dph) was affected by the diet, observing higher activity in larvae fed live feed compared with larvae fed formulated diets. As previously discussed this might be explained by differences in ingestion rates.

All diets used in the present study supported the growth and development of cod larvae, but growth was lower than reported in comparable studies reported by Folkvord (2005) when larvae were fed on live plankton. However, larval dry weight on 45 dph in the present study (approximately 540 day degrees) was comparable with early weaned cod larvae on 50 dph (approximately 530 day degrees) in studies described by Baskerville-Bridges and Kling (2000). Larval dry weight on 30-35 dph was comparable with larvae fed live feed reported by Galloway et al. (1999) and Hoehne (1999) under equal rearing conditions as the present study, illustrating the potential of improving formulated diets and for early weaning of cod larvae.

Conclusion

In conclusion, rearing of cod larvae by substituting *Artemia* completely with a formulated diet was successful. Larval growth and intestinal maturation suggested that the incorporation form of DHA and EPA (PL or NL) in the diet may be crucial for cod larval development; being more beneficial when DHA and EPA were present in the PL-fraction of the diets rather than the NL-fraction. These results should be taken into account in the formulation of compound diets for cod larvae. However, a more comprehensive gradient experiment is necessary to evaluate the optimal lipid composition of an early weaning diet for this species.

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Legends.

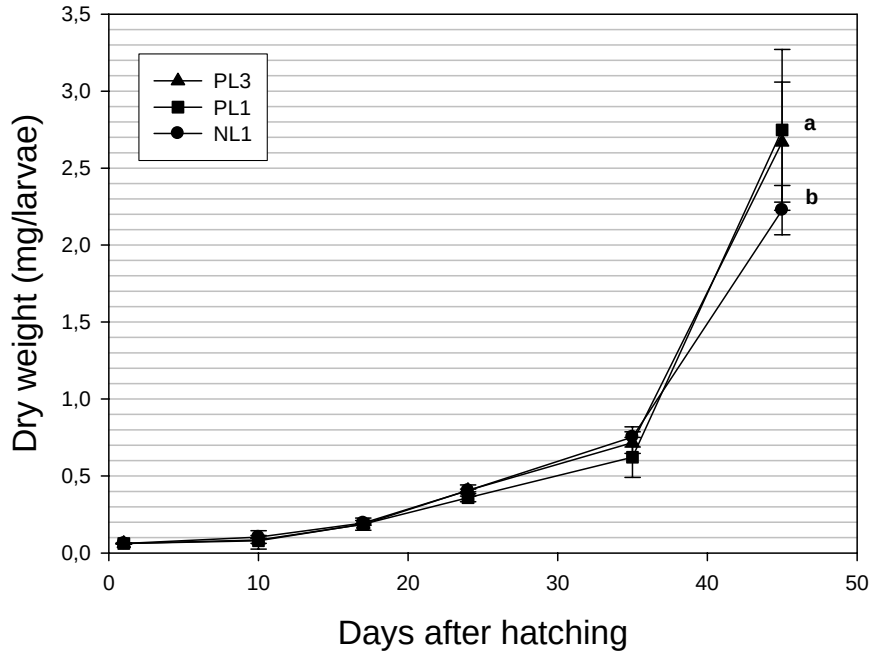
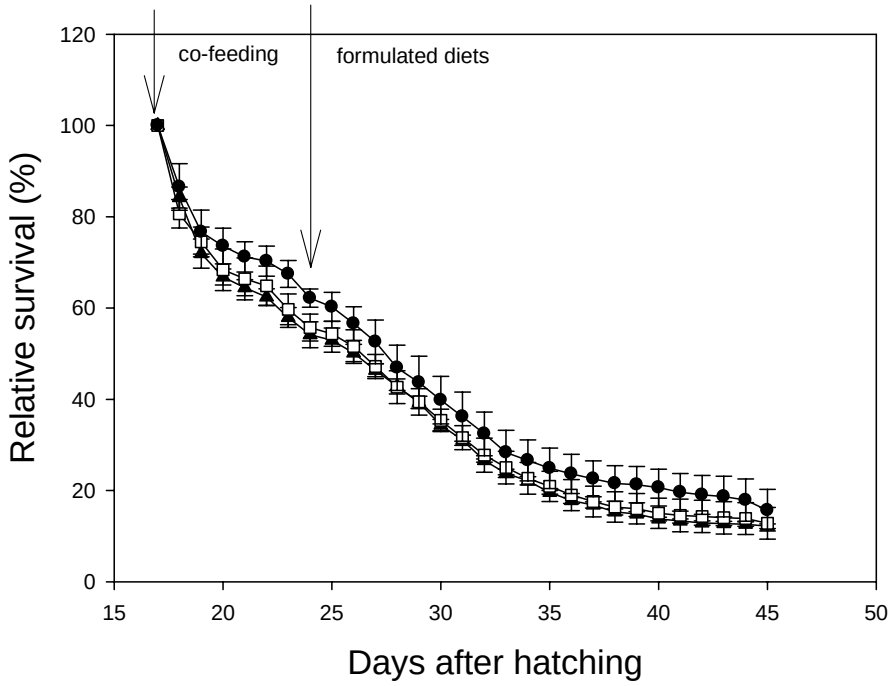


