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A Review on n-3 HUFA and Live Food Organism for Marine Fish Larvae Nutrition

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ABSTRACT

Marine fish farm industries face ongoing challenges due to a lack of quality seed, a low survival rate and a slow growth rate of marine fish larvae. One of the most sensitive problems is a nutritionally balanced quality feed for rearing these larval fish at the first feeding stage. Many studies have reported high requirements of n-3 highly unsaturated fatty acids, mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) for proper development, which have also been reported to increase the survival and growth status of larval fish. Marine fish larvae have difficulties accepting artificial feed at their weaning stage, so live food plays a vital role in the rearing process. *Artemia* is one of the most commonly used live food organisms in marine fish larvae production systems. However, they are deficient in EPA and DHA, which are most critical for larval development. Recent advancements in live food production systems have developed several techniques of bio-encapsulation and enrichment of nutrients in live food. But the instability of DHA and the high cost of enrichment procedures remain-bottlenecks for supplying proper nutrients through live food. This short review emphasizes challenges in marine fish larvae culture in terms of HUFAs nutrition with a comparative study on DHA requirements of marine larval fish and its availability in live food organism *Artemia*. We also highlighted several factors affecting DHA enrichment process and its degradation following enrichment procedures.

INTRODUCTION

Thanks to the impressive growth of world aquaculture, while capture fisheries have remained stagnant since the 1980s, culture fisheries have outpaced world population growth with an average 5.6% (by volume) annual growth rate (FAO, 2022). At present, global fish and shellfish production is about 178 million tonnes (2022), of which 90 million tons coming from capture fisheries that have remained exceptionally stable since last decades due to overfishing (Figure 1). Nevertheless, aquaculture shares a great proportion of world fish production with an increasing trend of production estimated 88 million tons (value USD 265 billion) contribution, which covered 49% of global fish production in 2020 (FAO, 2022). Finfish account for two-third of aquaculture production that is 57.5 million tons (valued USD 146.1 billion, 2022).

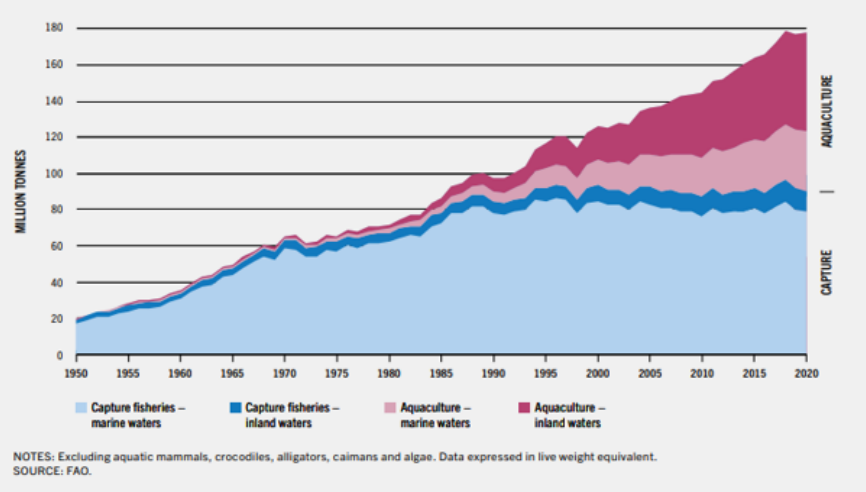

Figure 1: Trend in world fisheries and aquaculture production (adapted from FAO, 2022)

A big part of aquaculture production is coming from freshwater fish, constituting 49.1 million tonnes (valued USD 109.8 billion), whereas, marine fish culture is lagging with 8.3 million tonnes (USD 36.2 billion). Similarly, mollusk and crustacean contribute about 17.7 (USD 29.8 billion) and 11.2 million tonnes (USD 81.5 billion) respectively. All most all the aquaculture product are used for human consumption except the non-food use of it's by-product. Annual per capita fish consumption has been reached beyond 20.2 kg by 2020 increasing faster than the world population growth rate (Figure 2; FAO, 2020c). Aquaculture thus plays a significant role in world food security.

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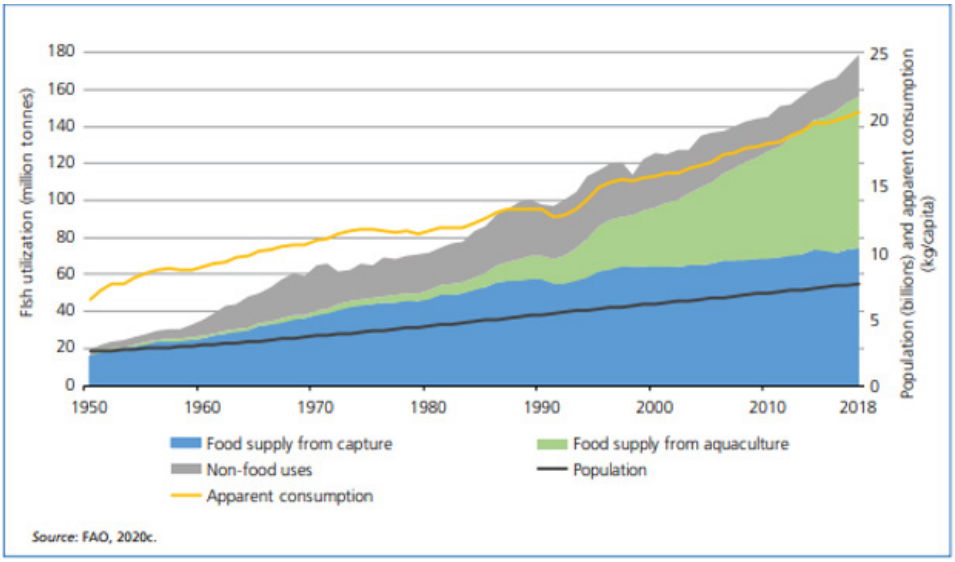

Figure 2: World fish utilization and apparent per capita consumption, 1950–2018 (adapted from FAO, 2020c)

Marine fisheries contribute 8.3 million tons of mariculture product and 78.8 million tons of capture fisheries to world marine production (112 million tons). Marine fish production reached a peak in 1996 (86.4 million tons) followed by 84.5 million tons in 2018, which declined by 1.6% in 2020, severely impacted by the COVID-19 pandemic. The world's marine fish stocks have decreased from 90% in 1974 to 68.6% in 2013, of which fully fished stocks accounted for 58.1% (31.4 % fished at a biologically unsustainable level) and 10.5 % under-fished stocks. Formulation of fish meal and oils from non-commercial fish leads to overfishing of many marine species. An estimation of 18 million tonnes of fish are processed annually for fish feed production, mainly small oily pelagic fishes that are rapidly declining in the wild. Marine finfish production contributes 13% of total aquaculture production but occupies 27 % of total value (FAO, 2020). The high inclusion rate of fish meal and oils, the requirement of high- priced live feed in an early stage, and high production costs elevate the price of marine fish. However, the major bottleneck of marine fish culture includes high mortality of larval fish and the poor quality of hatchery produced larvae compared to wild fish larvae. Marine fish larvae are very sensitive to biotic and abiotic factors at their early developmental stage and a lack of appropriate first feeding protocols makes marine larvae culture very challenging.

Among several factors, the nutritional quality of starter feed, especially n-3 highly unsaturated fatty acids (HUFA) such as 20:5n-3 (eicosapentaenoic acid), 22:6n-3 (docosahexaenoic acid) and 20:4n-6 (arachidonic acid) plays a significant role in the physiological development of marine larvae (Watanabe, 1982; Figueiredo *et al.*, 2012). Moreover, DHA (22:6n-3) is a prime concern during the first feeding stage among other n-3 HUFAs due to its superior role in larval growth and development. Marine fish larvae cannot *de novo synthesize* long chain n-3 HUFAs thus DHA must be supplied in their very first diet (Buzzi *et al.*, 1996; Figueiredo *et al.*, 2012; Tocher, 2015).

