LIVE FEED FOR EARLY ONTOGENETIC DEVELOPMENT IN MARINE FISH LARVAE

EDITED BY: Per Meyer Jepsen, Sami Souissi and Yen-Ju Pan
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Topic Editors:
Per Meyer Jepsen, Roskilde University, Denmark
Sami Souissi, Lille University of Science and Technology, France
Yen-Ju Pan, National Taiwan Ocean University, Taiwan

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Table of Contents

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Yngvar Olsen,
Norwegian University of Science and Technology, Norway

*CORRESPONDENCE
Yen-Ju Pan
panyj@mail.ntou.edu.tw

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Editorial: Live feed for early ontogenetic development in marine fish larvae

Yen-Ju Pan[1,2]*, Sami Souissi[3] and Per Meyer Jepsen[4]

[1]Department of Aquaculture, National Taiwan Ocean University, Keelung, Taiwan, [2]Center of Excellence for the Oceans, National Taiwan Ocean University, Keelung, Taiwan, [3]Université de Lille, The French National Centre for Scientific Research, Université du Littoral Côte d'Opale, French National Research Institute for Sustainable Development, Mixed Research Unit 8187 Laboratory of Oceanology and Geosciences, Station Marine de Wimereux, Lille, France, [4]Department of Science and Environment, Roskilde University, Roskilde, Denmark

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Editorial on the Research Topic:
Live feed for early ontogenetic development in marine fish larvae

1 Preface

Live feeds hold the key to a stable and expanding marine aquaculture. In this editorial, we briefly review the history of live feed production for marine larviculture and summarize the latest contribution issued in the research topic – Live Feed for Early Ontogenetic Development in Marine Fish Larvae. With the current research that were submitted to this research topic, we see trends into many different aspects of live feed production. We are ensured that some of the remaining bottlenecks will be solved in a near future, providing a diverse and ecological sound marine aquaculture sector to flourish.

2 Larviculture of marine fish species

The marine aquacultures are expanding with an increasing diversity of fish species across different regions. Most commercial marine fish larvae require live feed as first feeding diet, such as European seabass (*Dicentrarchus labrax*), gilthead sea bream (*Sparus aurata*), turbot (*Scophthalmus maximus*), Atlantic halibut (*Hippoglossus hippoglossus*) and cod (*Gadus* spp.) in Mediterranean and East Atlantic regions (Sweetman, 1992; Reitan et al., 1993; Moksness and Støle, 1997; Shields, 2001; Evjemo et al., 2003; Oie et al., 2017) milkfish (*Chanos chanos*), groupers (*Epinephelus* spp.), cobia (*Rachycentron canadum*), snappers (*Lutjanus* spp.), sea bass (*Lates calcarifer* and *Lateolabrax japonicus*), sea breams (*Acanthopagrus* spp. and *Pagrus* spp.) and pompano (*Trachinotus* spp.) in Asian Pacific and Oceanian regions (Chen and Long, 1991;

Fushimi, 2001; Liao et al., 2001; Marte, 2003; Le Moullac et al., 2003; Palmer et al., 2007); grey mullet (*Mugil cephalus*), Pacific threadfin (*Polydactylus sexfilis*), snook (*Centropomus* sp.) and red drum (*Sciaenops ocellatus*) in American regions (Lee and Ostrowski, 2001; Cerqueira and Tsuzuki, 2009). Besides the edible fish species, the larviculture of many ornamental reef fish are already or under commercialization stage, such as clownfish (*Amphiprion* spp.), tang fish (*Paracanthurus* spp. and *Zebrasoma* spp.) and angelfish (*Centropyge* spp. and *Pomacanthus* spp.). Furthermore, several emerging species with difficult early larval stages (i.e., very tiny mouth or perception toward mobile pattern of specific live feed) are targeted by specialised research institutions and conservation programs developed by aquariums, such as Rising Tide Conservation, Tropical Aquaculture Laboratory, University of Florida; Oceanic Institute of Pacific University in Hawaii (Degidio et al., 2017; Callan et al., 2018), James Cook University in Australia (Chen et al., 2020), National Museum of Marine Biology & Aquarium and National Dong Hwa University in Taiwan (Chiu and Leu, 2021; Leu et al., 2022). Private sectors, such as Bali Aquarich based in Indonesia, De Jong Marinelife in the Netherlands and Biota in the USA have also contributed extensively to the captive breeding of marine ornamental fish for the demand of the aquarium market.

Techniques of live feed production is a subject that is commonly interesting for larviculture industry worldwide, but the selection of live feed is related to what fish species and developmental stages they are targeted for. For warm water fish species, such as groupers (*Epinephelus* spp.), duration of larviculture (hatch to metamorphosis) is around 20-30 days. Live feeds selected for the three-stage larviculture are categorized as follows: (1) first feeding stage (3-10 day post hatch, dph): fertilized oyster egg (<70 μm), nauplii of small copepod species (<120 μm) and SS-type *Brachionus* rotifer (<150 μm); (2) secondary feeding stage (8-20 dph): S-type *Brachionus* rotifer (<200 μm), newly-hatched *Artemia* nauplii (<450 μm) and copepodites (200-600 μm); (3) pre-weaning stage (18-30 dph): *Artemia* metanauplii (<600 μm), adult copepods (800-1200 μm) and micro pellet diets (Marte, 2003; Zhang et al., 2015). This example indicates that the size and type of live feeds are primarily critical, and highlights that the establishments of live feed production should be diversified for various fish species. Most of the marine fish larvae are born with a small mouth gape and incomplete digestive system (Yúfera and Darias, 2007). Those larvae need tiny live feed with great bioavailable nutrients to sustain survival, growth and other metabolic functions. Indeed, live feeds are essential for larval ontogeny due to their superior nutritional value, palatability and mobility-triggered predatory attraction. This research topic aims to establish a collection of articles that tackles different issues related to the recent progress of live feed production, and their implications in marine larviculture.

3 Live feed production for marine larviculture

In general, marine fish have poor capability to synthesize essential fatty acids, which indicates that a dietary supplement of these nutrients is crucial (Coutteau et al., 1997; Rainuzzo et al., 1997; Mejri et al.). The specific requirement on highly unsaturated fatty acids (HUFAs) could be a consequence of evolution in marine trophic relationships. In nature, phytoplankton capture solar energy *via* photosynthesis, and biosynthesize HUFAs and other organic nutrients. These components are ingested, sometimes bio-transformed and accumulated by zooplankton, and then by larvae of most marine fish. Indeed, phytoplankton and zooplankton are natural food items for marine larvae, thus they are also produced as live feed for larval nourishment in marine hatchery (Reitan et al., 1997). Based on the species availability and climate condition, applications of live feeds (species selection and production techniques) are diverse and region-specific (Lee et al., 2008; Conceição et al., 2010; Nielsen et al., 2017; Pan et al.).

Phytoplankton has been long used as live feed for zooplankton and larvae of several marine organisms, and as water conditioning element (i.e., green water technique) (De Pauw et al., 1984; Borowitzka, 1997; Pan et al.). One of the focuses in research and development (R&D) for microalgae is the photobioreactor, which could facilitate an efficient and high-density microalgae production. Many companies and institutions have well demonstrated the industrial scale photobioreactor system for marine hatcheries (Naumann et al., 2013; Tibbetts et al., 2020). Several studies have showed that manipulations in culture conditions and composition of fertilizer could affect microalgal productivity and nutritional quality (Harrison et al., 1990; Reitan et al., 1994; Reitan et al., 1997; Cañavate, 2019; Han et al., 2019; Cañavate and Fernández-Díaz, 2022). In this research topic, Latsos et al. revealed a significant increase of fatty acid levels in the microalga *Rhodomonas* sp. inducing by nitrogen starvation. The authors proposed a two-phase culture system, which firstly accelerates the cell growth and subsequently induces PUFAs accumulation by nitrogen starvation in commercial scale cultivation.

Ciliates are a group of micrometre-sized protists that show great potential for feeding small-mouthed reef fish larvae, such as cleaner goby (*Gobiosoma evelynae*), purple fire-fish (*Nemateleotris decora*) and blue-striped angelfish (*Chaetodontoplus septentrionalis*) (Olivotto et al., 2005; Madhu and Madhu, 2014; Leu et al., 2015). Ciliate production relies on the suspending organic matters and bacteria as their dietary resources, which makes their cultivation relatively easy. In addition, the fact of ciliate's bacterivorous feature has facilitated their implication as bio-effector. In this research topic, Lin et al. revealed an innovative method of using the

ciliate *Strombidium* sp. to reduce pathogenic *Vibrio* sp., which leads to a significant increase of the survival rate in juvenile grouper *Epinephelus coioides*.

Rotifers in aquaculture largely belongs to the genus *Brachionus*, and it has been extensively produced as live feed for marine larviculture since the 1960-70s in Japan and Norway (Watanabe et al., 1983; Pejler, 1998; Lubzens et al., 2001). Rotifer cultures are well established, and intensive culture systems are available in the industry worldwide (Lubzens et al., 1989; Fu et al., 1997; Odo et al., 2015; Pan et al.). One of the advantages of rotifer production is the feasibility of reaching ultra-high densities. For instance, Yoshimura et al. (2003) reported a rotifer density over 10^5 individuals ml^{-1} in a culture equipped with a membrane filtration system. Research in rotifer enrichment and its effect on marine fish larviculture are still an area of interest. In this research topic Ghaderpour and Estevez shows a close relationship between the composition of the dominant phospholipids between Meager larvae (*Argyrosomus regius*) and its live feed, rotifers respectively. Another example from the research topic is the ongoing research in effects of novel enrichment protocols for rotifers. Safiin et al. showed promising results when incorporating palm oil into enrichment diets of rotifers that were fed to *L. calcarifer* larvae. Furthermore, Fu et al. also investigated the effects of different commercially available enrichment products on rotifers to reveal how the enrichment impact survival, growth, fatty acid composition and jaw deformities of Golden Pompano larvae (*Trachinotus ovatus*).

The harvest of brine shrimp *Artemia* dormant cysts or biomass was first commercialized in San Francisco Bay, California and the Great Salt Lake, Utah, USA during 1950s for the aquarium market, and further exploited for the increased demands from the marine larviculture industries in 1970s (Lavens and Sorgeloos, 2000). Until now, *Artemia* cyst production relies mainly on natural resources, thus new harvest sites (e.g., southern Siberia and central Asia) have been investigated and established for cyst production industry in the recent decades (Litvinenko et al., 2015; Le et al., 2019; Camara, 2020; Pan et al.). Another manner of brine shrimp cyst production is the cultivation in inland saltworks or hypersaline ponds (e.g., China, Kenya and Vietnam), which facilitates a better biological and environmental controls to achieve sustainable cyst production (Baert et al., 1997; Van Stappen et al., 2020). Owning the significant role of *Artemia* in the industry, two UN-recognized Artemia Reference Centres has been established at Ghent University, Belgium and Tianjin University of Science and Technology, China, where scientists are working on the pioneer aspects of genomic sequencing, genetic and microbial regulations, nutrition and exploitation of new cyst resources (Sorgeloos, 1980; FAO, 2017; Sorgeloos and Roubach, 2021; Duan et al., 2022). Based on the great scientific contributions, the supply chain of *Artemia* cysts and their relevant products are successfully marketed by several companies, such as INVE Aquaculture Co. in Belgium and Ocean Star International Co. in the USA. Research in *Artemia* is still very relevant when used as starter diets for fish larvae of some species. In this research topic, Planas et al. and Planas et al.) studies different breeder and pre-breeding diets, with *Artemia*, and their effect on new-born seahorses.

Copepods are natural food items for marine organisms, and are either harvested from field or intentionally cultivated for marine larviculture (Støttrup, 2000; Drillet et al., 2011; Hansen, 2017). The capacity of PUFA bioconversion or accumulation in copepod determines their nutritional benefit for larval feeding (Nielsen et al., 2019). Research protocols addressing nutritional manipulations of copepods are issued in this research topic (Camus et al.; Dayras et al.; Matsui et al.; Wang et al.). Focuses on other cultivation parameters are also included in the research topic, such as salinity, temperature, and photoperiod (Choi et al.; Wang et al.; Yoshino et al.). On the other hand, the risk of epibionts on copepods in the indoor intensive culture system was reported by Pan et. al. This study revealed a significant decline of egg production in diatom-infested *Acartia tonsa*, suggesting the prevention of epibiosis should be carried out in copepod cultures.

The intensive mono-species production of copepods is still in its' pre-industrial stage, but the commercial production is estimated economically feasible (Abate et al., 2015; Abate et al., 2016). Some temperate species, in particular the calanoid *Acartia tonsa* (strain DFH.AT1) has been intensively studied for aquaculture purposes (Støttrup et al., 1986; Drillet et al., 2006; Jepsen et al., 2007; Hagemann et al., 2016; Pan et al., 2020). Its capacity to easily produce resting eggs that can be stored at low temperature encouraged the R&D projects (e.g., IMPAQ in Denmark, COPREST and STARTRENS in Norway) and the industrialisation initiatives. Since few years, the C-FEED company based in Norway, produces resting eggs of *A. tonsa* that are easily shipped for larviculture. On the other hand, many local suppliers in Asia that provide live copepods (e.g., *Apocyclops royi* and *Pseudodiaptomus annandalei*) cultivated in outdoor ponds for commercial fish hatcheries (Su et al., 1997; Blanda et al., 2015; Grønning et al., 2019). The optimal mass culture protocols of the listed copepod species have been long developed and reviewed in several projects and publications (Drillet et al., 2011; Rasdi and Qin, 2016; Hansen, 2022). Although these millimetre-sized copepod species (adult size at around 1-2 mm) are commercially used, the research and development of novel and micrometre-sized copepod species (adult size < 800 µm) are needed. Based on the articles collected in our research topic, there are trends in that the search for the desirable copepod candidates are micrometre-sized calanoid (Camus et al.; Choi et al.; Yoshino et al.; Wang et al.) and cyclopoid copepods (Dayras et al.). These successful accomplishments, coupled with the previous contributions from Australia, Hawaii and South Korea (McKinnon et al., 2003; Kline and Laidley, 2015; Lee and Choi, 2016), have

implicated the potential breakthrough of using micrometre-sized copepods e.g. *Bestiolina* sp., *Parvocalanus* sp., and *Paracyclopina* sp. as first feeding diets for the emerging marine aquaculture fish species with particularly tiny mouth gapes.

4 Enrichment techniques in rotifer and *Artemia* for marine fish larviculture

One of the focuses addressed in this research topic is nutritional enrichment protocols for fish larval feeding (Fu et al.; Matsui et al.; Planas et al.; Planas et al.; Safiin et al.; Vo et al.). This is especially relevant for live feed organisms with poor capacity in bioconversion and accumulation of nutrients e.g., *Artemia* and rotifers. High availability and easy maintenance make these animals commercial live feeds, even though none of them appear naturally in the marine food web. As unnatural food items, *Artemia* and rotifer are often poor in essential fatty acids. Fortunately, these organisms demonstrate non-selective feeding behaviour which makes them excellent vectors of nutritious elements for farmed larvae (Léger et al., 1987; Fernández-Reiriz et al., 1993; Ghaderpour and Estevez). In this research topic, processing of alternative ingredients and enrichment protocols are reported for the larval feeding of many emerging fish species, such as Asian Seabass (*L. calcarifer*), Atlantic cod (*Gadus morhua*), Golden Pompano (*T. ovatus*), Long-snouted seahorse (*H. guttulatus*), Slender seahorse (*Hippocampus reidi*), Meager (*A. regius*), and Red Sea Bream (*Pagrus major*) (Fu et al.; Matsui et al.; Planas et al.; Planas et al.; Safiin et al.; Vo et al.). The choice of live feeds, enrichment products and protocols differ among these studies, and the suitability of various designs are highly correlated to the nutritional requirement of the farmed species. Another research focus is to replace fish oil in the enrichment emulsion for sustainability and cost-down management. Indeed, the alternative ingredients (e.g., palm oil) could reduce over-reliance in fish oil and open a new avenue in regional-specific industry of live feed enrichment products (Safiin et al.).

5 Perspectivation

Industrial production for micrometre-sized copepod is particularly worth to be invested for the supplement or replacement of the rotifer and the price-increasing *Artemia*. Selective breeding on preferable aquaculture traits (e.g., high productivity, nutritional value) could further improve the economic feasibility of the available live feed strains (Souissi et al., 2016; Pan et al., 2017). Genomic or genetic studies of live feed organisms should be addressed to clarify the effects of culture managements on their physiology (e.g., nutrient synthesis, stress tolerance) (Nielsen et al., 2019; Lee et al., 2022). The production of other live feed candidates, such as the recent successful larviculture project of ballan wrasse (*Labrus bergylta*) using cryo-preserved barnacle nauplii called "CryoPlankton" from Planktonic Co., Norway (Malzahn et al., 2022), and gelatinous zooplankton (e.g., flame jellyfish *Rhopilema esculentum* for feeding larval silver pomfret *Pampus argenteus* and larvacean for eel larvae) should be developed to support the successful larviculture of emerging aquaculture species (MochiokaIwamizu, 1996; Liu et al., 2015). Overall, the optimization of live feed production, conservation for shipment (e.g., cryopreservation or artificially-induced resting stages) and the improvement of enrichment techniques are still hot topics. Commercialization of the relevant research contributions are encouraged to be accomplished under collaborations between academia and industry (Hansen et al., 2017). Another important step is continuous meetings between academia and industry at international levels in relevant forums e.g., LARVI-conference and the European Aquaculture Society to further support the development of the marine aquaculture (Hansen and Møller, 2021).

Author contributions

All authors were responsible for the idea of this special volume, wrote, and reviewed this editorial topic. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Biological Protective Effects Against *Vibrio* Infections in Grouper Larvae Using the *Strombidium* sp. NTOU1, a Marine Ciliate Amenable for Scaled-Up Culture and With an Excellent Bacteriovorous Ability

Hung-Yun Lin[1†], Wei-Yu Yeh[1†], Sheng-Fang Tsai[2], Kuo-Ping Chiang[2,3], John Han-You Lin[4], Che-Chia Tsao[5] and Han-Jia Lin[1,3*]*

[1] Department of Bioscience and Biotechnology, National Taiwan Ocean University, Keelung City, Taiwan, [2] Institute of Marine Environment and Ecology, National Taiwan Ocean University, Keelung City, Taiwan, [3] Center of Excellence for the Oceans, National Taiwan Ocean University, Keelung City, Taiwan, [4] School of Veterinary Medicine, National Taiwan University, Taipei, Taiwan, [5] Department of Biological Sciences and Technology, National University of Tainan, Tainan, Taiwan

Bacterial infectious diseases cause a huge economic loss in aquaculture. Active biological control that uses bacteriovorous organisms to remove pathogens is an ecologically friendly approach for the cultural system to counteract the bacterial infection. The ciliate is one of the main predators of bacteria in aquatic ecosystems, but whether it can be effectively adopted to protect aquaculture organisms from bacterial pathogens remains to be investigated. In this study, we optimized the culturing method for a marine ciliate *Strombidium* sp. NTOU1 and analyzed its bacteriovorous properties. *Strombidium* sp. NTOU1 could feed on a variety of bacteria including pathogenic species. By controlling the amount of frozen bacteria *Erwinia* spp. in the medium, the ciliate grew to the maximum density within 4 days and could reach 1.2×10^5 cells/mL after the suction filtration enrichment. Ingested bacteria were observed in the food vacuole of the ciliate, and the average bacterial clearance rate of a single NTOU1 cell was ∼300 cells/hr. In the challenge trial which grouper larvae were exposed to an extreme environment containing a high density of the pathogen *Vibrio campbellii*, only 33% of the grouper larvae could survive after 5 days. However, preincubating with *Strombidium* sp. NTOU1 for an hour resulted in their survival rate to rise to 93%. Together, our results demonstrated that *Strombidium* sp. NTOU1 has the potential to become a biological control species to actively remove pathogens in aquaculture. In addition, the technical improvement to culture *Strombidium* sp. NTOU1 provides an advantage for this ciliate in the future academic research or biotechnological application.

Keywords: biological control, aquacultrure, marine ciliate, bacteriovorus ability, high-density cultivation

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***Correspondence:**
Che-Chia Tsao
cctsao@mail.nutn.edu.tw
Han-Jia Lin
hanjia@mail.ntou.edu.tw

† These authors have contributed equally to this work

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INTRODUCTION

As global capture fisheries resources are gradually depleted, aquaculture has become an important source of high-quality animal proteins to meet the need of the world's continuously growing population (Goldburg and Naylor, 2005; Brander, 2007). Modern intensive aquaculture improves production, but it also increases the risk of infectious disease outbreaks, which cause an economic loss of US$6 billion per year for the aquaculture industry (Bostock et al., 2010; Oliva-Teles, 2012; FAO, 2018). Among fish diseases, bacterial infection is the biggest threat.

To date, antibiotics are still the most effective way to fight bacterial infectious diseases, but their excessive use in aquaculture has become a serious problem (Cabello, 2006; Defoirdt et al., 2011). The abuse of antibiotics induces the resistance of bacterial pathogens in the environment, indirectly causing the prevalence of super bacteria, which in turn threatens human health (Sapkota et al., 2008; Heuer et al., 2009). In addition, antibiotics also harm probiotics in the environment and the intestine of animals, leading to an imbalance in microbial ecology that contributes to the instability of the aquaculture system (Martinez, 2011). Many countries have reached a consensus to ban the use of antibiotics in aquatic animals. Alternative methods, which can effectively control the infectious disease but also maintain the ecological balance of the culture, are urgently on demand (Pérez-Sánchez et al., 2018).

