Keshuai Li

Phospholipids in Atlantic cod (*Gadus morhua* L.) larvae rearing:

Incorporation of DHA in live feed and larval phospholipids and the metabolic capabilities of larvae for *de novo* synthesis

Thesis for the degree of Philosophiae Doctor

Trondheim, October 2015

Norwegian University of Science and Technology
Faculty of Natural Sciences and Technology
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Trondheim, Spring 2015

Keshuai Li

List of papers

Paper I.

Keshuai Li, Elin Kjørsvik, Maria Bergvik, Yngvar Olsen (2015) Manipulation of the fatty acid composition of phosphatidylcholine and phosphatidylethanolamine in rotifers *Brachionus* Nevada and *Brachionus* Cayman. Aquaculture Nutrition, 21: 85–97. doi: 10.1111/anu.12140

Paper II.

Yang Jin, **Keshuai Li**, Yngvar Olsen (2014) Time kinetics of fatty acid changes in phospholipids following enrichment and starvation of *Artemia franciscana* with a main focus on docosahexaenoic acid (DHA). Aquaculture Nutrition. doi: 10.1111/anu.12224

Paper III.

Keshuai Li, Yngvar Olsen (2015) Effect of enrichment time and dietary DHA and non-highly unsaturated fatty acid composition on the efficiency of DHA enrichment in phospholipids of rotifers (*Brachionus* Cayman). Submitted to Aquaculture

Paper IV.

Yngvar Olsen, Jan Ove Evjemo, Elin Kjørsvik, Harald Larssen, **Keshuai Li**, Ingrid Overrein, Jose Rainuzzo. (2014) DHA content in dietary phospholipids affects DHA content in phospholipids of cod larvae and larval performance. Aquaculture. 428-429, pp. 203-214

Paper V.

Keshuai Li, Mari-Ann Østensen, Kari Attramadal, Per Winge, Torfinn Sparstad, Atle M Bones, Olav Vadstein, Elin Kjørsvik, Yngvar Olsen (2015) Gene regulation of lipid and phospholipid metabolism in Atlantic cod (*Gadus morhua*) larvae. Submitted to Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology

Paper VI.

Keshuai Li, Rolf Erik Olsen, Mari-Ann Østensen, Dag Altin, Elin Kjørsvik, Yngvar Olsen (2015) Atlantic cod (*Gadus morhua*) larvae have enzymatic capabilities to biosynthesis phospholipid *de novo* from 2-Oleoyl-glycerol and glycerol precursors. Submitted to Fish physiology and biochemistry

My contributions:

I wrote Paper I, Paper III, Paper V and Paper VI, contributed significantly to the planning and running of the experiments, sample and data analysis.

For Paper II, I was a co-advisor for master student Yang Jin and contributed to the planning of the experiment, guiding lipid and data analysis and manuscript preparation.

For Paper IV, I contributed to lipid analysis of some experimental materials and involved in improving the discussion.

1. Introduction

Atlantic cod is among the most important species in North Atlantic fisheries, and an interest of culturing the species has been evident for decades. However, the quality of cultured juveniles has been variable, which gives poor predictability of production (Hamre, 2006). Meanwhile, the cod producers have suffered from low prices due to the competition of increased landings from cod fisheries, especially during 1980'ties, mid 1990'ties and in recent years (Figure 1). Nevertheless, research on Atlantic cod farming has made significant progress. The full genome of cod has been sequenced recently (Star *et al.*, 2011), and will contribute to improve knowledge of the species. Genome data and molecular tools will improve the possibilities of using cod as a model species in research and also enhance the potential to become a successful species in aquaculture in the future.

1.1 Phospholipid of live feed

Live feeds are important food supply for most species of interest in aquaculture during their early life stages. Rotifers (*Brachionus* Nevada and *Brachionus* Cayman) and brine shrimp (*Artemia* sp.) are the most commonly live feed organisms used in aquaculture due to their relatively cost-efficient protocols for stable mass production (Conceicao *et al.*, 2010). However, the content of n3 highly unsaturated fatty acids (HUFA), particularly docosahexaenoic acid (DHA, 22:6n3) and eicosapentanoic acid (EPA, 20:5n3), in both rotifers and *Artemia* are insufficient for many marine larval fish species. Techniques to produce and enrich live feed with lipids that contain high levels of n3 HUFA have therefore been established over the last decades (Coutteau and Sorgeloos, 1997; Dhert *et al.*, 2001), but there are still questions raised on the lipid quality of live feeds used for larval fish species that are attractive for cultivation in northern countries, such as Atlantic cod (*Gadus morhua* L.) (O'Brien-MacDonald *et al.*, 2006; Garcia *et al.*, 2008).

Juveniles produced using nauplii and copepodit stages of various copepod species as live deed generally grow better and are of better quality (Hamre, 2006). It has been suggested that the superiority of copepod nauplii and copepodites is mainly due to their high n3 HUFA levels, and specifically their high contents of DHA in glycerolphospholipid (PL) (Bell *et al.*, 2003; Tocher *et al.*, 2008), which may contain up to 40% DHA of their total fatty acids (Overrein, 2010). The n3 HUFAs seem to be

more available for larval growth and development when they are incorporated in PL than in neutral lipids, as shown for European sea bass (Gisbert *et al.*, 2005), sea bream (Izquierdo *et al.*, 2001) and for cod (Wold *et al.*, 2007; Kjørsvik *et al.*, 2009). The PL associated DHA and EPA has also showed higher bioavailability for rodents and humans (Cansell *et al.*, 2003; Schuchardt *et al.*, 2011; Rossmeisl *et al.*, 2012).

Rotifers, *Artemia* and copepods, contain PL in similar quantitative amounts (around 40-60 mg g^{-1} dry weight) (Rainuzzo *et al.*, 1994a; Harel *et al.*, 1999; Olsen, 2004; Bergvik *et al.*, 2012). It appears to be difficult to modify the quantitative contents of PL (e.g., mg PL g^{-1} dry weight) in live feed organisms since they are mainly structural lipids (Rainuzzo et al., 1994b; McEvoy et al., 1996; Coutteau and Sorgeloos, 1997). On the other hand, it appears possible, within limits, to manipulate the qualitative fatty acid composition of PL fatty acids, including the percentage of DHA (Rainuzzo et al., 1994a; McEvoy et al., 1996). Among the numerous studies of n3 HUFA enrichment in the live feed, very few have analysed enrichment of PL of live feed (Frolov *et al.*, 1991; FernandezReiriz and Labarta, 1996; Sargent *et al.*, 1999; Guinot *et al.*, 2013b; Olsen *et al.*, 2014). Fish larvae may have specific requirements to their

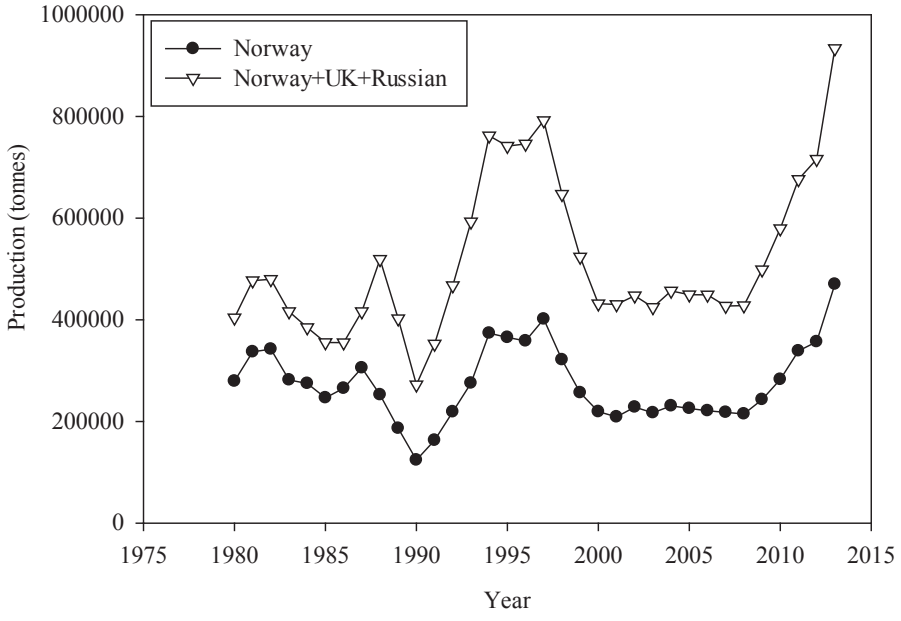

Figure 1. Catches of Atlantic cod (Data from http://www.fao.org/fishery/statistics/global-capture-production/query/en)

dietary PL composition, and there is an emerging need to learn more on how PL of live feed organisms can be efficiently enriched by n3 HUFA.

1.2 Essentiality of phospholipid for fish larvae

The importance of dietary PL for fish larvae was demonstrated in the early 1980's (Kanazawa *et al.*, 1981; Kanazawa *et al.*, 1983). The beneficial effects found were mainly associated with growth, survival rates, digestive functions, occurrence of deformities, and stress resistance in larval and juvenile stages of various species of fish (Coutteau *et al.*, 1997; Tocher *et al.*, 2008; Cahu *et al.*, 2009). Fish larvae seem dependent on a dietary supply of PL between 2-12 % of the diet (Tocher *et al.*, 2008) for normal growth and functional development. However, The PL requirements appear to decline as the fish grow bigger, and no requirements were generally observed in fish larger than 5 g (Tocher *et al.*, 2008; Cahu *et al.*, 2009). Intestinal steatosis (lipid droplet accumulation) occurred in much higher ratio (13 out of 16 *versus* 1 out of 18) in the fry stages (20 days post swim-up stage) compared to juvenile stages (146 days post swim-up stage) of rainbow trout when a PL-deficient diet was fed to the fish (Dapra *et al.*, 2011). The mechanism of essentiality of PL for larval and early juvenile fish is not clear, however, the requirement of dietary PL was suggested to be due to the limited ability to biosynthesize PL *de novo*, which is necessary for lipoprotein synthesis and transport from the enterocytes when dietary PL is insufficient (Tocher *et al.*, 2008; Cahu *et al.*, 2009). However, little is known about the PL biosynthesis capability in marine fish larvae.

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant PLs of eukaryotic membranes, accounting for more than 50% of the total PL species (Kent, 2005). For rotifers, PC and PE contributes up to 99 % of the total PL (Rainuzzo et al., 1994b). PC is also an important component of lipoproteins, which is required for chylomicrons assembly in the enterocytes and normal very low-density lipoprotein (VLDL) secretion from the hepatocytes, playing an important role in lipid absorption and transport. In mammals, the PC used in chylomicrons can originate from either dietary, biliary or *de novo* synthesis (Mansbach, 1977). The liver of healthy Caucasians produces 7 to 22 g PC every day that is excreted into the intestinal lumen through the bile (Northfield and Hofmann, 1975), and a 20 g mouse can secret 23 mg PC daily (Kuipers *et al.*, 1997).

However, early life stages of many fish species have incomplete developed livers during the period of first feeding and it has been suggested that these as mentioned have limited capacity for *de novo* synthesis of PL (Tocher *et al.*, 2008; Cahu *et al.*, 2009). Deficiency in endogenous PC could therefore lead to low lipoprotein synthesis and lipid accumulation in the enterocytes. Consequently, supplementation of dietary PL could improve the intestinal lipid absorption for many species of fish larvae (Fontagne *et al.*, 1998; Olsen *et al.*, 2003; Dapra *et al.*, 2011).

1.3 Pathways of phospholipid biosynthesis

The biochemically limiting steps for PL synthesis in fish larvae are unknown. However, the metabolic pathways appear to be the same as for mammals (Figure 2) (Sargent *et al.*, 2002). In mammals, the major biosynthesis pathway of PC is the CDP-choline (cytidine diphosphate-choline) pathway with choline kinase (CK) producing phosphocholine, followed by CTP:phosphocholine cytidylyltransferase (CT) producing CDP-choline, and accomplished by CDP-choline: *sn*-1,2-diacylglycerol cholinephosphotransferase (CPT) producing PC. The second step catalysed by CT is considered to be the rate-limiting step in the pathway under normal physiological conditions. However, the last step catalysed by CPT can become rate-limiting if the supply of di-acylglycerol (DAG) is restricted (Gibellini and Smith, 2010). Alternatively, PC can be synthesized by methylation of phosphatidylethanolamine (PE), catalysed by phosphatidylethanolamine N-methyltransferase (PEMT), which mainly operates in the liver and contributes to 30-40% of PC in hepatocytes (Sundler and Akesson, 1975; DeLong *et al.*, 1999; Reo *et al.*, 2002). PE is synthesized through the CDP-ethanolamine pathway, which is similar as the CDP-choline pathway. In mitochondria, PE is synthesized by decarboxylation of phosphatidylserine (Borkenhagen *et al.*, 1961).

DAG is an important intermediate common for the synthesis of both TAG and PC (PE). It can be generated by acylation of glycerol-3-phosphate (G-3-P) to phosphatidic acid (PA) which is subsequently dephosphorylated by phosphatidic acid phosphohydrolase (PAP or lipin), termed G-3-P pathway in the thesis. Alternatively, DAG can be formed by reacylation of *sn*-2-monoacylglycerol (2-MAG) by monoacylglycerol acyltransferase (MGAT), termed 2-MAG pathway.

Considering the metabolic mechanisms that can be involved, the fatty acid composition in PL could be modified through PL synthesis and modification through the pathway of *de novo* synthesis and the land cycle (PL to lyso-PL to PL) pathway, respectively. Many acylases and transacylases involved in these pathways do not have absolute specificities, which mean that the levels of fatty acids available from the diet might have a significant influence on the fatty acid composition in PL of the enriched rotifers (Sargent *et al.*, 1999). However, some degree of selectivity on fatty acids or

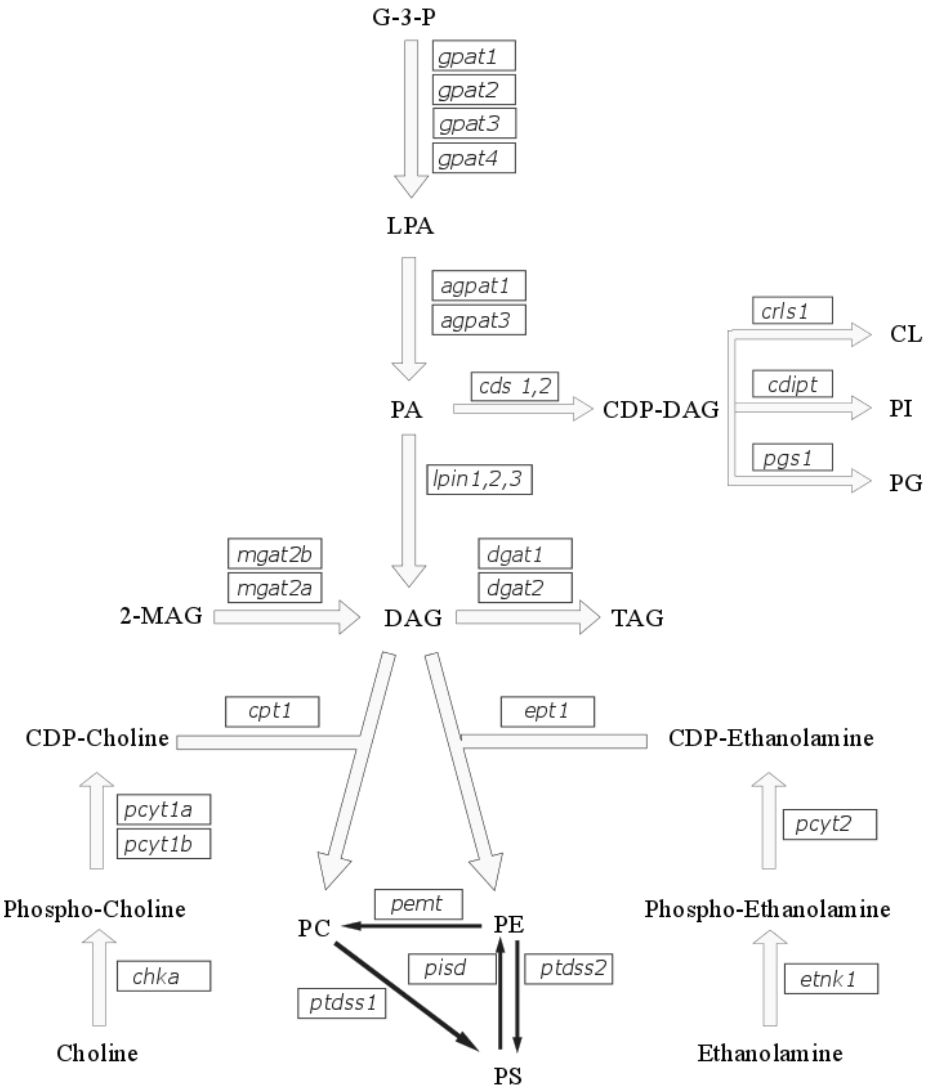

Figure 2. Pathways of phospholipid *de novo* biosynthesis (taken from **Paper V**)

fatty acid associated structures in phospholipid synthesis and modification has been reported, especially for the key enzymes involved in respective pathways, the CPT and lysophosphatidycholine acyltransferase (LPCAT).

Studies using rat liver microsomes have shown that CPT preferred 1-16:0-2-DHA-DAG for synthesis of PC over its *sn*-1-stearoyl counterparts, and 18:1n9 was potentially a strong competitor for the *sn*-2 position in this DAG structure (Holub, 1978). Human LPCAT3 enzyme showed preference towards 18:2n6-CoA and 20:4n6-CoA as acyl donors and lyso-PC with saturated fatty acid at the *sn*-1 position as substrates (Kazachkov *et al.*, 2008). Unfortunately, the selectivity of these enzymes in rotifers has not been described. If a similar selectivity exists in live feed organisms like rotifers and *Artemia*, the non-HUFA (non-highly unsaturated fatty acid) composition of the enrichment diets might affect the efficiency of DHA enrichment in phospholipids.

Choline is an essential nutrient for animals, and its main fate is the biosynthesis of PC via the CDP-choline pathway (Li and Vance, 2008). Inadequate choline intake can lead to fatty liver or muscle damage in humans (Fischer et al., 2007) and liver dysfunction, reduced growth and poor feed efficiency in many fish species (Millikin, 1982). Supplementation of 0.2% choline of dry weight in the diet can improve growth performance, intestinal enzymes activities and feed utilization of blunt snout bream fed high-lipid diet (Li et al., 2015a). One of the obvious functions of choline was the stimulating effect of PC biosynthesis as shown in isolated rat hepatocytes where supplementation of 0.5-2 mM choline could stimulate PC synthesis 2 to 3 folds via the CDP-choline pathway (Sundler and Akesson, 1975).

2. Aims of study

The main focus of the thesis is on two main challenges related to nutrition of cod and other marine fish larvae with the following main objectives:

- To increase the content of DHA in PC and PE in cultivated rotifers and *Artemia*
- To improve the understanding of the specific requirement of dietary PL in the early stages of cod larvae.

The thesis can be divided into two parts.

2.1 Part 1 - Manipulation of fatty acid profile in phospholipid of live feed

- **Paper I**

  The main objective of **Paper I** was to examine how variable lipid class and fatty acid composition of dietary lipids could affect the fatty acid composition of the PC and PE of two rotifer strains (*Brachionus* Nevada and *Brachionus* Cayman) after enrichment by a diet rich in marine PL or a diet containing mainly TAG. A second objective was to compare the fatty acid composition in PC and PE between enriched rotifers and copepodites harvested from natural waters, with a main focus on the DHA content.

  The paper shows fatty acid profile of PC and PE of two rotifer strains after a so called long-term enrichment by a diet either rich in PL or TAG. The correlation of DHA in PC and PE of enriched rotifers and the diet is investigated.

- **Paper II**

  The objective of **Paper II** was to study time kinetics of change in important HUFAs in PC and PE of *Artemia franciscana* nauplii and juveniles following enrichment and subsequent starvation.

  The paper shows fatty acid profile of PC and PE of *Artemia* nauplii, with focus on the change of DHA in PC and PE following enrichment and starvation. It also compares the content of DHA in *Artemia* nauplii and juvenile.

- **Paper III**

  The objective of **Paper III** was to investigate the effect of enrichment time and dietary non-HUFA composition on the efficiency of DHA enrichment in the main PL molecules PC and PE of rotifers. The stability of DHA post-enrichment was also tested under starving conditions at different temperature.

The paper shows the changes of DHA in PC and PE of *Brachionus* Cayman through enrichment and through starvation post-enrichment with commonly used commercial diet Multigain or DHA Selco. An enrichment method to obtain high DHA% in phospholipid of rotifers is suggested based on our overall results and the possible mechanism is discussed.

- **Paper IV**

  The objective of **Paper IV** was to examine if cod larvae could incorporate DHA from dietary PL more efficiently than from dietary TAG, by providing differently enriched rotifers and copepods.

  The paper describes enrichment methods to obtain different DHA% in phospholipid, but similar total lipid and fatty acid composition of rotifers.

2.2 Part 2 - Phospholipid metabolism in cod larvae

- **Paper IV**

  Paper IV also shows that the accumulation of DHA in larvae was related to DHA in live feed PL but not in TL, and therefore we conclude that PL from copepods is a better DHA source than PL from rotifers for larval cod.

- **Paper V**

  The main objective of **Paper V** was to obtain more knowledge on ontogenesis of lipid metabolism, especially the pathway of *de novo* phospholipid synthesis during early stages of cod larvae.

  It describes ontogenesis of genes involved in lipid metabolism and phospholipid *de novo* synthesis through the early life stages of cod.

- **Paper VI**

  The main objective of **Paper VI** was to investigate if cod larvae could synthesis PL from 2-MAG and glycerol precursors, which are the main products of TAG digestion. Moreover, the effect of supplemented choline on the biosynthesis of PL and TAG was also investigated.

  It shows that cod larvae are metabolically capable to synthesis PL from radiolabeled 2-MAG and glycerol precursors.

3. Main results and discussion

3.1 Manipulation of fatty acid in PC and PE of live feed

3.1.1. Rotifer enrichment

Marine PL versus TAG (Paper I) - Long-term enrichment techniques were used in this study (Rainuzzo *et al.*, 1994b), where efficient rotifer enrichment of n3 HUFA can be achieved by feeding a complete diet, with a relatively low lipid content and a high percentage n3 HUFA of total fatty acids, which can support fast growth and reproduction of the rotifer cultures. A steady state of growth and biochemical composition can be reached in 4-5 days of cultivation when > 90 % of the rotifer individuals are produced in the period, given high growth rate (~0.4 day^{-1}).

Rotifer cultures of *Brachionus* Nevada and Cayman were grown semi-continuously, fed live *Rhodmonas baltica* algae, and cultures were diluted daily (20 % day^{-1}). The enrichment diets Marol E (TAG-rich) or PL (cod roe emulsion, PL-rich) were added to both cultures (Nevada 40 ng ind^{-1} day^{-1} and Cayman 25 ng ind^{-1} day^{-1}, respectively). After 4 days of enrichment at a dilution rate of 20 % day^{-1}, Cayman rotifers fed Marol E (CM) had comparable (P>0.05) quantitative amounts of DHA to natural harvested copepods (Figure 3B), which were analysed for comparison, and the Cayman strain showed generally a more efficient incorporation of HUFAs than the Nevada strain.

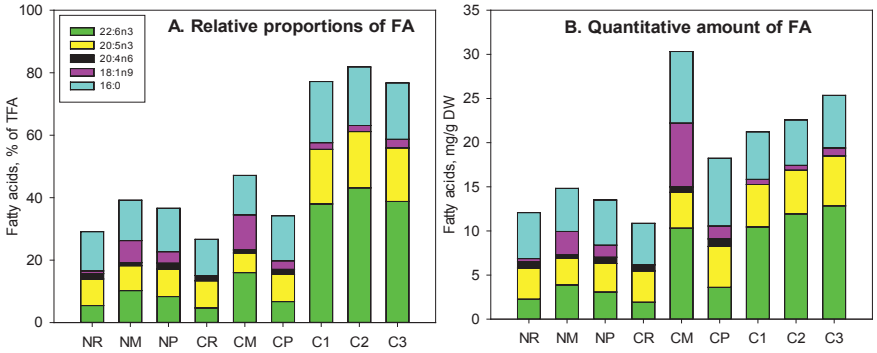

Figure 3. Selected fatty acid composition of enriched rotifers compared to copepods. NR, Nevada cultivated by R. *baltica*; NM, Nevada enriched by Marol E; NP, Nevada enriched by PL emulsion; CR, Cayman cultivated by R. baltica; CM, Cayman enriched by Marol E; CP, Cayman enriched by PL emulsion. C1, copepod size between 300-400 µm; C2, copepod size between 400-500 µm; C3, copepod size between 700-1000 µm (modified from **Paper I**).

However, the percentage DHA of the total fatty acids in copepods were much higher than in enriched rotifers ($P<0.05$) (Figure 3A).

Among the selected fatty acids shown in Figure 3B, the content of 18:1n9 was apparently higher in CM rotifers than in copepods. It might be possible to further increase the DHA % in total fatty acid and PC and PE by reducing competing fatty acids, such as 18:1n9 in the enrichment diets. The PC-DHA (DHA in PC) and PE-DHA (DHA in PE) levels of copepods were up to 50 %. For rotifers, the highest PC-DHA (12.5 %) and PE-DHA (5.1%) levels were found in CM rotifers. The increase of DHA in PL of rotifers was mainly due to an increase of DHA in PC, whereas the PE-DHA was only weakly affected by dietary lipids and the enrichment procedure. The percent PC-DHA levels in rotifers were positively related to the total dietary DHA levels ($P<0.0001$), independent of the PC-DHA content in the diets. Diets based on marine PL were not more efficient than diets based on marine TAG for the DHA manipulation of PC and PE of rotifers.

Multigain *versus* DHA selco (DSelco) Paper III - Knowing that 18:1n9 may compete with DHA in incorporating into PL, we compared the DHA enrichment efficiency of two commercial diets, Multigain containing low 18:1n9 and high 16:0 and Dselco containing high 18:1n9 and low 16:0.

Total lipid and the fatty acid composition in total lipid, PC and PE of rotifers were measured throughout the enrichment period of about 24 h. The highest DHA levels in PC (23.0 %) and PE (16.5 %) in Cayman were found after 24 h enrichment with Multigain, and levels were far higher than obtained in **Paper I** and **Paper IV**. The enriched DHA was stable at 10 ºC for at least 24 h post enrichment under starving conditions ($P>0.05$), whereas a significant ($P<0.05$) decrease of DHA was observed during starvation at 20 ºC.

It could be suggested that the higher percentage fraction of DHA provided using Multigain rather than DSelco explained why PC and PE enrichments were most efficient for incorporating DHA in PC and PE of the rotifer, but the DHA ration up to 12 h of enrichment was the same. An alternative explanation might be that a lipid emulsion, which constitutes mainly lipids, is more poorly assimilated and therefore metabolically less efficient for transferring DHA to rotifer PL than a slightly more complete enrichment feed, with some proteins added (13%, from algae and yeast) and

with high DHA level. The enhanced efficiency might be a combination of these effects, and a Multigain-type diet would then in all events be most efficient for PL

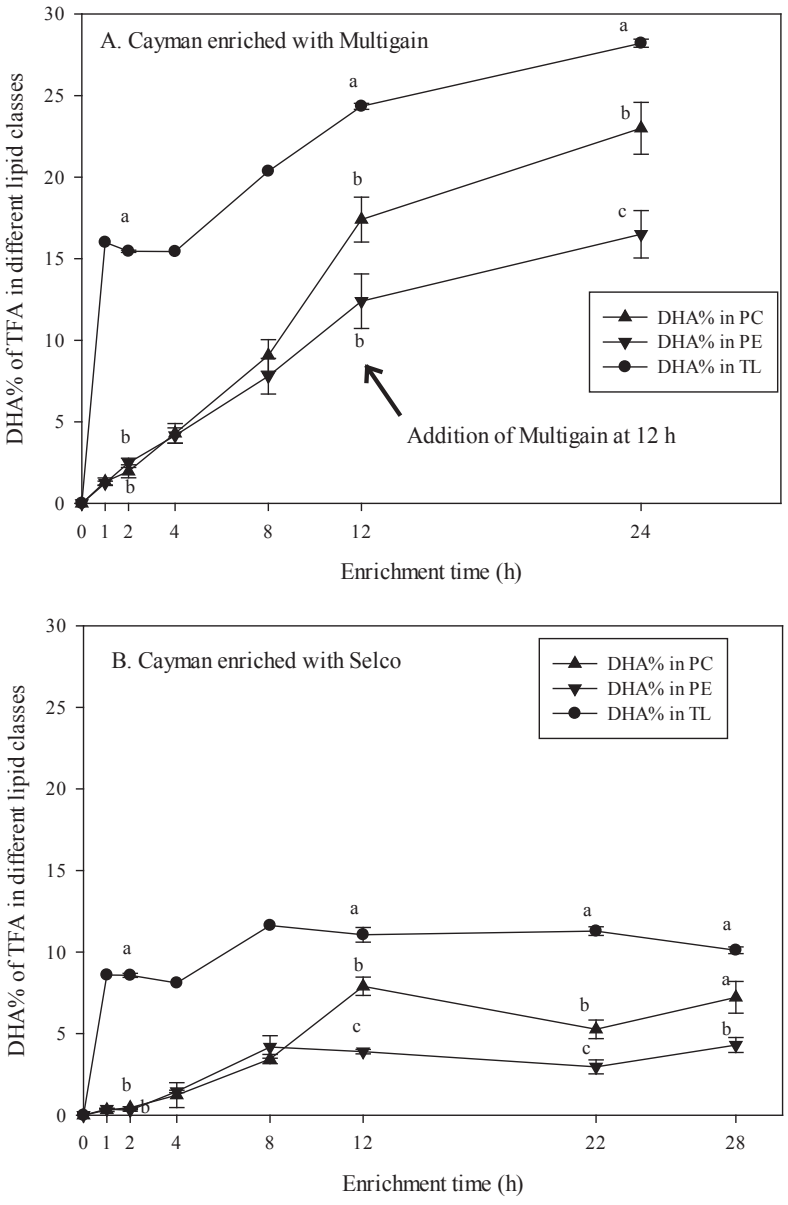

Figure 4. Changes in DHA% in total lipid (circles), PC (triangles) and PE (inverted triangles) as a function of time in *Brachionus* Cayman enriched with Multigain (A) and DSelco (B). Error bars express standard errors of the mean of replicates. Numbers of replicates were 1-6, see Appendix. Different letter inserted for data points for specific time indicate significantly different values ($P<0.05$) (Taken from **Paper III**).

enrichment of DHA and HUFA. The results of **Paper I and IV** put, however, some doubts about this simple explanation. The enrichment emulsion (Marol E) used in previous studies contained more than 40% DHA in total lipid, and the DHA% in total lipid of enriched rotifers could reach 22.3-26.2 %. However, the maximum DHA% in PL was still only about 12 % (**Paper I**, **Paper IV**). This suggested that provision of diets with high DHA content might not alone be enough to obtain high DHA levels in PL of rotifers.

Our results suggested that MUFA was replaced by PUFA in PL of rotifers during the enrichment process. The relatively lower DHA levels incorporated into PL in **Paper I** may be due to competition caused by high contents of 18:1n9 in the emulsion and short chain n3 PUFAs like 18:3n3 and 18:4n3 in *R. baltica*. Beside this, we suggest that the high 16:0 levels in Multigain may stimulate the biosynthesis of PL of growing and reproducing rotifers. It has been reported that 16:0 may increase phospholipid synthesis in cultured Caco-2 cells (Van Greevenbroek *et al.*, 1995) and reduce the intracellular lipid droplet formation in Arctic char (Olsen *et al.*, 2000). In addition, there are indications that 16:0 may facilitate the incorporation of dietary DHA into PC of human plasma (Subbaiah *et al.*, 1993). Moreover, Williams *et al*. (1999) suggested that 16:0-DHA-PC was preferably retained in the plasma membrane. We therefore suggest that an efficient enrichment diet should have high content of DHA and possibly high content of 16:0 and a low content of MUFA.

Enrichment time (Paper II, Paper III) - The enrichment time was another decisive factor for the rate of fatty acid replacement in phospholipids of the rotifers and *Artemia*. Previous studies have shown that prolonged enrichment time was more effective than increased oil rations in boosting n3 HUFA content in TL of rotifers (Rodriguez *et al.*, 1996). Rotifers are commonly enriched only for a short period (for example, 2 h) in some hatcheries and laboratory studies (Maehre *et al.*, 2013; Rehberg-Haas *et al.*, 2015). Our results revealed that total lipid content and DHA% in total fatty acids increased most efficiently during the first hour of enrichment, whereas the DHA in PC and PE showed a much slower rate of increase during the same time period (Figure 4). Because of this relatively slow increase of DHA in PC and PE compared to that in TL, a prolonged enrichment time (24 h) is suggested to obtain high DHA or HUFA content of rotifer PL. Contrary, very short enrichment time, for example 2 h, will not result in very much DHA or HUFA enrichment in the

PL of rotifers, which can be important for experimental purposes (**Paper IV, Paper V**).

Low feeding ration to keep rotifers lean (Paper III, Paper IV) - Short-term enrichment (within 24 h) strategies tend to produce rotifers with high lipid content (Rainuzzo *et al.*, 1994b), which has not been recommended for cold water fish larvae, because high rotifer mortality may occur when fat rotifers are transferred to cold water (Olsen *et al.*, 1993). Moreover, fat rotifers contain a higher TAG fraction than more lean rotifers, because the PL content per dry matter remains fairly constant independent of the total lipid contents. A high TAG content of the rotifers may not be optimal for early fish larvae (**Paper IV**). DHA is normally believed to be esterified in the *sn*-2 position of TAG (Sargent *et al.*, 1999; Sargent *et al.*, 2002). During digestion processes in fish larvae, the hydrolysed fatty acids from *sn*-1 and *sn*-3 positions of dietary TAG could then dilute the DHA% in the enterocyte, resulting in lower DHA levels in the resynthesized PL through lyso-PL (Lands cycle) pathway (**Paper IV**). To produce copepod-like live feed, it is therefore an issue to both minimise dietary lipid rations and rotifer lipid level besides having high DHA enrichment of PL, which may seem to be a contradiction, or at least a challenge.

Enrichment method to produce rotifers with high DHA in PL (Paper III) - Our study has shown that it is, nevertheless, possible to enrich the DHA levels in both PC and PE of rotifers to relatively high levels, and that these levels can be kept stable for at least 24 h at 10 ºC. Based on our overall results, we suggest the enrichment time should be at least 24 h, in order to maximise the efficiency of the PL enrichment. Beside this, because of the effect of high TAG mentioned above, the feeding rations of lipids should be as low as possible to reduce the total lipid contents of the rotifers. The enrichment diet should have high content of DHA. The possible stimulating effect of 16:0 and inhibiting effect of MUFA needs further investigation.

Method to produce live feed with variable DHA in PL (Paper II, PaperIII, Paper IV) - The main challenge of using live feed diets to study PL nutrition of fish larvae is to design diets where one principal component is varied whereas the remaining components are kept relatively constant over some time. **Paper IV** described a method to produce rotifers containing similar amount of total lipid, total fatty acids, DHA and n3 HUFA, and PL content per dry weight. The most striking difference was the variable DHA% in total PL fatty acids. This can be achieved by providing the

same feed components, but different feeding strategies involving a combination of long-term and short-term enrichment techniques as described above. The basis of the method is a "lag phase" in DHA accumulation in PL of rotifers and *Artemia* during enrichment (**Paper II, Paper III**). The effects of DHA in either forms of PL or TAG in live feed could therefore be conveniently investigated by feeding fish larvae with live feed organisms enriched using this principal method, which must be adapted to the specific experimental situation.

3.1.2 Artemia enrichment

Changes of HUFAs in PC and PE of *Artemia franciscana* nauplii and juveniles were studied following enrichment and subsequent starvation in **Paper II**. Multigain was provided in a concentration of 200 mg L^{-1} at the start of enrichment of the *Artemia* culture with a density of 120 ind mL^{-1}. The highest DHA levels were found at 8 h post enrichment in total lipid (8.00 %), PC (1.96 %) and PE (2.01 %) of *Artemia*. Most studies of DHA enrichment of PL in *Artemia* have shown that DHA ends up in TAG and that very little DHA are incorporated into PL (less than 3% of total fatty acid in PL) (Coutteau and Mourente, 1997; Sargent *et al.*, 1999; Guinot *et al.*, 2013a; Jin *et al.*, 2014).

An extreme lag phase of 1 h in the accumulation of DHA, EPA and ARA (arachidonic acid, 20:4 n6) in PC and PE after initiation of enrichment was found for *Artemia*, suggesting slower HUFA incorporation rate in PL compared to rotifers.

The enrichment time also affects the relative HUFA content and the overall fatty acid composition in total lipid relative to that of PL in *Artemia*, as shown for rotifers. A very short enrichment time will result in a relatively low HUFA enrichment of PL, while total lipid is enriched very rapidly. To obtain an optimum HUFA enrichment of PL, we suggest that the enrichment time of *Artemia* nauplii in commercial hatcheries should be at least 8 h. The lag phase in HUFA enrichment opens also possibilities to produce *Artemia* nauplli with variable relative HUFA enrichments in PL and TAG, as achieved for rotifers in **Paper IV**.

There is an apparent constrain of DHA enrichment in PL of *A. franciscana*, which is most likely related to strain or its genetic characteristics. One obstacle is their ability to retroconvert DHA into EPA both during enrichment and subsequent starvation (Navarro *et al.*, 1999), making it extremely difficult to achieve high DHA content in

PL of *Artemia*. On the other hand, the DHA content in total lipid decreased much faster than that in PL of *Artemia* upon starvation post-enrichment. The use of *Atremia* juveniles, which could contain higher levels of HUFA in PL in relative terms, may therefore be a good alternative for some marine fish species. Their relatively low levels of DHA in total lipid can additionally be manipulated by short-term enrichment by an emulsified lipid diet. However, the big size of *Artemia* juvenile and extra cost for cultivation will likely limit its application in commercial hatcheries.

3.2 Phospholipid metabolism in cod larvae

3.2.1 Implication of DHA% in live feed diet and cod larvae (Paper IV, Paper V)

First feeding experiments of cod larvae were carried out by feeding with short-term (2.1 % DHA in PL) or long-term (9.4 % DHA in PL) enriched rotifers, and cultivated nauplii of *Acartia tonsa* (30% DHA in PL) in **Paper IV**. An increasing gradient of DHA% in PL of live feeds was achieved as described above. Meanwhile, the DHA% in total lipid was similar (26.2, 22.3, and 28.4-39.4 % of total fatty acids, respectively).

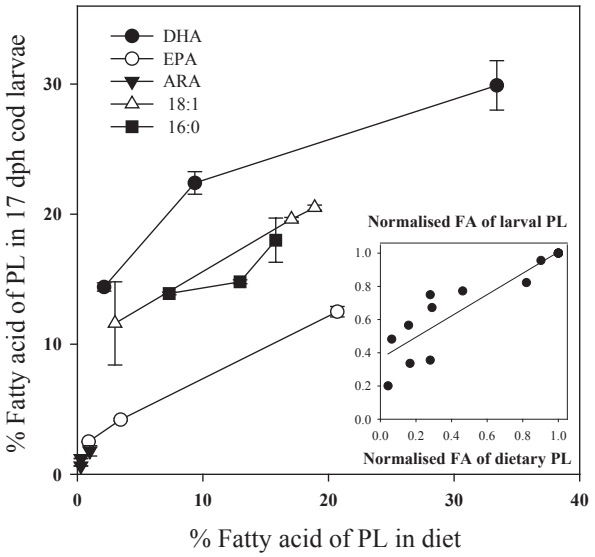

Figure 5. Composition of dominant fatty acids in PL (% of PL fatty acids) in 17 dph larvae as a function of the respective fatty acid composition in the dietary PL. Inset shows the similar relative composition after normalising contents of the individual fatty acid to the respective maximal value, with regression line and 95% CI included (taken from **Paper IV**).

The dominant fatty acids in PL of 17 dph larvae were highly correlated to the fatty acid composition in the dietary PL (Figure 5, **Paper IV**).

A similar study (**Paper V**), using the same enrichment strategies but emulsions containing lower DHA levels, resulted in lower DHA% in total lipid of rotifers (6.7-11.6 %). However, after 18 days of feeding, the DHA levels in cod larvae were not significantly different (P>0.05, 14.4-20.9 % **Paper IV** *versus* 15.3-17.1 % **Paper V**). The only dietary origin of DHA was via rotifers in both studies, which suggested that DHA was selectively incorporated and retained in PL of cod larvae, especially in the PE fractions, which contained close to 50 % DHA before first feeding and 30 % at 18 dph. We therefore suggest that a threshold DHA level to maintain membrane fluidity and other functions may exist in the PL of cod larvae. As a consequence, the correlation of DHA in PL of the diets and in PL of larvae found in **Paper IV** was not observed in **Paper V**, likely due to the low DHA levels of the rotifers. Besides, the analytical methods for separating lipid classes were different between the two experiments. The focus of **Paper V** is the major PL classes: PC and PE, whereas **Paper IV** separates polar lipids from neutral lipids. Although PC and PE are the main polar lipids, other components including phosphatidylinositol, phosphatidylserine, lyso-PC, lyso-PE, phosphatidic acid and cardiolipin could contribute up to 12-20 % of total polar lipids of cod larvae (Olsen *et al.*, 1991; Overrein, 2010). Different PL classes could have very different DHA content, as revealed for PC and PE in **Paper V**.

Our analytical data allow some further speculations on how the fatty acid composition and configuration of dietary PL and TAG may affect PL synthesis and performance in fish larvae. The main pathways of *de novo* synthesis of PL based on dietary TAG was summarised in Figure 2 above. If we assume that the n3 HUFA of a dietary TAG is mainly esterified at the *sn*-2 position (Sargent *et al.*, 1999; Sargent *et al.*, 2002), the n3 HUFA will be retained in the MAG after digestion in the intestine and in DAG after re-esterification in the enterocytes. Most of this DAG will be re-esterified further to TAG and a small proportion can be synthesized to PC via CDP-choline pathway (Lehner and Kuksis, 1996; Oxley *et al.*, 2007). If PL of tissues are *de novo* synthesised based on dietary marine TAG, we should expect a similar n3 HUFA composition in the dominant PL molecules of fish tissues compared to the diet and a positive correlation between percentage n3 HUFA content in dietary TAG or total lipids and tissue PL of fish. However, no such correlation was found for DHA of PL

(Paper IV). We therefore concluded that the *de novo* synthesis of PL is inefficient in fish larvae.

However, this was based on many assumptions and the digestion of TAG and *de novo* synthesis of PL are very complicated metabolic processes. First of all, the position of DHA in TAG of rotifers was assumed to be on the *sn*-2 position. Ando *et al*. (2004) found that rotifers tend to retain DHA in *sn*-3 position after enrichment with fish oil. If most of the DHA was esterified in *sn*-3 position of the rotifers provided, we would have no information on the fatty acid that remained in the *sn*-2 position after digestion. Secondly, the main neutral lipase in cod is bile activated lipase (BAL) and its specificity remains to be determined. It was found that the purified BAL of cod possessed 1, 3-specificity toward TAG (Gjellesvik, 1991; Gjellesvik *et al.*, 1992), whereas Lie and Lambertsen (1985) suggested that lipolytic activity in cod intestinal juice hydrolysed HUFAs in the *sn*-2 position of TAG. At last, but not least, the enzyme selectivity for DAG species to the end product of either TAG or PC is not considered in this assumption. This will be discussed in section 3.2.2.

The study carried out in **Paper IV** provided evidence for that DHA incorporated in PL, rather than TAG, was more beneficial to cod larvae for their new synthesis of tissue PL as most DHA originated from dietary PL. However, the conclusion on the ability of *de novo* synthesis of PL needs further investigation.

3.2.2 Expression of the related genes involved in the pathway (Paper V)

PL is found to be an essential dietary component for fish larvae (Tocher *et al.*, 2008; Cahu *et al.*, 2009). However, the genes involved in PL biosynthesis pathways in fish are poorly studied. Transcriptome analysis in different stages of cod larvae was therefore carried out using a recently developed cod microarray with the objective to obtain more knowledge on ontogenesis of lipid metabolism, especially the pathway of *de novo* PL synthesis during early stages of cod larvae (**Paper V**).

There are more than 100 genes involved in metabolism of PL. Most of the genes encoding *de novo* PL biosynthesis pathway, the Lands cycle pathway (PL to lyso-PL to PL) and the PL turnover pathway were not significantly up-regulated ($P>0.05$) up to 60 dph. One recent study (Dapra *et al.*, 2011) showed that the intestinal *cpt* (CDP-choine pathway), *lpcat* (Lands cycle pathway) and *dgat* (DAG to TAG) mRNA levels were not affected by PL deficiency in fry and juveniles of rainbow trout whereas only

apoB was up-regulated in the PL depleted treatment during fry stage. In **Paper V**, both *apoB* and *apoAI* showed significant up-regulation at 17 dph, and *apoAI* was also significantly up-regulated at 13 dph and 60 dph, suggesting that an elevated lipoprotein assembly might have occurred at these stages. The expression of *cpt* in cod larvae did not show any regulation up to 60 dph in **Paper V**. Other studies have indicated that the enzyme activity of CPT in cells was in excess (Vance, 2002; Gibellini and Smith, 2010), which likely means that transcriptional regulation of *cpt* is not necessary for the production of PC.

Of the PC synthesis genes, only *pcyt1b* showed a significant up-regulation at 17 dph in **Paper V**. *Pcyt1b* encodes for CTβ2 and CTβ3 in mice and CTβ1 and CTβ2 in humans. CTβ is expressed at the same level as CTα in the brain, whereas CTβ expression in other tissues is only about 10% of CTα (Gibellini and Smith, 2010). Recent studies with *Pcyt1b*$^{-/-}$ mice showed no obvious neurological problems, but reduced PC synthesis in distal axons and less neurite branching was observed in neurons cultured from CTβ2-deficient mice (Vance and Vance, 2009; Strakova *et al.*, 2011).

The up-regulation of *pcyt1b* in the present study may be related to the need for biosynthesis of PL in the brain of cod larvae due to low DHA levels of the PL in rotifers. There are evidences that lyso-PC may be a preferred form to pass through the blood-brain barrier and carry DHA to the brain (Lagarde *et al.*, 2001). The expression of *pcyt1b* of cod larvae was found to be down-regulated during the live feed period when they were fed copepods, their natural prey, which contains much higher DHA levels in PL compared to rotifers (Elin Kjørsvik communication, CODE project). To our knowledge, no information on CTβ has been reported in any fish species. Even in mammals, there is very little information about CTβ compared to the dominant CTα, which is encoded by *pcyt1a*. As they have similar membrane-binding domain, CTβ is probably also regulated by a similar translocation process as CTα (Sugimoto *et al.*, 2008).

An increase in PL content per individual larvae is expected due to cell division and growth, especially during the fast growth stages in the early life. However, very few genes involved in the *de novo* synthesis pathways of PL were significantly regulated throughout the live feed period and up to 60 dph. One reason for this maybe because the larvae had sufficient dietary phospholipid from live feed and commercial diets,

with no need for extensive *de novo* phospholipid synthesis. It has been suggested that exogenous PC can inhibit *de novo* PC synthesis (Mansbach, 1977). Secondly, non-transcriptional regulation of key genes involved in the pathway might be another explanation. For example, the activity of CTα involved in the rate-limiting step of PC biosynthesis can be regulated efficiently by translocation on and off membranes. The active form of CTα is membrane-bound and de-phosphorylation can promote activation of CTα activity (Sugimoto *et al.*, 2008). Therefore, cells could increase PC biosynthesis by translocation of CTα to membranes without changing the expression level of *pyct1a* (Golfman *et al.*, 2001). Finally, it is important to note that the gene expression levels of the current study were normalized to 1 dph. No up-regulation does not imply low gene expression level, meaning that the gene expression level of cod larvae may be high already at 1 dph or before hatching. A recent study reported gene expression levels of cod eggs in 6 stages (Kleppe *et al.*, 2014), and by analysing their raw data, we found that the expression of *pyct1a* (ENSGAUG00000007851) was generally higher in the egg stages compared to the larvae stages.

To summarize, our overall data suggested that there is no clear genetic constrains of undertaking *de novo* PL synthesis in cod larvae, and larvae appeared to have relatively high capability of PL biosynthesis already at 1 dph. This capability may regrettably be related to cell division and growth, rather than to lipoprotein assembly.

3.2.3 Biochemical study with radiolabelled tracers (Paper VI)

As mentioned in the previous section, many of the regulatory mechanisms of PC biosynthesis are at non-transcriptional levels (Sugimoto et al., 2008), and biochemical or enzymological studies are therefore needed to elucidate if cod larvae are capable to synthesis PL *de novo*. *In vitro* studies using intestinal segments were not applicable due to the small size of fish larvae. A tube feeding method, as described by Rønnestad et al. (2001), was used to deliver 2-Oleoyl-[1,2,3-^{3}H]glycerol and [^{14}C(U)] glycerol together with bovine serum albumin (BSA) bound 16:0 and DHA, with or without choline chloride, to the foregut of anesthetized cod larvae. The metabolism of these components in the larvae were thereafter monitored through short time (0-4 h) following injection (**Paper VI**). Anaesthesia can reduce digestive functions as has previously been reported in zebrafish (Hama et al., 2009), but the injected mixtures (**Paper VI)** would be absorbed directly without being further digested in the larvae.

The percentage of [^{3}H] label in 2-MAG was already very low after 0.5 h of incubation (Figure 6), indicating fast absorption and utilization of the 2-MAG precursor by cod larvae. *In vitro* studies using intestinal segments of rainbow trout (*Oncorhynchus mykiss*) have shown that only 0.5-1% of the initial radioactivity of fatty acids added to the luminal side passed through the intestinal epithelium after 110 min (Geurden *et al.*, 2009). It is likely that the biosynthesis of PL and TAG in the present study occurred mainly in the enterocytes of cod larvae within the first hour post injection.

Within the first hour, the molecular ratio of PC:TAG obtained from the 2-MAG and the G-3-P pathways were 0.44-0.74 and 1.02-2.06, respectively (Figure 6). It was

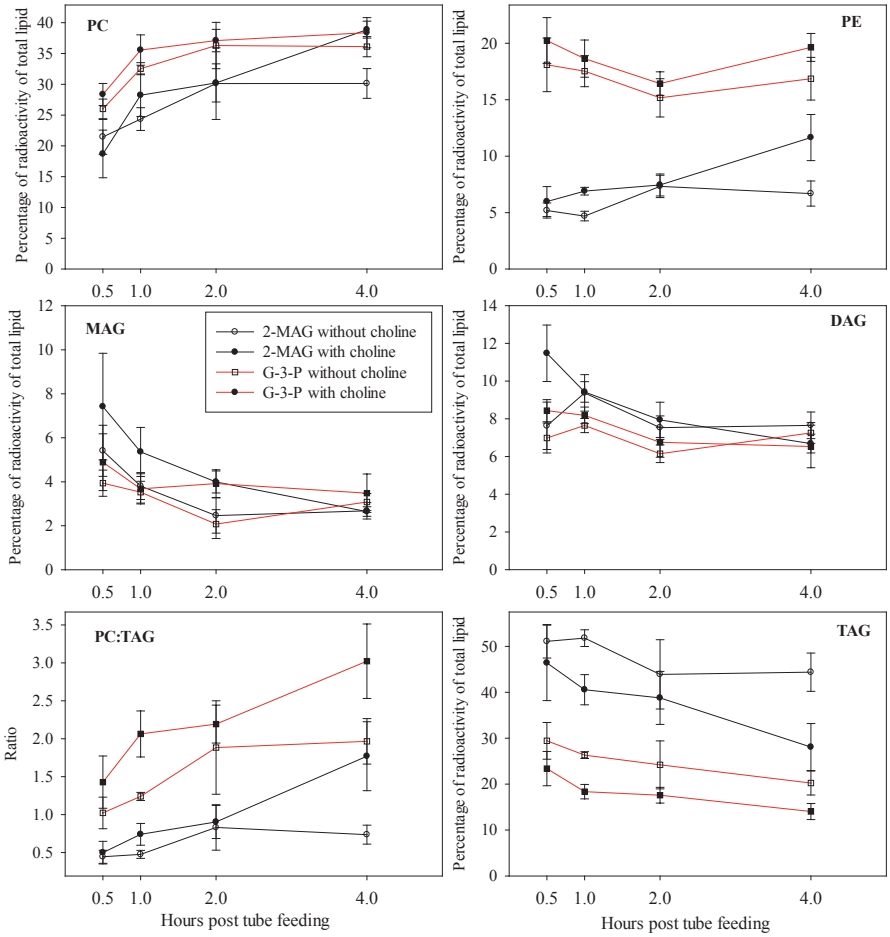

Figure 6. Percentage radioactivity of total lipid for each lipid class and PC:TAG ratio of cod larvae post tube feeding (Modified from **Paper VI**) .

relatively high compared to that found in other studies (0.04-0.22 derived from 2-MAG pathway, 0.27-1.43 derived from G-3-P pathway) (Lehner and Kuksis, 1992; Oxley *et al.*, 2007). DAG is a common precursor for both the 2-MAG and the G-3-P pathways. Its fatty acid composition is important for the selectivity of CPT channelling it further into PC production (Mantel *et al.*, 1993).

The fatty acids 16:0 and 22:6n3 provided in **Paper VI** were chosen as the combination believed to favour PC synthesis (**Paper III**). They are also the most abundant fatty acids in the natural prey for cod larvae (Li *et al.*, 2015b). The CPT of rat liver has showed highest selectivity for DAG species containing 16:0 in the *sn*-1 position and PUFA in the *sn*-2 position (Holub, 1978; Morimoto and Kanoh, 1978; Mantel *et al.*, 1993), and salmon intestine showed higher PC:TAG ratio when 16:0 was available as substrate (Oxley *et al.*, 2007). Moreover, addition of 16:0 to diets containing high linseed oil significantly reduced intestinal steatosis in Arctic char (*Salvelinus alpinus*) (Olsen *et al.*, 2000). The high PC:TAG ratio found after 2 h and 4 h of incubation in the present study probably related to TAG being used as an energy source whereas PC was used for membrane or structural purposes.

Paper VI clearly showed that cod larvae intestines have a high metabolic rate, and are fully capable to synthesize PL through both the 2-MAG and G-3-P pathways when dietary components that could be directly assimilated were injected. Furthermore, supplementation of choline chloride significantly increased the PC synthesis over TAG (PC:TAG ratio) ($P<0.05$). Cod larvae were able to synthesize PC to similar levels as TAG, agreeing with the relatively fast reacylation process of MAG to TAG in the enterocytes found for salmon juvenile (Oxley *et al.*, 2005; Oxley *et al.*, 2007). We therefore suggest that cod larvae might have relatively high ability to synthesize PC from 2-MAG and glycerol precursors under the conditions of the present study where these components were injected and might be assimilated directly without further need for digestion.

3.2.4 Final evaluation of the requirement of dietary PL of cod larvae

PL is an essential dietary component for marine fish larvae during their early life stages and the specific requirement of PL of fish larvae has been suggested to originate in an inefficient ability to biosynthesis PL *de novo* (Tocher *et al.*, 2008;

Cahu *et al.*, 2009). However, our studies (**Paper V and VI)** showed that cod larvae were able to biosynthesis PL *de novo* efficiently.

Early studies with Atlantic cod larvae suggested that they might have low ability to digest neutral lipid classes due to insufficient suitable lipases, bile acids or both (Olsen *et al.*, 1991). Bile activated lipase (BAL) was found to be the main functional lipase for digestion of neutral lipids, and its enzyme activity and gene expression was found to be low in the early stages of cod larvae, and "adult type" digestion of neutral lipid was suggested to occur when the pyloric caeca started to develop (from 20 mm SL) (Saele *et al.*, 2010; Kortner *et al.*, 2011).

Another study have reported that pre-digested dietary lipid only had minor effects on absorption and metabolism compared to intact lipid, suggesting that cod larvae were able to utilize both neutral lipid and polar lipid efficiently already from a SL of 9.75 (SD 0.63) mm (Hamre *et al.*, 2011). On the other hand, few data are available regarding the bile production in fish larvae. Our recent study from a cDNA-microarray analysis showed that the expression level of *Cyp7a1* (cholesterol 7-alpha-monooxygenase, the rate-limiting enzyme in the conversion of cholesterol to bile acids) of cod larvae increased from 1 dph (hatching) to 3 dph (mouth opening) where after it decreased all the way till 60 dph (Li et al. unpublished data). Studies with humans showed that the bile acid concentrations in the intestine of premature and full-term new-borns were insufficient for micelle formation (Murphy and Singer, 1974). It is possible that the digestive function for neutral lipid of cod larvae may suffer from both low BAL activity and low bile acid production in the very early stages.

The incorporation of radiolabeled isotopes (**Paper VI)** was made independent of digestion, and then the enzymatic capabilities could be studied without influence of the first enzymatic steps during digestion. It is possible that with normal feed the substrates for *de novo* PL synthesis in the enterocytes becomes limited and dietary PL becomes important. The requirements of dietary PL can also be a particular result of the high growth rate of fish larvae, the need of PL may not be fulfilled even with a relatively high capacity of *de novo* synthesis.

Paper V indicated that the alternative pathway for PC production in the liver via PEMT might not be applicable due to the low gene expression of *pemt* from hatching up to 60 dph of cod larvae. We therefore suggest that the requirement of dietary PC

for fish larvae may be a result of low input of bile PC from the immature liver, rather than a limited ability of *de novo* PC synthesis in the enterocytes.

In summary, Atlantic cod larvae at 30 dph (9.90 mm SL) showed comparable biosynthesis ability of PC and TAG from both 2-MAG and glycerol precursors within the first hour of tube feeding, suggesting that larvae have relatively high capacity of *de novo* PL synthesis under the conditions of **Paper VI**. We suggest that the intestinal steatosis frequently observed in fish larvae fed PL-deficient diets may originate in immature digestive functions of neutral lipid to provide substrate for *de novo* PC synthesis, the low input of biliary PC for the assembly of lipoproteins, or both.

3.3 Summary of main results

The present study has increased our knowledge of DHA enrichment in PC and PE of cultivated live feed organisms. It provides fatty acid composition of total lipid, PC and PE of most commonly used rotifer and *Artemia* strains as well as of natural harvested and cultivated copepods. The fatty acid profile of the main phospholipid components can be efficiently manipulated by the methods proposed in the present study for research or industrial purposes.

- The highest PC-DHA (12.5 %) and PE-DHA (5.1%) levels were found in Cayman rotifers enriched with Marol E by long-term enrichment strategy (**Paper I**), which also had comparable ($P>0.05$) quantitative amounts of DHA to natural harvested copepods whereas, the DHA levels in PC and PE of copepods were up to 50 %. The percent PC-DHA levels in rotifers were positively related to the total dietary DHA levels ($P<0.0001$), independent of the PC-DHA content in the diets.
- The highest DHA levels were found at 8 h post enrichment in total lipid (8.00 %), PC (1.96 %) and PE (2.01 %) of *Artemia* (**Paper II**). A pronounced lag phase of 1 h in the accumulation of DHA, EPA and ARA in PC and PE after initiation of enrichment was found for *Artemia*. *Artemia* juvenile shows lower DHA% in TL compared with nauplii, but the content of DHA in PC and PE was similar.
- The highest DHA levels in PC (23.0 %) and PE (16.5 %) in Cayman were found after 24 h enrichment with Multigain, and The enriched DHA was

stable at 10 ºC for at least 24 h post enrichment under starving conditions ($P>0.05$) (**Paper III**).

- Lipid of rotifers can be manipulated to contain similar total lipid and DHA% in total lipid, but different DHA% in PL (**Paper IV**).

Moreover, the present study has improved our understanding of the specific requirement of dietary PL in the early stages of cod larvae. Three different methods were used to elucidate the essentiality of PL, including fatty acid analysis of PC and PE, molecular studies of the genes involved in the pathway, and radio-labelled tracer study to investigate the metabolic pathway.

- The dominant fatty acids in the PL of larvae and PL of the diets were well correlated, especially for DHA. The DHA% in larval PL was also significantly positively correlated to larval dry weight at 17 dph (**Paper IV**).
- However, in **Paper V,** no significant differences ($P<0.05$) were found for the two rotifer diets (manipulated in the same way as described by **Paper IV,** but with a lower DHA emulsion) in the overall gene expression levels of cod larvae, their growth and survival, and their DHA levels in total lipid and PL fraction. The fatty acid data showed that DHA was selectively incorporated and retained in PL of cod larvae, especially in PE fractions. No up-regulation of the key regulating genes involved in the PL biosynthesis pathways were found throughout the sampling period (1 to 60 days post hatch).
- Our results in **Paper VI** showed that cod larvae intestines have a high metabolic rate, and are fully capable to synthesize PL through both the 2-MAG and G-3-P pathways. Furthermore supplementation of choline chloride significantly increased the PC synthesis over TAG (PC:TAG ratio) ($P<0.05$).

4. Conclusions and future perspectives

Live feed enrichment in PL and PL metabolism of cod larvae were studied in this thesis. The most important findings were:

- Diets based on marine PL were not more efficient than diets based on marine TAG for the DHA manipulation of PC and PE of rotifers by long-term enrichment stategy.
- A "lag phase" was found for DHA enrichment in PC and PE of both rotifers and *Artemia*, whereas total lipid content and DHA% in total fatty acids increased fast during the same period.
- Provision of diets with high DHA content might not alone be enough to obtain high DHA levels in PL of rotifers. We propose the hypothesis that 18:1n9 can inhibit DHA incorporation in PL of live feed through competition whereas 16:0 can facilitate DHA incorporation into PC.
- We suggest that the enrichment time should be at least 24 h and the feeding rations should be relatively low in order to maximise the efficiency of the PL enrichment and reduce the total lipid content of rotifers. The enrichment diets should have a high content of DHA. The suggested stimulating effect of 16:0 and the inhibiting effect of MUFA should be considered, but this needs further investigation.
- DHA incorporated in PL, rather than TAG, was more beneficial to cod larvae for their new synthesis of tissue PL.
- We suggest that there is no apparent genetically constraining step in the pathway of *de novo* PL synthesis in cod larvae, and they appeared to have relatively high capability of PL biosynthesis already at 1 dph, but this capability may be related to cell division and growth, rather than to lipoprotein assembly.
- We suggest that cod larvae might have relatively high ability to synthesize PC from 2-MAG and glycerol precursors and the requirement of dietary PC for fish larvae fed normal diets that need digestion was possibly due to the immature digestive functions for neutral lipid or the low input of bile PC from the immature liver, or both.

We suggested a method to enrich DHA in PL of rotifers in Chapter 3.1.1 that also considered specific dietary non-HUFA. However, the non-HUFA hypothesis needs to

be further verified. Although the commercial enrichment diets in **Paper III** contained significantly different levels of SFA and MUFA, these diets were not specifically manufactured for testing "non-HUFA" hypothesis and we cannot exclude the possible effects of other ingredients in the diets, such as proteins. Specific tailor-made emulsions with same characteristics and DHA level, but different 16:0 and 18:1n9 levels should be used to further confirm the hypothesis.

DHA enrichment in PL of *Artemia* to high levels remains problematic. New strains or genetic modified strains with less or no ability of retroconverting DHA to EPA may be needed in the future.

The DHA% in PL of enriched rotifers and *Artemia* needs to be further enhanced to obtain similar DHA levels as in PL of copepods or roes of marine fish larvae. It is important to continue efforts to establish a better understanding of mechanisms and enrichment strategies to increase the DHA levels in PL of live feeds.

Paper V and VI provided new insights into the mechanisms of dietary PL requirements of fish larvae. However, further studies are needed to quantify the enzyme activity involved in the CDP-choline pathway and factors affecting PC biosynthesis ability in fish larvae. Addition of choline clearly increased the PC:TAG ratio, however, its function either in simulating PC synthesis or TAG catabolism or both needs further investigation.

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Enclosure Paper I-VI

Paper I

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Manipulation of the fatty acid composition of phosphatidylcholine and phosphatidylethanolamine in rotifers *Brachionus* Nevada and *Brachionus* Cayman

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Abstract

Rotifer cultures of *Brachionus* Nevada and Cayman were grown semi-continuously, fed live *Rhodmonas baltica* algae, and cultures were diluted daily (20% day^{-1}). The enrichment diets Marol E (triacylglycerol-rich) and PL (cod roe emulsion, phospholipid-rich) were added to both cultures (Nevada 40 ng ind^{-1} day^{-1} and Cayman 25 ng ind^{-1} day^{-1}, respectively). After 4 days of enrichment (dilution rate: 20% day^{-1}), Cayman rotifers–fed Marol E had comparable ($P > 0.05$) quantitative amounts of DHA (docosahexaenoic acid, 22:6n-3) to natural harvested copepods, which were analysed for comparison, and the Cayman strain had generally more efficient incorporation of HUFAs (highly unsaturated fatty acids) than the Nevada strain. However, the percentage DHA of the total fatty acids in copepods were much higher than in enriched rotifers ($P < 0.05$). The PC-DHA (DHA in phosphatidylcholine) and PE-DHA (DHA in phosphatidylethanolamine) levels of copepods were up to 50%, which was highly unlikely for rotifers to reach. The increase of DHA in total phospholipids of rotifers was mainly due to an increase of DHA in PC, whereas the PE-DHA was only weakly affected by dietary lipids. The per cent PC-DHA levels in rotifers were positively related to the total dietary DHA levels ($P < 0.0001$), independent of the PC-DHA content in the diets.