Marine fish larvae faces difficulties to intake artificial feed at their weaning period, therefore, live food organisms such as copepods, *Artemia*, Rotifers etc. supplied to first feeding larvae to facilitate their digestion and development. However, *Artemia* won the race among live food organisms due to its well-established mass production techniques and ease of management. Nonetheless, it is deficient of DHA, and a negligible amount of EPA content is a major concern as a proper diet for marine fish larvae (Copeman *et al.*, 2002; Hawkyard *et al.*, (2016). Therefore, several nutrient enrichment protocols have been developed to modulate nutrient content in these live food organisms (Fehér *et al.*, 2013). However, DHA is very unstable in *Artemia* after the enrichment process (Triantaphyllidis *et al.*, 1995). A complete knowledge of ontogeny, feeding physiology, nutritional requirements at different stages of larvae, nutritional modification of live food organisms is required for proper development, growth and survival of marine fish larvae (Jafari *et al.*, 2011). This review includes an insight on fact of n-3 HUFA specifically DHA for marine fish larvae and its availability in live food organism *Artemia*.

Challenges in Mrine Fish Larviculture

The commencement of marine fish culture was based on the wild capture of fish fry. Later on, hatcheries were constructed in many countries with the purpose of developing fertilized eggs, embryos, and larvae for restocking the sea (Stottrup and Mcevoy, 2008). By 1890, Britain, the USA, Canada, and France had developed hatcheries for the propagation of commercial fish species such as cod (*Gadas moruha*), haddock *(Melanogrammus aeglefinus),* flounder (*Pleuronectes americanus*) and lobster (Homarus sp.), where larvae were maintained till the pro-larval stage and distributed for ocean restocking. No convenient food was proposed to maintain post larval survival and growth. Therefore, restocking of the ocean with such an early-life stage caused high mortality of larvae in nature. Development of production technology

has expanded hatcheries on a commercial scale. The progress in larviculture techniques has moved from extensive/semi-intensive to intensive and super-intensive rearing methods with high-density stocking such as 50–150 larvae/L for some fishes (Shields, 2001).

More than ten millions of fries for a commercial species per year in an industrial hatchery. But the mortality levels between hatchery reared and wild fish larvae are highly variable, such as around 5x109 hatchery produced salmon larvae released annually in wild but only 5 % survival to adulthood (Brown and Laland, 2001). Large quantities of larvae are produced but the survival and growth rate of larvae is often low or highly variable with quality problems, such as skeletal deformities. Only 4 % of wild caught larvae shows body deformities, whereas values are very high for hatchery-produced larvae (Cahu *et al*., 2003).

Sub-optimal nutrition level, difficulties in acceptance of inert microdiets, and lack of knowledge on specific nutritional requirements are the major causes of low quality and high mortality of marine larvae (Conceicao *et al*., 2010).

Marine fish larvae undergo major functional and morphological changes during developmental stages. After exhaustion of yolk sac, nutrients and energy required for the developmental changes depends on ingested food. The exogenous feeding of fish larvae depends on structural development of the organ for food uptake, appropriate quality of food, digestion and assimilation success of ingested food. Massive mortality in the first few days is mostly related to nutritional deficiency due to food deprivation and inappropriate food quality. This period is known as the "critical period" for marine fish larvae (Yufera and Darias, 2007).

Survival varies depending on fish species and it falls sharply if all the feeding requirements are not met properly. The development of anatomical features in fish larvae are directly correlated to their exogenous feeding. For instance, the organs for prey localization (eyes and chemosensory organs), capture (mouth, tail, and muscles), ingestion, and digestion (digestive system) develop first. Marine fish larvae are hatched with a rudimentary digestive system, without a mouth and unpigmented eyes. Visual organ eye becomes pigmented during mouth opening (Yufera and Darias, 2007).

However, marine fish are well known as visual feeders which are mostly confined to daylight period for detection and catching prey. The newly hatched larvae possess a very small mouth, an undifferentiated digestive tract, no stomach but a functional liver and pancreas (Dabrowski and Glogowski, 1977). Without a stomach, digestion of ingested food takes place in the intestine with alkaline pH, very low proteolytic activity, and without any amino peptidase or tyrosinase activity. Food ingestion at the beginning mostly depends on size of prey and mouth gape of fish larvae. Therefore, intensive research on the early incorporation of formulated diet to feed marine fish larvae has achieved a very little success due to the poor digestion capacity of fish, lack of digestive enzymes, and poor assimilation capacity (Infante *et al*., 2001). However, Several weaning strategies have been developed to supply exogenous enzymes to fish larvae through live feed (Kolkovski *et al*., 2001). In nature, a huge variety of small zooplankton organisms, especially copepods, believed to fulfill their nutritional demands by supplying proper nutrients and exogenous enzymes necessary for digestion.

Live feed for marine fish larvae

Live feeds are easily detectable by fish larvae due to their swimming movements in the water column. Hiowever, formulated diets tend to aggregate on the water surface or sink quickly, becoming less available to fish. Almost all marine fish species rely on live foods during their early stages. Live food organisms supply various pancreatic and intestinal enzymes which help to digest food. The maturational process of the digestive tract of larval fish delayed when they are fed to a formulated diet with a low nutritional profile (Infante and Cahu, 2001). Dabrowski and Glogowski (1977) estimated 40-80 % of the enzymatic activities (mainly the proteolytic activity) in fish larvae are donated by the live food organisms such as rotifers, molluscs, and *Artemia* (Table 1). Weaning of marine fish larvae to dry feed had been practiced as co-feeding with live food organism (Kolkovski, 2001). Live food organism contributes digestive enzymes which increase digestive activity to facilitate digestion process untill the larval alimentary system fully differentiated and developed (Kolkovski, 2001). Dabrowski and Glogowski (1977) tested enzymatic activity of different fish species such as common carp, *salmon Samol gairdneri*, whitefish *Coregonus larvaretus* and grass carp *Ctenopharyngodon idella* and reported that 40-80% of the enzymatic activity in marine larval fish derived from live food organism (Table 1).

In nature, a wide variety of phytoplankton and zooplankton shape the diet composition of fish larvae. Microalgae are used as a direct food source for fish, such as in green water technique, or as an indirect food source in the production of live food organisms such as rotifers, *Artemia* or copepods (Makridis *et al*., 2010). Microalgae rich in n-3 HUFA (*Thraustochytrid, Schizochytrium sp*., *Isochrysis galbana*, etc.) are fed to zooplankton to boost their nutritional composition, which in turn benefits fish larvae in subsequent feeding (Barclay and Zeller, 1996; Makridis *et al*., 2010). Incorporation of microalgae production facilities into fish farms represents 30% of a hatchery's operating cost with a variation in productivity (Coutteau and Sorgeloos, 1992).

Most commonly used zooplanktons in aquaculture are *Artemia* (200-500 µm), rotifers (*Brachionus plicatilis*; 50-200 µm) and Copepod (*Tigriopus sp*., *Acartia sp*., *Oithona sp*.). The rotifers, mainly *Brachionus plicatilis*, are an appropriate prey to start the first feeding of fish larvae right after the resorption of yolk reserves (Makridis *et al*., 2010). The smaller body size (between 70 and 350 µm) makes them suitable for small larvae, which being replaced afterward

Table 1: Contribution of digestive enzymes by live food organisms (Kolkovski, 2001)

Species	Live food organism	Contribution of digestive enzymes
Cyprinus carpio, Ctenopharyngodon idella, Salmo gairdneri, White fish *Coregonus larvaretus*	*Copepods, Cladocera*, Rotifers, *Artemia*	10-98% proteolytic activity from live food
Turbot *Scophtalmus maximus*	Rotifers, *Artemia*, *Copepods*	Exogenous digestive enzymes contribution: Protease 43-60%, esterase 89-94%; exonuclease 79-88%; amylase 15-27%
Herring *Clupea harengus*	*Copepods*	0.5% of total trypsin activity from live food
White fish Coregonus sp.	*Moina sp.*	70% trypsin activity derived from live food
Japanase sardine Sardinops melanoticus	Rotifers	0.6% of total protease activity derive from live food in larvae fish

by larger prey species, such as *Artemia* (Conceicao *et al.*, 2010).