Fig. 1. Dry weight (mg DW/larvae) of cod larvae during the experiment (mean ± s. e. n = 45-67). Different letters denote significant differences between dietary treatments.



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696 Fig. 2. Relative survival (in %) of cod larvae (mean ± s.e. n = 3) during the periods of co-

697 feeding and feeding with formulated diets exclusively.

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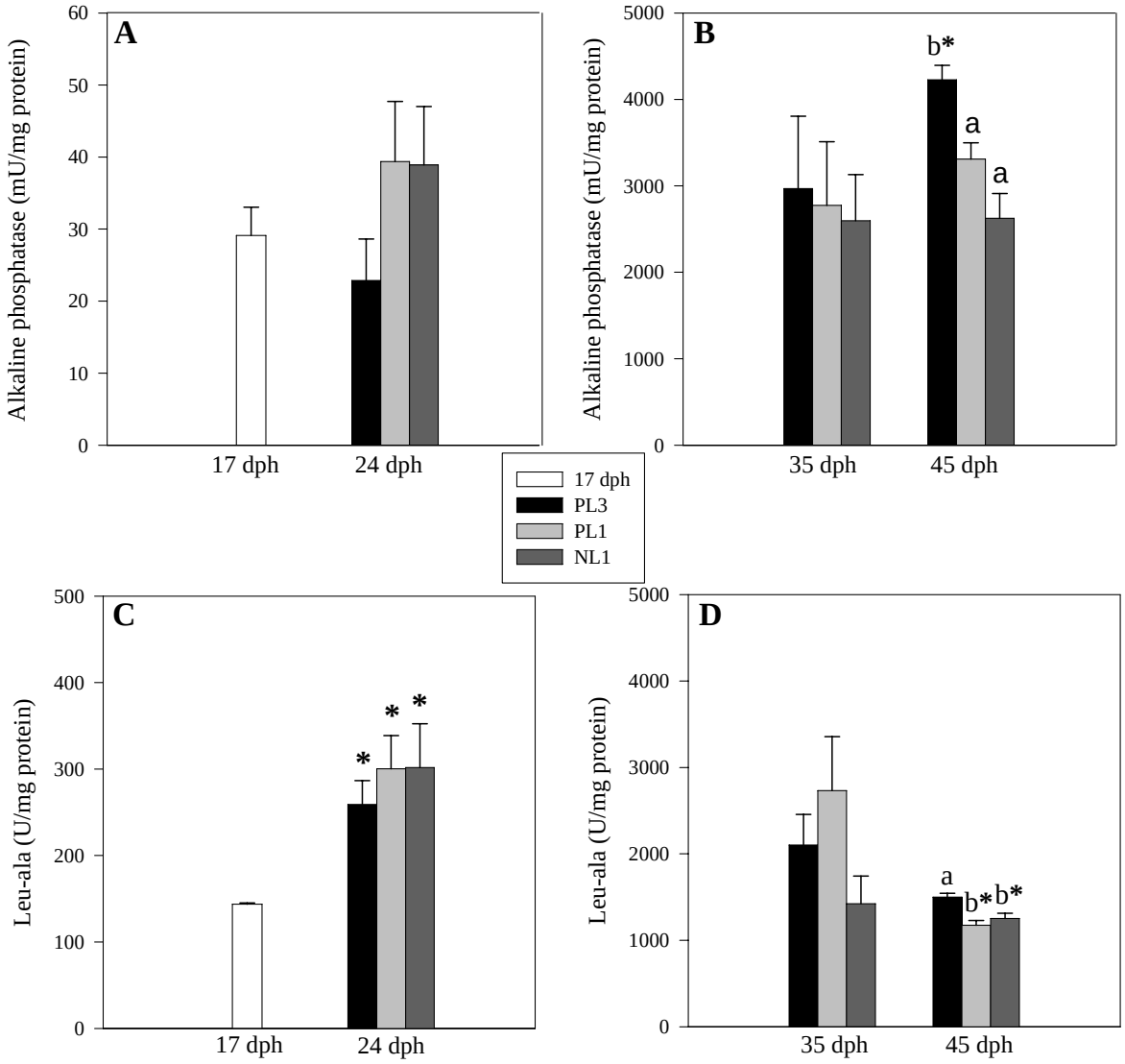
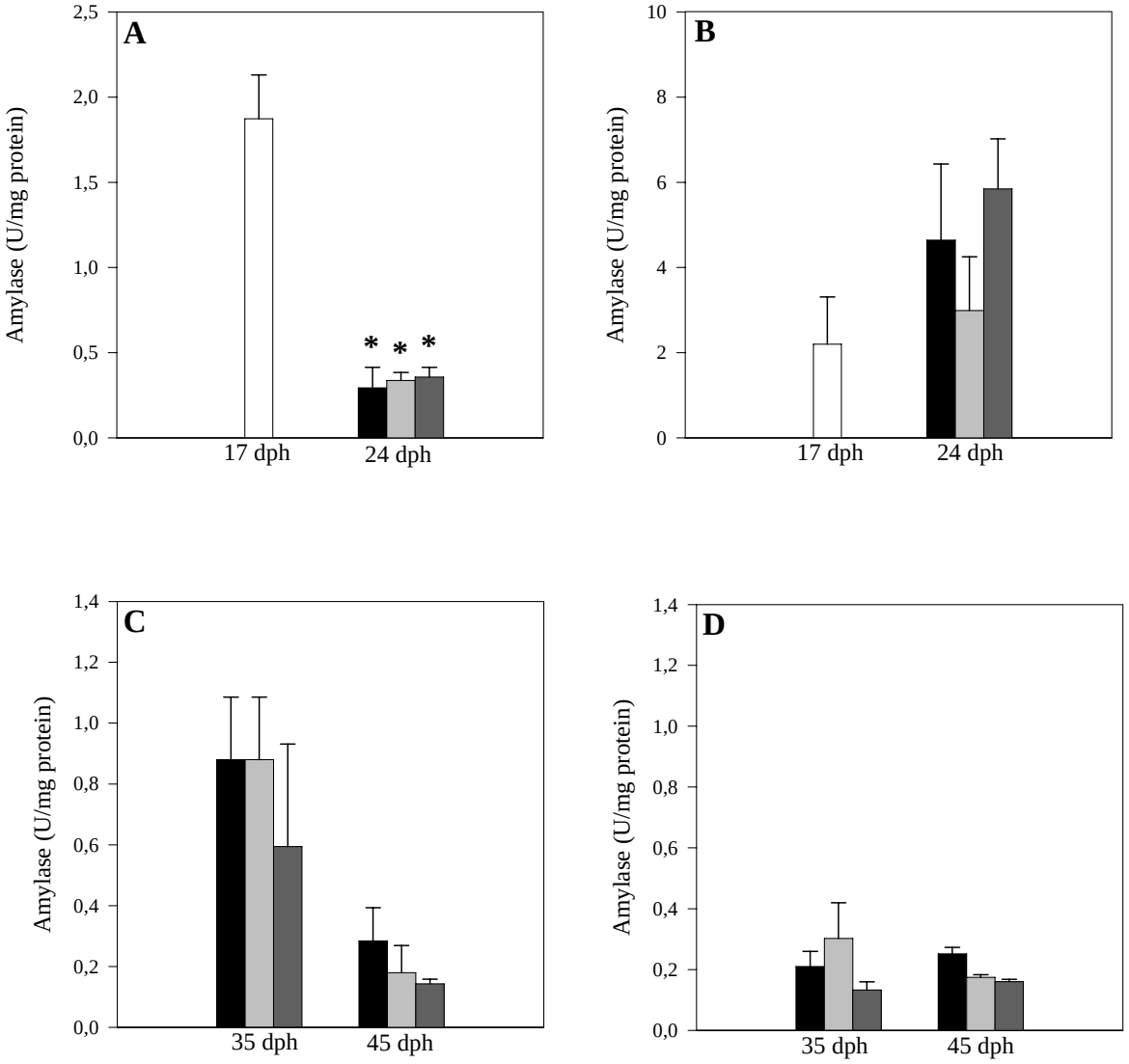