Biological control is using natural enemies of a harmful organism in the ecosystem to control its population density and to prevent the occurrence of harm. Passive biological control in aquaculture is to create an adverse environment for the pathogen, which, in turn, facilitates the prevention of the disease. For example, probiotics compete with bacterial pathogens for the resources needed for their growth (Verschuere et al., 2000). Probiotics and microalgae have also been reported to secrete antibacterial and immune-enhancing substances, which indirectly reduce the damage caused by bacterial pathogens (Muller-Feuga, 2000; Nayak, 2010). In general, passive biological control can prevent the growth of bacterial pathogens, but it is not able to completely avoid the outbreak of the disease. On the other hand, active biological control is aimed at directly removing harmful organisms from the environment. For example, the bacteriophage can be used to infect and destroy pathogenic bacteria to prevent disease outbreaks (Letchumanan et al., 2016). Nevertheless, it can only selectively eliminate the specific host, and it has yet to find a bacteriophage that possesses the ability to fight against diverse bacterial pathogens in the actual aquaculture environment (Kalatzis et al., 2018).

In addition to bacteriophages, ciliates are the main predators of bacteria in the microbial ecosystem (Sherr and Sherr, 2002; Becks et al., 2005). Bacteriovorous ciliates, such as *Halteria grandinella*, *Paramecium caudatum, Tetrahymena pyriformis,* and *Tetrahymena vorax,* were reported to have the ability to prey on microbes not restricted to any type of bacteria through the non-selective phagocytosis (Grønlien et al., 2002; Ali and Saleh, 2014). Therefore, ciliates have been applied to lower the bacterial density in the aquatic environment. For example, *Stentor roeselii* was used to control the bloom caused by the cyanobacteria *Microcystis aeruginosa* in freshwater (Kim et al., 2007). In an outdoor algal cultivation system, the *Colpoda* sp. was deployed to protect the green algae *Chlorella* sp. from the bacterial contamination dominated by *Microbacterium* sp. (Cho et al., 2019). However, applying a ciliate to antagonize bacterial pathogens in aquaculture has not yet been reported. It is probably because suitable ciliate species, which can be practically cultured on a large scale and have a property ideal for field utilization in aquaculture, remain largely underexplored.

An oligotrich ciliate, termed *Strombidium* sp. NTOU1 hereby, was isolated from the coastal water of Taiwan (Lee, 2015). As an ecologically successful species, it may have advantageous properties potentially adaptable for human utilization, such as a broad bacteriovorous ability. In this study, we established a robust and efficient culturing method for the *Strombidium* sp. NTOU1 and characterized its feeding and ability of bacterial clearance. The result from pathogen challenging assay demonstrated the effectiveness to use this ciliate as an active biological control species for grouper larvae to against *Vibrio campbellii*.

MATERIALS AND METHODS

Chemicals, Enzymes, and Antibodies

All general chemicals used in this work, including nutrient salts and antibiotics, were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, United States). PCR related reagents were provided from Ten Giga Bio (Keelung, Taiwan). Restriction enzymes were obtained from New England Biolabs (Ipswich, MA, United States). Immunostaining related reagents were purchased from Thermo Fisher Scientific Inc. (Wilmington, DE, United States).

Source of Bacterial Feeds

There are six strains of bacteria used in this study (**Supplementary Table S2**). *Escherichia coli* BL21 strain (DE3) was purchased from New England Biolabs. *Vibrio campbellii* and *Vibrio harveyi* were provided by Dr. Han-You Lin's laboratory at National Taiwan University (Wang et al., 2010; Lam et al., 2011). The *Erwinia* spp. and *Kluyvera* spp. were isolated from the rice medium (see below) made of 5 grains of raw rice in 500 mL of artificial seawater to enrich the natural bacterial populations. The identification of these bacterial species was in accordance with the API-20E commercial kit following the manufacturer's instructions.

To create the pET-EGFP *E. coli* strain, the enhanced green fluorescence protein (EGFP) DNA fragment was amplified by PCR (**Supplementary Table S1**) from the pCX-EGFP plasmid (Addgene, Watertown, MA, United States) and then ligated into the pET28a vector (Addgene) between the restriction enzyme sites of *Eco*RI and *Xho*I to yield the pET28-EGFP plasmid. Subsequently, the pET28-EGFP plasmid was transformed into *E. coli* BL21 strain to obtain the genetically engineered *E. coli* pET-EGFP strain. 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added in the log phase (OD600 = 0.4−0.6) cells for 5 h to induce the production of EGFP recombinant proteins.

The cell density of each bacterial strain is obtained from an actual number of colonies after plating. All bacteria frozen stocks were prepared by overnight cultured bacterial suspension containing 20% glycerol and then stored in a −80°C refrigerator.

Source and General Maintenance of the Ciliate

The marine oligotrich ciliate used in this study, termed *Strombidium* sp. NTOU1, was initially isolated in Prof. Kuo-Ping Chiang's laboratory (Lee, 2015). Wild ciliates were collected using a 20-μm mesh plankton net from the coastal waters of northeastern Taiwan (25° 08′ 30″ N; 121° 47′ 42″ E) on September 8, 2011. A single-cell-isolated strain was obtained and identified as a *Strombidium* sp. based on the morphological analysis using the protargol impregnated method (Ng and Nelsen, 1977).

The primary culture and general preservation of *Strombidium* sp. NTOU1 was carried out with the rice medium at a starting concentration of 1 cell/mL. The rice medium is an unboiled infusion filled with naturally grown bacterial feed and was prepared by adding one grain of raw rice to 100 mL of artificial seawater (ASW) and standing overnight at 25 °C.

High-Density Culture and Handling of *Strombidium* sp. NTOU1

The high-density culture of ciliates took place in 5 liters of sterile ASW with a starting density of 100 cells/mL. During the culturing period, ciliate cells were harvested daily for proper serial dilution and cell counts. The food source, frozen *Erwinia* spp., was daily applied to the culture flask in an amount of 10,000 times the number of ciliates. For the preparation of the frozen *Erwinia* spp., ∼5.00×10^{10} cells were washed twice with sterile artificial seawater after centrifugation (3,000 *g*, 10 min, 4°C) and stored in the form of pellet at −20°C. The bacterial density of frozen *Erwinia* spp. was adjusted using sterile ASW. After 5-day culture, these ciliates were further subjected to the suction filtration enrichment using the water-jet aspirator pump (A-1000S, Eyela, Tokyo, Japan) at a pressure of 5 cm/Hg with a pore size of 10 μM filter membrane (Whatman, Buckinghamshire, United Kingdom) to further concentrate the ciliate culture solution to a volume of 500 mL.

To determine the cell density of *Strombidium* sp. NTOU1, 10 mL of the culture medium were sampled into a six-well plate and 0.5 mL of Lugol's regent (final concentration 5%) was added for cell fixation. After the cells were serially diluted to the appropriate concentration, the total number of ciliates in the well was counted using a dissecting microscope (ZM-160A, Optima, Taichung, Taiwan) at a magnification of 40×. The number of ciliates in per milliliter of culture was calculated according to the counted numbers in the 10-mL culture and the folds of dilution.

Palatability and Clearance Rate for *Strombidium* sp. NTOU1 on Different Bacteria

Before the experiment, ciliates were moved into the sterile ASW for 24 h after 5 times of washes. Thereafter, 5 ciliates were randomly picked and inspected under the microscope to confirm that no obvious food vacuole was present in the cell.

For measuring the ciliate growth rate when feeding different bacteria, 100 ciliates were first moved into flasks with 100 mL sterile ASW using a mouth pipette under a dissecting microscope (Optima). Each bacteria strain was subsequently added into the flask to reach the concentration of 2.00×10^7 CFU/mL, respectively. The cell density of ciliates was calculated every 24 h during the 3-day culture. Then, the growth rates (μ; day^{-1}) were calculated as $\mu = \ln(N_t/N_0)/dt$ (Montagnesa and Lessard, 1999).

In this equation, N_0 and Nt are represent the initial cell number and cell density after culture, where dt is the time of culture. The above palatability test contained multiple independent setup groups, which was suitable for analyzing the differences among population mean values, such as one-way analysis of variance (ANOVA). Significance was accepted with $p < 0.05$.

The palatability of frozen bacterial fodder for *Strombidium* sp. NTOU1 was carried out in culture flasks with 100 mL sterilized ASW, with 2.00×10^7 CFU / mL live *Erwinia* spp. or frozen-treatment bacteria, respectively. The initial number of ciliates added to the culture flask was 100 cells/mL and ciliate concentrations were counted every 24 h for the next 3 days. Student's *t*-test was used to compare the mean of the two groups (corresponding to the control group) for statistical analysis.

The bacteria clearance rate of *Strombidium* sp. NTOU1 was measured using a bacterial strain that emits fluorescence and a pathogenic species, respectively. To visualize and to quantify the ingestion of bacteria, 50 and 150 cells of ciliates were inoculated in 3 mL of sterile artificial seawater and starved for 24 h followed by adding ∼3.75×10^5 cells of the *E. coli* pET-EGFP strain, which provided ∼3,000 fluorescence intensity. The fluorescence intensity (excitation/emission: 475/575, sensitivity 100) of each sample was measured using the SynergyMx microplate fluorescence reader (Biotek, Winooski, VT, United States) every hour for 12 h. In the same period, samples from the experiment with 150 ciliates feeding on fluorescent bacteria were taken at 1, 2, 4, and 6 h for observation using the BX60 fluorescence microscope (Olympus, Tokyo, Japan) at 1,000×magnification.

The clearance test of *V. campbellii* was carried out in a flask containing 20 mL of sterile ASW with 2.00×10^6 CFU/mL of vibrio cells and/or 5,000 cells/mL of *Strombidium* sp. 100 μl of the sample containing the ciliate or vibrio was taken every 20 min for 1 h for plate count to estimate the residual concentration of the bacteria.

Immunofluorescence Staining

Ciliate samples at the density of 100 cells/mL were washed 5 times with sterile artificial seawater. 200 ciliate cells were transferred to a coverslip by a mouth pipette under a dissecting microscope (Optima). An equal volume of 2× fixatives regent (8% paraformaldehyde, 0.1% Triton X 100 in PHEM buffer, pH 6.9) were added and allowed for air drying, following by soaking the coverslip with cells in PBS-0.1% Tween (PBST) and blocking solution (3% bovine serum albumin with PBST). The fixed cells were stained with the alpha-tubulin monoclonal antibody (DM1A, 1:1000), Alexa

Fluor 568-conjugated anti-mouse secondary antibody (1:2000), and DAPI staining solution (10 ng/mL). The coverslip was counted with 10 μL of DABCO^{TM} onto the glass slide. All specimens were stored in a dark box at 4℃ and observed with a fluorescence microscope (Olympus) at a magnification of 1,000 times.

Strombidium sp. NTOU1 in the Protection of Grouper Larvae

The grouper *Epinephelus coioides* larva about 5–8 cm in length were used to be the protective object in the challenge experiment. The pathogen *V. campbellii* was cultured in TSB medium with 3% NaCl to reach an OD_{600} of 1.00 and washed twice with aerated seawater before use. At the beginning of the protection test, three biological combinations were first adapted to a culture tank with 5 L of aerated seawater at room temperature for an hour, including a group of without additional bacteria, 3.00×10^6 CFU/mL of *V. campbellii* alone, and the same number of vibrio co-cultured with 5,000 cells/mL of *Strombidium* sp. NTOU1. Next, added 5 grouper larvae to each tank for a 1-h challenge test. After treatment, each fish was rinsed with 500 mL of aerated seawater and returned to the culture tank with 8 L of aerated seawater. These grouper larvae were monitored for 5 days and fish mortality were calculated every 24 h.

Groupers are high-commercial value fish species in Taiwan aquaculture and are well-considered challenge trial test species. To avoid cannibalism in the larva stage, a suitable culture density <1 fish/L or less was used in the challenge test to mimic the real culture condition. The same density was used in our challenge test to mimic the real culture condition. The challenge was repeated three times to bring up the total number to 15 larvae for each group, and we realized that under such experimental settings, individual differences in the immune response of each batch of grouper larvae did exist and might affect the statistical results. A nonparametric statistical analysis (Kruskal-Wallis *H*-test) was applied in the dataset of 3 challenge experiments. Using this method to convert the measured values into a ranking order, the difference in survival rates between three groups can be analyzed.

RESULTS

Strombidium sp. NTOU1 Feeds on a Variety of Bacterial Species in the Laboratory

Strombidium sp. NTOU1 could be cultured in the laboratory using the unboiled rice medium made of rice grains and artificial seawater (ASW) (Lee, 2015; **Supplementary Figure S1**). To distinguish whether the growth of the ciliate is dependent on the organic substance released from the rice grain and/or on the bacteria sustained in the rice medium, an additional preparation of the same infusion was autoclaved for the comparison. The ciliate cultured in the rice medium reached a growth rate at 0.215 day^{-1} but it did not grow in the sterilized rice medium (**Figure 1**). This result indicates that the bacterium from the rice grain and proliferating in the medium is required to provide *Strombidium* sp. NTOU1 with nutrients for survival.

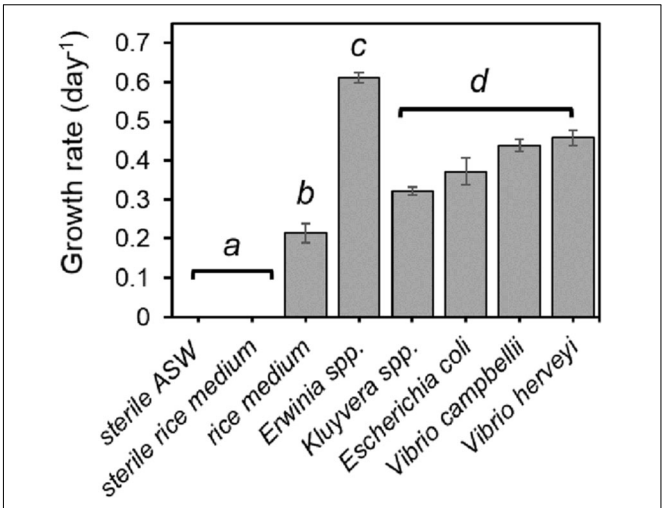

FIGURE 1 | The specific growth rate of *Strombidium* sp. NTOU1 fed with different bacteria. The error bar represents the standard deviation (SD) of the average from three independent experiments. The statistical significance of the growth rate between each group was analyzed by the ANOVA. Different letters (a, b, c, and d) indicate a significant difference (p <0.05) between different treatments.

Two main bacteria, an *Erwinia* spp. and a *Kluyvera* spp., were isolated from the rice medium. In order to verify whether these bacteria could be the nutrient source for *Strombidium* sp. NTOU1, the ciliate growth was monitored after either bacterium was added into the sterile ASW, respectively. The result confirmed that feeding either of the two bacteria alone was sufficient to culture the ciliate (**Figure 1**). To further investigate the bacteriovorous preference of *Strombidium* sp. NTOU1, other bacteria commonly found in the aquaculture environment, including *Escherichia coli*, *Vibrio harveyi,* and *Vibrio campbellii,* were also tested as the sole food source. At the initial bacterial concentration of 2.00×10^7 CFU/mL, all the bacteria we tested were able to sustain the culture of *Strombidium* sp., but the growth rate of ciliates was significantly higher when they were fed with the *Erwinia* spp. than in other conditions (**Figure 1**). Together, these results demonstrated that *Strombidium* sp. could utilize a variety of bacterial species as the food source, and the *Erwinia* spp. isolated from the rice medium is preferential in our culturing condition.

Establishment of a Standardized Procedure to Culture and Enrich *Strombidium* sp. NTOU1 in Large Scale

To optimize the utilization of bacterial food to culture *Strombidium* sp. NTOU1, *Erwinia* spp. received a pre-freezing treatment that allowed its batch-wise preparation and storage to reduce the possible variation in the nutritional status. As shown in **Figure 2**, freeze-treated *Erwinia* spp., similar to live bacteria, could be utilized to culture *Strombidium* sp. NTOU1 although the growth rate of ciliates was slightly reduced in 3 days ($\Delta\mu$ <0.05).

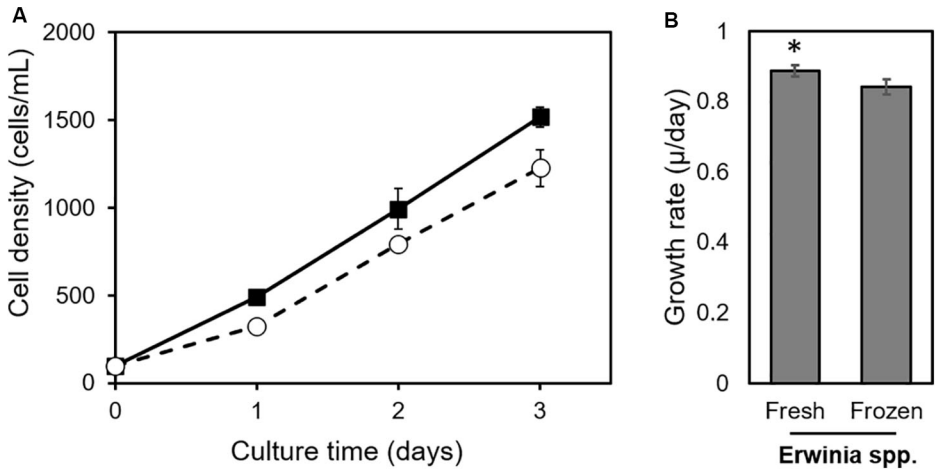

FIGURE 2 | Growth of ciliates fed with fresh or frozen *Erwinia* spp. **(A)** Cell density of *Strombidium* spp. NTOU1. Black rectangles, fed with fresh *Erwinia* spp. fodder; white circles, fed with frozen bacterial feeds. **(B)** Average growth rate of *Strombidium* spp. NTOU1. The asterisk indicates a significant difference between two groups (Student's *t*-test, $p < 0.05$). The error bars represent the standard deviations of the average from three independent experiments.

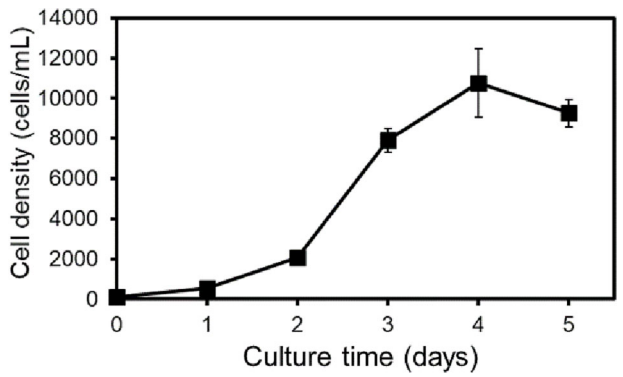

FIGURE 3 | Continuous culture of *Strombidium* sp. NTOU1 with frozen *Erwinia* spp. A continuous culture was carried out by adding 10,000 times the ciliate number of frozen *Erwinia* spp. as a food source with an initial ciliate density of 100 cells/mL. The error bar represents the SD of the average from three independent experiments.

This result indicated that the frozen *Erwinia* could be adopted as an amenable alternative of ciliate food.

Based on previous observation, it is noticed that excessive *Erwinia* spp. in the culture environment, especially when the initial ciliate density is low, might affect the growth rate of *Strombidium* sp. NTOU1 (**Figures 1**, **2B**). To minimize the number of environmental bacteria while maintaining enough nutrients for ciliates, a batch feeding approach was tested using less initial amounts of bacteria but adding the supplement daily. When *Strombidium* sp. NTOU1 was fed the daily bacteria dose about 10,000-fold of the ciliate number (less than 1/3 of the initial bacterial amount in the prior experiment), the ciliates with a starting density of 100 cells/mL were able to be raised to 1.07×10^4 cells/mL, approximately a hundred folds, through this batch feeding method in 4 days (**Figure 3**, $\mu = 1.21 \pm 0.033$).

In parallel, a suction filtration procedure was tested to enrich the ciliate density. Through a mild pressure control to effectively reduce the volume of culture medium, the concentration of the ciliates could be further increased by about 10 folds to reach 1.20×10^5 cells/mL. Microscopy observation showed that *Strombidium* sp. NTOU1 remained motile, and they could continue to grow in the subsequent culturing, suggesting that the suction filtration caused limited physical damages and the ciliate could recover from the enrichment process. In the further assessment, the cells were stained against alpha-tubulin, which is one of the major cytoskeleton proteins in the ciliate. The immunofluorescence observation also confirmed that the cilia structure of *Strombidium* sp. was not significantly impaired after our culture and enrichment processes (**Figure 4**). All these results demonstrated that we have established a feasible culturing and handling procedure in the laboratory.

Strombidium sp. NTOU1 Could Ingest and Remove Bacteria in the Culture Efficiently

To further characterize the ability of ciliates to ingest bacteria, the bacterial clearance rate of *Strombidium* sp. NTOU1. was estimated using a fluorescent *E. coli* pET-EGFP strain, in which an exogenous plasmid carrying the *EGFP* gene expression cassette was introduced to overexpress the traceable and quantifiable green fluorescent protein (GFP). After *Strombidium* sp. were starved for 24 h in advance to ensure no residual bacteria inside the ciliate cell, the ciliates were incubated with 3.75×10^5 cells of the *E. coli* pET-EGFP strain and the GFP fluorescent intensity in the culture was measured.

Without co-incubation with the ciliate, the fluorescence intensity of *E. coli* pET-EGFP strain in the ASW culture did not markedly change for 12 h (**Figure 5A**). When 50 or 150 cells of *Strombidium* sp. NTOU1 were added, however, the fluorescence decreased after 1 hr and the intensity continued to

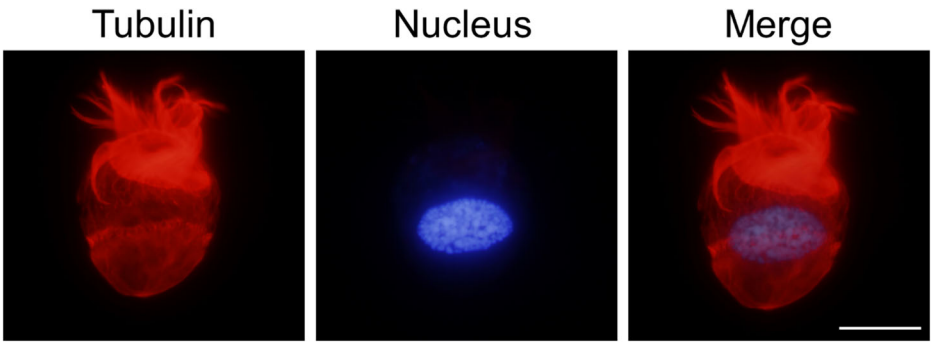

FIGURE 4 | Immunofluorescent staining of *Strombidium* sp. NTOU1. The microtubule structure and nucleus of *Strombidium* sp. NTOU1 were labeled using the anti-alpha tubulin antibody (red) and DAPI stain (blue), respectively. Scale bar, 50 μm.