KEY WORDS: copepods, enrichment, fatty acids, phosphatidylethanolamine, phosphatidylcholine, rotifers

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Introduction

Brachionus Nevada (Papakostas *et al*. 2006) and *Brachionus* Cayman (Baer *et al*. 2008) are among the most commonly used rotifer species in Norwegian marine fish hatcheries and in hatcheries around the world. The lorica length of Nevada is 250–330 μm, while the lorica size of Cayman is (168 ± 10) × (111 ± 9) × (81 ± 8) μm^3 (Baer *et al*. 2008). *De novo* synthesis of n-3 highly unsaturated fatty acids (n-3 HUFA) has been documented in rotifers, but their rate of synthesis is too slow to produce the levels needed in their tissues to meet the nutritional demands of predators like marine fish larvae (Lubzens *et al*. 1985). Techniques to produce and enrich live feed with lipids that contain high levels of n-3 HUFA have, therefore, been established over the last decades (Coutteau & Sorgeloos 1997; Dhert *et al*. 2001), but there are still questions on the lipid quality of rotifers used for fish species that are attractive for cultivation in northern countries, such as Atlantic cod (*Gadus morhua*) and turbot (*Scophthalmus maximus*) (Reitan *et al*. 1994; O'Brien-MacDonald *et al*. 2006; Garcia *et al*. 2008)

One of the main natural foods for marine fish larvae in Northern seas is thought to be copepod nauplii and small copepodite stages, and such nauplii have shown to be a superior live feed as compared with rotifers and *Artemia* (Evjemo *et al*. 2003; Turner 2004; Hamre 2006; van der Meeren *et al*. 2008). It has been suggested that this superiority of copepod nauplii and copepodites is mainly due to their high n-3 HUFA levels, and specifically, their high contents of DHA (docosahexaenoic acid, 22:6n-3) in phospholipids (PL) (Bell *et al*. 2003; Tocher *et al*. 2008), which may be up to 40% DHA of their total fatty acids (Overrein 2010). The n-3 HUFAs seem to be more beneficial to larval growth and development when they are incorporated into PL than in neutral lipids, as shown for European sea bass (Gisbert *et al*. 2005), sea bream (Izquierdo *et al*. 2001) and for cod (Wold *et al*. 2007; Kjørsvik *et al*. 2009). In a recent

experiment, we were able to cultivate *Brachionus* Nevada with different DHA content in their PL, and the cod larvae fed *Brachionus* Nevada with relatively high per cent DHA content in their PL showed better growth and higher DHA in larval PL at day 17 posthatching (dph) than those fed Nevada with lower DHA in PL (Olsen *et al*., in prep.).

Marine and freshwater fish species have essential dietary requirements for PL during early larval stages (Geurden *et al*. 1998; Cahu *et al*. 2003, 2009; Tocher *et al*. 2008). Copepods, rotifers and *Artemia* contain PL, which are mainly structural lipids, in similar quantitative amounts (around 40–60 mg g^{-1} DW) (Rainuzzo *et al*. 1994a; Harel *et al*. 1999; Olsen 2004; Bergvik *et al*. 2012). It appears to be difficult to modify the quantitative contents of PL (e.g. mg PL g DW^{-1}) in live feed organisms (Rainuzzo *et al*. 1994b; McEvoy *et al*. 1996; Coutteau & Sorgeloos 1997). On the other hand, it appears possible, within limits, to manipulate the qualitative fatty acid composition of PL fatty acids, including the percentage of DHA (Rainuzzo *et al*. 1994a; McEvoy *et al*. 1996). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most dominant lipid molecules in the phospholipids of rotifers, contributing up to 99% of the total phospholipids (Rainuzzo *et al*. 1994b). There are few, if any, reports on the fatty acid composition in PC and PE of rotifers and their dynamic relationship with dietary lipids and fatty acids. Moreover, most of the current enrichment procedures are developed using tri-acylglycerols (TAG) or fatty acid ethyl esters as dietary lipids, and enrichment diets with high PL content have only occasionally been used to enrich rotifers (Rainuzzo *et al*. 1994b).

The main objective of this study was to examine how variable lipid class and fatty acid composition of dietary lipids could affect the fatty acid composition of the PC and PE of rotifers after enrichment by a diet rich in marine PL or a diet containing mainly TAG. A second objective was to compare the fatty acid composition in PC and PE between enriched rotifers and copepodites harvested from natural waters, with a main focus on the DHA content.

Two different rotifer strains were included, because there may be differences in lipid metabolism between rotifer strains. Semi-continuous cultures of the rotifers were fed the microalgae *Rhodomonas baltica* alone or together with a PL-rich or a TAG-rich lipid emulsion, so-called long-term enrichment (Rainuzzo *et al*. 1994b; Olsen 2004). The harvested copepod samples contained early copepodite stages of *Calanus finmarchicus* of variable size (>95% purity).

Materials and methods

Production of Rhdomonas baltica

The seawater (35 g L^{-1}, 20 °C) used for the cultivation of *Rhdomonas baltica* (NIVA-5/91) was sterilized with sodium hypochlorite (0.25 mL L^{-1}, 10–15% NaOCl) overnight and thereafter dechlorinated with sodium thiosulphate (3 g for each 25 mL of sodium hypochlorite) for 6 h under heavy aeration. *Rhodmonas baltica* was grown in the pretreated seawater with Conwy medium (1.5 mL L^{-1}) (Walne 1974) in 200-L transparent plastic cylinders. Air containing 1–2% of CO_2 gas was added to the algal cultures that were illuminated with artificial light from three sides (GE Polylux XL830 F58W). *Rhodmonas baltica* was cultivated semi-continuously, and 50% of the culture was harvested daily and replaced with pretreated seawater (with added nutrient medium). This gave a constant growth rate and a constant biochemical composition, independent of time in the established phase of steady-state growth (constant cell numbers with time).

For the lipid analysis, duplicate *R. baltica* samples were harvested from steady-state cultures and centrifuged for 5 min at 1710 ***g*** (WIFUG 4000E; WIFUG Ltd, Bradford, UK) in 1-L containers to remove most of the water, after which they were transferred to 50-mL tubes and centrifuged (Hettich universal 32R; Hettich, Tuttlingen, Germany) for 3 min at 1640 ***g***. The concentrated algae were immediately frozen in liquid nitrogen. Frozen samples were freeze-dried and thereafter stored under N_2 gas at −80 °C before analysis.

Oil emulsions

The lipid emulsion Marol E (prepared by SINTEF Fisheries and Aquaculture, Norway), based on the marine oil DHASCO (Martek Biosciences, Columbia, MD, USA), was used as a TAG source rich in DHA. The phospholipid-rich emulsion (PL emulsion) was based on lipids extracted from cod roe and prepared in the same way as Marol E by SINTEF.

Before being added to the rotifer cultures, the emulsions were mixed with freshwater (5–20 mg mL^{-1}) using a blender (250 Watt; Braun GmbH, Frankfurter, Germany). The PL emulsion was also mixed with $NaHCO_3$ (10% by weight) to obtain smaller lipid particles when the emulsion was mixed with seawater.

Rotifer rearing and sampling

Brachionus Nevada and *Brachionus* Cayman were fed *R. baltica* in semi-continuous cultures (35 g L^{-1}, 25 °C, dilution

rate: 20%), with the oxygen level kept above 70% of saturation. To have the same biomass for the two rotifer strains, similar amount of *R. baltica* and emulsions were provided (Table 1). Nevada rotifers were cultured in 250-L conical fibreglass vessels, while Cayman rotifers were cultured in 100-L vessels. The live algae were continuously pumped into the systems (rations per individual rotifer, see Table 1). The enrichment diet Marol E and the PL emulsion were added to both cultures, respectively, after 4 days of dilution (Table 1). Three replicate rotifer cultures were run for each treatment.

Ninety litres of each of the rotifer cultures was sampled from the middle of the tanks, through a plastic pipe, avoiding sediments and big particles. The rotifers were concentrated using a sieve with a mesh size of 64 μm, and then, they were rinsed in filtered seawater and finally in tap water. The sieve was dried from underneath by paper towels. The concentrated rotifers were packed into 50-mL plastic tubes and immediately frozen in liquid nitrogen and finally freeze-dried and stored under N_2 gas at −80 °C before analysis.

Copepod sampling

The copepod samples were collected by trawling with plankton net from the Trondheim fjord in May 2009. Upon arrival on deck, planktonic organisms were graded in different size fractions (300–400 μm, 400–500 μm and 700–1000 μm) by filters. They were then packed into plastic bags, and immediately frozen in liquid nitrogen, and finally freezedried and stored under N_2 gas at −80 °C before analysis. The lipid content and fatty acid profile among copepods of different size groups (data not shown) were not significantly ($P > 0.05$) different, and therefore, they were combined as one in the results.

Biomass determination of Rhodmonas baltica *and rotifers*

For carbon/nitrogen analysis (CN), the *R. baltica* culture was filtered through a 25-mm GF/C precombusted (450 °C) glass microfibre filter (Whatman International Ltd., Maidstone, UK) until saturation of the filter. The filtered volume was registered, and the filter was placed in a Petri dish, marked and stored at −20 °C. The outer circle of the filter was cut off, and the inner circle with the algal sample was transferred into a tin capsule (4 × 6 mm) and dried at 60 °C for 48 h after being exposed for 2 h to concentrated HCl to remove carbonate from the harvested sample. Duplicate filters were analysed for carbon and nitrogen content in an Elemental combustion analyser (Costech Analytical Technologies Inc., Valencia, CA, USA) using acetanilide as an internal standard.

The cell numbers of *R. baltica* were measured by Beckman Multisizer™ 3 Coulter Counter® (Beckman Coulter Inc., Miami, FL, USA). The carbon content of *R. baltica* was calculated based on a premade standard curve of the relationship between cell numbers and the measured carbon. By assuming a constant carbon/dry-weight ratio of 0.4 for the phytoplankton (Parsons *et al.* 1984), the dry-weight (DW) of *R. baltica* could be estimated based on the measured carbon content (C):

$$\mathrm{DW} = C * 2.5$$

Samples for counting rotifers and their eggs were taken from different positions of each culture with a hollow plastic pipe to ensure that the rotifer density was assessed adequately. The samples were mixed well before each count-

Table 1 Rations of *Rhodmonas baltica* and lipid emulsions added, expressed as mean ± SE

	Treatment					
	Nevada			Cayman		
Variables	NR	NM	NP	CR	CM	CP
Rhodmonas baltica, mg C L^{-1} day^{-1}	14.0 ± 0.71^{abc}	16.8 ± 0.36^{b}	15.2 ± 0.29^{c}	13.4 ± 0.64^{ac}	13.9 ± 0.18^{a}	13.9 ± 0.18^{a}
Lipid emulsion, mg mg C^{-1} *Rhodmonas baltica*		0.51 ± 0.01^{a}	0.56 ± 0.01^{ab}		0.58 ± 0.01^{b}	0.60 ± 0.01^{b}
Rotifer density, ind mL^{-1}	259 ± 12.7^{a}	240 ± 15.3^{a}	211 ± 3.97^{a}	332 ± 14.8^{b}	330 ± 10.1^{b}	336 ± 11.0^{b}
Rhodmonas baltica, ng DW ind^{-1} day^{-1}	154 ± 7.28^{a}	187 ± 12.3^{a}	182 ± 4.98^{a}	102 ± 2.93^{b}	105 ± 3.75^{b}	107 ± 2.86^{b}
Lipid emulsion, ng ind^{-1} day^{-1}		38.5 ± 2.60^{a}	40.7 ± 0.84^{a}		24.4 ± 0.81^{b}	25.4 ± 0.78^{b}
Total lipid, ng ind^{-1} day^{-1}	23.7 ± 1.12^{ac}	55.1 ± 3.03^{b}	54.9 ± 1.21^{b}	15.7 ± 0.45^{a}	32.8 ± 0.73^{c}	33.2 ± 0.91^{c}
Days of feeding and dilution before harvest	4	4	4	3	4	4

NR, Nevada cultivated with *Rhodmonas baltica*; *NM*, Nevada enriched with Marol E; *NP*, Nevada enriched with PL emulsion; *CR*, Cayman cultivated with *Rhodmonas baltica*; *CM*, Cayman enriched with Marol E; *CP*, Cayman enriched with PL emulsion.
Values in the same row not sharing a superscript are significantly different ($P < 0.05$).

ing, and ten droplets of 0.05 mL were counted for rotifers and eggs under a stereo-microscope after fixation with acid Lugol's solution. One sample per tank was counted per day.

Lipid and fatty acid analyses

Lipids were extracted using a modified version of the method used by Bligh & Dyer (1959), and the lipid content was determined gravimetrically in triplicate. Fatty acid methyl esters were prepared according to Metcalfe *et al*. (1966) and analysed with a gas chromatograph (Perkin Elmer AutoSystem XL, Norwalk, CT, USA) with TotalChrom V.6.3.1 (Perkin Elmer Inc.) software. The instrumental conditions were identical to those used by Bergvik *et al*. (2012).

The content of PC and PE was analysed using thin-layer chromatography coupled with a flame ionization detector (TLC-FID, Iatroscan MK 6; Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan), based on the method described by Fraser *et al*. (1985). The instrumental conditions and analytical procedures are described in the study described Bergvik *et al*. (2012).

Lipids from 1–2 g of freeze-dried samples were extracted for the separation of PC and PE as described earlier. Chloroform was removed by a rotary evaporator (Laborota 4000-efficient, Heidolph, Schwabach, Germany) connected to a vacuum pump (Cvc 3000; VACUUBRAND GMBH + CO KG, Wertheim, Germany). The lipids were dissolved in chloroform/methanol (1/1, v/v) with a concentration of about 150 mg mL^{-1}. The concentration was measured by determining the lipids gravimetrically. The silica plates (PLC silica gel 60 F_{254}, 0.5 mm. 20 × 20 cm. MERCK KGaA, Darmstadt, Germany) were predeveloped in a glass chamber with chloroform/methanol/water (67/30/2.5, v/v) for 45 min and dried under a draught cupboard (Kilab AS, Jessheim, Norway) for 15 min. The concentrated lipids (90 μL) were then applied onto the silica plates (60 mm), together with commercial standards (20 mm, Soy PC and PE from Avanti Polar Lipids Inc., Alabaster, AL, USA) by using LINOMAT IV (CAMAG, Mutlenz, Switzerland). The silica plates with lipids were developed again in the same chamber to separate PC and PE for 45 min. The plates were exposed to UV to detect PC and PE, which were then scraped off the plates, eluted with 3 mL chloroform/methanol (2/1) and centrifuged for 2 min (5 °C, 1640 ***g*** Hettich universal 32R; Hettich, Tuttlingen, Germany). The upper layers without silica were collected and the elution was repeated twice more, after which the solvents were evaporated at 60 °C under N_2. Fatty acid methyl esters were prepared according to Metcalfe *et al*. (1966) and analysed as mentioned previously. Duplicate samples were analysed for each rotifer culture (three replicate cultures for one treatment).

Statistics

The experimental data were tested for statistical significance by using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test or Dunnet's T3 multiple comparison test if the P value of homogeneity of variance test was less than 0.05, and differences were considered significant at the $P < 0.05$ level. Slope coefficients were compared by using analysis of covariance (ANCOVA). All of the statistical tests were performed using SPSS 19.0 for windows (SPSS Inc., Armonk, NY, USA). All tables were made in Excel 2003 and figures by SIGMA PLOT 10.0 (Systat Software Inc., San Jose, CA, USA).

Results

Lipid content and fatty acid composition of diets

The lipid content and fatty acid composition of the rotifer diets are shown in Table 2. In addition, the content of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in the diets, and the PC and PE fatty acid profiles of *R. baltica* and PL emulsion are shown in Table 3 and Table 4. No PC or PE was detected in Marol E.

Lipid content and HUFA composition of rotifers and copepods

The lipid content of copepods were significantly ($P < 0.05$) lower than of all the rotifer groups (Table 5). The Nevada rotifers had a constant total lipid content after enrichment by Marol E (NM rotifers) and PL emulsion (NP rotifers). The total lipid content of Cayman rotifers increased after enrichment with Marol E (CM rotifers) and with PL emulsion (CP rotifers) compared with the Cayman rotifers fed *R. baltica* only (CR rotifers) (Table 5).

Following enrichment, the EPA content remained at a similar level in the Nevada strain, but it decreased significantly ($P < 0.05$) in CM rotifers, mainly due to the low EPA content in Marol E. The DHA level increased significantly ($P < 0.05$) after enrichment with both emulsions in both rotifer strains. The highest DHA levels were found in CM rotifers (Table 5).

After enrichment of both rotifer strains, the MUFA (mono unsaturated fatty acid) levels increased, whereas

Table 2 Total lipid and fatty acid composition (duplicate samples with one or two analyses) of rotifer diet components, expressed as mean ± SE

	Rhodmonas baltica	Marol E	PL-emulsion
	($n = 2 \times 2$) mg/g DW	($n = 2 \times 1$) mg/g emulsion	($n = 2 \times 1$)
Total lipid	154 ± 4.70^b	684 ± 2.00^c	659 ± 1.50^a
Total fatty acids	62.3 ± 2.90^a	509 ± 4.74^b	282 ± 4.63^c
Fatty acids	% of total fatty acids		
14:0	6.91 ± 0.04^a	13.1 ± 0.43^b	2.41 ± 0.02^c
16:0	5.54 ± 0.07^b	12.0 ± 0.40^c	19.8 ± 0.05^a
18:0	0.00 ± 0.00^c	0.00 ± 0.00^c	1.96 ± 0.01^a
∑SFA	12.4 ± 0.11^b	25.1 ± 0.83^c	24.2 ± 0.05^c
16:1n-7	0.67 ± 0.01^a	2.89 ± 0.09^b	3.63 ± 0.00^c
18:1n-7	3.05 ± 0.01^a	0.00 ± 0.00^b	4.52 ± 0.02^a
18:1n-9	0.61 ± 0.01^b	26.3 ± 0.88^c	13.5 ± 0.02^a
20:1n-9	0.00 ± 0.00^a	0.88 ± 0.03^b	2.93 ± 0.02^c
∑MUFA	4.32 ± 0.02^c	30.1 ± 0.99^a	24.5 ± 0.05^b
18:2n-6	5.31 ± 0.02^a	1.67 ± 0.49^b	1.16 ± 0.01^c
18:3n-6	0.69 ± 0.01^b	0.00 ± 0.00^c	0.00 ± 0.00^c
20:4n-6	0.59 ± 0.01^c	0.00 ± 0.00^b	1.28 ± 0.01^a
∑n-6	6.59 ± 0.03^b	1.67 ± 0.05^c	2.45 ± 0.01^a
18:3n-3	23.8 ± 0.03^a	0.00 ± 0.00^b	0.00 ± 0.00^b
18:4n-3	24.8 ± 0.06^c	0.00 ± 0.00^b	0.59 ± 0.05^a
20:4n-3	0.57 ± 0.01^b	0.00 ± 0.00^a	0.00 ± 0.00^a
20:5n-3	9.42 ± 0.01^a	0.72 ± 0.02^b	14.7 ± 0.05^c
22:5n-3	0.00 ± 0.00^b	0.00 ± 0.00^b	1.25 ± 0.01^a
22:6n-3	8.05 ± 0.05^a	41.1 ± 2.09^b	28.5 ± 0.11^c
∑n-3	66.6 ± 0.15^b	41.8 ± 2.07^a	44.9 ± 0.14^a
∑PUFA	73.2 ± 0.10^a	43.5 ± 2.02^b	47.4 ± 0.15^b
DHA/EPA	0.85 ± 0.01^a	57.4 ± 4.44^b	1.94 ± 0.00^a
Unknown	10.1 ± 0.03^b	0.48 ± 0.02^c	3.97 ± 0.21^a

∑SFA, sum of saturated fatty acid, including 14:0, 16:0 and 18:0; ∑MUFA, sum of mono unsaturated fatty acid, including 16:1n-7, 18:1n-7, 18:1n-9, 20:1n-9; ∑PUFA, sum of poly unsaturated fatty acid, including 18:2n-6, 18:3n-3, 18:3n-6, 18:4n-3, 20:4n-3, 20:4n-6, 20:5n-3, 22:5n-3, 22:6n-3.
Values in the same row not sharing a superscript are significantly different ($P < 0.05$).

the PUFA (polyunsaturated fatty acid) levels decreased. The SFA (saturated fatty acid) levels remained constant in the Nevada strain and increased in the Cayman strain. The SFA, EPA and especially the DHA levels of copepods were significantly ($P < 0.05$) higher than in rotifers. The 18:1n-9 contents in enriched Nevada and Cayman

Table 4 Fatty acid composition in dietary PC and PE, expressed as mean ± SE

	Rhodmonas baltica PC	*Rhodmonas baltica* PE	PL-PC	PL-PE
Fatty acid	% of TFA in PC and PE ($n = 2 \times 1$)			
14:0	6.23 ± 0.01	6.71 ± 0.44	1.49 ± 0.01	0.26 ± 0.00
16:0	9.06 ± 0.31	6.83 ± 0.45	23.3 ± 0.05	11.2 ± 0.00
18:0	0.00 ± 0.00	0.00 ± 0.00	1.64 ± 0.00	3.05 ± 0.00
∑SFA	15.3 ± 0.30	13.5 ± 0.90	26.4 ± 0.06	14.5 ± 0.00
16:1n-7	0.94 ± 0.01	1.03 ± 0.01	2.61 ± 0.00	0.72 ± 0.00
18:1n-7	0.00 ± 0.00	0.77 ± 0.06	3.65 ± 0.00	7.99 ± 0.00
18:1n-9	0.00 ± 0.00	0.00 ± 0.00	10.9 ± 0.02	13.8 ± 0.00
20:1n-9	0.00 ± 0.00	0.00 ± 0.00	1.95 ± 0.00	5.57 ± 0.00
∑MUFA	0.94 ± 0.01	1.80 ± 0.05	19.1 ± 0.02	28.1 ± 0.00
18:2n-6	5.87 ± 0.01	4.46 ± 0.23	0.82 ± 0.00	1.07 ± 0.00
20:4n-6	1.83 ± 0.01	0.00 ± 0.00	1.43 ± 0.00	1.26 ± 0.00
∑n-6	7.71 ± 0.02	4.46 ± 0.23	2.24 ± 0.00	2.33 ± 0.00
18:3n-3	18.5 ± 0.03	33.6 ± 1.63	0.00 ± 0.00	0.00 ± 0.00
18:4n-3	16.6 ± 0.26	35.0 ± 0.82	0.00 ± 0.00	0.00 ± 0.00
20:4n-3	1.96 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:5n-3	25.7 ± 0.11	1.58 ± 0.61	16.4 ± 0.00	15.6 ± 0.00
22:5n-3	0.00 ± 0.00	0.00 ± 0.00	1.16 ± 0.01	1.30 ± 0.00
22:6n-3	13.3 ± 0.07	9.25 ± 1.85	31.2 ± 0.12	36.8 ± 0.00
∑n-3	76.1 ± 0.27	79.5 ± 0.01	48.8 ± 0.11	53.7 ± 0.00
∑PUFA	83.8 ± 0.29	83.9 ± 0.22	51.1 ± 0.11	56.0 ± 0.00
DHA/EPA	0.52 ± 0.01	6.33 ± 1.27	1.90 ± 0.01	2.35 ± 0.00

PL-PC, phosphatidylcholine in the PL-emulsion; PL-PE, phosphatidylethanolamine in the PL-emulsion (duplicate samples with one analysis).

were significantly higher ($P < 0.05$) than those in copepods.

The quantitative DHA contents of CM rotifers (10.3 ± 0.31 mg g DW^{-1}) were comparable ($P > 0.05$) with that of copepods (11.7 ± 0.70 mg g DW^{-1}). However, due to the high content of other fatty acids in rotifers (such as 18:1n-9), the percentage of DHA was much lower ($P < 0.05$) in the CM rotifers than in copepods.

PC and PE content of rotifers

Table 6 shows the PC and PE contents of all rotifer treatments. The quantitative contents of PC of both rotifer strains remained constant and were quite similar for the two strains. For the PE content of Nevada, no significant ($P > 0.05$) changes were found after enrichment. However,

Table 3 Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) content in the diet components, expressed as mean ± SE (duplicate samples with duplicate or triplicate analyses)

	% DW		% of Lipids	
	PC	PE	PC	PE
Rhodmonas baltica ($n = 2 \times 2$)	1.27 ± 0.05	3.09 ± 0.10	9.70 ± 0.52	20.5 ± 0.60
PL-emulsion ($n = 2 \times 3$)	37.4 ± 0.32	8.39 ± 0.45	43.2 ± 0.38	8.40 ± 0.37
Marol E	0	0	0	0

Table 5 Total lipid and fatty acid composition of rotifers, expressed as mean ± SE of three replicate cultures with duplicate or triplicate analyses

	Nevada			Cayman			
	NR	NM	NP	CR	CM	CP	Copepod
	(n = 3 × 3)						n = 3 × 2
Total lipid mg g^{-1} DW	98.2 ± 1.45^{a}	91.7 ± 1.67^{a}	98.0 ± 0.95^{a}	106 ± 0.83^{b}	127 ± 2.14^{c}	115 ± 1.19^{d}	70.7 ± 2.87^{e}
TFA mg g^{-1} DW	41.8 ± 0.75^{a}	37.8 ± 0.75^{a}	36.9 ± 0.31^{a}	40.7 ± 0.80^{a}	64.4 ± 2.04^{b}	53.3 ± 1.84^{c}	29.4 ± 1.33^{d}
Fatty acid% of TFA (n = 3 × 2)							
14:0	4.88 ± 0.06^{a}	5.20 ± 0.10^{a}	3.79 ± 0.07^{c}	3.97 ± 0.05^{c}	5.95 ± 0.09^{b}	3.92 ± 0.03^{c}	5.34 ± 0.43^{abc}
16:0	12.6 ± 0.10^{a}	12.9 ± 0.10^{a}	13.9 ± 0.12^{b}	11.6 ± 0.15^{c}	12.6 ± 0.21^{a}	14.4 ± 0.07^{b}	18.8 ± 0.38^{d}
18:0	3.26 ± 0.09^{ac}	3.25 ± 0.04^{ac}	3.02 ± 0.04c^{a}	3.30 ± 0.08^{a}	2.44 ± 0.03^{b}	3.14 ± 0.05^{ac}	2.97 ± 0.10^{c}
∑SFA	20.7 ± 0.10^{a}	21.4 ± 0.15^{ac}	20.7 ± 0.21^{a}	18.8 ± 0.28^{b}	21.0 ± 0.17^{ac}	21.5 ± 0.05^{c}	27.1 ± 0.56^{d}
16:1n-7	1.34 ± 0.03^{a}	1.52 ± 0.02^{b}	1.64 ± 0.02^{bc}	1.12 ± 0.01^{d}	1.58 ± 0.02^{bc}	1.52 ± 0.02^{b}	1.75 ± 0.13^{abcd}
18:1n-7	2.78 ± 0.03^{ac}	2.09 ± 0.03^{b}	2.77 ± 0.02^{a}	2.65 ± 0.01^{ce}	1.67 ± 0.03^{d}	2.64 ± 0.03^{e}	1.54 ± 0.12^{bd}
18:1n-9	0.87 ± 0.04^{a}	7.06 ± 0.14^{b}	3.69 ± 0.05^{c}	0.00 ± 0.00^{d}	11.2 ± 0.39^{e}	2.74 ± 0.02^{f}	2.28 ± 0.15^{f}
20:1n-9	2.47 ± 0.09^{ab}	2.24 ± 0.0^{b}	2.37 ± 0.01^{a}	2.16 ± 0.03^{b}	2.21 ± 0.00^{b}	1.77 ± 0.02^{c}	
22:1n-9	0.00 ± 0.00^{a}	0.86 ± 0.04^{b}	0.70 ± 0.01^{b}	0.00 ± 0.00^{a}	0.83 ± 0.01^{b}	0.84 ± 0.03^{b}	
∑MUFA	7.46 ± 0.10^{a}	13.8 ± 0.14^{b}	10.9 ± 0.11^{c}	5.94 ± 0.05^{d}	17.5 ± 0.36^{e}	9.50 ± 0.05^{f}	5.57 ± 0.23^{d}
18:2n-6	8.59 ± 0.09^{a}	6.07 ± 0.21^{b}	8.40 ± 0.09^{a}	10.3 ± 0.12^{c}	7.81 ± 0.08^{d}	9.46 ± 0.02^{e}	0.86 ± 0.03^{f}
18:3n-6	0.73 ± 0.03^{a}	0.00 ± 0.00^{b}	0.69 ± 0.01^{a}	1.00 ± 0.01^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	
20:2n-6	0.44 ± 0.01^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.70 ± 0.02^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	
20:3n-6	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.86 ± 0.02^{b}	0.00 ± 0.00^{a}	0.57 ± 0.01^{c}	
20:4n-6	1.71 ± 0.02^{a}	0.97 ± 0.03^{b}	1.94 ± 0.03^{c}	1.69 ± 0.01^{a}	0.96 ± 0.02^{b}	1.59 ± 0.01^{d}	
∑n-6	11.3 ± 0.14^{a}	7.05 ± 0.23^{b}	11.0 ± 0.12^{a}	14.6 ± 0.16^{c}	8.78 ± 0.09^{d}	11.6 ± 0.01^{a}	0.86 ± 0.03^{e}
18:3n-3	17.8 ± 0.05^{a}	13.0 ± 0.18^{b}	13.4 ± 0.16^{b}	15.6 ± 0.15^{c}	10.6 ± 0.28^{d}	14.4 ± 0.03^{e}	1.51 ± 0.05^{f}
18:4n-3	7.71 ± 0.11^{a}	5.93 ± 0.12^{b}	5.98 ± 0.08^{b}	7.80 ± 0.21^{a}	5.52 ± 0.22^{b}	7.34 ± 0.05^{a}	4.20 ± 0.21^{c}
20:3n-3	1.17 ± 0.03^{a}	0.56 ± 0.01^{b}	0.57 ± 0.01^{b}	1.43 ± 0.01^{c}	0.57 ± 0.03^{b}	0.85 ± 0.01^{d}	
20:4n-3	9.62 ± 0.02^{a}	7.77 ± 0.06^{b}	7.40 ± 0.03^{c}	9.79 ± 0.10^{a}	5.09 ± 0.10^{d}	7.18 ± 0.05^{c}	
20:5n-3	8.52 ± 0.09^{a}	7.99 ± 0.14^{a}	8.75 ± 0.09^{a}	8.70 ± 0.16^{a}	6.27 ± 0.11^{b}	8.71 ± 0.06^{a}	17.6 ± 0.27^{c}
22:5n-3	1.49 ± 0.02^{ac}	1.67 ± 0.04^{b}	1.65 ± 0.08^{ab}	1.89 ± 0.03^{d}	1.47 ± 0.01^{c}	1.70 ± 0.02^{b}	
22:6n-3	5.47 ± 0.13^{a}	10.3 ± 0.41^{b}	8.34 ± 0.21^{c}	4.71 ± 0.10^{a}	16.0 ± 0.27^{d}	6.76 ± 0.06^{e}	40.0 ± 1.35^{f}
∑n-3	51.8 ± 0.30^{a}	47.1 ± 0.42^{b}	46.1 ± 0.12^{b}	49.9 ± 0.47^{c}	45.6 ± 0.53^{b}	47.0 ± 0.07^{b}	63.2 ± 1.11^{d}
∑PUFA	63.1 ± 0.30^{a}	54.1 ± 0.27^{b}	57.1 ± 0.14^{c}	64.5 ± 0.31^{a}	54.3 ± 0.62^{b}	58.6 ± 0.08^{d}	64.1 ± 1.09^{a}
DHA/EPA	0.64 ± 0.01^{a}	1.28 ± 0.04^{b}	0.95 ± 0.02^{c}	0.54 ± 0.01^{d}	2.56 ± 0.09^{e}	0.78 ± 0.01^{f}	2.28 ± 0.09^{e}
Unknown	8.74 ± 0.26^{a}	10.7 ± 0.26^{b}	11.3 ± 0.25^{b}	10.7 ± 0.09^{b}	7.20 ± 0.15^{c}	10.5 ± 0.11^{b}	3.23 ± 0.34^{d}

Values in the same row not sharing a superscript are significantly different ($P < 0.05$).

Table 6 The content of phosphatidylcholine and phosphatidylethanolamine of rotifers, expressed as mean ± SE of three replicates with duplicate analyses (n = 3 × 2)

		Nevada			Cayman		
		NR	NM	NP	CR	CM	CP
% DW	PC	2.25 ± 0.04^{ac}	2.21 ± 0.04^{ac}	2.12 ± 0.10^{c}	2.63 ± 0.61^{b}	2.50 ± 0.09^{ab}	2.50 ± 0.10^{ab}
	PE	1.95 ± 0.13^{a}	2.20 ± 0.09^{ac}	1.93 ± 0.05^{a}	2.34 ± 0.08^{c}	1.90 ± 0.09^{a}	2.10 ± 0.10^{ac}
	PC + PE	4.21 ± 0.16^{b}	4.42 ± 0.10^{bc}	4.09 ± 0.14^{b}	4.99 ± 0.14^{ac}	4.41 ± 0.18^{ab}	4.59 ± 0.15^{ab}
% of lipids	PC	23.1 ± 0.80^{ab}	24.5 ± 0.78^{a}	21.8 ± 1.16^{ab}	24.8 ± 0.68^{a}	19.7 ± 0.66^{b}	21.7 ± 0.90^{ab}
	PE	20.0 ± 1.51^{ac}	24.4 ± 1.44^{a}	19.9 ± 0.56^{ac}	22.2 ± 0.88^{ac}	15.0 ± 0.66^{b}	18.3 ± 0.94^{bc}
PE/PC		0.86 ± 0.04^{bc}	0.99 ± 0.03^{ab}	0.92 ± 0.03^{ab}	0.90 ± 0.02^{bc}	0.76 ± 0.02^{c}	0.85 ± 0.06^{ac}

Values in the same row not sharing a superscript are significantly different ($P < 0.05$).

the PE content of CM rotifers decreased significantly ($P < 0.05$) as compared with that of CR rotifers. The percentage of PC and PE of total lipids of CM rotifers also decreased significantly ($P < 0.05$) mainly because of the increase of total lipid after enrichment.

There was an insignificant ($P > 0.05$) increase in the PE/PC ratio in Nevada after enrichment and a small decrease in Cayman. A two-way ANOVA (with emulsion and strain as factors, and Tukey's multiple comparison test) showed significant differences ($P < 0.05$) of PE/PC ratio and PL

(PC + PE) DW^{-1} content between Nevada and Cayman; however, the changes of PE/PC ratio and PL content within the same strain were not significant ($P > 0.05$). The overall results suggested a fairly constant PE/PC ratio and PL content independent of dietary treatments.

HUFA composition in PC and PE of rotifers and copepods

The fatty acid composition in PC of the rotifer groups and of the copepods is shown in Table 7. After enrichment of both rotifer strains, the EPA and SFA levels remained constant, whereas the DHA and MUFA levels increased significantly ($P < 0.05$). The highest DHA contents of PC were found in CM rotifers (12.5%), following the contents in NM rotifers (9.95%). Copepods had significant ($P < 0.05$) lower levels of SFA, MUFA and n-6 fatty acids, but they contained significantly ($P < 0.05$) higher levels of EPA and especially DHA than all the rotifer groups.

The fatty acid composition in PE of the rotifer treatments and copepods are presented in Table 8. The EPA and SFA levels of Nevada did not show significant ($P > 0.05$) changes after enrichment, whereas for Cayman strains, the SFA levels increased ($P < 0.05$) and the EPA levels decreased significantly ($P < 0.05$). The DHA levels only increased slightly in both strains ($P > 0.05$). Copepods had significantly ($P < 0.05$) lower levels of MUFA and n-6 fatty acids, but they contained significantly ($P < 0.05$) higher levels of DHA and SFA than all the rotifer treatments. The EPA levels were as low as that in rotifers.

The results revealed a major difference in the fatty acid composition of copepods and rotifer phospholipids, with more than a tenfold higher percentage of DHA in the copepod PC and PE.

Relationship between HUFA composition in diets and rotifers

Dietary HUFA versus HUFA levels in total lipids of rotifers The regression analysis (Table 9) revealed a highly significant ($P < 0.0001$) positive relationship between DHA (Fig. 1a) and EPA (Fig. 1b) in total lipids of the diets and the respective contents in both Nevada and Cayman. The

Table 7 Fatty acid composition, given as percentage of TFA in PC of rotifers, expressed as mean ± SE of three replicates with duplicate analyses ($n = 3 \times 2$)

	Nevada			Cayman			Copepod
	NR	NM	NP	CR	CM	CP	
Fatty acid % of PC	($n = 3 \times 2$)						
14:0	5.15 ± 0.25^{ab}	5.32 ± 0.48^{b}	4.04 ± 0.11^{cd}	4.30 ± 0.16^{ac}	4.33 ± 0.26^{ac}	3.22 ± 0.18^{d}	1.85 ± 0.29^{e}
16:0	20.9 ± 0.18^{a}	21.3 ± 0.28^{ab}	23.2 ± 0.29^{bc}	21.2 ± 0.48^{a}	23.8 ± 0.43^{cd}	25.4 ± 0.41^{d}	17.0 ± 0.99^{a}
18:0	4.86 ± 0.35^{ab}	4.52 ± 0.70^{ab}	4.71 ± 0.24^{ab}	5.31 ± 0.24^{a}	3.75 ± 0.31^{b}	4.36 ± 0.28^{ab}	4.39 ± 0.21^{ab}
∑SFA	30.9 ± 0.56^{b}	31.2 ± 1.40^{b}	32.0 ± 0.51^{b}	30.8 ± 0.48^{b}	31.9 ± 0.67^{b}	33.0 ± 0.37^{b}	23.2 ± 1.23^{a}
16:1n-7	1.23 ± 0.04^{a}	1.54 ± 0.09^{b}	1.86 ± 0.08^{c}	1.27 ± 0.02^{a}	1.20 ± 0.06^{a}	1.31 ± 0.03^{a}	
18:1n-7	2.43 ± 0.05^{bc}	2.05 ± 0.02^{a}	2.55 ± 0.09^{abc}	2.62 ± 0.03^{c}	1.59 ± 0.04^{d}	2.39 ± 0.02^{b}	0.71 ± 0.02^{e}
18:1n-9	0.00 ± 0.00^{a}	5.33 ± 0.11^{bd}	3.32 ± 0.43^{bce}	0.00 ± 0.00^{a}	6.72 ± 0.33^{d}	1.99 ± 0.02^{c}	0.65 ± 0.08^{e}
20:1n-9	0.73 ± 0.01^{b}	1.12 ± 0.09^{a}	1.01 ± 0.12^{abc}	0.97 ± 0.01^{ac}	0.93 ± 0.04^{c}	0.65 ± 0.01^{bc}	
∑MUFA	4.39 ± 0.09^{a}	10.0 ± 0.25^{b}	8.75 ± 0.71^{c}	4.86 ± 0.03^{a}	10.4 ± 0.23^{b}	6.24 ± 0.08^{d}	1.36 ± 0.08^{e}
18:2n-6	7.15 ± 0.07ac	4.69 ± 0.25b	6.61 ± 0.24c	9.55 ± 0.17d	7.38 ± 0.15a	8.63 ± 0.12e	
18:3n-6	0.85 ± 0.02b	0.00 ± 0.00a	0.00 ± 0.00a	1.22 ± 0.01b	0.00 ± 0.00a	1.22 ± 0.01b	
20:3n-6	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	0.86 ± 0.03b	0.00 ± 0.00a	0.00 ± 0.00a	
20:4n-6	1.77 ± 0.13^{ab}	1.30 ± 0.14^{bc}	2.18 ± 0.14^{a}	1.74 ± 0.12^{ab}	1.16 ± 0.09^{c}	1.45 ± 0.15^{bc}	0.86 ± 0.07^{c}
∑n-6	9.78 ± 0.10^{a}	5.66 ± 0.57^{b}	8.78 ± 0.15^{d}	13.4 ± 0.10^{c}	8.54 ± 0.24^{d}	11.1 ± 0.21^{e}	0.86 ± 0.07^{f}
18:3n-3	15.0 ± 0.21^{a}	10.6 ± 0.35^{bc}	10.7 ± 0.45^{bc}	12.8 ± 0.07^{b}	9.23 ± 0.19^{c}	12.2 ± 0.17^{b}	0.51 ± 0.00^{d}
18:4n-3	8.10 ± 0.23^{a}	6.82 ± 0.58^{abc}	6.50 ± 0.29^{abc}	6.84 ± 0.12^{b}	5.45 ± 0.07^{c}	7.38 ± 0.11^{ab}	1.53 ± 0.07^{d}
20:3n-3	1.00 ± 0.03^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.93 ± 0.03^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	
20:4n-3	11.2 ± 0.37^{a}	9.06 ± 0.56^{bc}	7.77 ± 0.31^{bc}	10.9 ± 0.17^{a}	7.67 ± 0.21^{b}	9.05 ± 0.08^{c}	
20:5n-3	7.79 ± 0.16^{bc}	8.52 ± 0.39^{bc}	8.95 ± 0.14^{b}	6.19 ± 0.28^{a}	6.29 ± 0.27^{a}	7.26 ± 0.28^{ac}	20.8 ± 0.49^{d}
22:5n-3	2.81 ± 0.13^{a}	3.10 ± 0.28^{ab}	3.12 ± 0.11^{abc}	2.95 ± 0.04^{abc}	3.25 ± 0.11^{bc}	3.30 ± 0.04^{c}	
22:6n-3	4.29 ± 0.20^{b}	9.95 ± 1.11^{c}	6.58 ± 0.07^{a}	3.21 ± 0.19^{d}	12.5 ± 0.74^{e}	6.60 ± 0.23^{a}	50.8 ± 1.40^{f}
∑n-3	50.2 ± 0.98^{a}	48.0 ± 2.54^{ab}	43.6 ± 1.04^{b}	43.9 ± 0.60^{b}	44.4 ± 0.71^{b}	45.8 ± 0.38^{ab}	73.3 ± 1.46^{c}
∑PUFA	59.9 ± 0.95^{a}	53.7 ± 1.97^{b}	$52.4 \pm 1.13b^{cd}$	57.2 ± 0.51^{ad}	52.9 ± 0.60^{bc}	57.1 ± 0.47^{a}	74.2 ± 1.52^{e}
DHA/EPA	0.55 ± 0.04^{b}	1.18 ± 0.17^{bc}	0.73 ± 0.01^{c}	0.52 ± 0.01^{b}	2.01 ± 0.16^{a}	0.91 ± 0.04^{c}	2.45 ± 0.09^{a}
Unknown	4.73 ± 0.33^{ad}	5.12 ± 0.42^{ac}	6.90 ± 0.10^{bc}	7.10 ± 0.12^{bc}	4.93 ± 0.41^{ad}	3.90 ± 0.18^{d}	1.74 ± 0.24^{e}

Values in the same row not sharing a superscript are significantly different ($P < 0.05$).

Table 8 Fatty acid composition, given as percentage of TFA in PE of rotifers, expressed as mean ± SE of three replicates with duplicate analyses ($n = 3 \times 2$)

	Nevada			Cayman			
	NR	NM	NP	CR	CM	CP	Copepod
Fatty acid% of PE	($n = 3 \times 2$)						
14:0	2.65 ± 0.21^{ac}	2.92 ± 0.10^{a}	1.78 ± 0.08^{b}	1.73 ± 0.06^{b}	2.15 ± 0.21^{bc}	1.80 ± 0.14^{b}	0.63 ± 0.04^{d}
16:0	10.9 ± 0.43^{ac}	11.6 ± 0.57^{ab}	10.6 ± 0.28^{a}	7.95 ± 0.07^{d}	12.9 ± 0.59^{ab}	12.6 ± 0.33^{bc}	26.0 ± 1.05^{e}
18:0	2.76 ± 0.05^{a}	2.60 ± 0.09^{a}	2.51 ± 0.04^{a}	2.69 ± 0.03^{a}	3.50 ± 0.03^{c}	3.87 ± 0.06^{d}	7.26 ± 0.38^{b}
∑SFA	16.3 ± 0.68^{ab}	17.1 ± 0.74^{ab}	14.9 ± 0.36^{bc}	12.4 ± 0.10^{c}	18.5 ± 0.82^{ab}	18.3 ± 0.50^{a}	33.9 ± 1.34^{d}
16:1n-7	0.96 ± 0.07^{a}	1.19 ± 0.03^{bc}	1.39 ± 0.03^{c}	0.76 ± 0.02^{a}	1.01 ± 0.07^{ab}	1.22 ± 0.07^{bc}	
18:1n-7	2.50 ± 0.09^{b}	1.88 ± 0.02^{c}	2.24 ± 0.03^{a}	2.24 ± 0.01^{a}	1.52 ± 0.04^{d}	2.22 ± 0.07^{a}	
18:1n-9	0.83 ± 0.05^{a}	8.16 ± 0.22^{b}	4.55 ± 0.10^{c}	0.00 ± 0.00^{e}	10.0 ± 0.74^{b}	3.24 ± 0.03^{d}	1.07 ± 0.08^{a}
20:1n-9	7.15 ± 0.21^{c}	4.32 ± 0.17^{ae}	6.21 ± 0.21^{b}	5.59 ± 0.09^{bd}	4.98 ± 0.10^{ad}	3.85 ± 0.19^{e}	1.16 ± 0.05^{f}
22:1n-11	1.39 ± 0.08^{a}	1.16 ± 0.10^{a}	1.09 ± 0.12^{a}	1.09 ± 0.01^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	
22:1n-9	0.00 ± 0.00^{b}	1.84 ± 0.06^{a}	1.28 ± 0.13^{a}	0.00 ± 0.00^{b}	1.80 ± 0.05^{a}	0.00 ± 0.00^{b}	
∑MUFA	12.8 ± 0.13^{c}	18.6 ± 0.33^{b}	16.7 ± 0.49^{b}	9.67 ± 0.09^{a}	19.3 ± 0.94^{b}	10.5 ± 0.21^{a}	1.84 ± 0.38^{d}
18:2n-6	13.2 ± 0.21^{ac}	10.9 ± 0.57^{a}	14.5 ± 0.15^{b}	13.0 ± 0.09^{ac}	13.9 ± 0.41^{bc}	14.7 ± 0.11^{b}	
20:2n-6	0.66 ± 0.01^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.82 ± 0.01^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	
20:3n-6	0.56 ± 0.03^{a}	0.00 ± 0.00^{c}	0.54 ± 0.02^{a}	0.82 ± 0.02^{b}	0.56 ± 0.00^{a}	0.00 ± 0.00^{c}	
20:4n-6	1.17 ± 0.06^{a}	0.61 ± 0.01^{b}	1.41 ± 0.06^{e}	1.68 ± 0.03^{c}	0.76 ± 0.06^{d}	1.26 ± 0.05^{ae}	
∑n-6	15.6 ± 0.17^{b}	11.5 ± 0.57^{a}	16.4 ± 0.10^{b}	16.3 ± 0.09^{b}	15.2 ± 0.29^{b}	16.0 ± 0.07^{b}	
18:3n-3	18.7 ± 0.10^{c}	16.2 ± 0.30^{ad}	15.7 ± 0.13^{a}	17.0 ± 0.08^{d}	13.0 ± 0.20^{b}	16.6 ± 0.26^{d}	
18:4n-3	3.95 ± 0.05^{a}	3.27 ± 0.13^{d}	3.37 ± 0.14^{ad}	8.17 ± 0.15^{b}	3.56 ± 0.35^{ad}	5.94 ± 0.27^{c}	
20:3n-3	0.75 ± 0.03^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.86 ± 0.01^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	
20:4n-3	11.0 ± 0.48^{a}	8.73 ± 0.63^{abc}	8.25 ± 0.12^{bc}	8.67 ± 0.20^{b}	7.03 ± 0.27^{c}	7.70 ± 0.19^{bc}	
20:5n-3	5.83 ± 0.36 ^{cd}	4.44 ± 0.47^{bc}	5.84 ± 0.37 ^{cd}	10.2 ± 0.30^{a}	3.63 ± 0.45^{b}	6.58 ± 0.31^{d}	7.04 ± 0.26^{d}
22:5n-3	0.92 ± 0.08^{a}	0.60 ± 0.04^{b}	0.83 ± 0.03^{ab}	1.05 ± 0.03^{a}	0.92 ± 0.07^{a}	1.09 ± 0.08^{a}	
22:6n-3	1.79 ± 0.25^{a}	2.85 ± 0.43^{ab}	2.33 ± 0.15^{ab}	2.86 ± 0.08^{ab}	5.10 ± 0.53^{b}	2.91 ± 0.24^{ab}	52.1 ± 0.84^{c}
∑n-3	42.9 ± 1.14^{b}	36.1 ± 1.98^{bc}	36.3 ± 0.68^{c}	48.9 ± 0.38^{d}	33.3 ± 1.82^{ac}	40.8 ± 0.79^{ab}	59.1 ± 0.96^{e}
∑PUFA	58.5 ± 1.10^{a}	47.6 ± 1.42^{b}	52.7 ± 0.66^{ce}	65.2 ± 0.34^{d}	48.5 ± 1.68^{be}	56.8 ± 0.82^{ac}	59.1 ± 0.96^{a}
DHA/EPA	0.31 ± 0.03^{ac}	0.64 ± 0.04^{b}	0.40 ± 0.01^{a}	0.28 ± 0.01^{c}	1.40 ± 0.12^{d}	0.44 ± 0.03^{abc}	7.43 ± 0.26^{e}
Unknown	12.1 ± 0.42^{b}	16.9 ± 0.45^{a}	16.1 ± 0.34^{ac}	12.8 ± 0.24^{bc}	13.9 ± 0.24^{ab}	13.4 ± 0.79^{ab}	5.86 ± 1.65^{d}

Values in the same row not sharing a superscript are significantly different ($P < 0.05$).

slope coefficient (Table 9) of Cayman was significantly ($P < 0.05$) higher than that of Nevada for both DHA and EPA, suggesting a more efficient incorporation of fatty acids in the Cayman rotifers.

Dietary HUFA versus HUFA levels in PC and PE of rotifers Highly significant ($P < 0.0001$) relationships were also found between the percentage DHA in the diet and the per cent DHA content in PC for both Nevada and Cayman (Fig. 1c). The slope coefficient (Table 9) of Cayman was also significantly ($P < 0.05$) higher than that of Nevada. The EPA content of PC (Fig. 1d) was not significantly related to the dietary EPA levels of total lipids in any strain ($P > 0.05$, Table 9). The per cent DHA (Fig. 1e) and the EPA (Fig. 1f) in PE of both strains were significantly related ($P < 0.05$, Table 9) to the corresponding DHA and EPA contents in total dietary lipids. However, we did not find significant ($P > 0.05$) increase in DHA and EPA in PE of Nevada.

HUFA levels in PC and PE of diets versus HUFA levels in PC and PE of rotifers The correlations of the EPA in the PC of diets and PC of both rotifer strains (Fig. 2c) were statistically significant ($P < 0.05$) but exhibited a negative slope and a low r^2, indicating a weak negative relationship. The other correlations between dietary fatty acids of PC and PE and the respective PL in rotifers in Fig. 2a,b and d were not statistically significant ($P > 0.05$) (Table 9).

Discussion

The method used to enrich the rotifers in the present study involved n-3 HUFA enrichment during growth, and this method has been termed long-term enrichment (Rainuzzo *et al.* 1994b; Olsen 2004). In this way, efficient rotifer enrichment of n-3 HUFA can be achieved by feeding a complete diet, with a relatively low lipid ration consisting of a high n-3 HUFA dietary lipid ratio, which can support

Table 9 Regression analysis of dietary HUFA and HUFA levels in rotifers

	Nevada			
Relationship $f = y_0 + a*x$	Slope	Intercept	r^2	P
Dietary DHA% versus DHA% in Nevada	0.23 ± 0.02	3.78 ± 0.39	0.90	<0.0001
Dietary EPA% versus EPA% in Nevada	0.10 ± 0.02	7.61 ± 0.16	0.64	<0.0001
Dietary DHA% versus DHA% in PC in Nevada	0.27 ± 0.03	1.99 ± 0.67	0.82	<0.0001
Dietary DHA% versus DHA% in PE in Nevada	0.05 ± 0.02	1.38 ± 0.36	0.33	<0.05
Dietary EPA% versus EPA% in PC in Nevada	0.02 ± 0.05	8.18 ± 0.46	0.01	0.7785
Dietary EPA% versus EPA% in PE in Nevada	0.18 ± 0.07	3.82 ± 0.60	0.32	<0.05
Dietary DHA% in PC versus DHA% in PC in Nevada	0.00 ± 0.11	6.53 ± 2.08	0.00	0.9891
Dietary DHA% in PE versus DHA% in PE in Nevada	0.00 ± 0.03	2.23 ± 0.42	0.00	0.8776
Dietary EPA% in PC versus EPA% in PC in Nevada	−0.14 ± 0.05	11.6 ± 1.10	0.34	0.0278
Dietary EPA% in PE versus EPA% in PE Nevada	0.09 ± 0.07	5.06 ± 0.39	0.08	0.2598
	Cayman			
Relationship $f = y_0 + a*x$	Slope	Intercept	r^2	P
Dietary DHA% versus DHA% in Cayman	0.53 ± 0.05	−0.71 ± 1.00	0.88	<0.0001
Dietary EPA% versus EPA% in Cayman	0.31 ± 0.03	5.29 ± 0.29	0.86	<0.0001
Dietary DHA% versus DHA% in PC in Cayman	0.43 ± 0.03	−0.65 ± 0.69	0.91	<0.0001
Dietary DHA% versus DHA% in PE in Cayman	0.12 ± 0.03	1.22 ± 0.54	0.59	<0.05
Dietary EPA% versus EPA% in PC in Cayman	0.09 ± 0.05	5.78 ± 0.46	0.18	0.0783
Dietary EPA% versus EPA% in PE in Cayman	0.45 ± 0.16	2.75 ± 1.46	0.36	0.0145
Dietary DHA% in PC versus DHA% in PC in Cayman	−0.10 ± 0.17	9.19 ± 3.08	0.02	0.5567
Dietary DHA% in PE versus DHA% in PE in Cayman	−0.05 ± 0.05	4.09 ± 0.79	0.06	0.3635
Dietary EPA% in PC versus EPA% in PC in Cayman	−0.16 ± 0.05	10.3 ± 1.19	0.38	<0.05
Dietary EPA% in PE versus EPA% in PE in Cayman	0.00 ± 0.18	6.56 ± 1.02	0.00	0.9876

fast growth and reproduction of the rotifer cultures. A steady state of growth and biochemical composition can be reached in 4–5 days of cultivation when >90% of the rotifer individuals are produced in the period if the growth rate is high (~0.4 day^{-1}) (Olsen 2004). The method allows a very efficient control of the fatty acid composition of the rotifers, which are also leaner than those produced by so-called short-term enrichment (Rainuzzo *et al*. 1994b). The enrichment time of the present study was 4 days, and the fatty acid composition of total lipids is believed to be relatively constant at that time.

Relatively few studies have used phospholipid-based diets for lipid and fatty acid enrichment of rotifers, and little is known about the roles of dietry PC and PE on rotifer lipid accumulation (Rainuzzo *et al*. 1994b). The structural amphipathicity of phospholipids (PL) enables tri-acylglycerols (TAG) to be transported in aqueous environments by forming lipid/water interfaces (Tocher *et al*. 2008). In earlier experiments, the Nevada strain exhibited very high TAG accumulation during short-term enrichment by an oil emulsion extracted from the roe of Atlantic halibut (*Hippoglossus hippoglossus*) (Rainuzzo *et al*. 1994b), and the PL was effectively digested and incorporated as TAG in the rotifer body. A similar phenomenon was found in *Artemia*, in which the dietary PL was digested into free fatty acids and assimilated mainly as TAG (McEvoy *et al*. 1996).

A main objective of the present study was to examine whether it was more efficient to manipulate the DHA or n-3 HUFA in general in the PC and PE of rotifers by using enrichment diets based on marine PL rather than with diets based on marine TAG. The DHA composition of total lipid and PC in rotifers enriched by Marol E were generally higher than those enriched by the PL emulsion, and these differences were more obvious in Cayman than in Nevada. Although the total dietary lipids per individual rotifer were the same, the fatty acid profiles of the two emulsions were very different. Marol E had much higher DHA contents than PL emulsion.

We found that the DHA content of total lipids of rotifers and the DHA contents in rotifer PC and PE were both well correlated to the DHA contents of total dietary lipids, regardless of whether the dietary DHA originated from triacylglycerides or from phospholipids. However, no significant ($P > 0.05$) linear relationship was found between the dietary PC-DHA and rotifer PC-DHA content (Table 9). This suggested that the DHA contents of PC in the rotifers were mainly affected by the total dietary DHA levels, independent of the DHA contents in dietary PC.

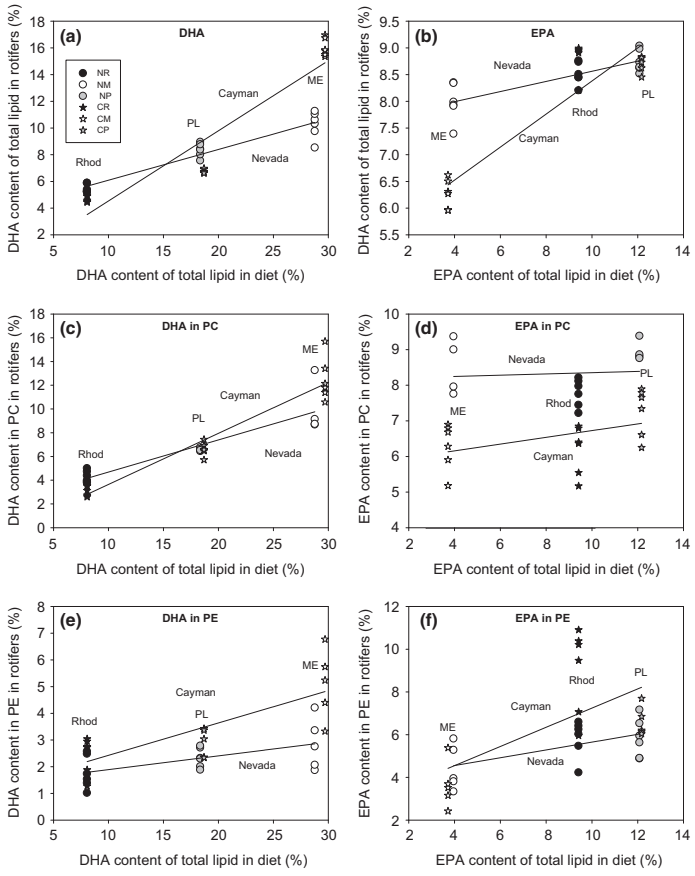

Figure 1 Relationship between dietary DHA, EPA and the DHA, EPA percentages of total lipid (a, b), PC (c, d) and PE (e, f) in rotifers. NR, Nevada cultivated with *R. baltica*; NM, Nevada enriched with Marol e; NP, Nevada enriched with PL emulsion; CR, Cayman cultivated with *R. baltica*; CM, Cayman enriched with Marol E; CP, Cayman enriched with PL emulsion.

TAG and PL are, therefore, likely to be fully digested and incorporated as monoacylglycerol (MAG) and Lyso PL in rotifer tissues in the same way as for fish and humans (Tocher *et al.* 2008). The released fatty acids compete for resynthesis of MAG to form TAG, PC and PE and re-acylation of Lyso PL to form PC and PE in rotifer tissues. This conclusion agrees with that for *Artemia* published by McEvoy *et al.* (1996) and implies that diets based on marine PL were no more efficient than diets based on marine TAG for manipulating DHA in PC and PE of rotifers.

The dietary lipid ration given per individual of Cayman was much lower than that of Nevada, as the body weight of Nevada strain was 3–4 times higher than that of Cayman (Lubzens & Zmora 2003). The dietary lipid ration given per biomass for Cayman was, therefore, still higher than for Nevada. This may be one reason for the higher incorporation efficiency of Cayman. Moreover, Nevada would have higher investment in body growth rather than reproduction compared to Cayman at a relatively high food level (>5 mg C L^{-1}) (Kostopoulou & Vadstein 2007), which means Cayman reproduce and grow faster, and they have more areas of stomach to incorporate lipid. However, the effect of giving Cayman a slightly higher lipid ration per biomass during enrichment will not have any effect on the phospholipid enrichment, because it is primarily dependent on the dietary fatty acid composition (percentage values), not the rations.

As discussed previously, the percentage DHA of TFA in total lipids and in PC increased after enrichment in all rotifer treatments, whereas the DHA content of PE remained stable, despite the high dietary DHA content of PE in the PL emulsions (Figs 1 & 2). The percentage DHA content in PE was accordingly more difficult to manipulate through enrichment than the DHA in PC. This may originate from

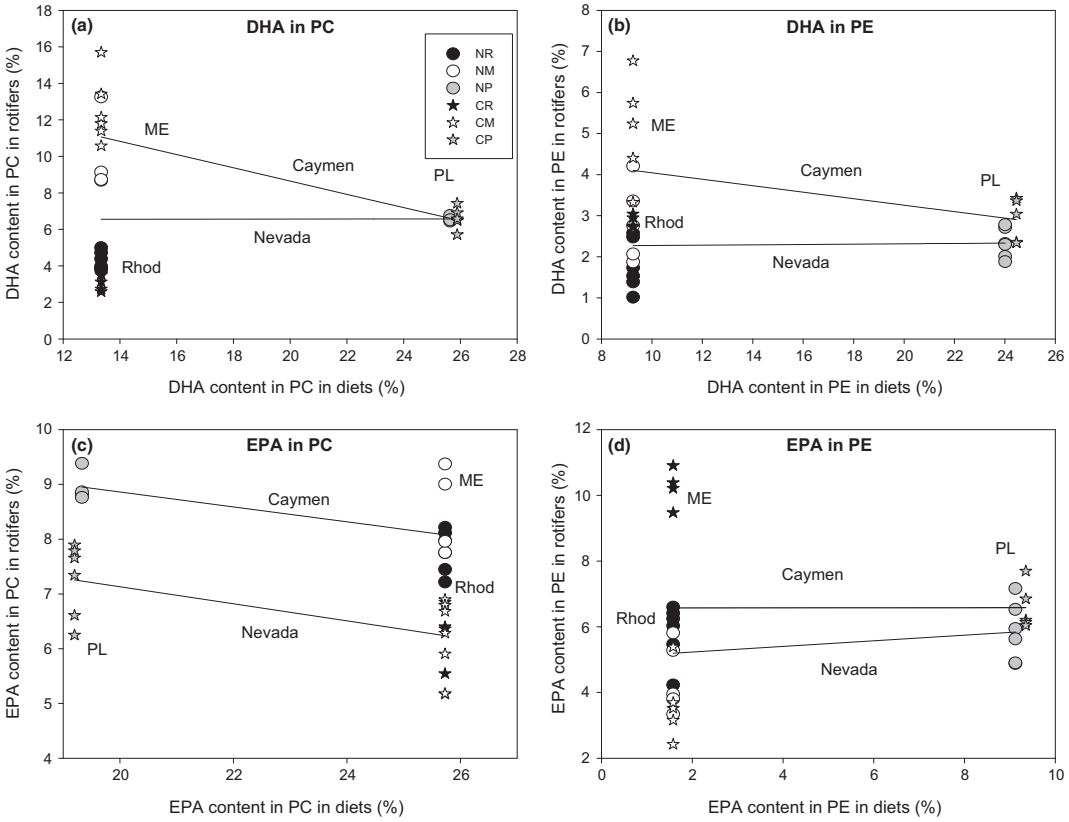

Figure 2 Relationship between dietary DHA, EPA of PC, PE and the DHA, EPA content of PC (a, b), PE (c, d) in rotifers.

the different functions, activities and distribution in cell membranes of PC and PE. PC is generally the most active component of phospholipids and is usually preferentially catabolized during the embryonic and yolk sac stages of marine fish larvae development, serving as a main source of energy and DHA (Rainuzzo *et al.* 1992; Coutteau *et al.* 1997).