Besides the size suitability, large scale production, high growth rate, ease of feeding, good tolerance, appropriate energy content, and reasonable nutritional value are important for commercial aquaculture application. Rotifers are nutritionally deficient in long-chain poly unsaturated fatty acids (LC-PUFA) which lowers their quality as first larval food. Thus, enrichment with microalgae, oil emulsion or commercial products to modify essential fatty acid (EFA) content are practiced with rotifer culture (Watanabe *et al.*, 1984)

In nature, copepods are the dominant food for most species of fish larvae and are used in hatchery production of fish larvae. Copepods are reported to improve the survival, growth, and quality of marine fish larvae compared to rotifers and *Artemia* (Shields, 2001; Stottrup and Mcevoy, 2008; Conceicao *et al.*, 2010; Makridis *et al.*, 2010). Most commonly used copepod groups for marine larvae are Calanoida, Harpacticoida and Cyclopoida e.g., *Acartia sp.*, *Eurytemora affinis*, *Centropages hamatus and Gladioferens imparipes*, *Tisbe sp.*, Euterpina acutifrons, Tigriopus japonicus and Nitokra sp. Copepods contain a high level of free amino acids and protein (4.3 %-8.9 %, and 32.7-53.6 % respectively), moderate lipids (6.9-22.5 % of DW) and low amounts of 20:4n-6 (0-2.6 %). The 20:5n-3 (8.3-24.6 % of total lipid) and 22:6n-3 (13.9-42.3 %) contents in copepods are approximately 0.8-fold higher than enriched rotifer and 0.3-fold of enriched *Artemia* (Table 2). The ratios of 20:5n-3/20:4n-6 above 20 and 22:6n-3/20:5n-3 above 2 are crucial for marine fish larvae, which are very difficult to achieve in rotifers and *Artemia* (Sargent *et al.*, 1999; Makridis *et al.*, 2010). Some extensive and intensive production of copepods has been practiced, but mass production under a cost-effective method is a major limitation for commercial aquaculture application. Copepods are mostly collected from the wild population for aquaculture use (Makridis *et al.*, 2010). However, a great practical problem of using *Artemia* and rotifers for marine fish larvae is their inadequate essential fatty acids profile, mainly 20:5n-3 and 22:6n-3. Ballan wrasse (*Labrus bergylta ascanius*) fed with copepod showed better growth performance compared to *Artemia* and rotifer diets, and the diet transition from rotifer to *Artemia* showed better performance (Almli, 2012).

A similar performance was also found for Cod (Koedijk *et al.*, 2010). In fact, superior pigmentation, survival, and retinal morphology have been documented in halibut larvae fed to copepods other than *Artemia* (Shields, 2001; Evjemo *et al.*, 2003). Recent studies on marine fish nutrition have emphasized on manipulation of fatty acid in the live feed, especially for first feeding larvae. The Copepod fatty acid profile is often used as a reference diet of marine fish larvae to enrich *Artemia* and rotifers (Meeren *et al.*, 2008).

Table 2: Average biochemical composition of copepod nauplii, copepods, enriched rotifers and enriched *Artemia* (Makridis *et al.*, 2010)

Parameters	Copepods nauplii	Rotifers	Copepods	*Artemia* M24
Dry weight (DW; µg/individual)	0.6	0.6	8.8	2.1
Protein (% DW)	30.03	25.0	43.0	29.0
FAA (% DW)	8.6	1.7	6.0	3.1
Total lipids (% DW)	8.6	15.0	11.0	25.0
Polar lipids (% DW)	5.4	6.1	6.2	4.5
DHA (% DW)	3.5	1.9	3.5	2.7
EPA (% DW)	1.4	1.1	1.8	1.9

DHA/EPA (% DW)	2.8	1.7	2.2	1.4
EPA/ARA (% DW)	27.7	3.7	24.0	4.0
Vitamin E (µg /g DW)	-	513	113.0	456.0
Vitamin C (µg /g DW)	-	220	515.0	446.0
Vitamin B1 (µg /g DW)	-	49	23.0	16.0
Iodine (µg /g DW)	-	4.7	121.0	1.1
Selenium (µg /g DW)	-	0.08	4.0	-

***Artemia*: Characteristic, Biology and Production**

The brine shrimp, *Artemia*, is a well-established live feed for marine fish and shrimp larvae culture in the aquaculture industry. It is an Anostracan branchiopod crustacean belonging to the phylum- Arthropoda and class-Crustacea, found worldwide in hypersaline inland salt lakes, ponds, and coastal lagoons. They can survive in a wide range of environments with salinity fluctuations approximately 10 to 340 g/L and a temperature regime of 6-35°C (Reis *et al*., 2017) and a wide variation of ionic composition (Persoone *et al*., 1980).

In addition, cyst production capacity helps with an easy adaptation to the harsh and unstable environment. The properly processed diapause cysts can be stored for several years and can easily terminate with environmental cues for hatching (Lavens and Sorgeloos, 1987). Several hundred species found to have bisexual or parthenogenetic populations produce either cysts and/or nauplii and switch over from one to the other mode depending on surrounding environmental changes. The new world bi-sexual populations have higher fecundity, a large number of offspring per brood, early sexual maturity, and tend to be dominant compared to the old world bi-sexual parthenogenetic population (Van Stappen, 2002).

No active dispersion, but vector insisted and manmade deliberate dispersion of cysts introduced them in different geographical areas. Cysts are metabolically inactive, activated by immersion into seawater in optimum condition to burst the outer membrane of the cyst. The emerging smaller size instar-I nauplius is used to feed first-feeding fish and shrimp larvae following a specific enrichment procedure to enhance its nutritional properties (Van Stappen *et al*., 2003). The filter feeding behavior of *Artemia* is used in the bio-encapsulation of *Artemia* with nutrients, therapeutics, antibiotics, etc. (Figure 3, right). The life cycle consists of cyst/egg, nauplii, metanauplii, and adults which take a generation time of nearly 14-28 days (Figure 3, left).

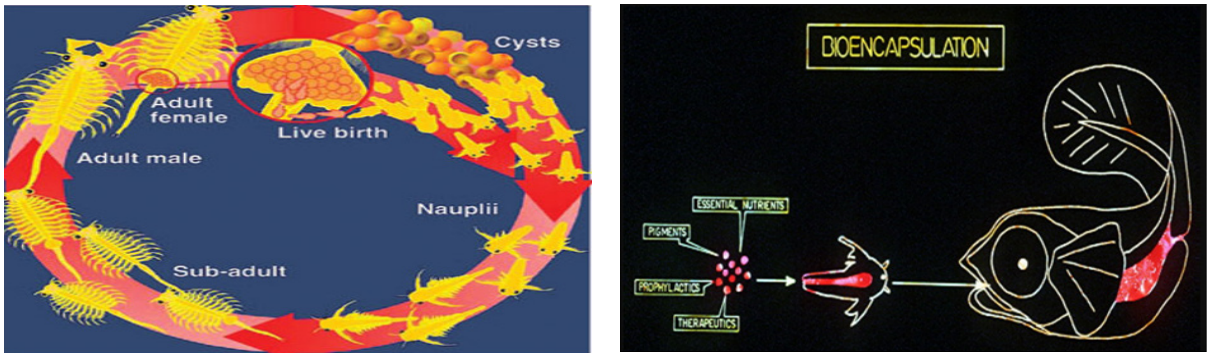

Figure 3: Life cycle of *Artemia* (left) and encapsulation of different nutrient components in *Artemia* (right) (Source: https://wildlife.utah.gov/gsl/brineshrimp/life_cycle.php)

Many research is ongoing on the partial replacement of *Artemia* by artificial diets, but little success has been achieved in feeding marine fish larvae (Dhont and Stappen, 2003). It occupies the high demand as an indispensable live food item in most of marine finfish and shellfish hatchery despite the consequence of its high cost and low 22:6n-3 content. The ease of mass production, availability of dormant cysts (remarkable 'shelf- life'), bio-encapsulation, and simplicity of hatching make it the most convenient live food available for aquaculture (Lavens and Sorgeloos 2000).