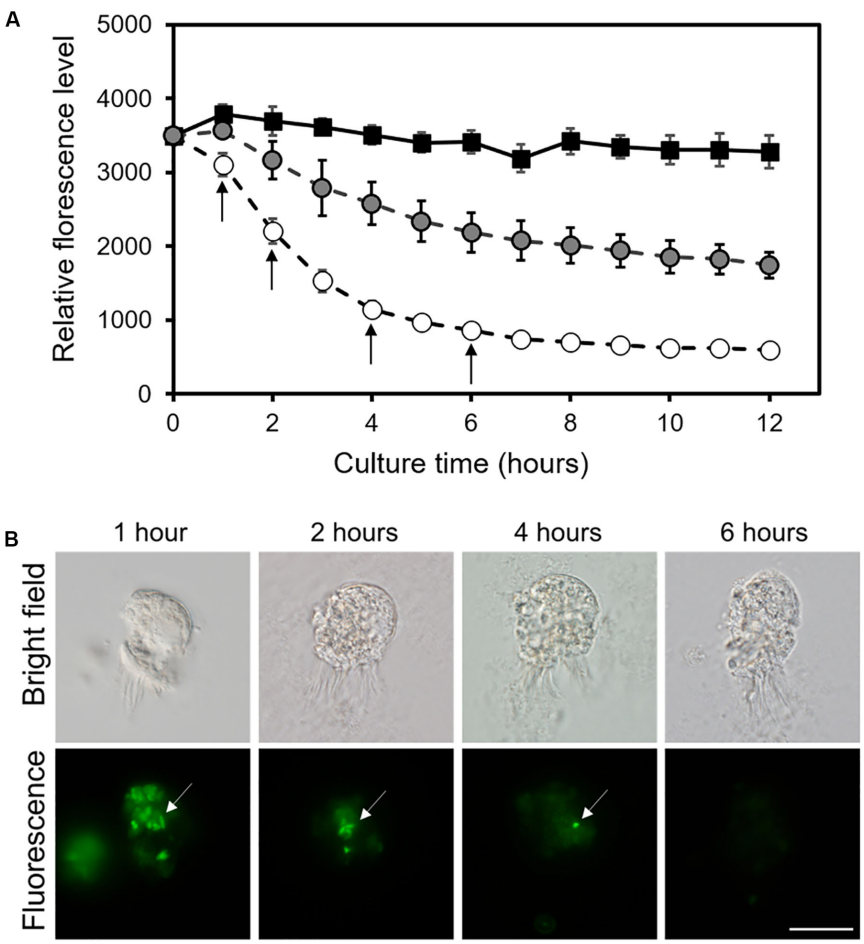

FIGURE 5 | The intensity and localization of the fluorescence from *E. coli* pET-EGFP strain after its feeding to *Strombidium* spp. NTOU1. **(A)** The fluorescent intensity of the *E. coli* pET-EGFP strain alone (black square) and *E. coli* with incubation with 50-cell (gray circle) or 100-cell (white circle) of ciliates at different time points. The error bar represents the SD of the average from three independent experiments. The black arrow indicates the time point for fluorescent microscopy observation. **(B)** Localization of fluorescent bacteria in the ciliate cell after 1, 2, 4, and 6 h of incubation. The white arrow indicates the food vacuole of *Strombidium* sp. NTOU1. Scale bar, 50 μm.

drop. Within 6 hrs, 150 cells of the ciliate could eliminate the fluorescent bacteria to reach an equilibrium state and to reduce the fluorescence intensity by ∼75.5 % (**Figure 5A**). Incubation with 50 ciliate cells also could lower the GFP fluorescence in the culture with a slower kinetic change. During this period, the fluorescent microscopy observation revealed that fluorescence

signals were accumulated into multiple 2-μm spheres in the ciliate cell, presumably the food vacuole of *Strombidium* sp., after 1 hr (**Figure 5B**). The number of food vacuoles in the ciliate, along with the vacuolar fluorescent intensity, gradually decreased within 6 hrs (**Figure 5**). Together, these results indicated that a significant fraction of pET-EGFP *E. coli* has been ingested and digested by the ciliate and removed from the culture. Calculating the number of fluorescent bacteria reduced during this period shows that the average clearance rate of each ciliate for fluorescent bacteria is 315 cells/hr.

Strombidium sp. NTOU1 Could Markedly Eliminate Vibrio in a Simulated Environment

That *Strombidium* sp. NTOU1 could lower the bacterial density in the culture prompted us to explore the potential to utilize this ciliate for biological control of marine pathogens in aquaculture. In order to verify whether *Strombidium* sp. NTOU1 possessed the ability to fight pathogenic bacteria in the actual environment, the bacterial clearance was measured under a milliliter-scale condition that mimicked the aquaculture environment. Aerated aquaculture seawater which contained 3,000–5,000 CFU/mL of bacteria (**Figure 6**), was chosen as the assaying background. In addition, based on the LC50 of the marine vibrio *V. campbellii* for grouper larvae reported in previous studies (Lam et al., 2011; Noor et al., 2019), a condition with a high dose of pathogens was set up to simulate the extreme situation in which vibrio infectious diseases have erupted.

In the background environment, the bacterial concentration was slightly fluctuated but remained at a low level $\sim 10^3$ CFU/mL. When *V. campbellii* was inoculated at a starting concentration of 2.00×10^6 CFU/mL, the pathogen further increased for more than one-fold to reach 4.38×10^6 CFU/mL in the aerated seawater (**Figure 6**). Adding 5,000 cells/mL of *Strombidium* sp. to such an environment, however, rapidly lowered the bacterial density within 20 mins and eliminated 80% of the bacteria in an hour (**Figure 6**). This result argues that it is possible to protect aquaculture fish from the vibrio infection using *Strombidium* sp. NTOU1.

Incubation With *Strombidium* sp. NTOU1 Lowered the Fish Lethality Caused by Vibrio Infection

To investigate whether *Strombidium* sp. NTOU1 could effectively alleviate the damage of fish caused by the actual vibrio infection, a pathogen challenge assay was performed using the grouper *Epinephelus coioides*, an economically important aquaculture fish in Taiwan, as the testing species. No vibrio, a high-dose of vibrio, or a high-dose of vibrio co-incubated with *Strombidium* sp. were, respectively, adapted into a tank with 5 L aerated water for an hour. 5 grouper larvae were added to those tanks and returned to normal condition after 1 h of the vibrio infection, and the fish mortality were monitored for 5 days.

Without challenging with vibrio (the control), grouper larvae were able to survive in aerated seawater without any mortality (**Table 1**). Once infected with *V. campbellii* at 3×10^6 CFU/mL, grouper larvae started to die after the 2nd day even after they returned to normal aerated seawater. The overall mortality rate was 66.67% after 5 days (**Figure 7**). In such an extreme environment, however, if additional 5,000 cells/mL of ciliates

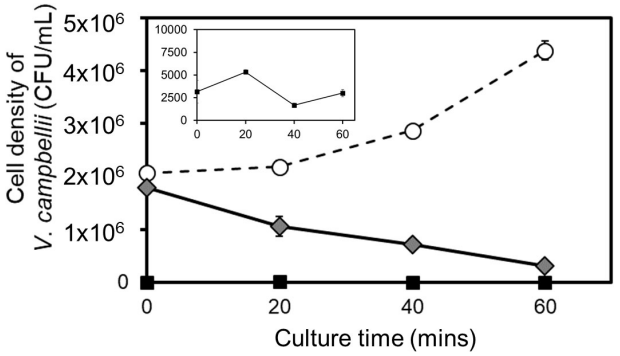

FIGURE 6 | Bacterial clearance by *Strombidium* spp. NTOU1. Black squares (also shown in the inset using an adjusted scale for clarity) indicate the number of bacteria in the aerated seawater as the background controlled group. White circles indicate the cell density of *V. campbellii* cultured alone. Gray diamonds represent the bacterial density after 5,000 cells/mL of ciliates were added. The error bar represents the S. D. of the average from three independent experiments.

TABLE 1 | Summary of the pathogen challenge assay from grouper larvae infected by *Vibro campbellii* with or without the addition of *Strombidium* sp. NTOU1.

Challenge assay						
Sample	**Assay 1 (*n* = 5)**	**Assay 2 (*n* = 5)**	**Assay 3 (*n* = 5)**	**Sum (*n* = 15)**	**Average percentage (%)**	***H*-test**
Control fish	5	5	5	15	100	–
Fish + VC	3	1	1	5	33	*
Fish + VC+ STM	5	5	4	14	93	–

VC, Vibro campbellii; STM, Strombidium sp. NTOU1; The asterisk indicates that this group is significantly different ($p < 0.05$) from the other two groups using Kruskal-Wallis H-test.

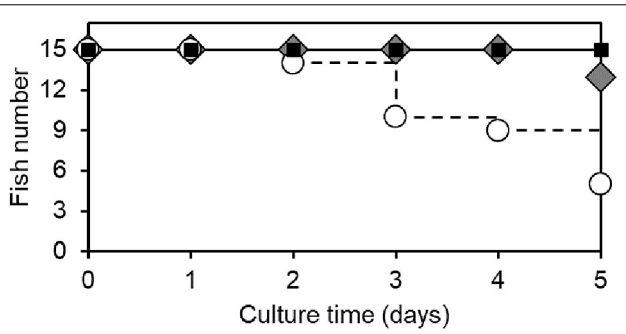

FIGURE 7 | Survival rate of grouper larvae infected with *Vibrio campbellii* with or without the addition of *Strombidium* spp. NTOU1. Black squares indicate the control group without either vibrio infection or addition of ciliates. White circles represent the group of vibrio-infected grouper without the addition of ciliates. Gray diamonds represent the group with the simultaneous addition of both vibrio and ciliates. The data is the sum of three independent experiments. Five fish larvae were used for each set of the experiment.

were co-cultured with the vibrio for an hour, the residual pathogen could not effectively infect the grouper larvae. In three sets of independent assays, only one grouper larva died on the 5th day after the challenge when they were protected by *Strombidium* sp., and the survival rate of groupers increased to 93.33% (**Figure 7** and **Table 1**). These experiments demonstrated the potential of *Strombidium* sp. NTOU1 to be applied to active biological control of pathogenic bacteria for grouper larvae.

DISCUSSION

We had established a robust procedure for large-scale culturing and handling of the marine ciliate *Strombidium* sp. NTOU1 and characterized its ability to ingest bacteria. The excellent bacteriovorous properties prompted us to investigate its potential for active biological control in aquaculture. Our result demonstrated that it could efficiently remove *Vibrio campbellii* from the environment and protect grouper larvae from infection.

As shown in **Figure 1**, *Strombidium* sp. NTOU1 could not survive in the sterilized medium, but it could utilize different types of bacteria as the nutritional source. Fluorescent microscopy observation confirmed that the preyed GFP-expressing bacteria appeared within food vacuoles in the ciliate, and the subsequent diminishing of the fluorescence suggested that the bacteria were degraded (**Figure 5B**). These results collectively argue that *Strombidium* sp. may mainly obtain nutrients from the preyed bacteria, which is similar to the bacteriovorous ciliate *Cyclidium glaucoma* (Posch et al., 2001). The growth rate of ciliates for feeding different bacteria was not significantly different except for *Erwinia* spp. and feeding *Strombidium* sp. with *Erwinia* spp. gave the maximum growth efficiency, indicating that *Erwinia* spp. can be used as a suitable nutrition provider in the routine culture of *Strombidium* sp. (**Figure 1**). Furthermore, the common marine bacterial pathogens in aquaculture, such as *V. campbellii* and *V. herveyi*, also could sustain *Strombidium* sp. NTOU1 growth, indicating that this ciliate did have the potential as a biological control species to prey on pathogens (**Figure 1**).

To develop an active biological control method for aquaculture, it is necessary to supply enough ciliate biomass to affect the pathogen population in the culture pond. Therefore, methods for improving the culture efficiency of *Strombidium* sp. is an important issue. In general, most culture methods for bacteriovorous ciliates rely on rice/wheat grain medium containing naturally grown bacteria or an organic medium rich in a single strain of bacteria (Christaki et al., 1998; Chen et al., 2013; Schaafsma and Peperzak, 2013; Jiang et al., 2019). Although the preparation in such methods is simple, the growth rate of ciliates was fluctuated due to the constant change of bacterial numbers in the medium (Christaki et al., 1998; Cho et al., 2019). Not only was it difficult to accurately control the growth rate of ciliates, but it also took a relatively long time to reach the maximum density of the culture. Here, we tested an alternative approach to supply the ciliate with a given amount of frozen bacteria. As shown in **Figure 2**, although the growth rate is slightly lower than that of the fresh bacteria feeding, the *Strombidium* sp. did use the frozen *Erwinia* spp. as a nutrient source. Another marine ciliate *Parauronema acutum* could also survive on dead bacteria-based medium (Soldo and Merlin, 1977; Hamsher et al., 2018), indicating that this method could be applied to culture other ciliates. Frozen bacteria can be stored and is convenient to use, and this approach also allows us to precisely control the total amount of bacterial food in the medium, making it feasible to consistently culture ciliates for practical applications.

Previous studies have shown that excess food organisms in the environment limited the growth of ciliates (Berk et al., 1976; Taylor, 1977). Ideally, the number of bacteria should be minimized while sufficient nutrients are provided. Therefore, a batch feeding approach was conducted to replenish the frozen *Erwinia* spp. daily with the cell number at the 10,000:1 ratio to the ciliate numbers during the culture process. From the bacterial clearance rate of *Strombidium* sp. for *E. coli* and *V. campbellii* (315/340 bacterial CFU/hr/ciliate; see **Figures 5**, **6**), the amount of bacterial feeding that we added was indeed close to the daily bacterial consumption of the ciliate. Through this optimized culture method, the growth rate of the ciliate gradually increased within 3 days and entered the stationary phase on the 4th day. It allowed ciliates to grow faster and to reach a higher cell density (1.07×10^4 cells/mL, **Figure 3**) than ciliates cultured in the rice medium (**Supplementary Figure S1**). Meanwhile, we tested an enrichment step that the ciliate density could be further concentrated by suction filtration to reach 1.20×10^5 cells/mL without apparent physical damages to the ciliate (**Figure 4**). Altogether, we made a critical technical improvement to culture and handle this ciliate, which lays down the foundation for utilizing *Strombidium* sp. NTOU1 strain in future academic research or biotechnology applications.

Feeding with the fluorescent *E. coli* confirmed that *Strombidium* sp. NTOU1 could rapidly remove bacteria from the water body by capturing bacteria and digesting them in food vacuoles (**Figure 5**). Several lines of evidence argue that this bacteriovorous property can be adopted for active biological control. The bacterial clearance rate of *Strombidium* sp. NTOU1 (∼300 bacteria/hr/ciliate) is slightly higher than that of other marine ciliates in previous reports (Berk et al., 1976; Christaki et al., 1998), indicating that this species potentially has advantages over other reported marine ciliates in biological protective effects against bacterial infections. In the simulated extreme environment at the milliliter scale, *Strombidium* sp. NTOU1 was able to effectively eliminate 85 % of bacterial pathogens *V. campbellii* within an hour (**Figure 6**). Increasing the testing scale to 5 L, *Strombidium* sp. also maintained the bacterial clearance ability. During three independent trials to protect grouper larvae from vibrio challenge, the survival rate was significantly increased to 93%, a striking contrast to 33% of the no-ciliate group (**Figure 7**). For the ciliate-protected group, only one grouper larva died on the last day of the 5-day observation. Furthermore, we noticed that no typical symptom

of vibrio infectious was observed in the sole dead larva from the ciliate-protected group while the other grouper larvae died from *V. campbellii* infection showed obvious white wounds (data not shown), implying that there might be other causes unrelated to vibrio infection. These results argue that the ability to limit vibrio growth by *Strombidium* sp. and its biological protection was as good as using bacteriophages (Wagner and Waldor, 2002). Also, utilizing ciliates for biological control of pathogenic bacteria offers several advantages. First, a bacteriophage may promote the pathogen to develop and select for antiviral mutations, and there is a risk of transduction-mediated horizontal gene transfer between bacteria (Labrie et al., 2010; Keen et al., 2017). Using ciliates is relatively safer. Second, due to the specificity between a phage to its host pathogen, it is necessary to evaluate the bacteria strains in the culture environment before selecting a suitable phage (Pereira et al., 2011), which limits its usage and may not be feasible in practical applications. *Strombidium* sp. NTOU1 could use different bacteria as the food source in our experiment (**Figure 1**), and the average bacterial clearance efficiency for *E. coli* and *V. campbellii* was similar (**Figures 5**, **6**). These results imply that *Strombidium* sp. NTOU1 has the potential to combat multiple pathogenic bacteria in the actual culture environment where the infection source is unknown and variable from case to case.

As shown in **Figure 5**, we found that even with the presence of enough *Strombidium* sp. NTOU1, bacteria at a density less than 2.5×10^4 cells/mL were unable to be further reduced. This suggests that, unlike the environmental hazard caused by antibiotics (Cabello, 2006; Martinez, 2011), the excess *Strombidium* sp. might not affect the original microbial population in the culture pond, which helps to maintain the ecological balance. In addition, while parasitic ciliates such as *Uronema nigricans*, *Ichthyophthirius multifiliis*, or *Cryptocaryon irritans* could pose a potential risk to infect aquatic organisms, to our knowledge, no studies have found that any ciliate in the genus *Strombidium* has the potential to affect the health of aquatic organisms (Montagnesa et al., 1996; Montagnesa and Lessard, 1999; Dolan, 2018; McManus et al., 2018). In fact, several studies have suggested that *Strombidium* ciliates are ideal biological feed for larvae of crustaceans and fish (Maeda and Liao, 1994; Thompson et al., 1999; Côrtes et al., 2013). *Strombidium* ciliates feeding on different food sources could accumulate specific nutrients, such as amino acids and unsaturated fatty acids, within their cells (Kramhøft et al., 1997; Wallberg et al., 1997; Fujibayashi et al., 2018). These studies and our results collectively support that there is great potential to use *Strombidium* for versatile applications in aquaculture.

In summary, we successfully overcome the technical limitations to utilize *Strombidium* sp. NTOU1 and establish a standard process for culturing and preparation. The concept of culture can be applied to increase the growth rate of other ciliates. In addition, the challenging assay result demonstrated that *Strombidium* sp. NTOU1 could protect aquatic organisms from the threat of bacterial disease. These studies provide a new perspective on the environmentally safe approach to reduce the biomass of harmful bacteria in aquaculture environments.

DATA AVAILABILITY STATEMENT

These data generated from this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee, College of Life Sciences, National Taiwan Ocean University (IACUC Approval No. 102025).

AUTHOR CONTRIBUTIONS

H-YL, C-CT, and H-JL: conceptualization. W-YY: experimental operation. H-YL: writing – original draft preparation. S-FT, C-CT, and H-JL: writing – review and editing. S-FT and K-PC: field sampling. S-FT: ciliate identification. JL: Vibro isolation and identification. H-JL: funding acquisition. All authors read and approved the final manuscript.

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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.2020.00373/full#supplementary-material

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A Multidisciplinary Experimental Study on the Effects of Breeders Diet on Newborn Seahorses (*Hippocampus guttulatus*)

Miquel Planas[1], Ike Olivotto[2], María Jesús González[3], Rosaria Laurà[4] and Matteo Zarantoniello[2]*

[1] Department of Ecology and Marine Resources, Instituto de Investigaciones Marinas (CSIC), Vigo, Spain, [2] Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona, Italy, [3] Marine Products Chemistry, Instituto de Investigaciones Marinas (CSIC), Vigo, Spain, [4] Dipartimento di Scienze Veterinarie, Università di Messina, Messina, Italy

The global trade in seahorses is increasing and new rearing techniques are currently available for a few species. One of the main bottlenecks in seahorse production is reproductive success, which is dependent upon the administration of high quality diets to breeders. However, little is known about the most adequate diets, on how they should be administered and on how they might affect newborn performance. In this study, three homogeneous groups of adult seahorses *Hippocampus guttulatus* were maintained in captivity. Each group was fed on one of the following diets since the onset of the breeding period: unenriched adult *Artemia* (diet A), unenriched adult *Artemia* and mysidaceans (diet AM) and mysidaceans (diet M). As a positive control, a wild pregnant male was used. The mixed diet AM provided the highest overall breeding success and reasonable fatty acid profiles, and better resembled newborn from a wild male. The use of mysidaceans solely (Diet M) provided the worse results in spite of the high DHA content. Significant differences were observed in newborn characteristics and fatty acid profiles among dietary groups and along the breeding season, especially in total n-3 HUFA and DHA. Monospecific diets including *Artemia* or mysidaceans very likely suffered nutritional deficiencies, which were reflected in morphological alterations of the muscle tissue (diet A) or abnormal early mortalities in newborn (diet M). Three progressive stages were identified along the whole breeding period: initial mixed capital-income period (100–120 days since the onset of the breeding period), followed by an inflection period (short transitional stage – income sources), and finally a long period characterized by the use of income sources and the progressive exhaustion of body reserves. Special attention should be deserved to the progressive changes in fatty acid profiles along the breeding season, resulting in a decrease in newborn performance. Considering the time required for a diet to be reflected in newborn, we recommend *Hippocampus guttulatus* breeders to be fed on a high quality diet for at least 3 months prior the breeding period.