The fatty acid composition of copepods and all rotifers from the different treatments differed at many critical points, in particular for the major PL components, PC and PE. The percentages of DHA in PC and PE fatty acids of copepods were around 50%, and higher than the DHA contents in their total lipids. On the other hand, the rotifers from all treatments showed far lower percentages of DHA in their PC and PE than the copepods. The percentage DHA of the dietary lipids of the rotifers (Fig. 2) was far higher than the percentage DHA contents obtained for rotifer PC and PE, suggesting that the DHA enrichment of rotifer PL was not as efficient as for their TAG. For *Brachionus* Nevada, there was actually an apparent maximum level of around 10% DHA of total PL fatty acids, even when the dietary percentage DHA exceeded 30% (Olsen *et al.*, in prep.). If the percentage DHA in phospholipids is an important feature for the nutritional value of the live feed for some species of marine fish larvae, the general adequacy of rotifers as live feed for these species may be questioned.

Dietary PL is an essential nutrient of fish larvae, and it has been suggested that this may imply that fish larvae base their PL synthesis on dietary PL and not on dietary TAG in early developmental stages (Gisbert *et al.* 2005; Tocher *et al.* 2008; Cahu *et al.* 2009). The mechanism of digestion and re-acylation of dietary PL in animals (Tocher *et al.* 2008; Olsen *et al.*, in prep.) does not allow major changes in fatty acid composition and configuration during the digestion and re-acylation of Lyso PL, which suggests that

the dietary fatty acid composition of PC and PE should be close to the normal composition of larval PL (Sargent *et al.* 1999). Nauplii and copepodites of calanoid copepods seem to represent an optimal food source for marine cold-water fish larvae and their fatty acid composition and configuration of PC and PE in this regards are likely optimal. Our results showed that rotifers and copepods were very different at this point, which suggests that PL of rotifers can be relatively far from being optimal for fish larvae. In support, Olsen *et al.* (in prep.) found a positive relationship between the percentage DHA of PL in the live feed, including two treatments of *Brachionus* Nevada and nauplii of *Acartia tonsa*, and in PL of 17 days posthatched cod larvae. Moreover, there was a positive relationship between the percentage DHA of larval PL and their growth and survival. We have also found a long-lasting effect on the growth and functionality of cod and ballan wrasse larvae, after relatively short feeding periods with cultivated *Acartia tonsa* nauplii (Øie, Kjørsvik *et al.*, in prep).

Based on data from Coutteau & Sorgeloos (1997), we suggest that *Artemia* nauplii may also suffer from a similar inadequate PL composition as the present strains of rotifers, but this should be further evaluated both for other rotifer strains and for *Artemia*. Then, there is a need to establish mitigating measures to obtain a more optimal PL available for the larvae through their rotifer and *Artemia* diets. At present, there are no other suitable live feed organisms readily available on a commercial scale. However, copepods such as *Acartia tonsa* can be cultured and produced in a controlled way (Støttrup 2000; Overrein 2010, Olsen *et al.*, in prep.). Today, their production costs are much higher than those of rotifers and *Artemia*, but technology for mass production of copepods should be further investigated.

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Paper II

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Time kinetics of fatty acid changes in phospholipids following enrichment and starvation of *Artemia franciscana* with a main focus on docosahexaenoic acid

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Abstract

The main objective was to study time kinetics of change in important highly unsaturated fatty acids (HUFAs) in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of *Artemia franciscana* nauplii and juveniles following enrichment and subsequent starvation. Samples of *Artemia* nauplii were taken at variable times (0.5–24 h) following enrichment and starvation. Samples of *Artemia* juveniles were taken after 2, 3 and 4 days of cultivation. No docosahexaenoic acid (DHA) was found in PC and PE of *Artemia* nauplii during the first hour of enrichment, while a significant ($P < 0.05$) increase was found in total lipids (TLs). The content of DHA in PC and PE increased thereafter steadily from 1 to 8 h of enrichment. DHA in PC and PE during enrichment (1–8 h) and following starvation (8–24 h), respectively, increased and decreased significantly ($P < 0.05$), but at a lower rate than that in TL. Moreover, juvenile *Artemia* (2–4 days) contained a relatively low level of DHA in TL compared with enriched *Artemia* nauplii, but the content of DHA in PC and PE was similar. The results open perspectives for both industry and science. For scientific studies, the lag phase in HUFA enrichment makes it possible to produce *Artemia* nauplii with variable relative HUFA enrichments in phospholipids and TL.

KEY WORDS: *Artemia*, DHA, enrichment, HUFA, phospholipids, starvation

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Introduction

The brine shrimp, *Artemia* sp. is widely used as a live feed organism for marine and freshwater fish larvae and juveniles in aquaculture all around the world. *Artemia* sp. is considered as an insufficient source of n-3 highly unsaturated fatty acid (HUFA) for marine larvae (Leger *et al.* 1986; Navarro *et al.* 1992; Bell *et al.* 2003), and the contents of these fatty acids in *Artemia* nauplii can be increased by enriching the *Artemia* with n-3 HUFA-rich products, such as microparticulate diets and lipid emulsions (Leger *et al.* 1986; Conceicao *et al.* 2010). The larger juvenile *Artemia* has been considered as a good alternative live feed for species such as seahorses *Hippocampus abdominalis* (Woods 2003) and Atlantic halibut *Hippoglossus hippoglossus* (Olsen *et al.* 1999b). *Artemia* nauplii are normally enriched by using a lipid-rich diet, whereas *Artemia* juveniles are normally fed by a balanced diet including microalgae and algae pastes, followed by short-term enrichment of lipid-rich diet (Olsen *et al.* 1999b; Smith *et al.* 2002; Guinot *et al.* 2013).

The importance and essential function of HUFA, such as docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6) in fish larval nutrition has been known for long time (Kanazawa 1997; Bell *et al.* 1999; Sargent *et al.* 1999; Lee 2001; Cahu *et al.* 2009; Tocher 2010). Phospholipids (PL) and triacylglyceride (TAG) are the main lipid classes in diets that may supply HUFA for marine fish larvae. Dietary PL is currently considered essential for early stages of fish larvae and has therefore become an important issue in larval rearing (Tocher *et al.* 2008; Cahu *et al.* 2009). Recent studies have also suggested that HUFA in PL are more available than HUFA in TAG for many species of fish larvae, such as herring (*Clupea harengus*), haddock (*Melanogrammus aeglefinus*), cod (*Gadus morhua*) and halibut (*Hippoglossus hippoglossus*) (Kanazawa 1997; Gisbert *et al.* 2005; Cahu *et al.* 2009; Olsen *et al.* 2014).

It has recently been shown that two strains of the rotifer *Brachionus plicatilitis* accumulated n-3 HUFA mainly in their TAG during n-3 HUFA enrichment and that it appeared difficult to obtain levels of DHA in PL higher than 100 g kg^{-1} of PL fatty acids (Li *et al*. 2014). There is ample evidence that the same situation will occur for *Artemia* nauplii and juveniles (Coutteau & Mourente 1997; Guinot *et al*. 2013). This may be important for the culturing of cold-water species, such as Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*), for which the supply of DHA during the first feeding appears to be a problem for larval quality (Evjemo *et al*. 2003; Wold *et al*. 2009). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most dominant lipid classes in the PL of *Artemia* (Navarro *et al*. 1991). We assume that the HUFA level of PC and PE, like that of total lipids (TLs) in *Artemia,* can be manipulatedusing an enrichment diet, but the rate of change and the quantitative levels that can be obtained of HUFA (DHA in particular) in PC and PE is unknown. The objective of this study was to obtain more information on the time kinetics of change in important HUFA in PC and PE of *Artemia franciscana* nauplii following enrichment and subsequent starvation, with a main focus on DHA.

Materials and methods

Production of nauplii and juvenile Artemia

Artemia franciscana cysts (Great Salt Lake, UT, USA; INVE Aquaculture, Belgium) were decapsulated using hypochlorite, followed by rinsing and transfer to conical plastic tanks with seawater (26 °C) and strong aeration for hatching (Sorgeloos *et al*. 1986). After 24 h, hatched *Artemia* were separated into three tanks for subsequent short-time enrichment.

Three conical plastic tanks with 60 L of seawater (34 g L^{-1}, 28 °C) and strong aeration from the bottom of each tank were used for the short-time enrichment of *Artemia* nauplii. The starting density was 120 ind mL^{-1}. *Artemia* were enriched with Multigain (BioMar Group, Brande, Denmark) given in a concentration of 200 mg L^{-1} at the beginning of the enrichment period (Time 0) that lasted for 8 h. The feed were consumed at that time, and *Artemia* were thereafter starved for another 16 h. The lipid and fatty acids analysis of the enrichment diet has shown that Multigain contained certain amount of PL, but no EPA and ARA were found in PL, and the content of DHA in PL was close to none (<1g kg^{-1} of the total DHA) (data not shown).

Juvenile *Artemia* were cultivated in three plastic tanks with 80 L of seawater at 28 °C for 4 days. The starting density was 30 ind mL^{-1}. *Artemia* were fed with a combined diet of the algae paste Pavlova 1800 (Brown Biflagellate *Prymnesiophyceae*, Instant Algae®, Reed Mariculture Inc., Campbell, CA, USA; 0.19 mL L^{-1} 24 h^{-1}) and Multigain (2.6 mg L^{-1} 24 h^{-1}). The fatty acid composition of the algae paste was declared by Reed Mariculture Inc. (Appendix Tables A1 and A2). Other research of *Pavlova* has shown that the algae contained about 14 g kg^{-1} of PL and 300 g kg^{-1} of DHA in PL (Guiheneuf *et al*. 2010). The fatty acid composition of the combined diet was estimated as the weighted mean of Pavlova 1800 and Multigain.

The density of *Artemia* was counted at the beginning and the end of the experiments for nauplii and every day after sampling for the juvenile *Artemia*. Samples were then taken from the centre of the tanks by a hollow plastic pipe and mixed well before counting. Ten 0.1-mL droplets were counted under a stereo-microscope to determine the number of live *Artemia* and the total numbers after fixation with Lugol's solution. Survival was calculated as the number of live *Artemia*/total number of *Artemia* at the beginning. Ten juvenile *Artemia* individuals were sampled every day for the measurement of body lengths under 25× magnification in a stereo-microscope.

Lipid and fatty acid analysis

Artemia samples for lipid and fatty acid analysis were sampled at times 0, 0.5, 1, 2, 4, 8, 12 and 24 h for *Artemia* nauplii, and on days 2, 3 and 4 for *Artemia* juveniles. *Artemia* were sampled by a sieve with a 64-μm mesh and rinsed with filtered seawater and tap water. Concentrated *Artemia* were scraped off into a 50-mL plastic tube and were immediately frozen by liquid nitrogen. All samples were freeze-dried and stored at −80 °C under nitrogen atmosphere before analysis.

Lipid extraction was carried out following a modified method of Bligh and Dyer (1959). Freeze-dried samples (~30 mg) were homogenized in a blender (T10 Basic Ultra-Turrax®, IKA®, Staufen, Germany) for 1 min with 0.8 mL of distilled water (dH_2O), 2 mL of methanol and 1 mL of chloroform. After that, 1 mL of chloroform was added to the mixture, which was blended for 20 s. After the addition of 1 mL of dH_2O, blending was continued for another 20 s. The homogenate was then centrifuged (Hettich Universal 32R; Hettich, Tuttlingen, Germany) at 1640 ***g*** for 10 min at 5 °C. After centrifugation, 1 mL of chloroform was transferred into a tube and dried under N_2 at 40 °C in the sample concentrator (DB3D Techne Dri-bolck® Bibby Scientific

Limited, Staffordshire, UK). Concentrated samples for TL were weighted on an analytical balance (UMX2 Automated-S Ultra-microbalance, Mettler-Toledo AS, Olso, Norway) after 24 h in the drying vessel.

Lipid separation of PC and PE was undertaken using thin-layer chromatography (TLC) based on a modified method of Fraser *et al.* (1985). A similar method according to Li *et al.* (2014) was used, and the PC and PE of *Artemia* were separated and stored at −80 °C under nitrogen atmosphere before analysis.

Extracted TL, PC and PE were then used to prepare fatty acid methyl esters (FAMEs) for fatty acid analysis following the method of Metcalfe *et al.* (1966). The FAMEs were determined quantitatively by gas chromatography (Auto system XL PERKIN ELMER, Norwalk, CT, USA). An internal standard (IS) 19 : 0 methyl ester was added to the samples before extraction for analysing the absolute amount of fatty acids in the TL. A modified method from Abdulkadir & Tsuchiya (2008) was used for analysing fatty acids in PC and PE of *Artemia* juveniles. To the scrapped PC and PE with silica was added 0.5 mL of isooctane, followed by 0.2 mL of 140 g kg^{-1} BF_3 in methanol, and the mixture was heated at 100 °C for 2 h. After cooling on ice, 0.1 mL of isooctane and 0.2 mL of distilled water were added into the tubes. After centrifugation for 3 min (5 °C, 1640 ***g*** Hettich Universal 32R), the upper layer was transferred and analysed on a gas chromatograph (Auto system XL PERKIN ELMER). The method of direct methylation of PC and PE with silica was validated by a comparison with the conventional methods of Fraser *et al.* (1985) and Metcalfe *et al.* (1966). Eight replicates were taken from one freeze-dried sample. TL was applied and sprayed on a silica plate for TLC. Separated PC and PE were scrapped and put into eight quick fit test tubes, four for conventional methods and four for direct methylation. No significant difference ($P > 0.05$, data not shown) was observed for the percentage of fatty acids in PC and PE by using the two methods.

Statistics

Regression analysis of DHA content in TL, PC and PE was taken from the equation:

$$\text{DHA content} = At + B$$

where t is the time (h), A is the slope of the regression line (h^{-1}) and B is the content of DHA at time 0.

All data were checked for statistical significance by using one-way ANOVA with Tukey's multiple comparison test, and differences were considered significant when $P < 0.05$. All the statistical tests were performed using SPSS 19.0 (IBM SPSS, Armonk, NY, USA) for windows. All figures were made in Sigma Plot 12.0 (Systat Software Inc., San Jose, CA, USA).

Results

Both *Artemia* diets used for short-term enrichment of *Artemia* nauplii (Multigain) and for cultivation of juvenile *Artemia* (combined Pavlova-Multigain diet) contained high contents of DHA and relatively low amounts of EPA and ARA (Table 1). Multigain contained 1.8 times higher TL, 2.1 times higher total fatty acids (TFA) content, and 5 times higher contents of DHA, EPA and ARA than the combined diet.

Artemia nauplii subjected to short-term enrichment by Multigain showed a significant ($P < 0.05$) increase in TL content from 166 ± 0.3 mg g^{-1} DW at 0 h to 182 ± 0.8 mg g^{-1} DW at 8 h, which then decreased to 135 ± 1.3 mg g^{-1} DW during starvation until 24 h. In juvenile *Artemia,* the content of TL decreased significantly ($P < 0.05$) from day 2 (121 ± 6.6 mg g^{-1} DW) up to day 3 (96.5 ± 7.9 mg g^{-1} DW), but increased again significantly ($P < 0.05$) on day 4 (109 ± 4.6 mg g^{-1} DW). The survival rates of *Artemia* were higher than 90% during both nauplii and juvenile cultivation.

Similar time courses were found for the relative contents of DHA, EPA and ARA in PC (Fig. 1a) and PE (Fig. 1b) of *Artemia* nauplii during short-term enrichment. The content of DHA remained at 0% during the first hour of the enrichment and increased gradually thereafter. A similar delay in response was found also for EPA and ARA in PC and PE; both these HUFAs were present in significantly higher amounts ($P < 0.05$) than DHA. In comparison, the absolute and relative contents of fatty acids in TL (Fig. 1c,d) increased immediately and significantly ($P < 0.05$) after provision of feed, and the content of DHA increased much faster than that of EPA and ARA, consistent with earlier reports (Dhert *et al.* 1993; Evjemo *et al.* 1997).

Table 1 Contents of highly unsaturated fatty acidss, TFA and total lipid (TL) of the diets and the combined diet expressed in terms of mg g^{-1} DW (% of TFA) ($n = 2$)

	DHA	EPA	ARA	TFA	TL
Multigain	122 (33.1)	5.3 (1.4)	6.0 (1.6)	367	427
Pavlova	14.2 (11.2)	0.3 (0.2)	0.5 (0.4)	126	200
Combined diet[1]	22.5 (15.5)	0.6 (0.4)	0.9 (0.6)	172	242

[1] Combined diet of Multigain and algae paste Pavlova (Weighted Average).

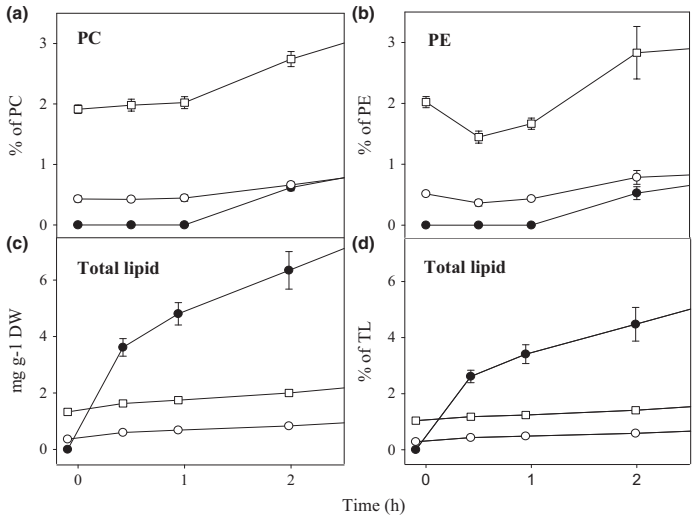

Figure 1 The contents of eicosapentaenoic acid (white square), arachidonic acid (white circle) and docosahexaenoic acid (black circle) in total lipid (TL), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of *Artemia franciscana* following the early phase of enrichment by Multigain (0–2 h). Error bars indicate SE from two replicate analyses at time 0 ($n = 2$) and three replicate cultures with two replicate analyses ($n = 6$) each in a later sampling.

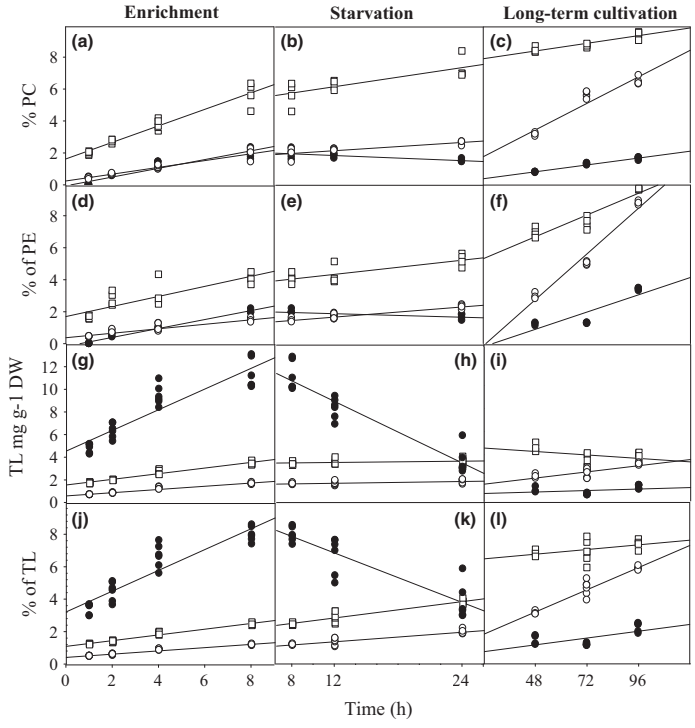

Figure 2 Change of eicosapentaenoic acid (white square), arachidonic acid (white circle) and docosahexaenoic acid (black circle) contents in total lipid (TL), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of *Artemia franciscana* during enrichment (1–8 h), starvation (8–24 h) and long-term cultivation (48–96 h). Error bars indicate SE from 2–3 replicate cultures with two replicate analyses ($n = 4$–6) each in a later sampling.

The percentages of DHA in PC and PE increased gradually beyond 1 h reaching a maximum content at 8 h (Fig. 2a,d, Table 2), which then decreased gradually reaching 1.54 ± 0.1% and 1.70 ± 0.1%, respectively, after starvation up to 24 h (Fig. 2b,e, Table 2). The percentage contents of DHA in PC and PE also increased during long-

Table 2 Regression analysis of docosahexaenoic acid (DHA) content in total lipid (TL), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of *Artemia franciscana* nauplii and juvenile [DHA content = At+ B; t is the time (h); A is the slope of the regression line (h^{-1})] (n = 4–6)

	A	r^2	P	n
A. franciscana nauplii (1–8 h)				
PC, % of TFA	0.27 ± 0.02^{c}	0.88	<0.001	4
PE, % of TFA	0.28 ± 0.01^{c}	0.96	<0.001	4
TL[1], mg g^{-1} DW	0.92 ± 0.09^{a}	0.83	<0.001	6
TL[2], % of TFA	0.64 ± 0.06^{b}	0.85	<0.001	6
A. franciscana nauplii (8–24 h)				
PC, % of TFA	-0.03 ± 0.01^{c}	0.49	0.011	4
PE, % of TFA	-0.02 ± 0.01^{c}	0.47	0.014	4
TL[1], mg g^{-1} DW	-0.46 ± 0.04^{a}	0.88	<0.001	6
TL[2], % of TFA	-0.26 ± 0.03^{b}	0.79	<0.001	6
A. franciscana juveniles (2–4 day)				
PC, % of TFA	0.02 ± 0.00^{b}	0.96	<0.001	4
PE, % of TFA	0.05 ± 0.01^{c}	0.76	<0.001	4
TL[1], mg g^{-1} DW	0.01 ± 0.01^{a}	0.10	0.260	4
TL[2], % of TFA	0.02 ± 0.01^{b}	0.42	0.010	4

Different superscript letters within a column indicate significant differences ($P < 0.05$).
[1] Absolute DHA content (mg g^{-1} DW).
[2] Relative DHA content (% of TL).

term cultivation of juvenile *Artemia*, reaching 130 and 173% of the initial level, respectively (Fig. 2c,f, Table 2). An increase in the EPA and ARA contents was also found in PC and PE of *Artemia* nauplii during short-term enrichment, starvation and in *Artemia* juveniles during long-term cultivation (Fig. 2a–f). The percentage contents of DHA in PC and PE were throughout significantly ($P < 0.05$) lower than the contents of EPA in nauplii and juvenile *Artemia*.

In comparison with the changes in PC and PE, the quantitative and relative contents of DHA in TL increased more rapidly with time during short-term enrichment (Fig. 2g,j, Table 2) and decreased more rapidly during starvation (Fig. 2h,k, Table 2), whereas in juvenile *Artemia* the contents of DHA increased only slightly between days 2 and 4 (Fig. 2i,l, Table 2). In addition, EPA and ARA increased steadily in TL during enrichment, starvation and long-term cultivation (Fig. 2g–l), but the absolute content of EPA in *Artemia* juveniles remained almost constant ($P > 0.05$) between days 2 and 4 (Fig. 2i). The contents of DHA were consistently higher than that of EPA and ARA in nauplii (Fig. 2g,h,j,k), but were significantly ($P < 0.05$) lower than EPA and ARA in juvenile *Artemia* (Fig. 2i,l).

The slope coefficients of the regression lines express the rate of increase (slope > 0) or decrease (slope < 0) of DHA in specific lipid classes (PC and PE) and TL (Table 2). The rate of increase in PC and PE of *Artemia* nauplii during short-term enrichment (1–8 h) and the starvation (8–24 h) period were similar ($P < 0.05$). Moreover, the rates of increase for DHA in PC and PE of *Artemia* juveniles were significantly ($P < 0.05$) lower than those of *Artemia* nauplii. The increase in PC and PE during enrichment and starvation were also significantly ($P < 0.05$) lower than those in TL within the same period. The rates of increase in DHA in PC and PE during enrichment were more than nine times higher than the rates of decrease during starvation period, which suggested that PLs retain HUFA more efficiently than TL.

Other major fatty acids in PC and PE of *Artemia* nauplii and juveniles were C18:3n-3, C18:1n-9 and C16:0 (Appendix Tables A3 and A4). In *Artemia* nauplii, the content of C18:3n-3 in PC increased during enrichment and starvation, whereas the content of C18:1n-9 remained almost unchanged. In PE, both fatty acids decreased during the enrichment and starvation. The percentage content of C16:0 in PC increased steadily during the enrichment and starvation periods. However, it varied unsystematically with time in PE. In *Artemia* juveniles, the contents of C18:1n-9 in PC and PE were consistently significantly higher than the contents of C18:3n-3 ($P < 0.05$). The percentage content of C16:0 significantly decreased in PC ($P < 0.05$), but remained unchanged ($P > 0.05$) in PE from days 2 to 4. A significantly ($P < 0.05$) higher percentage content of C16:0 was observed in PC (>14%) than in PE (<5%).

Discussion

Recent research has suggested that HUFAs of PL are better available for fish larvae in their early stages than HUFA incorporated in TAG and that PLs in fact appear to be essential for fish larvae (Tocher *et al.* 2008; Cahu *et al.* 2009). This also means that HUFA enrichment of PL, and not only enrichment of TLs, in live feed becomes an important issue (Guinot *et al.* 2013; Li *et al.* 2014). A main objective of the present study was to examine changes in HUFA composition of PL in nauplii and juvenile *Artemia* following HUFA enrichment, starvation and further on-growing and to compare this with the changes in HUFA in TL.

An important, although perhaps not surprising, result of our study was that there was a lag phase of 1 h in the accumulation of DHA, EPA and ARA in PC and PE after the initiation of feeding (Fig. 1a,b). The accumulation of these HUFA in TL started immediately, and DHA which was present in high amounts in the feed accumulated very

quickly during the first hour of enrichment. In addition, the different HUFA exhibited different patterns of variation during short-term enrichment, starvation and on-growing. The delay in HUFA accumulation in nauplii is compatible with the fact that it will take some time to digest, accumulate and re-acylate the consumed HUFA into PC and PE.

The content of DHA in TL increased more than two times faster than that in PC and PE (Table 2), which probably was because the incorporation of DHA into PL was slower and less efficient than the incorporation into TL. The rapid increase in DHA in TL may also to some extent originate in undigested lipid diet in the gut because these TLs reflect the sum of the lipids of the nauplii and those of the enrichment diet. The DHA contents of TL, PC and PE continued to increase up to 8 h and may still, like normally found for TL, increase somewhat after that (Evjemo *et al*. 1997). The DHA contents of PC and PE were around 2% of the total fatty acids in the respective PL component (Appendix Tables A3 and A4), which is consistent with the results of Coutteau & Mourente (1997) and Bell *et al*. (2003). The studies by Coutteau & Mourente (1997) and McEvoy *et al*. (1996) have shown that the TL of *Artemia* naupli contained about 35% of PL and 65% of neutral lipids, and the quantitative content of PC and PE in *Artemia* nauplii remained relatively stable after enrichment. We suggest that the increase in HUFA in PL reflects the fatty acid exchange between and within PL and TAG, because the quantitative PL content of the enriched *Artemia* nauplii will likely remain constant during short-term enrichment (Sargent *et al*. 1999; Tocher 2003).

The content of DHA in PC and PE of *Artemia* nauplii decreased, whereas the contents of EPA and ARA continued to increase during the starvation period from 8 to 24 h posthatching (Fig. 2), in accordance with the study carried out by Coutteau & Mourente (1997) and Navarro *et al*. (1999). Moreover, the content of DHA in TL became significantly less ($P < 0.05$), in accordance with Evjemo *et al*. (2001), and faster than that in PC and PE of the *Artemia* nauplii (Appendix Table A1). The slower loss rate of DHA in PC and PE than from TL may originate in the findings that HUFA like DHA in *Artemia* nauplii are preferentially esterified in the *sn*-2 position (Tocher *et al*. 2008), which will make DHA less available for being catabolized as compared with DHA esterified in the *sn-3* position in TAG (Ando *et al*. 2004). The percentages of EPA and ARA in PC and PE increased during the starvation period, in agreement with their concentration in TL, which was still high.

The slower accumulation rate of DHA found in *Artemia* juveniles compared with nauplii may be primarily due to the lower DHA concentration in the combined diet as compared to the enrichment diet (Table 1). Even though the content of DHA in TL of juvenile *Artemia* was lower than that in *Artemia* nauplii, a similar percentage of DHA was found in PC and PE of nauplii and juvenile *Artemia* (Fig. 2). This was probably because additional DHA was incorporated into PL through a synthetic pathway in juvenile *Artemia* because they must synthesize PL for new tissue during long-term cultivation, while fatty acid exchange between and within PL and TAG takes place at the same time (Sargent *et al*. 1999; Tocher 2003).

An important conclusion of our study is that HUFA enrichment of PL was not very efficient as compared to that of TL. We found a much lower percentage DHA content of PL of *Artemia* nauplii than for rotifers (~10%) and in particular for copepods (~40–50%) (Scott *et al*. 2002; Li *et al*. 2014). Recent finding of Guinot *et al*. (2013) has also shown that the content of DHA in PL of *Artemia* juveniles enriched with a PL-rich diet was only 1.9% of total fatty acids. Because HUFAs from PL seem to be more available from dietary PL than from dietary TAG (Kanazawa 1997; Gisbert *et al*. 2005; Cahu *et al*. 2009), this may contribute to explain why copepod nauplii, the natural live feed of many fish larval species, normally have provided much better growth and survival of fish larvae (Naess *et al*. 1995; McEvoy *et al*. 1998; Evjemo *et al*. 2003).

The present study has also revealed that the enrichment time used will affect the relative HUFA content and fatty acid composition in TL relative to that of PL in *Artemia* nauplii. A very short enrichment time will result in a relatively low HUFA enrichment of PL, while TL is enriched very rapidly. These results open perspectives for both industry and science. To obtain an optimum HUFA enrichment of PL, we suggest that the enrichment time of *Artemia* nauplii in commercial hatcheries should be 8 h or longer, which is for sure most common (Evjemo *et al*. 1997). This will ensure that a maximal amount of HUFAs, especially DHA, are incorporated into PL, which are beneficial for marine fish larvae (Sargent *et al*. 2002; Wold *et al*. 2007, 2009; Tocher *et al*. 2008; Cahu *et al*. 2009). For scientific studies, the lag phase in HUFA enrichment opens possibilities to produce *Artemia* nauplii with variable relative HUFA enrichments in PL and TAG. For example, through a 1-h enrichment of a high concentration of DHA oil, *Artemia* nauplii may get a high content of DHA concentrated in neutral lipids and very low in PL.

Artemia juveniles contained low levels of HUFA in TL (Table 1) and in relative terms, higher levels of HUFA in PL than in nauplii (Fig. 2), and it may therefore be a good alternative live feed for marine fish species. The relatively low levels of DHA in TL of *Artemia* juveniles can additionally be manipulated by short-time enrichment by emulsified diet, such as DHA Selco (containing HUFA-rich neutral lipids) and Marine lecithin LC60 (containing HUFA-rich PL), just before their use as live feed (Smith *et al*. 2002; Guinot *et al*. 2013). An obstacle for applying juvenile *Artemia* in marine fish larval rearing might be the relatively longer body length of juvenile *Artemia*, the higher bacteria load in culture (Olsen *et al*. 1999a), and the extra cost for cultivation.

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Appendix 1

Table A1 All fatty acids contents (mg g^{-1} DW) in total lipid of diets (Multigain and combination), *Artemia* nauplii (0–24 h) and juvenile (2–4 day)

	0 h (n = 2)	0.5 h (n = 6)	1 h (n = 6)	2 h (n = 6)	4 h (n = 6)	8 h (n = 6)	12 h (n = 6)	24 h (n = 6)	D2 (n = 4)	D3 (n = 6)	D4 (n = 5)	Mul (n = 2)	Pav	Comb (n = 2)
C14:0	0.73 ± 0.0	1.50 ± 0.1	1.65 ± 0.0	1.71 ± 0.1	1.84 ± 0.1	1.76 ± 0.2	1.54 ± 0.1	1.23 ± 0.2	1.18 ± 0.2	0.57 ± 0.0	0.71 ± 0.1	24.0 ± 0.9	15.8	16.5 ± 0.1
C16:0	13.6 ± 0.1^{a}	18.3 ± 0.8^{b}	19.0 ± 0.6^{b}	19.1 ± 0.7^{b}	19.4 ± 1.1^{b}	19.4 ± 1.5^{b}	18.5 ± 1.0^{b}	15.6 ± 0.9^{a}	8.14 ± 0.5^{c}	5.53 ± 0.2^{d}	6.11 ± 0.5^{d}	111 ± 1.8^{e}	11.7	19.3 ± 0.1^{b}
C18:0	6.41 ± 0.0	6.75 ± 0.5	6.85 ± 0.2	6.84 ± 0.2	6.95 ± 0.3	6.63 ± 0.3	6.28 ± 0.3	6.53 ± 0.6	6.00 ± 0.2	4.81 ± 0.3	3.89 ± 0.1	3.59 ± 0.2	0.38	0.63 ± 0.0
C20:0	0.12 ± 0.0	0.15 ± 0.0	0.15 ± 0.0	0.15 ± 0.0	0.15 ± 0.0	0.15 ± 0.0	0.15 ± 0.0	0.19 ± 0.0	0.13 ± 0.0	0.08 ± 0.0	0.08 ± 0.0	0.43 ± 0.0	0.00	0.03 ± 0.0
C22:0	0.18 ± 0.0	0.19 ± 0.0	0.22 ± 0.0	0.21 ± 0.0	0.25 ± 0.0	0.25 ± 0.0	0.23 ± 0.0	0.27 ± 0.0	0.21 ± 0.0	0.17 ± 0.0	0.18 ± 0.0	0.44 ± 0.0	0.00	0.03 ± 0.0
C16:1n7	2.43 ± 0.0	2.44 ± 0.2	2.39 ± 0.2	2.36 ± 0.2	2.32 ± 0.2	2.39 ± 0.2	2.32 ± 0.5	1.71 ± 0.2	1.12 ± 0.1	0.85 ± 0.1	1.13 ± 0.1	1.12 ± 0.1	7.99	7.46 ± 0.0
C18:1n7	6.94 ± 0.0	7.19 ± 0.5	7.16 ± 0.3	6.94 ± 0.6	6.96 ± 0.4	6.97 ± 0.3	6.82 ± 0.8	6.22 ± 0.5	5.31 ± 0.2	5.03 ± 0.5	4.60 ± 0.1	1.92 ± 0.1	2.03	2.02 ± 0.0
C18:1n9	21.5 ± 0.1^{a}	21.6 ± 1.6^{a}	21.5 ± 0.9^{a}	21.0 ± 1.5^{a}	20.4 ± 1.1^{a}	19.8 ± 1.3^{a}	18.4 ± 2.8^{ab}	14.3 ± 1.7b^{c}	11.5 ± 0.5^{cd}	10.2 ± 1.3^{d}	14.5 ± 0.2^{b}	9.98 ± 0.6^{d}	12.8	12.6 ± 0.0^{cd}
C20:1n9	0.59 ± 0.0	0.59 ± 0.1	0.59 ± 0.0	0.59 ± 0.0	0.59 ± 0.0	0.59 ± 0.0	0.55 ± 0.0	0.50 ± 0.1	0.31 ± 0.0	0.24 ± 0.0	0.24 ± 0.0	0.00 ± 0.0	2.16	1.99 ± 0.0
C18:2n6	7.63 ± 0.0	7.72 ± 0.6	7.79 ± 0.3	7.79 ± 0.4	7.58 ± 0.3	7.30 ± 0.5	6.55 ± 0.8	4.70 ± 0.6	5.3 ± 0.5	3.28 ± 0.2	3.61 ± 0.2	8.58 ± 0.4	7.23	7.33 ± 0.0
C18:3n3	42.2 ± 0.1^{a}	41.3 ± 3.5^{ab}	41.2 ± 1.4^{ac}	40.6 ± 1.7^{ac}	37.5 ± 1.5^{bcd}	35.1 ± 2.2^{bd}	31.0 ± 3.5^{d}	21.7 ± 2.7^{e}	11.5 ± 0.6^{f}	6.37 ± 0.5^{g}	4.68 ± 0.3^{h}	7.76 ± 0.6^{g}	6.97	7.03 ± 0.0^{g}
C18:4n3	8.26 ± 0.0	8.02 ± 0.7	7.98 ± 0.3	7.79 ± 0.4	7.00 ± 0.2	6.30 ± 0.5	5.19 ± 0.8	2.94 ± 0.5	2.90 ± 0.3	1.19 ± 0.1	0.82 ± 0.1	2.50 ± 0.2	22.6	21.0 ± 0.0
C20:2n6	0.26 ± 0.0	0.27 ± 0.0	0.27 ± 0.0	0.27 ± 0.0	0.28 ± 0.0	0.26 ± 0.0	0.25 ± 0.0	0.24 ± 0.0	0.21 ± 0.0	0.18 ± 0.0	0.16 ± 0.0	0.00 ± 0.0	0.13	0.12 ± 0.0
C20:3n3	1.69 ± 0.0	1.68 ± 0.1	1.71 ± 0.1	1.70 ± 0.0	1.65 ± 0.1	1.55 ± 0.1	1.37 ± 0.1	1.11 ± 0.2	0.52 ± 0.0	0.36 ± 0.0	0.22 ± 0.0	0.81 ± 0.1	1.14	1.12 ± 0.0
C20:3n6	0.14 ± 0.0	0.19 ± 0.0	0.20 ± 0.0	0.21 ± 0.0	0.24 ± 0.0	0.24 ± 0.0	0.21 ± 0.0	0.17 ± 0.0	0.14 ± 0.0	0.10 ± 0.0	0.15 ± 0.0	1.18 ± 0.0	0.13	0.21 ± 0.0
C20:4n3	1.32 ± 0.0	1.40 ± 0.1	1.43 ± 0.0	1.44 ± 0.0	1.39 ± 0.0	1.32 ± 0.1	1.11 ± 0.1	0.67 ± 0.1	0.40 ± 0.1	0.16 ± 0.0	0.15 ± 0.0	2.83 ± 0.1	0.13	0.33 ± 0.0
C20:4n6	0.37 ± 0.0^{a}	0.60 ± 0.0^{b}	0.68 ± 0.0^{c}	0.83 ± 0.0^{d}	1.28 ± 0.1^{e}	1.67 ± 0.1^{f}	1.71 ± 0.2^{f}	1.88 ± 0.2^{f}	2.16 ± 0.2^{f}	2.29 ± 0.4^{f}	3.19 ± 0.1^{g}	5.99 ± 0.3^{h}	0.51	0.92 ± 0.0^{d}
C20:5n3	1.33 ± 0.0^{a}	1.63 ± 0.1^{b}	1.75 ± 0.1^{b}	2.00 ± 0.0^{c}	2.73 ± 0.2^{d}	3.49 ± 0.2^{e}	3.67 ± 0.3^{e}	3.68 ± 0.3^{e}	4.70 ± 0.3^{f}	3.42 ± 0.6^{de}	4.01 ± 0.2^{ef}	5.25 ± 0.3^{f}	0.25	0.64 ± 0.0^{g}
C22:5n6	0.00 ± 0.0	1.40 ± 0.1	1.84 ± 0.1	2.38 ± 0.2	3.61 ± 0.3	4.47 ± 0.4	3.55 ± 0.5	1.74 ± 0.5	0.23 ± 0.0	0.20 ± 0.0	0.40 ± 0.1	47.5 ± 1.9	2.16	5.64 ± 0.1
C22:6n3	0.00 ± 0.0^{a}	3.61 ± 0.3^{b}	4.81 ± 0.4^{c}	6.34 ± 0.7^{d}	9.45 ± 0.9^{e}	11.4 ± 1.3^{e}	8.44 ± 0.9^{de}	3.67 ± 1.2^{f}	1.02 ± 0.3^{gh}	0.59 ± 0.1^{g}	1.22 ± 0.2^{h}	122 ± 5.2^{i}	14.2	22.5 ± 0.4^{j}
Unknow	12.5 ± 0.0	12.0 ± 1.0	12.0 ± 0.7	12.0 ± 0.7	11.0 ± 0.6	10.5 ± 0.7	9.60 ± 1.5	6.75 ± 0.9	6.17 ± 0.8	3.65 ± 0.2	4.56 ± 0.3	11.8 ± 0.2	14.6	14.4 ± 0.0
SFA	21.1 ± 0.2^{a}	26.9 ± 1.3^{bc}	27.9 ± 0.7^{b}	28.0 ± 1.0^{b}	28.6 ± 1.5^{b}	28.3 ± 1.9^{b}	26.8 ± 1.2^{bc}	23.9 ± 1.4^{ac}	15.7 ± 0.9^{d}	11.2 ± 0.6^{e}	11.0 ± 0.7^{e}	139 ± 2.9^{f}	28.8	37.3 ± 0.2^{g}
MUFA	31.5 ± 0.2^{a}	31.8 ± 2.2^{a}	31.7 ± 1.3^{a}	30.9 ± 2.3^{a}	30.3 ± 1.6^{a}	29.8 ± 1.8^{a}	28.1 ± 4.0^{ab}	22.8 ± 2.3^{bc}	18.2 ± 0.7^{ce}	16.4 ± 1.9^{e}	20.4 ± 0.1^{ce}	13.0 ± 0.8^{e}	25.0	24.1 ± 0.1^{b}
PUFA	63.2 ± 0.1^{a}	67.8 ± 5.3^{ab}	69.7 ± 1.9^{b}	71.3 ± 1.8^{b}	72.7 ± 2.1^{b}	73.0 ± 5.3^{ab}	63.1 ± 4.1^{ab}	42.5 ± 5.1^{c}	29.0 ± 2.0^{d}	18.2 ± 1.7^{e}	18.6 ± 0.6^{e}	204 ± 9.1^{f}	58.4	69.6 ± 0.7^{b}
TFA	128 ± 0.5^{a}	139 ± 9.6^{ab}	141 ± 4.2^{b}	142 ± 5.3^{b}	143 ± 5.4^{b}	142 ± 9.3^{ab}	128 ± 10^{ab}	95.9 ± 8.9^{c}	69.1 ± 3.8^{d}	49.3 ± 4.1^{e}	54.6 ± 1.5^{e}	368 ± 13^{f}	127	145 ± 1.0^{b}

Different superscript letters within a row indicate significant difference ($P < 0.05$). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids.

Table A2 All fatty acid content (% of total fatty acid) in total lipid of diets (Multigain and combination), *Artemia* nauplii (0–24 h) and juvenile (2–4 day)

	0 h (n = 2)	0.5 h (n = 6)	1 h (n = 6)	2 h (n = 6)	4 h (n = 6)	8 h (n = 6)	12 h (n = 6)	24 h (n = 6)	D2 (n = 4)	D3 (n = 6)	D4 (n = 5)	Mul (n = 2)	Pav	Comb (n = 2)
C14:0	0.57 ± 0.0	1.09 ± 0.0	1.17 ± 0.0	1.2 ± 0.0	1.29 ± 0.0	1.24 ± 0.0	1.21 ± 0.1	1.29 ± 0.2	1.69 ± 0.3	1.16 ± 0.1	1.31 ± 0.1	6.53 ± 0.0	12.5	11.3 ± 0.0
C16:0	10.7 ± 0.1^{a}	13.3 ± 0.6^{b}	13.5 ± 0.4^{b}	13.4 ± 0.4^{b}	13.6 ± 0.4^{b}	13.8 ± 0.5^{b}	14.6 ± 0.8^{bc}	16.4 ± 1.3^{c}	11.8 ± 0.1^{a}	11.2 ± 0.5^{a}	11.2 ± 0.6^{a}	30.1 ± 0.6^{d}	9.20	13.2 ± 0.1^{b}
C18:0	5.00 ± 0.0	4.87 ± 0.0	4.85 ± 0.0	4.81 ± 0.1	4.87 ± 0.1	4.68 ± 0.1	4.93 ± 0.2	6.82 ± 0.2	8.69 ± 0.3	9.77 ± 0.4	7.12 ± 0.1	0.98 ± 0.0	0.30	0.43 ± 0.0
C20:0	0.09 ± 0.0	0.11 ± 0.0	0.10 ± 0.0	0.11 ± 0.0	0.11 ± 0.0	0.10 ± 0.0	0.12 ± 0.0	0.19 ± 0.0	0.19 ± 0.0	0.17 ± 0.0	0.15 ± 0.0	0.12 ± 0.0	0.00	0.02 ± 0.0
C22:0	0.14 ± 0.0	0.14 ± 0.0	0.15 ± 0.0	0.14 ± 0.0	0.18 ± 0.0	0.18 ± 0.0	0.18 ± 0.0	0.28 ± 0.0	0.31 ± 0.0	0.34 ± 0.1	0.33 ± 0.1	0.12 ± 0.0	0.00	0.02 ± 0.0
C16:1n7	1.90 ± 0.0	1.77 ± 0.0	1.69 ± 0.1	1.66 ± 0.1	1.63 ± 0.1	1.69 ± 0.1	1.81 ± 0.3	1.79 ± 0.2	1.62 ± 0.0	1.73 ± 0.1	2.08 ± 0.1	0.30 ± 0.0	6.30	5.13 ± 0.0
C18:1n7	5.41 ± 0.0	5.19 ± 0.1	5.07 ± 0.1	4.88 ± 0.3	4.88 ± 0.1	4.93 ± 0.1	5.33 ± 0.2	6.5 ± 0.4	7.70 ± 0.3	10.2 ± 0.3	8.44 ± 0.4	0.52 ± 0.0	1.60	1.39 ± 0.0
C18:1n9	16.8 ± 0.0^{a}	15.6 ± 0.2^{b}	15.3 ± 0.3^{b}	14.8 ± 0.6^{bc}	14.3 ± 0.3^{c}	14.0 ± 0.1^{c}	14.4 ± 1.0^{bc}	14.9 ± 0.6^{bc}	16.6 ± 1.2^{abc}	20.7 ± 1.1^{d}	26.5 ± 0.5^{e}	2.71 ± 0.1^{f}	10.1	8.66 ± 0.0^{g}
C20:1n9	0.46 ± 0.0	0.43 ± 0.0	0.42 ± 0.0	0.42 ± 0.0	0.41 ± 0.0	0.42 ± 0.0	0.43 ± 0.0	0.53 ± 0.0	0.44 ± 0.0	0.48 ± 0.0	0.44 ± 0.0	0.00 ± 0.0	1.70	1.37 ± 0.0
C18:2n6	5.95 ± 0.0	5.57 ± 0.1	5.51 ± 0.1	5.47 ± 0.1	5.32 ± 0.0	5.16 ± 0.1	5.12 ± 0.2	4.9 ± 0.2	7.66 ± 0.4	6.67 ± 0.2	6.62 ± 0.2	2.33 ± 0.0	5.70	5.04 ± 0.0
C18:3n3	32.9 ± 0.1^{a}	29.8 ± 0.5^{b}	29.2 ± 0.2^{b}	28.5 ± 0.3^{c}	26.3 ± 0.3^{d}	24.8 ± 0.2^{e}	24.3 ± 0.8^{ef}	22.6 ± 0.9^{f}	16.6 ± 0.4^{g}	12.9 ± 0.7^{h}	8.59 ± 0.7^{i}	2.11 ± 0.1^{j}	5.50	4.84 ± 0.0^{k}
C18:4n3	6.44 ± 0.0	5.78 ± 0.1	5.65 ± 0.1	5.48 ± 0.1	4.91 ± 0.1	4.44 ± 0.1	4.05 ± 0.3	3.05 ± 0.2	4.19 ± 0.2	2.42 ± 0.2	1.51 ± 0.1	0.68 ± 0.0	17.8	14.5 ± 0.0
C20:2n6	0.21 ± 0.0	0.20 ± 0.0	0.19 ± 0.0	0.19 ± 0.0	0.19 ± 0.0	0.18 ± 0.0	0.19 ± 0.0	0.25 ± 0.0	0.30 ± 0.0	0.37 ± 0.0	0.29 ± 0.0	0.00 ± 0.0	0.10	0.08 ± 0.0
C20:3n3	1.32 ± 0.0	1.21 ± 0.0	1.21 ± 0.0	1.20 ± 0.0	1.16 ± 0.0	1.09 ± 0.0	1.08 ± 0.1	1.15 ± 0.1	0.76 ± 0.1	0.73 ± 0.0	0.41 ± 0.0	0.22 ± 0.0	0.90	0.77 ± 0.0
C20:3n6	0.11 ± 0.0	0.14 ± 0.0	0.14 ± 0.0	0.15 ± 0.0	0.17 ± 0.0	0.17 ± 0.0	0.17 ± 0.0	0.17 ± 0.0	0.21 ± 0.0	0.21 ± 0.0	0.27 ± 0.0	0.32 ± 0.0	0.10	0.14 ± 0.0
C20:4n3	1.03 ± 0.0	1.01 ± 0.0	1.01 ± 0.0	1.01 ± 0.0	0.98 ± 0.0	0.93 ± 0.0	0.87 ± 0.0	0.69 ± 0.0	0.57 ± 0.1	0.33 ± 0.0	0.27 ± 0.0	0.77 ± 0.0	0.10	0.23 ± 0.0
C20:4n6	0.28 ± 0.0^{a}	0.43 ± 0.0^{b}	0.48 ± 0.0^{c}	0.58 ± 0.0^{d}	0.90 ± 0.1^{e}	1.18 ± 0.0^{f}	1.35 ± 0.2^{fk}	1.96 ± 0.1^{g}	3.12 ± 0.1^{h}	4.62 ± 0.5^{i}	5.85 ± 0.1^{j}	1.63 ± 0.0^{k}	0.40	0.64 ± 0.0^{d}
C20:5n3	1.04 ± 0.0^{a}	1.18 ± 0.0^{b}	1.24 ± 0.0^{b}	1.41 ± 0.1^{c}	1.91 ± 0.1^{d}	2.47 ± 0.1^{e}	2.89 ± 0.3^{e}	3.85 ± 0.1^{f}	6.80 ± 0.2^{g}	6.91 ± 0.7^{g}	7.35 ± 0.3^{g}	1.43 ± 0.0^{c}	0.20	0.44 ± 0.0^{h}
C22:5n6	0.00 ± 0.0	1.01 ± 0.1	1.3 ± 0.1	1.68 ± 0.2	2.54 ± 0.3	3.15 ± 0.1	2.81 ± 0.5	1.82 ± 0.4	0.34 ± 0.0	0.41 ± 0.1	0.74 ± 0.2	12.9 ± 0.1	1.70	3.88 ± 0.0
C22:6n3	0.00 ± 0.0^{a}	2.61 ± 0.2^{b}	3.41 ± 0.3^{c}	4.47 ± 0.6^{d}	6.64 ± 0.7^{e}	8.00 ± 0.5^{e}	6.69 ± 1.2^{e}	3.83 ± 1.1^{bc}	1.46 ± 0.3^{fg}	1.19 ± 0.1^{f}	2.24 ± 0.3^{bg}	33.1 ± 0.3^{h}	11.2	15.5 ± 0.1^{i}
Unknow	9.72 ± 0.0	8.63 ± 0.2	8.47 ± 0.3	8.47 ± 0.2	7.68 ± 0.2	7.42 ± 0.2	7.50 ± 0.5	7.02 ± 0.4	8.91 ± 0.8	7.41 ± 0.2	8.50 ± 0.7	3.20 ± 0.2	11.5	9.88 ± 0.0
SFA	16.5 ± 0.1^{a}	19.5 ± 0.6^{b}	19.8 ± 0.5^{b}	19.7 ± 0.4^{b}	20.1 ± 0.4^{b}	20.0 ± 0.5^{b}	21.1 ± 1.0^{bc}	25.0 ± 1.4^{de}	22.7 ± 0.1^{cd}	22.7 ± 0.8^{cd}	20.6 ± 1.1^{b}	37.8 ± 0.5^{f}	22.7	25.6 ± 0.1^{e}
MUFA	24.5 ± 0.0^{a}	23.0 ± 0.2^{b}	22.4 ± 0.4^{b}	21.7 ± 0.9^{bc}	21.2 ± 0.5^{c}	21.0 ± 0.3^{c}	21.9 ± 1.4^{bc}	23.7 ± 1.1^{ab}	26.0 ± 1.6^{abc}	32.1 ± 2.6^{d}	36.6 ± 1.7^{e}	3.54 ± 0.1^{f}	19.7	16.6 ± 0.0^{g}
PUFA	49.3 ± 0.1^{ac}	48.9 ± 0.5^{ag}	49.4 ± 0.2^{ac}	50.1 ± 0.7^{ab}	51.0 ± 0.9^{bc}	51.6 ± 0.8^{b}	49.5 ± 1.4^{abg}	44.3 ± 1.7^{d}	42.2 ± 0.7^{d}	37.5 ± 2.2^{e}	34.7 ± 1.3^{e}	55.4 ± 0.6^{f}	46.1	47.9 ± 0.1^{g}

Different superscript letters within a row indicate significant difference ($P < 0.05$).

Table A3 All fatty acid content (% of total fatty acid) in phosphatidylcholine of *Artemia* nauplii (0–24 h) and juvenile (2–4 day)

	0 h (n = 2)	0.5 h (n = 4)	1 h (n = 6)	2 h (n = 4)	4 h (n = 6)	8 h (n = 4)	12 h (n = 4)	24 h (n = 4)	2 day (n = 4)	3 day (n = 4)	4 day (n = 4)
C14:0	0.40 ± 0.0	0.41 ± 0.0	0.45 ± 0.1	0.54 ± 0.1	0.62 ± 0.1	0.61 ± 0.1	0.72 ± 0.1	0.66 ± 0.1	1.40 ± 0.1	1.51 ± 0.1	1.28 ± 0.1
C16:0	12.0 ± 0.1^{a}	13.7 ± 0.8^{ab}	13.9 ± 1.0^{ab}	13.5 ± 0.6^{ab}	13.6 ± 1.1^{ab}	12.7 ± 1.4^{ab}	15.1 ± 1.0^{ac}	16.4 ± 2.1^{ac}	16.5 ± 0.3^{c}	14.9 ± 0.5^{b}	14.2 ± 0.8^{a}
C16:1n7	1.61 ± 0.0	1.65 ± 0.1	1.65 ± 0.1	1.63 ± 0.0	1.62 ± 0.1	1.66 ± 0.1	1.69 ± 0.1	1.86 ± 0.1	1.65 ± 0.1	1.66 ± 0.0	2.00 ± 0.0
C18:0	9.20 ± 0.3	9.69 ± 0.4	9.64 ± 0.5	9.99 ± 0.1	9.93 ± 0.3	9.53 ± 0.3	9.96 ± 0.4	9.87 ± 1.1	11.3 ± 0.1	11.4 ± 0.2	8.16 ± 0.2
C18:1n7	7.29 ± 0.1	7.08 ± 0.2	7.08 ± 0.2	7.12 ± 0.2	7.05 ± 0.1	7.35 ± 0.2	7.77 ± 0.3	8.57 ± 0.1	7.58 ± 0.2	9.46 ± 0.1	7.85 ± 0.2
C18:1n9	19.8 ± 0.2^{ab}	20.0 ± 0.2^{b}	20.0 ± 0.2^{b}	19.0 ± 0.1^{a}	18.7 ± 0.4^{a}	18.5 ± 0.3^{a}	18.3 ± 0.4^{a}	18.5 ± 0.9^{ab}	18.8 ± 0.9^{ab}	20.9 ± 1.1^{ab}	30.3 ± 0.9^{c}
C18:2n6	5.19 ± 0.0	5.16 ± 0.2	5.19 ± 0.2	5.23 ± 0.1	5.16 ± 0.1	5.27 ± 0.1	4.92 ± 0.2	4.77 ± 0.3	7.02 ± 0.5	5.44 ± 0.4	5.60 ± 0.2
C18:3n3	24.4 ± 0.2^{a}	22.5 ± 1.1^{ab}	22.6 ± 1.3^{ab}	22.7 ± 0.3^{b}	21.5 ± 0.7^{bc}	20.5 ± 0.4^{bc}	18.4 ± 1.1^{cd}	17.5 ± 1.0^{d}	11.5 ± 0.3^{e}	7.59 ± 0.1^{f}	4.14 ± 0.3^{g}
C18:4n3	8.94 ± 0.2	8.26 ± 0.3	8.14 ± 0.5	8.10 ± 0.2	7.13 ± 0.3	5.89 ± 0.3	4.49 ± 0.1	2.96 ± 0.4	0.20 ± 0.0	0.20 ± 0.0	0.13 ± 0.0
C20:1n9	0.64 ± 0.0	0.54 ± 0.0	0.54 ± 0.0	0.55 ± 0.0	0.57 ± 0.0	0.64 ± 0.1	0.62 ± 0.0	0.52 ± 0.0	0.39 ± 0.0	0.41 ± 0.0	0.39 ± 0.0
C20:2n6	0.30 ± 0.0	0.28 ± 0.0	0.28 ± 0.0	0.31 ± 0.0	0.31 ± 0.0	0.32 ± 0.0	0.35 ± 0.1	0.30 ± 0.0	0.30 ± 0.0	0.37 ± 0.0	0.30 ± 0.0
C20:3n3	1.55 ± 0.0	1.39 ± 0.0	1.33 ± 0.0	1.42 ± 0.1	1.36 ± 0.1	1.37 ± 0.1	1.31 ± 0.0	1.09 ± 0.1	0.59 ± 0.0	0.58 ± 0.0	0.29 ± 0.0
C20:4n3	0.96 ± 0.1	0.61 ± 0.1	0.61 ± 0.1	0.63 ± 0.1	0.70 ± 0.1	0.66 ± 0.1	0.53 ± 0.1	0.33 ± 0.0	0.30 ± 0.0	0.20 ± 0.0	0.14 ± 0.0
C20:4n6	0.43 ± 0.1^{a}	0.42 ± 0.0^{a}	0.44 ± 0.1^{a}	0.66 ± 0.1^{b}	1.13 ± 0.1^{c}	1.92 ± 0.4^{d}	2.23 ± 0.1^{d}	2.65 ± 0.1^{de}	3.08 ± 0.1^{e}	5.59 ± 0.2^{f}	6.46 ± 0.3^{g}
C20:5n3	1.91 ± 0.1^{a}	1.98 ± 0.1^{a}	2.02 ± 0.1^{a}	2.74 ± 0.1^{b}	3.81 ± 0.3^{c}	5.68 ± 0.8^{cd}	6.28 ± 0.3^{d}	7.33 ± 0.7^{de}	8.44 ± 0.2^{e}	8.73 ± 0.1^{f}	9.41 ± 0.2^{f}
C22:6n3	0.00 ± 0.0^{a}	0.00 ± 0.0^{a}	0.00 ± 0.0^{a}	0.62 ± 0.1^{b}	1.27 ± 0.2^{c}	1.96 ± 0.3^{cd}	1.86 ± 0.1^{d}	1.54 ± 0.1^{cd}	0.67 ± 0.0^{b}	1.19 ± 0.1^{c}	1.54 ± 0.1^{cd}
Unknown	5.40 ± 0.1	6.37 ± 0.7	6.16 ± 0.5	5.28 ± 0.6	5.42 ± 0.3	5.04 ± 0.1	5.08 ± 0.6	4.86 ± 0.3	6.68 ± 0.5	7.83 ± 0.6	6.81 ± 0.4
SFA	21.6 ± 0.4^{a}	23.8 ± 1.0^{ab}	24.0 ± 1.4^{ab}	24.1 ± 0.6^{ab}	24.1 ± 1.4^{ab}	22.9 ± 1.8^{ab}	25.8 ± 0.8^{bc}	27.0 ± 3.2^{ac}	29.4 ± 0.4^{c}	28.0 ± 0.7^{c}	23.8 ± 1.0^{a}
MUFA	29.3 ± 0.1^{ac}	29.3 ± 0.2^{ac}	29.2 ± 0.1^{ac}	28.3 ± 0.2^{b}	28.0 ± 0.3^{b}	28.2 ± 0.3^{b}	28.4 ± 0.3^{b}	29.4 ± 0.7^{abc}	28.4 ± 0.6^{b}	32.4 ± 1.2^{c}	40.5 ± 1.0^{d}
PUFA	43.7 ± 0.2^{a}	40.6 ± 1.6^{a}	40.6 ± 1.9^{a}	42.3 ± 0.7^{a}	42.5 ± 1.6^{a}	43.9 ± 1.8^{a}	40.8 ± 1.2^{a}	38.8 ± 2.6^{ab}	35.5 ± 1.0^{b}	31.7 ± 0.3^{c}	29.0 ± 0.6^{d}

Different superscript letters within a row indicate significant difference ($P < 0.05$).

Table A4 All fatty acid content (% of total fatty acid) in phosphatidylethanolamine of *Artemia* nauplii (0–24 h) and juvenile (2–4 day)

	0 h (n = 2)	0.5 h (n = 4)	1 h (n = 6)	2 h (n = 4)	4 h (n = 6)	8 h (n = 4)	12 h (n = 4)	24 h (n = 4)	2 day (n = 4)	3 day (n = 4)	4 day (n = 4)
C16:0	5.01 ± 1.0^{a}	8.67 ± 0.4^{b}	10.3 ± 0.4^{c}	4.05 ± 0.7^{a}	6.83 ± 3.1^{abc}	4.56 ± 0.9^{a}	6.23 ± 1.6^{abc}	7.71 ± 4.5^{abc}	4.40 ± 0.3^{a}	3.23 ± 0.0^{a}	3.90 ± 0.1^{a}
C16:1n7	0.54 ± 0.1	0.55 ± 0.0	0.53 ± 0.0	0.32 ± 0.1	0.42 ± 0.1	0.52 ± 0.2	0.60 ± 0.2	0.79 ± 0.3	0.49 ± 0.0	0.48 ± 0.0	0.50 ± 0.0
C18:0	8.06 ± 0.1	8.49 ± 0.4	8.09 ± 0.1	8.50 ± 0.6	8.48 ± 0.6	8.67 ± 0.1	9.13 ± 0.6	8.01 ± 2.0	11.5 ± 0.2	11.3 ± 0.4	9.69 ± 0.2
C18:1n7	9.85 ± 0.0	9.28 ± 0.3	9.37 ± 0.1	10.0 ± 0.1	9.75 ± 0.3	10.2 ± 0.2	10.5 ± 0.2	12.4 ± 0.9	12.9 ± 0.2	15.3 ± 0.2	13.5 ± 0.3
C18:1n9	26.3 ± 0.7^{ab}	26.0 ± 0.7^{b}	25.2 ± 0.3^{b}	25.7 ± 0.1^{b}	24.9 ± 0.4^{b}	24.5 ± 0.6^{b}	25.0 ± 1.2^{b}	24.4 ± 2.2^{bc}	25.6 ± 1.1^{b}	29.6 ± 1.1^{ac}	32.8 ± 0.6^{d}
C18:2n6	6.17 ± 0.0	5.97 ± 0.2	6.06 ± 0.0	6.07 ± 0.3	5.98 ± 0.3	6.06 ± 0.1	6.12 ± 0.1	5.93 ± 0.3	9.25 ± 0.4	8.61 ± 0.8	6.54 ± 0.3
C18:3n3	21.1 ± 0.0^{a}	20.0 ± 0.6^{b}	20.3 ± 0.2^{b}	20.3 ± 0.5^{ab}	19.5 ± 0.7^{abc}	18.8 ± 0.3^{cd}	18.0 ± 0.5^{cd}	16.4 ± 1.0^{d}	12.0 ± 0.2^{e}	7.50 ± 0.3^{f}	3.44 ± 0.2^{g}
C18:4n3	10.0 ± 0.6	8.70 ± 0.4	8.92 ± 0.1	10.3 ± 0.6	8.88 ± 1.2	7.25 ± 0.2	6.18 ± 0.9	3.71 ± 0.5	3.80 ± 0.3	1.86 ± 0.1	1.11 ± 0.1
C20:1n9	0.94 ± 0.0	0.76 ± 0.0	0.77 ± 0.0	1.00 ± 0.1	0.91 ± 0.1	1.00 ± 0.1	0.91 ± 0.1	0.90 ± 0.2	0.74 ± 0.0	0.21 ± 0.0	0.27 ± 0.0
C20:2n6	0.51 ± 0.0	0.39 ± 0.0	0.77 ± 0.0	0.61 ± 0.1	0.72 ± 0.1	0.55 ± 0.1	0.70 ± 0.2	0.70 ± 0.1	0.58 ± 0.0	0.63 ± 0.0	0.59 ± 0.0
C20:3n3	3.16 ± 0.2	2.27 ± 0.3	2.53 ± 0.1	3.53 ± 0.4	3.09 ± 0.7	3.05 ± 0.2	2.67 ± 0.2	2.49 ± 0.5	1.53 ± 0.1	1.20 ± 0.0	0.82 ± 0.1
C20:4n3	0.60 ± 0.0	0.39 ± 0.1	0.48 ± 0.0	0.67 ± 0.1	0.61 ± 0.1	0.59 ± 0.1	0.53 ± 0.1	0.43 ± 0.1	0.26 ± 0.0	0.25 ± 0.0	0.36 ± 0.0
C20:4n6	0.51 ± 0.0^{a}	0.36 ± 0.1^{b}	0.43 ± 0.0^{b}	0.78 ± 0.1^{ac}	0.95 ± 0.2^{c}	1.47 ± 0.1^{d}	1.63 ± 0.2^{d}	2.30 ± 0.1^{e}	3.03 ± 0.2^{f}	5.00 ± 0.1^{g}	8.76 ± 0.1^{h}
C20:5n3	2.02 ± 0.1^{a}	1.45 ± 0.1^{a}	1.67 ± 0.1^{a}	2.83 ± 0.4^{ab}	3.09 ± 0.8^{ab}	4.08 ± 0.3^{b}	4.26 ± 0.6^{bc}	5.24 ± 0.4^{c}	6.93 ± 0.3^{d}	7.53 ± 0.4^{d}	9.65 ± 0.0^{e}
C22:6n3	0.00 ± 0.0^{a}	0.00 ± 0.0^{a}	0.00 ± 0.0^{a}	0.53 ± 0.1^{b}	1.03 ± 0.2^{bc}	2.01 ± 0.1^{d}	1.81 ± 0.1^{d}	1.70 ± 0.1^{de}	1.24 ± 0.1^{ce}	1.28 ± 0.0^{ce}	3.39 ± 0.1^{f}
Unknown	5.23 ± 0.3	6.75 ± 1.5	4.57 ± 0.4	4.64 ± 0.2	4.19 ± 0.3	5.31 ± 0.3	4.55 ± 1.1	5.88 ± 0.7	5.54 ± 0.5	5.35 ± 0.8	3.96 ± 0.6
SFA	13.1 ± 1.2^{abc}	17.2 ± 0.5^{a}	18.4 ± 0.4^{b}	12.6 ± 1.3^{c}	15.3 ± 3.5^{abc}	13.2 ± 1.0^{c}	15.4 ± 2.1^{abc}	15.7 ± 6.4^{abc}	16.1 ± 0.5^{ac}	15.3 ± 0.4^{c}	14.3 ± 0.2^{c}
MUFA	37.6 ± 0.6^{abc}	36.6 ± 0.8^{bc}	35.9 ± 0.2^{b}	37.0 ± 0.2^{c}	36.0 ± 0.8^{bc}	36.2 ± 0.7^{bc}	37.0 ± 1.0^{abc}	38.5 ± 3.0^{abc}	39.7 ± 0.9^{a}	45.5 ± 1.1^{d}	47.1 ± 0.7^{e}
PUFA	44.1 ± 1.0^{ab}	39.5 ± 1.3^{b}	41.2 ± 0.2^{b}	45.8 ± 1.3^{a}	44.5 ± 2.7^{ab}	45.3 ± 0.9^{a}	43.1 ± 1.4^{ab}	39.9 ± 2.8^{abc}	38.6 ± 1.7^{bc}	33.9 ± 1.0^{c}	34.7 ± 0.3^{c}

Different subscript letters within a row indicate significant difference ($P < 0.05$).

