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Growth and Lipidomic Analyses of *Penaeus monodon* Larvae Supplemented With *Aurantiochytrium limacinum* BCC52274

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Long-chain polyunsaturated fatty acids (LC-PUFAs) are essential for growth and health of larval marine animals. Marine animals have a limited capability for LC-PUFA synthesis, and the larvae must obtain LC-PUFAs from diet. The protist *Aurantiochytrium limacinum* (AL) is abundant in 22:6 n-3 (docosahexaenoic acid, DHA), 22:5 n-3 (docosapentaenoic acid, DPA) and 16:0 fatty acids, which qualifies it as an LC-PUFA source for feed application. Therefore, in this study, a common feed containing lower amounts of total LC-PUFAs, *Thalasiosira weissflogii*, was replaced with AL at graded proportions and supplied to *Penaeus monodon* larvae from mysis (M) 1 to post-larval (PL) 2 stages to supplement LC-PUFAs in the diet. After that, all shrimp from PL2 to PL12 were continuously reared and subjected to the same diet regime, which was a combination of *Artemia* and commercial dried feed. The AL-supplemented PL2 shrimp demonstrated marked accumulation of the key fatty acids present in AL—16:0, DPA and DHA. The supplemented larvae showed no difference in growth during the supplementation period from M1 to PL2; however, average body weight and biomass were increased in PL12 shrimp that were fed earlier with AL. Lipidomic analysis revealed that profiles of fatty acids but not lipid classes/subclasses in PL shrimp reflected the supplied diet. The main saturated fatty acid (SFA, 16:0) predominantly accumulated in acylglycerols, which are energy-reserve lipids, in PL2 shrimp. Both LC-PUFAs (DHA and DPA) were preferentially deposited in phospholipids or structural lipids. Furthermore, while the amounts of both LC-PUFAs increased along with the amount of supplied AL, that of the SFA did not. This suggests that LC-PUFAs were prioritized to be stored over SFA when both types of fatty acids were present in high amounts. This analysis substantiates the importance of LC-PUFAs and provides an insight into how different types of the dietary fatty acids were differentially accumulated in lipid classes and subclasses for their biological functions.

Keywords: long-chain polyunsaturated fatty acids, lipids, lipidomics, shrimp larvae, *Aurantiochytrium limacinum*, diet supplement

INTRODUCTION

Lipids, represented by various classes and subclasses, perform various biological functions in living organisms. In aquatic animals, acylglycerols, particularly triglycerides, are the major source of energy for physical activities and cellular functions (Sargent et al., 1993; Tocher et al., 2008; Rey et al., 2018; Xie et al., 2020). Most energy in the form of ATP is produced from fatty acids associated with triglycerides through the beta-oxidation process (Berg et al., 2002a). In addition to energy provision, lipids play essential roles in cellular structure (Alberts et al., 2002). Phospholipids and sterols are key components of cell membrane structure, which is essential for the osmoregulation mechanism of marine animals (Chapelle et al., 1982; Sargent et al., 1993; Huang et al., 2019); moreover, phospholipids and sterols are also precursors of cellular signaling molecules and hormones that regulate growth, molting, the immune system and reproduction (Tocher et al., 2008).

The core of lipid molecules is esterified with diverse fatty acid constituents, which vary in chain length and saturation. Among the varieties of fatty acid types, n-3 and n-6 long-chain polyunsaturated fatty acids (LC-PUFAs) are essential for growth and health of marine animals, in particular at the larval stage (Glencross, 2009). They are an energy source and precursors of eicosanoids, which are local hormones that function in signaling and regulation of several biological mechanisms including immune response, growth and development (Bhathena, 2006; Wimuttisuk et al., 2013; Engelking, 2015; Stillwell, 2016). However, these fatty acids must be obtained from the diet because of the low activities of fatty acid elongation and desaturation in marine animals (Kanazawa et al., 1979; Glencross, 2009). Deficiency of LC-PUFAs causes abnormal development and growth of several marine animals (Watanabe, 1993; Harel et al., 2002; Samocha et al., 2010). Several studies have been carried out to supplement LC-PUFAs in the diet for marine animals (Ishizaki et al., 1998; Boglino et al., 2012; Lund et al., 2012; Watanabe et al., 2016; Visudtiphole et al., 2018).

Thraustochytrids are single-cell protists, which are abundant producers of LC-PUFAs including docosahexaenoic acid (DHA, 22:6 n-3), eicosapentaenoic acid (EPA, 20:5 n-3), docosapentaenoic acid (DPA, 22:5 n-3), and arachidonic acid (ARA, 20:4 n-6). They have been applied to aquafeed as an alternative LC-PUFA source to fish oil (Lewis et al., 1999; Unagul et al., 2017). In larviculture, they are used to enrich LC-PUFAs in live *Artemia* or rotifer, which are fed to marine animal larvae to promote growth and health (Leger et al., 1986; Barclay and Zeller, 1996; Harel et al., 2002). The effect of thraustochytrid enrichment on shrimp larviculture has been studied at the early post-larval stage (PL1), the earliest that can ingest enriched *Artemia* (Visudtiphole et al., 2018). However, supplementation of dietary LC-PUFAs for shrimp larvae at earlier developmental stages was not studied. Therefore, in this study, a thraustochytrid, *Aurantiochytrium limacinum* BCC52274 (AL), was applied to *Penaeus monodon* larvae from mysis 1 (M1) to PL2 to supplement DHA in the diet. To do so, a common feed for the larvae, *Thalasiosira weissflogii* (TW), which is deficient in LC-PUFAs, was replaced with AL at graded ratios and the effects of the supplementation on larval growth and lipidomics were then evaluated.

Lipidomics is the holistic study of lipid molecules found in biological organisms. With the complexity of lipid molecules, analytical tools in combination with lipid structure database are required to elucidate lipid molecules from unknown samples in both qualitative and quantitative analyses (Cajka and Fiehn, 2014). Lipidomic analysis using liquid chromatography-high resolution mass spectrometry (LC-HRMS) has been employed to understand the physiological processes of marine species including blue mussels and Pacific white shrimp (Huang et al., 2019; Laudicella et al., 2020). The technique has also been applied to study the diet effect on aquatic animals—for example, different lipid sources and fish meal replacement in swimming crab and Pacific white shrimp, respectively (Xie et al., 2020; Yuan et al., 2021). Therefore, as well as growth evaluation, lipidomic analysis using LC-HRMS was carried out to examine the effects of the AL supplement on lipid profiles in shrimp. Moreover, since AL was found to contain distinctly high amounts of not only LC-PUFAs (DHA and DPA) but also a saturated fatty acid (16:0), the acquired lipidomic data were further analyzed to understand how the two different types of fatty acids were differentially accumulated in lipid classes and subclasses of the shrimp. The obtained results substantiate different biological roles of the two types of fatty acids in shrimp larvae.

MATERIALS AND METHODS

Aurantiochytrium limacinum BCC52274 Preparation

AL strain BCC52274 (obtained from National Biobank of Thailand) was cultured at 28°C in a 10-L fermenter with aeration for 3 days (Unagul et al., 2017). The medium was composed of 1.5, 10, and 2% w/v sea salt (Sigma), Glucose (Difco), yeast extract (Difco), respectively. Cells were then centrifuged at 3,000 × *g* and washed twice with 0.9% normal saline. Cells were stored at 4°C in sealed vacuum plastic bags protected from light and used within 1 month. For feeding, AL cells were suspended in 30-ppt sea water through 100T polyester mesh before the suspension was poured into the larviculture.

Experimental Animals

Specific pathogen-free *P. monodon* larvae were obtained from Shrimp Genetic Improvement Center, Surat Thani, Thailand. Experiments were carried out in a hatchery at the same location.

Abbreviations: ABW, average body weight; AL, *Aurantiochytrium limacinum*; ARA, arachidonic acid; % CV, percent coefficient of length variation; DG, diacylglycerols; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; HCD, higher-energy collisional dissociation; LC-HRMS, liquid chromatography-high resolution mass spectrometry; LC-PUFA, long-chain polyunsaturated fatty acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; M, mysis; MS, mass spectrometry; MUFA, monounsaturated fatty acid; PC, phosphatidylcholine; PE, phosphatidylethnolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, post-larval; PRM, parallel reaction monitoring; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TFA, total fatty acid; TG, triacylglycerols; TW, *Thalasiosira weissflogii*.

Larvae were maintained from nauplius to zoea 3, during which zoea were fed solely with *Thalasiosira weissflogii* (TW). The experiment was started when the larvae entered the mysis 1 (M1) stage. All animal experiment protocols were approved by National Center for Genetic Engineering and Biotechnology Institutional Animal Care and Use Committee (Approval code: BT-Animal 25/2560) and carried out in accordance with the relevant guidelines and regulations.

Feeding Experiments

Five hundred-liter dark plastic tanks were each stocked with 40,000 M1 larvae and 300 L of 30-ppt water with aeration. The stocking tanks were randomly assigned to the dietary treatments ($N = 3$ tanks for each treatment group). The experimental design of treatment groups is shown in **Table 1**. Mysis were fed 8 times/day. Anesthetized frozen instar-I *Artemia* was supplied every other meal, alternating with either AL or TW. The numbers of meals with either TW or AL varied among treatment groups while the number of *Artemia* meals was the same for all groups. Each experimental group was fed to saturation. The feeding amount of each group was separately adjusted according to the unconsumed amount, which was assessed by a hematocrit after the feed time for 3 h. Supplementation experiments were carried out from M1 to PL2 (4.5 days).