Fig. 3: **A**. Specific activity of alkaline phosphatase (mU/mg protein) in whole cod larvae on 17 and 24 dph. **B.** Specific activity of alkaline phosphatase (mU/mg protein) in the isolated brush border from intestinal segments (35 and 45 dph). **C.** Specific activity of leu-ala aminopeptidase (U/mg protein) in whole larvae on17 and 24 dph. **D.** Specific activity of leu-ala in intestinal segments of dissected larvae 35 and 45 dph. n = 3. Means ± s.e.; n = 3. Different letters denote significant differences between treatments. Stars denote significant differences between different days for the same treatment (in the same graph).



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712 Fig. 4: Specific activity of α-amylase (U/mg protein) in cod larvae **A.** In the pancreas of
713 larvae on 17 and 24 dph. **B.** In the intestine of larvae on 17 and 24 dph. **C**. In the pancreatic
714 segment of larvae on 35 and 45 dph. **D.** In the intestinal segment of larvae on 35 and 45 dph.
715 n=3 (tanks). Means ± s.e. Different letters denote significant differences between treatments.
716 Stars denote significant differences between days for the same treatment in the same graph.

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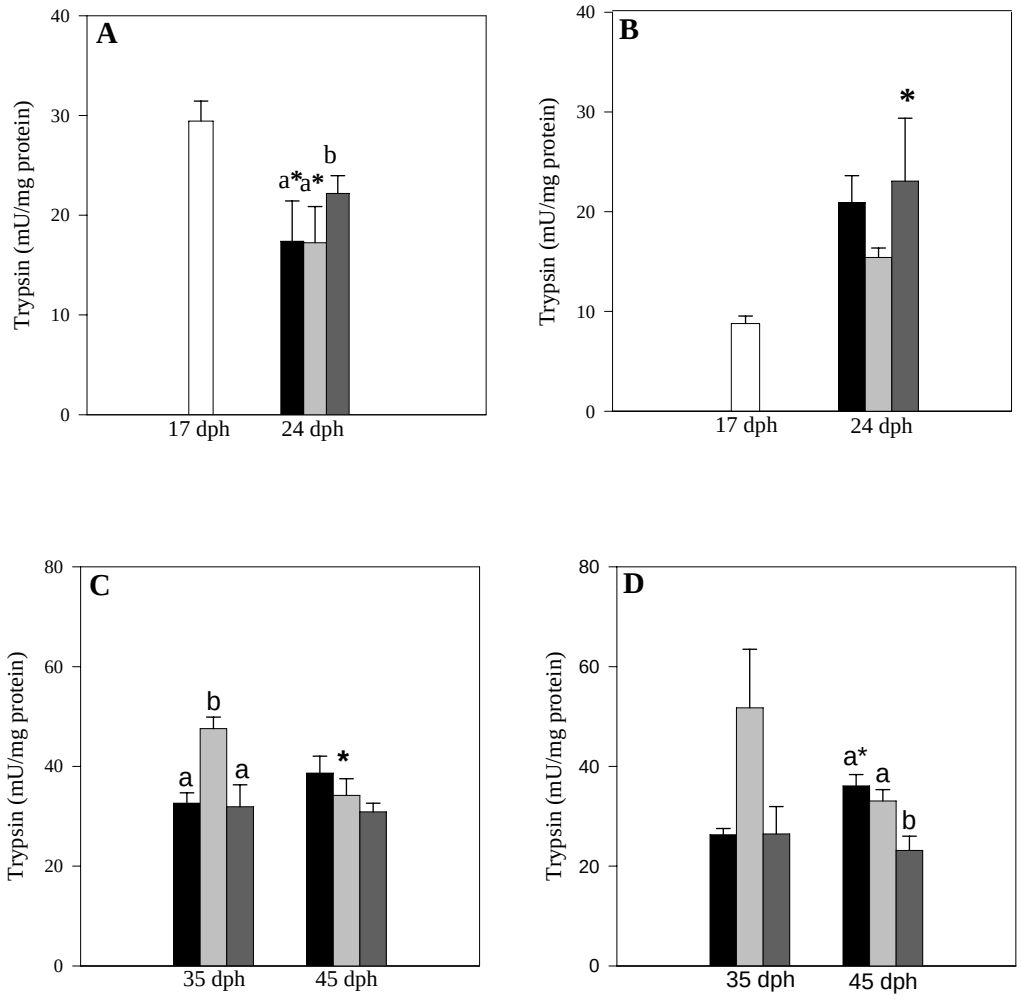

Fig. 5: Specific activity of trypsin (mU/mg protein) in cod larvae **A.** In the pancreas of larvae on 17 and 24 dph. **B.** In the intestine segment of larvae on 17 and 24 dph. **C**. In the pancreatic segment of larvae 35 and 45 dph. **D.** In the intestinal segment of larvae on 35 and 45 dph. n=3 (tanks). Means ± s.e. Different letters denote significant differences between treatments. Stars denote significant differences between days for the same treatment (in the same graph).