Artemia has been offered to a wide range of valuable organisms including fish, crustaceans, squid, polychaetes, insects, chaetognaths, decapod larvae, coelenterates, etc.

The demand for *Artemia* cysts is around 2000 metric per annum (Dhont and Sorgeloos, 2002; Dhont and Stappen, 2003) which is 40% of the total aquaculture feed demand in larvae culture. Since 1958, larvae of Penaeus japonicus successfully has been reared with *Artemia* nauplii, and till present, 80-85 % of total sale of *Artemia* cyst goes for shrimp hatcheries.

The remaining proportion of produced *Artemia* cyst being sold for feeding marine fish larvae. Currently, the most important source of *Artemia* cysts for aquaculture production is Great Salt Lake, Utah, which covers about 35-40% harvest of the international *Artemia* cyst market (Stappen, 2002). The total raw biomass converted into 2000-3000 tons of dried *Artemia* cysts annually. However, a very low harvest of *Artemia* was observed in years 1980s and 1990s and in 1988-1998. However, production increased steadily in years 2003-2004 with a fluctuation of price (Nielsen *et al*., 2017).

Nowadays, many commercial products of *Artemia* (relatively low quantity) are increasing in the market from different locations such as North and Central China, Southern Siberia, Lake Urmia in Iran, salt lakes in Argentina, San Francisco Bay, Southern Vietnam, Colombia and Northeastern Brazil.

Fatty Acids

Fatty acids are the main component of bio-membrane contain a chain of nonpolar hydrocarbon bond that terminates with the polar carboxylic group, confers the molecule with a polar, hydrophilic end, and a nonpolar, hydrophobic end, which become insoluble in water. They are grouped as saturated and unsaturated fat, usually derived from triglycerides or phospholipids. The metabolism of fatty acid produces large quantities of ATP, however, denoted as biofuel supplier. Saturated fatty acids have no double bond (single bonds -C-C-) between carbon atoms, such as Caprylic acid, Lauric Acid, Myristic acid etc. Most of the animal fats are saturated. Conversely, more than one double bond between carbon atoms found in unsaturated fatty acids, are the important component of bio-membrane, generally the plants and fish fats, such as 20:5n-3 (eicosapentaenoic acid), 22:6n-3(docosapentaenoic acid) 20:4n-6 (arachidonic acid), 18:3n-3 (linolenic acid).

In unsaturated fatty acid, two hydrogen atoms stick out on the same side of the fatty acid chain gives cis-isomer configuration, and a trans configuration, by contrast, two hydrogen atoms lie on the opposite sides of the chain. Naturally occurring unsaturated fatty acids generally have the Cis-configuration (Table 5).

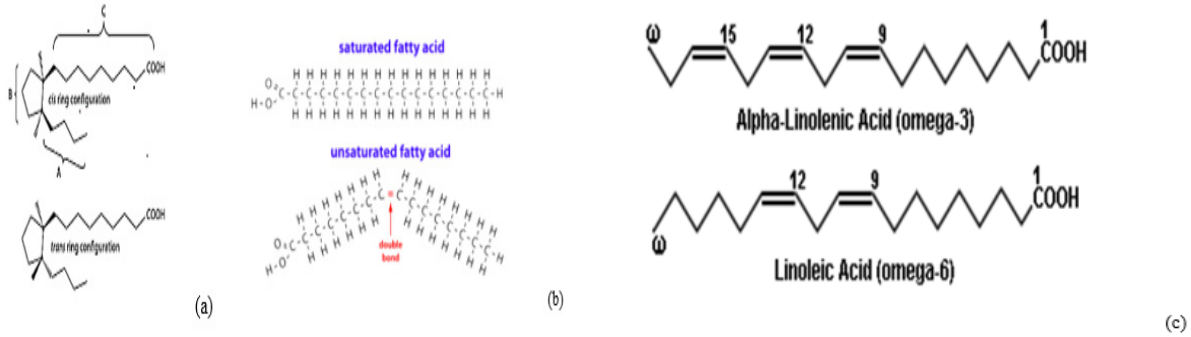

Figure 5: Trans-isomer and the cis-isomer configuration (a (Akoh, 2017); saturated and unsaturated fatty acid (b), omega 3 and omega 6 unsaturated fatty acids (c)
(*source: http://courses.washington.edu/conj/membrane/fattyacids.htm)*

Some fatty acid is synthesized chemically while other biologically produced in the cell cytoplasm of living animal through the lipogenic process with the action of fatty acid syntheses. Fatty acids containing phosphate group called phospholipid that makes up cell membrane and surround different cell organelles (mitochondria, nucleus, Golgi app20:4n-6tus etc.).

However, unsaturated fatty acids which cannot be synthesized by human or animal body but required for the maintenance of biological system and must be supplied with the diets, are denoted as essential fatty acids (22:6n-3, 20:5n-3, 20:4n-6). Unsaturated fatty acids having one double bond in the fatty acid chain are grouped as monounsaturated fatty acids denoted as Omega ω-5,7, 9, 10 based on the positions of the double bonds in a fatty acid chain. Poly unsaturated fatty acids (PUFAs) have more than one double bond (ω-3,6; Table 3 (a,b)). Fish and all vertebrates essentially require 20:5n-3, 22:6n-3 and 20:4n-6 for normal physiological functioning, the structural and functional integrity of cell membrane, the

Table 3 (a): A list of omega-6 (up) unsaturated fatty acids

Common name	Lipid name	Chemical name
Linoleic acid	18:2 (n-6)	*all-cis*-9,12-octadecadienoic acid
Gamma-linolenic acid (GLA)	18:3 (n-6)	*all-cis*-6,9,12-octadecatrienoic acid
Eicosadienoic acid	20:2 (n-6)	*all-cis*-11,14-eicosadienoic acid
Dihomo-gamma-linolenic acid (DGLA)	20:3 (n-6)	*all-cis*-8,11,14-eicosatrienoic acid
Arachidonic acid (AA)	20:4 (n-6)	*all-cis*-5,8,11,14-eicosatetraenoic acid
Docosadienoic acid	22:2 (n-6)	*all-cis*-13,16-docosadienoic acid
Adrenic acid	22:4 (n-6)	*all-cis*-7,10,13,16-docosatetraenoic acid
Docosapentaenoic acid (Osbond acid)	22:5 (n-6)	*all-cis*-4,7,10,13,16-docosapentaenoic acid
Tetracosatetraenoic acid	24:4 (n-6)	*all-cis*-9,12,15,18-tetracosatetraenoic acid
Tetracosapentaenoic acid	24:5 (n-6)	*all-cis*-6,9,12,15,18-tetracosapentaenoic acid

Table 3 (b): A list of omega-3 unsaturated fatty acids

Common name	Lipid name	Chemical name
Hexadecatrienoic acid (HTA)	16:3 (n-3)	*all-cis* 7,10,13-hexadecatrienoic acid
Alpha-linolenic acid (ALA)	18:3 (n-3)	*all-cis*-9,12,15-octadecatrienoic acid
Stearidonic acid (SDA)	18:4 (n-3)	*all-cis*-6,9,12,15,-octadecatetraenoic acid
Eicosatrienoic acid (ETE)	20:3 (n-3)	*all-cis*-11,14,17-eicosatrienoic acid
Eicosatetraenoic acid (ETA)	20:4 (n-3)	*all-cis*-8,11,14,17-eicosatetraenoic acid
Eicosapentaenoic acid (EPA, Timnodonic acid)	20:5 (n-3)	*all-cis*-5,8,11,14,17-eicosapentaenoic acid
Heneicosapentaenoic acid (HPA)	21:5 (n-3)	*all-cis*-6,9,12,15,18-heneicosapentaenoic acid
Docosapentaenoic acid (DPA, Clupanodonic acid)	22:5 (n-3)	*all-cis*-7,10,13,16,19-docosapentaenoic acid
Docosahexaenoic acid (DHA, Cervonic acid)	22:6 (n-3)	*all-cis*-4,7,10,13,16,19-docosahexaenoic acid
Tetracosapentaenoic acid	24:5 (n-3)	*all-cis*-9,12,15,18,21-tetracosapentaenoic acid
Tetracosahexaenoic acid (Nisinic acid)	24:6 (n-3)	*all-cis*-6,9,12,15,18,21-tetracosahexaenoic acid

precursor of eicosanoids, and reproduction (Sargent *et al.*, 1997).