Paper III

Effect of enrichment time and dietary DHA and non-highly unsaturated fatty acid composition on the efficiency of DHA enrichment in phospholipid of rotifer (*Brachionus* Cayman)

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Abstract

An increasing number of studies suggest that DHA associated to dietary phospholipid (PL) are better metabolically available for fish larvae. Knowledge on how to increase DHA levels in the major PL classes of the live feed are needed. *Brachionus* Cayman was cultured in conical fibreglass vessels, enriched with commonly used commercial diet Multigain or DHA Selco (DSelco) in flat bottom vessels at 20 ºC. The changes of DHA content in the PL of the rotifers through enrichment and through a starvation phase post-enrichment were investigated in order to define an appropriate enrichment time and to test the stability of DHA post-enrichment. The different dietary non-HUFA (non-highly unsaturated fatty acid) composition of DSelco (25.1 % SFA, 32.1 % MUFA) and Multigain (40.7 % SFA, 2.11 % MUFA) allowed an evaluation on how SFA and MUFA might affect the efficiency of DHA enrichment of phospholipid in the rotifers. Rotifer samples were collected at 0 h, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h after initiation of enrichment. Total lipid (TL) and the fatty acid composition in TL, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of rotifers and enrichment diets were analysed. The highest DHA levels in PC (23.0%) and PE (16.5%) in Cayman were found after 24 h enrichment with Multigain, and levels were far higher than obtained earlier. The enriched DHA was stable at 10 ºC for at least 24 h post enrichment under starving conditions ($p>0.05$), whereas a significant ($p<0.05$) decrease of DHA was observed during starvation at 20 ºC. In order to obtain high DHA levels in PC and PE in rotifers, the enrichment time is suggested to be at least 24 h to maximise the efficiency of DHA enrichment in PL and the enrichment diet should have a high DHA content. The feeding rations should be low to reduce the total lipid contents of the rotifers. The overall results allowed us to propose a hypothesis that 18:1n9 can inhibit DHA incorporation through competition whereas 16:0 can facilitate DHA incorporation into PL. The hypothesis needs to be further tested.

Key words: Rotifer, Enrichment, DHA, Phosphatidylcholine, Phosphatidylethanolamine

Introduction

The importance of dietary phospholipids (PL) for fish larvae was demonstrated in the early 1980's (Kanazawa *et al.*, 1981; Kanazawa *et al.*, 1983). The beneficial effects found were mainly associated with growth, survival rates, digestive functions, occurrence of deformities, and stress resistance in larval and juvenile stages of various species of fish (Coutteau *et al.*, 1997; Tocher *et al.*, 2008; Cahu *et al.*, 2009). Fish larvae appear to be dependent on a dietary supply of PL between 2-12% of the diet for normal growth and functional development (Tocher *et al.*, 2008).

Besides the quantitative requirement of PL for fish larvae, several studies have suggested that the fatty acid composition of PL, especially its DHA and EPA contents, is also of great importance for marine fish larvae. European sea bass (*Dicentrarchus labrax*) larvae showed better growth, survival and a more normal development when they were fed 2.3% EPA and DHA in the PL fraction of the diet compared to lower contents (Gisbert *et al.*, 2005). Atlantic cod (*Gadus morhua*) larvae obtained better growth, organ development and skeletal development when they were fed diets with n-3 HUFA incorporated in PL rather than in neutral lipids (Kjorsvik *et al.*, 2009; Wold *et al.*, 2009). Moreover, a recent study on cod larvae showed that the percentage of DHA of total fatty acids (DHA%) in larval PL was positively correlated to DHA% in their dietary PL, but not to the DHA% in their dietary total lipids. This further supported that DHA is better available to cod larvae when it is incorporated into dietary PL than in neutral dietary lipids (Olsen *et al.*, 2014). The PL form of DHA and EPA has also showed higher bioavailability for rodents and humans (Cansell *et al.*, 2003; Schuchardt *et al.*, 2011). Studies on mice fed high-fat diet showed that n-3 HUFA in PL were superior to that in TAG (triacylglycerol) with respect to prevention of glucose intolerance and reduction of obesity (Rossmeisl *et al.*, 2012).

The distribution of n-3 HUFA in PL and TAG of nauplii of copepods, important natural food for marine fish larvae in Northern seas, and cultured live feed like *Artemia* and rotifers are also very different. Nauplii of copepods may contain up to 50% DHA of total fatty acids in their PL (Li *et al.*, 2015) whereas DHA levels in PL of *Artemia* and rotifers can be relatively poor even after efficient n-3 HUFA enrichment (Jin *et al.*, 2014; Li *et al.*, 2015). The n-3 HUFA contents of total lipids in enriched rotifers and *Artemia* may be high, but the majority of the DHA and EPA are associated to TAG (Coutteau *et al.*, 1997, Jin *et al.*, 2014, Li *et al.*, 2015).

Among the numerous studies of n-3 HUFA enrichment in the live feed, most have analysed enrichment of total live feed lipids. Knowing now that fish larvae may have specific requirements to their dietary PL composition, there is an emerging need to learn more on how PL of live feed organisms can be efficiently enriched. There are indeed very few data available on the PL composition of live feed. Most relevant studies have focused on fatty acid composition in total PL of *Artemia* (Sargent *et al.*, 1999; Guinot *et al.*, 2013) and rotifers (Frolov *et al.*, 1991; FernandezReiriz and Labarta, 1996; Olsen *et al.*, 2014), whereas even fewer have reported on fatty acid composition in specific PL classes, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Coutteau and Mourente, 1997; Jin *et al.*, 2014; Li *et al.*, 2015). Most studies of DHA enrichment of PL in *Artemia* showed that DHA ended up in TAG and that very little DHA were incorporated into PL (less than 3% of total fatty acid in PL) (Coutteau and Mourente, 1997; Sargent *et al.*, 1999; Guinot *et al.*, 2013; Jin *et al.*, 2014). Our previous study on rotifer enrichment showed that the highest DHA% in PC and PE that could be reached was 12.5% and 5.1%, respectively, which was far lower than the DHA% found in PL of copepods (Li *et al.*, 2015). It is therefore important to continue efforts to establish a better understanding of mechanisms and enrichment strategies to increase the DHA levels in PL of both rotifers and *Artemia*.

Considering the metabolic mechanisms that can be involved, the fatty acid composition in PL could be modified through PL synthesis and modification: *de novo* synthesis pathway and land cycle (PL to lyso-PL to PL) pathway, respectively. Many acylases and transacylases involved in these pathways do not have absolute specificities, which mean that the levels of fatty acids available from the diet might have a significant influence on the fatty acid composition in PL of the enriched rotifers (Sargent *et al.*, 1999). However, certain selectivity on fatty acids or fatty acid associated structures in phospholipid synthesis and modification has been reported, especially for the key enzymes involved in respective pathways: CDP-choline: 1,2-diacyl-*sn*-glycerol cholinephosphotransferase (CPT) and lysophosphatidycholine acyltransferase (LPCAT). Studies using rat liver microsomes have shown that CPT preferred 1-16:0-2-DHA-DAG for synthesis of PC over its *sn*-1-stearoyl counterparts, and 18:1n9 was potentially a strong competitor for the *sn*-2 position in such a DAG structure (Holub, 1978). Human LPCAT3 enzyme showed preference towards 18:2n6-CoA and 20:4n6-CoA as acyl donors and lyso-PC with saturated fatty acid at the *sn*-1 position as substrates (Kazachkov *et al.*, 2008). Unfortunately, the selectivity of these enzymes in rotifers has not been described. If a similar selectivity exists in rotifers, the non-HUFA (non-highly unsaturated fatty acid)

composition of the enrichment diets might affect the efficiency of DHA enrichment in phospholipids.

The overall objective of our study was to investigate how to increase DHA levels in the main phospholipid molecules PC and PE of rotifers. Beside the dietary contents of DHA, the effect enrichment time, dietary non-HUFA composition and the stability of DHA post-enrichment were also focused in this study. In order to define an appropriate enrichment time and test the stability of DHA at different temperatures, the changes of DHA in PC and PE of the most commonly used rotifer strain in Norwegian hatcheries *Brachionus* Cayman (Baer *et al.*, 2008) were investigated through enrichment and through starvation post-enrichment. Moreover, two commonly used enrichment media Easy DHA Selco (DSelco, INVE Aquaculture, Belgium) and LARVIVA Multigain (BioMar, Denmark) were selected due to their different DHA composition (17.1 % *versus* 35.6 %) and non-HUFA compositions (SFA, 25.1 % *versus* 40.7 %; MUFA 32.1 % *versus* 2.11 %) which have appeared to give different enrichment efficiencies of DHA in PC and PE in our pilot studies.

Materials and methods

Rotifer rearing and enrichment

Brachionus Cayman (lorica size [168±10] × [111±9] × [81±8] μm^3) (Baer *et al.*, 2008) was cultivated on yeast (0.6 g l^{-1}) and an algae paste Rotifer Diet (RD, Reed Mariculture, 0.18 g l^{-1}) in semi-continuous cultures (35 g l^{-1} salinity, 20 ºC, dilution rate of 20 % day^{-1}) in 250-l conical fibreglass vessels. The enrichment was carried out in 100-l flat bottom vessels at 20 ºC. The rotifers were enriched with Easy DHA Selco (DSelco, INVE Aquaculture, Belgium) or LARVIVA Multigain (BioMar, Denmark) (Table 1). At 0 h, similar DHA ration from DSelco or Multigain was provided for the Cayman enrichment (Table 2), and for the Multigain treatment, another portion was added at 12 h to achieve similar total lipid ration. Three replicate cultures were run for each treatment.

Rotifer samples were collected at 0 h, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h (22 h and 28 h instead of 24 h for Cayman enriched with DSelco). Water samples were also collected at similar time intervals for particle counting (Beckman Multisizer™ 3 Coulter Counter®, Beckman Coulter Inc., Miami, FL, USA). For each sample, 10 litres of rotifer culture were pumped gently through two sieves with mesh sizes of 200 µm and 64 µm, and thereafter rinsed in filtered seawater and finally in cold tap water for 10 seconds. The sieve (64 µm) was dried from

underneath by paper towels, and the rotifers were packed into sample vials and immediately frozen in liquid nitrogen and finally freeze-dried and stored under N_2 at -80 ºC before analysis.

In normal cod larval rearing, the temperature is usually increased gradually from 6 to 12 ºC, which may not be suitable for rotifers raised at 20 ºC. In order to test the temperature effect on the DHA stability in PL post-enrichment, 10 l of rotifer culture from the Multigain treatment were collected through the sieves as described above, rinsed in and transferred back to filtered seawater, and transferred to a 10 ºC room for 24 hours' starvation post-enrichment. Another 10 l of rotifers were collected, rinsed, re-suspended in seawater and kept in 20 ºC for 24 hours' starvation post-enrichment. Three replicates were run for each treatment.

Lipid analyses

The total lipids were extracted by a modified Bligh and Dyer (1959) method, and lipid content was determined gravimetrically. Freeze-dried samples were homogenized in ice cold 0.8 ml water, 2 ml methanol and 1 ml chloroform for 1 min. 1 ml chloroform was then added to the mixture and blended for 20 sec. And finally, 1 ml of water was added and blended for 20 seconds. The homogenate was then centrifuged at 1,640 g for 10 min at 5 ºC. The lower phase was carefully transferred to a clean glass vial, and 1 ml of the lower phase was evaporated under N_2, and finally re-suspended in 100 µl chloroform:methanol (2:1, by volume).

30 µl of the lipid extracts was applied as 1 cm bands by LINOMAT IV (CAMAG, Mutlenz, Switzerland) on PLC silica plates (PLC silica gel 60 F254, 0.5 mm. 20 × 20 cm. Merck KGaA, Darmstadt, Germany). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were separated with the following solvent system, chloroform: methanol: water (67:30:2.5, by volume). Commercial PC and PE standards (Soy PC and PE, Avanti Polar Lipids Inc. Alabaster, AL, USA) were also applied on the plates, and they were then visualized under UV. The corresponding PC and PE bands were scraped off, and directly methylated by a modified method described by Abdulkadir and Tsuchiya (2008), with 0.2 ml BF3-methanol and 0.5 ml isooctane in N_2 atmosphere at 50 ºC overnight. 0.1 ml isooctane and 0.2 ml water were added to the mixture and then centrifuged at 1,640 g for 3 min at 5 ºC. The fatty acid methyl esters in the upper phase were analysed with a gas chromatograph (AutoSystem XL, Perkin Elmer, Waltham, MA) with Total Chrom Version 6.3.1 software. The system was equipped with an auto-injector (1 µl, inlet temperature 250 ºC) and a flame ionisation detector (FID, 280 ºC). The temperature program for the oven was 90 ºC for 1 min, then raised to 150 ºC at 30 min^{-1}

and finally raised to 225 ºC at 3 ºC min^{-1} and held for 7 min. Helium was used as the carrier gas and a fused silica capillary column coated with a chemically bonded polyethylene glycol (CP-Wax 52CB, 25 m × 0.25 mm i.d; Varian, Palo Alto, CA) was used.

Statistics

The experimental data were tested for statistical significance by using Independent- Samples T-test or one-way analysis of variance (ANOVA) with Tukey's multiple comparison test or Dunnet's T3 multiple comparison test if the *P* value of homogeneity of the variance test was less than 0.05. Differences were considered significant at the $P<0.05$ level. All of the statistical tests were performed using SPSS 20.0 for windows. All tables were made in Excel 2010 and figures by Sigma plot 12.5.

Results

The fatty acid composition of cultivation diets and enrichment medium are shown in Table 3. The percentage content of DHA (DHA%) of total fatty acids in the total lipid of DSelco and Multigain was 17.1 % and 35.6 %, respectively, and, the respective quantitative values were 117 *versus* 141 mg DHA g^{-1} DW. High levels of 16:0, 18:2n-6 and DHA were found in the PC fraction of Multigain, and the PE fraction was dominated by 16:0. 18:2n6 was the dominating fatty acid in PC and PE of DSelco. The algae paste RD fed to the rotifers contained high levels of EPA, especially in PE, but no DHA.

The enrichment of total lipid for both diets was most efficient during the first hour (Fig. 1). The total lipid content of rotifers enriched with DSelco was throughout significantly higher ($p<0.05$) than that of the Multigain treatment during the enrichment period. On the contrary, the DHA% of the Multigain treatment were significantly higher ($p<0.05$) than that of the DSelco treatment throughout the enrichment time (Fig. 2).

All values of DHA% were significantly higher in total lipid than in PC and PE throughout the enrichment period ($p<0.05$), except for DSelco treatment after 28 h when DHA% in TL and PC was not found to be significantly different ($p=0.06$). DHA% in both PC and PE started to increase from the beginning of the enrichment period in both treatments, but at a much slower rate compared to the rate of increases of DHA% in total lipid during the first hour (Fig.2.). Similar dietary DHA ration (19.5 *versus* 19.7 ng ind^{-1}) was provided for Cayman enrichment during the first 12 h, however, the Multigain treatment showed a significantly higher ($p<0.05$) DHA% in TL, PC and PE compared to that obtained in the DSelco treatment for Cayman up

214 to 12 h. In both treatments, DHA% in PC and PE showed similar levels until 8 h of
215 enrichment, but DHA% in PC was significantly higher (p<0.05) than that of PE in the DSelco
216 treatment at 12 h (Fig. 2B), whereas the difference at 12 h in the Multigain treatment was
217 close to significant (p=0.06) (Fig. 2A).

218 The contents of saturated fatty acids (SFA) remained relatively stable over time in PC and PE
219 for all the treatments (Fig. 3). A general increasing trend of polyunsaturated fatty acids
220 (PUFA) and decreasing trend of mono-unsaturated fatty acids (MUFA) was observed in both
221 PL fractions for all the enrichments. After 12 h, the same trend continued for the Multigain
222 treatment, whereas, it levelled off for the DSelco treatment. All the changes were significant
223 (p<0.05) at the end of enrichment period compared to their initial values (See Appendix).

224 Comparison of fatty acid composition in Cayman fed Multigain or DSelco at the end of
225 enrichment is shown in Table 4. There were no significant differences of total lipid and total
226 fatty acid contents. The contents of ARA, DPA-n6, DHA and PUFA in total lipid, PC and PE
227 were significantly higher (p<0.05) in Multigain treatment, whereas, MUFA levels were
228 significantly higher in DSelco treatment.

229 The stability of DHA in total lipids and PL was tested for the cultures of Cayman that had
230 been enriched by Multigain for 24h and thereafter starved at 10 ºC and 20 ºC for 24h (Fig. 4).
231 The total lipid levels remained constant during starvation at both temperatures (p>0.05), but
232 the total fatty acid content decreased significantly (p<0.05), by 16.7% and 25.3% for 10 ºC
233 and 20 ºC, respectively, for both temperatures (Appendix Table A.1). The PUFA levels in TL
234 and PC decreased significantly (p<0.05) after 24h of starvation at 20 ºC, whereas, the SFA
235 levels increased significantly (p<0.05). At 10 ºC, the percentage PUFA and SFA remained
236 relatively stable in TL, PC and PE (p>0.05). The MUFA content in TL, PC and PE remained
237 stable for 24 h at both 10 ºC and 20 ºC. The changes in DHA% were similar as that of PUFA,
238 and it decreased significantly (p<0.05) in TL, PC and PE at 20 ºC, whereas it remained
239 constant at 10 ºC (p>0.05). The percentage ARA and EPA levels in PC and PE remained
240 stable (p>0.05) in both starvation tests.

241

242 **Discussion**

243 Lipid enrichment studies of live feeds have mainly focused on n-3 HUFA levels in their total
244 lipids. However, an increasing number of studies now suggest that DHA associated to dietary
245 PL are better metabolically available for fish larvae (Gisbert *et al.*, 2005; Wold *et al.*, 2009;

246 Olsen *et al.*, 2014), which is suggested to base their PL synthesis on their dietary PL and not
247 on TAG. This hypothesis implies that the DHA or HUFA contents of the dietary PL of the fish
248 larvae then becomes important for larval growth and development, not necessarily the HUFA
249 contents of total lipids and TAG. These findings needs to be further verified, and this requires,
250 among others, further knowledge on fatty acid composition and enrichment efficiency of the
251 major phospholipid classes of the live feed during HUFA enrichment, for which data are
252 scarce.

253 The present study showed the highest DHA levels in PC (23.0%) and PE (16.5%) in Cayman
254 upon 24 h enrichment with Multigain. The enriched DHA was rather stable in 10 ºC for at
255 least 24 h post enrichment under starving conditions, suggesting that it was incorporated into
256 rotifer tissues. Theoretically, 23.0% and 16.5% of DHA in PC and PE, respectively, could
257 mean that 46% and 33% of the PC and PE molecules contain a DHA moiety, assuming DHA
258 were esterified in *sn*-2 position of phospholipids (Sargent *et al.*, 1999). These contents are
259 slightly lower than that found in phospholipids of copepods (~30-50%) (Li *et al.*, 2015), but
260 they nevertheless reveal a potential to enrich rotifers to high DHA levels in phospholipids.

261 *Enrichment diet*

262 It could be suggested that the higher percentage fraction of DHA provided using Multigain
263 (Table 3) explained why PC and PE enrichments were most efficient for incorporating DHA
264 in PC and PE of the rotifer. The conclusion might then be that the percentage fraction of DHA
265 in the diet determined the enrichment levels of DHA in PL of the rotifers. An alternative
266 explanation might be that a lipid emulsion, which constitutes mainly lipids, is more poorly
267 assimilated and therefore metabolically less efficient for transferring DHA to rotifer PL than a
268 slightly more complete enrichment feed, with some proteins added (13%, Table 1) and with
269 high DHA level (Cutts *et al.*, 2006). The enhanced efficiency might be a combination of these
270 effects, and a Multigain-type diet would then in all events be most efficient for PL enrichment
271 of DHA and HUFA.

272 The results of earlier experiments put, however, some doubts about this simple explanation
273 (Olsen *et al.*, 2014; Li *et al.*, 2015). In the efforts to enrich DHA in phospholipids of rotifers,
274 our previous studies using high DHA level emulsions (30-50% DHA of total fatty acid), with
275 lipids either in forms of TAG or PL, revealed a maximum DHA% in PL of enriched rotifers of
276 about 12%, whereas DHA% in total lipid could reach 22.3-26.2% (Olsen *et al.*, 2014; Li *et*
277 *al.*, 2015). This suggested that provision of diets with high DHA content was not enough to

get high DHA levels in PL of rotifers.

Multigain showed higher levels of SFA and lower levels of MUFA compared to DSelco, and our results gave some indications that the dietary non-HUFA composition may affect the DHA enrichment efficiency in PL. The results suggested an interesting mirror-image relationship between MUFA and PUFA during the period of enrichment in both PC and PE for all treatments (Fig. 3), which indicated that the MUFA was replaced by PUFA in PL during the enrichment process. It has been shown that ^{14}C-labeled 18:1n9 were very efficiently incorporated into PL in both digestive tract and body in 28-day-old gilthead sea bream larvae (Hadas *et al.*, 2003). Atlantic cod larvae fed with enriched rotifers showed two times higher MUFA levels in both total lipid and PL fractions compared to that in larvae fed *Arcartia tonsa* (Olsen *et al.*, 2014). Therefore, the high levels of 18:1n9 in the enrichment diet might have been a strong competitor which inhibited DHA or HUFA incorporation into phospholipids. The competition from high contents of 18:1n9 in the emulsion and short chain n-3 PUFAs like 18:3n3 and 18:4n3 from R. *baltica* might be the reason for the relatively lower DHA levels incorporated into phospholipids in our earlier study (Li *et al.*, 2015).

Beside this, we suggest that the high 16:0 levels in Multigain may stimulate the biosynthesis of PL of growing and reproducing rotifers. It has been reported that 16:0 may increase phospholipid synthesis in cultured Caco-2 cells (Van Greevenbroek *et al.*, 1995) and reduce the intracellular lipid droplet formation in Arctic char (Olsen *et al.*, 2000). In addition, there are indications that 16:0 may facilitate the incorporation of dietary DHA into PC of human plasma. After feeding marine lipid to normolipidemic males for 28 days, both DHA levels in PC and the PC species 16:0-DHA-PC in the plasma increased significantly. However, the 16:0 levels remained stable, which indicated that the increase of such PC species was mainly because another fatty acid in the *sn*-2 position was replaced by DHA, and 16:0 in the *sn*-1 position facilitated this process (Subbaiah *et al.*, 1993). Moreover, Williams (1999) suggested that 16:0-DHA-PC was preferably retained in the plasma membrane.

Although the commercial enrichment diets in the present study contained significantly different levels of SFA and MUFA, these diets were not specifically manufactured for testing "non-HUFA" hypothesis and we cannot exclude the possible effects of other ingredients in the diets. Specific tailor-made emulsions with same characteristics and DHA level, but different 16:0 and 18:1n9 levels should be used to further confirm the hypothesis.

Enrichment time

The enrichment time was another decisive factor for the rate of fatty acid replacement in phospholipids of the rotifers. Previous studies have shown that prolonged enrichment time was more effective than increased oil rations in boosting n-3 HUFA content in TL of rotifers (Rodriguez *et al.*, 1996). Rotifers are commonly enriched only for a short period (for example, 2 h) in some hatcheries and laboratory studies (Maehre *et al.*, 2013; Rehberg-Haas *et al.*, 2015). Our results revealed that total lipid content and DHA% in total fatty acids increased most efficiently during the first hour of enrichment, whereas the DHA in PC and PE showed a much slower increasing trend during the same time period (Fig. 1 and 2). Little DHA will then be present in the PL of rotifers after 2 h of enrichment.

A "lag phase" in the DHA accumulation of PL was observed in our recent study of *Artemia* enrichment (Jin *et al.*, 2014). No DHA was incorporated in PC or PE during the first hour of enrichment, whereas DHA in total lipid increased rapidly to high values. In the present study, the same clear "lag phase" was not apparent and the DHA levels in PC and PE of rotifers started to increase slowly already before 1 h of enrichment. There will likely be an influence from undigested diet in the guts of the rotifers in the early stages of the enrichment. The DSelco contained no DHA in PC or PE while Multigain contained no DHA in PE, only in PC. DHA was nevertheless found in PC and PE following enrichment by DSelco and in PE of the Multigain enrichment already after 1 h, which suggested that the incorporation of DHA into PC and PE of rotifers became apparent earlier than that in *Artemia* (Jin *et al.*, 2014).

The DHA% in PC and PE showed a linear pattern of increase during the first 8 h in all treatments, but the efficiency of DHA incorporation into PL was higher for Multigain, noting that similar dietary DHA ration was provided for the DSelco treatment (19.5 *versus* 19.7 ng ind^{-1}) during the first 12 h (Table 1). Due to the different DHA% of the two enrichment diet, a trade-off was made by adding another portion of diet in the Multigain treatment at 12 h to achieve similar total lipid rations for later evaluation. Therefore, from 0-12 h, the DHA ration was similar and from 12 h onwards, the total lipid ration was comparable between the two treatments. The number of particles in the enrichment tanks suggested that there were excess feed in all treatments at the end of enrichment (data not shown).

The increase of DHA% during the first 12 h was much more efficient compared to the increase between 12 h and 24 h, even with supplementation of extra Multigain at 12 h (Fig. 2A). At the end of enrichment, the rank order for DHA% appeared to be TL > PC >PE for all treatments (Table 4), which was in agreement with our previous study that the DHA% in PE was more difficult to manipulate compared to that in PC (Li *et al.*, 2015).

In conclusion, because of a relatively slow increase of DHA in PC and PE compared to that in TL, a prolonged enrichment time (24 h) is suggested to obtain high DHA or HUFA content of rotifer PL. Contrary, very short enrichment time, for example 2 h, will not result in very much DHA or HUFA enrichment in the PL of rotifers.

Stability of PL composition post-enrichment

We found a significant (p<0.05) decreases of PUFA and increases of SFA in PC of rotifers after starvation in 20 ºC (Fig. 4). These changes originated mainly in a decrease of DHA and an increase of 16:0. However, even after starvation for 24 h at 20 ºC, the DHA levels in PL, PC and PE were 21.4 %, 14.3 % and 10.5 % (Appendix), respectively, which can still be suitable for many warm water species cultured above 20 ºC. At 10 ºC only a minor decrease of DHA was observed and other studies have also shown a relatively stable fatty acid profile after starvation at low temperatures (Lubzens *et al.*, 1985; Rainuzzo *et al.*, 1989; Olsen *et al.*, 1993). Interestingly, the DHA levels in PE increased slightly during starvation, suggesting that the phospholipid head-group might have been restructured, replacing PC by PE when rotifers were acclimated to 10 ºC, which have been reported as a rapid process in rainbow trout (Hazel and Williams, 1990).

Concluding remarks

Short-term enrichment strategies tend to produce rotifers with high lipid content (Rainuzzo *et al.*, 1994), which has not been recommended for cold water fish larvae, because high rotifer mortality may occur when fat rotifers are transferred to cold water (Olsen *et al.*, 1993). Moreover, fat rotifers contain higher TAG fractions than more lean rotifers, because the PL content per dry matter remains fairly constant independent of the total lipid contents. A high TAG content of the rotifers may not be optimal for early fish larvae (Olsen *et al.*, 2014). DHA is normally esterified in the *sn*-2 position of TAG. During digestion processes in fish larvae, the hydrolysed fatty acids from *sn*-1 and *sn*-3 positions of dietary TAG could dilute the DHA% in the enterocyte, resulting in lower DHA levels in the resynthesized PL through lyso-PL (Lands cycle) pathway (Olsen *et al.*, 2014). To produce copepod-like live feed, it is therefore an issue to both minimise dietary lipid rations and rotifer lipid level besides having high DHA enrichment of PL, which may seem to be a contradiction, or at least a challenge.

Our study has shown that it is, nevertheless, possible to enrich the DHA levels in both PC and PE of rotifers to relatively high levels, and that these levels can be kept stable for at least 24 h at 10 ºC. Based on our overall results, we suggest that the enrichment time should be at least

24 h, in order to maximise the efficiency of the PL enrichment. Beside this, because of the effect of high TAG mentioned above, the feeding rations of lipids should be as low as possible to reduce the total lipid contents of the rotifers. The enrichment diet should have a high content of DHA. The suggested stimulating effect of 16:0 and the inhibiting effect of MUFA should be considered, but this needs further investigation.

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Figure 1

Time course of total lipid in rotifers enriched with DHA Selco (DSelco circles) and Multigain (triangles), respectively. Error bars express standard errors of the mean of replicates. Numbers of replicates are 2-4, see Appendix.

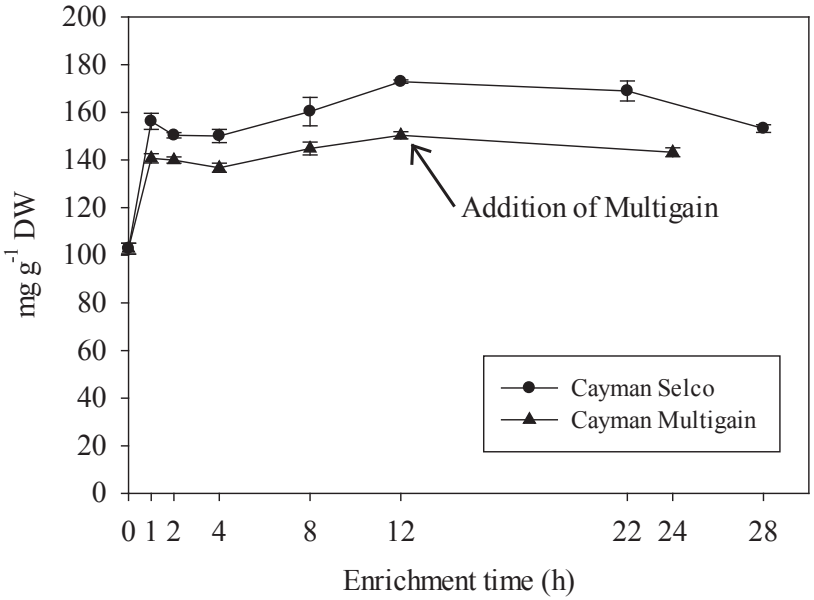
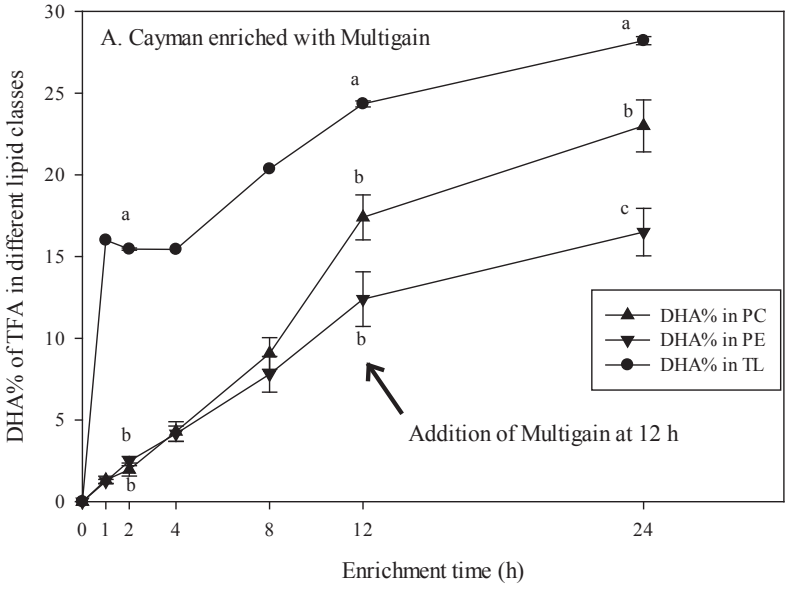


Figure 2

Changes in DHA% in total lipid (circles), PC (triangles) and PE (inverted triangles) as a function of time in *Brachionus* Cayman enriched with Multigain (A) and DSelco (B). Error bars express standard errors of the mean of replicates. Numbers of replicates were 1-6, see Appendix. Different letter inserted for data points for specific time indicate significantly different values ($p<0.05$).



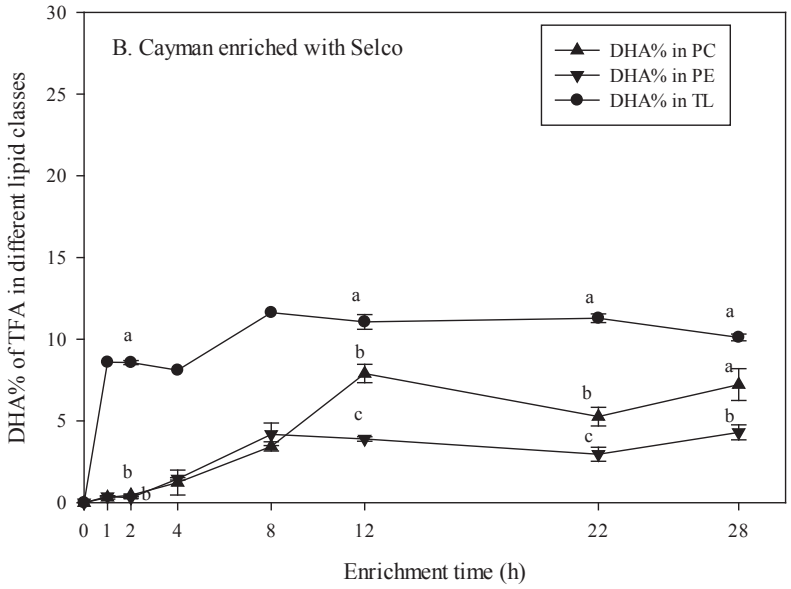


Figure 3

Fatty acid composition of PC and PE as a function of time in *Brachionus* Cayman enriched with Multigain (A, B) or DSelco (C, D) following start of enrichment (solid circles: SFA; open circles: MUFA; triangles: PUFA). Error bars express standard error of the mean of replicates (n=3-6, see Appendix).

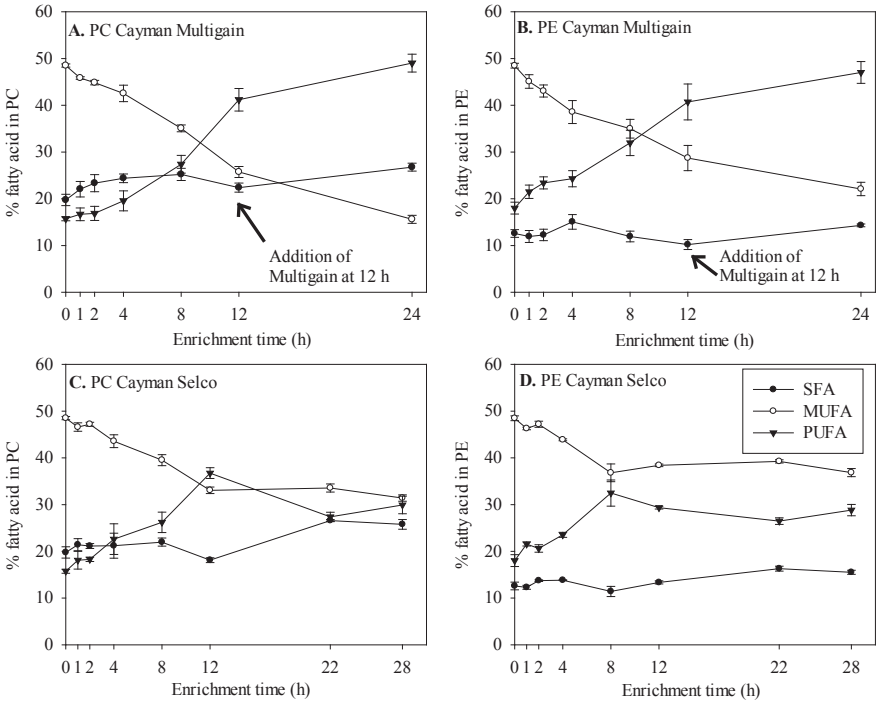


Figure 4

Fatty acid composition in PC (A), PE (B) and in TL (total lipids) (C) of *Brachionus* Cayman after enrichment with Multigain (left bar) and after 24 h starvation at 10 (middle bar) or 20 ^{o}C (right bar). Values assigned to different letter in same fatty acid group are significantly different at $p<0.05$. Numbers of replicates are 3-6, see Appendix.

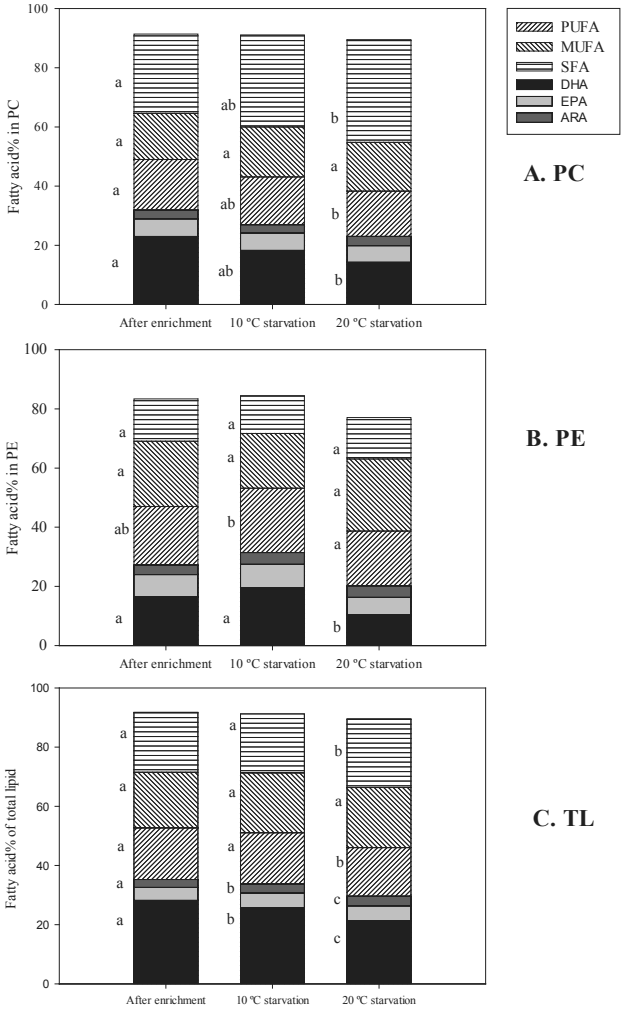


Table 1. Dietary composition as provided by the manufacture.

	Easy DHA Selco (DSelco)	LARVIVA Multigain
Ingreidents:	Water, fish oil, dried algae	Algae, yeast, maltodextrin, lecithin, yeast extract
Crude lipids	66%	44%
Crude proteins	0.50%	13%
Crude ash	0.50%	7.80%
Crude fibre	0.50%	2.60%
Vit. A	1,500,000 IU/kg	150,000 IU/kg
Vit. D3	150,000 IU/kg	50,000 IU/kg
Vit. E	3,600 mg/kg	6,600 mg/kg
Vit. C	800 mg/kg	33,300 mg/kg

Table 2. Experiment set up and feeding rations per individual rotifers.

	Cayman enriched with DSelco	Cayman enriched with Multigain
Cultivation		
Baker's yeast *Saccaromyces cerevisae*	0.6g $l^{-1}d^{-1}$	0.6g $l^{-1}d^{-1}$
Algae paste Rotifer diet (Reed Mariculture)	0.18 ml $l^{-1}d^{-1}$	0.18 ml $l^{-1}d^{-1}$
Cultivation density	600-800 ind ml^{-1}	600-800 ind ml^{-1}
Enrichment		
Enrichment diet (mg l^{-1})	115	75 (0h) + 75 (12 h)
Lipid ration (ng ind^{-1})	150	66 (0h) + 66 (12h)
DHA ration (ng ind^{-1})	19.7	19.5 (0h) + 19.5 (12h)
Enrichment density	~500 ind ml^{-1}	~500 ind ml^{-1}

Table 3. Lipid and fatty acid profile of cultivation and enrichment diets for rotifers. Fatty acid composition is expressed as percentage values of total fatty acids. Values represent Mean ± SE (standard error, n=3).

	DSelco TFA	DSelco PC	DSelco PE	Multigain TFA	Multigain PC	Multigain PE	RD TFA	RD PC	RD PE
Total lipid (mg g^{-1} DW)	893 ± 12.7			477 ± 17.4			127 ± 0.01		
Total fatty acid (mg g^{-1} DW)	687 ± 8.19			397 ± 12.1			64.1 ±0.14		
% of total fatty acids									
14:0	3.83 ± 0.01			8.11 ± 0.01	1.30 ± 0.00	0.30 ± 0.00	3.90 ± 0.22	3.54 ± 0.34	2.94 ± 0.96
16:0	16.8 ± 0.03	17.3 ± 1.08	18.2 ± 0.84	31.2 ± 0.20	34.1 ± 1.54	87.5 ± 8.69	16.2 ± 0.12	26.5 ± 1.37	18.2 ± 2.17
18:0	4.04 ± 0.00	20.1 ± 0.75	7.07 ± 0.03	0.65 ± 0.00	2.72 ± 0.03	1.56 ± 1.17	0.31 ± 0.03	0.73 ± 0.28	2.24 ± 0.00
SFA	25.1 ± 0.03	37.4 ± 1.83	25.3 ± 0.87	40.7 ± 0.21	38.2 ± 1.57	89.4 ± 9.87	20.4 ± 0.37	30.8 ± 1.99	23.4 ± 3.13
16:1n7	4.82 ± 0.00	13.0 ± 0.03	5.42 ± 0.93	0.16 ± 0.00	1.82 ± 0.00		15.0 ± 0.07	28.5 ± 0.53	23.8 ± 0.15
18:1n7	2.38 ± 0.02			0.15 ± 0.00	1.33 ± 0.00		0.91 ± 0.03	1.08 ± 0.07	0.76 ± 0.00
18:1n9	16.2 ± 0.10		15.8 ± 1.05	1.66 ± 0.02	9.93 ± 0.60	3.23 ± 2.34	3.56 ± 0.13	13.6 ± 0.20	2.39 ± 0.31
MUFA	32.1 ± 0.10		21.2 ± 1.99	2.11 ± 0.05	13.1 ± 0.60	3.23 ± 2.34	19.4 ± 0.22	43.2 ± 0.81	26.9 ± 0.45
18:2n6	5.74 ± 0.05	49.6 ± 1.85	38.3 ± 0.39	1.98 ± 0.01	27.3 ± 2.32	6.58 ± 4.36	4.70 ± 0.20	13.0 ± 1.26	5.68 ± 0.08
20:4n6	1.12 ± 0.01			1.60 ± 0.01	0.56 ± 0.00		2.88 ± 0.05	3.10 ± 0.82	2.06 ± 0.00
22:5n6	0.85 ± 0.01			13.2 ± 0.02	5.19 ± 0.61				
n-6	7.98 ± 0.03	49.6 ± 1.85	38.3 ± 0.39	17.3 ± 0.03	33.0 ± 2.93	6.58 ± 4.36	7.58 ± 0.25	16.1 ± 2.08	7.74 ± 0.08
18:3n3	1.49 ± 0.01		15.2 ± 1.50	0.24 ± 0.00	2.14 ± 0.16	1.87 ± 0.00	3.51 ± 0.13	1.34 ± 0.32	3.07 ± 0.55
18:4n3	2.00 ± 0.00			0.24 ± 0.01			1.17 ± 0.01		
20:5n3	6.16 ± 0.04			1.12 ± 0.02	1.49 ± 0.00		29.9 ± 0.53	4.53 ± 0.79	37.3 ± 5.42
22:6n3	17.1 ± 0.13			35.6 ± 0.08	15.5 ± 1.70				
n-3	26.7 ± 0.18		15.2 ± 1.50	38.4 ± 0.10	19.1 ± 1.86	1.87 ± 0.00	34.6 ± 0.66	5.87 ± 1.11	40.4 ± 5.97
PUFA	36.6 ± 0.14	49.6 ± 1.85	53.5 ± 1.12	55.7 ± 0.14	52.2 ± 4.79	8.45 ± 4.36	42.2 ± 0.91	22.0 ± 3.19	48.1 ± 6.05
DHA/EPA	2.78 ± 0.00			31.8 ± 0.48	10.4 ± 0.00				
n-3/n-6	3.34 ± 0.04			2.23 ± 0.00	0.56 ± 0.04		4.56 ± 0.06	0.36 ± 0.06	6.04 ± 0.13

DSelco, DHA Selco; *RD*, algae paste Rotifer Diet; *TFA*, total fatty acids; *SFA*, saturated fatty acid, including 14:0, 16:0, 18:0, 20:0; *MUFA*, monounsaturated fatty acid, including 16:1n7, 18:1n7, 18:1n9, 20:1n9, 22:1n9. *PUFA*, polyunsaturated fatty acid, including 18:2n6, 18:3n3, 18:4n3, 20:3n6, 20:4n3, 20:4n6, 20:5n3, 22:5n3, 22:5n6, 22:6n3.

Table 4. Lipid and fatty acid profile of rotifers after enrichment. Fatty acid composition is expressed as percentage values of total fatty acids. Values represent Mean ± SE (n=3-6, see Appendix).

	Cayman enriched with Multigain (24h)	Cayman enriched with DSelco (28h)
Total lipid (mg g^{-1} DW)	143 ± 1.85[a]	153 ± 1.62[a]
TFA (mg g^{-1} DW)	107 ± 4.28[a]	105 ± 2.19[a]
% of total fatty acids in total lipid		
SFA	20.3 ± 0.21[a]	17.7 ± 0.49[b]
MUFA	18.8 ± 0.39[a]	40.7 ± 0.12[b]
PUFA	52.7 ± 0.31[a]	30.8 ± 0.51[b]
ARA	2.65 ± 0.02[a]	1.28 ± 0.01[b]
EPA	4.41 ± 0.05[a]	6.55 ± 0.03[b]
DPA-n6	10.1 ± 0.09[a]	0.48 ± 0.01[b]
DHA	28.2 ± 0.25[a]	10.1 ± 0.20[b]
Fatty acid% in PC		
PC-SFA	26.8 ± 0.84[a]	25.8 ± 1.05[a]
PC-MUFA	15.6 ± 0.85[a]	31.4 ± 0.67[b]
PC-PUFA	49.0 ± 1.90[a]	29.9 ± 1.88[b]
PC-ARA	2.97 ± 0.14[a]	1.05 ± 0.11[b]
PC-EPA	5.96 ± 0.16[a]	6.68 ± 0.33[a]
PC-DPA-n6	7.29 ± 0.30	
PC-DHA	23.0 ± 1.59[a]	7.22 ± 0.97[b]
Fatty acid% in PE		
PE-SFA	14.3 ± 0.29[a]	15.5 ± 0.43[a]
PE-MUFA	22.1 ± 1.43[a]	36.9 ± 0.87[b]
PE-PUFA	47.0 ± 2.33[a]	28.8 ± 1.21[b]
PE-ARA	3.24 ± 0.14[a]	1.04 ± 0.06[b]
PE-EPA	7.49 ± 0.39[a]	6.53 ± 0.50[a]
PE-DPA-n6	7.70 ± 0.58	0
PE-DHA	16.5 ± 1.45[a]	4.30 ± 0.46[b]

Values in the same row not sharing a superscript are significantly different ($P < 0.05$).

Appendix

Table A.1. Lipid and fatty acid profile of Cayman enriched with Multigain, expressed as mean ± SE.

Total lipid	0h n=4	1h n=4	2h n=4	4h n=4	8h n=4	12h n=4	24h n=3	48c n=4	48r n=4
mg g^{-1} DW	103 ± 2.27^{a}	141 ± 1.97^{bcd}	140 ± 1.29^{bd}	137 ± 1.93^{b}	145 ± 2.68^{bcd}	150 ± 1.51^{c}	143 ± 1.85^{bcd}	136 ± 2.23^{b}	148 ± 2.80^{cd}
TFA Multigain	0h n=2	1h n=1	2h n=3	4h n=1	8h n=1	12h n=3	24h n=3	48c n=3	48r n=3
14:0	1.71 ± 0.03	4.62	4.42 ± 0.06	4.15	3.74	3.32 ± 0.06	3.56 ± 0.04	3.29 ± 0.01	3.25 ± 0.10
16:0	8.24 ± 0.48^{a}	17.0	16.7 ± 0.41^{b}	15.2	12.4	11.1 ± 0.21^{c}	13.6 ± 0.19^{d}	13.3 ± 0.13^{d}	16.2 ± 0.85^{b}
18:0	4.25 ± 0.32^{a}	2.74	2.76 ± 0.03^{b}	2.89	2.74	2.53 ± 0.01^{bc}	2.28 ± 0.02^{c}	2.48 ± 0.02^{bc}	2.71 ± 0.05^{b}
SFA	15.9 ± 0.75^{a}	25.7	25.1 ± 0.46^{b}	23.5	20.0	17.9 ± 0.27^{ac}	20.3 ± 0.21^{d}	19.9 ± 0.18^{cd}	23.2 ± 0.89^{b}
16:1n7	24.5 ± 1.60^{a}	13.4	13.5 ± 0.04^{b}	13.8	12.0	10.5 ± 0.16^{c}	7.00 ± 0.15^{d}	7.11 ± 0.11^{d}	6.85 ± 0.08^{d}
18:1n7	5.52 ± 0.22^{a}	3.15	3.13 ± 0.02^{b}	3.24	2.92	2.63 ± 0.04^{c}	2.16 ± 0.04^{d}	2.29 ± 0.03^{de}	2.57 ± 0.05^{ce}
18:1n9	18.6 ± 0.39^{a}	11.7	11.8 ± 0.04^{b}	12.3	11.1	9.96 ± 0.13^{c}	7.16 ± 0.13^{d}	7.61 ± 0.12^{d}	7.12 ± 0.20^{d}
20:1n9	3.35 ± 0.03	2.05	2.03 ± 0.02	2.21	2.10	1.97 ± 0.02	1.62 ± 0.02	1.83 ± 0.02	1.97 ± 0.03
MUFA	54.7 ± 1.47^{a}	32.0	31.8 ± 0.26^{b}	33.3	29.8	26.5 ± 0.32^{c}	18.8 ± 0.39^{d}	20.2 ± 0.29^{d}	20.3 ± 0.38^{d}
18:2n6	2.34 ± 0.06	1.88	2.11 ± 0.01	2.39	2.56	2.52 ± 0.01	2.53 ± 0.01	2.66 ± 0.01	2.74 ± 0.02
18:3n3	1.16 ± 0.26	0.60	0.63 ± 0.00	0.66	0.64	0.59 ± 0.00	0.46 ± 0.01	0.44 ± 0.00	0.39 ± 0.00
20:3n6	0.58 ± 0.02	0.50	0.51 ± 0.00	0.54	0.56	0.56 ± 0.00	0.54 ± 0.00	0.60 ± 0.00	0.65 ± 0.00
20:4n3	0.88 ± 0.01	0.89	0.91 ± 0.01	1.01	1.11	1.13 ± 0.00	1.12 ± 0.01	1.23 ± 0.01	1.29 ± 0.01
20:4n6	0.91 ± 0.01^{a}	1.37	1.39 ± 0.01^{b}	1.52	1.96	2.25 ± 0.01^{c}	2.65 ± 0.02^{d}	3.05 ± 0.03^{e}	3.30 ± 0.08^{f}
20:5n3	6.35 ± 0.11^{a}	4.35	4.43 ± 0.03^{b}	4.72	5.01	5.01 ± 0.08^{c}	4.41 ± 0.05^{b}	5.08 ± 0.06^{c}	4.98 ± 0.16^{c}
22:5n3	1.87 ± 0.21	1.47	1.54 ± 0.11	1.66	1.75	1.83 ± 0.03	2.12 ± 0.01	2.27 ± 0.01	2.53 ± 0.03
22:5n6	0^{a}	5.93	5.62 ± 0.07^{b}	5.36	6.59	7.85 ± 0.06^{c}	10.1 ± 0.09^{d}	9.59 ± 0.22^{d}	8.42 ± 0.11^{e}
22:6n3	0^{a}	16.0	15.5 ± 0.07^{b}	15.4	20.4	24.3 ± 0.18^{c}	28.2 ± 0.25^{d}	25.7 ± 0.63^{e}	21.4 ± 0.22^{e}
PUFA	14.4 ± 0.00^{a}	33.4	33.0 ± 0.10^{b}	33.8	41.1	46.7 ± 0.17^{c}	52.7 ± 0.31^{d}	51.1 ± 0.79^{d}	46.1 ± 0.42^{c}
n-3	10.5 ± 0.03^{a}	23.6	23.3 ± 0.11^{b}	23.9	29.4	33.4 ± 0.13^{c}	36.8 ± 0.22^{d}	35.1 ± 0.55^{e}	30.9 ± 0.24^{f}
n-6	3.97 ± 0.03^{a}	9.72	9.72 ± 0.06^{b}	9.87	11.7	13.3 ± 0.07^{c}	16.0 ± 0.09^{d}	16.0 ± 0.24^{d}	15.2 ± 0.19^{e}
n-3/n-6	2.63 ± 0.03	2.43	2.39 ± 0.02	2.42	2.51	2.52 ± 0.01	2.30 ± 0.00	2.20 ± 0.01	2.04 ± 0.01
DHA/EPA	0^{a}	3.68	3.49 ± 0.03^{b}	3.27	4.07	4.86 ± 0.10^{c}	6.40 ± 0.12^{d}	5.06 ± 0.18^{e}	4.30 ± 0.16^{e}
UNKNOWN	14.9 ± 2.22	8.91	10.1 ± 0.18	9.42	9.09	8.91 ± 0.13	8.23 ± 0.02	8.76 ± 0.34	10.5 ± 0.68
TFA mg g^{-1} DW	55.0 ± 2.45^{a}	83.6	78.8 ± 1.88^{be}	82.0	84.6	96.7 ± 3.53^{cd}	107 ± 4.28^{c}	90.3 ± 1.12^{de}	81.7 ± 4.81^{de}

Values in the same row not sharing a superscript are significantly different ($P < 0.05$). Fatty acid composition expressed as percentage values of total fatty acids.

48c, 24 hours starvation in 10 ºC post enrichment; *48r*, 24 hours starvation in 20 ºC post enrichment.

Table A.2. Lipid and fatty acid profile of Cayman enriched with DSelco, expressed as mean ± SE.

Total lipid	0h n=3	1h n=3	2h n=3	4h n=3	8h n=3	12h n=3	22h n=3	28h n=3
mg g-1 DW	103 ± 2.27^{a}	156 ± 3.36^{bd}	150 ± 1.15^{b}	150 ± 2.78^{b}	160 ± 5.99^{bcd}	173 ± 0.72^{c}	169 ± 4.17^{cd}	153 ± 1.62^{bd}
TFA Selco	0h n=2	1h n=1	2h n=3	4h n=1	8h n=1	12h n=3	22h n=3	28h n=3
14:0	1.71 ± 0.03	2.67	2.61 ± 0.02	2.60	2.89	2.47 ± 0.04	2.29 ± 0.05	2.26 ± 0.03
16:0	8.24 ± 0.48^{a}	12.6	12.3 ± 0.04^{b}	11.8	12.4	10.5 ± 0.18^{c}	10.5 ± 0.30^{c}	10.6 ± 0.30^{c}
18:0	4.25 ± 0.32^{a}	4.46	4.35 ± 0.02^{a}	4.19	3.86	3.49 ± 0.01^{b}	3.38 ± 0.15^{b}	3.38 ± 0.14^{b}
SFA	15.9 ± 0.75^{a}	21.4	20.9 ± 0.05^{b}	20.3	20.8	17.9 ± 0.23^{a}	17.5 ± 0.53^{a}	17.7 ± 0.49^{a}
16:1n7	24.5 ± 1.60^{a}	16.1	15.7 ± 0.05^{b}	15.7	12.3	13.4 ± 0.41^{bc}	12.6 ± 0.05^{c}	12.7 ± 0.16^{c}
18:1n7	5.52 ± 0.22^{a}	4.07	4.01 ± 0.01^{a}	1.43	0.97	2.06 ± 0.80^{b}	3.66 ± 0.01^{ab}	3.85 ± 0.02^{ab}
18:1n9	18.6 ± 0.39^{a}	17.4	17.5 ± 0.01^{b}	18.2	17.8	17.8 ± 0.10^{b}	18.0 ± 0.07^{ab}	18.1 ± 0.16^{ab}
20:1n9	3.35 ± 0.03	3.16	3.06 ± 0.01	3.08	2.95	2.84 ± 0.04	3.09 ± 0.02	3.28 ± 0.05
24:1	0.60 ± 0.00	0.64	0.59 ± 0.00	0.62	0.62	0.59 ± 0.00	0.51 ± 0.01	0.61 ± 0.03
MUFA	54.7 ± 1.47^{a}	44.4	43.6 ± 0.04^{b}	42.0	37.6	39.3 ± 1.29^{c}	39.9 ± 0.28^{c}	40.7 ± 0.12^{bc}
18:2n6	2.34 ± 0.06^{a}	3.60	4.06 ± 0.00^{b}	4.72	5.23	5.94 ± 0.09^{c}	6.24 ± 0.20^{c}	6.28 ± 0.21^{c}
18:3n3	1.16 ± 0.26	1.27	1.33 ± 0.00	1.43	1.42	1.55 ± 0.02	1.44 ± 0.03	1.37 ± 0.03
20:2n6	0.14 ± 0.05	0.16	0.20 ± 0.04	0.18	0.21	0.21 ± 0.00	0.28 ± 0.04	0.25 ± 0.00
20:4n3	0.88 ± 0.01	0.73	0.74 ± 0.00	0.80	0.81	0.96 ± 0.02	1.06 ± 0.02	1.11 ± 0.03
20:4n6	0.91 ± 0.01^{a}	1.10	1.11 ± 0.01^{b}	1.10	1.20	1.25 ± 0.01^{c}	1.29 ± 0.02^{c}	1.28 ± 0.01^{c}
20:5n3	6.35 ± 0.11^{a}	6.42	6.50 ± 0.03^{ac}	6.42	6.76	7.12 ± 0.08^{b}	6.77 ± 0.09^{c}	6.55 ± 0.03^{ac}
22:5n3	1.87 ± 0.21	1.55	1.68 ± 0.02	1.56	1.60	1.79 ± 0.01	2.02 ± 0.12	2.06 ± 0.04
22:5n6	0.00 ± 0.00	0.45	0.44 ± 0.01	0.40	0.56	0.49 ± 0.02	0.53 ± 0.01	0.48 ± 0.01
22:6n3	0^{a}	8.60	8.57 ± 0.12^{b}	8.10	11.6	11.1 ± 0.45^{c}	11.3 ± 0.27^{c}	10.1 ± 0.20^{c}
PUFA	14.4 ± 0.00^{a}	25.1	25.9 ± 0.17^{b}	26.0	30.9	31.9 ± 0.51^{c}	32.2 ± 0.61^{c}	30.8 ± 0.51^{c}
n-3	10.5 ± 0.03^{a}	19.4	19.7 ± 0.14^{b}	19.3	23.4	23.7 ± 0.48^{c}	23.5 ± 0.44^{c}	22.1 ± 0.34^{c}
n-6	3.97 ± 0.03^{a}	5.66	6.16 ± 0.04^{b}	6.75	7.49	8.22 ± 0.09^{c}	8.69 ± 0.21^{c}	8.66 ± 0.23^{c}
n-3/n-6	2.63 ± 0.03^{a}	3.43	3.21 ± 0.02^{b}	2.85	3.13	2.88 ± 0.06^{c}	2.71 ± 0.05^{acd}	2.55 ± 0.05^{ad}
DHA/EPA	0^{a}	1.34	1.32 ± 0.02^{b}	1.26	1.72	1.55 ± 0.06^{c}	1.67 ± 0.02^{c}	1.54 ± 0.03^{c}
UNKNOWN	14.9 ± 2.22	9.08	9.57 ± 0.14	11.7	10.7	10.8 ± 0.86	10.4 ± 0.22	10.9 ± 0.36
TFA (mg g^{-1} DW)	55.0 ± 2.45^{a}	104	100 ± 2.52^{b}	106	145	124 ± 3.27^{c}	111 ± 2.94^{bc}	105 ± 2.19^{b}

Values in the same row not sharing a superscript are significantly different ($P < 0.05$). Fatty acid composition expressed as percentage values of total fatty acids.

Table A.3. Fatty acid profile of PC in Cayman enriched with Multigain, expressed as mean ± SE.