Keywords: syngnathidae, seahorse, *Hippocampus*, diet, breeding, newborn, quality

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Yun-Zhang Sun,
Jimei University, China

****Correspondence:***
Miquel Planas
mplanas@iim.csic.es

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INTRODUCTION

The global trade in seahorses is estimated to exceed 20 million animals per year to sustain the aquarium trade, the traditional Chinese medicine and as souvenirs (Vincent, 1996; Planas et al., 2017a). In addition, because of the destruction and degradation of their coastal habitats (seagrasses, coral reefs, and mangroves), wild populations are progressively declining (Vincent, 1996; Martin-Smith et al., 2004). At present, many are the concerns over their long-term viability in nature (Vincent, 1996; Vincent et al., 2011; Kumaravel et al., 2012) and all seahorses species are included in Appendix II list of endangered species by CITES (Convention for the International Trade in Endangered Species).

Aquaculture represents a valid alternative to wild captured seahorses but *ex-situ* production still needs to face several biological and technical challenges (Cohen et al., 2016; Planas et al., 2017a). Both, low spawning quality and juvenile survival rates are the most critical factors for optimizing commercial seahorse aquaculture (Adams et al., 2001; Chang and Southgate, 2001; Lin et al., 2007).

Reproduction represents a key aspect in breeding success, and broodstock feeding/nutrition is doubtless, one of the key factors to sustain reproduction in teleost (Migaud et al., 2013). The dietary effect on the output performance (egg and fry quality and quantity) is well known in fishes, and many studies have reported the importance of lipids and fatty acids (FAs) (Rainuzzo, 1993; Bromage, 1995; Izquierdo et al., 2001; Sargent et al., 2002; Tocher, 2003, 2010; Pavlov et al., 2004). Broodstock preparation is thus a critical step in the success of the spawning process of a certain species. On this regard, many studies have been directed toward egg quality in oviparous fishes and the underlying characteristics to produce viable fry (Kjørsvik et al., 1990; Brooks et al., 1997). The egg's potential to produce viable fry is determined by several physical, genetic, and chemical factors, including the initial physiological processes occurring in the egg itself (Kjørsvik et al., 1990).

The ongoing process of oocytes maturation is one of the most metabolic demanding activities in fish and the quality of parental nutrition is thus of primary importance for the appropriate allocation of macromolecules into the oocytes and their maturation (Izquierdo et al., 2001; Volkoff and London, 2018). However, there is a scarcity of studies focused on breeding performance and egg or fry quality in fishes with male pregnancy such as syngnathids (pipefish and seahorses). Due to the uncommon nature of reproduction, syngnathids offer a unique opportunity to examine pathways of repercussion of breeder's dietary resources on breeding performance and the resulting newborn quality. Reproduction in seahorses has received increasing attention in recent years, mostly on the effect of environment (e.g., photoperiod regime and temperature) (Lin et al., 2006, 2008b; Planas et al., 2010, 2013) zootechnical conditions (e.g., sex ratio, aquaria design, and seahorse size) (Woods, 2000; Dzyuba et al., 2006; Faleiro et al., 2008, 2016; Planas et al., 2008) and biological / physiological features (Boisseau, 1967; Carcupino et al., 2002; Poortenaar et al., 2004; Stölting and Wilson, 2007; Scobell and MacKenzie, 2011; Zhang et al., 2019; Wittington and Friesen, 2020). Only a few studies have focused on the effects of feeding and dietary sources on breeding success and newborn features in seahorses under *ex-situ* conditions (Lin et al., 2007; Faleiro and Narciso, 2010; Binh and Serrano, 2012; Otero-Ferrer et al., 2012, 2016, 2020; Palma et al., 2012, 2017; Saavedra et al., 2015).

Seahorses (*Hippocampus* spp.) are batch spawners with cyclical egg maturation and repeated mates within the breeding season. The eggs produced by females are transferred to the brood pouch of males, where embryogenesis will take place for 2–4 weeks, depending on the species and temperature (Foster and Vincent, 2004). The sealed brood pouch of the male works as a pseudo-placenta, not only providing protection and oxygen to the developing embryos, but also nutrients (Wilson et al., 2001; Carcupino et al., 2002; Stölting and Wilson, 2007). As a consequence both female and male nutrition play a role on the embryonic and fry quality and development, but unfortunately, this topic remains one of the less studied subjects in seahorses (Olivotto et al., 2011b; Cohen et al., 2016).

The European long-snouted seahorse *Hippocampus guttulatus* Cuvier has been identified as a potential candidate for the marine ornamental trade (Cohen et al., 2016). Rearing techniques are currently available for this species but the breeding success (especially mating events) are still not optimized (Olivotto et al., 2011b; Planas et al., 2017a). Male competition is rather frequent resulting in mating interruption, and juvenile number and size decrease over the time in captive conditions (Faleiro et al., 2008). A bottleneck in breeding performance relies on both type and nutritional quality of diets. In captivity, both frozen and alive feed are usually used to nourish the broodstock, including mysid shrimps, amphipods and *Artemia* brine shrimps (Woods and Valentino, 2003; Olivotto et al., 2008; Palma et al., 2008; Murugan et al., 2009; Planas et al., 2017a). As concerns mysid shrimps, they are generally considered an optimal diet for seahorses (Woods and Valentino, 2003; Otero-Ferrer et al., 2012). Nutritionally enriched *Artemia* has received great attention and is presently widely employed (Woods, 2001; Wong and Benzie, 2003; Woods and Valentino, 2003; Planas et al., 2008) especially because it is easily cultured up to the adult stage, provides satisfactory n-3 HUFA and docosahexaenoic acid (DHA) contents after a long-time enrichment (Planas et al., 2017b) and supports high growth rates in *H. guttulatus* (Planas et al., 2008). However, it has been reported that the breeding performance in *H. hippocampus* decreased when fed on *Artemia* compared to diets including mysidaceans (Otero-Ferrer et al., 2012).

In the present study, the following questions have been addressed: (1) how experimental diets are assimilated (stable isotope analysis) by breeders and reflected in general features and biochemical composition of *H. guttulatus* newborn? (2) do mixed diets perform better than monospecific ones? and (3) Does *H. guttulatus* follow a mixed income-capital breeding strategy? The main objectives of the study were (1) to propose a breeder's diet enhancing reproduction performance in the selected species, and (2) to ascertain when and how long the diet should be administered to provide its beneficial effect. To our knowledge, this is the first multiapproach study on the effect of breeder's diet on reproductive performance and newborn quality in *H. guttulatus*.

MATERIALS AND METHODS

Microalgae and Live Prey

Microalgae (*Phaeodactylum tricornutum*, *Rhodomonas lens*, and *Isochrysis galbana*) were cultured at 22 ± 1°C in 80 L plastic bags containing sterilized seawater supplemented with F2P (100 g L^{-1}) media (VarAqua). Additionally, silicates were added to *P. tricornutum* cultures, and 200 μL F2P media to the *R. lens* culture flasks.

Artemia cysts (AF, Inve, Spain) were hatched at 28°C for 20 h in 20 L units, and the freshly hatched nauplii (614 ± 140 μm in length) gently rinsed with tapwater, collected on a 125 μm mesh, rinsed, and offered to seahorse juveniles.

Adult non-enriched *Artemia* was produced to feed adult seahorses during the breeding period. For that, the nauplii were hatched from MC450 cysts (Ocean Nutrition, Spain) and grown in 100 L units, at 26–28°C with gentle aeration and constant light. Adult *Artemia* was long-time enriched (3–6 days) to feed breeders prior to the start of the experiment but not during the experimental period. The enrichment was carried out in *Artemia* from day 16 onward on a mixture consisting on live microalgae *P. tricornutum* and *I. galbana* (10^7 cells mL^{-1}), red pepper (0.015 g L^{-1}), and dried *Spirulina* (0.03 g L^{-1}) (Planas et al., 2017a).

Copepods (*Acartia tonsa*; 614 ± 140 μm in length) were cultivated for the early feeding of seahorse juveniles in 700 L tanks at 26–27°C and 38 salinity, at an initial density of 1 copepod mL^{-1}. Copepods were fed every two days on the microalgae *R. lens* (10^3 cells mL^{-1}). Siphoning of the culture tanks and water renewals (10% of the total volume) were carried out three times per week.

Seahorse Breeding and Rearing

Animal capture, handling and sampling were conducted in compliance with all bioethics standards on animal experimentation of the Spanish Government (Real Decreto 1201/2005, 10th October 2005) and the Regional Government Xunta de Galicia (REGA ES360570202001/15/FUN/BIOL.AN/MPO01).

The adults of *H. guttulatus* seahorses used in the present study were reared in captivity or collected in Galicia (NW Spain) from August 2016 to January 2017 with permission of the Regional Government Xunta de Galicia. Prior to the start of the experiment, seahorses were maintained at the facilities of Instituto de Investigaciones Marinas (CSIC) in Vigo (Spain) in 320 L aquaria in a semi-closed system with a 10–14% daily renewal of seawater (Planas et al., 2008) and submitted to temperature and photoperiod natural-like regimes (Planas et al., 2010, 2013) fluctuating from 15°C (Winter) to 19°C (Summer) and from 10L:14D (Winter) to 16L:8D (Summer), respectively. Water quality was checked periodically for NO_2, NO_3, and NH_4/NH_3 content (0 mg L^{-1}). Salinity and pH levels were 37 ± 2 and 8.0 ± 0.2, respectively, for both species. Wastes and uneaten food were removed daily by siphoning the bottom of aquaria.

Males and females were maintained separate until the start of the experiment in mid-March 2017, when all seahorses were randomly distributed (1:1 sex ratio; six pairs per aquaria) in three 320 L aquaria and maintained for a whole breeding season (until early October 2017). All aquaria were maintained under the same conditions except for the diet administered. Temperature and photoperiod regimes were as described above. The average size (curved standard length) and weight of seahorse breeders (17.4 ± 2.6 cm and 11.50 ± 5.0 g, respectively) did not differ across experimental groups (ANOVA, p = 0.702, 17.4 ± 2.6 cm; ANOVA, p = 0.470, 11.5 ± 5.0 g). Seahorse broodstocks were continuously monitored to check for newborn release from male's pouch.

Prior to the breeding period, all breeders were fed for 2–5 months on a diet based on enriched adult *Artemia* and captured/frozen mysidaceans (*Siriella armata* and *Leptomysis* sp.). The length of the prey was 5–7 mm for *Artemia* and about 1–1.5 cm for mysidaceans. During the breeding period, each husbandry aquaria received two daily doses (*ad libitum*) of different diets (**Figure 1**):

- Diet A: Cultivated adult non-enriched *Artemia*.
- Diet M: Captured (*Siriella armata* and *Leptomysis* sp.) and frozen (*Neomysis* sp.; Ocean Nutrition, United States) mysidaceans (1:1).
- Diet AM: Mixture (1:1) of diets A and M.

Small portions of the breeders dorsal fin (partial fin-clipping; about 20 m^2 surface) were sampled at the onset and at the end of the breeding period, and properly stored for further stable isotopic analysis (SIA) (Valladares and Planas, 2012).

Newly released juveniles were carefully collected by siphoning, counted and transferred (2–5 juveniles L^{-1}) to 30 L pseudo-kreisel aquaria connected to a semi-opened recirculation system (Blanco et al., 2014). Each batch was cultivated in duplicate (whenever possible) until day 7 after male's pouch release (DAR) at 19°C under a constant 14L:10D photoperiod. The aquaria were filled with seawater filtered by a series of filter-cartridges (20, 10, 5, and 1 μm) and UV treated (76 w; 16 L min^{-1}) (JR1/50). The rearing system included a degasifying column and two 50 L chambers including mechanical (up to 20 μm) and biological filters (perforated plastic bio-balls) and aerators. From the biofilter unit, the seawater was pumped to 36 w UV units (AquaMedic®, Germany) and then to a 50 L reservoir aquarium, being finally routed by gravity toward the rearing aquaria (Planas et al., 2012).

Newborn seahorses were fed according to the following optimized schedule (Blanco and Planas, 2015):

- 0–5 DAR: Two daily doses of *Acartia tonsa* (0.67 copepods mL^{-1} $dose^{-1}$).
- 6–7 DAR: One daily dose of *Acartia tonsa* (0.67 copepods mL^{-1} $dose^{-1}$) and one daily dose of *Artemia* nauplii (1 *Artemia* mL^{-1} $dose^{-1}$).

Dead seahorses were removed (8:00 am and 15:00 pm) and counted daily. Final survivals of juveniles were recorded at 7 DAR (M_{50} at 18–21°C occurs at 6.3–6.7 DAR) (Planas et al., 2012).

For each experimental group, samples of newborn juveniles (see further sections for details) were randomly collected before

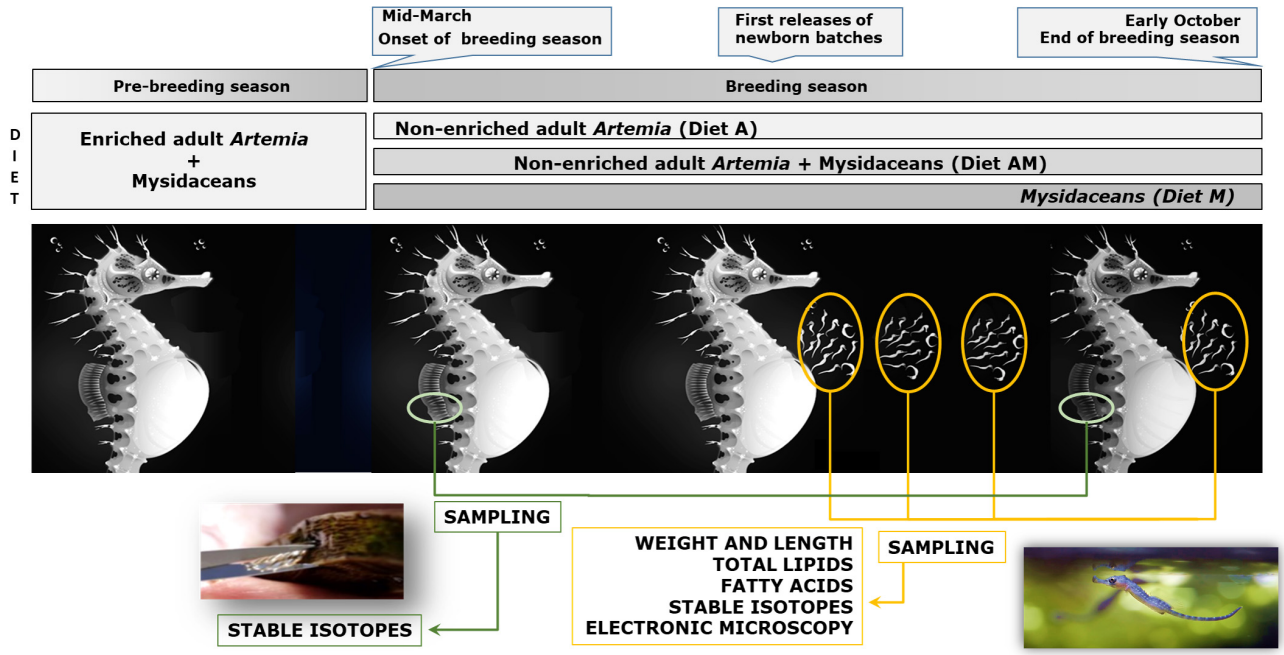

FIGURE 1 | General scheme of the experimental framework (breeders fed on diets A, AM, and M) and sampling (dorsal fin and newborn). Seahorse illustration by Sergio Picazo Ferro (https://sergiopicazo.com/).

first feeding, euthanized with Tricaine MS-222 (0.1 mg L^{-1}, Sigma Aldrich), rinsed with distilled water, and conserved at 80°C for further FAs and isotopic analyses.

For length and weight measurements, sampled seahorses (n = 15) were transferred to Petri dishes, photographed and weighed (pooled individuals) on a Sartorius microbalance MC210P (±0.01 mg). Curved standard lengths (SL = head + trunk + curved tail) were measured from digital photographs using an image processing software (NIS Elements, Nikon).

Sample Analysis

Total lipids from live prey and juveniles (pools of 10–20 mg dry weight per sample) (i.e., 25–40 newborn) were extracted according to Bligh and Dyer (1959). Aliquots of total lipid extracts with known lipid content were centrifuged, resuspended with 0.5 M ammonium formate solution, freeze-dried and stored at −80°C until further FAs analyses. Total lipid content was quantified gravimetrically (Herbes and Allen, 1983). FA composition of lipids was analyzed by gas-chromatography (GC) according to Christie (1982). Lipids were transmethylated (Lepage and Roy, 1986) and Fas analyzed by GC (Perkin Elmer, Clarus 500 gas chromatograph) as described in Planas et al. (2010). Samples were analyzed in duplicate.

Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope signatures and elemental composition (total C and N) were analyzed in both prey and seahorse samples (fin of breeders and bulk juveniles). Frozen juveniles (n > 5; pooled individuals) were dried for 48 h (60°C), weighted and manually homogenized. The analyses were performed on sub-samples of 0.2–1.0 mg dry weight biomass at Servizos de Apoio á Investigación (SAI) of the University of A Coruña (Spain) as reported by Valladares et al. (2016). As defatting of samples prior to isotopic analysis is recommended when lipid content exceeds 5% weight (C:N > 3.56) (Post, 2002) samples were not defatted but arithmetical corrected factors (**Supplementary Table 1**) were applied considering defatting (copepods, *Artemia* nauplii and metanauplii, and seahorse fins or juveniles) or defatting + acidification (10% HCl) (Mysidacea and adult *Artemia*). The samples were measured by continuous flow isotope ratio mass spectrometry using a FlashEA1112 elemental analyzer (Thermo Finnigan, Italy) coupled to a Delta Plus mass spectrometer (FinniganMat, Bremen, Germany) through a Conflo II interface. Stable isotope abundances are expressed as permil (‰) in conventional delta relative to VPDB (Vienna Pee Dee Belemnite) and Atmospheric Air. The precision (standard deviation) for the analysis of $\delta^{13}C$ and $\delta^{15}N$ of the laboratory standard (acetanilide) was ± 0.15‰ (1-sigma, n = 10). Standards were run every 10 biological samples.

Transmission electron microscopy (TEM) was applied to abdominal portions of seahorses (three newborns per dietary treatment). The samples were processed for Durcupan ACM (Fluka) resin embedding as follows: pieces fixed in 2.5% glutaraldehyde in 0.2 M phosphate-buffered saline (pH 7.4), for 2 h at 4°C, were washed repeatedly in the same buffer and postfixed in 1% osmium tetroxide in 0.2 M phosphate-buffered saline for 1 h at 4°C. Then, the tissues were dehydrated with increasing alcohol concentrations. The dehydrated pieces were embedded in Durcupan ACM (Squadrito et al., 2017). Finally, the sections were obtained with a Reichert Jung Ultracut E.

Semi-thin sections (1 μm) were stained with toluidine blue and examined with a light microscope. Ultrathin sections (740 Å) were obtained from selected areas of the semi-thin sections, stained with uranyl acetate and lead citrate, and examined and photographed with a transmission electron microscope (JEOL JEM 1400 Flash) (Viña et al., 2014).

Data Analysis

All statistical analyses were conducted in R v.3.6.1 (R Core Team, 2014). Variability across treatments was examined by ANOVA or non-parametric Kruskal–Wallis test for independent samples, depending on significant deviations from normality and homogeneity of variance, as examined by the Shapiro–Wilks and Levene tests, respectively (ggpubr v0.2.5 R package) (Kassambara, 2020a). When significant, A Kruskal–Wallis test followed by Wilcoxon range comparisons were applied (Pgirmess v1.6.9 package of R) (Giraudoux et al., 2018). Significance levels were set at $p < 0.05$.

The Jonckheere–Terpstra trend test (J–T test) was applied to individual FA data for an ordered alternative hypothesis within an independent sample (between-seahorse groups) design (clinfun v1.0.15 package in R) (Venkatraman, 2018). The test has more statistical power than the Kruskal–Wallis test, incorporating information about whether the order of the groups is meaningful (Siegel and Castellan, 1988). Hence, we applied the test to check whether the more proportions of mysidaceans in the diet (order: A – AM – M), the more the independent variable tested will go down or up.

Correlations between variables in the whole dataset and the resulting network plots of variables were explored with ggraph v2.0.2 (Pedersen, 2020) and corrr v0.4.2 (Kuhn et al., 2020) R packages. Hierarchical clustering of both variables and newborn batches was performed with Ward's method (provided the highest agglomerative coefficients) using factoextra v1.0.7 (Kassambara, 2020b) and ComplexHeatmap v3.11 (Gu et al., 2016) packages in R. Additionally, principal component analyses (PCA) were performed to summarize and visualize the information of the datasets. For that, we used factoMineR v2.3 (Husson et al., 2020), factoextra v1.0.7 (Kassambara, 2020b), and corrplot v0.8.4 (Wei et al., 2017) packages in R. The data values were standardized (mean = 0; sd = 1) for clustering and PCA.

Graphics were constructed using ggplot2 v3.3.0 (Wickham et al., 2020) and lattice v0.20-41 (Sarkar et al., 2020) packages in R.

RESULTS

Diet Assimilation

The protein content in breeder's diets increased from diets A to M, whereas lipid content decreased due to the lower lipid content in mysidaceans compared to adult *Artemia* (% C and C/N in **Table 1**). Prey (and diets) offered to seahorse breeders also differed notably in FA profiles (**Table 2** and **Supplementary Figure 1**). Compared to mysidaceans, adult non-enriched *Artemia* was richer in saturated FA (35.3%) (16:0, 17:0, and 18:0), n-6 FA (18:2n-6c and 18:3n-6c) (21.5%), and n-7 FA (18:1n-7) (18.8%). Captured and frozen mysidaceans were remarkably richer than *Artemia* in n-3 HUFA (42.6–45.2%, respectively) (EPA and especially DHA) and showed much higher DHA/EPA, DHA/ARA, and n-3/n-6 ratios.