The omega-3 and omega-6 PUFA with a carbon chain length of 20 or more while double bond 3 or more, are known as LC-PUFA or HUFA, mainly, ω-3/n-3, 20:5n-3, 22:6n-3 and ω-6/n-6 20:4n-6.

Requirements of n-3 HUFA in Marine Fish Larvae

Marine fish larvae have a low capability or unable to bio-convert 18 Carbon (C) fatty acids into 20-22C HUFA, neither bio-synthesize 22:6n-3 *de novo*, consequently the n-3 HUFA achieved most attention for their normal growth and survival. HUFA regulates properties of cell membrane and serve as precursors for hormone production in fish. Egg quality is an important factor for mass production of marine larvae with a better survival rate. Many authors reported decreased egg quality with low n-3 HUFA levels in broodstock diets, even though the effect also varies among different species (Watanabe *et al.*, 1984; Fernandez *et al.*, 1995). However, some authors have identified negative effects of excess amount of n-3 HUFA on egg quality (Fernandez *et al.*, 1995).

Determination of EFA requirement for marine fish larvae is difficult due to their poorly developed digestive system which deter the acceptance of micro-diets, therefore, necessity of live food increased in marine fish larvae nutrition (Zambonino Infante and Cahu 2001). The inadequacy of n-3HUFA in live feeds such as in rotifers and *Artemia* require further HUFA enrichment. Most often, the quantitative and semi-quantitative EFA requirements of fish larvae have been done with a combination of enriched live food and micro diets. Marine fish larvae requires higher n-3 HUFA than the juvenile and pre-adult fish and requirements also varies with the relative proportion of different HUFAs, such as 22:6n-3:/20:5n-3 ratio (Tocher, 2010).

However, the higher efficacy of 22:6n-3 than 20:5n-3 has been observed in fish larvae for their neural and visual development (Sargent *et al.*, 2003). On the other hand, dietary 20:4n-6 found to improves growth, survival and stress resistance in sea bream and Japanese flounder (Estevez *et al.*, 1997; Tocher, 2010). An increased 20:4n-6/20:5n-3 ratio with low n-3 HUFA has been reported for the malpigmentation problem in fish larvae. It is important to balance 22:6n-3/20:5n-3/20:4n-6 ratios in the larval diet (Hamre and Harboe 2008).

The high ratio of 22:6n-3/20:5n-3 reduces n-3 HUFA requirements in larval diet. Therefore, the EFA requirement often satisfied by 22:6n-3 than 20:5n-3. Nowadays, informations are available on qualitative and quantitative requirements of EFA for any new species of interest that provide a better chance of culture success.

Tocher (2010) The 22:6n-3 plays a major role in the development of the nervous system, brain, visual cells, vitality, stress resistance, and pigmentation of flat fish larval development. Bell *et al.* (1995) observed an impaired ability of turbot to capture prey at the low light intensity with a high mortality when fed to a diet containing low 22:6n-3/20:5n-3 ratio (0.07). However, eicosanoids are considered as essential elements of pigmentation in flatfish. Almost 100% normal pigmentation with 20:5n-3/20:4n-6 ratio 2.0; while 16–40% reduced pigmentation at a ratio of 0.2-0.4 was observed in Senegalese sole (Villalta *et al.* 2005). Furthermore, live food containing high level of 22:6n-3 through enrichment process supplied to marine flatfish larvae has been suggested to improve pigmentation, similar result reported for Japanese flounder, turbot and halibut fishes (Tocher, 2010).

Although, there are arguments on whether the malpigmentation related to the deficiency of 22:6n-3 or excess of 20:5n-3 (Sargent *et al.*, 1999). Manipulation of 22:6n-3 in the supplemented live food is almost impossible without simultaneously altering the level of 20:5n-3. Moreover, 20:5n-3 involved in producing eicosanoids (3-series prostanoids and 5-series leukotrienes) and

20:4n-6 is the major precursor of eicosanoids (2-series prostaglandins and 4-series leukotrienes) compete for each other for the enzymes during eicosanoid production (Tocher, 2010). Marine fish cannot convert C18 to C20 PUFA and 20:4n-6 is also an essential fatty acid in marine fish. Level of 20:5n-3 reduced by elevating the level of 22:6n-3 in the diet that alter the ratio of 20:5n-3/20:4n-6, and thereby causing competitive interactions in eicosanoid production. Thus, determining the exact ratio of 22:6n-3/20:5n-3 in fish diet remains difficult (Sargent *et al.*,1999). Moreover, HUFA can affect the skeletal deformities through peroxisome proliferator activated receptors (PPAR) that bind DNA as a heterodimer with the retinoid X receptor (RXR) that regulates genes involved in skeletal development during ontogenesis (Cahu *et al.*, 2003).

Gapasin and Duray (2001) reported a decrease of opercular deformities in milkfish *Chanos Chanos* which were fed to 22:6n-3 enriched live preys. However, the influence of dietary 22:6n-3 has been observed on behavioral character development in marine fish, such as the production of non-schooling fish at their schooling size of Yellowtail fish reared with a 22:6n-3 free diet due to the impaired development of their brain and nervous systems (Sargent *et al.*, 1999). Moreover, functional efficiency of HUFA can vary with its origins such as animal origin n-3HUFA showed better growth and survival performance of gilthead seabream (*Sparus aurata*) over the vegetal origin HUFA (Betancor *et al.*, 2012). The quantitative requirements of 22:6n-3/20:5n-3 ratio for different species need more intensive study as it varies considerably with different lipid source.

Poly unsaturated fatty acids (PUFA) in *Artemia*

Not by the protein quality or mineral composition, but the presence of EFA, especially 20:5n-3, 22:6n-3 and 20:4n-6 determine the food value of *Artemia* for marine fish (T. Watanabe *et al.*, 1980). Biochemical composition and quality of *Artemia* cyst often vary in different strains or even in different batch from same geographical origin with a general issue of high total lipid content and low content of LC-PUFA. Watanabe (1994) grouped *Artemia* as freshwater-type and marine-type. Freshwater-type Atemia contains 18:3n-3, 18:2n- 6 and some amount of 18:4n-3 as major PUFA but only traces amount of C20 PUFA that is suitable for freshwater fish. In contrast, marine-type *Artemia* are more suitable for marine fish; as these *Artemia* contains 20:5n-3 and 20:4n-6 as well as 18:3n-3, 18:2n-6 and 18: 4n-3 as major PUFA.