To examine the post-supplementation effect of AL, PL2 shrimp of all experimental groups were raised until PL12. To do so, 5,000 PL2 shrimp individuals from each tank were randomly taken and transferred to a dark plastic tank containing 150 L of 30-ppt sea water. During this period, all experimental groups were subjected to the same dietary regime and treatment. PL3-5 shrimp were fed with live *Artemia* for every other meal, alternating with a commercial flake feed (#2TNT, Advance Pharma, Thailand) while PL6-12 shrimp were fed only with PL300 flake (Inve). All PL shrimp were fed 8 meals/day. To mimic the actual common commercial practice, salinity of the culture water was gradually reduced. From PL3-6 and PL7-11, the culture water was exchanged by 20 and 30%, respectively, with 17-ppt water every day until the final salinity reached 17 ppt at PL12.

During the entire rearing experiment, the water quality of all tanks was controlled within the following ranges: pH at 7.5–8.5, temperature at 29–32°C, ammonia-N below 1.0 mg/L, nitrite-N below 2.0 mg/L, nitrate-N less than 3.0 mg/L and alkalinity between 140 and 220 mg $CaCO_3$/L.

Assessment of Growth Performance

At PL2 and PL12, growth performance assessment and sample collection were carried out, as previously described in Visudtiphole et al. (2018). Briefly, the animals were fasted by withholding a meal ahead of the process. Average body weight (ABW) was determined from the PL2 and PL12 individuals with a combined weight of 120 and 500 mg, respectively (2 count replicates/tank). Survival rate was calculated from the final biomass weight and average body weight data. Average body length was determined from 50 individuals for each tank, measuring from both ends of rostrum to telson. Percent coefficient of body length variation was calculated as standard deviation/mean × 100. Shrimp samples for the biochemical analyses were collected, immediately snap-frozen in liquid nitrogen and stored at -80°C until used.

Proximate Analysis

Crude protein, lipids, fiber and ash contents in the feed (2-g dried sample) were analyzed through the service provided by Animal Husbandry Department, Faculty of Veterinary Science, Chulalongkorn University (Thailand), using the standard methods of AOAC (Horwitz and Latimer, 2005). Total calories were determined through the service provided by Scientific and Technological Equipment Research Center, Chulalongkorn University (Thailand), using a Bomb Calorimeter AC-500 (Leco, United States).

Fatty Acid Profile Analysis by Gas Chromatography-Flame Ionization Detector

Fatty acids from freeze-dried samples (from 0.20 to 0.27 g fresh samples) were directly extracted and transesterified in a single step by heating at 90°C with 4% sulfuric acid in methanol for 1 h (Lepage and Roy, 1986). Heptadecanoic or nonadecanoic acid (17:0 and 19:0, respectively) was used as the internal standard (Sigma-Aldrich). The esterified samples were applied

TABLE 1 | Feeding scheme of the experimental group during Mysis1 to PL2.

Feeding time	Experimental group			
Meals supplied with AL/day	0	2	3	4
% of TW replacement with AL	0	50	75	100
TW: AL: *Artemia*	4:0:4	2:2:4	1:3:4	0:4:4
1:00	*Artemia*	*Artemia*	*Artemia*	*Artemia*
4:00	TW	AL	AL	AL
7:00	*Artemia*	*Artemia*	*Artemia*	*Artemia*
10:00	TW	TW	TW	AL
13:00	*Artemia*	*Artemia*	*Artemia*	*Artemia*
16:00	TW	AL	AL	AL
19:00	*Artemia*	*Artemia*	*Artemia*	*Artemia*
22:00	TW	TW	AL	AL

to GC (GC17A, Shimadzu), equipped with a 30 m × 0.25 mm Omegawax^{TM} 250 fused silica capillary column (Supelco), an automatic sampler and flame ionization detector (FID). The injector and detector temperatures were maintained at 250 and 260°C, respectively. Helium was used as a carrier gas at a linear velocity of 30 cm/s. The initial column temperature of 200°C was held for 10 min and then increased at the rate of 20°C/min to 230°C, which was then maintained for 17 min. Peak analysis (identification and quantification) was carried out based on the retention times relative to fatty acid methyl ester standards (Supelco 18919-1 AMP) (all from Sigma-Aldrich).

Lipidomic Analysis

Lipid Sample Preparation

Crude lipid was extracted from freeze-dried samples (from 13 ± 2 g wet weight), using chloroform/methanol (Folch et al., 1957). In brief, samples were homogenized in chloroform: methanol (2:1). After filtration to remove the debris, samples were washed with 0.15% NaCl and centrifuged to collect the lower lipid phase. The NaCl-wash step was repeated twice before the organic solvent was finally removed by evaporation with N_2. The dried lipid was then dissolved in chloroform, diluted with isopropanol: methanol (1:1) and filtered through PVDF membrane. After that, an internal standard (tributyrin) was added into the samples.

Lipidomics Acquisition Using Liquid Chromatography-High Resolution Mass Spectrometry

Liquid chromatography was performed by using an Acquity UHPLC HSST3 C18 column (Water, United States) with 100 × 2.1 mm inner diameter and 1.8 μm particle size. The column temperature was set at 45°C. Mobile phases were composed of solvent A (acetonitrile: water (60:40) in 0.1% formic acid with 10 mM ammonium formate) and solvent B (isopropanol: acetonitrile: water (88:10:2) in 0.1% formic acid with 10 mM ammonium formate). Elution was performed in 6 isocratic steps with graded concentration of solvent B: 40–55% at 0–7 min, 55–65% at 7–10 min, 65–70% at 10–19 min, 70–88% at 19–21 min, 88–95% at 21–23 min and 95–100% at 23–25 min and held for 5 min. The flow rate was 0.3 μL/min. Between each sample run, the system was re-equilibrated to the initial condition for 0.1 min (40% solvent B) and held for 4.9 min before starting the next run. LC-HRMS runs of pure lipid standards, triacylglycerol (TG 18:1/18:1/18:1), diacylglycerol (DG 18:1/18:1), lysophosphatidylcholine (LPC 18:0), phosphatidylcholine (PC 16:0,18:0,18:1,18:2), phosphatidylethnolamine (PE), phosphatidylglycerol (PG 18:0,18:1), phosphatidylinositol (PI), phosphatidylserine (PS) (Avanti Polar Lipids, United States), cholesteryl ester (with 18:1), and cholesterol (Sigma Aldrich, United States) were performed for validation of the lipid-class identification.

Electrospray ionization (ESI) was performed for mass spectrometry (MS), using both positive and negative ionization modes. The ion source settings were 3,000 V, 320°C for ion transferring and 300°C for vaporization. High-resolution MS data were acquired in two separate methods, namely, (i) full scan data dependent-tandem MS (full scan dd-MS2) method for untargeted lipids analysis through LipidSearch^{TM} 4.0 processing software (Thermo Fisher Scientific, United States) and (ii) parallel reaction monitoring (PRM) method for targeted lipids unavailable in LipidSearch^{TM}. Full scan dd-MS2 was used to collect MS and MS2 spectra over the mass range of 100–1,200 Da using an Orbitrap detector with a resolution of 120,000. The 10 most intense precursor ions of the spectra throughout the chromatographic run were fragmented using higher-energy collisional dissociation (HCD) at 25 ± 5% of collision energy with a resolution of 30,000. The PRM method was used to monitor cholesterol (369.3516 m/z) and tributyrin (320.2067 m/z) with 30 and 15% HCD, respectively, using an Orbitrap detector with a resolution of 60,000.

Data Processing and Analysis

The acquired MS data were imported to the LipidSearch^{TM} software and subjected to the following steps: (i) peak detection from high resolution mass spectral intensity of each sample, (ii) identification of lipid class by matching precursor and product ions to the database to define their structures, and (iii) alignment of the identified peaks across the samples for quantification. Analysis of the processed data generated lipid features (identified lipid compound with a specific retention time) and their abundances (areas under the corresponding peaks). After subtraction of the blank sample value, test sample intensity values were normalized to that of the internal standard, tributyrin. The normalized abundance data were then used for comparative analysis across the sample groups.

For analysis of 16:0, 22:5 n-3, and 22:6 n-3 fatty acids incorporated in lipid classes and subclasses, percent of sites esterified with the fatty acid of interest on the lipid molecule were determined, using the following equation:

$$\%\ esterified\ sites\ =\ \frac{\sum nA}{\sum NA}\ \times\ 100$$

in which A = normalized abundance.

n = the number of sites esterified with the fatty acid of interest.

N = the number of sites esterified with all fatty acids on the lipid molecule backbone (3 for TG; 2 for DG and phospholipids; 1 for lysophospholipids).

Statistical Analyses

Statistical analyses of the data were performed separately for each PL stage, using one-way analysis of variance (ANOVA) by Duncan's multiple range test (SPSS 11.5.0). Differences were considered to be significant when $P < 0.05$.