The incorporation of the experimental diets in breeder tissues was analyzed considering dietary isotopic profiles (**Table 1**) and the resulting isotopic changes in dorsal fins (initial vs final) (**Table 2**). Differences in isotopic signatures of diets corresponded approximately to one trophic level, with values ranging from −22.12 to −20.53‰ for $\delta^{13}C$ ($\Delta = 1.59$) and from 7.67 to 10.83‰ in $\delta^{15}N$ ($\Delta = 3.16$).

Initial isotope values for $\delta^{13}C$ and $\delta^{15}N$ in dorsal fin at the onset of the experimental breeding period did not differ significantly (ANOVA, $P = 0.192$ and 0.143, respectively) (**Table 2**). At the end of the experiment, changes in signatures for both isotopes in fins were inversely correlated (linear regression, $R^2 = 0.994$) across treatments (**Supplementary Figure 2**) and strongly agreed with isotope values of the corresponding diets, with significantly lower $\delta^{15}N$ signatures in the group fed uniquely on *Artemia* (K–W test, $P = 0.044$). Isotopic changes ($\Delta\delta$) in fins during the breeding season were 2.97, −0.20, and −1.89 for $\delta^{13}C$, and −1.42, 0.88, and 2.58 for $\delta^{15}N$, respectively (**Table 3**). Isotopic changes between final isotopic values in dorsal fins and diets A, AM, and M were 8.13, 4.14, and 2.50 for $\delta^{13}C$, and 2.62, 4.25, and 3.91 for $\delta^{15}N$, respectively.

Breeding Performance

The total amount of newborn batches produced in treatments A, AM, and M were 4, 7, and 3, respectively, and the total production of newborns were 534, 1,416, and 248, respectively (**Table 4**). Average batch size in treatment AM (202 ± 82 newborns; max = 343) was higher than in treatments A (134 ± 83) and M (84 ± 72) but differences across treatments were not significant (ANOVA, $P = 0.331$) due to the large standard deviations of means. Newborn production started earlier and extended over a longer period for group AM breeders (from day 72 to 210 of the breeding period). The opposite occurred in group M (from days 152 to 179). In group A, newborn batches were released from day 121 to day 202.

Newborn differed in length (K–W test, $p = 0.018$) but not in dry weight (0.60–0.74 mg) (K–W test, $P = 0.440$) (**Table 4** and **Supplementary Figure 3**). Newborns from treatment A (12.9 ± 0.7 mm) were significantly smaller in length than those from treatments AM (14.7 ± 0.7 mm) and M (14.1 ± 0.2 mm).

Survivals at 7 DAR were high but not significantly different across treatments (K–W test, $P = 0.066$) (**Table 4**), ranging from 94.0 ± 5.3% in treatment A to 61.4 ± 19.2% in treatment M. First mortalities in treatment M occurred at 2 DAR, with a peak at 4–5 DAR (**Figure 2**). Similarly to newborn reared from a batch released by a wild male, first mortalities in treatments A and AM were delayed until 3–4 DAR.

Newborn Characteristics

Pooled samples of newborn differed in $\delta^{15}N$ across treatments (K–W test, $P = 0.044$) but not in $\delta^{13}C$, ranging from 11.21 to 14.96‰ and from −17.31 to −13.86‰, respectively (**Table 2** and **Supplementary Figure 3**). Mean $\delta^{15}N$ value in group A was significantly lower than those from the other groups. However,

TABLE 1 | Average isotopic profiles ($\delta^{13}C‰$ and $\delta^{15}N‰$), total C and N, and C/N ratios in prey (A – *Artemia*, M_c – Captured mysidaceans, and M^f – Frozen mysidaceans) (n = 3) and in diets for breeders (diets A, AM, and M) of *Hippocampus guttulatus*.

		% C	% N	C/N	$\delta^{13}C‰$	$\delta^{15}N‰$
Prey	Adult *Artemia* (A)	43.8	10.3	4.25	−22.12	7.67
	Captured Mysidacea (M^c)	42.3	12.9	3.92	−24.34	12.71
	Frozen Mysidacea (M^f)	38.7	9.3	4.16	−16.72	8.96
Diet	A	43.8	10.3	4.25	−22.12	7.67
	AM	42.2	10.7	3.95	−21.32	9.25
	M ($M_c + M_f$)	40.5	11.1	3.65	−20.53	10.83

See text for further details on the composition of diets. Raw isotopic data were transformed considering conversions provided in ***Supplementary Table 1****.*

mean isotopic values did not reflect the pattern of change across treatments during the breeding period. The low number of batches released in treatments A and M did not allow a precise analysis of isotopic changes but a trend could be inferred from treatment AM, in which a progressive change in $\delta^{15}N$ toward those in the diet was noticed throughout the breeding season, with signals decreasing from 16.0 to 12.3‰.

Total lipids in newborn were similar in all experimental groups (12.17–12.99% DW) (K–W test, $P = 0.580$), but significant differences were noticed for some FA in total lipids (**Table 2**). Significant differences across treatments (K–W test, $P < 0.05$) were obtained for 15:0, 17:0, 18:1n-7, 20:1n-9, 20:2n-6, 20:4n-6, and 22:6n-3, total polyunsaturated FA and DHA/EPA ratio. J–T test revealed that increasing proportions of mysidaceans in the diet of breeders promoted significant increasing trends in newborn for 14:0, 15:0, 17:0, 18:1n-9t, 20:1n-9, 20:2n-6, 20:4n-6, 22:6n-3, and n-3 HUFA and DHA/EPA ratio, and declines in 16:1n-7, 18:1n-7, and 18:2n-6c (**Table 2**). The FA profile of newborn released by a wild male better resembled those from groups AM or M.

Global Assessment

From the correlation network developed for treatment AM (**Figure 3**) it can be observed that time (days elapsed from the onset of the breeding season) negatively affected survival, the relative content in saturated FA (particularly 14:0) and $\delta^{15}N$ values, but increased the content in total n-6 FA (mainly 18:2n-6c) and 18:3n-3. Length and dry weight of newborn were positively correlated but they did not correlate with time. Size and weight were negatively correlated with $\delta^{13}C$. In addition, weight was negatively and positively correlated with total monoenes and 18:0, respectively. The full results on correlation analyses are provided in **Supplementary Figures 4**, **5**. Sample similarities were remarkably high ($R^2 > 0.7$). However, early released newborn batches from group A were more similar to intermediate batches from group AM, and late released batches were similar, independently of the group considered. The only batch available by a wild male was similar to early batches released by males fed on diet AM.

The results obtained with PCA differed depending on the dataset used (**Figure 4** and **Supplementary Figure 6**). PCA performed with the datasets including general newborn characteristics (**Figure 4A**) and FA (**Figure 4B**) showed the proximity of newborn from the wild male to males from groups AM and, to a lesser extent, M. The first two components of PCA accounted for 58.1 and 50.4% of total variation in the dataset. The first component PC1 clearly separated samples from group A (left side) from the others (right side). The most significantly associated variables to PC1 (increasing order of significance) were $\delta^{15}N$, polyunsaturated FA, DHA/AA ratio, n-3 HUFA, DHA/EPA ratio and SL for positive associations, and monounsaturated FA, $\delta^{13}C$ and time for negative associations (**Supplementary Figures 4A,B**). Main FA relevant to PC1 were 20:2n-6, 22:6n-3, 15:0, 20:1n-9, 17:0, 18:1n-9t, and SL (right side), and 16:1n-7, 20:5n-3 and survival at 7 DAR (left side). For PC2, the most relevant proximate variables were n-3/n-6, lipid content, C/N ratio (positive association), and n-6 FA and SL (negative association). For FA, main positive associations with PC2 were time, 20:0, 20:1n-9, 18:1n-9t, 20:3n-6, and 18:2n-6c (positive), and 24:0 and survival (negative).

Variability explained by factors PC1 and PC2 increased up to 69.8% in the PCA performed on all diets and including exclusively significant variables in K-M and J–T tests (**Figure 4C**). In increasing order of importance, the most significantly associated variables (**Supplementary Figure 4C**) to PC1were DHA, 20:2n-6, total polyunsaturated FA, total N, $\delta^{15}N$, DHA/EPA ratio, 17:0, AA and 20:1n-9 for positive associations, and 18:1n-7, 16:1n-7 and survival for negative associations. Survival, 14:0 and saturated FA were positively associated to PC2, with negative association for time and monounsaturated 18:1n-9t and 20:1n-9.

Finally, the PCA performed for selected variables in AM dataset (other groups were not analyzed due to insufficient data) explained 69.2% of total variability with PC1 and PC2 (**Figure 4D**). PC1 was positively associated to DHA, 20:2n-6, total polyunsaturated FA, 15:0, $\delta^{15}N$, DHA/EPA ratio, 17:0, 20:4n-6 and 20:1n-9, and negatively associated to 16:1n-7, 18:1n-8 and survival (**Supplementary Figure 4D**). For PC2, the mostly positive contributions were due to survival, 14:0, and total saturated FA, with time, 20:1n-9 and 18:1n-9 as negatively associated factors.

Transmission Electron Microscopy

Ultrathin sections of abdominal portions of newborn seahorses were observed by TEM in order to analyze the ultrastructural features of muscular tissue. Generally, TEM micrographs of skeletal muscle sections revealed a regular morphology with a normal arrangement of sarcomeres, resulting in a regularly striated appearance with a normal alternation of dark anisotrope band A (myosin and actin) and light isotrope band I (actin

TABLE 2 | Total lipids (% dry weight) and fatty acids (relative percentage) in prey (n = 2), diets and newborn seahorses.

	Prey for seahorse breeders			Diets for seahorse breeders			Batches - Seahorse juveniles (0 DAR)				
Fatty acids (%)	**Adult *Artemia***	**Frozen Mysidacea**	**Captured Mysidacea**	**Diet A**	**Diet AM**	**Diet M**	**Diet A (n = 3)**	**Diet AM (n = 7)**	**Diet M (n = 2)**	**W (n = 1)**	**J-T test**
14:0	1.43 ± 0.09	3.41 ± 0.02	0.62 ± 0.02	1.43 ± 0.09	1.72 ± 0.18	2.02 ± 1.97	1.81 ± 0.51	1.53 ± 0.36	1.13 ± 0.14	1.35	↗**
15:0	0.46 ± 0.05	0.84 ± 0.03	0.56 ± 0.01	0.46 ± 0.05	0.58 ± 0.01	0.70 ± 0.20	0.36 ± 0.05^{a}	0.45 ± 0.04^{ab}	0.51 ± 0.08^{b}	0.53	↗**
16:0	17.92 ± 0.92	21.63 ± 0.15	21.30 ± 0.07	17.92 ± 0.92	19.69 ± 0.22	21.46 ± 0.24	17.84 ± 1.05	17.68 ± 0.61	18.07 ± 1.00	17.91	n.s.
16:1n-7	8.31 ± 0.17	9.06 ± 0.04	0.83 ± 0.01	8.31 ± 0.17	6.63 ± 1.00	4.95 ± 5.82	6.57 ± 2.11	4.02 ± 0.90	3.30 ± 0.83	2.61	↘**
17:0	3.22 ± 0.10	1.23 ± 0.03	1.57 ± 0.06	3.22 ± 0.10	2.31 ± 0.02	1.40 ± 0.24	1.21 ± 0.15^{a}	1.41 ± 0.08^{ab}	1.55 ± 0.12^{b}	1.24	↗**
17:1	0.06 ± 0.01	0.12 ± 0.09	0.91 ± 0.12	0.06 ± 0.01	0.29 ± 0.00	0.51 ± 0.56	0.18 ± 0.05	0.12 ± 0.03	0.14 ± 0.04	0.13	n.s.
18:0	11.44 ± 0.56	2.65 ± 0.04	4.04 ± 0.01	11.44 ± 0.56	7.39 ± 0.55	3.34 ± 0.98	13.00 ± 0.78	12.35 ± 0.90	11.24 ± 1.97	11.25	n.s.
18:1n-7	10.39 ± 3.98	4.11 ± 0.00	3.21 ± 0.02	10.39 ± 3.98	7.02 ± 2.53	3.66 ± 0.64	5.27 ± 0.37^{b}	4.42 ± 0.37^{ab}	3.74 ± 0.08^{a}	5.32	↘**
18:1n-9t	6.97 ± 0.50	5.99 ± 0.05	8.56 ± 0.03	6.97 ± 0.50	7.12 ± 0.91	7.27 ± 1.82	9.07 ± 0.43	9.65 ± 0.53	11.01 ± 0.75	7.99	↗***
18:2n-6c	12.55 ± 0.68	1.17 ± 0.01	1.77 ± 0.00	12.55 ± 0.68	7.01 ± 0.29	1.47 ± 0.42	3.37 ± 1.46	2.82 ± 0.88	1.42 ± 0.17	1.32	↘**
18:3n-3	0.86 ± 0.04	1.31 ± 0.01	1.05 ± 0.01	0.86 ± 0.04	1.02 ± 0.01	1.18 ± 0.19	0.57 ± 0.47	0.40 ± 0.28	0.24 ± 0.05	0.23	n.s.
18:3n-6c	6.36 ± 0.36	0.22 ± 0.00	0.18 ± 0.04	6.36 ± 0.36	3.28 ± 0.01	0.20 ± 0.02	0.23 ± 0.12	0.22 ± 0.08	0.16 ± 0.04	0.32	n.s.
18:4n-3	0.15 ± 0.03	0.18 ± 0.02	0.15 ± 0.00	0.15 ± 0.03	0.16 ± 0.00	0.17 ± 0.02	0.13 ± 0.13	0.22 ± 0.15	0.17 ± 0.18	0.34	n.s.
20:0	0.64 ± 0.01	0.20 ± 0.03	0.22 ± 0.01	0.64 ± 0.01	0.43 ± 0.00	0.21 ± 0.01	0.70 ± 0.18	0.70 ± 0.03	0.78 ± 0.10	0.55	n.s.
20:1n-9	0.30 ± 0.01	0.84 ± 0.01	0.97 ± 0.06	0.30 ± 0.01	0.60 ± 0.00	0.91 ± 0.09	0.36 ± 0.06^{a}	0.46 ± 0.06^{ab}	0.57 ± 0.07^{b}	0.31	↗***
20:2n-6	0.31 ± 0.00	0.52 ± 0.02	1.28 ± 0.03	0.31 ± 0.00	0.61 ± 0.00	0.90 ± 0.54	0.19 ± 0.07^{a}	0.30 ± 0.04^{ab}	0.40 ± 0.08^{b}	0.25	↗***
20:3n-6	0.66 ± 0.06	0.27 ± 0.00	0.41 ± 0.03	0.66 ± 0.06	0.50 ± 0.01	0.34 ± 0.10	0.27 ± 0.04	0.27 ± 0.04	0.27 ± 0.01	0.22	n.s.
20:4n-3	0.24 ± 0.00	0.35 ± 0.02	0.27 ± 0.03	0.24 ± 0.00	0.28 ± 0.00	0.31 ± 0.05	0.07 ± 0.03	0.13 ± 0.12	0.09 ± 0.05	0.44	n.s.
20:4n-6 (AA)	1.45 ± 0.05	2.33 ± 0.01	6.34 ± 0.10	1.45 ± 0.05	2.89 ± 0.15	4.33 ± 2.84	4.93 ± 0.48^{a}	5.74 ± 0.55^{b}	5.93 ± 0.72^{b}	6.85	↗***
20:5n-3 (EPA)	15.43 ± 0.76	22.19 ± 0.04	21.60 ± 0.03	15.43 ± 0.76	18.66 ± 0.31	21.89 ± 0.41	9.93 ± 4.44	5.98 ± 0.94	6.44 ± 1.48	8.16	n.s.
22:4n-6	0.12 ± 0.01	0.28 ± 0.01	0.31 ± 0.11	0.12 ± 0.01	0.21 ± 0.00	0.29 ± 0.02	0.79 ± 0.08	0.69 ± 0.11	0.78 ± 0.04	1.13	n.s.
22:5n-3	0.00 ± 0.00	0.43 ± 0.08	0.54 ± 0.04	0.00 ± 0.00	0.24 ± 0.00	0.49 ± 0.08	5.01 ± 1.15	4.31 ± 0.57	3.98 ± 0.37	4.77	n.s.
22:5n-6	0.00 ± 0.00	0.83 ± 0.05	0.36 ± 0.03	0.00 ± 0.00	0.30 ± 0.00	0.59 ± 0.33	0.51 ± 0.11	1.61 ± 0.54	1.39 ± 0.29	0.86	n.s.
22:6n-3 (DHA)	0.51 ± 0.01	19.69 ± 0.03	22.81 ± 0.02	0.51 ± 0.01	10.88 ± 0.02	21.25 ± 2.20	17.22 ± 6.14^{a}	24.15 ± 2.22^{ab}	26.31 ± 0.97^{b}	25.47	↗***
24:0	0.20 ± 0.02	0.15 ± 0.01	0.14 ± 0.03	0.20 ± 0.02	0.17 ± 0.00	0.14 ± 0.01	0.42 ± 0.18	0.37 ± 0.12	0.37 ± 0.17	0.49	n.s.
Saturated	35.3	30.1	28.4	35.32	32.30	29.28	35.33	34.50	33.65	33.30	n.s.
Monounsaturated	26.0	20.1	14.5	26.03	21.66	17.30	21.45	18.67	18.77	16.35	n.s.
Polyunsaturated	38.7	49.8	57.1	38.65	46.04	53.42	43.22^{a}	46.83^{ab}	47.58^{b}	50.35	↗***
n-3	17.2	44.2	46.4	17.20	31.24	45.29	32.93	35.18	37.24	39.40	↗**
n-6	21.5	5.6	10.7	21.46	14.79	8.13	10.29	11.65	10.34	10.95	n.s.
n-3 HUFA	16.2	42.6	45.2	16.18	30.06	43.94	32.22	34.56	36.82	38.83	↗**
DHA/EPA	0.03	0.89	1.06	0.03	0.58	0.97	2.11^{a}	4.16^{b}	4.22^{b}	3.12	↗**
DHA/ARA	0.35	8.45	3.60	0.35	3.76	4.90	3.43	4.23	4.46	3.72	n.s.
n-3/n-6	0.80	7.87	4.36	0.80	2.11	5.57	3.27	3.04	3.63	3.60	n.s.
Total lipids	10.39	7.99	6.70	10.39	8.87	7.35	12.99	12.17	12.56	15.85	n.s.

Sample size: 10–20 mg dry weight. J–T test: Jonckheere–Terpstra test. The medians of the groups ascend (↑) or descend (↓) in the order specified by the coding variable (order: increasing proportions of mysidaceans in the diet – A, AM, and M). Seahorse juveniles: different superscript letters indicate significant differences. W, newborn from a pregnant wild male; n.s., not significant.

TABLE 3 | Isotope profiles ($\delta^{13}C‰$ and $\delta^{15}N‰$) in dorsal fins (start and end of the experimental breeding period) of *Hippocampus guttulatus* breeders fed on diets A (*Artemia*), AM (*Artemia* + Mysidacea) and M (Mysidacea), and in newborn juveniles.

		Breeder's dorsal fin			Newborn juveniles	Pooled batches
	Group	Initial	Final	Δδ		
$\delta^{13}C$	A	−17.37 ± 1.47 (7)	−13.99 ± 1.05 (7)	2.97	−13.86 ± 1.64 (4)	
	AM	−16.98 ± 2.00 (11)	−17.18 ± 1.64 (11)	−0.20	−17.31 ± 1.13 (6)	
	M	−16.04 ± 0.87 (10)	−18.03 ± 1.38 (10)	−1.89	−16.80 ± 1.27 (3)	
	Wild	−16.00 – (1)	−18.00 – (1)	–	−14.10 – (1)	
$\delta^{15}N$	A	11.94 ± 0.81 (7)	10.28 ± 0.91 (7)	−1.43	11.21 ± 0.88 (4)	
	AM	12.79 ± 1.11 (11)	13.50 ± 0.98 (11)	0.88	14.96 ± 1.50 (6)	
	M	12.19 ± 0.72 (10)	14.74 ± 0.86 (10)	2.58	14.28 ± 2.35 (3)	
	Wild	12.00 – (1)	14.40 – (1)	–	14.10 – (1)	

Enrichment factors (Δδ) for fins (final − initial) are provided. Sample size (n) is given in brackets. $\delta^{13}C$ and $\delta^{15}N$ values in newborn from a batch released by a wild pregnant male are also given for comparative purposes.

TABLE 4 | Batches produced, batch size, standard length (mm), dry weight (mg), and survival percentage (7 DAR) of newborn juveniles released by *Hippocampus guttulatus* breeders fed on diets A (*Artemia*), AM (*Artemia* + Mysidacea), and M (Mysidacea).

Diet	Batches produced	Total juveniles	Batch size Mean sd	Length (mm) Mean sd	Dry weight (mg) Mean sd	Survival (%) Mean sd
A	4	534	134^{a} ± 83	12.9^{a} ± 0.7	0.60^{a} ± 0.16	94.0^{a} ± 5.3
AM	7	1,416	202^{a} ± 82	14.7^{b} ± 0.7	0.74^{a} ± 0.11	86.9^{a} ± 9.5
M	3	248	84^{a} ± 72	14.1^{b} ± 0.2	0.73^{a} ± 0.06	61.4^{a} ± 19.2
W	1	–	210	14.1	0.78	94.9

W: Newborn from a wild pregnant male. Different letter superscripts indicate significant differences ($P < 0.05$).

filament) (**Figures 5A,B,D**). In the longitudinal sections of TEM micrographs of skeletal muscle sections, the myofibrils appeared tightly packed in the scarce amount of cytoplasm. The nuclei were located in the peripheral layer of the fiber and mitochondria beneath the sarcolemma and among the myofibrils. Besides, the regular repeating pattern of myofilaments in the myofibrils were detected displaying dense Z lines, forming a regular zigzag (**Figures 5A,B,D**) at the center of the light I bands. The A bands showed a central light H zone divided by a dark M line (**Figure 5A**). In addition, the organization of the sarcoplasmic reticulum and the sarcoplasmic triad, consisting of two terminal cisterns and the transverse tubule T (formed by the invagination of the sarcolemma) were also observed (**Figures 5A,C**). Scattered satellite cells surrounded by volumetrically modest cytoplasm were often seen in muscle tissue (**Figures 5E,6B**).