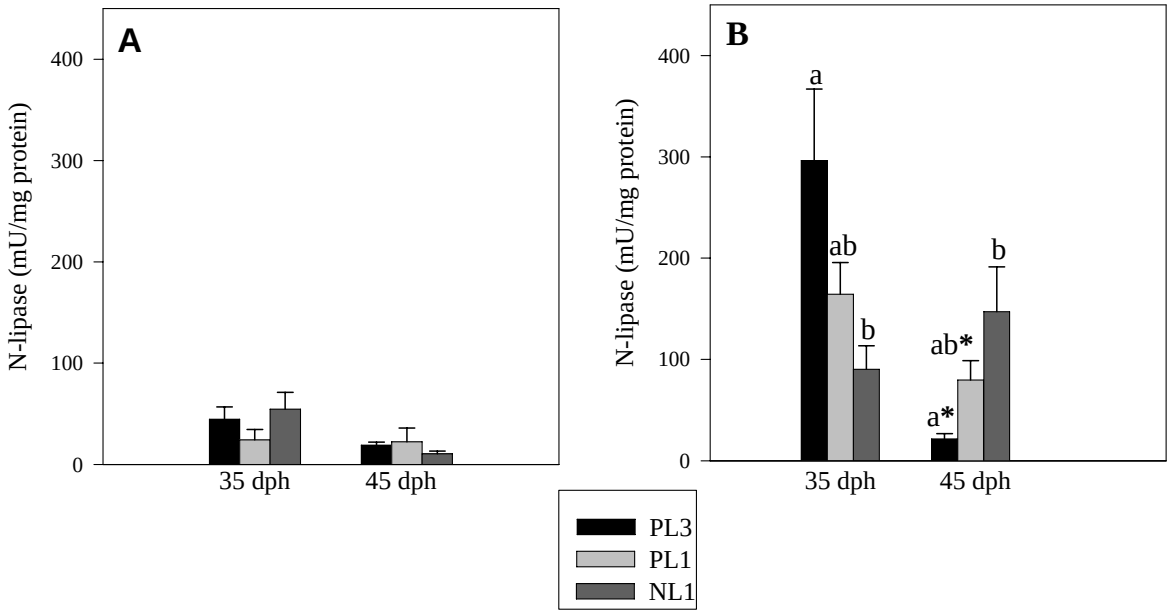

Fig. 6: Specific activity of neutral lipase in cod larvae (mU/mg protein) in pancreatic (**A**) and intestinal parts (**B**) of dissected cod larvae. n = 6-10. Means ± s.e. Different letters denote significant differences between treatments.

752 Table 1
753 Composition of experimental diets (%).
754
755

Diet Ingredients[a]	**PL3**	**PL1**	**NL1**
<u>Lipid ingredients (g/100g)</u>			
Cod liver oil	0	0	7
Marine lecithin[b]	14	7	0
Soybean lecithin[c]	7	14	14
<u>Proximate composition (%)</u>			
Proteins (N x 6.25)	61.5	58.3	57.7
Lipids	16.1	16.4	17.9
Phospholipids	**12.5**	**12.4**	**10.7**
EPA + DHA in PL	**2.3**	**1.1**	**0.3**
Neutral lipids	**3.7**	**4.4**	**6.8**
EPA + DHA in NL	**0.3**	**0.3**	**1.3**
Ash	17.4	17.5	17.4
Moisture	7.5	7.1	7.2
Energy[d]	1634	1592	1639

756

757 [a]All dietary ingredients were commercially available. Fish meal (La Lorientaise, Lorient,
758 France), hydrolyzed fish meal (CPSP, Soluble Fish Protein Concentrate; Sopropêche,
759 Boulogne sur Mer, France), cod liver oil (La Lorientaise), marine lecithin (LC60,
760 Phosphomins^{TM};