RESULTS

Analysis of the Experimental Feed

The nutritional contents of the TW and AL feeds were determined before comparing the effects of different feeds on the larvae. In line with the crude lipid content, the total fatty acid (TFA) content of AL was also much higher than that of

TW (**Supplementary Table 1** and **Table 2**). The predominant fatty acids in AL were palmitic acid (16:0) and DHA, which were present in much lower amounts in TW. In addition to DHA, DPA was also present in AL but absent in TW. In contrast, the amount of EPA in TW was slightly higher than that in AL. When comparing fatty acid types, AL contained more saturated fatty acids (SFAs), n-3 polyunsaturated fatty acids (PUFAs) and LC-PUFAs but had less monounsaturated fatty acids (MUFAs), and n-6 PUFAs than TW.

The contents of total acylglycerols, diacylglycerols (DG), and triacylglycerols (TG) in AL were higher than those of TW (**Figure 1**). However, the phospholipid and lysophospholipid contents in AL were extremely low, compared with those in TW (**Figures 2**, **3**). This indicates that most fatty acids in AL were present as acylglycerols. Only two types of phospholipids, i.e., phosphatidylcholine (PC) and phosphatidylethanolamine (PE), were detected in AL while PC, PE, phosphatidylglycerol (PG), and phosphatidylserine (PS) were all found in TW (**Figure 2**). Cholesterol was absent in both AL and TW whereas only a small amount of cholesteryl esters was present in AL (**Figure 4**).

Larval Growth Performance

Replacement of TW with AL did not affect growth of the larvae from mysis to PL2. All growth parameters of all treatment groups among PL2 shrimp were not significantly different (**Table 3**). However, a significant effect of AL on growth was observed during the post-supplementation period from PL2 to PL12. Average body weight and biomass of PL12 shrimp significantly increased with increasing amounts of AL supplied earlier to the larvae.

Analysis of Post-larval Shrimp's Fatty Acid Compositions

Fatty acid profiles of PL2 shrimp differed according to the diet supplied to the larvae during M1 to PL2 (**Table 4**). Replacement of TW with AL led to significantly increased total amounts of SFAs, PUFAs, n-3, and LC-PUFAs in PL2 shrimp, predominantly resulting from the increased levels of 16:0, DPA, and DHA, respectively. Nevertheless, levels of the three less complex n-3 PUFAs i.e., linolenic acid (18:3), eicosastrienoic acid (20:3), and EPA were significantly lower when AL was supplied. The total n-6 PUFA amount in PL2 shrimp was significantly decreased in treatments with AL, which can be attributed to the low content of linoleic acid (18:2 n-6) in AL. Ratios of n-3/n-6 and DHA/EPA in PL2 shrimp, as a result, were higher, reflecting the higher proportion of AL:TW in the diet. Finally, the TFA content in PL2 shrimp significantly increased with

TABLE 2 | Fatty acid composition of the feed analyzed by GC-FID.

Fatty acids	TW		AL		TNT		P300	
	%TFA	%DW	%TFA	%DW	%TFA	%DW	%TFA	%DW
14:0	14.78	0.57	3.37	1.54	6.13	0.56	6.68	0.47
15:0	2.72	0.11	6.53	2.99	0.64	0.04	0.43	0.05
16:0	26.29	1.02	44.17	20.22	26.92	2.00	24.05	2.06
18:0	nd	nd	0.98	0.45	6.54	0.26	3.07	0.50
20:0	4.04	0.16	0.19	0.09	0.31	0.02	0.20	0.02
∑ SFA	47.83	1.86	55.24	25.29	40.54	2.88	34.43	3.1
16:1	0.97	0.04	nd	nd	4.74	0.64	7.66	0.36
18:1	1.08	0.04	nd	nd	20.86	1.59	19.03	1.60
20:1	nd	nd	nd	nd	0.92	0.24	2.86	0.07
∑ MUFA	2.05	0.08	nd	nd	26.52	2.47	29.55	2.03
18:3 n-3	nd	nd	nd	nd	1.94	0.17	1.99	0.15
20:3 n-3	nd	nd	nd	nd	nd	nd	0.11	0.01
20:5 n-3	39.56	1.52	0.45	0.21	4.15	0.80	9.64	0.32
22:5 n-3	nd	nd	7.55	3.46	0.63	0.02	0.18	0.05
22:6 n-3	10.14	0.39	35.03	16.03	8.54	0.87	10.42	0.65
∑ n-3	49.70	1.91	43.03	19.70	15.26	1.86	22.34	1.18
18:2 n-6	0.41	0.02	nd	nd	16.60	1.09	13.08	1.27
20:4 n-6	nd	nd	nd	nd	1.09	0.05	0.59	0.08
∑ n-6	0.41	0.02	nd	nd	17.69	1.14	13.67	1.35
∑ PUFA	50.11	1.93	43.03	19.70	32.95	3.00	36.01	2.53
∑ HUFA	49.70	1.91	43.03	19.70	14.41	1.74	20.94	1.11
n-3/n-6	121.22		–		0.86		1.63	
DHA/EPA	0.26		77.84		2.06		1.08	
∑TFA (%DW)	3.86		44.99		7.65		8.33	

Data are presented in % of total fatty acids (TFA) and % dry weight (DW). AL and TW are A. limacinum and T. weissflogii. TNT and P300 are the commercial names of feed supplied to the shrimp during PL2-PL12, described in Materials and methods. Nd indicates not-detectable. SFA, MUFA, PUFA, and HUFA are abbreviations for saturated, monounsaturated, polyunsaturated, highly unsaturated and total fatty acids, respectively.

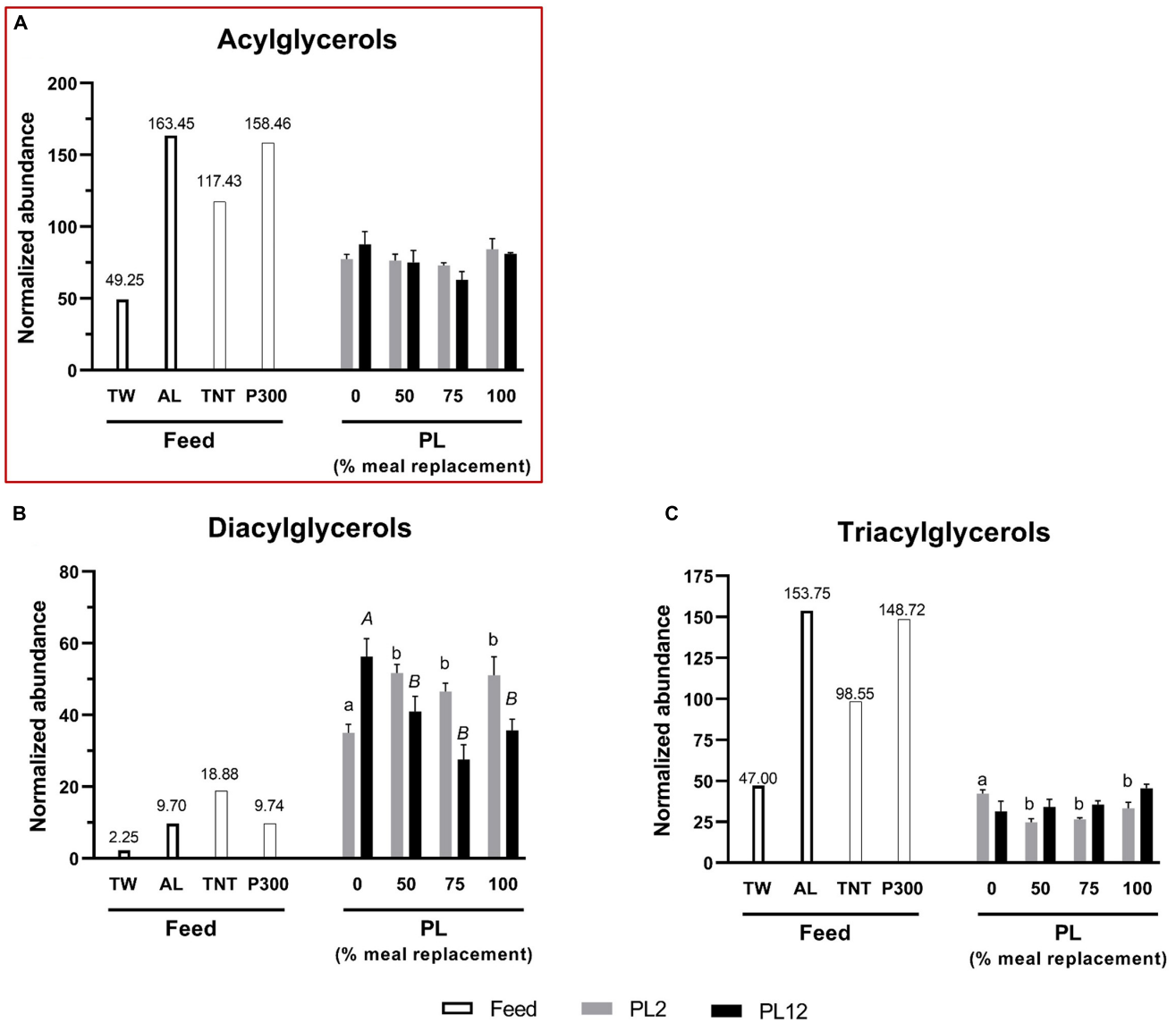

FIGURE 1 | Analysis of acylglycerols in feed and PL shrimp by LC-HRMS. Percent of meal replacement was percent of the number of meals for which TW was replaced by AL. Normalized abundance was derived from the peak intensity value normalized to that of tributyrin, the internal standard. Error bars represent standard errors ($N = 3$). Statistical analyses of the PL2 and PL12 data sets were performed separately, using ANOVA. Significant differences among subsets of treatment groups were determined by Duncan's multiple range test ($P < 0.05$); subsets with means not significantly different from one another are identified with the same letter above the bar. Small- and big-capital letters are individually for the PL2 and PL12 data sets, respectively. Bars with no specified letters mean no significant differences were identified among the groups ($P > 0.05$).

the increasing AL amounts, most likely due to the high TFA content in AL.