In the cross sections, the disposition of the two sets of myofilaments in different bands of the sarcomere were analyzed. The myofibrils were well delineated by the close apposition of the sarcoplasmic elements (**Figures 6A,B**). The nuclei were located in the peripheral layer of the fiber and mitochondria beneath the sarcolemma and among the myofibrils.

The general morphology observed in newborn released by males from diet A appeared similar to the other groups (M and AM). However, some ultrastructural muscle changes were noticed. In particular, group A displayed regressive changes in some muscle fibers characterized by a disarrangement of myofibrils (**Figure 5B**), with evident myofibrillary interstitial spaces. Besides, a discontinuity of the sarcolemma and myelin-like bodies were present (**Figure 6C**). In addition, a disorganization of the sarcoplasmic elements and of the invagination of the sarcolemma (i.e., tubules T) was also noticed. An interruption of the Z line was also occasionally evidenced (**Figures 5E,F**) and neuromuscular junctions were observed between motor nerve endings and muscle fibers. Particularly, the terminal axons of the motor neuron were spherical or oval in shape and fit into smooth depressions in each muscle fiber (**Figures 6D–F**). At the presynaptic zone, several mitochondria and small clear synaptic vesicles were noticed. Other morphological alteration were not detected at the level of the above structures.

DISCUSSION

Breeding Performance

In this study, the isotopic results achieved in dorsal fins of the seahorse *H. guttulatus* confirmed the effective assimilation of experimental diets by breeders, with effects on average isotopic signatures and FA profiles in newborn seahorses across groups. The overall breeding performance (extension of the effective breeding period, batches produced, and newborn released) resulted enhanced in parents fed on the mixed diet AM (non-enriched *Artemia* and mysidaceans), suggesting a better nutritional condition than in groups A and M. Unexpectedly, the use of mysidaceans as only prey (diet M) resulted in the worse breeding success among the three experimental diets.

Brood size in some seahorse species (e.g., *H. kuda* and *H. erectus*) has been related to the length of brood pouches

of males and trunk of females (Dzyuba et al., 2006; Lin et al., 2012). The newborn size might be correlated to brood size, gestation time and nutrient supply in the same males and females' investment (Lin et al., 2008b, 2012). Besides this, larger *H. erectus* males (larger brood pouches) do not produce larger broods, and male size can be positively correlated with juvenile size (Lin et al., 2012). In *H. guttulatus*, it has been pointed out that the size of newborn seahorses can be, in part, paternally determined (limited carrying capacity of the male), as male size is negatively correlated with embryo density and positively correlated with newborn size (Faleiro et al., 2016). As breeders average size did not differ across our experimental groups, we assume that differences between treatments in our study were due to dietary dissimilarities. Differences in the capability of seahorses to digest and assimilate a given prey is another subject to consider when assessing diet quality. The monitoring of prey in feces of *H. guttulatus* by 18SrDNA amplification showed that gut passage times of crustaceans (*Artemia*, *Palaemonetes*, and mysidaceans *Leptomysis* sp., and *Siriella* sp.) are species-dependent (longer passages times in mysidaceans), probably due to differences in their digestibility (lower digestibility in *Palaemonetes*) (Corse et al., 2015).

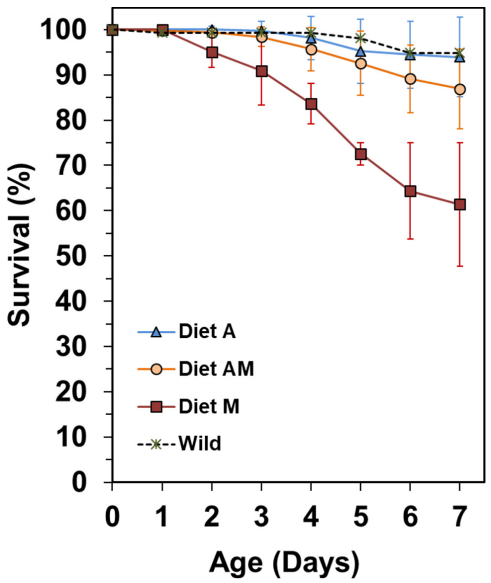

FIGURE 2 | Initial survival of newborn seahorses released by a wild male and by captive males fed on diets A, AM, and M.

Mysidaceans have been considered an excellent prey for the on-growing and breeding of some seahorse species (e.g., *H. abdominalis* and *H. hippocampus*). Otero-Ferrer et al. (2012) studied the effect of feeding *H. hippocampus* breeders exclusively on *Artemia* enriched with DHA or captured mysids (*Leptomysis* sp.). The authors reported better results on reproduction (spawning events and brood size) and newborn size in breeders fed on mysids, presumptively due to a higher nutritional quality of mysidaceans (basically in DHA content). Those results strongly agree with those with diet AM, but not with the poor results achieved with diet M. we also recommended (but not tested) the use of a mixed diet including *Artemia* and mysids.

In the present study, premature newborn release or egg clutch dropping were not observed. Such issues were observed in previous studies performed on *H. hippocampus* fed on *Artemia* (Otero-Ferrer et al., 2012) or commercial mysidaceans (i.e., much lower DHA and HUFA levels than in the present study) (Saavedra et al., 2015) and in *H. guttulatus* fed on suboptimal quality of *Artemia* (i.e., high lipid and low DHA content) (Planas et al., 2009b, 2010). In the later, the issue was solved by optimizing

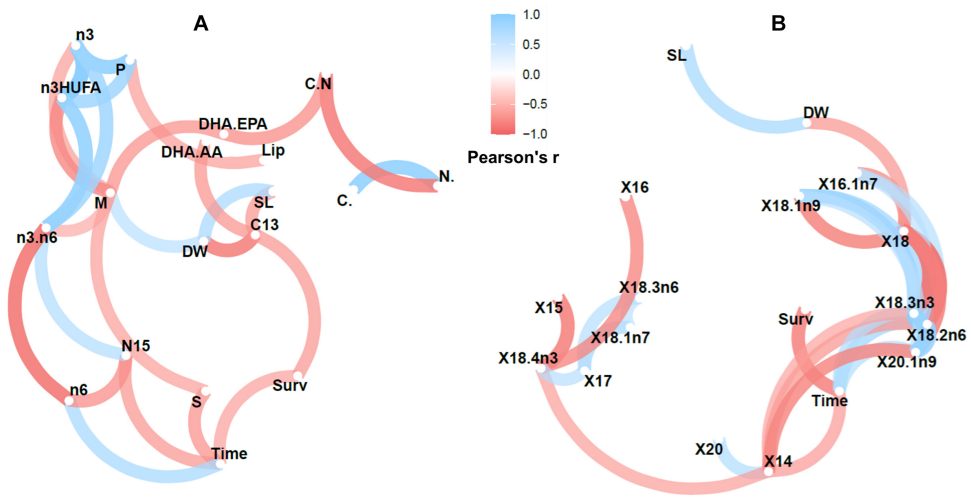

FIGURE 3 | Correlation network plots for newborn seahorses (Diet AM) showing the more highly correlated variables (Pearson's $r > 0.6$), which appear closer together and joined by stronger paths. Paths are also colored by their sign (blue for positive and red for negative). The proximity of the points were determined using multidimensional clustering. Original variable names were modified by R software. Variables: **(A)** time (days elapsed since the onset of the breeding period), SL, W, Survival at 7 DAR (Surv), total C (C.), total N (N.), C/N, δ^{13}C (C13), δ^{15}N (N15), total lipids (Lip.DW), saturated FA (S), monounsaturated FA (M), polyunsaturated FA (P), n-3 HUFA, n-3 FA, n-6 FA, n-3/n-6 (n3.n6), DHA/EPA (DHA.EPA) and DHA/AA (DHA.AA); **(B)** time, SL, W, Survival at 7 DAR (Surv) and individual FA.

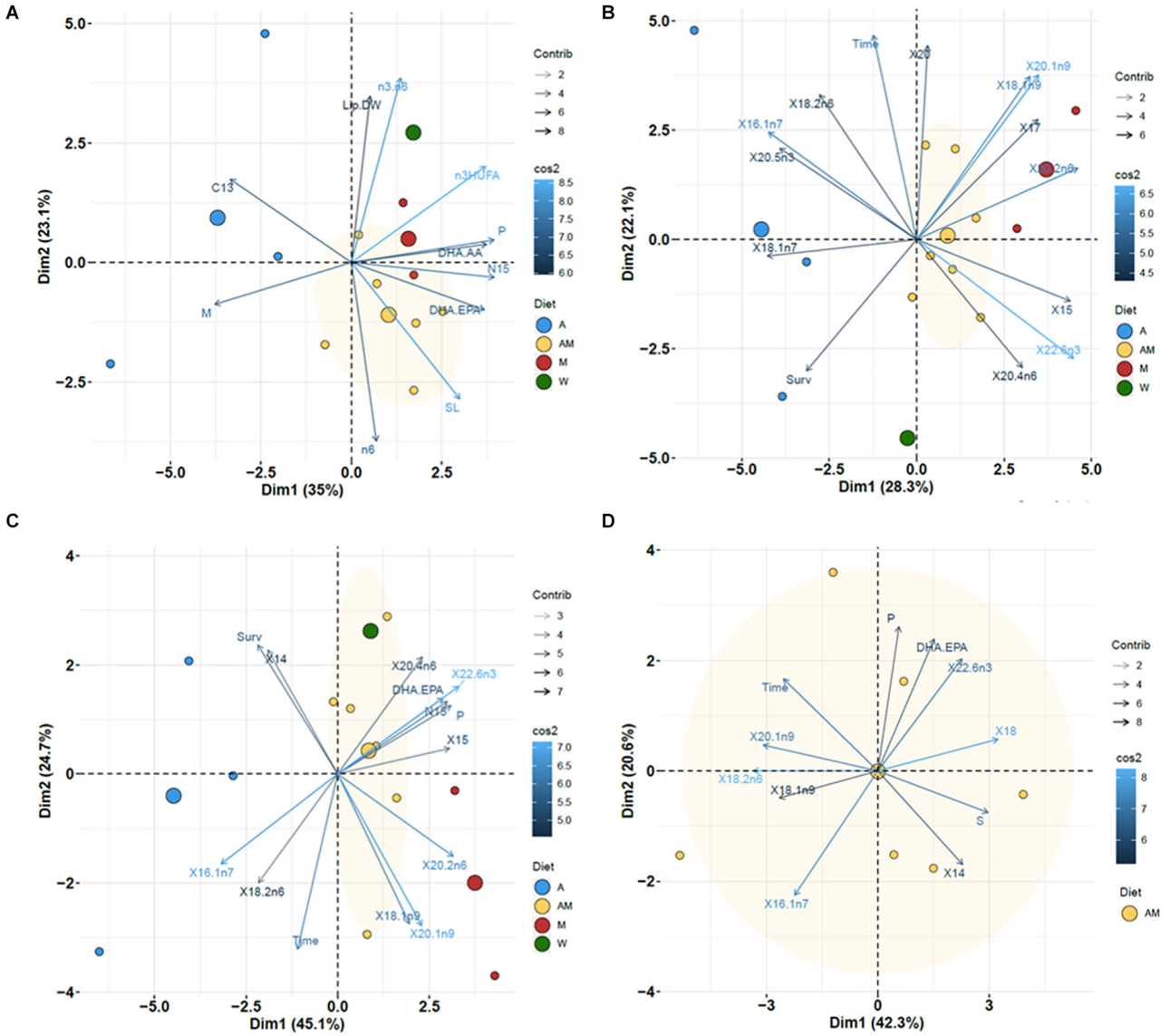

FIGURE 4 | Factor score plots for the principal component analysis (PCA) on newborn seahorses released by wild (W) or cultivated (A, AM, and M) adults. Only the variables with the highest contributions (cos2 > 0.6) on factors 1 and 2 maps are shown. Analyses performed on datasets **(A)** – all diets and general characteristics of newborn, **(B)** – all diets and all individual FA, **(C)** – all diets and variables selected according to K-M and J–T test, and **(D)** – diet AM (Same as for **C**). Original variable names were automatically modified by R software. Variables: days elapsed since the onset of the breeding period (Time), SL, W, Survival at 7 DAR (Surv), total C (C.), total N (N.), C/N (C.N), δ^{13}C (C13), δ^{15}N (N15), total lipids (Lip.DW), saturated FA (S), monounsaturated FA (M), polyunsaturated FA (P), n-3 HUFA, n-3 FA, n-6 FA, n-3/n-6 (n3.n6), DHA/EPA, DHA/AA, and individual FA. The ellipse corresponding to 95% confidence for group AM is shown in yellow shadow. Other ellipses were not computed due to insufficient data.

DHA and EPA content in *Artemia* (Planas et al., 2017b) and isolating pregnant males a few days prior to newborn release (Olivotto et al., 2011b).

Small initial lengths and reduced batch sizes in newborn from group A were accompanied by high survivals at 7 DAR. This result was unexpected as newborn survival commonly depends on the dietary quality provided to broodstock. Interestingly, it has been reported in *H. reidi* that reducing the nutritional quality of a male's diet since one month before conception resulted in abnormally large and heavy newborns with extremely reduced survivals, independently of the dietary quality in females (Otero-Ferrer et al., 2016). These findings partially agree with those reported in the pipefish *Syngnathus typhle*, in which a higher nutritional condition in males brooding heavier embryos was correlated with a higher embryo survival (Sagebakken et al., 2017).

Even though the growth of adult *H. guttulatus* fed on *Artemia* may be satisfactory (Planas et al., 2008, 2009a; Segade et al., 2015), it seems that its high lipid content and suboptimal FA profile might result in an excessive accumulation of lipids in the liver

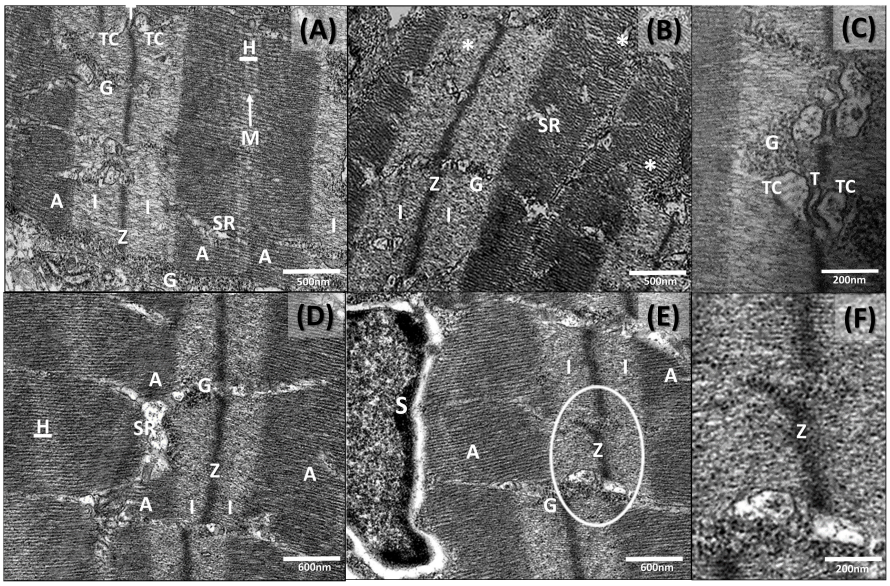

FIGURE 5 | Transmission electron micrographs of newborn seahorse muscle (longitudinal sections) from groups M, AM and A. **(A)** Group AM: normal sarcomere with light isotrope band (I); Z line; dark anisotrope band **(A)**, central H-zones and in the middle M lines (arrow); triad located at the Z line level. **(B)** Group A: myofibrils with a slight disarrangement. **(C)** Group AM: higher magnification. Note tubule T between two terminal cisternae close to each other just forming the longitudinal connection. **(D)** Group M: a regular zigzag configuration of the Z line of the sarcomere. **(E)** Group A: interruption of Z line (circled). **(F)** Higher magnification interruption of above circled Z line. SR, sarcoplasmatic reticule; G, glycogen; Tc, terminal cisternae; T, Tubule T; S, satellite cells.

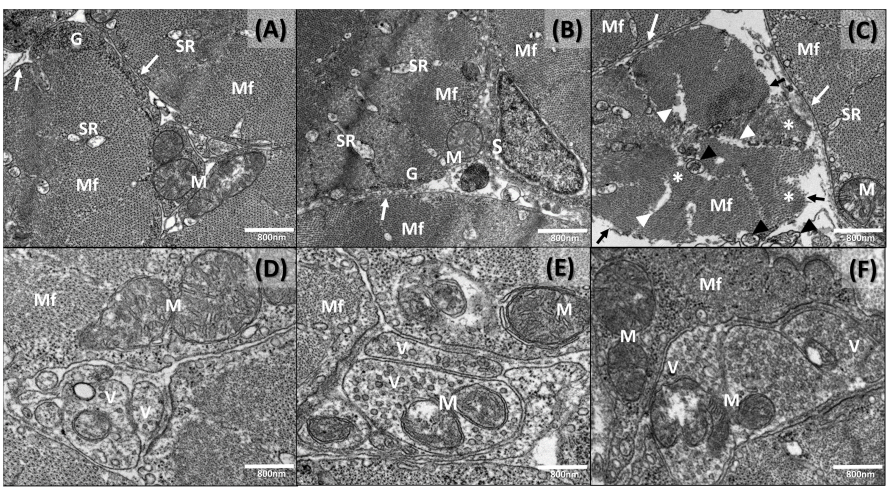

FIGURE 6 | Transmission electron micrographs of newborn seahorse muscle (cross sections) from groups AM, M, and A. **(A)** Group AM: normal structure. **(B)** Group M: normal structure. **(C)** Group A: disarrangement of myofibrils (asterisks); myofibrillary interstitial spaces (white arrow heads); discontinuity of the sarcolemma (black arrows); myelin-like bodies (black arrow heads). **(D)** Group AM: nerve ending. **(E)** Group M: nerve ending. **(F)** Group A: nerve ending. S, satellite cells; SR, sarcoplasmic reticule; Sarcolemma (white arrows); Mf, myofibrils; M, mitochondria; G, glycogen; V, vesicles.

and an abnormal (not necessarily pathological) displacement of hepatocytes core to the periphery of the cells (Segade et al., 2016). The presence of ultrastructural muscle alterations at the level of the contractile component and presence of myelin-like bodies (expression of the lysis of the membranes involved by the regressive phenomena) in newborns released by males fed on diet A can be related to some nutritional deficiencies in unenriched *Artemia*. Polyunsaturated fatty acids (PUFAs) are essential FAs in regulating normal growth and development in many fish species (Anderson et al., 1990; Olivotto et al., 2011a) and promoting muscle fiber development (Cong-cong et al., 2019). Specifically, DHA is a structural key component of cell membranes involved in their biosynthesis (Olivotto et al., 2011a; Randazzo et al., 2018). The low levels of DHA in diet A might thus be responsible for the alterations found in some muscle fibers. Furthermore, an interruption, observed only occasionally of the Z line, could

be the expression of the structural disarrangement of myofibrils demonstrated in muscle fibers with regressive phenomena.

The supposed superior quality of several small species of marine crustaceans (e.g., amphipods and mysidaceans) over *Artemia* has been reported in seahorse culture (Palma et al., 2008; Woods, 2009; Otero-Ferrer et al., 2012; Buen-Ursue et al., 2015; Vargas-Abúndez et al., 2018). Those sources are among the mostly consumed preys by wild seahorses (Manning et al., 2019). However, the main dietary sources for *H. guttulatus* adults in our nearby region (NW Iberian Peninsula) are amphipoda (caprellids and gammarids), whereas mysidaceans are insignificant prey in the bulk diet composition (Valladares et al., 2016). We recently demonstrated that diets including mysidaceans (alone or mixed with *Artemia*) provide the best trophic enrichment factors (laboratory derived) to infer the composition of bulk diet in wild syngnathids by means of isotopic signatures (Planas et al., 2020). Hence, even though mysidaceans might be considered a high quality prey for some seahorse species, it is very likely not suitable for breeding *H. guttulatus* when administered as the sole prey. Unfortunately, nutritional requirements in seahorses are unknown, and provision of suitable food sources for *ex-situ* culture often represents a serious constraint (Chang and Southgate, 2001) as not all species seem to have the same nutritional needs, as suggested by inter-specific differences in FA profiles (Lin et al., 2008a). Consequently, mixed diets (present study) and/or dietary supplementation would potentially reduce some nutritional deficiencies of single prey (Palma et al., 2012, 2017).