PC Cayman Multigain	0 n=3	1h n=4	2h n=4	4h n=4	8h n=4	12h n=4	24h n=6	48h Cold n=4	48h Room n=4
14:0	1.44 ± 0.20	1.64 ± 0.22	2.00 ± 0.22	2.80 ± 0.23	2.81 ± 0.17	2.16 ± 0.14	1.95 ± 0.13	2.53 ± 0.19	2.79 ± 0.20
16:0	14.0 ± 0.54^{a}	16.0 ± 0.92^{ab}	16.9 ± 0.86^{ab}	17.9 ± 0.82^{bc}	18.0 ± 0.51^{bc}	16.4 ± 0.75^{ab}	20.9 ± 0.64^{c}	25.1 ± 0.53^{d}	28.0 ± 0.32^{d}
18:0	4.29 ± 0.61	4.46 ± 0.55	4.44 ± 0.77	3.65 ± 0.26	4.39 ± 0.75	3.85 ± 0.59	3.92 ± 0.21	3.46 ± 0.17	3.92 ± 0.43
SFA	19.8 ± 1.20^{a}	22.1 ± 1.66^{ab}	23.3 ± 1.84^{ab}	24.4 ± 0.92^{ab}	25.2 ± 1.31^{abc}	22.4 ± 0.97^{ab}	26.8 ± 0.84^{bc}	31.1 ± 0.58^{cd}	34.7 ± 0.56^{d}
16:1n7	26.2 ± 0.74^{a}	23.5 ± 0.54^{a}	23.0 ± 0.35^{a}	22.8 ± 1.44^{a}	17.4 ± 0.58^{b}	11.5 ± 0.70^{c}	6.01 ± 0.49^{d}	6.92 ± 0.52^{d}	6.94 ± 0.23^{d}
18:1n7	5.42 ± 0.18	5.21 ± 0.10	5.10 ± 0.11	4.60 ± 0.16	4.18 ± 0.03	3.52 ± 0.09	2.69 ± 0.06	2.69 ± 0.03	2.91 ± 0.07
18:1n9	15.0 ± 0.85^{abc}	15.3 ± 0.30^{a}	15.0 ± 0.41^{a}	13.7 ± 0.61^{ac}	12.1 ± 0.14^{c}	9.50 ± 0.33^{b}	5.87 ± 0.26^{d}	6.19 ± 0.22^{d}	5.57 ± 0.12^{d}
20:1n9	1.89 ± 0.09	1.83 ± 0.12	1.73 ± 0.13	1.45 ± 0.13	1.44 ± 0.15	1.26 ± 0.11	1.04 ± 0.07	1.08 ± 0.08	1.12 ± 0.08
MUFA	48.5 ± 0.35^{b}	45.8 ± 0.27^{a}	44.9 ± 0.49^{a}	42.5 ± 1.76^{abc}	35.1 ± 0.73^{c}	25.7 ± 1.19^{d}	15.6 ± 0.85^{e}	16.9 ± 0.80^{e}	16.5 ± 0.34^{e}
18:2n6	2.45 ± 0.14	3.04 ± 0.20	3.12 ± 0.24	3.15 ± 0.18	3.00 ± 0.12	2.68 ± 0.07	2.48 ± 0.07	2.65 ± 0.05	2.64 ± 0.03
18:3n3	1.17 ± 0.16	0.98 ± 0.05	0.97 ± 0.07	0.92 ± 0.04	0.76 ± 0.03	0.66 ± 0.02	0.42 ± 0.01	0.39 ± 0.02	0.34 ± 0.01
18:4n3	0.18 ± 0.03	0.18 ± 0.00	0.18 ± 0.00	0.31 ± 0.00	0.30 ± 0.02	0.33 ± 0.03	0.31 ± 0.00	0.29 ± 0.02	0.19 ± 0.00
20:3n6	0.70 ± 0.07	0.68 ± 0.07	0.66 ± 0.08	0.64 ± 0.06	0.72 ± 0.05	0.85 ± 0.05	0.81 ± 0.01	0.82 ± 0.02	0.78 ± 0.03
20:4n3	1.11 ± 0.11	1.11 ± 0.15	1.14 ± 0.17	1.25 ± 0.11	1.56 ± 0.14	2.02 ± 0.15	1.89 ± 0.02	1.84 ± 0.06	1.67 ± 0.08
20:4n6	0.62 ± 0.12^{a}	0.62 ± 0.14^{a}	0.83 ± 0.14^{a}	1.13 ± 0.06^{a}	1.33 ± 0.18^{a}	1.90 ± 0.21^{ab}	2.97 ± 0.14^{b}	2.78 ± 0.12^{b}	3.12 ± 0.09^{b}
20:5n3	6.60 ± 0.23^{ab}	5.96 ± 0.29^{ab}	5.62 ± 0.27^{ab}	5.21 ± 0.68^{a}	5.67 ± 0.17^{ab}	6.75 ± 0.26^{b}	5.96 ± 0.16^{ab}	5.88 ± 0.19^{ab}	5.61 ± 0.26^{ab}
22:5n3	2.90 ± 0.16^{abc}	2.53 ± 0.53^{ac}	2.15 ± 0.45^{a}	2.27 ± 0.32^{ad}	2.70 ± 0.46^{ac}	4.10 ± 0.37^{bc}	4.19 ± 0.25^{b}	3.95 ± 0.35^{bcd}	4.61 ± 0.28^{b}
22:5n6		0.36 ± 0.05^{a}	0.54 ± 0.07^{a}	1.13 ± 0.14^{b}	2.36 ± 0.16^{c}	4.55 ± 0.35^{d}	7.29 ± 0.30^{e}	6.33 ± 0.29^{ef}	5.13 ± 0.16^{d}f
22:6n3		1.33 ± 0.23^{a}	1.97 ± 0.40^{ab}	4.30 ± 0.60^{b}	9.07 ± 0.97^{c}	17.4 ± 1.38^{de}	23.0 ± 1.59^{d}	18.3 ± 1.07^{de}	14.3 ± 0.67^{e}
PUFA	15.7 ± 0.42^{a}	16.7 ± 1.36^{a}	16.9 ± 1.51^{a}	19.6 ± 2.14^{ab}	27.4 ± 1.90^{b}	41.2 ± 2.40^{cd}	49.0 ± 1.90^{c}	43.1 ± 1.85^{cd}	38.3 ± 1.25^{d}
n-3	12.0 ± 0.32^{ab}	12.0 ± 1.18^{a}	11.9 ± 1.30^{a}	14.1 ± 1.75^{ab}	20.0 ± 1.72^{b}	31.2 ± 2.01^{cd}	35.8 ± 1.81^{c}	30.6 ± 1.54^{cd}	26.7 ± 1.18^{d}
n-6	3.76 ± 0.10^{a}	4.69 ± 0.19^{ab}	4.94 ± 0.23^{ab}	5.47 ± 0.39^{b}	7.41 ± 0.20^{c}	9.98 ± 0.48^{d}	13.3 ± 0.29^{e}	12.6 ± 0.34^{ef}	11.7 ± 0.09^{f}
n-3/n6	3.18 ± 0.03^{a}	2.54 ± 0.15^{ab}	2.40 ± 0.17^{ab}	2.54 ± 0.17^{ab}	2.68 ± 0.17^{ab}	3.13 ± 0.13^{ab}	2.70 ± 0.14^{ab}	2.42 ± 0.07^{b}	2.29 ± 0.09^{b}
DHA/EPA		0.22 ± 0.03^{a}	0.34 ± 0.06^{a}	0.82 ± 0.03^{b}	1.59 ± 0.14^{b}	2.56 ± 0.11^{c}	3.87 ± 0.30^{c}	3.10 ± 0.11^{c}	2.56 ± 0.08^{c}
UNKNOWN	16.0 ± 0.69	15.4 ± 0.46	14.9 ± 0.31	13.5 ± 0.28	12.3 ± 0.15	10.7 ± 0.34	8.58 ± 0.26	8.89 ± 0.59	10.4 ± 0.44

Values in the same row not sharing a superscript are significantly different ($P < 0.05$). Fatty acid composition expressed as percentage values of total fatty acids.

Table A.4. Fatty acid profile of PE in Cayman enriched with Multigain, expressed as mean ± SE.

PE Caymen Multigain	0 n=3	1h n=4	2h n=4	4h n=4	8h n=4	12h n=4	24h n=6	48h Cold n=4	48h Room n=4
14:0	1.03 ± 0.24	0.79 ± 0.18	0.91 ± 0.17	1.40 ± 0.29	0.99 ± 0.18	0.77 ± 0.15	0.66 ± 0.10	0.60 ± 0.03	0.85 ± 0.18
16:0	7.76 ± 0.69^{ab}	7.64 ± 0.86^{ab}	7.92 ± 0.80^{ab}	9.48 ± 0.88^{ab}	7.80 ± 0.71^{ab}	6.82 ± 0.61^{a}	10.6 ± 0.20^{b}	9.26 ± 0.46^{ab}	10.5 ± 1.26^{b}
18:0	3.79 ± 0.19	3.51 ± 0.24	3.45 ± 0.28	4.20 ± 0.82	3.16 ± 0.25	2.82 ± 0.25	3.10 ± 0.06	3.16 ± 0.16	2.89 ± 0.18
SFA	12.6 ± 0.83^{a}	11.9 ± 1.28^{a}	12.3 ± 1.23^{a}	15.1 ± 1.57^{a}	11.9 ± 1.14^{a}	10.2 ± 1.07^{a}	14.3 ± 0.29^{a}	12.7 ± 0.77^{a}	14.0 ± 1.68^{a}
16:1n7	16.6 ± 1.10^{a}	12.0 ± 1.98^{ab}	11.7 ± 1.79^{ab}	10.7 ± 2.47^{abc}	8.62 ± 1.85^{bcd}	6.04 ± 1.56^{bcd}	4.42 ± 0.31^{cd}	2.81 ± 0.20^{d}	4.29 ± 1.07^{cd}
18:1n7	4.05 ± 0.12	3.24 ± 0.22	3.10 ± 0.21	2.79 ± 0.27	2.41 ± 0.26	1.95 ± 0.27	1.93 ± 0.08	1.59 ± 0.06	2.08 ± 0.29
18:1n9	19.5 ± 0.54^{a}	20.3 ± 0.39^{a}	19.6 ± 0.40^{a}	17.6 ± 0.74^{a}	16.4 ± 1.02^{ab}	13.3 ± 1.51^{bc}	11.0 ± 0.75^{cd}	7.95 ± 0.43^{d}	9.55 ± 1.08^{cd}
20:1n9	6.31 ± 0.31	6.60 ± 0.25	5.74 ± 0.20	4.76 ± 0.29	4.76 ± 0.15	4.46 ± 0.09	4.29 ± 0.16	3.55 ± 0.07	4.27 ± 0.10
22:1n11	0.53 ± 0.06	1.85 ± 0.23	1.91 ± 0.15	1.85 ± 0.11	2.12 ± 0.00	2.82 ± 0.24		2.37 ± 0.08	2.38 ± 0.62
22:1n9	1.48 ± 0.03	2.03 ± 0.54	1.97 ± 0.49	1.73 ± 0.48	1.79 ± 0.48	2.14 ± 0.86	0.70 ± 0.12	1.45 ± 0.71	2.46 ± 0.50
MUFA	48.5 ± 0.52^{a}	45.1 ± 1.45^{ab}	43.1 ± 1.30^{abc}	38.6 ± 2.47^{bc}	35.0 ± 2.00^{cd}	28.7 ± 2.70^{de}	22.1 ± 1.43^{ef}	18.5 ± 1.01^{f}	24.4 ± 1.21^{ef}
18:2n6	3.99 ± 0.18	4.50 ± 0.07	4.53 ± 0.04	4.37 ± 0.04	4.51 ± 0.13	4.10 ± 0.30	4.82 ± 0.26	4.12 ± 0.25	5.69 ± 0.49
18:3n3	1.82 ± 0.27	1.30 ± 0.02	1.28 ± 0.02	1.06 ± 0.08	0.99 ± 0.04	0.84 ± 0.08	0.69 ± 0.03	0.50 ± 0.04	0.54 ± 0.07
20:3n6	0.89 ± 0.05	1.05 ± 0.10	1.02 ± 0.08	0.95 ± 0.07	1.09 ± 0.09	1.17 ± 0.08	1.18 ± 0.01	1.19 ± 0.05	1.07 ± 0.04
20:4n3	1.46 ± 0.10	1.73 ± 0.19	1.75 ± 0.17	1.73 ± 0.14	2.06 ± 0.19	2.30 ± 0.17	2.52 ± 0.02	2.62 ± 0.10	2.23 ± 0.11
20:4n6	0.80 ± 0.07^{a}	0.90 ± 0.08^{a}	0.97 ± 0.08^{a}	1.07 ± 0.09^{ab}	1.61 ± 0.17^{bc}	2.28 ± 0.27^{c}	3.24 ± 0.14^{d}	3.94 ± 0.10^{d}	3.83 ± 0.32^{d}
20:5n3	6.85 ± 0.68^{a}	7.18 ± 0.76^{a}	7.13 ± 0.66^{a}	6.65 ± 0.62^{a}	7.41 ± 0.93^{a}	8.86 ± 1.14^{a}	7.49 ± 0.39^{a}	7.88 ± 0.18^{a}	5.83 ± 0.68^{a}
22:5n3	2.02 ± 0.12	2.65 ± 0.35	2.68 ± 0.28	2.55 ± 0.28	2.92 ± 0.40	3.52 ± 0.42	3.33 ± 0.21	4.27 ± 0.06	2.95 ± 0.36
22:5n6		0.53 ± 0.04^{a}	0.86 ± 0.12^{ab}	1.49 ± 0.15^{bc}	2.89 ± 0.40^{cd}	4.97 ± 0.70^{de}	7.70 ± 0.58^{de}	9.11 ± 0.29^{e}	5.53 ± 0.84^{cde}
22:6n3		1.25 ± 0.12^{a}	2.49 ± 0.28^{ab}	4.16 ± 0.47^{bc}	7.80 ± 1.09^{cd}	12.4 ± 1.67^{de}	16.5 ± 1.45^{ef}	19.6 ± 0.64^{f}	10.5 ± 1.63^{d}
PUFA	18.0 ± 1.27^{a}	21.5 ± 1.42^{a}	23.4 ± 1.30^{ab}	24.3 ± 1.73^{ab}	32.0 ± 2.70^{bce}	40.7 ± 3.84^{cde}	47.0 ± 2.33^{de}	53.2 ± 0.78^{d}	38.7 ± 3.12^{e}
n-3	12.1 ± 1.06^{a}	14.1 ± 1.38^{ae}	15.3 ± 1.38^{ae}	16.1 ± 1.49^{ae}	21.2 ± 2.56^{abd}	27.9 ± 3.29^{bcd}	30.5 ± 2.00^{bcd}	34.9 ± 0.63^{c}	22.0 ± 2.69^{de}
n-6	5.86 ± 0.21^{a}	7.39 ± 0.09^{ab}	8.08 ± 0.15^{b}	8.16 ± 0.31^{b}	10.8 ± 0.19^{c}	12.8 ± 0.61^{d}	16.5 ± 0.50^{e}	18.3 ± 0.32^{f}	16.7 ± 0.48^{ef}
n-3/n6	2.07 ± 0.11	1.91 ± 0.18	1.90 ± 0.19	1.97 ± 0.13	1.96 ± 0.21	2.16 ± 0.18	1.84 ± 0.10	1.90 ± 0.04	1.31 ± 0.13
DHA/EPA		0.18 ± 0.01	0.35 ± 0.01	0.62 ± 0.01	1.05 ± 0.02	1.39 ± 0.04	2.18 ± 0.08	2.49 ± 0.10	1.78 ± 0.08
UNKNOWN	20.9 ± 0.52	21.5 ± 1.29	21.3 ± 1.25	22.1 ± 2.13	21.1 ± 0.73	20.3 ± 0.70	16.5 ± 0.63	15.5 ± 0.27	22.8 ± 0.91

Values in the same row not sharing a superscript are significantly different ($P < 0.05$). Fatty acid composition expressed as percentage values of total fatty acids.

Table A.5. Fatty acid profile of PC in Cayman enriched with DSelco, expressed as mean ± SE.

PC Cayman DSelco	0 n=3	1h n=3	2h n=3	4h n=3	8h n=4	12h n=3	22h n=2	28h n=4
14:0	1.44 ± 0.20	1.18 ± 0.14	1.23 ± 0.07	1.37 ± 0.26	1.73 ± 0.22	1.14 ± 0.07	2.02 ± 0.03	1.80 ± 0.21
16:0	14.0 ± 0.54^{a}	13.7 ± 0.93^{a}	15.2 ± 0.39^{a}	14.4 ± 1.34^{a}	15.6 ± 1.00^{a}	13.6 ± 0.27^{a}	20.1 ± 0.59^{b}	19.7 ± 0.44^{b}
18:0	4.29 ± 0.61	6.53 ± 0.24	4.72 ± 0.09	5.47 ± 1.10	4.70 ± 0.63	3.31 ± 0.22	4.48 ± 0.33	4.33 ± 0.54
SFA	19.8 ± 1.20^{abc}	21.4 ± 1.30^{abc}	21.1 ± 0.54^{ac}	21.2 ± 2.68^{abc}	22.0 ± 0.88^{abc}	18.1 ± 0.52^{a}	26.6 ± 0.22^{b}	25.8 ± 1.05^{bc}
16:1n7	26.2 ± 0.74^{a}	25.1 ± 1.13^{ab}	24.9 ± 0.37^{ab}	22.4 ± 2.14^{ab}	20.1 ± 1.33^{bc}	14.0 ± 0.71^{c}	14.5 ± 0.44^{cd}	13.0 ± 0.55^{c}
18:1n7	5.42 ± 0.18	5.38 ± 0.05	5.43 ± 0.12	5.32 ± 0.19	4.46 ± 0.08	4.25 ± 0.03	4.01 ± 0.02	3.95 ± 0.05
18:1n9	15.0 ± 0.85^{abc}	14.3 ± 0.09^{a}	15.3 ± 0.29^{ab}	14.6 ± 0.18^{ab}	13.5 ± 0.09^{b}	13.4 ± 0.17^{abc}	13.3 ± 0.26^{abc}	12.7 ± 0.11^{c}
20:1n9	1.89 ± 0.09	1.86 ± 0.27	1.55 ± 0.04	1.32 ± 0.42	1.41 ± 0.22	1.36 ± 0.02	1.83 ± 0.14	1.80 ± 0.11
MUFA	48.5 ± 0.35^{a}	46.6 ± 0.90^{ab}	47.2 ± 0.42^{ab}	43.6 ± 1.38^{bc}	39.5 ± 1.18^{c}	33.1 ± 0.70^{d}	33.6 ± 0.87^{d}	31.4 ± 0.67^{d}
18:2n6	2.45 ± 0.14^{a}	3.86 ± 0.08^{b}	4.96 ± 0.17^{b}	6.31 ± 0.43^{c}	6.66 ± 0.25^{cd}	7.75 ± 0.04^{d}	7.03 ± 0.24^{cd}	7.32 ± 0.26^{cd}
18:3n3	1.17 ± 0.16	1.71 ± 0.08	1.85 ± 0.06	2.19 ± 0.18	2.03 ± 0.09	2.23 ± 0.01	1.52 ± 0.04	1.45 ± 0.04
18:4n3	0.18 ± 0.03	0.35 ± 0.03	0.45 ± 0.05	0.72 ± 0.16	0.97 ± 0.02	1.30 ± 0.05	0.65 ± 0.08	0.65 ± 0.08
20:3n6	0.70 ± 0.07	0.60 ± 0.09	0.70 ± 0.04	0.52 ± 0.20	0.60 ± 0.07	0.73 ± 0.04	0.57 ± 0.01	0.54 ± 0.01
20:4n3	1.11 ± 0.11	1.06 ± 0.14	1.32 ± 0.07	1.24 ± 0.47	1.63 ± 0.12	2.46 ± 0.14	1.78 ± 0.06	1.77 ± 0.10
20:4n6	0.62 ± 0.12^{a}	1.26 ± 0.03^{a}	0.69 ± 0.03^{a}	0.94 ± 0.23^{a}	0.96 ± 0.31^{a}	0.78 ± 0.08^{a}	1.06 ± 0.15^{a}	1.05 ± 0.11^{a}
20:5n3	6.60 ± 0.23^{a}	7.49 ± 0.72^{a}	6.43 ± 0.19^{a}	8.03 ± 0.34^{a}	7.61 ± 1.28^{a}	9.15 ± 0.42^{a}	6.58 ± 0.11^{a}	6.68 ± 0.33^{a}
22:5n3	2.90 ± 0.16	1.56 ± 0.64	1.43 ± 0.08	1.44 ± 1.04	2.61 ± 0.17	4.45 ± 0.07	2.95 ± 0.58	3.52 ± 0.39
22:6n3		0.32 ± 0.10^{ab}	0.46 ± 0.06^{af}	1.23 ± 0.76^{abc}	3.44 ± 0.28^{be}	7.90 ± 0.56^{c}	5.26 ± 0.57^{cdef}	7.22 ± 0.97^{cde}
PUFA	15.7 ± 0.42^{a}	18.1 ± 1.90^{ab}	18.3 ± 0.47^{ab}	22.6 ± 3.28^{abd}	26.2 ± 2.19^{bd}	36.8 ± 1.16^{c}	27.4 ± 0.99^{bc}	29.9 ± 1.88^{cd}
n-3	12.0 ± 0.32^{a}	12.0 ± 1.69^{a}	11.5 ± 0.34^{a}	14.1 ± 2.74^{ac}	17.3 ± 1.67^{ac}	26.2 ± 1.13^{b}	18.1 ± 1.17^{ab}	20.6 ± 1.78^{bc}
n-6	3.76 ± 0.10^{a}	5.72 ± 0.19^{b}	6.35 ± 0.18^{b}	7.77 ± 0.44^{abcd}	7.93 ± 0.51^{bcd}	9.26 ± 0.05^{c}	8.65 ± 0.10^{cd}	8.64 ± 0.07^{d}
n-3/n-6	3.18 ± 0.03^{a}	2.09 ± 0.23^{ab}	1.81 ± 0.02^{b}	1.79 ± 0.25^{ab}	2.17 ± 0.09^{b}	2.83 ± 0.13^{ab}	2.09 ± 0.16^{ab}	2.39 ± 0.20^{ab}
DHA/EPA		0.03 ± 0.01^{a}	0.07 ± 0.01^{ab}	0.15 ± 0.09^{acd}	0.48 ± 0.06^{c}	0.86 ± 0.04^{cd}	0.80 ± 0.07^{bcd}	1.07 ± 0.10^{d}
UNKNOWN	16.0 ± 0.69	13.9 ± 0.36	13.4 ± 0.30	12.6 ± 0.81	12.3 ± 0.25	12.1 ± 0.12	12.5 ± 0.10	12.9 ± 0.23

Values in the same row not sharing a superscript are significantly different ($P < 0.05$). Fatty acid composition expressed as percentage values of total fatty acids.

Table A.6. Fatty acid profile of PE in Cayman enriched with DHA Selco, expressed as mean ± SE.

PE Cayman DSelco	0 n=3	1h n=3	2h n=3	4h n=3	8h n=4	12h n=3	22h n=2	28h n=4
14:0	1.03 ± 0.24	0.66 ± 0.04	0.71 ± 0.01	1.03 ± 0.04	0.57 ± 0.17	0.83 ± 0.05	0.96 ± 0.19	0.79 ± 0.12
16:0	7.76 ± 0.69^{a}	7.12 ± 0.27^{a}	8.28 ± 0.10^{a}	8.72 ± 0.06^{ab}	7.30 ± 0.78^{a}	8.47 ± 0.20^{a}	11.4 ± 0.42^{bc}	11.2 ± 0.24^{c}
18:0	3.79 ± 0.19	4.51 ± 0.12	4.73 ± 0.09	4.08 ± 0.03	3.54 ± 0.16	4.05 ± 0.10	3.98 ± 0.10	3.55 ± 0.11
SFA	12.6 ± 0.83^{ac}	12.3 ± 0.43^{a}	13.7 ± 0.18^{ab}	13.8 ± 0.09^{ab}	11.4 ± 1.10^{a}	13.3 ± 0.33^{ab}	16.3 ± 0.52^{b}	15.5 ± 0.43^{bc}
16:1n7	16.6 ± 1.10^{a}	14.3 ± 0.57^{ab}	14.1 ± 0.17^{ab}	13.9 ± 0.10^{ab}	9.00 ± 1.46^{c}	10.7 ± 0.27^{bc}	10.2 ± 0.59^{bc}	8.90 ± 0.80^{c}
18:1n7	4.05 ± 0.12	3.93 ± 0.05	4.04 ± 0.04	3.58 ± 0.02	2.81 ± 0.18	3.04 ± 0.04	3.21 ± 0.04	3.12 ± 0.13
18:1n9	19.5 ± 0.54^{abd}	20.8 ± 0.16^{ac}	22.6 ± 0.34^{a}	19.8 ± 0.02^{ab}	18.0 ± 0.70^{bcd}	19.6 ± 0.13^{b}	19.3 ± 1.03^{abd}	17.4 ± 0.24^{d}
20:1n9	6.31 ± 0.31	6.33 ± 0.12	6.12 ± 0.20	5.16 ± 0.29	4.67 ± 0.27	4.56 ± 0.06	5.51 ± 0.76	4.95 ± 0.25
22:1n11	0.53 ± 0.06	0.27 ± 0.11		0.38 ± 0.05	1.05 ± 0.27			1.07 ± 0.15
22:1n9	1.48 ± 0.03	0.78 ± 0.34	0.55 ± 0.26	1.00 ± 0.03	1.31 ± 0.16	0.73 ± 0.01	0.73 ± 0.54	1.43 ± 0.08
MUFA	48.5 ± 0.52^{a}	46.3 ± 0.27^{a}	47.2 ± 0.66^{ab}	43.9 ± 0.20^{bc}	36.8 ± 1.90^{abcd}	38.4 ± 0.28^{d}	39.3 ± 0.35^{cd}	36.9 ± 0.87^{d}
18:2n6	3.99 ± 0.18^{a}	5.28 ± 0.07^{a}	6.49 ± 0.08^{b}	6.36 ± 0.02^{b}	7.32 ± 0.23^{bc}	8.03 ± 0.08^{c}	9.13 ± 0.68^{abcd}	9.65 ± 0.24^{d}
18:3n3	1.82 ± 0.27	2.23 ± 0.03	2.43 ± 0.02	2.21 ± 0.02	2.25 ± 0.08	2.19 ± 0.02	1.99 ± 0.17	1.88 ± 0.04
18:4n3		0.31 ± 0.01	0.29 ± 0.00	0.43 ± 0.03	0.57 ± 0.02	0.49 ± 0.02	0.34 ± 0.05	0.31 ± 0.03
20:3n6	0.89 ± 0.05	0.96 ± 0.03	0.93 ± 0.04	0.90 ± 0.01	0.97 ± 0.12	0.95 ± 0.01	0.80 ± 0.04	0.75 ± 0.01
20:4n3	1.46 ± 0.10	1.68 ± 0.05	1.81 ± 0.03	1.77 ± 0.02	2.37 ± 0.19	2.34 ± 0.02	2.23 ± 0.22	2.31 ± 0.09
20:4n6	0.80 ± 0.07^{a}	0.88 ± 0.02^{a}	0.84 ± 0.05^{a}	0.86 ± 0.04^{a}	1.30 ± 0.16^{a}	1.07 ± 0.03^{a}	0.95 ± 0.04^{a}	1.04 ± 0.06^{a}
20:5n3	6.85 ± 0.68^{a}	7.67 ± 0.17^{ab}	6.54 ± 0.44^{a}	7.26 ± 0.35^{ab}	10.7 ± 1.48^{b}	7.74 ± 0.13^{ab}	6.03 ± 0.14^{a}	6.53 ± 0.50^{a}
22:5n3	2.02 ± 0.12	2.18 ± 0.09	1.18 ± 0.18	2.30 ± 0.03	3.26 ± 0.48	2.68 ± 0.10	2.05 ± 0.20	2.53 ± 0.17
22:6n3		0.37 ± 0.03^{a}	0.30 ± 0.05^{a}	1.46 ± 0.07^{b}	4.18 ± 0.69^{abc}	3.89 ± 0.13^{c}	2.96 ± 0.43^{abc}	4.30 ± 0.46^{bc}
PUFA	18.0 ± 1.27^{ab}	21.6 ± 0.44^{ac}	20.6 ± 0.81^{a}	23.5 ± 0.53^{ac}	32.5 ± 2.80^{abc}	29.4 ± 0.30^{bd}	26.5 ± 0.71^{abc}	28.8 ± 1.21^{cd}
n-3	12.1 ± 1.06^{ab}	14.5 ± 0.33^{a}	12.3 ± 0.77^{a}	15.4 ± 0.45^{a}	23.4 ± 2.79^{ab}	19.3 ± 0.31^{b}	15.6 ± 0.05^{a}	17.8 ± 1.21^{ab}
n-6	5.86 ± 0.21^{a}	7.12 ± 0.11^{b}	8.26 ± 0.10^{c}	8.12 ± 0.08^{bc}	9.11 ± 0.36^{cd}	10.0 ± 0.10^{de}	10.9 ± 0.75^{e}	11.1 ± 0.20^{e}
n-3/n-6	2.07 ± 0.11^{ab}	2.03 ± 0.02^{ab}	1.49 ± 0.09^{a}	1.90 ± 0.04^{ab}	2.58 ± 0.35^{b}	1.93 ± 0.04^{ab}	1.44 ± 0.10^{a}	1.61 ± 0.12^{a}
DHA/EPA		0.05 ± 0.00^{a}	0.03 ± 0.02^{a}	0.20 ± 0.01^{b}	0.39 ± 0.01^{c}	0.50 ± 0.01^{d}	0.49 ± 0.08^{abcde}	0.66 ± 0.02^{e}
UNKNOWN	20.9 ± 0.52	19.8 ± 0.26	18.5 ± 0.19	18.8 ± 0.59	19.3 ± 0.24	18.9 ± 0.27	18.0 ± 0.54	18.8 ± 0.23

Values in the same row not sharing a superscript are significantly different ($P < 0.05$). Fatty acid composition expressed as percentage values of total fatty acids.

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DHA content in dietary phospholipids affects DHA content in phospholipids of cod larvae and larval performance

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ABSTRACT

The objectives of the study were to examine if early cod larvae could incorporate long-chain highly unsaturated fatty acids like docosahexaenoic acid (22:6 n − 3, DHA) from dietary phospholipids (PL) more efficiently than from dietary triacylglycerides (TAG), and to investigate if because of this, PL from copepods is a better DHA source than PL from rotifers for larval cod. Two diets of *Brachionus plicatilis* Nevada were compared in one first feeding experiment. Their lipid composition was similar, but %DHA of PL fatty acids was different; 2.1% for *Brachionus*-Low and 9.4% for *Brachionus*-High. A second experiment compared the *Brachionus*-High diet (9.4% DHA in PL) and cultivated nauplii of *Acartia tonsa* that contained 30% DHA of PL-fatty acids. The total lipid per fry dry weight (DW) and the lipid class composition were similar for all larval groups at 17 days post-hatching (dph). The %DHA of total fatty acids in PL of newly hatched larvae (2 dph) was 26%, and the values for fed groups varied from 17 to 30% DHA of PL fatty acids. The dominant fatty acids in the PL of larvae and PL of the diets were well correlated ($r^2 = 0.84$, $p < 0.05$), specifically for %DHA ($r^2 = 0.90$). The %DHA in larval PL was also significantly positively correlated to larval DW at 17 dph, whereas the relationship to survival was positive, but not significantly correlated across the three diets. We conclude that cod larvae exhibited a low capacity for de novo PL synthesis based on dietary TAG, the normal pathway of adult fish and humans. The 17 dph cod larvae instead synthesised their PL through re-acylation of digested dietary PL. It then follows that only a dietary PL composition close to the PL composition of larval cod, like in PL of many copepods, can secure high DHA incorporation in larval PL, and ultimately high larval growth and survival. We suggest that the DHA requirement of cod larvae cannot be easily met with *B. plicatilis* Nevada and call for new approaches of larval feeding.

1. Introduction

The interests of culturing cold water marine finfish in Europe emerged in the 1970s, and the production of viable juveniles became the ultimate everlasting challenge for most species (e.g., Moksness et al., 2004). Some of the pioneering work on first feeding of Atlantic cod (*Gadus morhua*) larvae was undertaken using harvested zooplankton as live larval feed (Folkvord et al., 1994; Oiestad et al., 1985; Olsen et al., 1999), but cod juveniles have later been produced commercially using rotifers and *Artemia* as the principal live feed. Cultivation of Atlantic halibut (*Hippoglossus hippoglossus*) attracted major attention in northern countries from the early 1990s, and larval rearing of the species was undertaken using copepods and later, *Artemia* and rotifers as live feed (McEvoy et al., 1998; Olsen et al., 1999). Atlantic halibut and Atlantic cod have had a relatively strong focus with major investments made to solve the problems of juvenile production, but the global production of both species in 2011 was still relatively low, with 16,000 tons of cod and 2900 tons of halibut (www.fao.org).

There are presumably many reasons why both these species have not become more successful. The halibut has a very complex life cycle (Mangor-Jensen et al., 1998; Olsen et al., 1999) and cod aquaculture has suffered from variable juvenile quality as well as the competition with increased landings from cod fisheries. Nevertheless, the growth potential, survival, and viability of the juveniles are apparently not fully realised for these species when they are fed rotifers and *Artemia* at the larval stage. Larval feeding by copepods tends to produce juveniles of better quality, and it is still not completely understood why (Busch et al., 2010; Evjemo et al., 2003; McEvoy et al., 1998).

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Phosphoacylglycerides, here referred to as phospholipids (PL), have been shown to be essential ingredients of fish larval diets (Cahu et al., 2009; Coutteau et al., 1997; Tocher et al., 2008). These conclusions have been drawn based on knowledge on digestive and metabolic functions of lipids and PL (Iijima et al., 1990; Sargent et al., 1989; Tocher, 1995) and because of the practical problems of supplying nutritionally adequate PL to larvae (Cahu et al., 2003; Gisbert et al., 2005; Wold et al., 2007). Both *Artemia* and rotifers have high PL contents (Harel et al., 1999; Olsen, 2004; Rainuzzo et al., 1994a), but the fatty acid composition and configuration of the typical marine PL molecule are different from those of the enriched rotifers and *Artemia* (Coutteau and Mourente, 1997; Li et al., 2014; Sargent et al., 1989). It has been shown that the content of PL per dry weight (DW) of rotifers cannot be manipulated much, but that the percentage PL of total lipid varies dynamically with the total lipid content of the rotifers (Li et al., 2014; Olsen et al., 1999). It is also apparent that the fatty acid composition of PL of rotifers can be manipulated to some degree (Li et al., 2014; Rainuzzo et al., 1994b).

The present study was motivated by an early observation made for Atlantic halibut larvae fed short-term enriched *Artemia* and harvested copepods (Evjemo et al., 2003). It appeared that the halibut larvae were less capable of incorporating docosahexaenoic acid (DHA; 22:6 n − 3) from *Artemia* than from copepods into their tissues. This observation was not understood at that time, but we now suggest that this was the case, because DHA in enriched rotifers and *Artemia* are mainly associated with triacylglycerides (TAG) (Coutteau and Mourente, 1997; Li et al., 2014). We therefore suggest that early cod larvae cannot efficiently utilise DHA from digested TAG components for de novo PL synthesis and that they instead base their PL synthesis on dietary PL. This probably reflects a limited ability of early larvae to carry through de novo synthesis of PL based on dietary TAG, and is in agreement with the finding that PL appears to be essential for fish larvae in their very early stages (Cahu et al., 2009; Coutteau et al., 1997; Tocher et al., 2008).

The objective of the present study was accordingly to examine if cod larvae could incorporate DHA from dietary PL more efficiently than from dietary TAG in the early stages of feeding and growth. This might explain why copepods are generally a better live feed source than rotifers and *Artemia* for some cold water fish larvae, because copepods, contrary to rotifers and *Artemia*, have high DHA contents in their PL (Li et al., 2014). We compared DHA retention in PL of 17 day post-hatching (dph) cod larvae fed two rotifer-based diets and one copepod-based diet, all with a different percentage of DHA in PL. For Experiment 1, we designed a method to produce two rotifer diets that were almost equally composed except for a difference in percentage content of DHA in PL and we compared these diets in a first feeding trial. In Experiment 2, we compared one of the rotifer diets with cultured nauplii of *Acartia tonsa* exhibiting not only a higher percentage of DHA in their PL than the rotifers, but also other differences in their lipid composition.

2. Material and methods

2.1. Experimental description

Two first feeding experiments with larval cod (*G. morhua*) from egg stage to 17 dph were undertaken, and the procedures to produce the live feed diets consisting of rotifers or nauplii of the copepod *A. tonsa* represented the main effort of both experiments.

2.1.1. Microalgae

Rhodomonas baltica (NIVA 5/91: 8–9 μm) for feeding of copepods was grown semi-continuously at 27 °C in seawater autoclaved at 120 °C for 20 min and, thereafter, 1.5 mL L^{-1} Conwy nutrient mixture was added (Walne, 1974). Cultures (10 L glass bottles and 1.5 L PET bottles) were given air containing 1–3% CO_2 and were illuminated from one side (Philips TLD 36 W, 965). Around 60–70% of the volume of steady state cultures was harvested daily for use and replaced by a new medium. *Isochrysis galbana* (T-ISO strain), used as a supplement, was added directly to larval tanks ('green water treatment'). It was grown semi-continuously in polycarbonate cylinders (200 L) with 35% of volume harvested daily and replaced with treated water with the addition of nutrient mixture. Otherwise, *I. galbana* was treated in a similar way to *R. baltica*. Finally, the cultivation diet of the rotifers included *Nannochloropsis* sp. concentrate (Reed Mariculture, USA).

2.1.2. Rotifers

Rotifers (*Brachionus plicatilis* — Nevada type) were cultivated in 250 L glass fibre tanks at 20–22 °C and 20 ppt salinity. Feed sources used for the rotifer was Baker's yeast (*Saccharomyces cerevisiae*), a concentrate of *Nannochloropsis* sp. (Reed Mariculture), and an emulsified lipid diet Marol E (prepared by SINTEF Fisheries and Aquaculture, Norway) based on the marine oil DHASCO (Martek Biosciences, USA) (see details in Table 1). Cultures were diluted by 20% of volume day^{-1} and Baker's yeast, *Nannochloropsis* concentrate, and Marol E (for the *Brachionus*-High diet) were added continuously by membrane pumps (Iwaki Ltd., EW-F20VC-20EPF2, Japan). Rotifer cultures were used as live feed after reaching a steady state of growth, judged by an establishment of a constant rotifer density with time. The quality of the rotifer cultures was monitored daily (eggs $female^{-1}$, turbidity, swimming pattern, Olsen, 2004).

Two different rotifer diets were prepared (Table 1). *Brachionus*-High was prepared as described above with Marol E added continuously along with the other feed components, representing the standard rotifer diet of the laboratory (control). The rotifer culture becomes enriched by the n − 3 highly unsaturated fatty acids (HUFA) of the emulsion during normal growth, and the biochemical composition becomes constant with time. This method has been termed long-term enrichment or combined growth and enrichment (Olsen, 2004; Olsen et al., 1993; Rainuzzo et al., 1994b), and is well suited for use at low temperature. *Brachionus*-Low was grown without a continuous addition of Marol E, but was instead short-term enriched by this emulsion for 2 h just before its use as live feed (Table 1). The ration of Marol E given and the incubation time were adapted to yield a similar enrichment of total lipids and n − 3 HUFA, particularly DHA, for the two rotifer diets. The rotifer cultures were carefully rinsed by exchanging the water before their use for first feeding.

2.1.3. Acartia

A. tonsa eggs, obtained from Roskilde University (Denmark, identification code DFH-ATI) (Støttrup et al., 1986), were hatched and grown to adults in seawater (34 ppt salinity, 20 °C, slightly aerated) under constant light (2000 lux) in white polyethylene flat bottom tanks (100 L). The culture water was partially removed and exchanged with seawater every 3–4 days (30–40%). The copepods were fed *R. baltica* three times a day. Eggs were collected daily for a period of 3.5 months by siphoning the bottom material, selecting the particulate fraction between 38 and 120 μm, rinsing with chilled autoclaved seawater, and finally storing at 1.5–4 °C (20 ml glass tubes). The number of eggs was estimated from sub-samples (100 μl) that were diluted (10 or 20 ml) and counted under a microscope (10 × 50 μl). The average daily yield through the harvesting period of two consecutive stocking series was 1170 ± 530 eggs L^{-1} day^{-1}. The harvested eggs were kept refrigerated at 1.5–4 °C and the hatching was maintained through 3 months of storage (70–83%).

In the second phase of live feed production, the required amount of eggs needed for live feed for each day in the experiment was hatched in separate tanks (20–40 nauplii mL^{-1}) and further cultured for 2–3 days (nauplii stages I–III) or for 4–6 days (nauplii stages III–IV) using the same cultivation conditions as described above.

Table 1
Schematic description of methods used to produce live feed diets for the two first feeding experiments. The rotifer *Brachionus plicatilis*, strain Nevada (former SINTEF strain), was grown in semi-continuous culture in 250 L tanks, 20–22 °C, 20 ppt salinity, and fed and harvested once daily (n = 3–4). *Acartia* nauplii were cultured in 100 L tanks based on produced resting eggs, 20 °C, 34 ppt salinity (n = 2). For more details, see text.

Larval live feed diets	Production procedure
Brachionus-Low	
Brachionus plicatilis Nevada	Cultivation feed: Baker's yeast *Saccharomyces cerevisiae* (1.2 μg FW ind^{-1} day^{-1}) and
Low percentage DHA in PL	*Nannochloropsis* sp. paste (Reed Mariculture), 0.5 mL 10^{-6} ind day^{-1}
Short-term n−3 HUFA enrichment just before application	Feed for short-term enrichment: lipid emulsion Marol E (SINTEF), 0.16 μg emulsion ind^{-1}, 2 h of incubationRotifer concentration at steady state: 130–150 ind mL^{-1}
Brachionus-High	
Brachionus plicatilis Nevada	Cultivation feed: Baker's yeast *Saccharomyces cerevisiae* (1.2 μg FW ind^{-1} day^{-1}),
High percentage DHA in PL	*Nannochloropsis* sp. paste (Reed Mariculture), 0.5 ml 10^{-6} ind day^{-1} and lipid emulsion Marol E (SINTEF), 0.09 μg emulsion ind^{-1} day^{-1}
Long-term n−3 HUFA enrichment during cultivation	Rotifer concentration at steady state: 160–180 ind mL^{-1}
Acartia nauplii	
Acartia tonsa, identification code DFH-ATI, Roskilde University, Denmark (Støttrup et al., 1986)	Production and storage of eggs Hatching of eggs and cultivation of nauplii Cultivation feed: *Rhodomonas baltica* NIVA 5/91: 8–9 μm Stages I–III, 2–3 days of growth, length 110–190 μm Stages III–IV, 4–6 days of growth, length 190–310 μm

2.1.4. First feeding trials

Seawater was taken from 70 m depth in the Trondheim fjord and was passed through a sand filter (20 μm) and a bio-filter unit for re-colonisation of bacteria prior to use. Fertilised eggs of Atlantic cod (*G. morhua*) from Marine Breed (Sunndalsøra, Norway) were disinfected (glutaraldehyde; 400 ppm, exposure for 6 min, Salvesen et al., 1997) before distribution to the larval rearing tanks. In each experiment, six tanks were stocked with cod eggs (40 eggs L^{-1}) 2 days prior to hatching (Table 2). The microalga *I. galbana* was added to larval tanks before eggs and live feed and a slow exchange of water was initiated at 3 dph. Tanks were equipped with skimmers. The bottom precipitate was removed three times a week and dead larvae were counted. *A. tonsa* nauplii concentrations in the fish tanks were kept at 5 ind ml^{-1} and rotifers at 5–7 ind ml^{-1}. Live feed organisms and microalgae were added to the tanks three times daily, and the live feed concentration was then adjusted to the specified concentrations.

2.2. Sampling and analytical methods

The DW of larvae was analysed at 2 and 17 dph. Larvae were anaesthetised with metacaine (Finquel®, Argent Laboratories, Redmond, USA), rinsed for a few seconds in fresh water, and transferred to individually pre-weighted tin capsules (n = 12 for each tank and sampling day). Capsules were dried for a minimum of 48 h at 60 °C before weight determination. Values are given as mean weight of larvae from the respective treatment tanks ± standard error (SE).

Larval survival was estimated from the initial number of eggs corrected for % hatching rate, the number of larvae at the end of the experiment, and counts of sampled and dead larvae removed during the experiment.

2.3. Lipid analyses

Samples for lipid analyses were taken from feed components and steady state cultures of *R. baltica*, *Brachionus*-Low and *Brachionus*-High, and from cultures of *A. tonsa* nauplii grown for 2–3 and 4–6 days. Larvae for lipid analyses were anaesthetised, rinsed in freshwater (6 °C), and transferred to sample tubes added N_2 and immediately frozen at −80 °C.

Freeze-dried samples were crushed and homogenised. The lipids were extracted by the Bligh and Dyer method (Bligh and Dyer, 1959), and lipid content was determined gravimetrically. Fatty acid methyl esters were produced as described by Metcalfe et al. (1966) and analysed as described by Standal et al. (2008).

Table 2
Experimental conditions and sampling schedule used for cod larvae fed the three live feed diets (Table 1). Larval feeding was initiated on 3 dph and the experiment lasted till day 17, which is the normal period of rotifer feeding of cod larvae used in our laboratory. Larvae were fed three times daily; the density of live feed was then increased to the density indicated. The concentration of *Isochrysis galbana* was controlled daily and maintained within 1–1.5 mg C L^{-1}. n indicates the number of larval tanks of the treatment.

Days post-hatching (dph)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Temperature, °C	6	6	7	7	8	8	9	9	10	11	12	12	12	12	12	12	12
Light (40 W)	Dark	→	Continuous light conditions														←
Water exchange, day^{-1}			→	1×							←	→	4×				←
Microalgal addition	→	*Isochrysis galbana* (1–1.5 mg C L^{-1})															←
Experiment 1																	
Low-B larvae, n = 3			→	*Brachionus*-Low[a] (5–7 ind mL^{-1})													←
High-B larvae, n = 3			→	*Brachionus*-High[a] (5–7 ind mL^{-1})													←
Sampling of larvae		x							x								x
Experiment 2																	
High-B larvae, n = 4			→	*Brachionus*-High[a] (5–7 ind mL^{-1})													←
AT larvae, n = 2			Nauplii I–III[b]				→ Nauplii III–IV[c] (5 ind mL^{-1})										←
Sampling of larvae		x															x

[a] Length of rotifers was 130–340 μm.
[b] Nauplii stages I–III, 2–3 days of growth, length 110–190 μm.
[c] Nauplii stages III–IV, 4–6 days of growth, length 190–310 μm.

Lipid class determination was performed by high performance thin-layer chromatography (HPTLC), as described in Olsen and Henderson (1989) with a few modifications. A known amount of lipids was applied in bands, making use of Linomat IV (CAMAG, Muttenz, Switzerland) equipped with an N_2 spray unit and a Hamilton syringe (100 μl). Non-polar lipids were separated with a one-dimensional double development system adapted from Skipski et al. (1965). The first development was in diisopropylether:acetic acid (96:4, v/v) and the second was in petroleum ether (fraction 60–70 °C):diethyl ether:glacial acetic acid (90:10:1, v/v/v). PL were developed using methyl acetate:2-propanol:chloroform:methanol:0.25% potassium chloride (25:25:25:10:9, v/v/v/v) (Olsen and Henderson, 1989). All solvents were pro analysis quality or higher. Twin trough chambers (CAMAG Muttenz, Switzerland) lined with filter paper on one side were used for plate development and all solvents used were pro analysis grade or higher.

Developed plates were dried (air; 10 min, 20 °C) before they were submerged in 3% Cu (II) acetate in 8% phosphoric acid (w/v) (Fewster et al., 1969), followed by air drying, the first 10–15 min flat on a table and thereafter, 10 min with a hairdryer (20 °C), and finally charring at 140 °C for 40 min (CAMAG plate heater). The plates were analysed by photodensitometry in absorption mode at 325 nm using Scanner 3 (CAMAG) and winCATS software (1.42). Samples and standards were always applied at regular intervals on the plates in addition to a blank track. Replicate analyses were performed on four separate plates, and the lipid classes were quantified against calibration curves (Olsen and Henderson, 1989).

PL fatty acid composition was analysed using thin-layer chromatography (TLC) according to Fried and Sherma (1999). Extracted lipid samples was applied on TLC glass plates (SILICA GELslica gel 60 F254, MERCK KGaA, Darmstadt, Germany type (MERC)) using a CAMAG Linomat IV application machine and Hamilton-syringe (100 μL), the same instruments as described above. The plates were developed using a nonpolar solvent (hexane:diethyl ether:acetic acid, 80:20:1, v/v/v). The polar lipids remained in the application field, and the procedure was repeated. The plates were thereafter dried in a ventilation cabinet. The PL spots, identified by ultraviolet (UV) light, were scraped and analysed for percentage of fatty acid composition after methylation as described above.

2.4. Statistical analysis

Differences in fatty acid composition between the larval groups and the feed organisms were tested at the 5% level of significance using one way ANOVA. Larval weights from each tank were tested for normality by D'Agostino and Pearson test, and the two treatments were tested for differences by unpaired *t*-test at the 5% level of significance. Significant outliers were tested by use of Grubbs test. Differences in survival were tested using unpaired *t*-test at the 5% level of significance. Correlations between components in feed and larvae were assessed using linear regression. Statistical testing was performed using Sigmaplot version 12.3.

3. Results

3.1. Live feed diets

The rotifers were fed Baker's yeast, *Nannochloropsis* paste, and Marol E; the latter represented the main lipid source of the food of the rotifers, showing variable contents of lipids and n−3 HUFA (Table 3). DHA was the dominant fatty acid of the Marol E emulsion. *R. baltica* showed similar contents of eicosapentaenoic acid (EPA; 20:5 n−3) and DHA and was used alone for the cultivation of *A. tonsa* nauplii.

The contents of total lipids and total fatty acids in the two rotifer larval diets (*Brachionus*-Low and *Brachionus*-High) were similar whereas the *Acartia* nauplii (stages III–IV) exhibited lower lipid and fatty acid contents (Table 4, Fig. 1A). The three live feed diets analysed showed a PL content of around 7% of DW (range 6.6–7.3%) (Fig. 1B), corresponding to 42% of total lipids (range 41–43%) for the rotifer diets and 73% for the *Acartia* nauplii (Fig. 1C). TAG were the second most dominant lipid class of the rotifer diets, but TAG were only present in low amounts in *Acartia* nauplii, which instead

Table 3
Lipid composition (mean ± SE) of diet components for production of rotifers and *Acartia tonsa*. ∑SFA is the sum of saturated fatty acids, ∑MUFA is the sum of mono-unsaturated fatty acids, ∑n−6 is the sum of n−6 polyunsaturated fatty acids, ∑n−3 is the sum of n−3 polyunsaturated fatty acids, ∑PUFA is the sum of n−6 and n−3 polyunsaturated fatty acids, DHA is docosahexaenoic fatty acid (22:6 n−6), EPA is eicosapentaenoic fatty acid (20:5 n−3), unknown is the sum of unidentified fatty acids, n is the number of replicate analysis (Marol E, see text).

	Baker's yeast *Saccharomyces cerevisiae* (n = 2)	*Nannochloropsis* paste (n = 4)	*Rhodomonasbaltica* (n = 3)	Marol E (n = 3)
Total lipids, mg g DW^{-1}	7.34 ± 0.13	118 ± 6.26	154 ± 4.70	684 ± 2.00
Total fatty acids, mg g DW^{-1}	3.16 ± 0.07	56.1 ± 1.88	62.3 ± 2.90	509 ± 4.74
Fatty acids, % of total FA				
14:0	2.45 ± 0.47	2.82 ± 0.02	6.91 ± 0.04	13.1 ± 0.43
16:0	12.1 ± 0.25	19.5 ± 0.09	5.54 ± 0.07	12.0 ± 0.40
18:0	4.26 ± 0.28	0.44 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
∑SFA	19.0 ± 0.46	22.7 ± 0.10	12.4 ± 0.11	25.1 ± 0.83
16:1 n−7	30.8 ± 2.66	21.4 ± 0.13	0.67 ± 0.01	2.89 ± 0.09
18:1 n−7	1.74 ± 0.22	0.79 ± 0.01	3.05 ± 0.01	0.00 ± 0.00
18:1 n−9	32.1 ± 1.00	3.22 ± 0.01	0.61 ± 0.01	26.3 ± 0.88
20:1 n−9	2.27 ± 0.75	0.00 ± 0.00	0.00 ± 0.00	0.88 ± 0.03
∑MUFA	69.8 ± 1.87	25.4 ± 0.12	4.32 ± 0.02	30.1 ± 0.99
18:2 n−6	2.92 ± 0.26	2.77 ± 0.02	5.31 ± 0.02	1.67 ± 0.49
18:3 n−6	0.00 ± 0.00	0.00 ± 0.00	0.69 ± 0.01	0.00 ± 0.00
20:4 n−6	0.00 ± 0.00	3.34 ± 0.03	0.59 ± 0.01	0.00 ± 0.00
∑n−6	3.14 ± 0.33	6.63 ± 0.06	6.59 ± 0.03	1.67 ± 0.05
18:3 n−3	0.87 ± 0.15	0.13 ± 0.00	23.8 ± 0.03	0.00 ± 0.00
18:4 n−3	0.28 ± 0.16	0.11 ± 0.00	24.8 ± 0.06	0.00 ± 0.00
20:4 n−3	0.14 ± 0.05	0.00 ± 0.00	0.57 ± 0.01	0.00 ± 0.00
20:5 n−3	0.63 ± 0.19	32.4 ± 0.25	9.42 ± 0.01	0.72 ± 0.02
22:5 n−3	0.19 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
22:6 n−3	0.75 ± 0.29	0.16 ± 0.01	8.05 ± 0.05	41.1 ± 2.09
∑n−3	2.85 ± 0.88	32.8 ± 0.26	66.6 ± 0.15	41.8 ± 2.07
∑PUFA	5.99 ± 1.21	39.4 ± 0.30	73.2 ± 0.10	43.5 ± 2.02
DHA:EPA	1.67 ± 0.11	0.00 ± 0.00	0.85 ± 0.01	57.4 ± 4.44
Unknown	5.23 ± 1.23	12.5 ± 0.08	10.1 ± 0.03	0.48 ± 0.02

Table 4
Total lipid and total fatty acid contents and fatty acid composition (mean ± SE) of the live feed diets. For definitions of live feed categories, see legend of Table 1. Lipid acronyms are defined in legend of Table 3. n: number of replicate cultures. nd: not detected. nm: not measured.

Live feed	*Brachionus*-Low (n = 2)	*Brachionus*-High (n = 2)	*Acartia tonsa* NI–III (n = 2)	*Acartia tonsa* NIII–VI (n = 3)
Total lipids (mg g DW^{-1})	175 ± 1	168 ± 3	73 ± 3.0	94 ± 5
Total fatty acids (mg g DW^{-1})	107 ± 5	109 ± 2	nm	47.1 ± 16.5
% of total FA				
12:0	2.94 ± 0.17	0.88 ± 0.02	nd	nd
14:0	9.39 ± 0.55	6.32 ± 0.03	3.3 ± 0.1	4.7 ± 0.3
16:0	10.2 ± 0.53	8.59 ± 0.05	12.6 ± 0.8	14.7 ± 1.5
18:0	2.27 ± 0.08	2.51 ± 0.01	6.1 ± 0.5	3.9 ± 1.2
∑SFA	24.8 ± 0.79	18.3 ± 0.06	22.0 ± 1.3	23.3 ± 2.5
16:1 n−7	9.61 ± 0.49	9.54 ± 0.02	0.9 ± 0.6	0.6 ± 0.2
18:1 n−7	1.98 ± 0.10	2.18 ± 0.00	3.2 ± 1.3	3.0 ± 0.3
18:1 n−9	19.3 ± 1.05	20.1 ± 0.01	0.7 ± 0.4	0.4 ± 0.3
20:1 n−7 + 9	1.93 ± 0.10	3.29 ± 0.01	0.2 ± 0.1	0.1 ± 0.1
22:1 n−9 + 11	0.59 ± 0.03	1.00 ± 0.01	0.5 ± 0.1	0.4 ± 0.3
∑MUFA	33.4 ± 1.17	36.1 ± 0.03	5.5 ± 2.4	4.5 ± 0.3
18:2 n−6	4.48 ± 0.21	4.49 ± 0.01	4.3 ± 0.1	1.9 ± 0.6
18:3 n−6	nd	nd	0.5 ± 0.1	0.2 ± 0.1
20:2 n−6	0.34 ± 0.02	0.38 ± 0.04	1.0 ± 0.1	0.4 ± 0.1
20:4 n−6	0.19 ± 0.00	0.17 ± 0.01	1.3 ± 0.1	0.5 ± 0.1
∑n−6	5.01 ± 0.21	5.04 ± 0.04	7.1 ± 0.5	3.0 ± 0.8
18:3 n−3	1.14 ± 0.05	0.99 ± 0.00	5.2 ± 0.4	10.4 ± 3.8
18:4 n−3	nd	nd	2.2 ± 0.1	9.5 ± 5.4
20:3 n−3	0.62 ± 0.04	0.17 ± 0.00	0.2 ± 0.0	0.5 ± 0.1
20:4 n−3	nd	nd	0.3 ± 0.1	0.8 ± 0.2
20:5 n−3	0.96 ± 0.04	3.67 ± 0.00	16.0 ± 0.2	18.7 ± 1.9
22:5 n−3	0.71 ± 0.18	1.73 ± 0.42	nd	0.9 ± 0.3
22:6 n−3	26.2 ± 1.55	22.3 ± 0.09	39.4 ± 1.3	28.4 ± 6.1
∑n−3	29.6 ± 1.56	28.9 ± 0.43	63.3 ± 2.1	69.2 ± 2.2

contained more free fatty acids (FFA). Cholesterol was slightly more abundant in rotifers than in the copepod nauplii.

The fatty acid composition of the total lipids of rotifers was almost equal for both rotifer diets (Table 4). Saturated fatty acids were slightly more abundant and monounsaturated fatty acids were slightly less abundant in *Brachionus*-Low than in *Brachionus*-High, whereas the contents of both n−3 and n−6 polyunsaturated fatty acids (PUFAs) were equal ($p > 0.05$, one way ANOVA). *Acartia* nauplii (stages I–III and III–IV) exhibited a similar percentage content of saturated and essential n−6 fatty acids as the rotifers, but showed a much lower content of monounsaturated fatty acids and two times higher contents of essential n−3 fatty acids.

Among the dominant fatty acids of total lipids (Fig. 2), DHA showed slightly higher ($p < 0.05$, one way ANOVA) and EPA slightly lower ($p < 0.05$, one way ANOVA) contents (Fig. 2A, Table 4) and fractions (Fig. 2B) in *Brachionus*-Low than in *Brachionus*-High. EPA contents were relatively low in both rotifer types, leading to a DHA:EPA ratio of 22 and 6.1 for *Brachionus*-Low and *Brachionus*-High, respectively.

The quantitative DHA contents of *Acartia* nauplii (stages I–III and III–IV) were lower than those of the rotifers, constituting on average 53% of that found in rotifers (range 48–59%). The EPA contents were variable among the samples, but always highest in the *Acartia* nauplii. The percentage contents of DHA of total fatty acids in the *Acartia* nauplii were, however, equal to or higher than that of the rotifers, but the content in nauplii stages III–IV, which was used through most of the feeding period in Experiment 2 (Table 2), was not significantly different from that of the rotifer diets ($p > 0.05$, one way ANOVA). The content of arachidonic acid (ARA; 20:4 n−6) was 0.2 and 0.2–0.5 mg g DW^{-1} in rotifers and *Acartia* nauplii, respectively. Finally, the content of 18:1 n−9 was far higher in both rotifer diets than in the *Acartia* nauplii (Fig. 2, Table 4).

The data revealed a gradient in percentage DHA contents in the PL fraction of the rotifer larval diets and in *Acartia* nauplii (stages III–IV) (Fig. 3), with the lowest value for *Brachionus*-Low, intermediate value for *Brachionus*-High, and highest value for *Acartia* nauplii ($p < 0.05$, see legend). EPA contents in PL showed a similar significant pattern as DHA, whereas 16:0 and 18:1 fatty acids were found in more equal and variable amounts. The percentage content of ARA in PL was 0.4 and 0.9% of total PL fatty acids in rotifers and *Acartia* nauplii, respectively.

3.2. Cod larvae

The contents of total lipids and total fatty acids were equal for newly hatched yolk sac larvae (2 dph) and for 17 dph larvae fed the different live feed diets ($p > 0.05$, one way ANOVA) (Fig. 4A, Table 5). The contents of PL per DW (Fig. 4B) and per total lipids (Fig. 4C) were also similar, constituting on average 98 mg PL g DW^{-1} (range 90–102 mg g DW^{-1}) and 64% PL of total lipids (range 60–68%), respectively. The contents of TAG and FFA were low throughout and the cholesterol content was lowest in larvae fed *Acartia* (AT larvae).

The contents of saturated fatty acids were similar for all groups, but were slightly higher for newly hatched larvae than for the 17 dph larvae (Table 5). Monounsaturated fatty acids, dominated by 18:1 n−9, were found at the highest levels in larvae fed rotifers, whereas AT larvae maintained their initial level of monounsaturated fatty acids. Larvae fed *Brachionus*-Low (Low-B larvae) showed higher 18:2 n−6 and Σn−6 contents than the other groups, whereas ARA contents was lowest in larvae fed *Brachionus*-High (High-B larvae). The mean ARA content of all groups was 1.4% of total fatty acids and 1.3 mg g DW^{-1} (range 0.6–1.8 mg g DW^{-1}). The Σn−3 fatty acids became significantly reduced in 17 dph larval groups fed rotifers ($p < 0.05$, one way ANOVA) compared to that at 2 dph, whereas it increased significantly, by more than 20%, in AT larvae ($p < 0.05$, one way ANOVA). DHA was the dominant n−3 fatty acid, and both DHA and EPA exhibited similar patterns of variation as Σn−3 fatty acids in total lipids of the cod larvae.

The composition of the dominant fatty acid in PL for the 17 dph larval groups (Fig. 5) revealed a significant gradient in percentage DHA and EPA contents in PL ($p < 0.05$, one way ANOVA), with the lowest values in Low-B larvae, intermediate values in High-B larvae, and the highest values in AT larvae, which maintained the initial DHA and EPA levels of the 2 dph larvae (Fig. 5). The contents of 16:0 and 18:1 fatty acids varied more unsystematically and were similar for all feed groups. The percentage content of ARA in PL was 0.4 and 0.9% of PL fatty acids in rotifer- and *Acartia*-fed larvae, respectively.

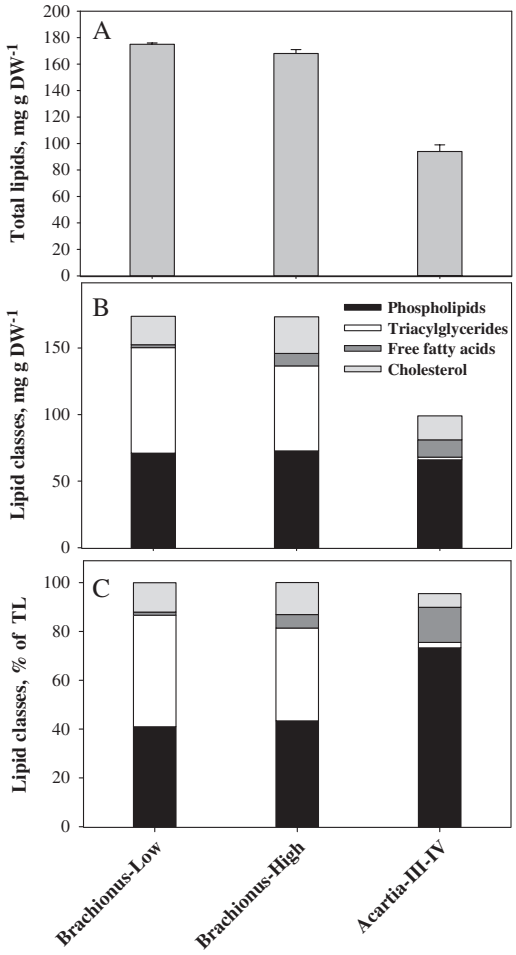

Fig. 1. Total lipids (A: mg g DW^{-1}) and lipid class composition (B: mg g DW^{-1} and C: % of total lipids) of the three live feed diets. Bars express 1 SE.

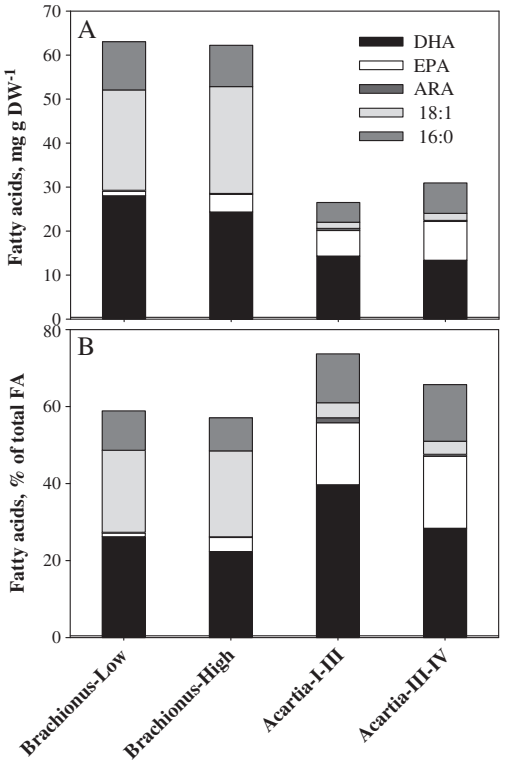

Fig. 2. Quantitative contents (A: mg g DW^{-1}) and composition (B: % of total fatty acids) of dominant fatty acids in total lipids of the three live feed diets, including *Acartia* nauplii stages I–III.