When AL was no longer supplemented and all the experimental groups were fed with the same diet during later stages of development from PL2 to PL12, levels of most fatty acids, including 16:0 and DHA, in PL12 shrimp among all treatment groups were not significantly different (**Table 4**). Both 16:0 and DHA contents of all the groups fed at earlier stages with AL decreased to the same level in PL12 shrimp after AL was no longer supplied. The contents of some fatty acids such as 20:3 n-3 and DPA and the n-3/n-6 ratio were still significantly different among the treatment groups of PL12 shrimp; however, the degree of difference was much less than that in PL2 shrimp.

Analysis of Lipid Classes and Subclasses in Post-larval Shrimp

While TG constitutes the main subclass of acylglycerols in both TW and AL feeds, DG and TG in the shrimp were present at the comparable levels (**Figures 1B,C**). The DG content in PL2 shrimp was significantly increased by the AL diet replacement (**Figure 1B**). This was in line with the higher DG content in AL

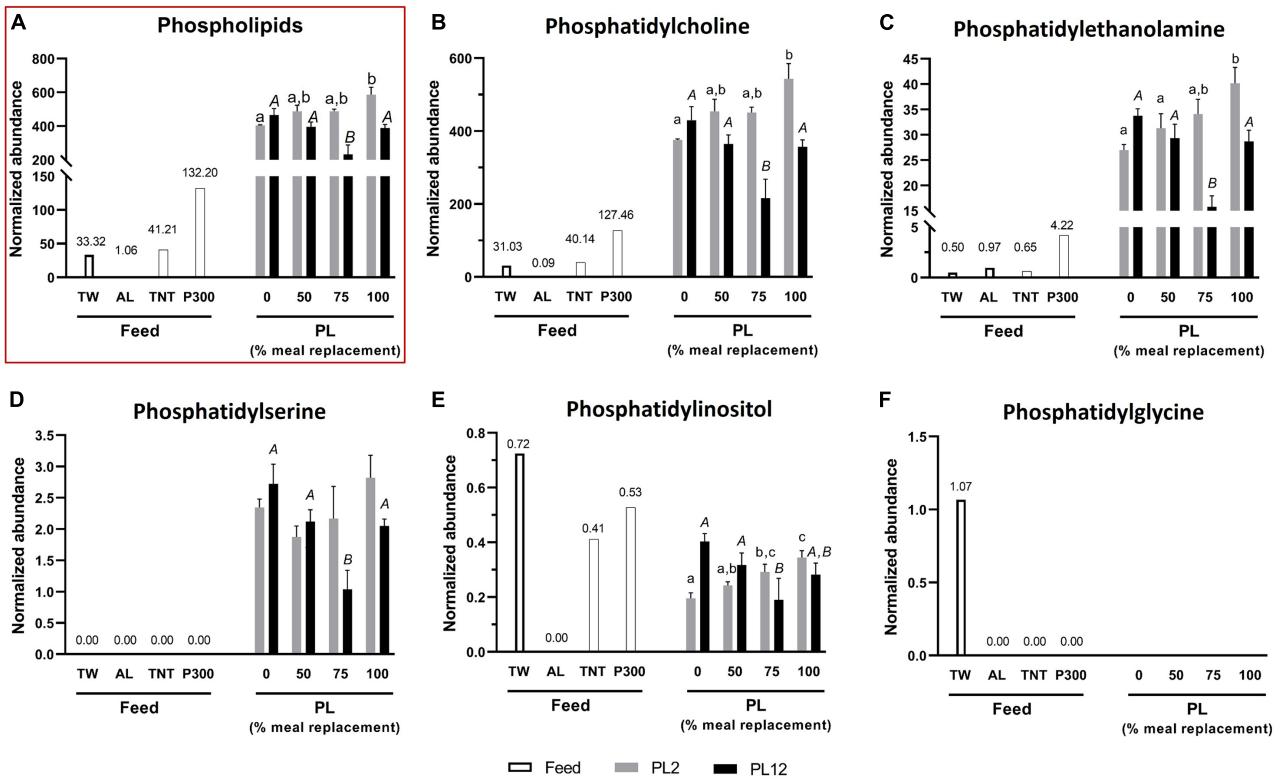

FIGURE 2 | Analysis of phospholipids in feed and PL shrimp by LC-HRMS. Percent of meal replacement was percent of the number of meals for which TW was replaced by AL. Normalized abundance was derived from the peak intensity value normalized to that of tributyrin, the internal standard. Error bars represent standard errors ($N = 3$). Statistical analyses of the PL2 and PL12 data sets were performed separately, using ANOVA. Significant differences among subsets of treatment groups were determined by Duncan's multiple range test ($P < 0.05$); subsets with means not significantly different from one another are identified with the same letter above the bar. Small- and big-capital letters are individually for the PL2 and PL12 data sets, respectively. Bars with no specified letters mean no significant differences were identified among the groups ($P > 0.05$).

than that in TW. Nevertheless, the TG content in PL2 shrimp was significantly decreased by the replacement although the level of TG in AL feed was higher than that in TW (**Figure 1C**). The opposite trends of changes in DG and TG contents resulted in insignificant differences of the total acylglycerols levels in PL2 shrimp among all groups (**Figure 1A**).

The content of total phospholipids in PL2 shrimp of the 100% replacement group was significantly higher than those of all the other groups (**Figure 2A**). The most and second most abundant phospholipids in the PL shrimp were PC and PE (**Figures 2B,C**), respectively, both of which are the major components of cell membranes in juvenile shrimp and also other species (Ronnestad et al., 1995; Coutteau et al., 1997; Caers et al., 2000a; Ju et al., 2011; Rey et al., 2015; Huang et al., 2019). PS and PI phospholipids were also detected in the PL shrimp (**Figures 2D,E**); however, they were present in much lower amounts than PC and PE. The PC, PE, and PI contents in PL2 shrimp were significantly increased with the increasing amounts of AL (**Figures 2B–E**). In contrast, the PS content in PL2 shrimp was not significantly different across all treatment groups (**Figure 2D**).

Lysophosphatidylcholine (LPC) constitutes the major subclass of lysophospholipids in PL2 and PL12 shrimp. The contents of LPC and total lysophospholipids were not significantly different among the treatment groups (**Figures 3A,B**). In contrast, the content of lysophosphatidylethanolamine (LPE) in PL2 shrimp increased as more AL was supplied to the larvae (**Figure 3C**).

The cholesterol content in PL2 shrimp was significantly higher as the larvae were fed with more AL (**Figure 4A**). In contrast, levels of cholesteryl esters in PL2 shrimp were not significantly different; however, no cholesteryl esters were found in the PL2 shrimp fed with no AL (**Figure 4B**).

After the post AL-supplementation period, the contents of most lipid classes and subclasses in PL12 shrimp were not significantly different across the treatment groups. Nevertheless, PL12 shrimp fed earlier with AL during their mysis development contained lower DG than those fed with only TW (**Figure 1B**).

Analysis of Palmitic Acid (16:0), Docosahexaenoic Acid (22:6 n-3), and Docosapentaenoic Acid (22:5 n-3) Incorporated in Lipid Classes and Subclasses

The fatty acid profile result analyzed by GC in **Table 4** showed that increased ingestion of AL resulted in significant accumulation of three dietary fatty acids, 16:0, 22:5 n-3,