Nutritional composition and proper size of *Artemia* nauplii are important factors to be considered for feeding fish larvae. For instance, A. *tibetiana* (instar-I) nauplii is the highest size nauplii (length 667 um) among all parthenogenetic and bisexual species. It contains a very high 20:5n-3 (44 mg/g DW) content which is an important EFA for marine fish larvae (Stappen *et al.*, 2003) but it's greater size limits its application for first feeding larvae. Abatzopoulos *et al.* (2006) reported a lower level of EPA (1.8 to 7.2 mg/g), a higher level of linolenic acid (32.7 to 54.7 mg/g DW) and a trace amount of DHA in A. *urmiana* nauplii collected from Urmia lake, Iran. Similarly, Dhont and Sorgeloos (2002) reported that linolenic acid (LA) content in A. *urmiana* nauplii is higher than *Artemia* species from GSL, China, and Argentina (29-40 mg/g, 4-39 mg/g and 17 mg/g of DW respectively). *Artemia* strains from San Francisco Bay, Brazil, and China varies considerably in 20:5n-3 and less variation has been observed in *Artemia* from Canada and Utah (Leger *et al.*, 1986). Most of *Artemia* strains contain a high level of 18:3n-3, very few strains contain a moderate level of 20:5n-3 but almost all strains contain no or a trace amount of 22:6n-3. The deficiency of 20:5n-3 generally associated with an abundance of 18:3n-3 in *Artemia*. Ruiz *et al.* (2007) investigated fatty acid profiles in cysts of *Artemia* populations from Argentina. The author reported that the presence of high proportions of n-3HUFA in marine-type compared to freshwater-type cysts, DHA was generally absent, or present only in a trace amount in few samples.

Navarro *et al.* (1993) investigated fatty acid profile in a coastal and inland population of *Artemia* from Spain. This author also reported no correlation between the presence of PUFA and geographical location and a general pattern such as of 20:5n-3 was present in coastal populations and 18:3n-3 was high in inland population. It has been known that coexisting species sharing the same environment and food shows a different fatty acid profile in *Artemia*. Several authors have been suggested that the variation of HUFA content among cyst batches might relate to the composition of microalgae present in the habitat of their parental populations (Leger *et al.*, 1986; Ruiz *et al.*, 2007).

Biosynthesis of Long chain (LC) PUFA in Crustacean live Food Organism

The study of PUFA biosynthesis and metabolism in live food organism is an important issue in fish nutrition. Deep sea bacteria, these are responsible for LC-PUFA synthesis is found in the intestines of cold-water invertebrates, bivalves, and amphipods. The biosynthesis pathways of LC-PUFA varies among species. PUFA metabolism in crustacean organisms do not follow the same pattern and there is no direct correlation for PUFA synthesis in crustacean with their phylogenetics (classes/subclasses) or habitats (freshwater/marine; Monroig *et al.*, 2013). The desaturases and elongases have been demonstrated in harpacticoid copepods. But calanoid copepods or other zooplanktons *Dropanopus forcipatus* and *Euphausia superba* do not possess this ability of PUFA synthesis. Similarly, endogenous production of 22:6n-3 had been reported in freshwater cyclopoid, *Eucyclops serrulatus* when they were fed a diet free of 22:6n-3.

On the other hand, an increased long chain PUFA content was reported in branchiopods *Daphnia pulex* when they were exposed to the low temperature (Schlechtriem, Arts, and Zellmer 2006). Some ability to convert n-6 to n-3 fatty acid found in *Artemia* but not much study has been

done on its enzymatic system (Schauer and Simpson 1985). The molecular study of gene expression reported the presence of sequences with high homology to n-3 desaturases in copepod *Caligus rogercresseyi*. The delta-5 fatty acid desaturase and elongases found in copepod that is homologous to vertebrate Elovl2 (gb|ACO10776.1|) and Elovl4 (gb|ACO11542.1|) genes (Monroig, Tocher, and Navarro 2013). Fish obtain their PUFA requirement by trophic upgrading from the higher trophic level but invertebrate organisms are in between the primary producers and fish in aquatic ecosystems. However, information on biosynthesis process of LC-PUFA and metabolic activity in live food organism is very limited.

Enrichment of n-3 highly unsaturated fatty acids in *Artemia*

Artemia has been used intensively in marine fish culture due to its availability and ease of culture protocols rather than its nutritional value. *Artemia* does not possess the endogenous capacity to synthesize HUFA. The concentration of n-3 HUFA mainly, 20:5n-3 and 22:6n-3 determine the dietary value of *Artemia* which are essential nutrients for marine fish. Bio-encapsulation processes are introduced in live food culture to enhance their nutritional value prior to feeding fish.

Instar-I nauplii depend on yolk reserve, do not eat, and developed into instar-II after 7-8 hours of hatching. Instar-II nauplii are non-selective filter feeders which are used in nutrient enrichment. In enrichment process, Instar-II nauplii are collected in a tank containing neutralized seawater and hypochlorite medium under 25°C temperature in a density of 100-300 nauplii/ml. Enrichment product is added every 12 h at a dose of 0.3 g/L for a duration of 24 h. During enrichment, strong aeration is required to maintain dissolved oxygen in the tank. After 24 or 48 h, enriched nauplii are harvested, thoroughly rinsed and stored at <100°C temperature to reduce metabolism or immediately supplied to fish larvae to assure that the HUFAs are not metabolized during storage.

The n-3 HUFA levels 50–60 mg/g DW are obtained after 24 h enrichment with the emulsified concentrates. Shorter enrichment time is better to keep size appropriate for feeding small larval fish. Other fatty acids and vitamins content of *Artemia* also improved in the same way. Short-term enrichment includes high concentration 100–250 mg/L of n-3 HUFA rich diets for <8 h and long term enrichment performed with yeast, algae and lipid emulsifier (Coutteau and Sorgeloos, 1997).

Different enrichment procedures have been developed by British, Japanese, and Belgian researchers with different types of products such as selected microalgae and/or micro-encapsulated products, yeast and/or emulsified product, self-emulsifying concentrates and/or micro-particulate products (Leger *et al.*, 1986).

British techniques include an algal suspension mostly *Isochrysis galbana* rich in 22:6n-3 content, where nauplii are cultured for 24 h at a density of 10000 nauplii/L. Japanese techniques also include microalgae for culturing nauplii, later they adopted an omega-yeast (0.38 mg/ml) as a substitute of algae to pre feed *Artemia* before supplying to fish. Another technique of using emulsified fish oils in combination with a beaker yeast to pre feed nauplii, was developed by Watanabe *et al.* (1982). A duration of 6-12 h achieved maximum n-3 HUFA level in *Artemia*. Enrichment of n-3 HUFA seems difficult when comparing lower incorporation rate 20% in *Artemia* using a emulsified methyl ester containing 85% n-3 HUFA that achieved 60% incorporation in rotifers (Leger *et al.*, 1986). Approximately 0.3% n-3HUFA in *Artemia* after enrichment could be a satisfactory level suggested by Watanabe *et al.* (1982).

However, another method has been introduced by French researcher using compound diets such as powder algae, cholesterol, cod liver oils, vitamin premix, minerals etc. to pre feed nauplii for 2 days with/without a subsequent 30 min. enrichment with another compound diet. This showed satisfactory improvement of nutritional values in *Artemia* than other methods. Belgian enrichment techniques included the use of n-3HUFA coated micro-particles to pre feed nauplii which involved fish oils to coat micronized rice bran. But technique is complex and expensive to produce a micro-particle diet. Self-emulsifying enrichment became more effective technique later. It is a product mixture of n-3 HUFA, vitamins, carotenoids, steroids, phospholipid and emulsifier. This product is diluted in water and dispersed into small globules using aeration.

Nauplii are incubated for 12-24 h at 28°C and ingest these globules easily. This method was further improved by incorporation of other techniques where highest concentration reached 14 mg/g after 12 h , 16.4 mg/g after 24 h and 57.8 mg/g after 48 h enrichment (Leger *et al.*, 1986). Recently, marine phospholipids have been intensively used for live food enrichment due to its high content of 22:6n-3 and 20:5n-3 in an easily digestible and highly bioavailable form. PUFA are presented to *Artemia* through liposomes which are lipid vesicles and microscopic carriers for nutrients. Use of liposome over emulsion provides more advantages which can encapsulate n-3 PUFA, vitamin C, vitamin A, free amino acids and water soluble antibiotics during *Artemia* nauplii enrichment (Lu *et al.*, 2011).