3.3. Live feed versus cod larvae

Summarising our data, a positive relationship between both DHA and EPA in PL of the diets and in PL of 17 dph larvae was apparent (Figs. 3 and 5). It is noteworthy that the quantitative PL contents and the percentage DHA of total lipids in all diets were similar (Fig. 1 and *Acartia*-II–IV in Fig. 2); the percentage DHA content of total fatty acids was in fact lowest for *Brachionus*-High (Fig. 2).

The relationship between percentage contents of the dominant fatty acids and ARA in the live feed versus those of the corresponding 17 dph larvae is further illustrated in Fig. 6. The results suggested a close relationship between the composition of dominant PL fatty acids in the diet and in the larvae. There was a significant positive relationship for all data after normalising values of the individual fatty acid contents to their respective maximum value (see inset, $r^2 = 0.84$, $p < 0.001$, $n = 15$), suggesting that 84% of the variability in the PL composition of cod larvae could be explained by the variation in PL composition of their feed (90% for DHA). The relationship for normalised data for percentage fatty acids in total lipids of the diets and that of PL of the larvae showed a significant positive relationship as well ($r^2 = 0.66$, $p < 0.001$, $n = 15$), but the DHA contents in total dietary lipids were similar for all diets and did not correlate with DHA in PL of the larvae ($r^2 = 0.12$).

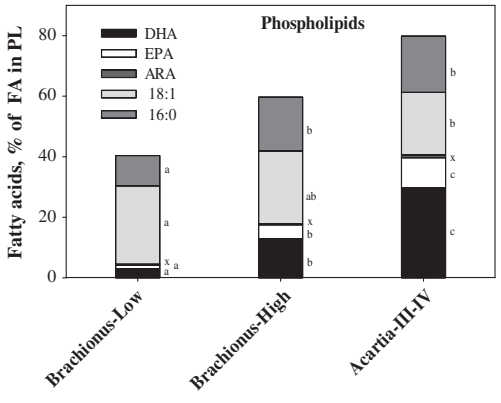

Fig. 3. Composition of dominating fatty acids in PL of the three live feed diets (% of PL fatty acids). Equal indexes for the individual fatty acids indicate that percentages are not significantly different ($p > 0.05$, one way ANOVA). Index x for ARA indicates that ANOVA coefficients could not be estimated.

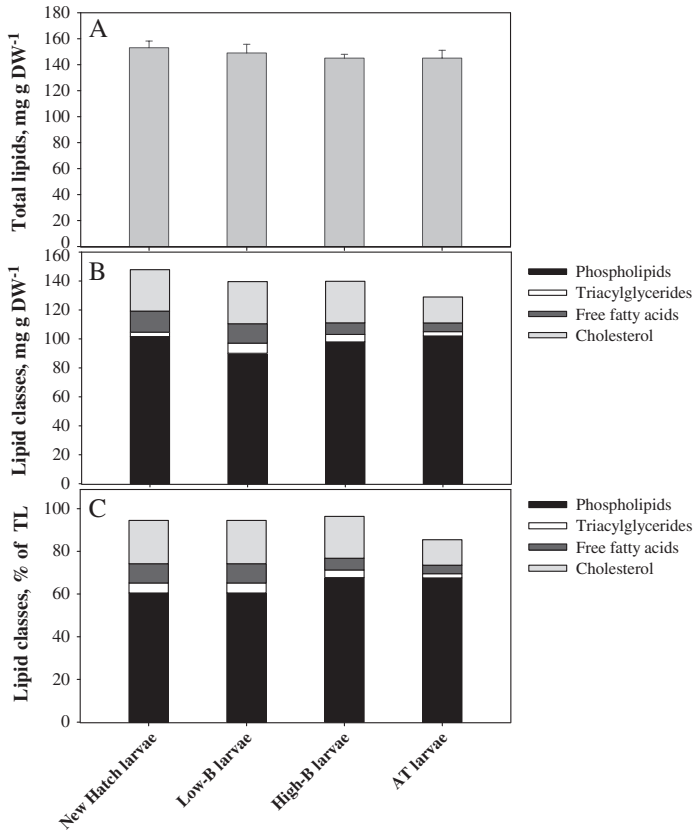

Fig. 4. Total lipids (A: mg g DW^{-1}) and lipid class composition (B: mg g DW^{-1} and C: % of total lipids) of 2 dph yolk sac larvae of cod (NewHatch larvae) and of larvae fed different live feed diets till 17 dph. Bars express 1 SE.

3.4. Growth and survival of cod larvae

High-B larvae were significantly bigger than Low-B larvae at 17 dph in Experiment 1 ($p < 0.05$, *t*-test), and AT larvae were significantly bigger than High-B larvae in Experiment 2 ($p < 0.05$, *t*-test) (Table 6). *Brachionus*-High acted as a control diet across both experiments. The size of the cod egg was different for the experiments, as reflected by the larval biomass at 2 dph (Table 6), and the results across the two experiments were therefore not directly comparable. Fig. 7 shows pooled results for the two experiments after normalising the results to their respective control treatment. The final DW biomass of the larvae was significantly different between all treatments ($p < 0.05$ for all, *t*-test). The survival of AT larvae at 17 dph was significantly higher than that of High-B larvae (Experiment 2, $p < 0.05$), but no significant difference was apparent in survival of Low-B and High-B larvae (Experiment 1).

4. Discussion

The main questions addressed in the present study were if larvae of cod incorporated DHA into their tissue PL more efficiently from dietary PL than from dietary TAG in the early stages of first feeding and if they therefore grew and performed better. We have found that 17 dph Atlantic cod larvae fed different diets of the rotifer *B. plicatilis* Nevada and nauplii stages III–IV of *A. tonsa* showed a positive relationship between percentage DHA and EPA in cod larval PL and percentage DHA and EPA in PL of the live feed ($p < 0.05$, one way ANOVA). Moreover, high DHA and EPA contents of PL of larval tissues coincided with the higher larval growth rate at 17 dph. The results suggested that the availability of HUFA from PL and TAG must have been different and that dietary PL was the main source of PL synthesis through digestion and re-acylation of lyso-PL in the enterocytes of the larvae. These results support the view that de novo synthesis of PL from diacylglyceride (DAG) and cytidine diphosphate (CDP)-activated polar groups (e.g., choline, ethanolamine) or from CDP-activated DAG and an inactivated polar group (Tocher et al., 2008) was inefficient. The better availability of n − 3 HUFA for PL synthesis from dietary PL than from neutral lipids (e.g., TAG) and a very inefficient de novo synthesis of PL have been demonstrated using formulated diets for many marine and freshwater species (see reviews by Cahu et al., 2009; Coutteau et al., 1997; Tocher et al., 2008). A limited capacity of de novo synthesis of PL in fish larvae was first described by Kanazawa (e.g., Kanazawa, 1985), and there is ample evidence that this is a general feature.

In this regard, it is important that both rotifers and *Artemia* appear to have low n − 3 HUFA contents in their PL (Coutteau and Mourente, 1997; Li et al., 2014), whereas copepod nauplii has PL as the main lipid class (Fig. 1) and exhibit very high DHA and EPA contents in their PL (Fig. 3) (Evjemo et al., 2003; Li et al., 2014; McEvoy et al.,

Table 5
Total lipid and total fatty acid contents and fatty acid composition (mean ± SE) of newly hatched yolk sac larvae (2 dph) and 17 dph cod larvae fed the different diets. Low-B larvae is fed *Brachionus*-Low (see Table 1), High-B larvae is fed *Brachionus*-High (see Table 1), AT-Larvae is fed *Acartia* nauplii (see Table 1). Lipid acronyms are defined in the legend of Table 3.

Cod larvae	Newly hatched 2 dph larvae (n = 8)	Low-B larvae (17 dph) (n = 6)	High-B larvae (17 dph) (n = 6)	AT larvae (17 dph) (n = 4)
Total lipids (mg g DW^{-1})	153 ± 5.2	149 ± 6.7	145 ± 3.0	145 ± 6.1
Total fatty acids (mg g DW^{-1})	95.1 ± 6.2	96.8 ± 4.3	94.0 ± 2.0	98.9 ± 2.4
% of total FA				
14:0	1.0 ± 0.3	2.11 ± 0.07	2.45 ± 0.04	1.9 ± 0.4
16:0	24.1 ± 0.5	13.9 ± 0.08	14.8 ± 0.15	18 ± 1.7
18:0	5.2 ± 0.25	8.29 ± 0.24	8.04 ± 0.08	6.1 ± 0.7
20:0	0.1 ± 0.01	0.12 ± 0.05	0.09 ± 0.1	nd
∑SFA	30.4 ± 0.63	24.4 0.27	25.3 0.20	26.0 ± 1.9
16:1 n−7	2.0 ± 0.2	7.38 ± 0.25	6.14 ± 0.08	3.7 ± 1.8
18:1 n−7	4.0 ± 0.1	5.67 ± 0.14	4.87 ± 0.05	4.0 ± 0.4
18:1 n−9	11.2 ± 1.3	14.8 ± 0.14	15.0 ± 0.13	7.6 ± 3.4
20:1 n−9	1.8 ± 0.4	2.11 ± 0.01	2.46 ± 0.03	0.6 ± 0.4
∑MUFA	19.0 ± 1.38	30.0 0.32	28.5 0.163	15.9 ± 3.9
18:2 n−6	1.9 ± 0.3	7.46 ± 0.14	4.02 ± 0.02	3.0 ± 1.4
18:3 n−6	0.1 ± 0.1	nd	nd	0.2 ± 0.2
20:2 n−6	0.3 ± 0.1	1.0 ± 0.01	0.54 ± 0.02	0.7 ± 0.5
20:3 n−6	0.3 ± 0.05	1.0 ± 0.1	0.29 ± 0.05	nd
20:4 n−6	1.8 ± 0.1	1.21 ± 0.02	0.64 ± 0.01	1.8 ± 0.4
∑n−6	4.3 ± 0.35	10.7 ± 0.14	5.5 ± 0.03	5.7 ± 1.6
18:3 n−3	0.3 ± 0.1	1.56 ± 0.08	0.96 ± 0.01	4.5 ± 1.5
18:4 n−3	0.2 ± 0.1	nd	nd	2.7 ± 0.4
20:3 n−3	0.2 ± 0.05	1.01 ± 0.03	0.63 ± 0.1	0.3 ± 0.1
20:4 n−3	0.2 ± 0.05	nd	nd	0.8 ± 0.3
20:5 n−3	13.4 ± 0.9	2.51 ± 0.02	4.2 ± 0.1	12.5 ± 0.4
22:5 n−3	1.2 ± 0.1	0.51 ± 0.01	1.38 ± 0.03	1.1 ± 0.3
22:6 n−3	26.7 ± 1.6	14.4 ± 0.32	20.9 ± 0.59	29.9 ± 1.9
∑n−3	42.1 ± 1.85	20.0 ± 0.33	28.0 ± 0.60	51.8 ± 2.5

1998). Our study has again confirmed that both copepod nauplii and newly hatched cod larvae have PL as the main lipid class, very low TAG contents, and a similar fatty acid composition of their PL. This means that DHA and EPA are mainly associated with PL, and this is likely why cod larvae fed copepod nauplii grow faster and perform better. On the contrary, as rotifers and *Artemia* both appear to have most of their n−3 HUFA associated with TAG following enrichment, we suggest that low availability of the enriched n−3 HUFA of the larvae may be one reason why the quality of Atlantic cod and Atlantic halibut juveniles fed rotifers and *Artemia* is still relatively poor while copepods give better growth and performance of the fry (McEvoy et al., 1998). We therefore question if it is possible to modify the PL composition of rotifers and *Artemia* to make them fully adequate as live feed for the mentioned fish species. In this regard, it may also be important that high DHA in TAG has appeared to result in high mortality for sea bass (Cahu et al., 2009).

4.1. Live feed diets

The problem of using experimental live feed diets to study PL nutrition of fish larvae is well recognised (e.g., Tocher et al., 2008). The main general challenge is to design diets where one principal component is varied whereas the remaining components are kept relatively constant over some time, like for formulated feeds. Regarding PL and live feed, it

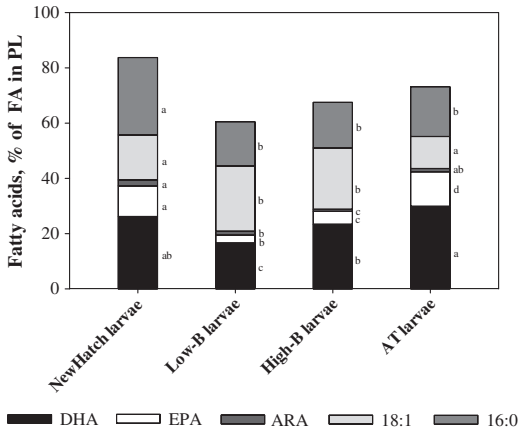

Fig. 5. Composition of dominating fatty acids in PL of 2 dph yolk sac larvae (NewHatch larvae) and of larvae fed different live feed diets till 17 dph (% of PL fatty acids). Equal indexes for the individual fatty acids indicate that percentages are not significantly different ($p > 0.05$, one way ANOVA).

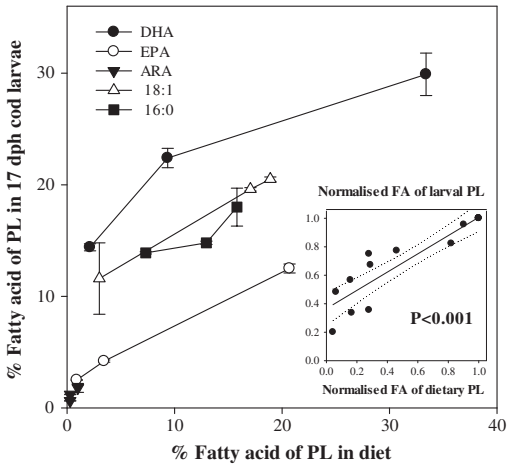

Fig. 6. Composition of dominant fatty acids in PL (% of PL fatty acids) in 17 dph larvae as a function of the respective fatty acid composition in the dietary PL. Inset shows the similar relative composition after normalising contents of the individual fatty acid to the respective maximal value, with regression line and 95% *CI* included.

Table 6
Larval weights at 2 and 17 dph and survival at 17 dph for treatments and experiments (mean ± standard error). n: number of replicate larval tanks. Larval acronyms are defined in the legend of Table 5.

Experiment	Biomass at 2 dph (μg DW ind^{-1})	Biomass at 17 dph (μg DW ind^{-1})	Survival 17 dph (% of initial)
Experiment 1			
Low-B larvae, n = 3	72	335 ± 26	41.9 ± 0.1
High-B larvae, n = 3		423 ± 23	42.7 ± 4.4
Experiment 2			
High-B larvae, n = 4	60	240 ± 26	71.7 ± 7.6
AT-larvae, n = 2		324 ± 36	86.3 ± 2.3

is not possible to vary the PL content per DW of live feed very much, whereas PL per lipid is variable and dependent on the total lipid contents of the live feed (Li et al., 2014; Rainuzzo et al., 1994b). It is also possible to manipulate the fatty acid composition of the PL of live feed, and we have demonstrated a technique to produce larval live feed diets based on rotifers that are characterised by different percentages of n−3 HUFA in PL while most other factors remain relatively constant. It is difficult to prepare a larval diet based on copepods with the same lipid characteristics as the rotifer diets, but it is possible to prepare

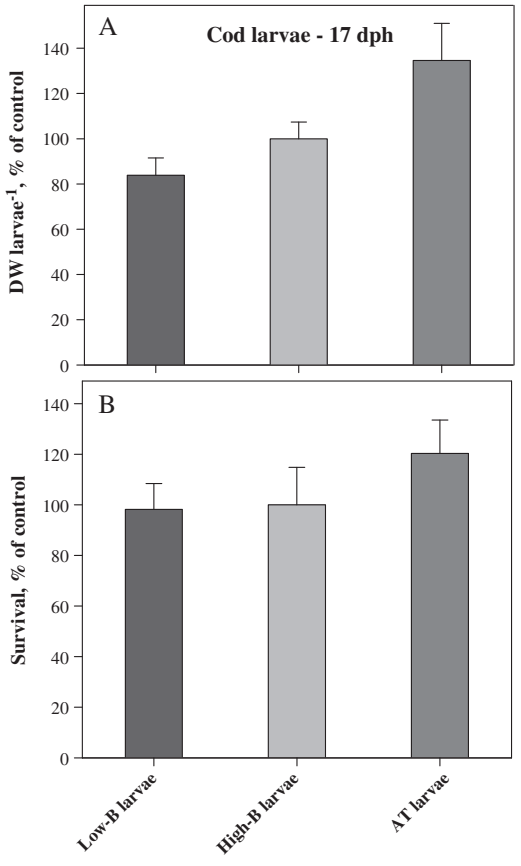

Fig. 7. A compilation of growth (final DW larvae^{-1}) and survival (% of initial stock) of 17 dph cod larvae fed different live feed diets in the two first feeding experiments. Values of DW and survival in the two experiments were normalised to the respective control (High-B larvae). Bars express 1 SE.

similar diets in studies where a few or one essential fatty acid is given priority.

The *Acartia*-based live feed diet exhibited lipid and fatty acid contents that were slightly different from the two rotifer diets, but some key characteristics were similar. The percentage DHA in PL of *Acartia*-based live feed diet was higher than that of both rotifer diets (30%) and the lipid content was lower, but PL per DW was the same. Moreover, the nauplii contained very little TAG and slightly less cholesterol than the rotifer diets. The quantitative DHA content of the rotifer treatments was also higher than that of the copepod nauplii, but the percentage DHA of total fatty acids was the same; the EPA content was throughout higher, whereas the content of 18:1 fatty acid was lower.

The feed components used for *Brachionus*-Low in the present study were the same as those used for *Brachionus*-High; the only difference was in how the lipid emulsion was provided during production (Table 1). The lipid characteristics of the two rotifer diets were almost identical; their total lipids, total fatty acids, DHA and n−3 HUFA, and PL content per DW were almost equal, and their percentage DHA of total fatty acids was similar, although slightly higher in the *Brachionus*-Low diet that still had lower DHA in PL. The EPA content of rotifer diets and accordingly also the DHA:EPA ratio were not strictly the same, but EPA was relatively low (<4%) and the DHA:EPA ratio was above that of the copepods (>1.5) for both rotifer treatments. The most striking difference of the diets was the higher percentage DHA of total PL fatty acids of *Brachionus*-High (9.4% of total PL fatty acids) compared with that of *Brachionus*-Low (2.1%). The higher percentage DHA was not accompanied by a comparable reduction in a single other fatty acid in the PL of the rotifers. In conclusion, the three larval diets formed a gradient in percentage DHA and EPA in PL, with very little differences in most other fatty acids, including ARA (Fig. 3).

It was a surprising result that the percentage DHA in *Brachionus*-High did not reach higher levels than 9.4% of total PL fatty acids, which is only 23% of the DHA content of Marol E. Others have recently reported a similar result for rotifers (Li et al., 2014; Overrein, 2010), and we suggest that this low level of DHA in the PL of rotifers may be genetically or metabolically constrained, therefore representing a maximum level for the present rotifer strain, a level that is still far lower than that found for copepods (Li et al., 2014; McEvoy et al., 1998). This conclusion implies that DHA and n−3 HUFA enrichment of rotifer PL was relatively inefficient compared with that of total lipids or TAG.

Brachionus-High was produced using the so-called long-term enrichment method (Olsen, 2004; Rainuzzo et al., 1994b), where the lipid source is given along with a more nutritionally complete cultivation feed during cultivation (Table 1). The fatty acid composition of the rotifers will then be directly related to that of their dietary lipids, and DHA and EPA can be brought to high levels by using an emulsion with high percentage contents of these n−3 HUFA (Olsen, 2004). These rotifers, grown at steady state for >5 generations (i.e., doubling of biomass) in semi-continuous culture (Olsen, 2004) fed constant feed and daily rations, are believed to exhibit a constant biochemical composition independent of time, with a percentage n−3 HUFA content of total fatty acids comparable to, although slightly lower, than that of their diet (Olsen et al., 1993). In the present case, using very high DHA in the diet, the rotifers reached a threshold of 22% DHA of total fatty acids (Table 4), which was 54% of the content in Marol E (Table 3).

4.2. First feeding experiments

The two feeding trials were undertaken with larval groups exhibiting different individual DW for 2 dph larvae. The weight in Experiment 2 was 83% of that in Experiment 1 (Table 6). This reflects eggs of different sizes, and perhaps also of different qualities (Kamler, 2005; Kjørsvik et al., 1990). Both larval groups showed, however,

normal development and a similar growth response in the initial phase, suggesting that both groups were performing well. The difference in initial DW per larvae was, however, maintained till 17 dph. Survival was also different at 17 dph, but still relatively high for both experiments as compared to the normal result of similar experiments in our laboratory. It is important that the final growth and survival yields cannot be deduced at 17 dph, as nutritional implications can become manifest at a later stage. The fact that responses in growth and survival in Experiment 2 were already found at 17 dph most likely indicates that the diets were, and would remain, strong drivers of a continuing divergence in growth and survival beyond 17 dph.

The overall pattern after normalisation of DW and survival at 17 dph to the control treatment of the individual experiment (*Brachionus*-High) suggest that larval weights were positively related to the DHA content of PL in diets and in the larvae themselves. A stepwise evaluation of the experiments one by one yields the same conclusion overall, with the DW of Low-B larvae < High-B larvae and High-B larvae < AT larvae, meaning that the DW of Low-B larvae < High-B larvae < AT larvae. This supports our suggestion that the DHA content in dietary PL can be a critical factor across all three diets.

4.3. Pathways of lipid metabolism

Our analytical data allow some further speculations on how the fatty acid composition and configuration of dietary PL and TAG may affect PL synthesis and performance in fish larvae. We do this based on the current knowledge on pathways of lipid metabolism in marine fish larvae (e.g., Cahu et al., 2009; Daprà et al., 2011; Gisbert et al., 2005; Tocher et al., 2008).

The main pathways of de novo synthesis of PL based on dietary TAG and the alternative synthesis of PL through re-acylation of lyso-PL originating from dietary PL are schematically summarised in Fig. 8. The anabolic processes of de novo synthesis of PL and re-acylation of lyso-PL take place in the enterocytes, from which TAG and PL are transported via the blood to the tissues as complex lipoproteins.

If we assume that the n−3 HUFA of a dietary TAG is mainly esterified at the sn−2 position (Fig. 8) (Sargent et al., 1989, 1999), the n−3 HUFA will be retained in the monoacylglyceride (MAG) after digestion in the intestine and in DAG after re-acylation in the enterocytes. The n−3 HUFA will then become incorporated in the de novo synthesised PL, synthesised either from DAG and CDP-activated polar groups choline or from ethanolamine, forming phosphatidylcholine (PC) and phosphatidylethanolamine (PE), respectively (Fig. 8), or from CDP-activated DAG and serine or inositol, forming phosphatidylserine (PS) and phosphatidylinositol (PI), respectively (not shown) (Tocher et al., 2008). Other exchange mechanisms in between the PL molecules may take place as well, but it follows that both de novo synthesised PL and TAG will retain a majority of their original n−3 HUFA at the sn−2 position because the n−3 HUFA at sn−2 position will never be removed from the glycerol skeleton (Fig. 8). If PL of tissues are mainly de novo synthesised based on dietary marine TAG, we should expect high retention of dietary n−3 HUFA in both the dominant PL molecules and in TAG of fish tissues and a positive correlation between percentage n−3 HUFA content in dietary TAG or total lipids and tissue PL of fish.

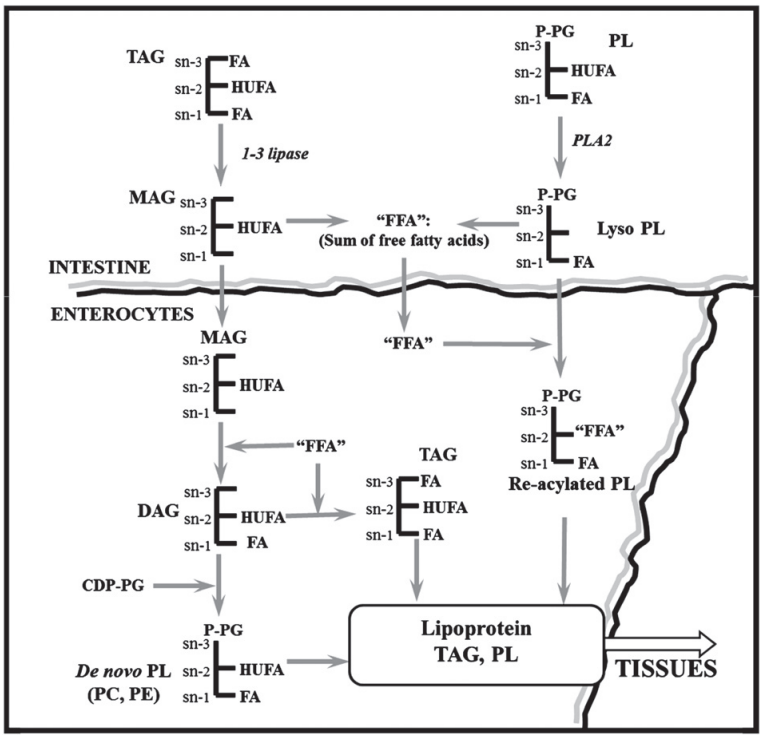

Fig. 8. Schematic view of the digestion and metabolism of TGA and PL in the intestine and enterocytes. TAG: triacylglycerides (with HUFA at the sn−2 position); MAG: monoacylglycerides; DAG: diacylglycerides; PL: phospholipid (with HUFA at the sn−2 position); PG: polar group (choline, ethanolamine, inositol, and serine); CDP: cytidine diphosphate; CDP-PG: activated choline cytosine with polar group (here choline or ethanolamine); PC: phosphatidylcholine; PE: phosphatidylethanolamine; de novo PL: phospholipids synthesised based on dietary TAG; re-acylated PL: phospholipids synthesised based on dietary PL; ΣFFA: pool of free fatty acids, saturated, monounsaturated, and polyunsaturated, including HUFA; 1–3 lipase and PLA2 are digestive enzymes.

We found no such correlation for DHA, the dominant n − 3 HUFA of PL ($r^2 = 0.12$).

The results therefore agree with the findings that de novo synthesis of PL is inefficient in fish larvae and that consequently, PL must be supplied in the feed. Many authors (e.g., Cahu et al., 2009; Daprà et al., 2011; Gisbert et al., 2005; Kanazawa, 1985; Tocher et al., 2008) have evaluated the principal mechanism of this apparent limitation, but there is so far no clear conclusion. It appears that re-acylation and most other anabolic processes take place in the enterocytes, but that the formation of lipoproteins and transport out of the enterocytes are in some ways inefficient (Daprà et al., 2011). Tocher et al. (2008) mention CDP-choline and CDP-ethanolamine phosphotransferases as possible limiting steps, but suggest that the limiting step may be further back in these pathways of de novo PL synthesis.

It follows that the principal precursor of PL synthesis in larvae is dietary PL, forming lyso-PL and a FFA after digestion. These components are taken up in enterocytes and lyso-PL undergoes re-acylation of FFA to form PL (Fig. 8). With this as the principal process of PL synthesis in larvae, we suggest that the fatty acid composition and configuration of dietary PL are very important for the fatty acid composition and configuration of larval PL, and accordingly also for larval membrane functionality, cellular metabolism, and ultimately for growth and performance of larvae and juveniles. It is generally believed that phospholipase A2 (PLA2) is the active digestive enzyme, and PLA2 removes the sn − 2 acyl chain from the PL molecule. Because the n − 3 HUFA of marine PL are normally esterified at the sn − 2 position (Sargent et al., 1989, 1999), PLA2 removes the most vulnerable fatty acids from the dietary PL molecule. In the acylation process in the enterocytes, the composition of FFA available (ΣFFA, the pool of FFA, includes saturated, monounsaturated, and polyunsaturated fatty acids, including HUFA) and the specificity of the enzymes catalysing the re-acylation process affect the n − 3 HUFA content of the PL. The concentration and composition of ΣFFA are related to the total lipid content of the diet or its TAG content. If total lipids/TAG is low, like in copepod nauplii, the sn − 2 fatty acid of the dietary PL will dominate ΣFFA. If total lipids/TAG is high, sn − 1 and sn − 3 fatty acids from TAG will dominate.

With these considerations in mind, we deduce that for dietary PL with a very low DHA content, independent of TAG content (e.g., *Brachionus*-Low), ΣFFA will include fatty acids from the sn − 1 and sn − 3 positions in dietary TAG and from the sn − 2 position of dietary PL. The fraction of DHA will then always be low. The PL of *Brachionus*-Low contained around 2% DHA, meaning that 4 out of 100 PL molecules contained a DHA molecule. The PL of *Brachionus*-High contained 9.4% DHA, meaning that around 2 out of 10 PL molecules contained a DHA molecule. Therefore, the DHA content of the re-acylated PL molecule in both cases will be questionable, and high TAG contents may strengthen this negative effect.

In the other extreme case, if the dietary PL has a high DHA content and TAG content is low (e.g., *Acartia* nauplii), ΣFFA will have a much higher fraction of DHA. The PL of *Acartia* nauplii had a three times higher DHA content than *Brachionus*-High, with around 30% the DHA content of the nauplii, meaning that 6 out of 10 PL molecules contained DHA. Harvested nauplii and copepodides in northern waters may readily have up to 50% DHA in their PL (Li et al., 2014), meaning that all PL molecules may contain a DHA molecule. The DHA content of the re-acylated PL molecule will then likely be much higher. If TAG content becomes higher, the fraction of DHA in ΣFFA will become reduced. Finally, HUFA/PUFA are selected for during re-acylation of lyso-PL at the sn − 2 position (Sargent et al., 1999), but the final DHA content of the re-acylated PL will likely still be lower than that in the dietary PL.

The above considerations imply that the live feed of species like cod, and we add halibut, should be fed diets with fatty acid contents and a configuration of PL similar to their own requirements, judged by the PL composition in newly hatched larvae. Moreover, the TAG content should be as low as possible, to maximize the DHA fraction in ΣFFA, the pool of FFA available in lyso-PL re-acylation. High DHA in PL and low TAG content are typical characteristics of copepod nauplii and copepodides.

There are some questions raised regarding the activity of PLA2 in fish larvae. Larvae of both gilthead sea bream and sea bass did not show PLA2 activity in early larval stages (Izquierdo and Henderson, 1998; Zambonino Infante and Cahu, 2001). Gene expression studies in whole larval cod have shown a stable level of PLA2 during the first 20 days and, thereafter, a rapid increase in the expression of PLA2 genes up to day 60 (Kortner et al., 2011). However, these studies do not specify the baseline level at hatching, only the increase in gene expression. Saele et al. (2011) showed that expression of the PLA2 enzyme species secreted from the pancreas remained low in Atlantic cod (*G. morhua*) until day 62, whereas the expression of total PLA2 in the larvae showed higher values. These results at least question the general idea that PLA2 is the principal enzyme of PL digestion in fish larvae.

Alternative digestion enzymes of PL are PLA1 and 1–3 lipase, which both remove the sn − 1 acyl chain. The 1–3 lipase is present at hatching in fish larvae (Cahu et al., 2009), but it is not known if it can cleave PL. PLA1 is present in fish ovaries (Hiratsuka et al., 2008), but its presence and activity in the larval intestine during the early developmental stages are not documented. Nevertheless, if the sn − 1 acyl moiety is removed from PL during digestion, the re-acylated PL molecules will exhibit a similar HUFA content as the dietary PL, because the HUFA will remain associated with the phosphoglycerol skeleton throughout. The potential interference of high TAG may then be less critical.

5. Concluding remarks

As early fish larvae primarily retain their n − 3 HUFA from dietary PL while the n − 3 HUFA enrichment techniques primarily increase the n − 3 HUFA contents of TAG of rotifers, it follows that the traditional enrichment techniques may not be efficient for species like Atlantic cod. The n − 3 HUFA enrichment of rotifer PL is low compared with that of rotifer TAG. We believe that the DHA (or n − 3 HUFA) requirements of cod larvae cannot be easily met using *B. plicatilis* Nevada as the only live food, and the strain *B. plicatilis* Cayman has shown similar characteristics (Li et al., 2014). These findings may contribute to explaining the problems of rearing juveniles of cold water species like Atlantic cod and Atlantic halibut, and call for new approaches in larval feeding and live feed technology for such species. Co-feeding by formulated diets and a minimised use of mass cultured copepod nauplii or copepodides are approaches that should be further elaborated.

We also speculate that the efficiency of de novo synthesis of PL, and thereby the ability to utilise HUFA in TAG of the live feed, may vary among larval fish species. First feeding is known to be easier for some larval species than for others (Moksness et al., 2004), and we postulate that a species-specific ability to use HUFA from TAG from an early developmental stage may be decisive for these differences.

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Paper V

Gene regulation of lipid and phospholipid metabolism in Atlantic cod (*Gadus morhua*) larvae

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Abstract

The main objective of the present study was to obtain more knowledge on ontogenesis of lipid metabolism, especially the pathway of *de novo* phospholipid (PL) synthesis during early life stages of Atlantic cod. Cod larvae were sampled at 1, 3, 8, 13, 17, 18, 30, 42 and 60 days post hatch (dph) for transcriptome analysis using a recently developed microarray. The fatty acid profile and gene expression levels of cod larvae at 17 dph were compared after feeding differently enriched rotifers, which contained different lipid and DHA levels. No significant differences ($P<0.05$) were found for the two rotifer diets in the overall gene expression level of cod larvae, their growth and survival, and their DHA levels in total lipid and PL fraction. The fatty acid data suggested that dietary EPA was elongated to DPA by cod larvae. Almost no up-regulation of the key regulating genes involved in the PL biosynthesis pathways were found up to 60 dph, however, the up-regulation of *pcyt1b* needs further attention, due to its possible roles in regulating PC biosynthesis in the brain of cod larvae. Our overall data suggest that there is no apparent genetically constraining step in the pathway of *de novo* PL synthesis in cod larvae, and they appeared to have relatively high capability of PL biosynthesis already at 1 dph, but this capability may be related to cell division and growth, rather than to lipoprotein assembly. Further studies are needed to elucidate the enzyme activity involved in the pathways.

Key words:

Atlantic cod larvae, microarray, phospholipid metabolism, elongase, desaturase

1. Introduction

Atlantic cod (*Gadus morhua* L.) is among the most important species in North Atlantic fisheries, and there has for long been an interest of culturing the species. The quality of cultured juveniles has, however, been variable with poor predictability in the production (Hamre, 2006). Meanwhile, the cod producers have suffered from low prices due to the competition of increased landings from cod fisheries, especially during 1980'ties, middle 1990'ties and in recent years. Nevertheless, research on Atlantic cod farming has made significant progress. The full genome of cod has been sequenced recently (Star *et al.*, 2011), and will contribute to improve the fundamental

biological knowledge of the species. Genome data and molecular tools will improve the possibilities of using cod as a model species in research and also enhance the potential to become a successful species in aquaculture in the future.

High mortality and low growth rate are commonly found for early life stages of marine fish larvae in aquaculture, including Atlantic cod, as a result of suboptimal nutrition. Most producers use rotifers (*Brachionus* spp.) and *Artemia* spp. that are enriched with essential long chain highly-unsaturated n3 fatty acids (n3 HUFA) to feed cod larvae during the live feed period (Hamre, 2006; Garcia *et al.*, 2008; Olsen *et al.*, 2014). One major obstacle has been to meet the high n3 HUFA requirement of the larvae. Most studies on n3 HUFA requirements of fish larvae were conducted with live prey enriched with triacylglycerides (TAG) or fatty acid esters (Izquierdo *et al.*, 2001). Dietary phospholipids (PLs) have only more lately been subject to particular interest (Harel *et al.*, 1999; Li *et al.*, 2015).

Phospholipid is an essential dietary component for normal development and growth in fish larvae (Cahu *et al.*, 2009). Dietary PL can enhance transport of neutral lipids, supply essential components such as choline and inositol, and PL in cell membranes can generate second messengers such as diacylglycerol (DAG), phosphatidic acid and arachidonic acid (ARA). Moreover, DHA (docosahexaenoic acid) and EPA (eicosapentanoic acid) incorporated in PL have showed higher bioavailability for fish larvae, rodents and humans than that in TAG (Cansell *et al.*, 2003; Schuchardt *et al.*, 2011; Olsen *et al.*, 2014). The PL requirements appear to decline as the fish grow bigger, and no requirements were generally observed in fish larger than 5 g (Tocher *et al.*, 2008; Cahu *et al.*, 2009). Intestinal steatosis occurred in much higher ratio in the fry stages (13 out of 16, 20 days post swim-up stage) compared to the juvenile stages (1 out of 18, 146 days post swim-up stage) of rainbow trout when a PL-deficient diet was fed to the fish (Dapra *et al.*, 2011).

A main hypothesis is that fish larvae need dietary PL because they have limited ability to biosynthesize PL *de novo*, which is necessary for lipoprotein transport from the enterocytes (Tocher *et al.*, 2008). The biochemically limiting steps for PL synthesis in fish larvae are unknown, but the metabolic pathways for digestion and biosynthesis of PL appear to be the same as for mammals (Sargent *et al.*, 2002). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant phospholipids of eukaryotic membranes, accounting for 40-60 % and 25 % of the total phospholipid species, respectively (Henneberry *et al.*, 2002; Kent, 2005). In mammalian cells, the

major pathways of biosynthesis of PC is the CDP-choline (cytidine diphosphate-choline) pathway, also known as the Kennedy pathway (Figure 1), and it is accomplished by three steps: choline is firstly phosphorylated to phosphocholine by choline kinase (CK); CMP (cytidine monophosphate) is then transferred from CTP (cytidine triphosphate) to phosphocholine to form CDP-choline, catalysed by CTP:phosphocholine cytidylyltransferase (CT). Finally, CDP-choline is transferred to diacylglycerol (DAG) to form PC, catalysed by CDP-choline: *sn*-1, 2-diacylglycerol cholinephosphotransferase (CPT). Alternatively, PC can be synthesized by methylation of PE, catalysed by phosphatidylethanolamine *N*-methyltransferase (PEMT). This pathway mainly operates in the liver, and it could contribute to 30-40 % of PC in hepatocytes (Sundler and Akesson, 1975; DeLong *et al.*, 1999; Reo *et al.*, 2002). PE is synthesized through the CDP-ethanolamine pathway, which is similar as the CDP-choline pathway. In mitochondria, PE is synthesized by decarboxylation of phosphatidylserine (Borkenhagen *et al.*, 1961) (Figure 1).

A recent study on cod larvae (Olsen *et al.*, 2014) showed that the percentage of DHA of total fatty acids (DHA %) in larval PL was positively correlated to DHA% in their dietary PL, but not to the DHA% in total lipid. It was therefore suggested that the Atlantic cod larvae exhibited low capacity of *de novo* PL syntheses through the normal pathway from digested TAG precursors, such as monoacylglycerides (MAG). The 17 dph (day post hatch) cod larvae instead obtained their PL through re-acylation of digested dietary PL, or by direct absorption of intact PL through pinocytosis as shown in larval zebrafish using fluorescent phospholipid (Farber *et al.*, 2001).

Transcriptional regulation of PL biosynthesis has been described in mammals and yeast (Sugimoto *et al.*, 2008), but little is known in fish. Our overall objective of this study was to obtain more knowledge of ontogenesis of PL synthesis capability in Atlantic cod larvae. For this purpose, transcriptome analysis of larvae in different stages was carried out using a recently developed Atlantic cod microarray. The fatty acid profile and gene expression levels of cod larvae were studied in larvae fed two differently enriched rotifers, which contained different lipid and DHA levels.

2. Materials and methods

2.1 Live feed rearing

Rotifers (*Brachionus* 'Cayman') (Gomez *et al.*, 2002) were cultivated in 250 L fiberglass tanks at 23-25 ºC and 36 ppt salinity. Cultures were diluted by 20% of

127 volume day^{-1} to obtain steady state of growth. In Experiment 1, the rotifers were fed
128 Baker's yeast (*Saccharomyces cerevisiae*), a concentrate of Rotifer diet (RD, Reed
129 Mariculture) and an emulsified lipid diet Easy DHA Selco (DSelco, INVE
130 Aquaculture, Belgium). They were either short-term (ST, 2 h) or long term (LT)
131 enriched continuously during growth (Rainuzzo *et al.*, 1994) (Table 1), and named as
132 Cayman-ST and Cayman-LT, respectively. Rotifer samples were collected by
133 pumping culture through two sieves with mesh sizes of 200 µm (to remove particles)
134 and 64 µm (to retain rotifers), thereafter rinsed in filtered seawater and finally in cold
135 tap water for 10 seconds. The sieve (64 µm) was dried from underneath by paper
136 towels, the rotifers were transferred to sample vials, immediately frozen in liquid
137 nitrogen, and finally freeze-dried and stored under N_2 at -80 ºC before analysis.
138 In Experiment 2, the enrichment diets were replaced by LARVIVA Multigain
139 (BioMar, Denmark), and the rotifers were only short-term enriched (2 h). *Artemia*
140 *franciscana* cysts (Great Salt Lake, Utah, USA; INVE Aquaculture, Belgium) were
141 hatched and enriched with Multigain (24 h) before fed to the larvae.
142
143 *2.2 Fish larvae rearing*
144 Atlantic cod (*Gadus morhua*) eggs were obtained from Nofima marine national
145 breeding station, Havbruksstasjonen i Tromsø AS, and transported by air to NTNU
146 Sealab. They were disinfected in 400 ppm glutardialdehyde upon arrival at 6.9 ºC
147 according to Salvesen and Vadstein (1995). Thereafter, the eggs were kept in darkness
148 at 7 ºC in a 250 L cone bottomed incubator. Two days before hatching, eggs were
149 transferred to 100 L cone bottomed tanks with a density of 100 eggs L^{-1} (6.5 ºC). Day
150 0 was defined as the day when 90% of the embryos hatched.
151 In Experiment 1, cod larvae were maintained in darkness the first 3 days (until mouth
152 opening) and thereafter in continuous light. *Nannochloropsis oculata* algae paste
153 (Reed Mariculture) was added (1 mg C L^{-1}) to the fish tanks at feeding from day 2 to
154 the end of the experiment. Rotifers were fed to larval tanks by a robot system (Storvik
155 Aqua AS) 4 to 6 times a day to maintain the concentration of 5 000 to 12 000 rotifers
156 L^{-1}. Four replicate tanks (100 L, water exchange rate 2 to 3 d^{-1}) were used for each
157 treatment.
158 Experiment 2 is already described in detail by Rehberg-Haas et al. (2015). In brief a
159 feeding robot distributed enriched rotifers (2-4 times, 3-23 dph), *Artemia* (1-4 times,

19-38 dph) and dry feed (2-10 g d^{-1}, from 33 dph, Gemma Micro Diamond 300, Skretting, Norway) to the larval rearing tanks each day.

2.3 Weight development of larvae

All larvae sampled were sedated with an overdose (500 mg L^{-1}) of Tricaine Methanesulfonate (MS222). In Experiment 1, 12 larvae were randomly picked from each tank at 1, 3, 8, 13 and 17 dph, rinsed in deionised water, and transferred into individual pre-weighted tin capsules. The dry weight was calculated based on the C/N analysis by using a CHNSO analyser (ECS 4010, Costech instruments, Elemental combustion system (series number 260610079). Assuming the carbon content was 43%, a conversion factor of 2.34 was applied for the calculation (Reitan *et al.*, 1993). In Experiment 2, samples were collected in the same way for C/N analysis and RNA extraction at 1, 3, and 18 dph. At 30, 42 and 60 dph, the larvae were randomly picked and dried at 60 ºC for 48 h and then weighted.

2.4 Larval survival

Survival was calculated from the number of cod larvae alive at 18 dph compared to the initial number of hatched eggs and corrected for sampling and tank cleaning.

2.5 Lipid and fatty acid analyses

Cod larvae were sampled from the incubator at 1 and 3 dph and from each experimental tank at 18 dph after counting of survival. The total lipids were extracted by a modified Bligh and Dyer (1959) method, and lipid content was determined gravimetrically as described by Li *et al* (2015). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were separated on PLC silica plates (PLC silica gel 60 F254, 0.5 mm, 20 × 20 cm, Merck KGaA, Darmstadt, Germany) with chloroform: methanol: water (67:30:2.5, by volume) as the solvent system. Commercial PC and PE standards (Soy PC and PE, Avanti Polar Lipids Inc. Alabaster, AL, USA) were also applied on the plates, and they were then visualized under UV. The corresponding PC and PE bands were scraped off, and directly methylated with BF_3-methanol and isooctane in N_2 atmosphere at 50 ºC overnight. Isooctane and distilled water were added to the mixture and then centrifuged at 1640 x g for 3 min at 5 ºC. The fatty acid methyl esters in the upper phase were analysed with a gas

chromatograph (AutoSystem XL, Perkin Elmer, Waltham, MA, USA) with TotalChrom Version 6.3.1 software.

2.6 RNA isolation

After sedating with MS222, cod larvae were collected on 1-42 dph with the rear end of a pasteur pipette and spotted on a 1.5 x 1.5 cm^2 plankton net (100 µm) placed on a piece of tissue towel to drain off excess seawater. The net was immediately put into a SafeSeal micro tube (Sarstedt®), flash frozen in liquid Nitrogen, and stored at -80°C until RNA extraction.

Total RNA was extracted by use of RNeasy mini kit® from Qiagen. Immediately prior to RNA extraction the frozen sample was flushed off the plankton net into the micro tube with 600 µl RLT buffer (RNeasy Lysis Buffer, Qiagen) with 1% β-mercaptoethanol (ME) added. The samples were homogenized for 4 min at 25 Hz in a TissueLyzer® (Qiagen) using 5 mm stainless steel beads. For the samples 0-18 dph, all of the resulting lysate was used for RNA extraction. To avoid overloading of the RNA binding column, samples from 30 and 42 dph were diluted in RLT buffer (1:2 and 1:4, respectively) and 600 µl of the solutions were used in the further steps.

Sedated cod larvae at dph 60 were frozen individually in liquid nitrogen and kept in micro centrifuge tubes at -80 C° until dissection. Prior to dissection the cod larvae were put into liquid nitrogen to minimize thawing and RNA damage during the procedure. Using a scalpel, a small cut was made from gills to gut along the side of the larvae, and the abdominal wall was lifted up to expose the intestines. All intestines were removed while the lump was still frozen and immediately put into liquid nitrogen. The remaining parts of the larvae were put into liquid nitrogen in a separate tube. Pooled samples of 10 larvae were made both from intestines and the remaining tissues, and the samples were kept at -80°C until RNA extraction. The tissues were homogenized for 2 min at 25 Hz in frozen condition using TissueLyzer and stainless steel beads at -80°C and without RLT buffer. Then 600 µl RLT buffer containing 1% β-ME and a room tempered stainless steel bead was added to 25 mg homogenized tissue and mixed well for another 2 min at 25 Hz.

From this point all samples were treated the same way and according to the supplier's protocol "Purification of Total RNA from Animal Cells using Spin Technology" including the optional "on-column DNase treatment" as described in the kit manual. RNA was eluted in 50 µl RNase-free water, and RNasin® (Promega) was added to a

final concentration of 1 U/µl. The RNA was quantified by measuring the absorbance at 260 nm, using a NanoDrop® ND-1000 spectrophotometer (NanoDrop technologies, Wilmington, DE, USA). The RNA integrity from samples 1-42 dph was evaluated by denaturing agarose gel electrophoresis and ethidium bromide staining, showing no signs of degradation. The RNA integrity in samples from larvae 60 dph was analyzed by Agilent RNA 6000 Nanochip© and Agilent Bioanalyzer© (Agilent Technologies), showing RIN (RNA Integrity Number) values >9.

2.7 Microarray design and hybridization

A custom, Agilent 44 k oligo microarray (A-MEXP-2226, ArrayExpress, EMBL-EBI) described by Kleppe *et al*. (2014) was used. This microarray design is partly based on the Atlantic cod gene set described in Star *et al*., (2011) as well as EST sequences from various cod tissues / developmental stages. 200ng total RNA was used to synthetize Cy3 labelled cRNA, using the Low Input Quick Amp Labeling Kit One-Color (Agilent Technologies cat.No. 5190-2305, Santa Clara, CA, USA). cRNA concentration and Cy3 incorporation were measured with a NanoDrop® ND-1000 spectrophotometer. 1.65 µg labelled cRNA was fragmented and hybridized on 4x44k Custom Gene Expression arrays for 18 hours at 65°C, using Gene Expression Hybridization Kit (cat.no.5188-5242) and hybridization oven (G2545A, all from Agilent Technologies). Slides were washed with buffers from Gene Expression Wash Buffer Kit (Agilent cat. No. 5188-5327) and immediately scanned with Agilent scanner (G2505BG25) and data was extracted from the resulting tif images with Feature Extraction software version 4.5.1. Number of biological replicated analyzed by microarray is shown in Table 2.

2.8 Microarray data analyses

The Limma (Linear Models for Microarray Data) package (version 3.18.13) (Smyth, 2004) and R version 3.0.3 were used for statistical analysis and identification of significant differentially expressed genes. Single colour feature expression files from the microarray scans were generated with Feature Extraction Software version 9.5.1 (Agilent Technologies, Inc). Median signal intensities were used and weak or not detected spots were given reduced weight, using the Limma weight function. The data were normalized using the quantile method, no background subtraction was

performed. A design matrix was created and pair-wise comparisons between the samples were performed using samples from 1 dph as a reference. The method of Benjamini and Hochberg (1995) was used to estimate the false discovery rate. Genes with adjusted p-value < 0.05 were considered as significantly differentially expressed and were included in the analysis if all probes for each gene had a mean adjusted p-value < 0.05.

2.9 Statistics

The other experimental data (growth, lipid and fatty acid) were tested for statistical significance by using Independent- Samples T-test or one-way analysis of variance (ANOVA) with Tukey's multiple comparison test or Dunnet's T3 multiple comparison test if the P value of homogeneity of the variance test was less than 0.05. Differences were considered significant if $p<0.05$. All of the statistical tests were performed using SPSS 20.0 for windows. All tables were made in Excel 2010 and figures by Sigma plot 12.5.

3. Results

3.1 Larval growth and survival

Survival at 18 dph for Cod-ST (cod larvae fed Cayman-ST) and Cod-LT (cod larvae fed Cayman-LT) were 43.3 ± 2.5 % and 45.8 ± 4.6 %, respectively, with no significant differences between the treatments. The dry weight of Cod-LT at 8 and 13 dph was significantly higher than that of Cod-ST (Table 3), but there were no significant differences in dry weight at 17 dph.

3.2 Larval feed

The enrichment diet DHA-Selco (DSelco) contained 17.1% DHA of total fatty acid, whereas the algae paste *Nannochloropsis* (NC) added into the larval tanks contained no DHA. Its dominating fatty acids were 16:1n7 and EPA. No DHA was found in PC or PE of DSelco (Table 4 and 5). Low levels of n3 DPA (22:5n3) were found in both rotifer treatments and DSelco. The Cayman-LT showed significantly lower DHA content compared to the Cayman-ST. However, the DHA content in PC and PE of Cayman-LT were significantly higher than that of Cayman-ST (Table 4 and 5).

3.3 Lipid and fatty acid profile of cod larvae

294 *3.3.1 Total lipid*

295 Total lipid and total fatty acids (TFA) decreased significantly in cod larvae at 18 dph
296 compared to their initial values (Table 6). Saturated fatty acid (SFA) and
297 polyunsaturated fatty acid (PUFA) levels also decreased significantly, while
298 monounsaturated fatty acid (MUFA) levels increased significantly. There was a
299 significant ($p<0.05$) decline in n3 HUFA (DHA and EPA) levels, especially for DHA.
300 The ARA and DPA contents increased significantly ($p<0.05$). The DHA levels in
301 Cod-LT and Cod-ST at 18 dph were not insignificantly different.

302

303 *3.3.2 PC*

304 The fatty acid profile in PC showed similar trends as that in total lipid (Table 7). Fatty
305 acids were dominated by 16:0, EPA and DHA in cod larvae at 1 and 3 dph. The
306 PUFA levels, including DHA and EPA, decreased significantly ($p<0.05$) with time,
307 whereas ARA and DPA as well as MUFA levels, especially 16:1n7 and 18:1n9
308 increased significantly ($p<0.05$) in the larvae at 18 dph. The SFA levels decreased
309 slightly, but significantly ($p<0.05$) and the decrease of PUFA levels were almost
310 equal to the increase of the MUFA levels at 18 dph. The fatty acid profile of PC was
311 similar for Cod-LT and Cod-ST at 18 dph.

312

313 *3.3.3 PE*

314 The fatty acids in PE of larvae at 1 and 3 dph were dominated by DHA and EPA
315 (Table 8). The DHA levels decreased significantly with time, while the EPA levels
316 remained relatively stable in the larvae at 18 dph. The PUFA levels in PE remained
317 relatively stable, but DHA decreased by 15-20% in the PE profile of 18 dph larvae.
318 The decrease of DHA was apparently compensated by an increase of ARA, DPA and
319 other PUFAs. SFA and MUFA in 18 dph larvae showed relatively stable levels
320 compared to their initial values. The Cod-LT showed slightly, but significantly higher
321 ARA and DPA levels compared to the Cod-ST, whereas their DHA and EPA levels
322 were similar.

323

324 *3.4 Transcriptomic analysis of cod larvae*

325 The $\log_2$-transformed gene expression ratios at 17 dph between were not significantly
326 different for Cod-LT and Cod-ST. Values were therefore combined in the further
327 treatment.

328 Figure 2 shows the number of significantly regulated genes at each sampling day
329 relative to day 1. Among the 23,857 unique genes represented with probes on the
330 microarray, 6214 genes showed significant regulations at least at one sampling point
331 (Figure 2). The lowest and highest number of differentially regulated genes was found
332 at 3 dph and 60 dph, respectively. In general, more genes were down-regulated than
333 up-regulated throughout the sampling period. The largest difference was found at 3
334 dph, with 344 more down-regulated genes than up-regulated genes. The smallest
335 difference was detected at 18 dph, where only 3 more genes were down-regulated
336 than up-regulated.
337 There are more than 100 genes involved in the PL metabolism. Table 9 shows the PL
338 related genes which were significantly ($p<0.01$) up or down regulated at least in one
339 sampling point (marked red in Figure 1). Most of the genes encoding *de novo* PL
340 biosynthesis pathway, the Lands cycle pathway (PL to lyso-PL to PL) and PL
341 turnover pathway were not significantly up-regulated ($p>0.05$) up to 60 dph. The
342 main neutral lipid digestive enzyme bile salt-activated lipase (*bal*) was significantly
343 up-regulated at 13, 17, 42 ($p<0.05$) and 60 dph ($p<0.01$). Monoacylglycerol lipase
344 (*abhd12*) and hepatic triacylglycerol lipase (*lipc*) was significantly up-regulated from
345 8 dph to 60 dph ($p<0.01$). Group 10 secretory phospholipase A2 (*pla2g10*) did not
346 show significant up-regulation until 60 dph. However, *pla2g1b* showed significant
347 up-regulation at 13, 30, 42 and 60 dph and phospholipase B1 (*plb1*) showed
348 significantly up-regulation all the way from 3 dph.
349 Many of the genes regulating apolipoprotein assembly showed significant changes:
350 *apoA-I* was up-regulated at 13 ($p<0.05$), 17 and 60 dph ($p<0.01$); *apoB-100* was
351 significantly down-regulated in many sampling points ($p<0.01$), and only one
352 moderate up-regulation occurred at 17 dph ($p<0.05$); *mttp* and *apoA-IV* only showed
353 significantly down-regulation at 42 dph ($p<0.01$).
354 The expression of Δ6 desaturase (*fadsd6*) showed significant up-regulation from 17 to
355 60 dph ($p<0.01$). The expression of *elovl1* (elongation of very-long-chain-fatty acids)
356 was down-regulated throughout the sampling period, whereas *elovl4* showed
357 significant up-regulation at 13 ($p<0.05$), 30, 42 and 60 dph ($p<0.01$) and *elovl7* was
358 significantly up-regulated at 17, 60 ($p<0.05$) and 42 dph ($p<0.01$). The remaining
359 desaturases and elongases did not show any significant changes.
360 Figure 3 shows the comparison of gene expression levels of gut and liver and the rest
361 of body of cod larvae at 60 dph. The up-regulated genes in gut and liver are mainly

involved in lipid digestion (*pla2g1b* and *bal*) and lipoprotein assembly (apolipoprotein family and *mttp*). The expression of *dgat1*, *dgat2* and *mgat2a* regulating the biosynthesis of TAG were also up-regulated in the gut and liver. Genes regulating the committing step of *de novo* synthesis of PC and PE, *chka* and *etnk1*, respectively, showed 5.46 and 3.43 fold higher expression levels in the gut and liver compared to the rest of the body. However, most of the genes involved in the *de novo* synthesis pathway of PL did not show any up-regulation in the gut and liver.

4. Discussion

The main objective of the present study was to obtain more knowledge on ontogenesis of lipid metabolism, with a focus on the pathway of *de novo* phospholipid synthesis during early stages of cod larvae. High throughput microarray data throughout the live feed period and up to 60 dph of cod larvae, together with fatty acid analysis of the major PL classes in the early stages provided valuable information for a better understanding of these mechanisms.

The DHA levels of rotifers in the present study were rather low (6.7-11.6 %) compared to our previous studies (22.3-26.2 %) (Olsen *et al.*, 2014); however, after 18 days of feeding, the DHA levels in cod larvae were not significantly different (15.3-17.1 % *versus* 14.4-20.9 %) between the two studies. This indicated that DHA was selectively incorporated and retained in PL of cod larvae, especially in the PE fractions, which contained close to 50 % DHA before first feeding and 30 % at 18 dph. We suggest that a threshold DHA level to maintain membrane fluidity and other functions may exist in the PL of cod larvae. As a consequence, Cod-ST larvae showed the same DHA levels in TL (15.3 %), PC (11.7 %) and PE (31.1 %) as Cod-LT larvae (17.1 %, 12.0 % and 30.7 %, respectively) at 18 dph, despite significantly different DHA levels in PC and PE of the Cayman-ST and Cayman-LT. The correlation of DHA in PL of the diets and in PL of larvae found in our previous study (Olsen *et al.*, 2014) was not observed here, likely due to the low DHA levels of the rotifers. Besides, the analytical methods for separating lipid classes were different between the two experiments. The focus of the present study was the major PL classes: PC and PE, whereas the previous study separated polar lipids from neutral lipids. Although PC and PE are the main polar lipids, other components including phosphatidylinositol, phosphatidylserine, lyso-PC, lyso-PE, phosphatidic acid and cardiolipin could contribute up to 12-20 % of total polar lipids of cod larvae (Olsen *et al.*, 1991;

396 Overrein, 2010). Different PL classes could have very different DHA content, as
397 revealed for PC and PE in this study.
398
399 *4.1 Desaturases and elongases*
400 Under conditions of low dietary DHA level, genes encoding desaturases and
401 elongases involved in the HUFA biosynthesis pathway can be up-regulated and
402 function as a compensation mechanism to secure endogenous synthesis of DHA and
403 (n3) DPA. Studies have shown that both the fatty acid composition of PL and the
404 expression of *fadsd6*, *fadsd5*, *elovl 2* and *elovl 5* of post-smolt Atlantic salmon were
405 affected by the dietary DHA levels, with liver being the most sensitive tissue
406 (Betancor *et al.*, 2014). Hepatocytes obtained from salmon juveniles fed low DHA
407 diets showed high capability of synthesizing DHA from ^{14}C labeled EPA, however,
408 both the synthesizing ability of DHA and the gene expression of *fadsd6*, *fadsd5* and
409 *elovl 2* decreased significantly when DHA was supplemented in the diet (Thomassen
410 *et al.*, 2012).
411 It has been suggested that an adequate total amount of 22-carbon PUFAs is needed for
412 development of the early nervous system (Moriguchi *et al.*, 2000). In the present
413 study, significantly elevated levels of (n3) DPA in TFA, PC and especially in PE were
414 found in 18 dph larvae compared to their initial values before first feeding. Due to the
415 low dietary levels of DPA in both enriched rotifers and algae paste *N. oculata* (NC),
416 we suggest that this increase was caused by elongation of dietary EPA, originating
417 mainly from NC which contained 32.4 % EPA (Table 4). However, genes encoding
418 elongases *elovl1, 4, 5* and *6* did not show any significant changes in expression levels
419 before 17 dph, except for *elovl7*, which was 1.78 fold up-regulated in 17 dph larvae
420 compared to 1 dph larvae. *elovl7* is mainly expressed in stomach and gill of cod (Xue
421 *et al.*, 2014), but its function in cod remains unknown. Interestingly, *fadsd6* of cod
422 larvae at 17 dph showed 1.85 ($p<0.01$) fold up-regulation compared to 1 dph larvae.
423 The expression of *fadsd6* was found to be significantly higher in liver of cod fed
424 camelina oil replaced diets with defatted fish meal compared to that found for fish oil
425 treatment (Xue *et al.*, 2014). Tocher (2006; 2010) has suggested that the expression of
426 transcripts for *Δ6* desaturase in marine fish may be related to the desaturation of EPA
427 rather than 18:3n3, especially in neural tissues, to maintain membrane DHA levels.
428 Due to absence of a *Δ5* desaturase and an *elovl2* gene, marine fish species including
429 cod are unable to produce DHA from 18:3n3, but they may be able to produce DHA

from EPA when dietary DHA is extremely low, with possible elongation functions of *elovl4* compensating for the absence of *elovl2* (Agaba *et al.*, 2005; Monroig *et al.*, 2011; Xue *et al.*, 2014).

4.2 Lipid digestion related genes

As mentioned above, the dietary effects on fatty acid composition of PL and gene expression are tissue specific. The present study used whole fish due to the challenges of accurate dissection in the early stages. Whole larval homogenates were also used in the molecular ontogenesis studies of digestive capability in Atlantic cod larvae carried out by Kortner (2011a; 2011b) and Sæle (2009; 2010; 2011). Sæle (2009) suggested that whole larvae samples could be suitable to study relative expression of genes that are expressed only in certain tissues by using qRT-PCR. However, microarray was used in the present study and it should be noted that as the larvae grow bigger, the proportion of each organ will change, for example, the GI-tract (gastrointestinal tract) of total body wet weight decreased from 18.5 % at 34 dph to 6.48 % at 62 dph (Saele *et al.*, 2010). The mRNA levels in GI-tract in later stages would therefore be diluted as we compared the gene expression level with that at 1 dph. Anyway, the genes regulating the main lipolytic enzymes BAL and PLA2 showed comparable trends to that reported in other studies. BAL exhibited similar expression trend as shown by Kortner (2011b), except that at 60 dph when the expression levels remained high in the present study.

Another ontogenic *bal* study by Sæle (2010) showed a low and stable expression level until 62 dph. The secretory phospholipase A2 (*pla2g10*) did not show any significant changes until 60 dph in the present study, in agreement with Sæle (2011). However, the expression of *pla2g1b* (ENSGAUG00000017210 and ENSGAUG00000001398, Table 9) was already significantly up-regulated at 13 dph. In general, it appeared that cod larvae were able to utilize both neutral lipid and polar lipid efficiently already from 40 dph (standard length 9.75 mm), because pre-hydrolyzed dietary lipid showed only minor effects on absorption and metabolism compared to intact lipid (Hamre *et al.*, 2011).

4.3 De novo synthesis of PC

Biosynthesis of PC via the CDP-choline pathway and PA intermediate in fish was first confirmed by Holub (1975a; 1975b), using ^{14}C labelled CDP-choline and ^{14}C-

glycerol-3-phosphate precursors, respectively, with liver microsomes of rainbow trout. This took place almost 20 years after the discovery of the CDP-choline pathway by Kennedy and Weiss (1956). Phospholipid is found to be an essential dietary component for fish larvae. However, the genes involved in phospholipid biosynthesis pathways in fish are poorly studied compared to the related digestive genes. One recent study (Dapra *et al.*, 2011) showed that the intestinal *cpt*, *lpcat* and *dgat* mRNA levels were not affected by PL deficiency in rainbow trout fry and juveniles and only *apoB* was up-regulated in the PL depleted treatment during fry stage. The expression of *cpt* in cod larvae did not show any regulation up to 60 dph in the present study. Most studies have indicated that the enzyme activity of CPT in cells was in excess (Vance, 2002; Gibellini and Smith, 2010), which likely means that transcriptional regulation of *cpt* is not necessary for the production of PC.