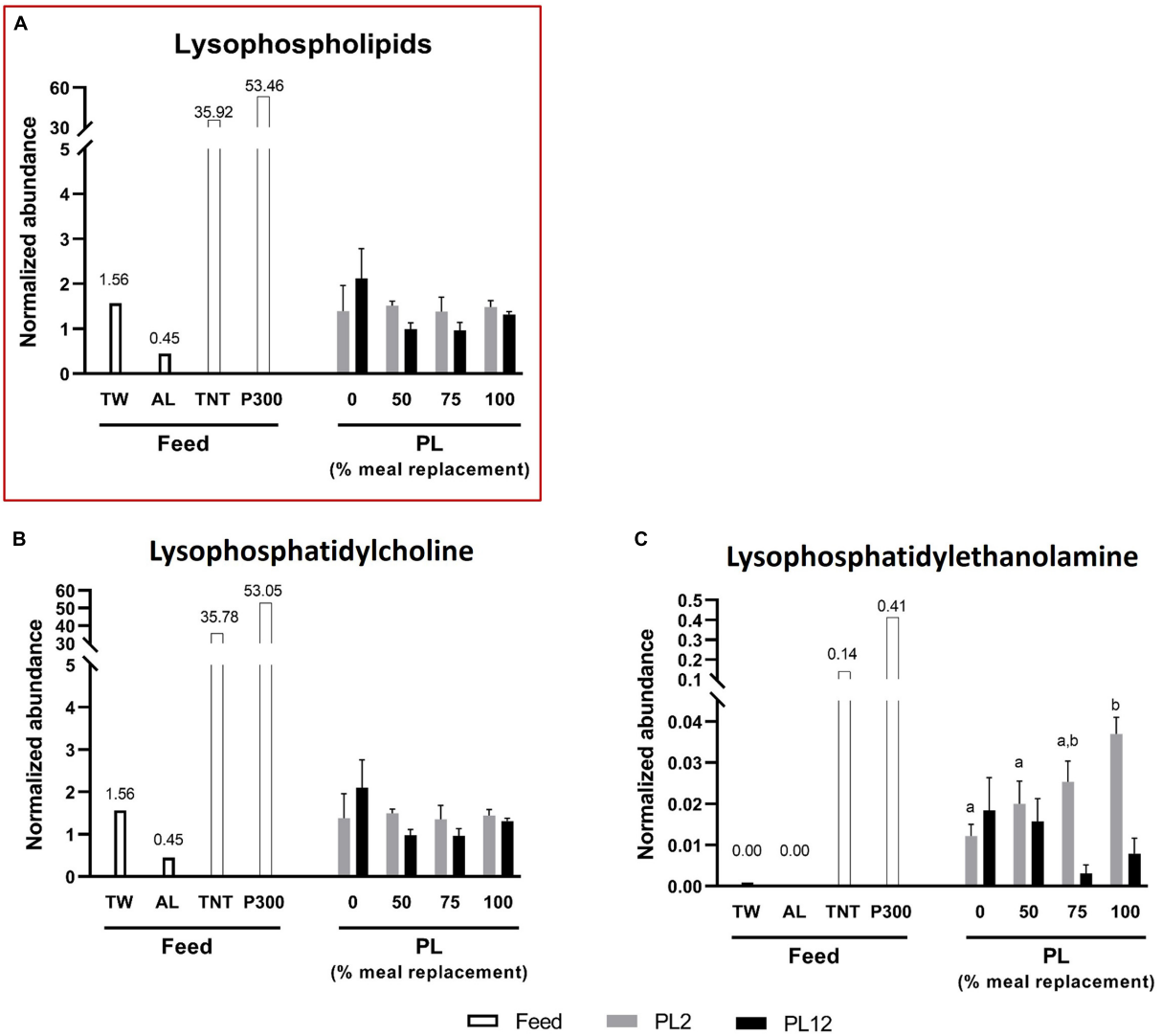

FIGURE 3 | Analysis of lysophospholipids in feed and PL shrimp by LC-HRMS. Percent of meal replacement was percent of the number of meals for which TW was replaced by AL. Normalized abundance was derived from the peak intensity value normalized to that of tributyrin, the internal standard. Error bars represent standard errors ($N = 3$). Statistical analyses of the PL2 and PL12 data sets were performed separately, using ANOVA. Significant differences among subsets of treatment groups were determined by Duncan's multiple range test ($P < 0.05$); subsets with means not significantly different from one another are identified with the same letter above the bar. Small- and big-capital letters are individually for the PL2 and PL12 data sets, respectively. Bars with no specified letters mean no significant differences were identified among the groups ($P > 0.05$).

and 22:6 n-3, in PL2 shrimp. Therefore, the lipidomic data were further analyzed to reveal the accumulation of these fatty acids distributed among lipid classes and subclasses in PL shrimp.

16:0 Fatty Acid

The percent of 16:0-esterified sites on acylglycerol backbones in PL2 shrimp of the AL-supplemented groups were significantly greater than that of the 0% meal replacement control group (**Figure 5A**). However, among the AL-supplemented groups, the percent of 16:0-esterified sites were not different or did not demonstrate further increases as the percent of the diet replacement was increased above 50%. A similar trend was also observed for the TG subclass whereas the percent of 16:0-esterified sites on DG were not significantly different among any of the treatment groups.

Unlike that of acylglycerols, the percent of 16:0-esterified sites on phospholipids in PL2 shrimp were not significantly different among all treatment groups (**Figure 5B**). At the subclass level, the percent of 16:0-esterified sites on the major phospholipid, PC, were reduced in groups with higher 16:0 content in the diet. On the other hand, the percent of 16:0-esterified sites on PE and PI increased while the percent of those on PS were unchanged. However, because the PE and PI contents in PL shrimp were far lower than that of PC (**Figure 2**), the increase of 16:0 on PE and PI had no effect on the 16:0 percent of total phospholipids.

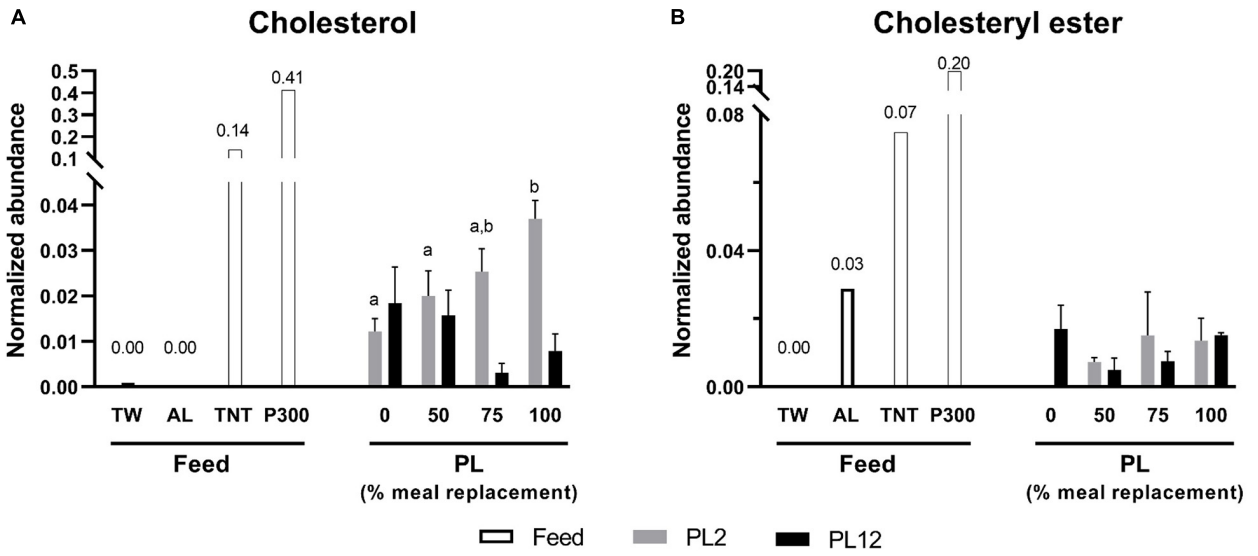

FIGURE 4 | Analysis of cholesterol and cholesteryl esters in feed and PL shrimp by LC-HRMS. Percent of meal replacement was percent of the number of meals for which TW was replaced by AL. Normalized abundance was derived from the peak intensity value normalized to that of tributyrin, the internal standard. Error bars represent standard errors ($N = 3$). Statistical analyses of the PL2 and PL12 data sets were performed separately, using ANOVA. Significant differences among subsets of treatment groups were determined by Duncan's multiple range test ($P < 0.05$); subsets with means not significantly different from one another are identified with the same letter above the bar. Small- and big-capital letters are individually for the PL2 and PL12 data sets, respectively. Bars with no specified letters mean no significant differences were identified among the groups ($P > 0.05$).

TABLE 3 | Growth parameters of PL2 and PL12 shrimp fed with graded percent of meals supplied with AL in replacement of TW.

	PL2				PL12			
	0%	50%	75%	100%	0%	50%	75%	100%
ABW (mg)	0.99 ± 0.03	1.00 ± 0.02	1.02 ± 0.07	0.99 ± 0.04	8.63 ± 0.24^{a}	9.81 ± 0.26^{b,c}	8.74 ± 0.33^{a,b}	10.13 ± 0.57^{c}
% Survival	60.36 ± 2.86	58.77 ± 1.21	61.69 ± 1.77	57.52 ± 2.10	84.04 ± 4.76	80.61 ± 4.84	95.38 ± 3.40	90.77 ± 2.37
Biomass (g)	23.87 ± 0.80	23.42 ± 0.49	25.03 ± 1.64	22.62 ± 0.54	36.18 ± 1.76^{a}	39.37 ± 1.55^{a}	41.56 ± 0.15^{a,b}	45.91 ± 2.49^{b}
Length (mm)	7.1 ± 0.1	7.2 ± 0.1	7.3 ± 0.1	7.3 ± 0.1	12.7 ± 0.1	12.9 ± 0.2	12.4 ± 0.22	13.0 ± 0.1
% CV	6.77 ± 0.62	7.01 ± 0.93	6.54 ± 0.24	7.98 ± 0.54	9.43 ± 0.84	9.79 ± 0.33	8.97 ± 0.51	8.17 ± 0.49

Data are presented as means ± standard error. Significant differences among the groups within the same stage are marked by different superscripted letters ($P < 0.05$). ABW and % CV are average body weight and percent coefficient of length variation, respectively.