Factors affecting DHA (22:6n-3) degradation in *Artemia* after enrichment

Bioconversion of HUFA

Enrichment can improve the fatty acid profile of live food organism but biochemical composition and energy content are notoriously unstable after enrichment. Live food organism maintains their own biological processes during enrichment. It has been reported that 20:5n-3 & 20:4n-6 preferentially esterified into phospholipid (PL) during enrichment which increases their bioavailability to fish larvae. In contrast, 22:6n-3 content highly esterified into TAG form that makes it unavailable to fish larvae

(Reis *et al*., 2017).

The emulsion products supplied to *Artemia* in ethyl ester form mainly incorporated into PL and TAG during enrichment. HUFA delivered in PL form have more beneficial effect on marine fish than neutral lipid form. Studies with radio-labeled 22:6n-3 demonstrated that 22:6n-3 actively trans-located into neutral lipids rather than PL in *Artemia* (Reis *et al*., 2017). Guinot *et al.* (2013) demonstrated that *Artemia* meta-nauplii enriched with marine Lecithin 60 accumulated up to 1.9% of 22:6n-3 in the PL form but 22% in TAG. Presence of monoacyl-glycerides and diacyl-glycerides in neutral lipid before the formation of TAG also confirm that 22:6n-3 has been translocated into neutral lipids. Naturally, *Artemia* contains relatively higher linolenic acid which competes with 22:6n-3 for PL position together with the inherent 22:6n-3 conversion capacity of *Artemia* from PL to neutral lipid. Therefore, it remains difficult to maintain high 22:6n-3 level in enriched *Artemia* even during first few hours of enrichment (Guinot *et al*., 2013).

Furthermore, *Artemia* retro-convert 22:6n-3 into 20:5n-3 during enrichment and subsequent starvation periods that quickly reduces 22:6n-3 in *Artemia* after enriched. Barclay and Zeller (1996) reported an increased ratio of 20:5n-3/22:6n-3 in *Artemia* nauplii with reduction of 22:6n-3 and 22:5n-6 into 20:5n-3 and 20:4n-6 after few hours of enrichment.

Ito and Simpson (1996) demonstrated bioconversion of n-3 PUFA from 18:2n-6 in *Artemia*. Information on specific enzymatic activity likely phospholipases, acyl-transferases and phosphatases activity believed to involved in PL catabolism, has not been documented in *Artemia* yet. However, catabolism of 22:6n-3 varies among strains. All of the *Artemia* species reported to have a high tendency of 22:6n-3 reduction with time but the reduction is considerably higher in A. *franciscana*. Degradation of 22:6n-3 related to organism's own energy metabolism pathway. *Artemia* rapidly catabolizes lipid as an energy source while other live food organisms such as copepods and rotifers use protein rather than lipid (Evjemo *et al*., 2001).

Starvation and Ambient Temperature

Temperature and starvation time are inevitably correlated to 22:6n-3 stability in *Artemia*. After enrichment, *Artemia* nauplii are stored for further use in ambient condition before fed to fish. No information available about consumption time of fish larvae when live food presented to them. It has been reported that *Artemia* nauplii and meta-nauplii catabolize higher proportion of lipid during starvation compared to rotifers which show higher lipid content throughout the enrichment and subsequent starvation (Naz, 2008).

Artemia nauplii cannot synthesize 22:6n-3 and they metabolize long chain PUFA very rapidly. During the starvation, *Artemia* metabolizes fatty acids that are incorporated into neutral lipids for energy production, structural maintenance and growth (Estévez *et al*., 1998). However, the free amino acid content in *Artemia* nauplii increases throughout the enrichment period and it tended to decrease during starvation after enrichment in metanauplii. This indicates that *Artemia* nauplii use lipids as an energy source during enrichment while protein degradation may take place at a later stage (Naz, 2008).

Reduction of 22:6n-3 in *Artemia* meta-nauplii is higher than that of nauplii indicates differences in energy metabolism. *Artemia* nauplii accumulate 22:6n-3 at the highest level during enrichment and reduces it throughout the starvation periods when it reached to metanauplii stage. The 22:6n-3 retention in rotifers could be improved by supplying a 22:6n-3 rich algae after enrichment but it has hardly any effects on *Artemia*. Naz (2008) reported an increase of 20:5n-3 level in *Artemia* during enrichment and it remains constant after enrichment period under a storage condition of 4°C. The retention of high 20:5n-3 in *Artemia* nauplii can be explained by finding of Navarro *et al*. (1999) who has demonstrated the conversion of 22:6n-3 into 20:5n-3 in *Artemia* nauplii throughout the enrichment and starvation period. Estevez *et al*. (1998) reported 70% catabolism of assimilated 22:6n-3 with reduction of 20:5n-3 and 20-30% catabolism of the initial content of 20:4n-6 by *Artemia* nauplii after 24 h of enrichment at 20°C temperature. The longer starvation period under high temperature largely increases catabolism of essential fatty acids in *Artemia*. The elevated 22:6n-3 level of enriched *Artemia* may not be available to fish if the starvation period is longer before feeding to fish.

Enriched *Artemia* nauplii are stored at low temperature (4°C) to reduce metabolic activity and to prevent degradation of assimilated FA during storage. However, 22:6n-3 retention during starvation varies considerably among strains, for instance, the degradation rate of 22:6n-3 during starvation at high temperature was higher in A. *franciscana* and reduced at low temperature but A. *sinica* able to retain a higher level of 22:6n-3 during starvation at different temperature (Evjemo *et al*., 1997).

This indicates that A. *sinica* uses other fatty acids rather than 22:6n-3 for energy production. Danielsen *et al*. (1995) reported 92% reduction of 22:6n-3 at >25° C in A. *franciscana* during a 24-h starvation period. Loss of 22:6n-3 is higher in enriched *Artemia* compared to the other live food organisms. Rotifer *Brachionus plicatilis* also shows a reduction of DHA under high temperature during starvation but it is lower than *Artemia*. Whiles, enriched copepods organisms show an increase of DHA% in total fatty acids and higher retention rate during starvation (Evjemo *et al*., 2001).

Autoxidation of Enrichment Products

Enrichment of fatty acids in *Artemia* performed with vigorous aeration and illumination at 27°C temperature which is reported to accelerate autoxidation of PUFA and the formation of oxidation products (Mcevoy *et al*., 1995). There are different types of enrichment product such as microalgae, spray-dried cells of *Schizochytrium sp*., microcapsules and oil emulsion which contain significant

levels 20:5n-3, 22:6n-3 and 20:4n-6 (Monroig *et al.*, 2007). Commercial enrichment product varies in formulae, essential nutrients, and physical form. The PUFA-rich enrichment products are highly susceptible to oxidative degradation under enrichment conditions which produce terminal toxic compounds can be accumulated in *Artemia*. The oxidation process consists of hydrogen abstraction, rearrangement of double bonds and the addition of triplet oxygen leading to highly reactive peroxy radicals which further react with other PL causes fragmentation of PL, further generation of truncated PL and other low molecular weight products such as aldehyde and ketones. The reduction of 22:6n-3 and 20:5n-3 increase with longer enrichment period which could be related to higher autoxidation of product. The oxidation of PUFA has been reported in all most all enrichment product after a certain duration mostly after 23 h of enrichment (Mcevoy *et al.*, 1995).

The stability of 22:6n-3 in enriched *Artemia* depends on the types of products used. Monroig *et al.* (2007) reported higher oxidative stability of liposome than lipid emulsions used in the enrichment of live food. The liposomes are mostly comprised of phospholipids and the commercial emulsions are composed of triacylglycerides (TAG) and fatty acid ethyl esters (FAEE). Marine phospholipid based liposomes have been modified with the incorporation of antioxidant to improve its oxidative stability (Monroig *et al.*, 2007). Relatively higher degradation of 22:6n-3 has been observed in *Artemia* which were enriched with tuna orbital oil than the cod liver oil after 23 hours of enrichment period, and moreover, a relatively slow oxidation of 22:6n-3 found with Super Selco enrichment product containing antioxidant, was reported by Mcevoy *et al.* (1995).