Breeding Strategy

Seahorses are synchronous batch spawners, as several oocyte populations are present during the reproductive season and clutches of oocytes develop, mature, and are released simultaneously (Fernández-Palacios et al., 2011). During the last part of the secondary growth of oocytes (vitellogenesis), lipids and yolk proteins are incorporated and stored in large amounts in the yolk. That process and further embryogenesis of fertilized eggs in males cyclically occurs along the breeding season, requiring a continuous expenditure of energy and nutrients. Seahorse females and males are time-synchronized for egg batch transfer from females to males and newborn release in males, and in *H. guttulatus* kept at 19–20°C both processes occur at 25–27 days time-intervals (Planas et al., 2010). On this regard, it is very important to ascertain the origin (capital, income or mixed capital-income reproduction strategy) of the energy and nutrients necessary for reproduction (Williams et al., 2017) and to evaluate whether and when egg/fry composition can be affected by the type of diet provided. Hence, the identification of both diet quality and effective feeding periods (the time period needed to change the biochemical composition of eggs/fry) for optimum broodstock performance is of pivotal importance in enhancing newborn quality (Fernández-Palacios et al., 2011; Griffen, 2018). The present study and the pattern of isotopic changes discussed elsewhere (Planas et al., 2020) suggest that the reproduction type followed by *H. guttulatus* corresponds to a mixed capital-income strategy. This strategy usually implies a progressive drop in newborn quality in successive batches (McBride et al., 2015; Griffen, 2018). In order to properly assess the breeding strategy followed by *H. guttulatus*, the effects of experimental diets must be analyzed over the entire breeding period. Given that the pattern of change in the biochemical components analyzed in newborn did not follow a mathematical model for fitting, the effective feeding periods were inferred from the smoothed trends in the most remarkable components along the breeding period (particularly for group AM) (**Figure 7**). In most cases, trend inflexions occurred within the period comprising 100 and 140 days, depending on the variable and seahorse group considered. We identified three stages along the whole breeding period:

- Stage 1 (mixed capital-income sources): progressive changes occur since the dietary shift at the onset of the breeding season. Energy and nutritional resources would mostly originate from resources gained prior to the breeding period (capital breeding), accompanied by an increasing use of sources derived from the experimental diet (income breeding). This stage would extend until days 100–140 since the onset of the breeding period.
- Inflection period (income sources): this is a transitory period indicating the end of the capital-income breeding strategy and the use exclusively of resources from the experimental diet. In this period, differences among treatment were clearly perceptible.
- Stage 2 (income sources and exhaustion of body reserves): in most cases, differences among groups increased or decreased (depending on the variable considered) beyond the inflection period. Maximal divergences between treatments were reached during this period, notably by the end of the stage. The trend followed by certain variables (e.g., DHA and DHA/EPA) in late newborn batch releases suggests a more or less intense exhaustion of body resources, accompanied by decreases in survivals at 7 DAR and newborn size. This statement is also supported by the trends in newborn isotopic signatures (Planas et al., 2020).

Fry quality can be manipulated to a certain extend by modifying the nutritional composition of the diet during the breeding season of fishes (Fernández-Palacios et al., 2011) including seahorses (Otero-Ferrer et al., 2020). However, it is also important to assess the effect of preconception diet on reproduction success and the contribution of males and females, as reported by Otero-Ferrer et al. (2016) in *H. hippocampus*. According to our above hypothesis on the stages recognized along the breeding season, the diet provided preceding the reproduction season would contribute for a certain period to the initial composition and quality of *H. guttulatus* newborn. Hence, the initial quality of eggs/newborn could be improved by feeding the breeders on a high quality diet for at least 3 months in advance of the onset of the breeding season, which is in agreement with previous findings in the tropical species *H. reidi* (Planas and Chamorro, 2019). That finding is of primary importance to female diets as it is noteworthy that embryos are mostly dependent on maternal nutrition (yolk quality) until later stages

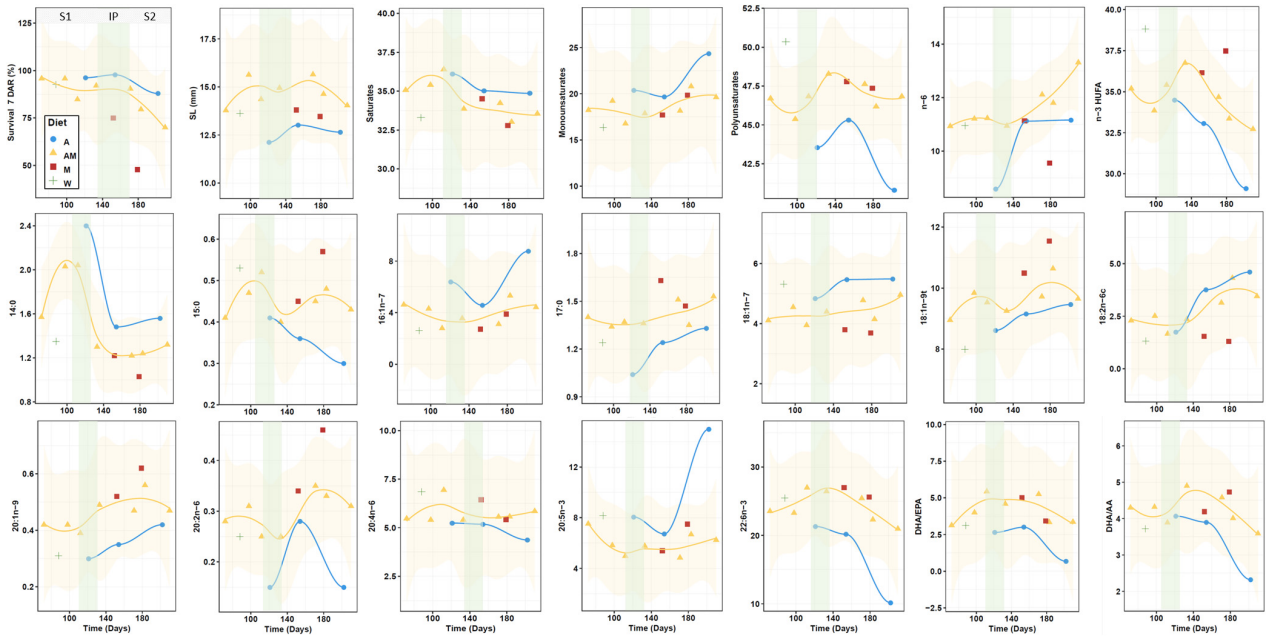

FIGURE 7 | Progressive changes in survival at 7 DAR, length (SL), fatty acids (area percentage), and FA ratios along the breeding period in newborn released by seahorses (Wild and groups A, AM and M). Smoothed trends are shown for groups AM (95% confidence intervals also shown) and A. Green shadowed areas show inflection periods indicating a remarkable change (transition from stage 1 to stage 2). Stages: S1 (Stage 1), IP (Inflection period), and S2 (Stage 2).

of development, when the embryos are mostly dependent on paternal contribution (Otero-Ferrer et al., 2020).

Newborn Composition

Breeders diet must be optimized to ensure good early development (i.e., growth, survival, and health) (Izquierdo et al., 2001; Migaud et al., 2013). Currently, nutrients requirements are rather well known in many marine species with interest in ornamental trade, and optimal dietary formulations are available (Craig et al., 2017). However, there are two main issues in the feeding of seahorses in captivity: the lack of knowledge on their nutritional needs and the rejection of inert formulated diets (Olivotto et al., 2011b; Palma et al., 2012; Planas et al., 2017a). As the broodstock diet is strongly reflected in the composition of eggs/fry, particularly for lipids (Wiegand, 1996), the composition of eggs or fry can be used as a proxy to infer nutritional requirements in fishes to improve breeding success (Sargent et al., 1989, 1999; Rainuzzo, 1993; Fernández-Palacios et al., 1995; Rainuzzo et al., 1997). The content in lipids and the FA profiles of *H. guttulatus* eggs under different dietary regimes were reported by Planas et al. (2008, 2010) and Faleiro and Narciso (2010). Essentially, FA profiles in eggs and newborn do not differ significantly when the content is referred to relative percentage of total FA, but they markedly differ when the content is given as dry weight percentage (Faleiro and Narciso, 2010). In the present study, we focused exclusively on newborn, including a batch released by a wild pregnant male for comparative purposes.

The similarity between the wild batch (released early in the season) and the early and mid-term batches released from group AM was mainly due to DHA, total polyunsaturated FA, $\delta^{15}N$, survival at 7 DAR, DHA/EPA ratio, AA and, to a lesser extent, to minority 20:1n-9 and 20:2n-6 (<1%). The DHA/EPA ratio reflected the higher and more stable content in DHA thorough the breeding season, compared to treatment A. The essentiality of DHA, EPA, and AA in early developmental stages of marine fish has been previously discussed in depth (Rainuzzo, 1993; Bromage, 1995; Wiegand, 1996; Rainuzzo et al., 1997; Estévez et al., 1999; Tocher, 2010). There is no doubt about the nutritional essentiality of certain HUFAs in marine fish. These FA are important sources of energy in fish during early embryogenesis (Sargent et al., 1999) when DHA and AA are highly conserved in most teleost (Watanabe et al., 1989). However, Faleiro and Narciso (2010) analyzed the lipid and FA dynamics during early development of *H. guttulatus* (breeders fed on adult *Artemia*; *Artemia* diet not provided) and reported that EPA, DHA, and palmitic acid were not preferentially retained in embryos as they constitute the main energy sources, with EPA being catabolized at a high rate. Consequently, an exceptionally low lipid content (5.0% DW) and extremely low EPA levels (5.0%) were reported in newborn. Compared to that study, EPA content and total lipids in newborn from our study were two-fold higher, in agreement with previous results in freshly released eggs (Planas et al., 2008, 2010).

In spite of the extremely low levels of DHA in diet A, its average content in newborns was high (with a sharp decrease in late batches) although lower than in groups AM and M. This finding indicates the essentiality of DHA in seahorses. Overall, FA profile and DHA/EPA ratio in newborns released from the wild male showed a higher similarity with newborns released by males fed on diets including mysidaceans. Hence, the

variable effect of breeder's diet on the allocation of FA resources in newborn from different experimental assays is confirmed in *H. guttulatus*, deserving further attention, especially with regard to dietary DHA requirements and further effects on survival and size of newborn.

Higher dietary contents in total n-3 HUFA and DHA does not necessarily imply higher quality. Excessive levels of dietary n-3 HUFA can reduce eggs production and survival, and cause yolk sac hypertrophy in fish larvae (Fernández-Palacios et al., 1995). As the TEM did not show any morphological alteration in tissues of newborn from diet M, we suspect that n-3 HUFA and DHA levels in diet M were below detrimental levels, and that the low breeding success in that treatment was due in part to energetic and/or nutritional dietary deficiencies. It is known that egg viability may decrease in lower quality diets (Saavedra et al., 2015) which is in agreement with the reduced newborn production in diets A and M. As eggs dropping did not occur in our study and female-male egg transfers were not checked, viability in eggs transferred to males is not available. Hence, the reasons for that low performance are uncertain. In addition to potential negative effects of low quality diets on mating displays, a drop in reproductive success due to the strategy of cryptic choice should not be discarded. According to this strategy, males could increase rates of offspring abortion in pregnancies to absorb nutrients nutrients for future reproductive opportunities (Paczolt and Jones, 2010; Sagebakken et al., 2010).

CONCLUSION

In the experimental study presented here, isotopic signatures of experimental diets supplied during the breeding season were reflected in breeder's dorsal fins (efficient assimilation of diets) and subsequently in newborn, with higher $\delta^{15}N$ signals in breeders and newborn from the diets including mysidaceans. Monospecific diets including *Artemia* or mysidaceans very likely suffered from nutritional deficiencies, reflected in morphological alterations of the muscle tissue or abnormal early mortalities in newborn. Those deficiencies seemed to be overcome with the mixed diet AM (unenriched *Artemia* and mysidaceans), which provided the highest overall breeding success and reasonable FA profiles, and better resembled newborn from a wild male. However, special attention deserve the progressive changes in FA profiles along the breeding season, resulting in a decrease in newborn performance. Considering the time required for a diet to be reflected in newborn, we recommend that *H. guttulatus* breeders be fed on a high quality diet at least 3 months in advance of the onset of the breeding period.

In order to further optimize captive breeding and rearing of seahorses, further research should focus on wild populations with particular emphasis on broodstock feeding habits, on how feeding preferences possibly change during the breeding season and on potential shifts depending on the geographical distribution of the fish. Data collected from the wild will help scientist in the selection of the more suitable diet able to sustain the best egg and fry quality.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study can be found in the Pangaea collection (https://doi.pangaea.de/10.1594/PANGAEA.919218). Data not listed in this collection is available from the authors upon reasonable request.

ETHICS STATEMENT

The animal study was reviewed and approved by Regional Government Xunta de Galicia (REGA ES360570202001/15/FUN/BIOL.AN/MPO01).

AUTHOR CONTRIBUTIONS

MP as leading author has done the conception of the work, breeding and rearing, sampling, lipid and stable isotope analysis, interpretation of data, and most part in drafting. IO and RL assisted in manuscript writing. MG was responsible for fatty acid analysis. RL and MZ were responsible for TEM and this part of the draft. All authors revised the manuscript. 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.2020.00638/full#supplementary-material

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The Effect of Nitrogen Starvation on Biomass Yield and Biochemical Constituents of *Rhodomonas* sp.

Christos Latsos[1], Jasper van Houcke[1] and Klaas R. Timmermans[1,2]*

[1] Research Group Aquaculture, HZ University of Applied Sciences, Vlissingen, Netherlands, [2] Department of Estuarine and Delta Systems, Royal Netherlands Institute for Sea Research (NIOZ), Utrecht University, Yerseke, Netherlands

The microalgae *Rhodomonas* sp. is known as an excellent feed source for live feed organisms such as copepods. The main benefits of feeding *Rhodomonas* to live feed animals are attributed to the relative high polyunsaturated fatty acid (PUFA) level, the combination of containing both docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), and the ratio between these fatty acids (FA). It has been shown that microalgae are able to accumulate valuable metabolites, such as lipids, under adverse conditions. The easiest and most inexpensive method to induce stress to microalgae is through nitrogen (N) starvation. In this study, the effect of N-starvation on biomass concentration, cell volume, and cellular composition, such as fatty acid concentration and composition, and phycoerythrin (PE) concentration of *Rhodomonas* sp. during a period of 8 days, was investigated. The research was divided into two stages. In the first (growth) stage, *Rhodomonas* sp. was cultivated in small 400 ml photobioreactors (Algaemist-S) under optimal conditions in turbidostat mode, which reached a biomass concentration of 1.5 gDW L^{-1} and dilution rate of 1.3 d^{-1}. Samples were taken every 24 h for cell density and volume and productivity measurements in order to ensure a healthy and stable culture. In the next stage (N-starvation), the biomass was washed and transferred in a reactor filled with N-depleted medium. During N-starvation, samples were taken for biomass concentration, cell volume, PE and FA composition. The results of this study demonstrate that the lipid content increased significantly from 9% (t = 0 h) to 30% (t = 120 h) of the dry weight. After 120 h of N-starvation, the total FA content of *Rhodomonas* sp. remained stable for the remainder of the experiment (next 72 h). The highest increase of the FA concentration was represented by C16:0, C:18:1, C18:2, and C18:3, with highest concentrations after 120 h of starvation. The maximum EPA and DHA concentrations were observed after 48 h of starvation, while the maximum DHA to EPA ratio was detected at the end of the starvation.

Keywords: *Rhodomonas* sp., nitrogen starvation, cell volume, fatty acids, phycoerythrin, PUFA, DHA, EPA

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****Correspondence:***
Christos Latsos
christos.latsos@hz.nl

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INTRODUCTION

In the aquaculture industry, microalgae are mainly used as a live feed. Microalgae are usually used in the cultivation of bivalves, crustaceans, marine fish and zooplankton. The main benefit attributed to using live microalgae is their high nutritional value (Borowitzka, 1997). The application for animal feed accounts for about 30% of the algal production in the world (Becker, 2007). The cost of feed

contributes significantly to the final cost price of aquaculture products. High priority is, therefore, given to improving feed efficiency in industrial systems (Patil et al., 2007). It is stated that algae could increase aquaculture production up to 4-fold and as a result decrease the fish production cost by €0.10 to €0.21 kg^{-1} (Brune, 2012).

Microalgae nutritional value as aquaculture feed needs to be high in order to guarantee the best production of cultured animals (Volkman et al., 1989). The nutritional quality is connected with cell size, digestibility, and biochemical composition of microalgae, especially the fatty acid concentration and composition and the relative proportions of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Chu and Dupuy, 1980; Watanabe et al., 1983; Brown et al., 1997; Muller-Feuga, 2000). Most aquatic animals are not able to synthesize polyunsaturated fatty acids (PUFAs), such as EPA and DHA (Kanazawa et al., 1979). PUFAs are crucial for growth and development in penaeid prawns and shrimps and oyster species (Langdon and Waldock, 1981; González-Araya et al., 2012). Even marine animals that do not require EPA or DHA have demonstrated higher growth rates and larval survival rates when these PUFAs were included in their diet (Brown and Blackburn, 2013). For instance, *C. gigas* larvae did not show any boosted growth when the feed was enriched with DHA more than 2% of total fatty acids (Thompson et al., 1993), while also clams do not require PUFAs in their diet (Helm and Bourne, 2004). Except for the quantity of DHA and EPA, the ratio of these fatty acids is nutritionally important in the larval performance of mussel and fish larvae (Rodríguez et al., 1998; Pettersen et al., 2010). Pettersen et al. (2010) study correlated positively the *Mytilus galloprovincialis* larvae settlement and metamorphosis with the EPA and DHA ratio of the microalgae diets. Rodríguez et al. (1998) reported that for fish larvae diet DHA/EPA ratio should be 1:1 to 2:1. Decreases of DHA/EPA ratios in rotifers feed resulted in lower larval growth performance, while the most deficient larval growth was obtained in rotifers fed with the lowest DHA/EPA ratio (Rodríguez et al., 1997).

The main benefits of feeding *Rhodomonas* sp. to live feed animals are attributed to the relative high PUFAs level, the combination of containing both DHA and EPA, and the ratio between these fatty acids. *Rhodomonas* sp. has a high content of essential fatty acids (PUFAs) and nutrients, lipid accounts for about 10–30%, and protein takes about 30–60% of the dry weight (Renaud et al., 1999; Seixas et al., 2009; Costard et al., 2012; Coutinho et al., 2020). The variability of lipid and protein concentration depends on cultivation conditions. The high protein concentration is attributed to by another characteristic of *Rhodomonas* sp., the high phycoerythrin (PE) content. PE is a water soluble, colored and fluorescent phycobiliprotein that harvests light in the green wavelength (λ_{max} = 545 nm) and provides energy to Photosystem II (PSII) (Doust et al., 2006). So, live *Rhodomonas* sp. can be an excellent feed for aquaculture. It has been previously proved to constitute a high-quality diet to rear calanoid copepods *Acartia sinjiensis* and *Artemia* sp. (Knuckey et al., 2005; Seixas et al., 2009; Coutinho et al., 2020).

It has been shown that microalgae are able to accumulate lipids under adverse conditions. The easiest and most inexpensive method to induce stress to microalgae is through N-starvation. The availability of nitrogen can influence the lipid quantity and quality of many microalgae (Richardson et al., 1969; Breuer et al., 2012; Simionato et al., 2013). A reduction of photosynthetic performance is also observed under N-depleted growth medium, because microalgae decrease their light harvesting efficiency and energy transduction (Berges et al., 1996; Young and Beardall, 2003). Especially cryptophytes under N-starvation lose their PE antenna entirely, while the chlorophyll a/c and PS II are affected as well (Rhiel et al., 1986; Bartual et al., 2002). *Rhodomonas* cells in N-starvation conditions decrease their PE concentration and their fluorescence capacity up to 90% (da Silva et al., 2009). As a result, PE acts as an internal N reserve for cells in order to ensure their continuation of growth.

The aim of our study is to investigate the effect of N-starvation on biomass yield cell volume, lipid concentration and composition, and PE concentration (as a proxy for protein concentration) of *Rhodomonas* sp. The effect of an N stress time period of 8 days on the total fatty acids (TFA) and PUFAs content of *Rhodomonas* sp. is explored. The change of the absolute and also the relative DHA and EPA levels during the N-starvation period is determined.

MATERIALS AND METHODS

Strain, Growth Medium, and Pre-cultivation

Rhodomonas sp. was supplied by the Dutch aquaculture industry, as a strain used in commercial application. The strain was characterized by 18S sequencing and confirmed as *Rhodomonas* sp. (data not shown). *Rhodomonas* sp. inoculum for the experiments was pre-cultured in 300 ml pre-sterilized (20 min at 120°C) Erlenmeyer flasks in a conditioned shaker incubator. Cool white TL fluorescent tubes continuously provided a photon flux intensity of 100 μmol photons m^{-2} s^{-1} and the temperature was kept stable at 20 ± 1°C. Algae were maintained in 20 times concentrated nutrients of filtrated (0.2 μm pore size) f/2 medium, to maintain a nutrient-rich condition. The final concentration of $NaNO_3$ and $NaH_2PO_42H_2O$ was 1.5 g L^{-1} and 113 mg L^{-1}, respectively, and a salinity of 30 g L^{-1}. Air enriched with 5% CO_2 V/V_{air} was supplied in the headspace of the Erlenmeyer flasks. The growth of the cultures was monitored by measuring the cell abundance with a Coulter counter (Beckman coulter Z1) in order to ensure that the inoculum was in the exponential phase before it was used in the experiments.

Experimental Setup

In the experiments, *Rhodomonas* sp. was continuously cultivated in flat panel airlift-loop photobioreactor (PBR) (Algaemist-S, Technical Development Studio, Wageningen University, Netherlands) with 0.4 L working volume, 14 mm light path and 0.028 m^2 total illuminated area (Breuer et al., 2013). Warm light was continuously provided by Bridgelux LED lamps (BXRAW1200, Bridgelux, United States) from one side of the Algaemist-S system. Unintentional exposure to other light sources was prevented by a black cover on the other side of

the reactor. The primary light intensity was firstly set at around 100 μmol photons m^{-2} s^{-1} after the algae were inoculated to resemble the environment of the orbital incubator where the algae grew at the pre-culture phase. Then the light intensity was raised gradually until reaching 300 μmol photons m^{-2} s^{-1}, which is reported as non-limiting for *Rhodomonas* sp. (Vu et al., 2016). The temperature was maintained at 22°C using a water jacket, attached to the culture compartment of the photobioreactor. The pH was set at 7.5 ± 0.1 and maintained constant by mixing CO_2 with the airflow on demand. For the experiment, a two-phase N starvation strategy (growth and N-starvation, respectively) was applied. The experimental setup was performed in duplicate.