For lysophospholipids, 16:0 fatty acid was detected to esterify with LPC and LPE (**Figure 5C**). The percent of LPC and LPE esterified with 16:0 fatty acid were not significantly different across the treatment groups, and all LPE molecules were esterified with 16:0.

22:6 n-3 and 22:5 n-3 Fatty Acids

22:6 n-3 and 22:5 n-3 LC-PUFAs accumulated in lipid classes and subclasses in patterns that were different from those of the 16:0 SFA. The percent of DHA- or DPA esterified sites on phospholipids and their subclasses in PL2 shrimp of the AL-supplemented groups were significantly greater than that of the control group but did not further increase as the percent of the replacement was increased above 50% (**Figures 6B**, **7B**). However, with an exception, more DHA incorporated in PC when the replacement was increased to 100%. DHA but not DPA was detected accumulating on PS and LPC (**Figure 6C**). Unlike that of 16:0, the percent of DHA-esterified sites on LPC were significantly increased by AL feeding. LPE, which was shown above to be specific to 16:0, was not esterified by DHA and DPA.

In contrast to those on phospholipids, the patterns of the DPA and DHA accumulation on acylglycerols were different. The percent of DPA- or DHA-esterified sites on total acylglycerols and TG in PL2 shrimp were significantly different in treatments with higher amounts of AL supplied to the larvae (**Figures 6A**, **7A**). DHA was also found to accumulate on DG with a similar pattern while DPA was not detected on the DG molecule. Furthermore, all cholesteryl ester molecules in both PL2 and PL12 shrimp were esterified specifically only with DHA (**Figure 6D**).

Similar to the aforementioned lipid class and subclass results, after the feed was changed to the same diet for all test groups during PL2–PL12, the percent of sites esterified with 16:0, 22:5 n-3, or 22:6 n-3 fatty acid in all lipid classes and subclasses were not significantly different among any of the sample groups (**Figures 5–7**).

TABLE 4 | Fatty acid profiles (% dry weight) of shrimp fed with different diets analyzed by GC-FID.

Fatty acids	PL2				PL12			
	0%	50%	75%	100%	0%	50%	75%	100%
14:0	0.018 ± 0.000^{a}	0.022 ± 0.000^{b}	0.024 ± 0.000^{b}	0.028 ± 0.000^{c}	0.041 ± 0.004	0.048 ± 0.006	0.057 ± 0.006	0.0054 ± 0.002
15:0	0.015 ± 0.000^{a}	0.049 ± 0.002^{b}	0.054 ± 0.001^{b}	0.076 ± 0.004^{c}	0.016 ± 0.001	0.017 ± 0.001	0.017 ± 0.000	0.017 ± 0.000
16:0	1.042 ± 0.009^{a}	1.279 ± 0.022^{b}	1.322 ± 0.010^{b}	1.482 ± 0.045^{c}	0.919 ± 0.023	0.938 ± 0.028	0.999 ± 0.021	0.957 ± 0.012
18:0	0.692 ± 0.003^{a}	0.670 ± 0.008^{b}	0.677 ± 0.012^{b}	0.677 ± 0.008^{c}	0.425 ± 0.007	0.407 ± 0.008	0.419 ± 0.004	0.401 ± 0.004
20:0	0.049 ± 0.000^{a}	0.046 ± 0.000^{b}	0.045 ± 0.000^{c}	0.047 ± 0.001^{c}	0.028 ± 0.000	0.028 ± 0.000	0.03 ± 0.000	0.029 ± 0.000
∑ SFA	1.816 ± 0.013^{a}	2.065 ± 0.031^{b}	2.122 ± 0.021^{b}	2.310 ± 0.059^{c}	1.429 ± 0.033	1.438 ± 0.041	1.521 ± 0.031	1.458 ± 0.014
16:1	0.080 ± 0.001^{a}	0.056 ± 0.003^{b}	0.056 ± 0.003^{b}	0.052 ± 0.002^{c}	0.043 ± 0.003	0.039 ± 0.004	0.040 ± 0.002	0.037 ± 0.001
18:1	1.202 ± 0.019^{a}	0.932 ± 0.030^{b}	0.939 ± 0.052^{b}	0.881 ± 0.016^{c}	0.806 ± 0.039	0.833 ± 0.048	0.893 ± 0.035	0.856 ± 0.016
20:1	0.053 ± 0.000^{a}	0.042 ± 0.001^{b}	0.042 ± 0.001^{b}	0.040 ± 0.000^{c}	0.058 ± 0.003^{a}	0.060 ± 0.002^{a}	0.066 ± 0.003^{b}	0.066 ± 0.002^{b}
∑ MUFA	1.335 ± 0.019^{a}	1.031 ± 0.033^{b}	1.038 ± 0.057^{b}	0.972 ± 0.018^{b}	0.907 ± 0.045	0.932 ± 0.054	0.999 ± 0.038	0.958 ± 0.018
18:3 n-3	1.449 ± 0.031^{a}	1.088 ± 0.039^{b}	1.095 ± 0.075^{b}	0.998 ± 0.030^{c}	0.539 ± 0.037	0.554 ± 0.046	0.578 ± 0.036	0.537 ± 0.008
20:3 n-3	0.209 ± 0.000^{a}	0.129 ± 0.004^{b}	0.128 ± 0.009^{b}	0.110 ± 0.002^{c}	0.071 ± 0.001^{a}	0.065 ± 0.001^{b}	0.067 ± 0.002^{b}	0.066 ± 0.001^{b}
20:5 n-3	0.497 ± 0.005^{a}	0.379 ± 0.002^{b}	0.373 ± 0.005^{b}	0.390 ± 0.007^{b}	0.533 ± 0.006	0.506 ± 0.003	0.521 ± 0.004	0.506 ± 0.008
22:5 n-3	0.006 ± 0.000^{a}	0.122 ± 0.003^{b}	0.135 ± 0.005^{b}	0.186 ± 0.012^{c}	0.010 ± 0.000^{a}	0.014 ± 0.000^{b}	0.015 ± 0.000^{b}	0.014 ± 0.000^{b}
22:6 n-3	0.218 ± 0.005^{a}	0.836 ± 0.013^{b}	0.908 ± 0.022^{b}	1.109 ± 0.046^{c}	0.477 ± 0.004	0.517 ± 0.005	0.553 ± 0.010	0.536 ± 0.005
∑ n-3	2.379 ± 0.022^{a}	2.555 ± 0.038^{b}	2.640 ± 0.054^{b}	2.793 ± 0.058^{c}	1.629 ± 0.041	1.657 ± 0.048	1.735 ± 0.046	1.659 ± 0.019
18:2 n-6	0.377 ± 0.005^{a}	0.292 ± 0.009^{b}	0.294 ± 0.017^{b}	0.273 ± 0.005^{c}	0.369 ± 0.015	0.381 ± 0.022	0.417 ± 0.015	0.395 ± 0.007
20:4 n-6	0.123 ± 0.001^{a}	0.151 ± 0.001^{b}	0.151 ± 0.002^{b}	0.165 ± 0.004^{b}	0.122 ± 0.004	0.116 ± 0.002	0.113 ± 0.002	0.116 ± 0.001
∑ n-6	0.500 ± 0.005^{a}	0.442 ± 0.009^{b}	0.444 ± 0.014^{b}	0.438 ± 0.004^{b}	0.491 ± 0.017	0.497 ± 0.021	0.530 ± 0.015	0.511 ± 0.008
∑ PUFA	2.879 ± 0.028^{a}	$2.997 \pm 0.047^{a,b}$	$3.084 \pm 0.068^{b,c}$	3.231 ± 0.061^{c}	2.120 ± 0.059	2.154 ± 0.069	2.265 ± 0.061	2.170 ± 0.024
∑ HUFA	1.054 ± 0.011^{a}	1.617 ± 0.016^{b}	1.695 ± 0.025^{b}	1.960 ± 0.066^{c}	1.212 ± 0.008^{a}	1.219 ± 0.008^{a}	1.269 ± 0.011^{b}	1.238 ± 0.013^{a}
n-3/n-6	4.754 ± 0.005^{a}	5.778 ± 0.040^{b}	5.949 ± 0.075^{b}	6.376 ± 0.094^{c}	3.320 ± 0.034	3.340 ± 0.047	3.274 ± 0.020	3.248 ± 0.046
DHA/EPA	0.438 ± 0.006^{a}	2.207 ± 0.036^{b}	2.434 ± 0.029^{c}	2.843 ± 0.085^{d}	0.895 ± 0.017^{a}	1.022 ± 0.007^{b}	1.061 ± 0.019^{b}	1.059 ± 0.014^{b}
∑ TFA	6.031 ± 0.058^{a}	6.093 ± 0.105^{a}	$6.244 \pm 0.145^{a,b}$	6.514 ± 0.124^{b}	4.455 ± 0.137	4.524 ± 0.164	4.785 ± 0.127	4.586 ± 0.053

Data are presented as means ± standard error. Significant differences among the groups within the same stage are marked by different superscripted letters ($P < 0.05$). SFA, MUFA, PUFA, and HUFA are abbreviations for saturated, monounsaturated, polyunsaturated, highly unsaturated and total fatty acids, respectively.