Another recent study showed that both *chk* and *pcyt1a*, together with lipoprotein assembly related genes (*mttp, apoB, apoAI* and *apoAIV*), were up-regulated in pyloric caeca of post-smolt Atlantic salmon fed plant (lupin) meal compared to that of fish fed fish meal (Gu *et al.*, 2014). Gu and co-authors suggested that up-regulation of the capability for lipoprotein assembly acted as a compensatory mechanism to transport excessive lipid droplets that accumulated in pyloric caeca and liver (Gu *et al.*, 2014). In the present study, both *apoB* and *apoAI* showed significant up-regulation at 17 dph, and *apoAI* was also significantly up-regulated at 13 dph and 60 dph, suggesting that elevated lipoprotein assembly might have occurred at these stages. Of the PC synthesis genes, only *pcyt1b* showed a significant up-regulation at 17 dph in our study. *Pcyt1b* encodes for CTβ2 and CTβ3 in mice and CTβ1 and CTβ2 in humans. CTβ is expressed at same level as CTα in the brain, whereas CTβ expression in other tissues is only about 10% of CTα (Gibellini and Smith, 2010). CTβ2 disrupted mice was viable with no brain defects, but showed reduced fertility with gonadal dysfunction (Jackowski *et al.*, 2004). Recent studies with *Pcyt1b*$^{-/-}$ mice showed no obvious neurological problems, but reduced PC synthesis in distal axons and less neurite branching was observed in neurons cultured from CTβ2-deficient mice (Vance and Vance, 2009; Strakova *et al.*, 2011). The up-regulation of *pcyt1b* in the present study may be related to the need for biosynthesis of PL in the brain of cod larvae due to low DHA levels of the PL in rotifers. There are evidences that lyso-PC may be a preferred form to pass through blood-brain barrier and carry DHA to the brain (Lagarde *et al.*,

2001). The expression of *pcyt1b* of cod larvae was found to be down-regulated during the live feed period when they were fed copepods, their natural prey, which contains much higher DHA levels in PL compared to rotifers (Elin Kjørsvik communication, CODE project). To our knowledge, no information of CTβ has been reported in any fish species. Even in mammals, there is very little information about CTβ compared to the dominant CTα, which is encoded by *pcyt1a*. As they have similar membrane-binding domain, CTβ is probably also regulated by similar translocation process as CTα (Sugimoto *et al.*, 2008).

The cod larvae increased their body dry weight more than 400 times from hatching to 60 dph in the present study. An increase in phospholipid content per individual must then be expected due to cell division and growth. However, very few genes involved in the *de novo* synthesis pathways of PL were significantly regulated throughout the live feed period and up to 60 dph. One reason for this maybe because the larvae had sufficient dietary phospholipid from live feed and commercial diets, with no need for extensive *de novo* phospholipid synthesis. It has been suggested that exogenous PC could inhibit *de novo* PC synthesis (Mansbach, 1977). Non-transcriptional regulation of key genes involved in the pathway might be another explanation. For example, the activity of CTα involved in the rate-limiting step of PC biosynthesis can be regulated efficiently by translocation on and off membranes. The active form of CTα is membrane-bound and de-phosphorylation can promote activation of CTα activity. In the cell cycle, the transcription of CTα was found to increase two-fold during the S phase (Golfman *et al.*, 2001), whereas the maximal total CT activity and enhanced PC biosynthesis were found during the G1 phase prior the S phase (Sugimoto *et al.*, 2008). Therefore, cells could increase PC biosynthesis by translocation of CTα to membranes without changing the expression level of *pyct1a*.

It is finally important that the gene expression levels of the current study were normalized to 1 dph. No up-regulation does not imply low gene expression level; on the contrary, the gene expression level of cod larvae may be already high at 1 dph and before hatching. In support for this, the CTα mRNA level in the liver of rat was high before birth and decreased after birth, maybe due to reduced activity in cell division (Sesca *et al.*, 1996). A recent study reported gene expression levels of cod eggs in 6 stages (Kleppe *et al.*, 2014), and by analysing their raw data (accession number E-MTAB-2170, http://www.ebi.ac.uk/arrayexpress), we found that the expression of *pyct1a* (ENSGAUG00000007851) was generally higher in the egg stages compared to

the larvae stages (Figure 4). The two experiments were using the same microarray, same labelling protocol and scanner, and therefore the comparison can be relevant.

A recent study using tube feeding method to inject lipid slurry into cod larvae showed that they (30 dph, standard length 9.90 mm) could biosynthesize PL from both 2-Oleoyl-[1,2,3-^{3}H]glycerol (2-MAG) and [^{14}C(U)] glycerol. PC and TAG were mostly synthesized from 2-MAG precursors and the [^{3}H] labelled PC:TAG ratio was 0.44 and 0.74 after 1 h and 4 h of incubation, respectively (Li *et al*. submitted manuscript). Given adequate digestion, it is likely that cod larvae could biosynthesis PL *de novo* in the intestine based on the substrates provided, but their ability need further investigation.

Anyway, the comparison of gene expression level between gut and liver and the rest of body in cod larvae at 60 dph showed that most of the genes involved in PL biosynthesis were expressed at similar level (Figure 3), except for *chka* and *etnk1*, suggesting higher needs for PC and PE biosynthesis in the gut and liver, likely due to the need for assembly of lipoproteins to transport lipid out from the intestine and liver. The alternative PC biosynthesis pathway through PE methylation by PEMT might not be applicable in the cod larvae due to immature liver and the expression level of *pemt* was very low throughout (data not shown). When fish larvae are fed a high fat and low PL diet, limited PL would be available for the production of lipoprotein. Therefore, steatosis (fat droplet accumulation) is frequently observed in their intestine and liver (Dapra *et al.*, 2011).

4.4 Conclusion

The present study provided evidence for that cod larvae could elongate EPA to DPA, and maybe even further to DHA. The function of *elovl4* and *elovl7* in cod larvae may need further investigation, especially *elovl4* due to its possible roles involved in DHA biosynthesis.

The live feed diet provided sufficient PL for cod larvae and almost no up-regulation of the key regulating genes were found throughout the sampling period. However, the up-regulation of *pcyt1b* needs further attention, because of its possible roles in regulating PC biosynthesis in the brain of cod larvae.

Our overall data suggested that there is no clear genetic constrains of undertaking *de novo* PL synthesis in cod larvae, and they appeared to have relatively high capability of PL biosynthesis already at 1 dph, but this capability may be related to cell division

565 and growth, rather than to lipoprotein assembly. The essentiality of dietary PL for fish
566 larvae may not be related to their limited *de novo* PC synthesis ability in the intestine,
567 but to other metabolic constrains, such as limited digestive functions for neutral lipid
568 in the early stages, or low input of bile PC for the lipoprotein assembly as a result of
569 immature liver. Further studies are needed to elucidate the enzyme activity involved
570 in the pathways.
571

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577

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751

752 **Legends**

753 **Figure 1. Pathways of phospholipid (PL) *de novo* biosynthesis.**

754 Red color indicates genes significantly regulated ($p<0.01$) in at least one of the
755 sampling points. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS,
756 phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; CL,
757 cardiolipin; G-3-P, glycerol-3-phosphate; LPA, 2-acyl-glycerol-3-phosphate; PA,
758 phosphatide acid; 2-MAG, 2-monoacylglycerol; DAG, diacylglycerol; TAG,
759 triacylglycerol. CHKA, choline kinase α; PCYT1A/B, phosphate cytidylyltransferase
760 1 α/β; chpt1, choline phosphotransferase 1; ETNK1, ethanolamine kinase; PCYT2,

761 phosphate cytidylyltransferase 2; EPT1, ethanolamine phosphotransferase 1; PEMT,
762 phosphatidylethanolamine N-methyltransferase; PISD, phosphatidylserine
763 decarboxylase; Ptdss1, phosphatidylserine synthase 1; Ptdss2, phosphatidylserine
764 synthase 2; CRLS1, cardiolipin synthase 1; Cdipt, CDP-diacylglycerol-inositol 3-
765 phosphatidyltransferase; PGS1, phosphatidylglycerophosphate synthase 1;
766 GPAT1,2,3,4, sn-1-glycerol-3-phosphate acyltransferase; AGPAT1,2,3, acyl-CoA:1-
767 acylglycerol-3-phosphate acyltransferase; Lipin 1,2,3, PA phosphatase; MGAT, MAG
768 acyltransferase; DGAT, DAG acyltransferase.

769

770 **Figure 2. Numbers of up-regulated and down-regulated genes of each sampling**
771 **points compared to 1 day post hatch. Cut-off p<0.05.**

772

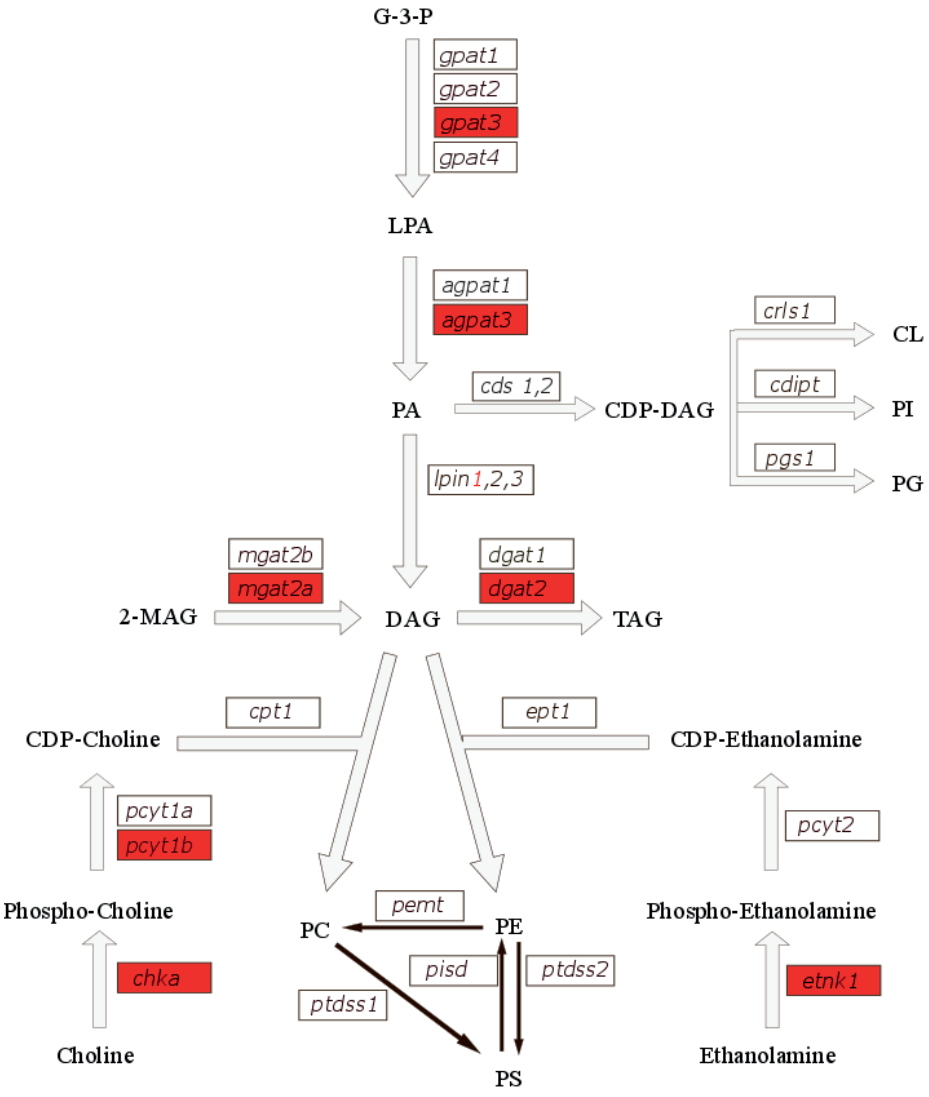
773 **Figure 3. Heat map of the expression of selected genes involved in phospholipid**
774 **metabolism.** Values are log_2-transformed comparing the gene expression between gut
775 & liver versus the rest of body of larvae at 60 dph. Red and blue colours represent up-
776 and down-regulation of genes, respectively.

777

778 **Figure 4. Expression of *chka* and *pcyt1a* throughout egg stages until 60 dph.**
779 Values are log_2-transformed and normalized to 1 dph. Pv, pre-vitellogenic follicles;
780 Ev, early-vitellogenic follicles; Lv, late-vitellogenic follicles; Oo, unfertilized egg; Bl,
781 blastula stage; Ga, gastrula stage; numbers indicating days post hatch.

782

Figure 1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gpat3
gpat4
LPA
agpat1
agpat3
PA
cds 1,2
CDP-DAG
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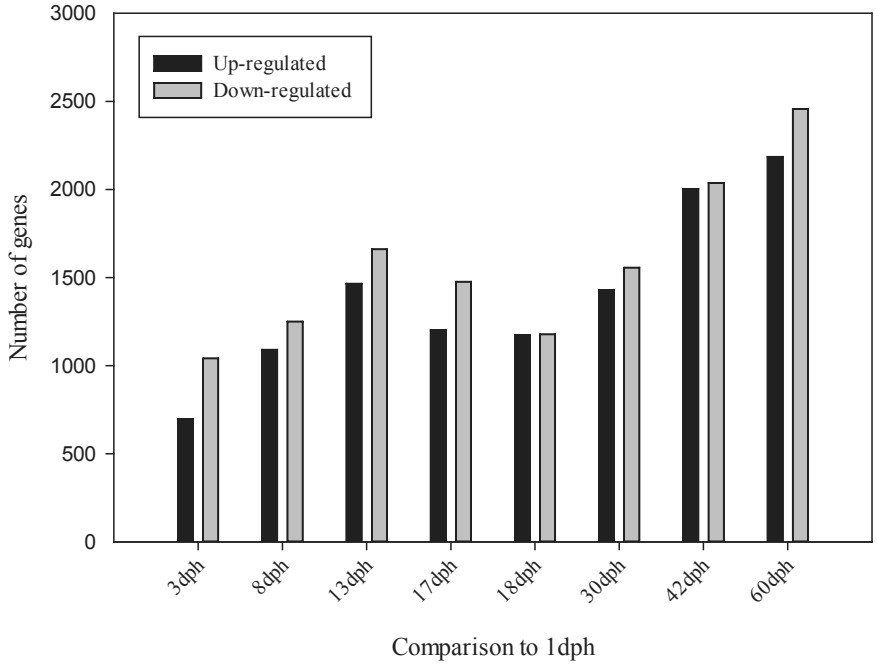
CL
cdipt
PI
pgs1
PG
lpin1,2,3
mgat2b
mgat2a
2-MAG
DAG
dgat1
dgat2
TAG
cpt1
CDP-Choline
ept1
CDP-Ethanolamine
pcyt1a
pcyt1b
Phospho-Choline
pcyt2
Phospho-Ethanolamine
pemt
PC
PE
chka
pisd
ptdss2
ptdss1
etnk1
Choline
PS
Ethanolamine

Figure 2


3000
2500
2000
1500
1000
500
0
Number of genes
Up-regulated
Down-regulated
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8dph
13dph
17dph
18dph
30dph
42dph
60dph
Comparison to 1dph

Figure 3

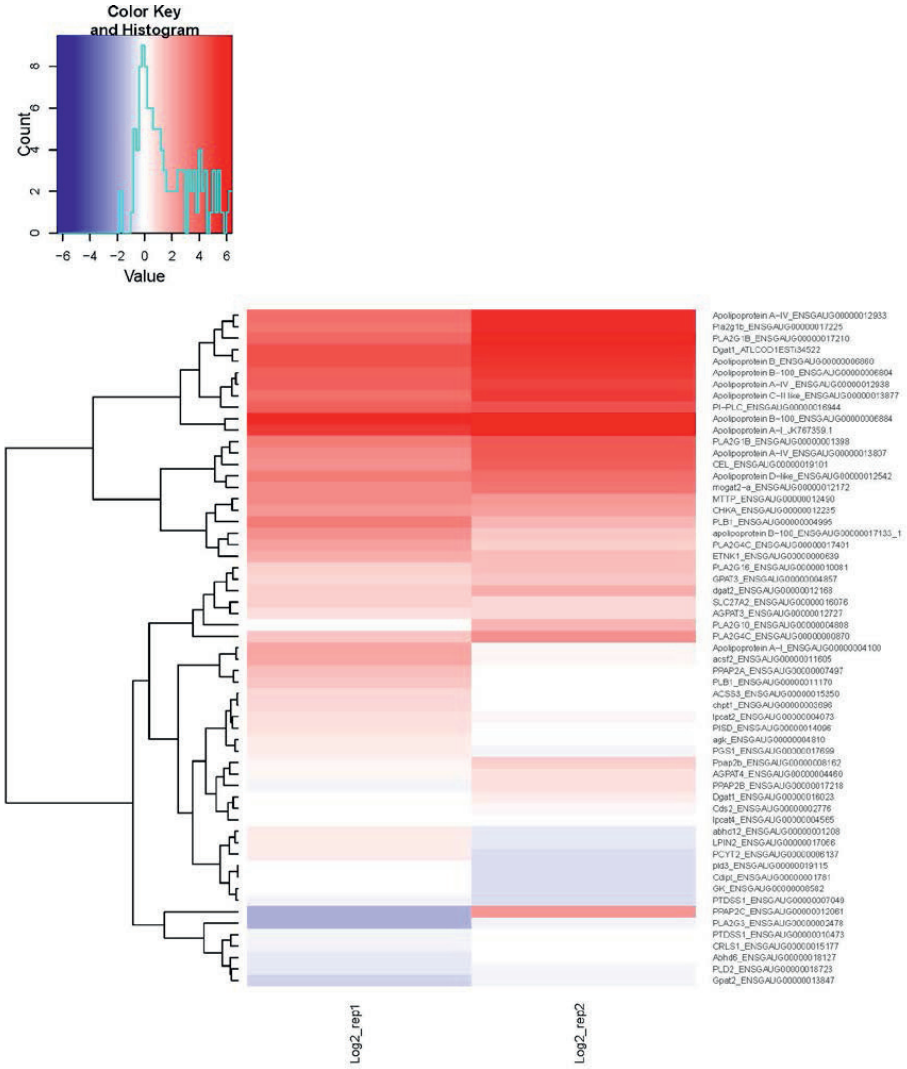
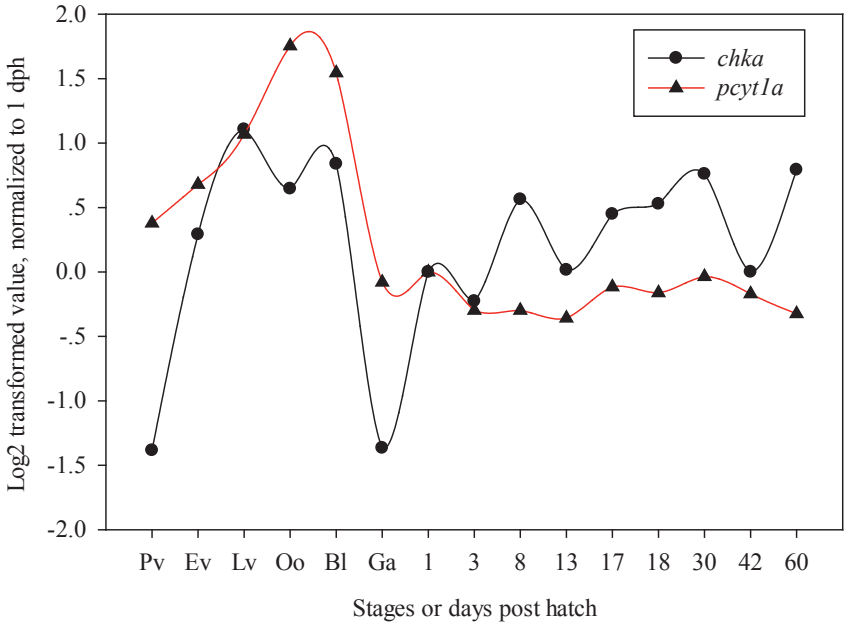


Figure 4


2.0
1.5
1.0
.5
0.0
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Log2 transformed value, normalized to 1 dph
chka
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Ev
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1
3
8
13
17
18
30
42
60
Stages or days post hatch

Table 1. Experiment set up and feeding rations for rotifers. Cayman-ST, Cayman short-term enriched with DSelco; Cayman-LT, Cayman long-term enriched with DSelco; Cayman-Multi, Cayman enriched with Multigain.

Feed source	Experiment 1		Experiment 2
	Cayman-ST	Cayman-LT	Cayman-Multi
Baker's yeast *Saccharomyces cerevisiae*	1.0 µg FW $ind^{-1}d^{-1}$	1.0 µg FW $ind^{-1}d^{-1}$	0.9 µg FW $ind^{-1}d^{-1}$
Rotifer diet algae paste (Reed Mariculture)	0.3-0.5 ml 10^6 ind^{-1} d^{-1}	0.3-0.5 ml 10^6 ind^{-1} d^{-1}	0.3-0.4 ml 10^6 ind^{-1} d^{-1}
Easy DHA Selco (DSelco)	0.23 µg ind^{-1}, 2h of incubation	0.05 µg ind^{-1} d^{-1}, added continuously along with the other feed components	
Multigain			0.15 µg ind^{-1}, 2h of incubation

Table 2. Number of replicates for microarray analyses (dph, days post hatch)

	Number of biological replicates (samples from different tanks)	Number of larvae in each sample
1 dph	3	40
3 dph	8	40
8 dph	6	25
13 dph	4	17
17 dph	8	10
18 dph	3	10
30 dph	3	10
42 dph	3	10
60 dph	2	10
60 dph gut&liver	2	10
60 dph larvae without gut& liver	2	10

Table 3. Cod larvae survival at 18 dph and dry weight (μg ind^{-1}). Values represent Mean ± SE (standard error).

	Experiment 1		Number of replicates	Experiment 2	Number of replicates
	Cod-LT	Cod-ST			
Survival, %	45.8 ± 4.58	43.3 ± 2.50	4		
Dry weight larvae^{-1}					
1 dph	53.2 ± 0.22	53.2 ± 0.22	12	54.4 ± 4.23	10
3 dph	53.2 ± 0.22	53.2 ± 0.22	24		
*8 dph	68.2 ± 1.55	56.3 ± 1.50	48		
*13 dph	101 ± 2.87	86.2 ± 3.21	48		
17 dph	155 ± 6.83	157 ± 5.58	48		
18 dph				161 ± 54.6	10
30 dph				640 ± 57.1	10
42 dph				1180 ± 77.8	10
60 dph				22100 ± 491	450

*indicates significantly different dry weights of larvae at $p<0.05$ level

Table 4. Total lipid and fatty acid composition of rotifers, enrichment diet DHA-Selco (DSelco) and algae paste *Nannochloropsis oculata* (NC), expressed as Mean ± SE.

	Cayman-LT	Cayman-ST	DSelco	NC
	n=6	n=6	n=2	n=2
Total lipid mg g^{-1} DW*	117 ± 1.16	163 ± 3.05	893 ± 12.7	11.8 ± 0.31
Total fatty acid mg g^{-1} DW*	66.3 ± 1.98	105 ± 8.68	687 ± 8.19	56.1 ± 1.88
Fatty acid as percentage of total fatty acid				
14:0*	1.34 ± 0.08	2.24 ± 0.09	3.83 ± 0.01	2.82 ± 0.02
16:0*	10.9 ± 0.05	13.1 ± 0.59	16.8 ± 0.03	19.5 ± 0.09
18:0	3.89 ± 0.16	4.15 ± 0.36	4.04 ± 0.00	0.44 ± 0.00
20:0*	0.09 ± 0.01	0.21 ± 0.02	0.25 ± 0.00	
22:0		0.13 ± 0.01	0.17 ± 0.00	
SFA*	**16.2 ± 0.09**	**19.8 ± 0.56**	**25.1 ± 0.03**	**22.7 ± 0.10**
16:1n7	15.8 ± 0.45	13.7 ± 1.25	4.82 ± 0.00	21.4 ± 0.13
18:1n7	3.62 ± 0.06	3.51 ± 0.14	2.38 ± 0.02	0.79 ± 0.01
18:1n9*	21.7 ± 0.55	19.4 ± 0.26	16.2 ± 0.10	3.22 ± 0.01
20:1n9	3.23 ± 0.19	2.87 ± 0.04	3.83 ± 0.01	
22:1n9	1.05 ± 0.03	0.57 ± 0.02	3.92 ± 0.01	
MUFA*	**46.5 ± 0.85**	**41.6 ± 1.59**	**32.1 ± 0.10**	**25.4 ± 0.12**
18:2n6*	5.27 ± 0.02	3.93 ± 0.14	5.74 ± 0.05	2.77 ± 0.02
20:4n6	1.38 ± 0.02	1.23 ± 0.06	1.12 ± 0.01	3.34 ± 0.03
22:5n6*	0.42 ± 0.02	0.62 ± 0.06	0.85 ± 0.01	0.17 ± 0.00
n6*	**7.87 ± 0.07**	**6.37 ± 0.24**	**7.98 ± 0.03**	**6.63 ± 0.06**
18:3n3	1.04 ± 0.01	1.05 ± 0.01	1.49 ± 0.01	0.13 ± 0.00
18:4n3*	0.26 ± 0.02	0.67 ± 0.05	2.00 ± 0.00	0.11 ± 0.00
20:4n3*	0.98 ± 0.04	0.63 ± 0.02	0.53 ± 0.00	
20:5n3*	6.07 ± 0.14	6.63 ± 0.05	6.16 ± 0.04	32.4 ± 0.25
22:5n3*	2.55 ± 0.09	1.70 ± 0.01	1.21 ± 0.01	
22:6n3*	6.67 ± 0.40	11.6 ± 1.07	17.1 ± 0.13	0.16 ± 0.01
n3*	**17.4 ± 0.69**	**21.7 ± 1.11**	**26.7 ± 0.18**	**32.7 ± 0.26**
PUFA	**25.5 ± 0.76**	**28.8 ± 1.40**	**36.6 ± 0.14**	**39.4 ± 0.30**
DHA/EPA*	1.09 ± 0.04	1.74 ± 0.15	2.78 ± 0.00	0.00 ± 0.00
n3/n6*	2.21 ± 0.07	3.40 ± 0.04	3.34 ± 0.04	4.94 ± 0.03
UNKNOWN	11.7 ± 0.21	9.80 ± 0.35	6.17 ± 0.01	12.5 ± 0.08

SFA, including 14:0, 16:0, 18:0, 20:0, 22:0;

MUFA, including 16:1n7, 18:1n7, 18:1n9, 20:1n9, 22:1n11, 22:1n9, 24:1;

n6, including 18:2n6, 20:2n6, 20:3n6, 20:4n6, 22:5n6

n3, including 18:3n3, 18:4n3, 20:3n3, 20:4n3, 20:5n3, 22:5n3, 22:6n3;

*indicates significant difference between ST-Cayman and LT-Cayman ($p<0.05$)

Table 5. Fatty acid composition of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of rotifers and algae paste *Nannochloropsis oculata* (NC), expressed as Mean ± SE.

	PC			PE		
	Cayman-LT	Cayman-ST	NC	Cayman-LT	Cayman-ST	NC
	n=6	n=6	n=2	n=6	n=6	n=2
14:0	0.86 ± 0.06*	1.24 ± 0.06	1.41 ± 0.11	0.46 ± 0.06	0.57 ± 0.03	2.13 ± 0.12
16:0	19.3 ± 0.50*	14.8 ± 0.83	22.9 ± 0.76	8.91 ± 0.55¤	6.64 ± 0.22	16.7 ± 0.88
18:0	4.37 ± 0.54	5.03 ± 0.42	0.76 ± 0.10	3.81 ± 0.10	3.90 ± 0.16	
SFA	**24.6 ± 0.23***	**21.1 ± 1.00**	**25.1 ± 0.97**	**13.2 ± 0.60**	**12.2 ± 0.31**	**18.8 ± 0.99**
16:1n7	13.1 ± 0.20*	23.6 ± 0.98	22.5 ± 0.27	8.19 ± 0.32¤	14.4 ± 0.94	18.0 ± 0.54
18:1n7	3.02 ± 0.05*	4.69 ± 0.25	1.33 ± 0.00	2.64 ± 0.13¤	3.33 ± 0.03	
18:1n9	15.7 ± 0.46	16.6 ± 0.35	12.9 ± 0.00	20.9 ± 0.50¤	23.5 ± 0.14	1.33 ± 0.09
20:1n9	1.56 ± 0.05	1.61 ± 0.10		5.38 ± 0.09¤	5.01 ± 0.07	
22:1n11	0.12 ± 0.01	0.00 ± 0.00		1.88 ± 0.12	0.00 ± 0.00	
22:1n9	0.26 ± 0.01*	0.33 ± 0.01		2.20 ± 0.08	1.95 ± 0.11	
24:1	0.23 ± 0.03	0.24 ± 0.01		0.76 ± 0.08¤	0.55 ± 0.04	
MUFA	**33.9 ± 0.70***	**47.1 ± 1.48**	**36.7 ± 0.27**	**41.3 ± 0.88¤**	**48.7 ± 0.87**	**19.3 ± 0.46**
18:2n6	5.62 ± 0.06*	4.09 ± 0.46	16.6 ± 0.29	9.41 ± 0.25¤	5.02 ± 0.24	9.17 ± 0.46
20:2n6	0.21 ± 0.01*	0.12 ± 0.03		0.13 ± 0.01	0.14 ± 0.04	
20:3n6	0.65 ± 0.02	0.60 ± 0.03		0.80 ± 0.04	0.87 ± 0.03	
20:4n6	1.29 ± 0.20*	0.68 ± 0.08	3.54 ± 0.25	1.42 ± 0.09¤	0.93 ± 0.09	1.84 ± 0.12
n6	**8.08 ± 0.15***	**5.49 ± 0.54**	**20.2 ± 0.04**	**12.0 ± 0.30¤**	**6.91 ± 0.39**	**11.0 ± 0.34**
18:3n3	0.98 ± 0.02	1.09 ± 0.12	5.36 ± 0.07	1.42 ± 0.03	1.41 ± 0.05	6.91 ± 0.17
18:4n3	0.45 ± 0.02	0.33 ± 0.08		0.17 ± 0.01	0.21 ± 0.02	
20:4n3	1.40 ± 0.09*	0.77 ± 0.06		1.81 ± 0.11¤	1.10 ± 0.04	
20:5n3	7.18 ± 0.25*	5.67 ± 0.59	8.28 ± 1.11	5.62 ± 0.41¤	7.33 ± 0.26	36.1 ± 2.16
22:5n3	4.94 ± 0.27*	2.57 ± 0.28		2.62 ± 0.15	2.28 ± 0.11	
22:6n3	6.99 ± 0.38*	0.90 ± 0.17	1.09 ± 0.17	3.54 ± 0.26¤	0.68 ± 0.09	

n3	**21.5 ± 0.74***	**11.1 ± 1.12**	**14.7 ± 1.01**	**15.0 ± 0.88¤**	**12.8 ± 0.43**	**43.0 ± 1.99**
PUFA	**30.1 ± 0.73***	**16.9 ± 1.71**	**34.9 ± 1.06**	**27.1 ± 1.11¤**	**19.9 ± 0.71**	**54.0 ± 1.65**
DHA/EPA	0.98 ± 0.07*	0.16 ± 0.02	0.14 ± 0.04	0.63 ± 0.02¤	0.09 ± 0.01	0.00 ± 0.00
n3/n6	2.67 ± 0.11*	2.01 ± 0.05	0.73 ± 0.05	1.25 ± 0.06¤	1.87 ± 0.09	3.91 ± 0.30
UNKNOWN	11.4 ± 0.18	14.9 ± 1.17	3.35 ± 0.18	18.4 ± 0.11	19.3 ± 0.51	7.88 ± 0.20

SFA, including 14:0, 16:0, 18:0, 20:0, 22:0;

MUFA, including 16:1n7, 18:1n7, 18:1n9, 20:1n9, 22:1n11, 22:1n9, 24:1;

n6, including 18:2n6, 20:2n6, 20:3n6, 20:4n6, 22:5n6

n3, including 18:3n3, 18:4n3, 20:3n3, 20:4n3, 20:5n3, 22:5n3, 22:6n3;

*indicates significant difference between Cayman-LT and Cayman-ST in PC ($p<0.05$)

¤indicates significant difference between Cayman-LT and Cayman-ST in PE ($p<0.05$)

Table 6. Total lipid and fatty acid composition of cod larvae, expressed as Mean ± SE.

TFA	1 dph	3 dph	18 dph Cod-LT	18 dph Cod-ST
	n=2	n=2	n=8	n=8
Total lipid mg g^{-1} DW	150±0.50^{a}	153±2.65^{a}	134±2.27^{b}	134±2.17^{b}
Total fatty acid mg g^{-1} DW	91.7±1.05^{a}	88.5±0.57^{a}	71.9±1.62^{b}	78.1±0.62^{c}
14:0	1.02±0.01^{a}	0.80±0.02^{b}	0.62±0.03^{c}	0.79±0.02^{b}
16:0	19.6±0.06^{a}	19.2±0.06^{a}	15.4±0.14^{b}	14.3±0.14^{c}
18:0	4.67±0.06^{a}	5.50±0.04^{b}	7.61±0.13^{c}	7.77±0.04^{c}
SFA	**25.3±0.10^{a}**	**25.6±0.08^{a}**	**23.6±0.22^{b}**	**22.9±0.17^{c}**
16:1n7	2.05±0.02^{a}	1.82±0.04^{a}	5.04±0.34^{b}	6.80±0.09^{c}
18:1n7	3.69±0.02^{a}	3.62±0.04^{a}	3.89±0.02^{b}	4.45±0.02^{c}
18:1n9	10.1±0.07^{a}	9.50±0.12^{a}	12.5±0.17^{b}	12.5±0.08^{b}
20:1n9	1.36±0.01^{a}	1.30±0.02^{a}	1.92±0.05^{b}	1.83±0.04^{b}
24:1	0.13±0.01^{a}	0.13±0.01^{a}	0.42±0.03^{b}	0.37±0.01^{b}
MUFA	**15.3±0.09^{a}**	**14.6±0.18^{a}**	**18.8±0.28^{b}**	**19.4±0.14^{b}**
18:2n6	2.35±0.03^{a}	2.09±0.04^{a}	3.42±0.11^{b}	3.19±0.03^{b}
20:2n6	0.26±0.00^{a}	0.28±0.00^{a}	0.47±0.01^{b}	0.42±0.01^{c}
20:3n6	0.07±0.00^{a}		0.66±0.01^{b}	0.70±0.01^{b}
20:4n6	1.64±0.00^{a}	2.03±0.03^{b}	3.60±0.06^{c}	3.09±0.03^{d}
22:5n6	0.25±0.00^{a}	0.25±0.00^{a}	0.51±0.02^{b}	0.42±0.01^{c}
n6	**4.53±0.07^{a}**	**4.66±0.06^{a}**	**8.65±0.06^{b}**	**7.82±0.06^{c}**
18:3n3	0.24±0.01^{a}	0.19±0.00^{a}	0.39±0.02^{b}	0.53±0.00^{c}
18:4n3	0.22±0.00^{a}	0.16±0.00^{ab}		0.12±0.00^{b}
20:4n3	0.32±0.00^{a}	0.29±0.00^{a}	0.87±0.02^{b}	0.79±0.00^{c}
20:5n3	16.2±0.07^{a}	16.0±0.10^{a}	11.7±0.42^{b}	13.5±0.17^{c}
22:5n3	1.88±0.01^{a}	1.92±0.00^{a}	4.97±0.06^{b}	4.15±0.03^{c}
22:6n3	30.0±0.17^{a}	31.2±0.28^{a}	17.1±0.86^{b}	15.3±0.28^{b}
n3	**48.8±0.08^{a}**	**49.7±0.17^{a}**	**35.0±0.59^{b}**	**34.5±0.23^{b}**
PUFA	**53.4±0.01^{a}**	**54.4±0.11^{a}**	**43.7±0.54^{b}**	**42.3±0.25^{b}**
DHA/EPA	1.85±0.02^{ac}	1.96±0.03^{c}	1.49±0.12^{ab}	1.14±0.03^{b}
UNKNOWN	6.04±0.03^{a}	5.53±0.01^{a}	13.9±0.52^{b}	15.5±0.13^{c}

Values in the same row not sharing a superscript are significantly different ($p < 0.05$).

Table 7. Fatty acid composition of phosphatidylcholine (PC) of cod larvae, expressed as Mean ± SE.

PC	1 dph	3 dph	18 dph Cod-LT	18 dph Cod-ST
	n=3	n=2	n=7	n=5
14:0	0.93±0.04^{a}	0.84±0.03^{ac}	0.59±0.03^{b}	0.70±0.06^{bc}
16:0	26.0±0.41^{a}	27.8±0.29^{a}	21.4±0.54^{b}	20.2±1.04^{b}
18:0	3.74±0.14^{a}	3.98±0.03^{a}	5.46±0.34^{b}	6.16±0.49^{b}
SFA	**30.7±0.57^{a}**	**32.6±0.35^{a}**	**27.5±0.45^{b}**	**27.1±0.73^{b}**
16:1n7	1.83±0.09^{a}	1.96±0.08^{a}	5.25±0.23^{b}	6.47±0.36^{c}
18:1n7	2.66±0.03^{a}	2.55±0.02^{a}	3.05±0.03^{b}	3.59±0.03^{c}
18:1n9	8.09±0.26^{a}	9.12±0.21^{a}	14.3±0.56^{b}	13.8±0.44^{b}
20:1n9	0.78±0.05^{a}	0.83±0.00^{a}	1.50±0.06^{b}	1.28±0.06^{b}
MUFA	**13.4±0.37^{a}**	**14.5±0.31^{a}**	**24.6±0.66^{b}**	**25.4±0.78^{b}**
18:2n6	2.19±0.02^{a}	2.16±0.02^{a}	4.01±0.14^{b}	3.57±0.05^{b}
20:2n6	0.21±0.00^{a}	0.23±0.00^{a}	0.42±0.00^{b}	0.39±0.01^{c}
20:4n6	1.79±0.03^{b}	2.05±0.02^{c}	3.60±0.15^{a}	3.06±0.16^{a}
22:5n6	0.27±0.01^{a}	0.26±0.01^{a}	0.43±0.02^{b}	0.38±0.03^{ab}
n6	**4.46±0.02^{a}**	**4.69±0.01^{b}**	**9.25±0.11^{c}**	**8.20±0.18^{d}**
18:3n3	0.22±0.00^{a}	0.18±0.00^{a}	0.44±0.03^{b}	0.56±0.01^{c}
18:4n3	0.17±0.00^{a}	0.15±0.00^{a}	0.09±0.01^{b}	0.15±0.01^{a}
20:4n3	0.32±0.00^{a}	0.29±0.01^{a}	0.85±0.03^{b}	0.78±0.02^{b}
20:5n3	19.4±0.74^{a}	18.4±0.27^{a}	12.6±0.50^{b}	14.0±0.49^{b}
22:5n3	1.78±0.06^{a}	1.59±0.04^{a}	4.14±0.10^{b}	3.45±0.19^{c}
22:6n3	26.1±0.30^{a}	24.0±0.43^{a}	12.0±0.53^{b}	11.7±0.84^{b}
n3	**48.0±1.03^{a}**	**44.6±0.74^{a}**	**30.2±0.69^{b}**	**30.7±1.50^{b}**
PUFA	**52.4±1.05^{a}**	**49.3±0.75^{a}**	**39.4±0.77^{b}**	**38.9±1.66^{b}**
DHA/EPA	1.35±0.04^{a}	1.31±0.00^{a}	0.97±0.06^{b}	0.84±0.04^{b}
n3/n6	10.7±0.18^{a}	9.51±0.13^{b}	3.26±0.06^{c}	3.74±0.11^{d}
UNKNOWN	3.56±0.13^{a}	3.63±0.09^{a}	8.52±0.24^{b}	8.61±0.17^{b}

Values in the same row not sharing a superscript are significantly different ($p < 0.05$).

Table 8. Fatty acid composition of phosphatidylethanolamine (PE) of cod larvae, expressed as Mean ± SE.

PE	1 DPH	3 DPH	18 dph Cod-LT	18 dph Cod- ST
	n=3	n=2	n=7	n=6
14:0	0.15±0.04		0.12±0.01	0.13±0.00
16:0	7.36±0.27^{b}	6.25±0.15^{ab}	5.29±0.33^{a}	4.82±0.14^{a}
18:0	5.71±0.04^{a}	6.19±0.04^{a}	9.10±0.10^{b}	9.48±0.18^{b}
SFA	**13.2±0.28^{ab}**	**12.4±0.19^{a}**	**14.5±0.40^{b}**	**14.4±0.25^{b}**
16:1n7	0.69±0.14	0.41±0.00	0.77±0.08	0.90±0.03
18:1n7	5.24±0.07^{b}	5.19±0.02^{bc}	4.42±0.06^{a}	4.89±0.05^{c}
18:1n9	8.65±0.21^{a}	8.16±0.10^{a}	6.94±0.18^{b}	6.97±0.10^{b}
20:1n9	2.19±0.02^{a}	2.12±0.05^{a}	2.21±0.05^{a}	1.87±0.02^{b}
MUFA	**16.6±0.52^{a}**	**15.7±0.04^{ab}**	**14.2±0.38^{b}**	**14.5±0.19^{b}**
18:2n6	2.06±0.06	1.78±0.03	2.04±0.07	1.92±0.06
20:2n6	0.49±0.02^{a}	0.52±0.02^{ab}	0.55±0.01^{b}	0.49±0.00^{a}
20:3n6			0.63±0.02	0.68±0.01
20:4n6	0.91±0.06^{a}	0.89±0.00^{a}	3.91±0.04^{b}	3.41±0.04^{c}
22:5n6	0.29±0.01^{a}	0.30±0.00^{a}	0.79±0.02^{b}	0.72±0.02^{b}
n6	**3.75±0.04^{a}**	**3.49±0.04^{a}**	**7.92±0.09^{b}**	**7.23±0.08^{c}**
18:3n3	0.16±0.01		0.28±0.01	0.36±0.00
20:4n3	0.49±0.02^{a}	0.46±0.01^{a}	1.38±0.04^{b}	1.25±0.02^{b}
20:5n3	14.1±0.54^{ab}	12.4±0.03^{b}	14.6±0.51^{a}	16.3±0.20^{a}
22:5n3	2.34±0.06^{a}	2.35±0.00^{a}	8.47±0.16^{b}	7.61±0.06^{c}
22:6n3	46.7±0.49^{a}	50.7±0.27^{b}	30.7±0.89^{c}	31.1±0.68^{c}
n3	**63.8±0.45^{a}**	**66.0±0.31^{a}**	**55.7±0.92^{b}**	**56.9±0.60^{b}**
PUFA	**67.6±0.42^{ac}**	**69.4±0.35^{c}**	**63.6±0.87^{b}**	**64.1±0.52^{ab}**
DHA/EPA	3.33±0.15^{a}	4.08±0.01^{a}	2.13±0.12^{b}	1.91±0.06^{c}
n3/n6	17.0±0.29^{a}	18.9±0.15^{b}	7.04±0.17^{c}	7.89±0.17^{d}
UNKNOWN	2.73±0.17^{a}	2.46±0.12^{a}	7.67±0.39^{b}	6.94±0.40^{b}

Values in the same row not sharing a superscript are significantly different ($p< 0.05$).

Table 9. Summary of the significantly regulated genes ($p<0.05$, at least in one of the sampling points) that were related to phospholipid metabolism. The data are log2-transformed gene expression ratios normalized to 1dph.

Accession	symbol	description	3 dph	8 dph	13 dph	17 dph	18 dph	30 dph	42 dph	60 dph
***De novo* PL synthesis**										
ENSGAUG00000004857	*gpat3*	1-acyl-sn-glycerol-3-phosphate acyltransferase theta-B	0.164	0.077	-0.169	-0.059	0.01	-0.026	1.32**	0.452
ENSGAUG00000004460	*agpat4*	1-acyl-sn-glycerol-3-phosphate acyltransferase delta-like	0.009	0.57	1.203*	0.296	0.438	0.302	0.54*	0.592*
ENSGAUG00000012061	*ppap2C*	lipid phosphate phosphohydrolase 2-like	-0.121	0.417	-0.025	0.13	0.467	0.64*	0.435	1.017**
ENSGAUG00000017066	*lpin2*	phosphatidate phosphatase LPIN2-like	-0.601*	-1.055**	-0.915**	-0.667**	-0.871**	-0.809**	-0.71**	-0.816**
ENSGAUG00000007572	*lpin1*	phosphatidate phosphatase LPIN1-like	1.301**	0.483*	-0.015	0.412*	0.432	-0.114	1.279**	-0.347
ENSGAUG00000012172	*mogat2-a*	2-acylglycerol O-acyltransferase 2-A-like	0.311	0.136	0.276	0.276	0.104	-0.879	-1.282**	-0.067
ENSGAUG00000003992	*dgat1*	diacylglycerol O-acyltransferase 1	0.449	0.139	-0.626*	-0.044	-0.008	-0.194	-0.042	-1.092**
ENSGAUG00000016023	*dgat1*	diacylglycerol O-acyltransferase 1-like	-0.675**	-1**	-0.409*	-1.303**	-0.996**	-1**	-0.855**	0.243
ENSGAUG00000012168	*dgat2*	diacylglycerol O-acyltransferase 2-like	0.365	0.728**	0.17	0.687**	1.242**	0.831**	0.993**	1.932**
ENSGAUG00000002261	*pcyt1b*	choline-phosphate cytidylyltransferase B-like	0.254	-0.074	0.561	1.042**	0.017	-0.112	-0.328	0.115
ENSGAUG00000000639	*etnk1*	ethanolamine kinase 1-like	0.153	-0.059	0.327	0.568	0.333	0.296	0.151	1.449**

Lipase

ENSGAUG00000019101	*bal*	Bile salt-activated lipase-like protein	-0.315	0.739	2.646*	2.519*	2.156	1.619	2.656*	5.034**
ENSGAUG00000015559	*lipc*	Hepatic triacylglycerol lipase	-0.272	1.521**	2.064**	2.466**	2.415**	1.787**	1.454**	3.484**
ENSGAUG00000001398	*pla2g1b*	Phospholipase A2	-1.252	1.833	3.894*	2.606	2.146	2.826	3.644*	4.844**
ENSGAUG00000017210	*pla2g1b*	phospholipase A2-like	-0.107	1.061	2.136**	0.966	1.069	1.839**	4.943**	7.876**
ENSGAUG00000017225	*pla2g1b*	phospholipase A2-like	0.035	0.322	0.401	0.164	0.038	0.157	3.691**	7.442**
ENSGAUG00000004995	*plb1*	Phospholipase B1, membrane-associated-like	1.818**	2.675**	2.531**	2.835**	2.491**	1.285*	0.617	2.407**
ENSGAUG00000011170	*plb1*	Phospholipase B1, membrane-associated-like	1.277**	1.789**	0.947**	1.19**	1.436**	-0.095	0.016	-0.2
ENSGAUG00000001208	*abhd12*	monoacylglycerol lipase ABHD12-like	0.158	0.764**	0.77**	0.97**	0.926**	1.053**	0.996**	1.187**
ENSGAUG00000011605	*acsf2*	acyl-CoA synthetase family member 2, mitochondrial-like	0.589*	0.641*	0.722*	0.924**	0.888**	0.824**	0.497	1.083**
ENSGAUG00000015350	*acss3*	acyl-CoA synthetase short-chain family member 3, mitochondrial-like	0.879**	-0.006	0.252	0.173	-0.357	-0.934**	-1.258**	-1.418**
ENSGAUG00000016076	*slc27a2*	Very long-chain acyl-CoA synthetase	0.437	0.745*	0.676	1.087**	0.818	0.405	-0.022	0.881**
ENSGAUG00000019115	*pld3*	phospholipase D3-like	-0.388	-0.651*	-1.275**	-0.575*	-0.411	-0.409	-0.112	-1.043**
ENSGAUG00000002478	*pla2g3*	group 3 secretory phospholipase A2 precurso	-0.071	0.189	0.309	0.181	0.492	0.99**	0.955**	1.965**
ENSGAUG00000000870	*pla2g4c*	cytosolic phospholipase A2 zeta-like	-0.579	-1.026*	-0.061	0.805*	0.678	0.659	-0.088	2.168**

ENSGAUG00000004227	*plcg2*	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma-2	-0.262	0.285	1.408*	-0.278	0.167	0.183	0.479	-0.105
ENSGAUG00000016944	*plcA*	1-phosphatidylinositol phosphodiesterase-like	-0.441	1.232	2.728**	1.927*	1.634	1.674	2.438*	4.034**
ENSGAUG00000004808	*pla2g10*	group 10 secretory phospholipase A2-like	-0.627*	-0.956**	-0.399	-0.74**	-0.415	0.743*	0.554	2.365**
ENSGAUG00000010269	*pnpla8*	calcium-independent phospholipase A2-gamma-like	0.263	0.08	0.076	-0.011	0.185	-0.502	2.143**	-1.026*
Lipoprotein										
ENSGAUG00000012490	*mttp*	microsomal triglyceride transfer protein large subunit-like	-0.053	-0.133	0.133	0.442	-0.132	-0.33	-1.506**	-0.315
ENSGAUG00000006804	*apoB*	apolipoprotein B-100-like	-1.913**	-0.071	0.736	1.107*	0.72	0.742	-0.396	0.863
ENSGAUG00000006860	*apoB-100*	Apolipoprotein B-100	-0.053	-0.669*	-0.129	-0.116	-0.464	-1.319**	-1.9**	-0.962**
ENSGAUG00000012933	*apoA-IV*	Apolipoprotein A-IV	-0.88	-0.625	-0.303	-0.024	-0.646	-0.351	-2.758**	0.021
ENSGAUG00000012938	*apoA-IV*	apolipoprotein A-IV-like	-0.428	-0.047	0.231	0.835	-0.148	-0.154	-1.883**	-0.232
ENSGAUG00000013807	*apoA-IV*	Apolipoprotein A-IV precursor	0.312	0.487	0.378	0.835	0.56	0.361	-1.454*	-0.129
ENSGAUG00000004100	*apoA-I*	Apolipoprotein A-I-like	-0.892	0.568	1.563*	2.615**	1.664	1.451	0.248	2.023*
Desaturases and elongases										
ENSGAUG00000017746	*fadsd6*	delta-6 fatty acyl desaturase	0.116	0.354	0.282	0.829**	0.566*	0.95**	0.741**	1.179**
ENSGAUG00000010702	*fadsd6*	fatty acid desaturase 6-like	-0.044	-0.585*	-0.687	-0.584**	-1.059**	-1.409**	-1.073**	-0.811**
ENSGAUG00000018234	*elovl1*	elongation of very long chain fatty acids protein 1-like	-1.657**	-1.396*	-1.603*	-1.155	-1.13	-0.593	-0.882	-0.989

ENSGAUG00000018557	*elovl4*	elongation of very long chain fatty acids protein 4-like	-0.248	0.114	0.483*	0.188	0.482	0.899**	1.198**	1.952**
ENSGAUG00000007839	*elovl7*	elongation of very long chain fatty acids protein 7-like	0.052	0.349	0.444	0.889*	0.771	0.729	1.402**	1.218*

*indicates significant difference at $p<0.05$ level;

**indicates significant difference at $p<0.01$ level

Paper VI

Atlantic cod (*Gadus morhua*) larvae have enzymatic capabilities to biosynthesis phospholipid *de novo* from 2-Oleoyl-glycerol and glycerol precursors

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Abstract

The dietary requirement of phospholipid (PL) of fish larvae has been suggested to originate in an inefficient ability to *de novo* biosynthesis of PL based on dietary triacylglycerol (TAG). The main objective of the present study was to investigate if cod larvae could synthesis PL from *sn*-2-mono-acylglycerol (2-MAG) and glycerol precursors. A tube feeding method was used to deliver equal molar aliquots of 2-Oleoyl-[1,2,3-^{3}H]glycerol and [^{14}C(U)] glycerol together with bovine serum albumin (BSA) bound 16:0 and docosahexaenoic acid (DHA), with or without choline chloride to the foregut of anesthetized cod larvae and thereafter monitoring the metabolism of these components in the larvae through short time following injection. Our results showed that cod larvae intestines have a high metabolic rate, and are fully capable to synthesize PL through both the 2-MAG and glycerol-3-phosphate (G-3-P) pathways. Furthermore supplementation of choline chloride significantly increased the phosphatidylcholine (PC) synthesis over TAG (PC:TAG ratio) ($p<0.05$). We therefore suggest that cod larvae might have relatively high ability to synthesize PC from 2-MAG and glycerol precursors and the requirement of dietary PC for fish larvae fed normal diets that need digestion was possibly due to the immature digestive functions for neutral lipid and the low input of bile PC from the immature liver. However, further studies are needed to quantify the enzyme activity involved in the CDP-choline (cytidine diphosphate-choline) pathway, factors affecting PC synthesis ability and bile acid production in fish larvae.

Key words: Atlantic cod larvae, phospholipid, *de novo* synthesis, tube feeding

Introduction

Phospholipids (PL) are essential dietary components for normal development and growth in fish larvae, whereas the PL requirements appear to decrease as the fish grow bigger (Tocher *et al.*, 2008; Cahu *et al.*, 2009). One typical observation in larvae from many fish species fed PL-deficient diets is the accumulation of lipid droplets (steatosis) in the intestine (Fontagne *et al.*, 1998; Olsen *et al.*, 1999; Olsen *et al.*, 2003; Dapra *et al.*, 2011).

Phosphatidylcholine (PC) is the most abundant phospholipid of eukaryotic membranes, accounting for 40-60% of the total phospholipid species (Kent, 2005). PC is also an important component of lipoproteins and thus transport of lipid throughout the body. In lipoproteins, PC surrounds the neutral core of triacylglycerol (TAG) and other nonpolar lipids while apolipoproteins are assembled into the PC monolayer. The PC used in the lipoprotein assembly in enterocytes can originate from dietary, biliary or *de novo* synthesis (Mansbach, 1977). The liver of healthy Caucasians produces 7 to 22 g PC every day that is excreted into the intestinal lumen through the bile (Northfield and Hofmann, 1975), and a 20 g mouse can secret 23 mg PC daily (Kuipers *et al.*, 1997). However, early life stages of many fish species have incomplete developed livers during the period of first feeding and it has been suggested that these have limited capacity for *de novo* synthesis of PL (Tocher *et al.*, 2008; Cahu *et al.*, 2009). Thus deficiency in endogenous PC could lead to low lipoprotein synthesis and lipid accumulation in the enterocytes. Consequently, supplementation of dietary PL could improve the intestinal lipid absorption for many species of fish larvae (Fontagne *et al.*, 1998; Olsen *et al.*, 2003; Dapra *et al.*, 2011).

The biochemically limiting steps for PL synthesis in fish larvae are unknown. However, the metabolic pathways appear to be the same as for mammals (Sargent *et al.*, 2002). In mammals, the major biosynthesis pathway of PC is the CDP-choline (cytidine diphosphate-choline) pathway with choline kinase (CK) producing phosphocholine, followed by CTP:phosphocholine cytidylyltransferase (CT) producing CDP-choline, and accomplished by CDP-choline: *sn*-1,2-diacylglycerol cholinephosphotransferase (CPT) producing PC. The second step catalysed by CT is considered to be the rate-limiting step in the pathway under normal physiological conditions. However, the last step catalysed by CPT can become rate-limiting if the supply of di-acylglycerol (DAG) is restricted (Gibellini and Smith, 2010).

Alternatively, PC can be synthesized by methylation of phosphatidylethanolamine (PE), catalysed by phosphatidylethanolamine *N*-methyltransferase (PEMT), which mainly operates in the liver and contributes to 30-40% of PC in hepatocytes (Sundler and Akesson, 1975; DeLong *et al.*, 1999; Reo *et al.*, 2002).

Choline is an essential nutrient for animals, and its main fate is the biosynthesis of PC via the CDP-choline pathway (Li and Vance, 2008). Inadequate choline intake can lead to fatty liver or muscle damage in humans (Fischer *et al.*, 2007) and liver dysfunction, reduced growth and poor feed efficiency in many fish species (Millikin, 1982). Supplementation of 0.2% choline of dry weight in the diet can improve growth performance, intestinal enzymes activities and feed utilization of blunt snout bream (*Megalobrama amblycephala*) fed high-lipid diet (Li *et al.*, 2015a). One of the obvious functions of choline was the stimulating effect of PC biosynthesis as shown in isolated rat hepatocytes where supplementation of 0.5-2 mM choline could stimulate PC synthesis 2 to 3 folds via the CDP-choline pathway (Sundler and Akesson, 1975).

Atlantic cod is one of the most important species in North Atlantic, and significant progress has been made in research on Atlantic cod cultivation, and the full genome of cod has recently been sequenced (Star *et al.*, 2011). Our recent studies showed that most of the genes involved in the *de novo* PL synthesis pathways remained the same expression level from hatching to 60 days post hatch (Li et al. in prep). Many of the regulatory mechanisms of PC biosynthesis are at non-transcriptional levels (Sugimoto *et al.*, 2008), and biochemical or enzymological studies are therefore needed to elucidate if cod larvae could synthesis PL *de novo*.

The main objective of the present study was to investigate if cod larvae could synthesis PL from 2-MAG and glycerol precursors, which are the main products of TAG digestion. Moreover, the effect of supplemented choline on the biosynthesis of PL and TAG was also investigated. *In vitro* studies using intestinal segments were not applicable due to the small size of fish larvae. A tube feeding method as described by Rønnestad *et al.* (2001) was used to deliver 2-Oleoyl-[1,2,3-^{3}H]glycerol and [^{14}C(U)] glycerol together with bovine serum albumin (BSA) bound 16:0 and DHA, with or without choline chloride to the foregut of anesthetized cod larvae and thereafter monitoring the metabolism of these components in the larvae through short time following injection.

Material and methods

Experimental fish

Atlantic cod (*Gadus morhua*) eggs were obtained from Nofima marine national breeding station, Havbruksstasjonen i Tromsø AS, hatched and cultivated in a flow through system according to the standard procedure at NTNU Sealab (Attramadal *et al.*, 2014). From 3 dph (day post hatch) to 17 dph, the larvae were fed Multigain enriched rotifers (Li and Olsen, submitted) 3 times a day, thereafter, Multigain enriched *Artemia* (Jin *et al.*, 2014) 3 times a day until the end of the experiment. During the transfer from rotifers to *Artemia*, the fish larvae were co-fed for 5 days.

Preparation of solutions

Fatty acid-free bovine serum albumin (BSA) was dissolved in distilled water to make 50 mg ml^{-1} BSA solution. Fatty acids were added to the BSA solution in molar ratio of fatty acid: BSA of 4:1. The solutions were sonicated for 5 min under nitrogen atmosphere at room temperature and thereafter sterilized by filtration through 0.2 μm cellulose acetate membrane filters (Cat no. 28145-477, VWR) (Lynch, 1980).

A stock solution of 0.5 mg ml^{-1} cold 2-Oleoyl-glycerol (2-MAG) was solubilized in chloroform. Radiolabelled 2-Oleoyl-[1,2,3-^{3}H]glycerol (60 Ci $mmol^{-1}$, American Radiolabeled Chemicals Inc. St. Louis, MO, USA) was diluted with cold 2-MAG to form a specific radioactivity of 1.08 Ci $mmol^{-1}$ of the solution. The 2-MAG-chloroform solution was then dried under a stream of nitrogen and resuspended in freshly prepared BSA solution (see composition above) by sonication for 5 min under nitrogen at room temperature. The BSA-2-MAG solution was used immediately to minimize the effect of isomerization.

For the control treatment without choline-chloride added, equal molar solutions of DHA-BSA, 16:0-BSA, 2-Oleoyl-[1,2,3-^{3}H]glycerol-BSA and [^{14}C(U)] glycerol (150 mCi $mmol^{-1}$ in distilled water, American Radiolabeled Chemicals Inc. St. Louis, MO, USA) were mixed. For the choline treatment, a stock solution of 0.5 mg ml^{-1} choline-chloride was dissolved in ethanol, dried under a stream of nitrogen, resuspended in [^{14}C(U)] glycerol-water solution, and mixed with DHA-BSA, 16:0-BSA and 2-Oleoyl-[1,2,3-^{3}H]glycerol-BSA solutions. The final concentrations are reviewed in Table 1.

150 *Tube-feeding and post incubation*

151 One day before undertaking tube-feeding, larvae were transferred into a tank
152 containing 30 L of seawater, and the larvae were starved 12-16 h before tube-feeding.
153 The tube-feeding experiment was performed for 30 dph larvae kept in the same room
154 as the rearing tank. Individual larva was anesthetized for 2-5 min with MS-222 (30 µg
155 ml^{-1}, Tricaine methanesulfonate, Sigma-Aldrich, St. Louis, MO, USA) until
156 swimming ceased and then placed under a dissecting microscope attached with a
157 camera. A picture was taken before they were tube-fed 66 nl (approximately 13% of
158 the gut volume) (Kamisaka and Ronnestad, 2011) of the different solutions shown in
159 Table 1. A 0.19 mm plastic capillary tube (Sigma-Aldrich, St. Louis, MO, USA)
160 connecting to a 10 µl syringe mounted with a manual nanolitre injector (Sutter
161 Instrument Corp.) was used. The standard length (SL 9.90, with SD 1.03 mm) of
162 individual larvae was calculated by software ImageJ (Version 1.48. National Institute
163 of Health, Maryland, US), and there were no significant differences between the two
164 treatments.

165 After tube feeding, each larva was rinsed by successive transfers to 3 vials containing
166 5 ml clean seawater. Thereafter, each larva was kept in individual incubation vials
167 with 5 ml of filtered (0.2 µm) seawater. A modified CO_2 trap containing 5 ml 0.5 M
168 KOH was connected to each incubation vial as described by Rønnestad *et al* (2001).
169 The incubation vials and the CO_2 traps were connected by a multi-channel tubing
170 pump (Watson Marlow Inc., Model 205) to create an air flow of 2 ml min^{-1}. Six larvae
171 for each treatment were sampled after 0.5, 1, 2 and 4 h of incubation. Larval survival
172 following the tube-feeding procedures was 90% and the dead ones were replaced by
173 new tube-fed larvae. The samples were collected by transferring each larva with a
174 droplet of seawater to micro centrifuge tubes and immediately frozen in liquid
175 nitrogen and stored at -80 ℃. Total CO_2 expired was collected in the trap after a final
176 acidification of the seawater with 0.05 ml 1M HCl. One ml of incubation water and
177 KOH-solution were transferred to scintillation vials, respectively, and 10 ml
178 scintillation cocktail was added for radioactivity counting.

179 *Lipid analyses*

180 The samples were thawed on ice and total lipid extracted by a modified Folch method
181 (Folch *et al.*, 1957). Individual larva was homogenised in 2.5 ml of

chloroform:methanol (2:1, v/v), rinsed with 0.625 ml of 0.88% KCl and centrifuged at 1640 *g* for 10 min at 4 °C. 0.5 ml of the upper layer was transferred into scintillation vials containing 10 ml of scintillation cocktail for ^{3}H and ^{14}C counting. The lower layer was transferred into GC vials, dried under a stream of nitrogen, and re-suspended in 30 µl of chloroform:methanol (2:1, v/v).

Lipid classes were separated by double-development HPTLC (Olsen and Henderson, 1989). HPTLC silica gel 60 plates without fluorescent indicator (10 × 10 cm) were purchased from Merck KGaA (Darmstadt, Germany). The plates were activated at 110 °C for 1 h and 10 µl of the extracted lipid sample was applied as a 1 cm streak by a glass micro-syringe, along with MAG and DAG standards. The plates were first developed to a distance of 4.5 cm from the origin using methyl acetate: isopropanol: chloroform: methanol: 0.25% KCl (25:25:25:10:9, v/v) solvent system. The plates were evaporated by a hair drier for 10 sec and then placed in a desiccator over dry NaOH for 30 min. Subsequently, plates were developed in hexane: diethyl ether: glacial acetic acid (80:20:2, v/v) to 8.8 cm from the origin. Lipid bands were visualized by exposure to iodine vapour and quickly marked with a pencil. Each lipid bands were scraped into respective scintillation vials containing 10 ml of scintillation cocktail and the amount of radioactivity was determined on ^{3}H and ^{14}C channels by a liquid scintillation counter.

Statistics

The experimental data were tested for statistical significance by using Independent-Samples T-test or two-way analysis of variance (ANOVA) with time and choline treatment as the two factors. Differences were considered significant if $p<0.05$. All of the statistical tests were performed using SPSS 20.0 for windows. All tables were made in Excel 2010 and figures by Sigma plot 12.5.

Results

Distribution of radioactivity in the system

The percentage of total radioactivity in the different system components is shown in Figure. There was a decreasing trend of radioactivity in fish (without lipid) and lipid

along with an increasing trend in the incubation water and CO_2 from 0.5 h to 4 h of incubation. Two-way ANOVA showed that the percentages of total radioactivity of lipid, fish and water were significantly affected by incubation time and choline supplementation (Table 2). Both [^{3}H] and [^{14}C] radioactivity decreased significantly in lipid and fish in the choline supplemented treatment compared to the control (without choline). However, the percentage of radioactive CO_2 from [^{14}C] glycerol incorporation did not show any significant differences between the two treatments. The percentage of radioactivity in lipid found for [^{3}H] 2-MAG (26.16 ± 2.16 %) was significantly higher ($p<0.05$, T-test) than that for [^{14}C] glycerol (6.37 ± 0.82%). No significant differences were, however, found between [^{3}H] and [^{14}C] radioactivity in fish tissues and incubation water.