Oxidative stability of 22:6n-3 increases in PL in a combination of antioxidant compared to triacylglycerides (TAG) and fatty acid ethyl esters (TG). The degree of unsaturation, the acyl chain, and length of fatty acids influences lipid oxidation. The soybean liposomes (Lext SPC:CHO and Ldet SPC) are more prone to oxidation than krill liposomes (Ldet KPEand Ldet KPE:vitA) which contain longest and the highest unsaturated fatty acids. The Lext DPPC:CHO containing saturated fatty acids (palmitic acid, 16:0) showed a higher oxidative stability (Monroig *et al.*, 2007). Oxidative stability of free DHA increase when it is included in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in the structure of the phospholipids Lyberg *et al.* (2005). Combination of enrichment product with antioxidant increase oxidative stability of PUFA but exposure to aggressive pro-oxidant conditions of enrichment process disrupt this antioxidant protection (Mcevoy *et al.*, 1995).

The retinyl palmitate is an antioxidant substance which is added in enrichment products but could not offer the oxidative stability of enrichment product under enrichment condition (Monroig *et al.*, 2007). The synergistic effects of alpha-tocopherol and PL have been reported in different organ tissue of mackerel and sardine which has been used to prepare fish oil based enrichment product. Henna Lu *et al.* (2011) mentioned some ingredients that improve autoxidation stability of marine phospholipid based liposome such as tocopherol, cholesterol, chicken egg albumin, soy protein etc. Tocopherol has high antioxidative effects on the liposome, moreover, cholesterols condense PL bilayer over phase transition that increases physical stability of PUFA, furthermore, chicken egg albumin and soy protein reported to protect bilayer of liposome from the attack of free radicals (Henna Lu *et al.*, 2011).

Prospective research approach on impact of temperature and maternal nutritional profile on fatty acids composition of crustacean live food organism

Some studies have been conducted on copepods and *Daphnia* with a purpose of better understanding the fatty acid composition in response to temperature change. The fatty acid utilization pattern of crustacean zooplankton reflects their adaptation to varying conditions in nature. Lipid breakdown for energy production and fatty acid composition of live food organism greatly influenced by temperature and food availability. Storage lipid is the major source of energy in copepods and cladocerans inhibiting in extreme environment. It supports the survival of animal supplying energy during food shortage or temperature fluctuation. Membrane polar lipids are more conserved than storage lipids during short term starvation and low temperature exposure.

The conservative nature of membrane lipids in crustacean zooplankton is mainly to maintain the structural component of cell membrane. Food deprived condition leads to degradation of both membrane and storage lipids that might be related to the degradation of cell organelles during prolong starvation which has been reported in starved *Daphnia magna*.

Copepods contain higher 22:6n-3 in their membrane lipids while enriched *Artemia* contains 22:6n-3 in neutral lipid. In natural condition storage lipid remains higher in low temperature and decrease with increasing temperature. While short term heat stress condition causes suppression of physiological processes and causes a change in the composition of fatty acids. Short term suppression of physiological process results in an imbalance between oxygen demand by tissue and oxygen supply by cardiovascular system, therefore generate an anaerobiosis condition which reduces oxidative degradation of PUFA in storage lipid (Werbrouck *et al.*, 2017). Notably, information on the mechanism of selective 20:5n-3/22:6n-3 catabolism in crustacean zooplankton is limited.

The long-term heat stressed food deprived condition can potentially increase the PUFA degradation. Therefore, some studies hypothesized relevance of the mitochondrial reactive oxygen species (ROS) that increase with increasing temperature has potential to cause damage to cellular lipid, protein, and DNA (Schulte 2015). PUFA are more susceptible to oxidative damages. Thus, the 20:5n-

3 and 22:6n-3 on mitochondrial membrane increase susceptibility to damage by ROS. Werbrouck *et al.* (2016) reported higher degradation of PUFA, particularly 20:5n-3 in heat stressed condition which decreased under low temperature. The author also mentioned the higher structural requirement of EPA under cold conditions in *Daphnia pulex* and harpacticoid copepods. *Platychelipus littoralis* exposed to short-term food deprivation showed a preferential retention of 22:6n-3 under low temperature. The initial 22:6n-3 retention under all temperature remains same during food deprived condition that indicates the requirement of 22:6n-3 in adverse condition to maintain cellular biochemical competency but longer exposure to heat stress gradually reduces 22:6n-3 from storage lipid. Utilization of fatty acid classes varies among crustaceans. However, few studies have been conducted on crustacean zooplankton to determine the effect of parental exposure to different temperature on fatty acid composition of offspring population.

Sperfeld and Wacker (2012) reported that *Daphnia magna* grew in 20:5n-3 free diet showed no 20:5n-3 in body and eggs at 20°C but it was present at 15°C temperature which indicates a breakdown of stored PUFA to supply 20:5n-3 to survive at low temperature. The author also reported a higher n-3 PUFA content in cladoceran *Daphnia magna* reared at 15°C than in 20°C and 25°C while saturated fatty acid (SFA), mono unsaturated fatty acid (MUFA) and n-6 PUFA was higher at 25°C. Similarly, stearidonic acid (SDA), 20:5n-3 and 22:6n-3 found 1.4-1.5 times higher in 15 °C than at 20 °C.

The exposure to environmental stress and nutritional profile of maternal population has great influence on offspring fitness. Mother cladoceran *Daphnia magna* fed with 20:5n-3 supplemented diet showed higher 20:5n-3 in offspring at 15°C than 20°C (Sperfeld and Wacker, 2012). Another cladoceran *Daphnia pulex* has grown on 20:5n-3 free and α-linolenic acid rich diet showed increased 20:5n-3 in body tissue at the expense of α-linolenic acid at low temperature (Schlechtriem *et al.*, 2006).

Other live feed organisms, crustacean cladocerans are able to accumulate a large amount of 20:5n-3 & 20:4n-6 from a diet rich in HUFA but hardly accumulate 22:6n-3 as they readily convert 22:6n-3 into 20:5n-3. They are able to synthesize both 20:5n-3 and 20:4n-6 from alpha linolenic acid and linolenic acid when exposed to a low temperature to maintain membrane fluidity. On the other hand, increasing level of 20:5n-3 & 20:4n-6 are highly conserved in animals exposed to a low temperature for a longer duration to survive in starvation. But no 22:6n-3 has been detected at low temperature in this cladoceran (Schlechtriema *et al.*, 2006).

Nanton and Castell (1999) reported that the copepods cultured with *Isocrysis galbana* at different temperature showed the different concentration of HUFA in relation to temperature and 22:6n-3 content was increased in cold stress condition while the saturated fatty acid was higher at high temperature. In nature, aquatic organisms are more prone to adapt to frequent environmental changes from generation to generation that evolve their adaptation against these changes. Short transient exposure to toxic substances or environmental stress results the inheritance of traits to an unexposed next generation. Epigenetic influence on *Artemia* mainly investigated on the elevated immune response in heat stress condition. Norouzitallab *et al.* (2014) reported an enhanced immune response in each generation of *Artemia* against environmental heat stress as a transgenerational trait transfer from parental population to its offspring. However, modification of the fatty acid composition in subsequent generation of *Artemia* influenced by induced ambient temperature not studied yet. The prominent effect of temperature change on PL profile has been reported in many live feed organisms which indicates a possibility of inducing a change in the fatty acid profile in offspring population of *Artemia* by manipulating the ambient temperature and diet composition in the parental population.

CONCLUSION

Nutrient profile of live food organism is very crucial for first feeding marine fish larvae. Stabilizing concentration of essential fatty acids (n-3 HUFAs) content in *Artemia* through enrichment processing is difficult due to organism's own metabolism and difficulties of marine fish larvae to accept formulated diet at their weaning stage. Therefore, natural modification of n-3 HUFA content in *Artemia* under several ambient condition, diet modification throughout their life cycle followed by several generations, an in-depth knowledge on associated factors for DHA degradation in *Artemia* followed by enrichment processing could be promising research approach in this context.

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