DISCUSSION

Previously, dietary supplementation of AL through *Artemia* enrichment had been studied to promote growth performance of *P. vannamei* post-larvae from PL1 to PL19 (Visudtiphole et al., 2018). In the present study, AL supplementation was conducted in *P. monodon* larvae from M1 to PL2 by direct suspension of the AL cells into the larviculture. This approach is more efficient than the enrichment technique because all fatty acids in AL are taken in directly by the shrimp and not retroconverted by the enriched *Artemia* (Navarro et al., 1999; Han et al., 2001; Visudtiphole et al., 2018). The supplementation resulted in accumulation of the key fatty acids present in AL i.e., 16:0, DPA, and DHA, in PL2 shrimp. No effect on the growth of larvae was observed during the supplementation period; however, the effect on growth was exhibited later in the post-supplementation period from PL2 to PL12. Average body weight and biomass were increased in PL12 shrimp that had been fed earlier with higher amounts of AL. Dietary supplementation using other high-n-3 LC-PUFA substances, such as fish oil, was also shown to enhance growth performance of shrimp at the PL stage (Leger et al., 1985; Immanuel et al., 2001). Taken together with the previous results in *P. vannamei* PL and from the other studies, dietary n-3 LC-PUFAs may be more important for growth of shrimp during PL than the larval stage. This result could be ascribed for two reasons. First, the supplementation period during the mysis stage might not be long enough for difference in growth to be exhibited. Second, development of penaeid mysis is secondary lecithotrophy, in which the larvae partially rely on the nutrients from maternal reserves in eggs and also from the food (Mourente et al., 1995; Palacios et al., 2001). The supplemented LC-PUFAs were thus accumulated during the mysis stage and subsequently used for growth during the later PL stage, when AL was no longer supplied. This is also supported by the indifferent levels of most LC-PUFAs in PL12 shrimp.

The fatty acid profile of the aquatic animal normally reflects that of the ingested diet (Glencross, 2009; Kumar et al., 2018a). Likewise, fatty acid profiles of the PL shrimp in this study reflected those of the supplied diet, both at PL2 in which marked differences in fatty acid profiles were observed among groups with different feeding regimens, and later at PL12 when fatty acid profiles became more similar after all groups were later fed with the same diet. In contrast to the fatty acid profile, lipid class and subclass profiles of the PL shrimp and feed showed weaker correspondence. This could be accounted for digestion of macro-lipid molecules in the digestive tract and subsequent modification of lipid molecules in the animal. Macro-lipid molecules, especially triacylglycerols, have to be digested to smaller-sized molecules before being absorbed through the

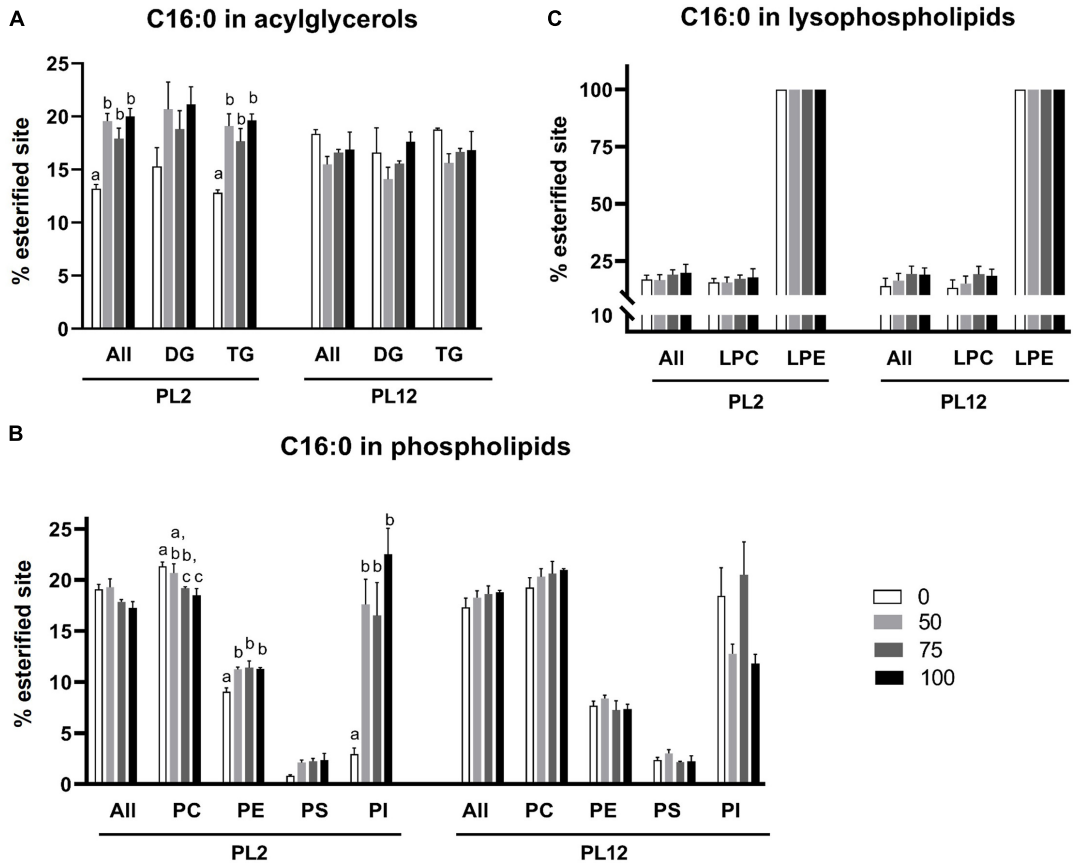

FIGURE 5 | Analysis of the 16:0 fatty acid distribution in lipid classes and subclasses by LC-HRMS. Percent of meal replacement was the percent of meals for which TW was replaced by AL. Percent esterified site is the percent of sites esterified with 16:0 compared with all the sites esterified with any types of fatty acids on the backbone. Error bars represent standard errors ($N = 3$). Statistical analyses of the PL2 and PL12 data sets were performed separately, using ANOVA. Significant differences among subsets of treatment groups were determined by Duncan's multiple range test ($P < 0.05$); subsets with means not significantly different from one another are identified with the same letter above the bar. Bars with no specified letters mean no significant differences were identified among the groups ($P > 0.05$).

intestine and subsequently repacked and modified to various lipid class products (Senior, 1964; Iqbal and Hussain, 2009).

Interestingly, increased ingestion of TG present in AL was associated with a lower TG level in PL2 shrimp. This result is consistent with a study on dietary LC-PUFA supplements for juvenile grass carp, in which reduced TG in the supplemented animals was explained by down-regulated expression of *diacylglycerol O-acyltransferase*, encoding the key enzyme that synthesizes TG from DG (Tian et al., 2017). Therefore, the downregulation of conversion from DG to TG might be the reason for the decrease of TG and the accumulation of DG in PL2 shrimp supplemented with the high DHA-containing AL in this study. It is known that DG acts as a second messenger for cell signaling by increasing the affinity of protein kinase C to bind with substrates; DG is thus involved in the signal transduction of several pathways related to immunity, cell activation, regulation, and function (Berg et al., 2002b; Singh and Kambayashi, 2016).

Analysis of the fatty acid distribution in lipid classes and subclasses showed differential accumulation of two distinct types of fatty acids present in AL i.e., SFA (16:0) and n-3 LC-PUFAs (DPA and DHA), among lipid classes of the shrimp. Increased 16:0 levels in the diet were associated with a greater percent of sites esterified with the fatty acid on acylglycerols but not phospholipids and lysophospholipids, consistent with the role of 16:0 fatty acid as a reserve for energy and substrate for the synthesis of other fatty acids (Caers et al., 2000b; Rey et al., 2018). In contrast, both of the LC-PUFAs accumulated on phospholipids and acylglycerols. The percent of DHA- and DPA-esterified sites on phospholipids in PL2 shrimp of the AL supplemented groups were higher than the percent of those of the control group. However, they did not increase further with more AL in the diet (**Figures 6B**, **7B**) whilst the percent of those on acylglycerols increased along with the amount of AL meal replacement (**Figures 6A**, **7A**). This suggests that DHA and DPA were prioritized to be incorporated in phospholipids, which are the main component of cell membrane (Tian et al., 2017; Rey et al., 2018). Nonetheless, if phospholipids with excess LC-PUFA esterified sites are present, they cannot be packed easily into the membrane bilayer structure and may disrupt the membrane's integrity owing to too much fluidity (Chapelle et al., 1982; Rajamoorthi et al., 2005; Bradbury, 2011; Parisi et al., 2019). The