Distribution of radioactivity of lipid classes

2-MAG pathway

After 0.5 h of incubation, the percentage of total [^{3}H] label in 2-MAG was already very low in both control and choline treatments (Table 3). The highest proportion of [^{3}H] radioactivity was found in TAG, followed by PC at 0.5 h post incubation. There was an increasing trend in [^{3}H] PC and a decreasing trend in [^{3}H] TAG, and the PC:TAG ratio increased with increasing incubation time. The choline supplementation did not increase the percentage of [^{3}H] PC significantly, but the percentage of [^{3}H] TAG was reduced significantly due to the increased radioactivity of other phospholipids, including PE, PS and PI. There was no interaction between time and treatment (choline) on incorporation into any lipid class.

G-3-P pathway

The predominant lipid classes derived from [^{14}C] glycerol were TAG, followed by PC and PE. A significant ($p<0.05$) increase in [^{14}C] PC and a decreasing percentages of [^{14}C] TAG over time were also observed. There was generally a higher recovery of [^{14}C] in PC, PE, and PS & PI in the choline treated fish compared to controls at all the sampling times, but these effects were not significant. However, significant reductions in [^{14}C] TAG for the choline treated fish resulted in an increase in the PC:TAG ratio.

T-tests were performed to compare the distribution of label in lipid classes between the two pathways. The percentages incorporation of radioactivity in all the

phospholipid classes: PC, PE and PS & PI for the G-3-P pathway were significantly higher than that derived from 2-MAG pathway. Accordingly, significantly higher PC:TAG ratio and significantly lower radioactivity in TAG was found in G-3-P pathway compared to 2-MAG pathway.

Discussion

Dietary PL is an essential dietary component for marine fish larvae during their early life stages and the specific requirement of PL of fish larvae has been suggested to originate in an inefficient ability to biosynthesis PL *de novo* based on dietary TAG (Tocher *et al.*, 2008; Cahu *et al.*, 2009). However, to our knowledge, no studies have described the PL biosynthesis capability in marine fish larvae. The main objective of the present study was to investigate if cod larvae could synthesize PL from 2-MAG and glycerol precursors. Anaesthesia can reduce digestive functions as has previously been reported in zebrafish (Hama *et al.*, 2009), but the injected mixtures in the present study would be absorbed directly without being further digested in the larvae. Tube feeding of herring (*Clupea harengus*) larvae (Morais *et al.*, 2005) showed that some larvae (3-4 out of 12 larvae) evacuated the injected slurry shortly after feeding. This may explain the high variances in the distribution of radioactivity in fish larvae and incubation water, and more specifically the high radioactivity of the incubation water at 0.5 h in Figure D.

Studies of enzyme activity and substrate uptake efficiency should be undertaken using excess substrate, but this was not possible in the present study due to the limited injected volume, the limited binding cites of BSA for fatty acids (Spector *et al.*, 1969), and the relatively low water solubility of BSA. The concentrations of substrates shown in Table 1 ensured already saturation of the binding capacity and solubility of BSA. This was why we carried out a pulse-chase study to follow the distribution pattern of radioactivity as the components moved through the lipid metabolic pathways.

Our results clearly showed that cod larvae intestines have a high metabolic rate, and are fully capable to synthesize PL through both the 2-MAG and G-3-P pathways when dietary components that could be directly assimilated were injected. Furthermore supplementation of choline chloride significantly increased the PC

277 synthesis over TAG (PC:TAG ratio) ($p<0.05$). Cod larvae were able to synthesize PC
278 to similar levels as TAG, and the reacylation process of MAG to TAG is relatively
279 fast in the enterocytes found for salmon juvenile (Oxley *et al.*, 2005; Oxley *et al.*,
280 2007). We therefore suggest that cod larvae might have relatively high ability to
281 synthesize PC from 2-MAG and glycerol precursors under the conditions of the
282 present study where these components were injected and might be assimilated directly
283 without further need for digestion.

284 *Effects of choline*

285 Supplementation of choline chloride to the injected slurry reduced the incorporation
286 of radioactivity into the lipid fractions significantly ($p<0.001$), especially for the G-3-
287 P pathways. It is known that the uptake of glycerol and choline can proceed through
288 Na^+ dependent active transporters (Vance and Vance, 2004; Kato *et al.*, 2005).
289 Respective transporters of choline and glycerol may compete for the same Na^+ pool
290 and it is possible that choline caused a competitive inhibition of the added glycerol in
291 the present study where the molar ratio of choline:glycerol was 2:1. On the other hand,
292 choline is essential for PC synthesis and the assembly of lipoproteins and therefore for
293 lipid transport to tissues (Tso *et al.*, 1978; Lehner and Kuksis, 1992; Li and Vance,
294 2008). Recent studies with human hepatic C3A cells has shown that choline promote
295 lipid catabolism partially by up-regulating genes involved in fatty acid β-oxidation
296 (peroxisomal proliferator-activated receptor alpha and carnitine palmitoyl transferase-
297 I) and down-regulation of fatty acid synthesis (fatty acid synthase) (Zhu *et al.*, 2014).
298 This is in agreement with the present study showing that supplementation of choline
299 significantly reduced the percentage of radioactivity in the lipid fraction for both
300 pathways. However, addition of choline (1-3%) in a feeding study with carp larvae
301 showed reduced survival and growth (Geurden *et al.*, 1995). This, on the other hand,
302 may be caused by the effect of excess of choline since supplementation of 1% choline
303 was more than 3 times higher than the estimated requirement for juvenile carp (Ogino
304 *et al.*, 1970). Another study with weanling pigs showed reduced weight gain when
305 0.7 % of choline was supplemented in the diet (Southern *et al.*, 1986). The effect of
306 excess choline is rarely reported, but it has been found toxic for rats and humans, with
307 half lethal dose for rats ranging from 3.4 to 6.1 g kg^{-1}, and an upper limit of daily
308 intake for human adults of 3.5 g (Jukes, 1947; Food and Nutrition Board, 1998; Li and
309 Vance, 2008).

The 2-MAG pathway predominates over the G-3-P pathway

The percentage of [^{3}H] label in 2-MAG was already very low after 0.5 h of incubation, indicating fast absorption and utilization of the 2-MAG precursor by cod larvae. *In vitro* studies using intestinal segments of rainbow trout (*Oncorhynchus mykiss*) have shown that only 0.5-1% of the initial radioactivity of fatty acids added to the luminal side passed through the intestinal epithelium after 110 min (Geurden *et al.*, 2009). It is likely that the biosynthesis of PL and TAG in the present study occurred mainly in the enterocytes of cod larvae within the first hour post injection.

It has been well documented that the 2-MAG pathway predominates over the G-3-P pathway in the synthesis of TAG in the intestine of both mammals and fish (Yang and Kuksis, 1991; Lehner and Kuksis, 1996; Oxley *et al.*, 2007). The *de novo* synthesis of PC has generally been assumed to proceed through the G-3-P pathway, until Lehner and Kuksis (1992) demonstrated that endogenous diacylglycerols (DAG) released by phospholipase C shared the same pool of DAG as generated from exogenous 2-MAG and that these DAG also could be utilized for the synthesis of TAG. Oxley *et al* (2007) further demonstrated that the PC synthesized via the 2-MAG pathway was 3.8 fold higher than that synthesized via the G-3-P pathway in the intestine segments of Atlantic salmon. In the present study, we obtained an average of 3.02 fold higher PC productions from the 2-MAG pathway compared to the G-3-P pathway and supplementation of choline increased this ratio to 22.7 (data not shown). However, as noted above, this may be due to a lower uptake of glycerol in the choline treatment.

PC:TAG ratio

The typical PC:TAG ratio of chylomicron and VLDL (very low density lipoprotein) is 0.09 and 0.36, respectively (Jonas, 2002; Torstensen *et al.*, 2004). Within the first hour, the PC:TAG ratio obtained from the 2-MAG and the G-3-P pathways were 0.44-0.74 and 1.02-2.06, respectively. These ratios were higher than the requirement for assembly of chylomicron and VLDL, and it was relatively high compared to other studies (0.04-0.22 derived from 2-MAG pathway, 0.27-1.43 derived from G-3-P pathway) (Lehner and Kuksis, 1992; Oxley *et al.*, 2007). DAG is a common precursor for both the 2-MAG and the G-3-P pathways. Its fatty acid composition is important for the selectivity of CPT channelling it further into PC production (Mantel *et al.*, 1993).

342 The fatty acids 16:0 and 22:6n3 provided in the present study were chosen as the
343 combination believed to favour PC synthesis. They are also the most abundant fatty
344 acids in the natural prey for cod larvae (Li *et al.*, 2015b). The CPT of rat liver has
345 higher selectivity for DAG species containing 16:0 in the *sn*-1 position and PUFA in
346 the *sn*-2 position (Holub, 1978; Morimoto and Kanoh, 1978; Mantel *et al.*, 1993), and
347 salmon intestine showed higher PC:TAG ratio when 16:0 was available as substrate
348 (Oxley *et al.*, 2007). Moreover, addition of 16:0 to diets containing high linseed oil
349 significantly reduced intestinal steatosis in Arctic char (*Salvelinus alpinus*) (Olsen *et al.*,
350 2000). The high PC:TAG ratio found after 2 h and 4 h of incubation in the present
351 study probably related to TAG being used as an energy source whereas PC was used
352 for membrane or structural purposes.

353 *Final evaluation and concluding remarks*

354 Early studies with Atlantic cod larvae suggested that they might have low ability to
355 digest neutral lipid classes due to insufficient suitable lipases, bile acids or both
356 (Olsen *et al.*, 1991). Bile activated lipase (BAL) was found to be the main functional
357 lipase for digestion of neutral lipids and its enzyme activity and gene expression was
358 low in the early stages of cod larvae, and "adult type" digestion of neutral lipid was
359 suggested to occur when the pyloric caeca started to develop (from 20 mm SL) (Saele
360 *et al.*, 2010; Kortner *et al.*, 2011). However, another study reported that pre-digested
361 dietary lipid only had minor effects on absorption and metabolism compared to intact
362 lipid, indicating cod larvae were able to utilize both neutral lipid and polar lipid
363 efficiently already from a SL of 9.75 (SD 0.63) mm (Hamre *et al.*, 2011). On the other
364 hand, few data are available regarding the bile production in fish larvae. Our recent
365 study from a cDNA-microarray analysis showed that the expression level of *Cyp7a1*
366 (cholesterol 7-alpha-monooxygenase, the rate-limiting enzyme in the conversion of
367 cholesterol to bile acids) of cod larvae increased from 1 dph (hatching) to 3 dph
368 (mouth opening) then decreased all the way till 60 dph (Li et al. unpublished data).
369 Studies with humans showed that the bile acid concentrations in the intestine of
370 premature and full-term new-borns were insufficient for micelle formation (Murphy
371 and Singer, 1974). It is possible that the digestive function for neutral lipid of cod
372 larvae may suffer from both low BAL activity and low bile acid production in the
373 very early stages.

The incorporation of radiolabeled isotopes in the present experiment was made independent of digestion, and then the enzymatic capabilities could be studied without influence of the first enzymatic steps during digestion. It is possible that with normal feed the substrates for *de novo* PL synthesis in the enterocytes becomes limited and dietary PL becomes important. The requirements of dietary PL can also be a particular result of the high growth rate of fish larvae, the need of PL may not be fulfilled even with a relatively high capacity of *de novo* synthesis.

Our recent study has indicated that the alternative pathway for PC production in the liver via PEMT was likely not applicable due to the low gene expression of *pemt* from hatching up to 60 dph of cod larvae (Li *et al* in prep.). We therefore suggest that the requirement of dietary PC for fish larvae may be a result of low input of bile PC from the immature liver, rather than a limited ability of *de novo* PC synthesis in the enterocytes. However, further studies are needed to quantify the enzyme activity involved in the CDP-choline pathway and factors affecting PC biosynthesis ability in fish larvae. Addition of choline clearly increased the PC:TAG ratio, however, its function either in simulating PC synthesis or TAG catabolism or both needs further investigation.

In summary, Atlantic cod larvae at 30 dph (9.90 mm SL) showed comparable biosynthesis ability of PC and TAG from both 2-MAG and glycerol precursors within the first hour of tube feeding, indicating they might have relatively high capacity of *de novo* PL synthesis under the conditions of the present study. We suggest that the intestinal steatosis frequently observed in fish larvae fed PL-deficient diets may be caused by the immature digestive functions of neutral lipid to provide substrate for *de novo* PC synthesis, the low input of biliary PC for the assembly of lipoproteins, or both.

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Figure. Distribution of radioactivity as percentage of total recovered radioactivity for different incubation times and treatments. Values not sharing a common letter are significantly different ($P < 0.05$). Letters in lower case show comparison for the 2-MAG pathway (A and B), and capital letters show comparison for the Glycerol-3-phosphate pathway (C and D).

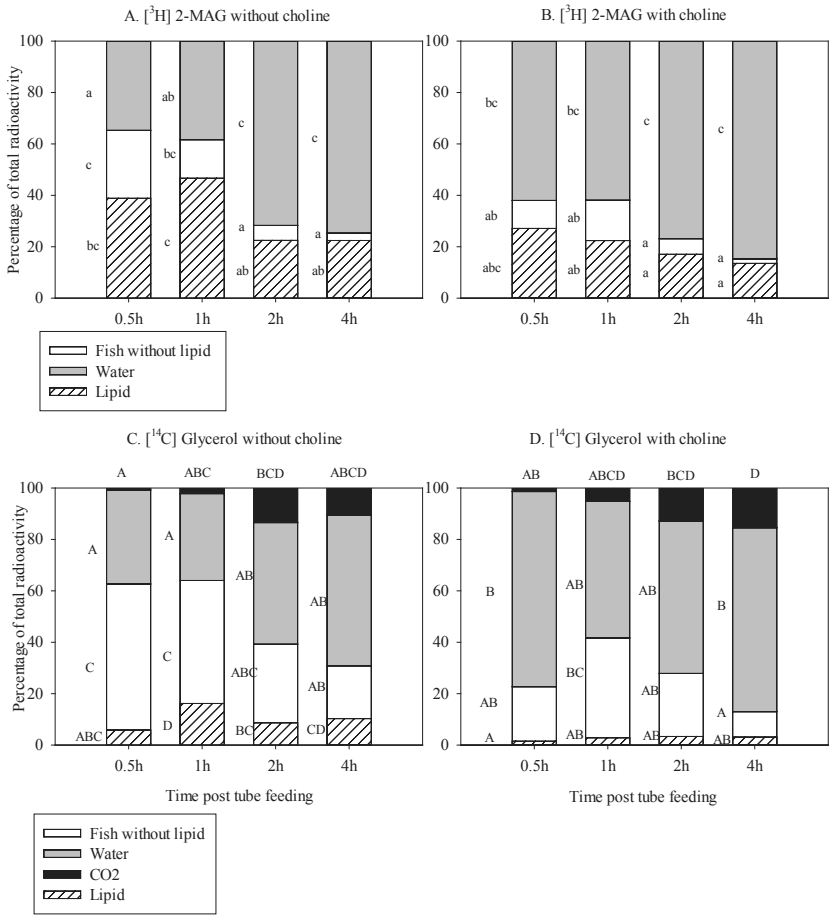

Table 1. Substrate concentration of the stock solutions. mM, mmol L^{-1}

	2-MAG-BSA [^{3}H]	Glycerol [^{14}C]	16:0-BSA	DHA-BSA	Choline-chloride
Control (without choline) (mM)	0.37	0.41	0.41	0.41	
Choline treatment (mM)	0.37	0.41	0.41	0.41	0.82
Specific radioactivity (Bq $pmol^{-1}$)	40	5.56			

Table 2. Two way ANOVA for the distribution of radioactivity in the incubation system and fish larvae. T×T, interaction between the two factors (time and treatment).

[^{3}H]	p value		
	Time	Treatment (choline)	T×T
Lipid	<0.001	<0.001	0.211
Fish	<0.001	<0.001	0.157
Water	<0.001	<0.001	0.157
[^{14}C]			
Lipid	0.002	<0.001	0.010
Fish	<0.001	<0.001	0.154
Water	0.044	<0.002	0.241
CO_2	<0.001	0.291	0.717

Table 3. Distribution of radioactivity in lipid classes, expressed in terms of percentage of radioactivity in total lipid ± standard error (n=6). Values in the same row not sharing a superscript are significantly different ($P < 0.05$). The factors for two-way ANOVA were time and treatment (choline). T×T, interaction of the two factors. Posteriori tests were performed when significant differences were found in the time factor.

[^{3}H]	0.5h		1h		2h		4h		p value		
	Control	Choline	Control	Choline	Control	Choline	Control	Choline	Time	Treatment	T×T
PC	21.5 ± 2.83^{a}	18.7 ± 3.87^{a}	24.4 ± 1.85^{a}	28.2 ± 3.45^{ab}	30.1 ± 5.82^{ab}	30.2 ± 3.10^{ab}	30.1 ± 2.41^{ab}	38.9 ± 1.37^{b}	<0.001	0.267	0.271
PE	5.19 ± 0.67^{a}	5.98 ± 1.33^{a}	4.69 ± 0.43^{a}	6.90 ± 0.34^{ab}	7.32 ± 0.99^{ab}	7.45 ± 0.97^{ab}	6.69 ± 1.11^{a}	11.6 ± 2.04^{b}	0.009	0.019	0.170
MAG	5.41 ± 1.16	7.42 ± 2.42	3.81 ± 0.61	5.36 ± 1.11	2.47 ± 0.80	3.99 ± 0.50	2.68 ± 0.37	2.65 ± 0.22	0.006	0.111	0.778
DAG	7.63 ± 1.26^{ab}	11.5 ± 1.50^{b}	9.38 ± 0.97^{ab}	9.43 ± 0.54^{ab}	7.53 ± 0.62^{ab}	7.95 ± 0.93^{ab}	7.66 ± 0.71^{ab}	6.69 ± 0.50^{a}	0.030	0.219	0.068
TAG	51.1 ± 3.65^{b}	46.4 ± 8.22^{ab}	51.8 ± 1.81^{b}	40.6 ± 3.29^{ab}	43.9 ± 7.55^{ab}	38.8 ± 5.77^{ab}	44.4 ± 4.16^{ab}	28.1 ± 5.11^{a}	0.072	0.014	0.611
PC:TAG	0.44 ± 0.09^{a}	0.50 ± 0.15^{a}	0.48 ± 0.05^{a}	0.74 ± 0.14^{a}	0.83 ± 0.30^{ab}	0.90 ± 0.22^{ab}	0.74 ± 0.13^{a}	1.77 ± 0.45^{b}	0.007	0.038	0.115
[^{14}C]	0.5h		1h		2h		4h		p value		
	Control	Choline	Control	Choline	Control	Choline	Control	Choline	Time	Treatment	T×T
PC	26.0 ± 1.60^{a}	28.4 ± 1.79^{ab}	32.5 ± 0.99^{abc}	35.6 ± 2.46^{bc}	36.3 ± 3.76^{bc}	37.1 ± 1.82^{bc}	36.1 ± 1.64^{bc}	38.4 ± 2.45^{c}	<0.001	0.171	0.967
PE	18.1 ± 2.38	20.2 ± 2.03	17.5 ± 1.37	18.6 ± 1.65	15.2 ± 1.69	16.4 ± 1.05	16.9 ± 1.89	19.6 ± 1.24	0.333	0.155	0.959
MAG	3.94 ± 0.60	4.89 ± 1.29	3.53 ± 0.49	3.68 ± 0.68	2.08 ± 0.66	3.92 ± 0.63	3.08 ± 0.39	3.48 ± 0.88	0.284	0.122	0.711
DAG	6.99 ± 0.79	8.43 ± 0.59	7.65 ± 0.37	8.19 ± 0.44	6.15 ± 0.47	6.76 ± 0.80	7.25 ± 0.55	6.54 ± 1.13	0.182	0.366	0.488
TAG	29.4 ± 3.99^{b}	23.4 ± 3.73^{ab}	26.3 ± 0.74^{ab}	18.4 ± 1.57^{ab}	24.2 ± 5.22^{ab}	17.6 ± 1.72^{ab}	20.2 ± 2.60^{ab}	14.0 ± 1.75^{a}	0.018	0.002	0.986
PC:TAG	1.02 ± 0.21^{a}	1.43 ± 0.34^{a}	1.24 ± 0.05^{a}	2.06 ± 0.30^{ab}	1.88 ± 0.62^{ab}	2.19 ± 0.25^{ab}	1.97 ± 0.30^{ab}	3.02 ± 0.49^{b}	0.004	0.012	0.671

Doctoral theses in Biology
Norwegian University of Science and Technology
Department of Biology

Year	Name	Degree	Title
1974	Tor-Henning Iversen	Dr. philos Botany	The roles of statholiths, auxin transport, and auxin metabolism in root gravitropism
1978	Tore Slagsvold	Dr. philos Zoology	Breeding events of birds in relation to spring temperature and environmental phenology
1978	Egil Sakshaug	Dr. philos Botany	"The influence of environmental factors on the chemical composition of cultivated and natural populations of marine phytoplankton"
1980	Arnfinn Langeland	Dr. philos Zoology	Interaction between fish and zooplankton populations and their effects on the material utilization in a freshwater lake
1980	Helge Reinertsen	Dr. philos Botany	The effect of lake fertilization on the dynamics and stability of a limnetic ecosystem with special reference to the phytoplankton
1982	Gunn Mari Olsen	Dr. scient Botany	Gravitropism in roots of *Pisum sativum* and *Arabidopsis thaliana*
1982	Dag Dolmen	Dr. philos Zoology	Life aspects of two sympartic species of newts (*Triturus, Amphibia*) in Norway, with special emphasis on their ecological niche segregation
1984	Eivin Røskaft	Dr. philos Zoology	Sociobiological studies of the rook *Corvus frugilegus*
1984	Anne Margrethe Cameron	Dr. scient Botany	Effects of alcohol inhalation on levels of circulating testosterone, follicle stimulating hormone and luteinzing hormone in male mature rats
1984	Asbjørn Magne Nilsen	Dr. scient Botany	Alveolar macrophages from expectorates – Biological monitoring of workers exosed to occupational air pollution. An evaluation of the AM-test
1985	Jarle Mork	Dr. philos Zoology	Biochemical genetic studies in fish
1985	John Solem	Dr. philos Zoology	Taxonomy, distribution and ecology of caddisflies (*Trichoptera*) in the Dovrefjell mountains
1985	Randi E. Reinertsen	Dr. philos Zoology	Energy strategies in the cold: Metabolic and thermoregulatory adaptations in small northern birds
1986	Bernt-Erik Sæther	Dr. philos Zoology	Ecological and evolutionary basis for variation in reproductive traits of some vertebrates: A comparative approach
1986	Torleif Holthe	Dr. philos Zoology	Evolution, systematics, nomenclature, and zoogeography in the polychaete orders *Oweniimorpha* and *Terebellomorpha*, with special reference to the Arctic and Scandinavian fauna
1987	Helene Lampe	Dr. scient Zoology	The function of bird song in mate attraction and territorial defence, and the importance of song repertoires
1987	Olav Hogstad	Dr. philos Zoology	Winter survival strategies of the Willow tit *Parus montanus*
1987	Jarle Inge Holten	Dr. philos Botany	Autecological investigations along a coust-inland transect at Nord-Møre, Central Norway
1987	Rita Kumar	Dr. scient Botany	Somaclonal variation in plants regenerated from cell cultures of *Nicotiana sanderae* and *Chrysanthemum morifolium*

1987	Bjørn Åge Tømmerås	Dr. scient Zoology	Olfaction in bark beetle communities: Interspecific interactions in regulation of colonization density, predator - prey relationship and host attraction
1988	Hans Christian Pedersen	Dr. philos Zoology	Reproductive behaviour in willow ptarmigan with special emphasis on territoriality and parental care
1988	Tor G. Heggberget	Dr. philos Zoology	Reproduction in Atlantic Salmon (*Salmo salar*): Aspects of spawning, incubation, early life history and population structure
1988	Marianne V. Nielsen	Dr. scient Zoology	The effects of selected environmental factors on carbon allocation/growth of larval and juvenile mussels (*Mytilus edulis*)
1988	Ole Kristian Berg	Dr. scient Zoology	The formation of landlocked Atlantic salmon (*Salmo salar* L.)
1989	John W. Jensen	Dr. philos Zoology	Crustacean plankton and fish during the first decade of the manmade Nesjø reservoir, with special emphasis on the effects of gill nets and salmonid growth
1989	Helga J. Vivås	Dr. scient Zoology	Theoretical models of activity pattern and optimal foraging: Predictions for the Moose *Alces alces*
1989	Reidar Andersen	Dr. scient Zoology	Interactions between a generalist herbivore, the moose *Alces alces*, and its winter food resources: a study of behavioural variation
1989	Kurt Ingar Draget	Dr. scient Botany	Alginate gel media for plant tissue culture
1990	Bengt Finstad	Dr. scient Zoology	Osmotic and ionic regulation in Atlantic salmon, rainbow trout and Arctic charr: Effect of temperature, salinity and season
1990	Hege Johannesen	Dr. scient Zoology	Respiration and temperature regulation in birds with special emphasis on the oxygen extraction by the lung
1990	Åse Krøkje	Dr. scient Botany	The mutagenic load from air pollution at two work-places with PAH-exposure measured with Ames Salmonella/microsome test
1990	Arne Johan Jensen	Dr. philos Zoology	Effects of water temperature on early life history, juvenile growth and prespawning migrations of Atlantic salmion (*Salmo salar*) and brown trout (*Salmo trutta*): A summary of studies in Norwegian streams
1990	Tor Jørgen Almaas	Dr. scient Zoology	Pheromone reception in moths: Response characteristics of olfactory receptor neurons to intra- and interspecific chemical cues
1990	Magne Husby	Dr. scient Zoology	Breeding strategies in birds: Experiments with the Magpie *Pica pica*
1991	Tor Kvam	Dr. scient Zoology	Population biology of the European lynx (*Lynx lynx*) in Norway
1991	Jan Henning L'Abêe Lund	Dr. philos Zoology	Reproductive biology in freshwater fish, brown trout *Salmo trutta* and roach *Rutilus rutilus* in particular
1991	Asbjørn Moen	Dr. philos Botany	The plant cover of the boreal uplands of Central Norway. I. Vegetation ecology of Sølendet nature reserve; haymaking fens and birch woodlands
1991	Else Marie Løbersli	Dr. scient Botany	Soil acidification and metal uptake in plants
1991	Trond Nordtug	Dr. scient Zoology	Reflectometric studies of photomechanical adaptation in superposition eyes of arthropods
1991	Thyra Solem	Dr. scient Botany	Age, origin and development of blanket mires in Central Norway
1991	Odd Terje Sandlund	Dr. philos Zoology	The dynamics of habitat use in the salmonid genera *Coregonus* and *Salvelinus*: Ontogenic niche shifts and polymorphism

1991	Nina Jonsson	Dr. philos Zoology	Aspects of migration and spawning in salmonids
1991	Atle Bones	Dr. scient Botany	Compartmentation and molecular properties of thioglucoside glucohydrolase (myrosinase)
1992	Torgrim Breiehagen	Dr. scient Zoology	Mating behaviour and evolutionary aspects of the breeding system of two bird species: the Temminck's stint and the Pied flycatcher
1992	Anne Kjersti Bakken	Dr. scient Botany	The influence of photoperiod on nitrate assimilation and nitrogen status in timothy (*Phleum pratense* L.)
1992	Tycho Anker-Nilssen	Dr. scient Zoology	Food supply as a determinant of reproduction and population development in Norwegian Puffins *Fratercula arctica*
1992	Bjørn Munro Jenssen	Dr. philos Zoology	Thermoregulation in aquatic birds in air and water: With special emphasis on the effects of crude oil, chemically treated oil and cleaning on the thermal balance of ducks
1992	Arne Vollan Aarset	Dr. philos Zoology	The ecophysiology of under-ice fauna: Osmotic regulation, low temperature tolerance and metabolism in polar crustaceans.
1993	Geir Slupphaug	Dr. scient Botany	Regulation and expression of uracil-DNA glycosylase and O^6-methylguanine-DNA methyltransferase in mammalian cells
1993	Tor Fredrik Næsje	Dr. scient Zoology	Habitat shifts in coregonids.
1993	Yngvar Asbjørn Olsen	Dr. scient Zoology	Cortisol dynamics in Atlantic salmon, *Salmo salar* L.: Basal and stressor-induced variations in plasma levels ans some secondary effects.
1993	Bård Pedersen	Dr. scient Botany	Theoretical studies of life history evolution in modular and clonal organisms
1993	Ole Petter Thangstad	Dr. scient Botany	Molecular studies of myrosinase in Brassicaceae
1993	Thrine L. M. Heggberget	Dr. scient Zoology	Reproductive strategy and feeding ecology of the Eurasian otter *Lutra lutra*.
1993	Kjetil Bevanger	Dr. scient Zoology	Avian interactions with utility structures, a biological approach.
1993	Kåre Haugan	Dr. scient Botany	Mutations in the replication control gene trfA of the broad host-range plasmid RK2
1994	Peder Fiske	Dr. scient Zoology	Sexual selection in the lekking great snipe (*Gallinago media*): Male mating success and female behaviour at the lek
1994	Kjell Inge Reitan	Dr. scient Botany	Nutritional effects of algae in first-feeding of marine fish larvae
1994	Nils Røv	Dr. scient Zoology	Breeding distribution, population status and regulation of breeding numbers in the northeast-Atlantic Great Cormorant *Phalacrocorax carbo carbo*
1994	Annette-Susanne Hoepfner	Dr. scient Botany	Tissue culture techniques in propagation and breeding of Red Raspberry (*Rubus idaeus* L.)
1994	Inga Elise Bruteig	Dr. scient Botany	Distribution, ecology and biomonitoring studies of epiphytic lichens on conifers
1994	Geir Johnsen	Dr. scient Botany	Light harvesting and utilization in marine phytoplankton: Species-specific and photoadaptive responses
1994	Morten Bakken	Dr. scient Zoology	Infanticidal behaviour and reproductive performance in relation to competition capacity among farmed silver fox vixens, *Vulpes vulpes*
1994	Arne Moksnes	Dr. philos Zoology	Host adaptations towards brood parasitism by the Cockoo
1994	Solveig Bakken	Dr. scient Botany	Growth and nitrogen status in the moss *Dicranum majus* Sm. as influenced by nitrogen supply

1994	Torbjørn Forseth	Dr. scient Zoology	Bioenergetics in ecological and life history studies of fishes.
1995	Olav Vadstein	Dr. philos Botany	The role of heterotrophic planktonic bacteria in the cycling of phosphorus in lakes: Phosphorus requirement, competitive ability and food web interactions
1995	Hanne Christensen	Dr. scient Zoology	Determinants of Otter *Lutra lutra* distribution in Norway: Effects of harvest, polychlorinated biphenyls (PCBs), human population density and competition with mink *Mustela vision*
1995	Svein Håkon Lorentsen	Dr. scient Zoology	Reproductive effort in the Antarctic Petrel *Thalassoica antarctica*; the effect of parental body size and condition
1995	Chris Jørgen Jensen	Dr. scient Zoology	The surface electromyographic (EMG) amplitude as an estimate of upper trapezius muscle activity
1995	Martha Kold Bakkevig	Dr. scient Zoology	The impact of clothing textiles and construction in a clothing system on thermoregulatory responses, sweat accumulation and heat transport
1995	Vidar Moen	Dr. scient Zoology	Distribution patterns and adaptations to light in newly introduced populations of *Mysis relicta* and constraints on Cladoceran and Char populations
1995	Hans Haavardsholm Blom	Dr. philos Botany	A revision of the *Schistidium apocarpum* complex in Norway and Sweden
1996	Jorun Skjærmo	Dr. scient Botany	Microbial ecology of early stages of cultivated marine fish; inpact fish-bacterial interactions on growth and survival of larvae
1996	Ola Ugedal	Dr. scient Zoology	Radiocesium turnover in freshwater fishes
1996	Ingibjørg Einarsdottir	Dr. scient Zoology	Production of Atlantic salmon (*Salmo salar*) and Arctic charr (*Salvelinus alpinus*): A study of some physiological and immunological responses to rearing routines
1996	Christina M. S. Pereira	Dr. scient Zoology	Glucose metabolism in salmonids: Dietary effects and hormonal regulation
1996	Jan Fredrik Børseth	Dr. scient Zoology	The sodium energy gradients in muscle cells of *Mytilus edulis* and the effects of organic xenobiotics
1996	Gunnar Henriksen	Dr. scient Zoology	Status of Grey seal *Halichoerus grypus* and Harbour seal *Phoca vitulina* in the Barents sea region
1997	Gunvor Øie	Dr. scient Botany	Eevalution of rotifer *Brachionus plicatilis* quality in early first feeding of turbot *Scophtalmus maximus* L. larvae
1997	Håkon Holien	Dr. scient Botany	Studies of lichens in spurce forest of Central Norway. Diversity, old growth species and the relationship to site and stand parameters
1997	Ole Reitan	Dr. scient Zoology	Responses of birds to habitat disturbance due to damming
1997	Jon Arne Grøttum	Dr. scient Zoology	Physiological effects of reduced water quality on fish in aquaculture
1997	Per Gustav Thingstad	Dr. scient Zoology	Birds as indicators for studying natural and human-induced variations in the environment, with special emphasis on the suitability of the Pied Flycatcher
1997	Torgeir Nygård	Dr. scient Zoology	Temporal and spatial trends of pollutants in birds in Norway: Birds of prey and Willow Grouse used as
1997	Signe Nybø	Dr. scient Zoology	Impacts of long-range transported air pollution on birds with particular reference to the dipper *Cinclus cinclus* in southern Norway
1997	Atle Wibe	Dr. scient Zoology	Identification of conifer volatiles detected by receptor neurons in the pine weevil (*Hylobius abietis*), analysed by gas chromatography linked to electrophysiology and to mass spectrometry

1997	Rolv Lundheim	Dr. scient Zoology	Adaptive and incidental biological ice nucleators
1997	Arild Magne Landa	Dr. scient Zoology	Wolverines in Scandinavia: ecology, sheep depredation and conservation
1997	Kåre Magne Nielsen	Dr. scient Botany	An evolution of possible horizontal gene transfer from plants to sail bacteria by studies of natural transformation in *Acinetobacter calcoacetius*
1997	Jarle Tufto	Dr. scient Zoology	Gene flow and genetic drift in geographically structured populations: Ecological, population genetic, and statistical models
1997	Trygve Hesthagen	Dr. philos Zoology	Population responces of Arctic charr (*Salvelinus alpinus* (L.)) and brown trout (*Salmo trutta* L.) to acidification in Norwegian inland waters
1997	Trygve Sigholt	Dr. philos Zoology	Control of Parr-smolt transformation and seawater tolerance in farmed Atlantic Salmon (*Salmo salar*) Effects of photoperiod, temperature, gradual seawater acclimation, NaCl and betaine in the diet
1997	Jan Østnes	Dr. scient Zoology	Cold sensation in adult and neonate birds
1998	Seethaledsumy Visvalingam	Dr. scient Botany	Influence of environmental factors on myrosinases and myrosinase-binding proteins
1998	Thor Harald Ringsby	Dr. scient Zoology	Variation in space and time: The biology of a House sparrow metapopulation
1998	Erling Johan Solberg	Dr. scient Zoology	Variation in population dynamics and life history in a Norwegian moose (*Alces alces*) population: consequences of harvesting in a variable environment
1998	Sigurd Mjøen Saastad	Dr. scient Botany	Species delimitation and phylogenetic relationships between the Sphagnum recurvum complex (Bryophyta): genetic variation and phenotypic plasticity
1998	Bjarte Mortensen	Dr. scient Botany	Metabolism of volatile organic chemicals (VOCs) in a head liver S9 vial equilibration system in vitro
1998	Gunnar Austrheim	Dr. scient Botany	Plant biodiversity and land use in subalpine grasslands. – A conservtaion biological approach
1998	Bente Gunnveig Berg	Dr. scient Zoology	Encoding of pheromone information in two related moth species
1999	Kristian Overskaug	Dr. scient Zoology	Behavioural and morphological characteristics in Northern Tawny Owls *Strix aluco*: An intra- and interspecific comparative approach
1999	Hans Kristen Stenøien	Dr. scient Botany	Genetic studies of evolutionary processes in various populations of nonvascular plants (mosses, liverworts and hornworts)
1999	Trond Arnesen	Dr. scient Botany	Vegetation dynamics following trampling and burning in the outlying haylands at Sølendet, Central Norway
1999	Ingvar Stenberg	Dr. scient Zoology	Habitat selection, reproduction and survival in the White-backed Woodpecker *Dendrocopos leucotos*
1999	Stein Olle Johansen	Dr. scient Botany	A study of driftwood dispersal to the Nordic Seas by dendrochronology and wood anatomical analysis
1999	Trina Falck Galloway	Dr. scient Zoology	Muscle development and growth in early life stages of the Atlantic cod (*Gadus morhua* L.) and Halibut (*Hippoglossus hippoglossus* L.)
1999	Marianne Giæver	Dr. scient Zoology	Population genetic studies in three gadoid species: blue whiting (*Micromisistius poutassou*), haddock (*Melanogrammus aeglefinus*) and cod (*Gradus morhua*) in the North-East Atlantic

1999	Hans Martin Hanslin	Dr. scient Botany	The impact of environmental conditions of density dependent performance in the boreal forest bryophytes *Dicranum majus*, *Hylocomium splendens*, *Plagiochila asplenigides*, *Ptilium crista-castrensis* and *Rhytidiadelphus lokeus*
1999	Ingrid Bysveen Mjølnerød	Dr. scient Zoology	Aspects of population genetics, behaviour and performance of wild and farmed Atlantic salmon (*Salmo salar*) revealed by molecular genetic techniques
1999	Else Berit Skagen	Dr. scient Botany	The early regeneration process in protoplasts from *Brassica napus* hypocotyls cultivated under various g-forces
1999	Stein-Are Sæther	Dr. philos Zoology	Mate choice, competition for mates, and conflicts of interest in the Lekking Great Snipe
1999	Katrine Wangen Rustad	Dr. scient Zoology	Modulation of glutamatergic neurotransmission related to cognitive dysfunctions and Alzheimer's disease
1999	Per Terje Smiseth	Dr. scient Zoology	Social evolution in monogamous families:
1999	Gunnbjørn Bremset	Dr. scient Zoology	Young Atlantic salmon (*Salmo salar* L.) and Brown trout (*Salmo trutta* L.) inhabiting the deep pool habitat, with special reference to their habitat use, habitat preferences and competitive interactions
1999	Frode Ødegaard	Dr. scient Zoology	Host spesificity as parameter in estimates of arhrophod species richness
1999	Sonja Andersen	Dr. scient Zoology	Expressional and functional analyses of human, secretory phospholipase A2
2000	Ingrid Salvesen	Dr. scient Botany	Microbial ecology in early stages of marine fish: Development and evaluation of methods for microbial management in intensive larviculture
2000	Ingar Jostein Øien	Dr. scient Zoology	The Cuckoo (*Cuculus canorus*) and its host: adaptions and counteradaptions in a coevolutionary arms race
2000	Pavlos Makridis	Dr. scient Botany	Methods for the microbial econtrol of live food used for the rearing of marine fish larvae
2000	Sigbjørn Stokke	Dr. scient Zoology	Sexual segregation in the African elephant (*Loxodonta africana*)
2000	Odd A. Gulseth	Dr. philos Zoology	Seawater tolerance, migratory behaviour and growth of Charr, (*Salvelinus alpinus*), with emphasis on the high Arctic Dieset charr on Spitsbergen, Svalbard
2000	Pål A. Olsvik	Dr. scient Zoology	Biochemical impacts of Cd, Cu and Zn on brown trout (*Salmo trutta*) in two mining-contaminated rivers in Central Norway
2000	Sigurd Einum	Dr. scient Zoology	Maternal effects in fish: Implications for the evolution of breeding time and egg size
2001	Jan Ove Evjemo	Dr. scient Zoology	Production and nutritional adaptation of the brine shrimp *Artemia* sp. as live food organism for larvae of marine cold water fish species
2001	Olga Hilmo	Dr. scient Botany	Lichen response to environmental changes in the managed boreal forset systems
2001	Ingebrigt Uglem	Dr. scient Zoology	Male dimorphism and reproductive biology in corkwing wrasse (*Symphodus melops* L.)
2001	Bård Gunnar Stokke	Dr. scient Zoology	Coevolutionary adaptations in avian brood parasites and their hosts
2002	Ronny Aanes	Dr. scient Zoology	Spatio-temporal dynamics in Svalbard reindeer (*Rangifer tarandus platyrhynchus*)
2002	Mariann Sandsund	Dr. scient Zoology	Exercise- and cold-induced asthma. Respiratory and thermoregulatory responses

2002	Dag-Inge Øien	Dr. scient Botany	Dynamics of plant communities and populations in boreal vegetation influenced by scything at Sølendet, Central Norway
2002	Frank Rosell	Dr. scient Zoology	The function of scent marking in beaver (*Castor fiber*)
2002	Janne Østvang	Dr. scient Botany	The Role and Regulation of Phospholipase A_2 in Monocytes During Atherosclerosis Development
2002	Terje Thun	Dr. philos Biology	Dendrochronological constructions of Norwegian conifer chronologies providing dating of historical material
2002	Birgit Hafjeld Borgen	Dr. scient Biology	Functional analysis of plant idioblasts (Myrosin cells) and their role in defense, development and growth
2002	Bård Øyvind Solberg	Dr. scient Biology	Effects of climatic change on the growth of dominating tree species along major environmental gradients
2002	Per Winge	Dr. scient Biology	The evolution of small GTP binding proteins in cellular organisms. Studies of RAC GTPases in *Arabidopsis thaliana* and the Ral GTPase from *Drosophila melanogaster*
2002	Henrik Jensen	Dr. scient Biology	Causes and consequenses of individual variation in fitness-related traits in house sparrows
2003	Jens Rohloff	Dr. philos Biology	Cultivation of herbs and medicinal plants in Norway – Essential oil production and quality control
2003	Åsa Maria O. Espmark Wibe	Dr. scient Biology	Behavioural effects of environmental pollution in threespine stickleback *Gasterosteus aculeatur* L.
2003	Dagmar Hagen	Dr. scient Biology	Assisted recovery of disturbed arctic and alpine vegetation – an integrated approach
2003	Bjørn Dahle	Dr. scient Biology	Reproductive strategies in Scandinavian brown bears
2003	Cyril Lebogang Taolo	Dr. scient Biology	Population ecology, seasonal movement and habitat use of the African buffalo (*Syncerus caffer*) in Chobe National Park, Botswana
2003	Marit Stranden	Dr. scient Biology	Olfactory receptor neurones specified for the same odorants in three related Heliothine species (*Helicoverpa armigera, Helicoverpa assulta* and *Heliothis virescens*)
2003	Kristian Hassel	Dr. scient Biology	Life history characteristics and genetic variation in an expanding species, *Pogonatum dentatum*
2003	David Alexander Rae	Dr. scient Biology	Plant- and invertebrate-community responses to species interaction and microclimatic gradients in alpine and Artic environments
2003	Åsa A Borg	Dr. scient Biology	Sex roles and reproductive behaviour in gobies and guppies: a female perspective
2003	Eldar Åsgard Bendiksen	Dr. scient Biology	Environmental effects on lipid nutrition of farmed Atlantic salmon (*Salmo Salar* L.) parr and smolt
2004	Torkild Bakken	Dr. scient Biology	A revision of Nereidinae (Polychaeta, Nereididae)
2004	Ingar Pareliussen	Dr. scient Biology	Natural and Experimental Tree Establishment in a Fragmented Forest, Ambohitantely Forest Reserve, Madagascar
2004	Tore Brembu	Dr. scient Biology	Genetic, molecular and functional studies of RAC GTPases and the WAVE-like regulatory protein complex in *Arabidopsis thaliana*
2004	Liv S. Nilsen	Dr. scient Biology	Coastal heath vegetation on central Norway; recent past, present state and future possibilities
2004	Hanne T. Skiri	Dr. scient Biology	Olfactory coding and olfactory learning of plant odours in heliothine moths. An anatomical, physiological and behavioural study of three related species (*Heliothis virescens, Helicoverpa armigera* and *Helicoverpa assulta*)

2004	Lene Østby	Dr. scient Biology	Cytochrome P4501A (CYP1A) induction and DNA adducts as biomarkers for organic pollution in the natural environment
2004	Emmanuel J. Gerreta	Dr. philos Biology	The Importance of Water Quality and Quantity in the Tropical Ecosystems, Tanzania
2004	Linda Dalen	Dr. scient Biology	Dynamics of Mountain Birch Treelines in the Scandes Mountain Chain, and Effects of Climate Warming
2004	Lisbeth Mehli	Dr. scient Biology	Polygalacturonase-inhibiting protein (PGIP) in cultivated strawberry (*Fragaria* x *ananassa*): characterisation and induction of the gene following fruit infection by *Botrytis cinerea*
2004	Børge Moe	Dr. scient Biology	Energy-Allocation in Avian Nestlings Facing Short-Term Food Shortage
2005	Matilde Skogen Chauton	Dr. scient Biology	Metabolic profiling and species discrimination from High-Resolution Magic Angle Spinning NMR analysis of whole-cell samples
2005	Sten Karlsson	Dr. scient Biology	Dynamics of Genetic Polymorphisms
2005	Terje Bongard	Dr. scient Biology	Life History strategies, mate choice, and parental investment among Norwegians over a 300-year period
2005	Tonette Røstelien	ph.d Biology	Functional characterisation of olfactory receptor neurone types in heliothine moths
2005	Erlend Kristiansen	Dr. scient Biology	Studies on antifreeze proteins
2005	Eugen G. Sørmo	Dr. scient Biology	Organochlorine pollutants in grey seal (*Halichoerus grypus*) pups and their impact on plasma thyrid hormone and vitamin A concentrations
2005	Christian Westad	Dr. scient Biology	Motor control of the upper trapezius
2005	Lasse Mork Olsen	ph.d Biology	Interactions between marine osmo- and phagotrophs in different physicochemical environments
2005	Åslaug Viken	ph.d Biology	Implications of mate choice for the management of small populations
2005	Ariaya Hymete Sahle Dingle	ph.d Biology	Investigation of the biological activities and chemical constituents of selected *Echinops* spp. growing in Ethiopia
2005	Anders Gravbrøt Finstad	ph.d Biology	Salmonid fishes in a changing climate: The winter challenge
2005	Shimane Washington Makabu	ph.d Biology	Interactions between woody plants, elephants and other browsers in the Chobe Riverfront, Botswana
2005	Kjartan Østbye	Dr. scient Biology	The European whitefish *Coregonus lavaretus* (L.) species complex: historical contingency and adaptive radiation
2006	Kari Mette Murvoll	ph.d Biology	Levels and effects of persistent organic pollutans (POPs) in seabirds, Retinoids and α-tocopherol – potential biomakers of POPs in birds?
2006	Ivar Herfindal	Dr. scient Biology	Life history consequences of environmental variation along ecological gradients in northern ungulates
2006	Nils Egil Tokle	ph.d Biology	Are the ubiquitous marine copepods limited by food or predation? Experimental and field-based studies with main focus on *Calanus finmarchicus*
2006	Jan Ove Gjershaug	Dr. philos Biology	Taxonomy and conservation status of some booted eagles in south-east Asia
2006	Jon Kristian Skei	Dr. scient Biology	Conservation biology and acidification problems in the breeding habitat of amphibians in Norway
2006	Johanna Järnegren	ph.d Biology	Acesta Oophaga and Acesta Excavata – a study of hidden biodiversity

2006	Bjørn Henrik Hansen	ph.d Biology	Metal-mediated oxidative stress responses in brown trout (*Salmo trutta*) from mining contaminated rivers in Central Norway
2006	Vidar Grøtan	ph.d Biology	Temporal and spatial effects of climate fluctuations on population dynamics of vertebrates
2006	Jafari R Kideghesho	ph.d Biology	Wildlife conservation and local land use conflicts in western Serengeti, Corridor Tanzania
2006	Anna Maria Billing	ph.d Biology	Reproductive decisions in the sex role reversed pipefish *Syngnathus typhle*: when and how to invest in reproduction
2006	Henrik Pärn	ph.d Biology	Female ornaments and reproductive biology in the bluethroat
2006	Anders J. Fjellheim	ph.d Biology	Selection and administration of probiotic bacteria to marine fish larvae
2006	P. Andreas Svensson	ph.d Biology	Female coloration, egg carotenoids and reproductive success: gobies as a model system
2007	Sindre A. Pedersen	ph.d Biology	Metal binding proteins and antifreeze proteins in the beetle *Tenebrio molitor* - a study on possible competition for the semi-essential amino acid cysteine
2007	Kasper Hancke	ph.d Biology	Photosynthetic responses as a function of light and temperature: Field and laboratory studies on marine microalgae
2007	Tomas Holmern	ph.d Biology	Bushmeat hunting in the western Serengeti: Implications for community-based conservation
2007	Kari Jørgensen	ph.d Biology	Functional tracing of gustatory receptor neurons in the CNS and chemosensory learning in the moth *Heliothis virescens*
2007	Stig Ulland	ph.d Biology	Functional Characterisation of Olfactory Receptor Neurons in the Cabbage Moth, (*Mamestra brassicae* L.) (Lepidoptera, Noctuidae). Gas Chromatography Linked to Single Cell Recordings and Mass Spectrometry
2007	Snorre Henriksen	ph.d Biology	Spatial and temporal variation in herbivore resources at northern latitudes
2007	Roelof Frans May	ph.d Biology	Spatial Ecology of Wolverines in Scandinavia
2007	Vedasto Gabriel Ndibalema	ph.d Biology	Demographic variation, distribution and habitat use between wildebeest sub-populations in the Serengeti National Park, Tanzania
2007	Julius William Nyahongo	ph.d Biology	Depredation of Livestock by wild Carnivores and Illegal Utilization of Natural Resources by Humans in the Western Serengeti, Tanzania
2007	Shombe Ntaraluka Hassan	ph.d Biology	Effects of fire on large herbivores and their forage resources in Serengeti, Tanzania
2007	Per-Arvid Wold	ph.d Biology	Functional development and response to dietary treatment in larval Atlantic cod *(Gadus morhua* L.) Focus on formulated diets and early weaning
2007	Anne Skjetne Mortensen	ph.d Biology	Toxicogenomics of Aryl Hydrocarbon- and Estrogen Receptor Interactions in Fish: Mechanisms and Profiling of Gene Expression Patterns in Chemical Mixture Exposure Scenarios
2008	Brage Bremset Hansen	ph.d Biology	The Svalbard reindeer (*Rangifer tarandus platyrhynchus*) and its food base: plant-herbivore interactions in a high-arctic ecosystem
2008	Jiska van Dijk	ph.d Biology	Wolverine foraging strategies in a multiple-use landscape
2008	Flora John Magige	ph.d Biology	The ecology and behaviour of the Masai Ostrich (Struthio camelus massaicus) in the Serengeti Ecosystem, Tanzania

2008	Bernt Rønning	ph.d Biology	Sources of inter- and intra-individual variation in basal metabolic rate in the zebra finch, (*Taeniopygia guttata*)
2008	Sølvi Wehn	ph.d Biology	Biodiversity dynamics in semi-natural mountain landscapes - A study of consequences of changed agricultural practices in Eastern Jotunheimen
2008	Trond Moxness Kortner	ph.d Biology	"The Role of Androgens on previtellogenic oocyte growth in Atlantic cod (*Gadus morhua*): Identification and patterns of differentially expressed genes in relation to Stereological Evaluations"
2008	Katarina Mariann Jørgensen	Dr. scient Biology	The role of platelet activating factor in activation of growth arrested keratinocytes and re-epithelialisation
2008	Tommy Jørstad	ph.d Biology	Statistical Modelling of Gene Expression Data
2008	Anna Kusnierczyk	ph.d Biology	*Arabidopsis thaliana* Responses to Aphid Infestation
2008	Jussi Evertsen	ph.d Biology	Herbivore sacoglossans with photosynthetic chloroplasts
2008	John Eilif Hermansen	ph.d Biology	Mediating ecological interests between locals and globals by means of indicators. A study attributed to the asymmetry between stakeholders of tropical forest at Mt. Kilimanjaro, Tanzania
2008	Ragnhild Lyngved	ph.d Biology	Somatic embryogenesis in *Cyclamen persicum.* Biological investigations and educational aspects of cloning
2008	Line Elisabeth Sundt-Hansen	ph.d Biology	Cost of rapid growth in salmonid fishes
2008	Line Johansen	ph.d Biology	Exploring factors underlying fluctuations in white clover populations – clonal growth, population structure and spatial distribution
2009	Astrid Jullumstrø Feuerherm	ph.d Biology	Elucidation of molecular mechanisms for pro-inflammatory phospholipase A2 in chronic disease
2009	Pål Kvello	ph.d Biology	Neurons forming the network involved in gustatory coding and learning in the moth *Heliothis virescens:* Physiological and morphological characterisation, and integration into a standard brain atlas
2009	Trygve Devold Kjellsen	ph.d Biology	Extreme Frost Tolerance in Boreal Conifers
2009	Johan Reinert Vikan	ph.d Biology	Coevolutionary interactions between common cuckoos *Cuculus canorus* and *Fringilla* finches
2009	Zsolt Volent	ph.d Biology	Remote sensing of marine environment: Applied surveillance with focus on optical properties of phytoplankton, coloured organic matter and suspended matter
2009	Lester Rocha	ph.d Biology	Functional responses of perennial grasses to simulated grazing and resource availability
2009	Dennis Ikanda	ph.d Biology	Dimensions of a Human-lion conflict: Ecology of human predation and persecution of African lions (*Panthera leo*) in Tanzania
2010	Huy Quang Nguyen	ph.d Biology	Egg characteristics and development of larval digestive function of cobia (*Rachycentron canadum*) in response to dietary treatments - Focus on formulated diets
2010	Eli Kvingedal	ph.d Biology	Intraspecific competition in stream salmonids: the impact of environment and phenotype
2010	Sverre Lundemo	ph.d Biology	Molecular studies of genetic structuring and demography in *Arabidopsis* from Northern Europe
2010	Iddi Mihijai Mfunda	ph.d Biology	Wildlife Conservation and People's livelihoods: Lessons Learnt and Considerations for Improvements. Tha Case of Serengeti Ecosystem, Tanzania

2010	Anton Tinchov Antonov	ph.d Biology	Why do cuckoos lay strong-shelled eggs? Tests of the puncture resistance hypothesis
2010	Anders Lyngstad	ph.d Biology	Population Ecology of *Eriophorum latifolium*, a Clonal Species in Rich Fen Vegetation
2010	Hilde Færevik	ph.d Biology	Impact of protective clothing on thermal and cognitive responses
2010	Ingerid Brænne Arbo	ph.d Medical technology	Nutritional lifestyle changes – effects of dietary carbohydrate restriction in healthy obese and overweight humans
2010	Yngvild Vindenes	ph.d Biology	Stochastic modeling of finite populations with individual heterogeneity in vital parameters
2010	Hans-Richard Brattbakk	ph.d Medical technology	The effect of macronutrient composition, insulin stimulation, and genetic variation on leukocyte gene expression and possible health benefits
2011	Geir Hysing Bolstad	ph.d Biology	Evolution of Signals: Genetic Architecture, Natural Selection and Adaptive Accuracy
2011	Karen de Jong	ph.d Biology	Operational sex ratio and reproductive behaviour in the two-spotted goby (*Gobiusculus flavescens*)
2011	Ann-Iren Kittang	ph.d Biology	*Arabidopsis thaliana* L. adaptation mechanisms to microgravity through the EMCS MULTIGEN-2 experiment on the ISS:– The science of space experiment integration and adaptation to simulated microgravity
2011	Aline Magdalena Lee	ph.d Biology	Stochastic modeling of mating systems and their effect on population dynamics and genetics
2011	Christopher Gravningen Sørmo	ph.d Biology	Rho GTPases in Plants: Structural analysis of ROP GTPases; genetic and functional studies of MIRO GTPases in Arabidopsis thaliana
2011	Grethe Robertsen	ph.d Biology	Relative performance of salmonid phenotypes across environments and competitive intensities
2011	Line-Kristin Larsen	ph.d Biology	Life-history trait dynamics in experimental populations of guppy (*Poecilia reticulata*): the role of breeding regime and captive environment
2011	Maxim A. K. Teichert	ph.d Biology	Regulation in Atlantic salmon (*Salmo salar*): The interaction between habitat and density
2011	Torunn Beate Hancke	ph.d Biology	Use of Pulse Amplitude Modulated (PAM) Fluorescence and Bio-optics for Assessing Microalgal Photosynthesis and Physiology
2011	Sajeda Begum	ph.d Biology	Brood Parasitism in Asian Cuckoos: Different Aspects of Interactions between Cuckoos and their Hosts in Bangladesh
2011	Kari J. K. Attramadal	ph.d Biology	Water treatment as an approach to increase microbial control in the culture of cold water marine larvae
2011	Camilla Kalvatn Egset	ph.d Biology	The Evolvability of Static Allometry: A Case Study
2011	AHM Raihan Sarker	ph.d Biology	Conflict over the conservation of the Asian elephant (*Elephas maximus*) in Bangladesh
2011	Gro Dehli Villanger	ph.d Biology	Effects of complex organohalogen contaminant mixtures on thyroid hormone homeostasis in selected arctic marine mammals
2011	Kari Bjørneraas	ph.d Biology	Spatiotemporal variation in resource utilisation by a large herbivore, the moose
2011	John Odden	ph.d Biology	The ecology of a conflict: Eurasian lynx depredation on domestic sheep
2011	Simen Pedersen	ph.d Biology	Effects of native and introduced cervids on small mammals and birds
2011	Mohsen Falahati-Anbaran	ph.d Biology	Evolutionary consequences of seed banks and seed dispersal in *Arabidopsis*

2012	Jakob Hønborg Hansen	ph.d Biology	Shift work in the offshore vessel fleet: circadian rhythms and cognitive performance
2012	Elin Noreen	ph.d Biology	Consequences of diet quality and age on life-history traits in a small passerine bird
2012	Irja Ida Ratikainen	ph.d Biology	Theoretical and empirical approaches to studying foraging decisions: the past and future of behavioural ecology
2012	Aleksander Handå	ph.d Biology	Cultivation of mussels (*Mytilus edulis):*Feed requirements, storage and integration with salmon (*Salmo salar*) farming
2012	Morten Kraabøl	ph.d Biology	Reproductive and migratory challenges inflicted on migrant brown trour (*Salmo trutta* L) in a heavily modified river
2012	Jisca Huisman	ph.d Biology	Gene flow and natural selection in Atlantic salmon
	Maria Bergvik	ph.d Biology	Lipid and astaxanthin contents and biochemical post-harvest stability in *Calanus finmarchicus*
2012	Bjarte Bye Løfaldli	ph.d Biology	Functional and morphological characterization of central olfactory neurons in the model insect *Heliothis virescens*.
2012	Karen Marie Hammer	ph.d Biology	Acid-base regulation and metabolite responses in shallow- and deep-living marine invertebrates during environmental hypercapnia
2012	Øystein Nordrum Wiggen	ph.d Biology	Optimal performance in the cold
2012	Robert Dominikus Fyumagwa	Dr. Philos Biology	Anthropogenic and natural influence on disease prevalence at the human –livestock-wildlife interface in the Serengeti ecosystem, Tanzania
2012	Jenny Bytingsvik	ph.d Biology	Organohalogenated contaminants (OHCs) in polar bear mother-cub pairs from Svalbard, Norway. Maternal transfer, exposure assessment and thyroid hormone disruptive effects in polar bear cubs
2012	Christer Moe Rolandsen	ph.d Biology	The ecological significance of space use and movement patterns of moose in a variable environment
2012	Erlend Kjeldsberg Hovland	ph.d Biology	Bio-optics and Ecology in *Emiliania huxleyi* Blooms: Field and Remote Sensing Studies in Norwegian Waters
2012	Lise Cats Myhre	ph.d Biology	Effects of the social and physical environment on mating behaviour in a marine fish
2012	Tonje Aronsen	ph.d Biology	Demographic, environmental and evolutionary aspects of sexual selection
	Bin Liu	ph.d Biology	Molecular genetic investigation of cell separation and cell death regulation in *Arabidopsis thaliana*
2013	Jørgen Rosvold	ph.d Biology	Ungulates in a dynamic and increasingly human dominated landscape – A millennia-scale perspective
2013	Pankaj Barah	ph.d Biology	Integrated Systems Approaches to Study Plant Stress Responses
2013	Marit Linnerud	ph.d Biology	Patterns in spatial and temporal variation in population abundances of vertebrates
2013	Xinxin Wang	ph.d Biology	Integrated multi-trophic aquaculture driven by nutrient wastes released from Atlantic salmon (*Salmo salar*) farming
2013	Ingrid Ertshus Mathisen	ph.d Biology	Structure, dynamics, and regeneration capacity at the sub-arctic forest-tundra ecotone of northern Norway and Kola Peninsula, NW Russia
2013	Anders Foldvik	ph.d Biology	Spatial distributions and productivity in salmonid populations
2013	Anna Marie Holand	ph.d Biology	Statistical methods for estimating intra- and inter-population variation in genetic diversity
2013	Anna Solvang Båtnes	ph.d Biology	Light in the dark – the role of irradiance in the high Arctic marine ecosystem during polar night

2013	Ane Kjersti Vie	ph.d Biology	Molecular and functional characterisation of the IDA family of signalling peptides in *Arabidopsis thaliana*
2013	Marianne Nymark	ph.d Biology	Light responses in the marine diatom *Phaeodactylum tricornutum*
2014	Jannik Schultner	ph.d Biology	Resource Allocation under Stress - Mechanisms and Strategies in a Long-Lived Bird
2014	Craig Ryan Jackson	ph.d Biology	Factors influencing African wild dog *(Lycaon pictus*) habitat selection and ranging behaviour: conservation and management implications
2014	Aravind Venkatesan	ph.d Biology	Application of Semantic Web Technology to establish knowledge management and discovery in the Life Sciences
2014	Kristin Collier Valle	ph.d Biology	Photoacclimation mechanisms and light responses in marine micro- and macroalgae
2014	Michael Puffer	ph.d Biology	Factors influencing African wild dog *(Lycaon pictus*) habitat selection and ranging behaviour: conservation and management implications
2014	Gundula S. Bartzke	ph.d Biology	Effects of power lines on moose *(Alces alces)* habitat selection, movements and feeding activity
2014	Eirin Marie Bjørkvoll	ph.d Biology	Life-history variation and stochastic population dynamics in vertebrates
2014	Håkon Holand	ph.d Biology	The parasite *Syngamus trachea* in a metapopulation of house sparrows
2014	Randi Magnus Sommerfelt	ph.d Biology	Molecular mechanisms of inflammation – a central role for cytosolic phospholiphase A2
2014	Espen Lie Dahl	ph.d Biology	Population demographics in white-tailed eagle at an on-shore wind farm area in coastal Norway
2014	Anders Øverby	ph.d Biology	Functional analysis of the action of plant isothiocyanates: cellular mechanisms and in vivo role in plants, and anticancer activity
2014	Kamal Prasad Acharya	ph.d Biology	Invasive species: Genetics, characteristics and trait variation along a latitudinal gradient.
2014	Ida Beathe Øverjordet	ph.d Biology	Element accumulation and oxidative stress variables in Arctic pelagic food chains: Calanus, little auks (alle alle) and black-legged kittiwakes (Rissa tridactyla)
2014	Kristin Møller Gabrielsen	ph.d Biology	Target tissue toxicity of the thyroid hormone system in two species of arctic mammals carrying high loads of organohalogen contaminants
2015	Gine Roll Skjervø	dr. philos Biology	Testing behavioral ecology models with historical individual-based human demographic data from Norway
2015	Nils Erik Gustaf Forsberg	ph.d Biology	Spatial and Temporal Genetic Structure in Landrace Cereals
2015	Magni Olsen Kyrkjeeide	ph.d Biology	Genetic variation and structure in peatmosses (*Sphagnum*)
2015	Bjørnar Sporsheim	ph.d Biology	Quantitative confocal laser scanning microscopy: optimization for *in vivo* and *in vitro* analysis of intracellular transport
2015	Leila Alipanah	ph.d Biology	Integrated analyses of nitrogen and phosphorus deprivation in the diatoms *Phaeodactylum tricornutum* and *Seminavis robusta*
2015	Keshuai Li	ph.d Biology	Phospholipids in Atlantic cod (*Gadus morhua* L.) larvae rearing: Incorporation of DHA in live feed and larval phospholipids and the metabolic capabilities of larvae for *de novo* synthesis