Phosphotech, Saint Herblain, France).
[b]Contains 60% phospholipids (with 45% PC, 20% PE, 16% PI), 5% TAG, 15% cholesterol, and 1 mg/g natural tocopherols as antioxidant.
[c]Contains 95% phospholipids (with 26% PC, 20% PE, and 14% PI).
[d]Calculated as: fat x 37.7 J/kg; protein x 16.7 J/kg.

Table 2
Daily weight increase (DWI) (%, means ± s.e.: n = 3) during different time intervals. Different letters denote significant differences in growth during different time intervals in the same dietary treatment.

	PL3	**PL1**	**NL1**
	Daily weight increase (% ± s.e.)		
17-24 dph (co-feeding)	11.8 ± 0.4[a]	9.8 ± 1.7[a]	11.1 ± 1.7[a]
24-35 dph (form. diets)	5.2 ± 1.0[b]	5.1 ± 1.2[a]	5.8 ± 1.6[a]
35-45 dph (form. diets)	13.3 ± 2.2[a]	16.2 ± 2.1[b]	11.4 ± 0.6[a]

Table 3
Ratio of segmental activity of alkaline phosphatase in the brush border membrane related to segmental activity of the cytosolic enzyme leu-ala peptidase (x 1000). Means ± s.e. (n = 3). Different letters denote significant differences in the same row.

	PL3	PL1	NL1
35 dph	46.6 ± 3.02[a]	36.5 ± 11.69[b]	18.2 ± 4.17[b]
45 dph	76.5 ± 10.69[b]	114.6 ± 14.12[a]	57.5 ± 15.71[b]