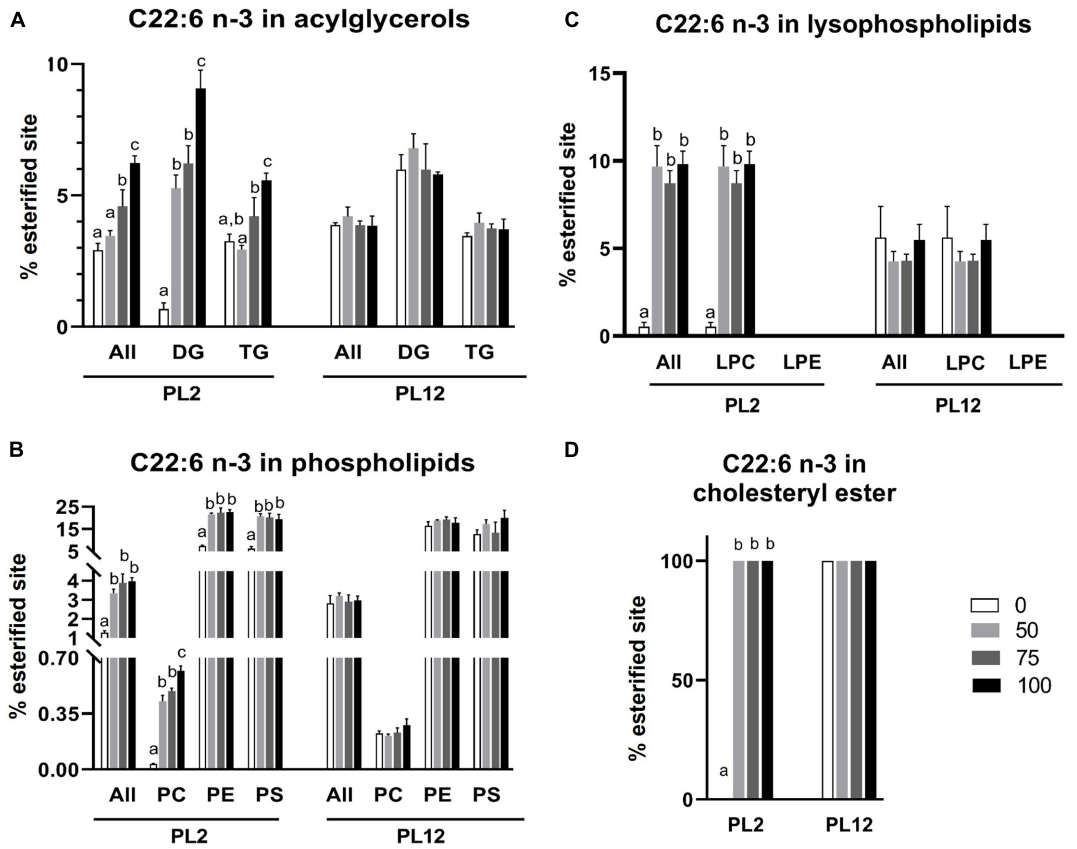

FIGURE 6 | Analysis of the 22:6 n-3 fatty acid distribution in lipid classes and subclasses by LC-HRMS. Percent of meal replacement was the percent of meals for which TW was replaced by AL. Percent esterified site is the percent of sites esterified with 16:0 compared with all the sites esterified with any types of fatty acids on the backbone. Error bars represent standard errors ($N = 3$). Statistical analyses of the PL2 and PL12 data sets were performed separately, using ANOVA. Significant differences among subsets of treatment groups were determined by Duncan's multiple range test ($P < 0.05$); subsets with means not significantly different from one another are identified with the same letter above the bar. Bars with no specified letters mean no significant differences were identified among the groups ($P > 0.05$).

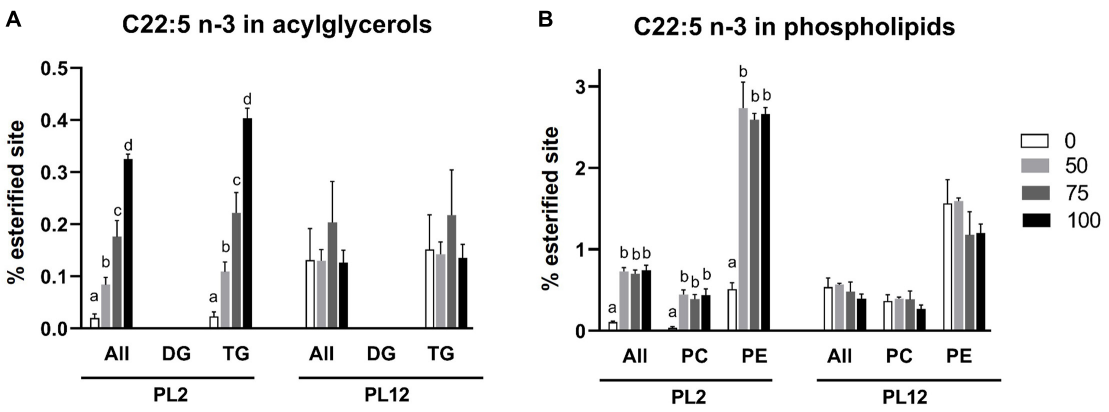

FIGURE 7 | Analysis of the 22:5 n-3 fatty acid distribution in lipid classes and subclasses by LC-HRMS. Percent of meal replacement was the percent of meals for which TW was replaced by AL. Percent esterified site is the percent of sites esterified with 16:0 compared with all the sites esterified with any types of fatty acids on the backbone. Error bars represent standard errors ($N = 3$). Statistical analyses of the PL2 and PL12 data sets were performed separately, using ANOVA. Significant differences among subsets of treatment groups were determined by Duncan's multiple range test ($P < 0.05$); subsets with means not significantly different from one another are identified with the same letter above the bar. Bars with no specified letters mean no significant differences were identified among the groups ($P > 0.05$).

integration of simpler fatty acids into the membrane structure is also required to maintain the membrane's stability (Huang et al., 2019). From our data, it can be inferred that dietary DHA and DPA were first preferentially deposited in the membrane and the excess was then stored with acylglycerols since the percent of sites esterified with these fatty acids continuously increased with the higher amounts of supplied AL. This result was in agreement with those in *Crassostea gigas* spat and juvenile *Mytilus edulis* fed with high LC-PUFA diets (Caers et al., 2000b; Laudicella et al., 2020). In contrast to that of the LC-PUFAs, deposition of 16:0 fatty acid in acylglycerols did not increase further with the higher amounts of supplied AL (**Figure 5A**). This result additionally suggests that LC-PUFAs were prioritized to be reserved over SFA when both types of fatty acids were present in high amounts in the diet. The preferential accumulation of LC-PUFAs over other types of fatty acids was also observed in other marine larvae (Caers et al., 1999, 2000b). The importance of LC-PUFAs is also corroborated by their selective retention in animals that were starved or fed with DHA-deficient diet (Coutteau et al., 1996; Knauer and Southgate, 1997).

The 16:0 fatty acid was markedly more abundant on PC than DHA or DPA (**Figures 5B**, **6B**, **7B**). In contrast, DHA was the most common modification of PE and PS. Moreover, unlike the 16:0 fatty acid, both n-3 LC-PUFAs were not detected to associate with PI, which is a minor component on the cytosolic side of cell membrane. Instead, the percent of 16:0-esterified sites on PI were significantly greater in PL2 shrimp fed with AL (**Figure 5B**). These results are in line with those in other species. Namely, DHA is more abundant on PE and PS than on PC and PI, and DHA supplementation resulted in the highest DHA accumulation on PE (Gillis and Ballantyne, 1999; Axelsen and Murphy, 2010; Rey et al., 2015; Bascoul-Colombo et al., 2016; Huang et al., 2019). In fact, PE is regarded as the most highly unsaturated phospholipid in marine animals (Ronnestad et al., 1995; Huang et al., 2019). Furthermore, PI is more specific to LC-PUFAs with a Δ5 double bond, like ARA and EPA, than those with a Δ3 double bond, which are DHA and DPA (Bascoul-Colombo et al., 2016). This also agrees with our result, in which the only LC-PUFAs detected to associate with PI were ARA and EPA (see **Supplementary Table 2**). Additionally, the high content of 16:0 fatty acid in PC coincides with a role of PC as a secondary energy source when TG is nearly depleted (Sasaki et al., 1986).

Cholesteryl ester was detected only in PL2 shrimp supplemented with AL and associated only with DHA (**Figure 6D**). Esterification of cholesterol with long-chain fatty acids makes cholesterol become more hydrophobic, which can be packed more easily inside the core of lipoproteins for the transportation in plasma (Maranhão and Freitas, 2014). AL supplementation in PL2 shrimp resulted in higher levels of cholesterol and the presence of cholesteryl ester (**Figure 4**). The presence of the latter indicates cholesterol transport between organs in shrimp supplemented with AL since cholesterol is transported in plasma as cholesteryl esters. Cholesterol is the precursor of steroid hormones that activate molting and growth such as ecdysteroids, which are synthesized specifically in the Y-organ of shrimp (Huberman, 2000; Kumar et al., 2018b). This suggests an additional role of DHA as a chaperone for cholesterol transportation in plasma to the target organ for the steroid hormone synthesis.

CONCLUSION

In conclusion, supplementation of AL as a source of dietary n-3 LC-PUFAs was demonstrated for the first time in shrimp larvae during the mysis developmental stage. The growth results from this and other previous studies suggests that dietary n-3 LC-PUFAs are more important and beneficial to PL shrimp than to the larvae at earlier stages. Analysis of differential accumulation of different fatty acid types provides an additional evidence on the importance and biological roles of LC-PUFAs in the larval diet.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

VV: experimental design and investigation, data analysis, and manuscript preparation. JK, PC, JS, and SL: shrimp rearing and feeding experiments. SP, AK, TR, KS, LK, and NR: laboratorial experiments. PU: experimental design and investigation. UU: experimental design and investigation, manuscript preparation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.771929/full#supplementary-material

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