Growth Phase

The PBR was filled with 20 times concentrated nutrients of filtrated (0.2 μm pore size) f/2 medium, to ensure nitrogen sufficient conditions. After inoculation, the reactor was operated in batch mode until the outgoing light intensity equaled 15 μmol photons m^{-2} s^{-1} and the biomass concentration reached 1.4 g L^{-1}. Then, the turbidostat mode was applied and the secondary light PAR sensor of the systems ensured stable outgoing light of 15 μmol photons m^{-2} s^{-1} and continuous operation. The growth phase was continued for 5 days with a stable dilution rate. Samples were taken every 24 h in order to ensure a healthy and stable culture.

N-Starvation Phase

After the growth phase, the biomass of the reactor was harvested and centrifuged in 2000 rpm for 15 min. The supernatant was discarded and the cells were washed with N-depleted medium in order to remove nitrogen residuals. The procedure was repeated. After washing, the biomass was transferred in a PBR filled with N-depleted medium. Excepting the nitrogen concentration of the medium, the growth conditions were the same as in the growth phase. During N-starvation, samples were taken at 0, 8, 16, 24, 48, 72, 96, 120, 144, 168, and 192 h after changing to the N-depleted medium. Samples were taken for biomass concentration, cell density and volume, PE and FA composition.

Culture Analysis

Growth Phase

Optical density was measured at 750 nm ($OD_{750\ nm}$) in a spectrophotometer (DR 5000, HACH, United States), from which biomass concentration (C_x) was calculated according to Oostlander et al. (2020). Cell volume was measured in Coulter Counter (Multisizer 3, Beckman Coulter, United States) in the size range 7–14 μm. The growth rate (μ) was calculated when the PBR was running in turbidostat mode as the dilution rate (D) according to equation 1, where V_H is the harvested volume in a Δt period of time and V_R the reactor volume. The biomass production rate (r_x) for the turbidostat mode was calculated from the growth rate and the biomass concentration (C_x, eq. 2).

$$\mu = D = \frac{\frac{V_H}{\Delta t}}{V_R} \quad (1)$$

$$r_x = \mu \times C_x \quad (2)$$

N-Starvation Phase

In the N starvation phase, the cell density and volume and the biomass concentration were measured in triplicates as according to the method described in the section "Growth Phase." Additionally, the daily cell death rate (k_d) was calculated according to eq. 3.

$$k_d = -\frac{ln\frac{Cx_{(t+\Delta t)}}{Cx_t}}{\Delta t} \quad (3)$$

The biomass of each sample was centrifuged at 2500 rpm for 15 min, washed twice with 0.5 M ammonium formate, stored at −80°C and freeze-dried. The lyophilized biomass was used in PE and FA analysis.

Phycoerythrin analysis

The phycobilin pigments were extracted by a freeze-thawing process in 0.05 M phosphate buffer (containing equal volumes of 0.1M 0.1 M K_2HPO_4 and KH_2PO_4 and pH 6.7). The samples were kept in −80°C for 48 h and then for 24 h in 5°C for thawing. A centrifugation step at 4000 rpm for 10 min was followed to discard the biomass pellets from the tubes. The supernatant was analyzed using UV-VIS spectroscopy at 545 nm, according to Bennett and Bogobad (1973) and Lawrenz et al. (2011). PE was calculated in (g L^{-1}) according to eq. 4, where A is the absorbance at 545 nm, ε is the molar extinction coefficient (for PE: 2.41 × 10^6 $L{\cdot}mol^{-1}{\cdot}cm^{-1}$), *d* represents the path length of the cuvette and MW stands for the molecular weight of phycobilin (for PE: 240,000 g mol^{-1}).

$$\mathrm{PE} = \frac{A}{\varepsilon d}^{*} MW \quad (4)$$

Fatty acid analysis

Fatty acids were quantified in 10 mg of lyophilized biomass, according to Breuer et al. (2012). Cell disruption was performed in beat beater tubes ("Lysing matrix E," MP biomedicals, United States) using a Bead Beater (Precellys 24, Bertin Instruments, France) in the presence of glass beads (150–212 μm diameter). Three cycles of 60 s at 2500 rpm with 120 s interval were performed. The lipids were extracted using a chloroform:methanol (1:1.25 v/v) mixture and methylated in a MeOH solution containing 5% H_2SO4 for 3 h at 70°C in a block heater. The fatty acid methyl esters (FAME) quantification was performed in gas chromatography (7890, Agilent, United States) using a 30 m column (Supelco Nukol™) with Helium as carrier gas. FA/FAMEs were identified based on retention time data of known standards. Tripentadecanoin (C15:0 TAG) was used as internal standard for fatty acid quantification. TFA was calculated as the sum of all individual FA. FA were also processed as saturated fatty acids (SFA), monounsaturated fatty acids (MFA), PUFAs and DHA/EPA ratio.

Statistical Analysis

All data measurements are shown as average +/− standard deviation (±SD) of three independent replicates. Data were tested for normal distribution (Kolmogorov-Smirnov goodness of fit test) before being analyzed by one-way analyses of variance (ANOVA) with α = 0.05, using SPSS 15.0. Mann–Kendall test was

performed to determine whether the results have a monotonic trend over the period of N-starvation.

RESULTS

Growth Phase

During the growth phase, the PBR was in a steady state for 6 days (**Figure 1**). The volumetric productivity was stable, 1.5 ± 0.1 g L^{-1} d^{-1}, while the dilution rate was 1.3 ± 0.1 d^{-1}. The biomass concentrations in the PBR was 1.2 ± 0.1 g L^{-1}, which equals 9.2 ± 1.2 × 10^6 cells ml^{-1}. In the time of the growth phase, the cell volume was stable, 535 ± 18 μ m^3.

N-Starvation Phase

Biomass Yield: Cell Density and Cell Volume

The cell density and volume of *Rhodomonas* sp. changed when grown under N-starvation. The cell density reduced significantly ($P < 0.01$) from 6.3 × 10^6 cells ml^{-1} at 0 h until 32 h of N-starvation phase, with a death rate of 0.24 d^{-1}, to 4.6 ± 0.1 × 10^6 cells ml^{-1} (**Figure 2**). From 32 to 120 h of N-starvation, no significant difference ($P > 0.05$) was observed on the cell density. The highest death rate was observed at 144 h, 0.46 d^{-1}, where the cell density decreased to 3.1 × 10^6 cells ml^{-1}. From then on, the cell density continued declining significantly ($P < 0.01$) with a rate of 0.46 d^{-1}. When the experiment stopped, after 192 h in N-starvation phase, the cell density was 2.5 × 10^6 cells ml^{-1}. The cell volume increased significantly after N starvation from 556 ± 65 μm^3 at $t = 0$ h to 660 ± 67 μm at $t = 32$ h ($P < 0.01$). After 32 h the cell volume reduced gradually to 304 ± 36 μm at $t = 192$ h ($P < 0.01$).

Phycoerythrin

The PE concentration in *Rhodomonas* sp. when the N-starvation phase started was 19.1% of total DW and remained stable for the first 8 h (**Figure 3**). After 8 h, PE declined significantly ($P < 0.01$) until 32 h after N-starvation, when it was equal to 4.5% of total DW. From 32 until 74 h after N-starvation, there was no significant change in PE concentration ($P > 0.05$).

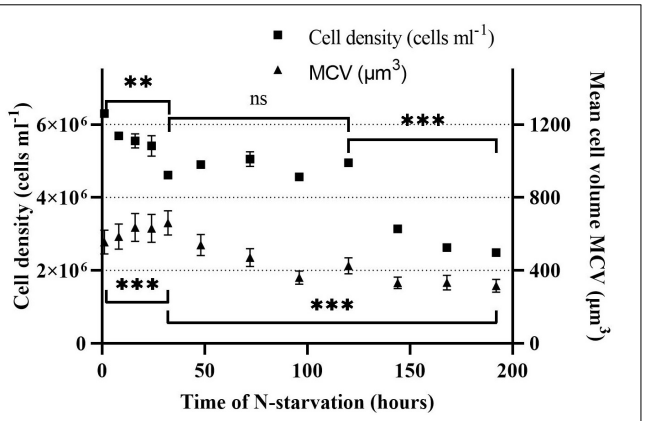

FIGURE 2 | Cell density and mean cell volume (MCV) of *Rhodomonas* sp. during a 192 h N-starvation period of time. Data are expressed as the average of three replicates ± SD. *Indicates the significant difference after ANOVA *post hoc* test. ns $P > 0.05$, **$P < 0.01$, ***$P < 0.001$.

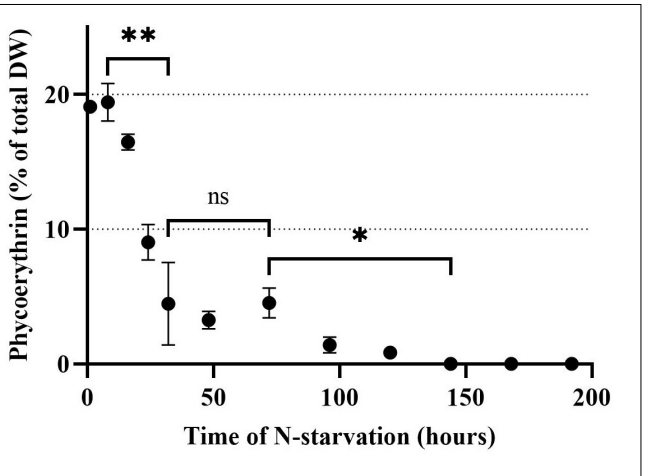

FIGURE 3 | Phycoerythrin concentration of *Rhodomonas* sp. during a 192 h N-starvation period of time. Data are expressed as the average of three replicates ± SD. *Indicates the significant difference after ANOVA *post hoc* test. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$.

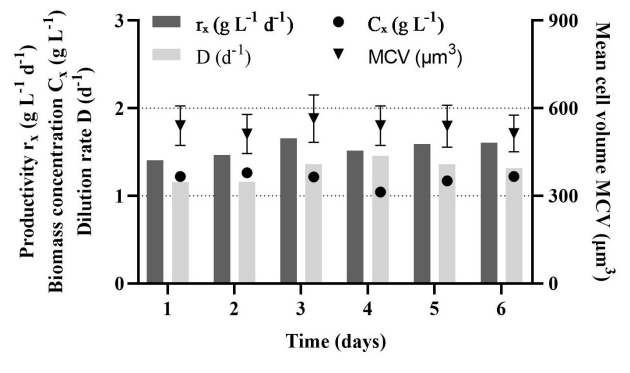

FIGURE 1 | Steady state volumetric productivity (rx), dilution rate (D), biomass concentration (Cx) and mean cell volume (MCV) of *Rhodomonas* sp. in a photobioreactor during the growth phase.

After 74 h of N-starvation, PE concentration declined further significantly ($P < 0.01$) until 144 h after N starvation, when no PE could be measured.

Fatty Acid Analysis

Rhodomonas sp. accumulated FA 32 h after the transfer into the N-depleted medium (**Figure 4**). There was no significant difference in TFA the first 24 h of N-starvation phase ($P > 0.05$). TFA increased significantly from 9.2% of total DW at 24 h to 30.3% of total DW at 120 h ($P < 0.01$). After 120 h no significant ($P > 0.05$) increase of TFA was measured up until 192 h of N- starvation. SFA did not change significantly in the first 24 h of N-starvation ($P > 0.05$). A significant increase ($P < 0.01$) of SFA concentration was observed after 24 h and continued until 120 h of N-starvation, from 2.3% to 12.4% of total DW, respectively. PUFA content followed the same pattern as SFA

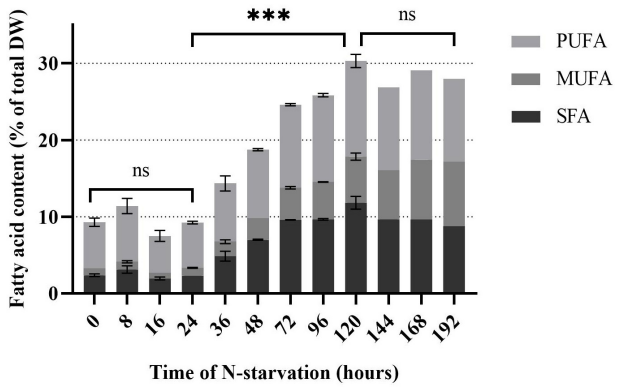

FIGURE 4 | Fatty acid concentration of *Rhodomonas* sp. during a 192 h N-starvation period. SFA: Saturated fatty acids, MUFA: Monounsaturated fatty acids and PUFA: Polyunsaturated fatty acids. Data are expressed as the average of three replicates ± SD. *Indicates the significant difference after ANOVA *post hoc* test. ns $P > 0.05$, ***$P < 0.001$.

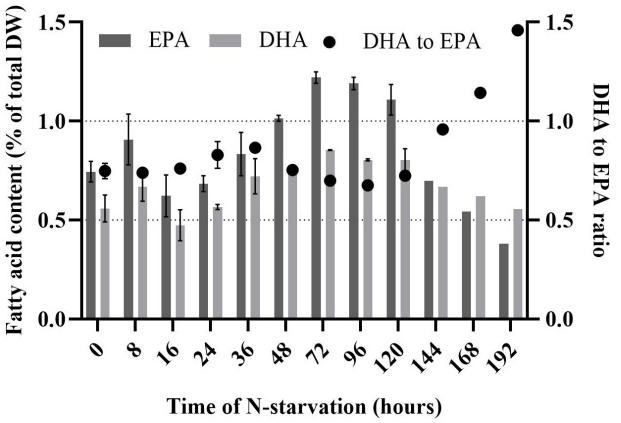

FIGURE 5 | Eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and DHA to EPA ratio of *Rhodomonas* sp. during a 192 h N-starvation period. Data are expressed as the average of three replicates ± SD.

(**Figure 4**). PUFAs content started at 6% of total DW before the N-starvation phase and raised to 12.5% of total DW 120 h after the N-starvation started. The same pattern was followed by the MUFA rising from 1% at 24 h to 6.4% of total DW at 120 h of N-starvation. After 120 h the SFA and PUFA showed a decrease, while the MUFA kept increasing until 192 h of N starvation period to 8.4% of total DW. EPA and DHA showed a peak after 72 h, 1.2 and 0.9% of total DW, respectively. The initial concentration was 0.7% and 0.6% of total DW for EPA and DHA (**Figure 5**). After 192 h, the EPA and DHA concentration dropped to 0.4% and 0.6% of total DW, respectively. The DHA to EPA ratio did not show a significant difference ($P > 0.05$) the first 120 h after N-starvation and remained around 0.72. At 144 h, the DHA to EPA ratio more than doubled ($P < 0.01$), to 1.46 at 192 h.

The FA with the highest concentration were linolenic (18:3, 1.8% of total DW), stearidonic (18:4, 1.5% of total DW), palmitic acid (16:0, 1.2% of total DW) and linoleic (18:2, 1.2% of total DW) acids before the starvation period. After 120 h of N-starvation the highest FA in *Rhodomonas* sp. were palmitic acid (6.1% of total DW), oleic acid (18:1, 5.5% of total DW) and linolenic acid (3.7% of total DW) (**Supplementary Table S1**).

DISCUSSION

The nutritional value of microalgae as aquaculture feed is an essential key factor for the aquaculture sector. This study established that *Rhodomonas* sp. quality can be maintained stable in a PBR, but it can also be manipulated. An N-starvation application can modify the quality of *Rhodomonas* sp. by inducing the FA accumulation and protein consumption (PE as proxy).

Growth Phase

During the growth phase *Rhodomonas* sp. was stable in the PBR in the sense of biomass productivity and cell volume. The growth rate that was observed during a period of 6 days, 1.3 ± 0.1 d^{-1}, was higher than most of the maximum growth rates that have been reported for *Rhodomonas* species in the literature, 0.8 to 1.0 d^{-1} (Bartual et al., 2002; Lafarga-De la Cruz et al., 2006; Vu et al., 2016). Only Fernandes et al. (2016) reported a higher growth rate of 1.6 d^{-1} for the strain of *Rhodomonas marina*. It has to be noted that the maximum growth rate data from these studies were obtained in batch cultures during exponential phase. In our study, we present the steady state growth rates during a turbidostat mode, not the maximum growth rate of the strain. Moreover, interspecific variation can explain the variation of the growth phase data of this study with the data given in other studies. For example, Guevara et al. (2016) studied two *Rhodomonas salina* species and obtained significant differences in growth and nutritional quality between the species (protein, total lipids, EPA, and DHA).

N-Starvation Phase

Cell Volume and Phycoerythrin

The cell volume of *Rhodomonas* sp. changed under N-starvation, with cells being bigger the first 32 h of N-starvation phase, thereafter observing a significant cell decrease. The volume increase at the beginning of the N-starvation phase could be explained by carbohydrate accumulation, which is reported to start earlier than lipid accumulation. Jia et al. (2015) cultivated *Nannochloropsis oceanica* in N-deplete medium for 14 days and observed a 2-fold increase of free glucose and mannitol the first and second day of starvation, respectively. Previous research on *Rhodomonas* sp. (da Silva et al., 2009) illustrated that N-starved cells increased their volume in the first 3 days of the N-starvation phase. The decrease of cell volume that followed after 48 h in N-free medium in our experiment is supported in many studies. Kilham et al. (1997) found that the volume per cell of *Ankistrodesmus falcatus* decreased as a function of N-limitation compare to non-limited cells. Lynn

et al. (2000) also observed smaller cell sizes for N-limited *Stephanodiscus minululus* cultures as compared to non-limited cultures. Rhee (1978) observed that *Scenedesmus* sp. cells size was smaller when growing in N-limited medium and explained it in terms of lower protein content. The decrease of protein content has been reported for many algae species grown under N-starvation conditions (Harrison et al., 1990; Lynn et al., 2000). Jia et al. (2015) reported a decrease in protein concentration of *Nannochloropsis oceanica*, which started 48 h after N-starvation phase.

The reduction of cell volume and the correlation with protein content can be explained in our study by the decrease of PE concentration (here used as a proxy for cellular protein concentration), which started 16 h after the N-starvation started. It has also been demonstrated in other studies that cells of *Rhodomonas* sp., as in some other cryptomonads, showed a drastic decrease of the protein and phycoerythrin content upon N-starvation (Sciandra et al., 2000; Bartual et al., 2002; Vu et al., 2016). Yamamoto et al. (2020) reported for *Rhodomonas* sp. a 75% reduction of PE concentration between the exponential and late stationary phase, where no N-source is available. In our research, the PE concentration was reduced by 75% 32 h after N-starvation, while after 4 days of starvation, the PE reduction was 93%. The different light intensity conditions that were used in Yamamoto et al. research can clarify the differences in the PE concentrations with our results. Consistent with Vu et al. (2016) study, N-starvation conditions caused a reduction in PE. Proteolysis of phycobiliproteins in cyanobacteria under N-starvation maintain the protein turnover (Grossman et al., 1994). These studies suggest that PE behaves as cell reserves in N-deprivation conditions providing amino acids for new protein synthesis.

FA Concentration

Many publications support that microalgae decrease their cell division and start to accumulate lipid under nutritional limitation (Shifrin and Chisholm, 1981; Piorreck et al., 1984) or starvation (Tornabene et al., 1983; Converti et al., 2009). Illman et al. (2000) showed the response of five *Chlorella* species to N-limitation. Lipid content doubled for all *Chlorella* species in low N medium. PUFA content increases relative to the increasing nutrient limitation (Janssen et al., 2019). In our study, the FA (SFA, MUFA, and PUFA) increase is in line with the literature for *Rhodomonas* sp. in N-starvation phase (Yamamoto et al., 2020). The highest increase during N-starvation was obtained for oleic (C18:1), palmitic (C16:0), and linoleic (C18:2) acid, 10-fold, 5-fold and 4-fold, respectively. PUFAs, which are the most important FA for aquaculture, indicated a 2-fold increase in the first 96 h of N-starvation. Similar increased PUFA content (65% of total FA) was reported by Coutinho et al. (2020) for *Rhodomonas lens* maintained under nitrate saturated conditions. DHA and EPA presented a peak in concentration 72 h after N-starvation. However, at the end of the experiment (120 h in N-starvation), DHA concentration returned to the initial value (t = 0 h), while EPA showed a 2-fold decrease compared to the initial concentration.

Fatty Acid Composition

The major FA's of *Rhodomonas* sp. in this study were palmitic (C16:0), linoleic (C18:2), linolenic (C18:3), and stearidonic (C18:4) acid. These FA's are in line with the literature results for *Rhodomonas* species (van Houcke et al., 2017; Vu et al., 2019; Coutinho et al., 2020). However, differ from lipid profiles of other microalgae that are used in aquaculture, for example, *Tisochrysis lutea*, *Chaetoceros neogracile*, *Skeletonema marinoi*, *Pavlova lutheri*, *Nitzschia* sp., *Thalassiosira* sp. *Tetraselmis* sp., *Dictyosphaerium pulchellum, Stichococcus* sp., *Chlorella* sp. and *Scenedesmus* sp. (Pratoomyot et al., 2005; González-Araya et al., 2012; González-Araya and Robert, 2018). The SFA composition or *Rhodomonas* sp. varies from 25 to 39% of TFA, which is similar to Yamamoto et al. (2020) research on *Rhodomonas* sp. The most significant difference is noticed in PUFA, which is 65% of TFA in *Rhodomonas* sp., while in other marine algae varies from 25 to 47% of TFA. This difference is defined mainly by the higher DHA content, 6% of TFA, while the EPA concentration is 8% of TFA, higher than *Tisochrysis lutea* and *Tetraselmis* sp., but lower than *Pavlova lutheri*, *Chaetoceros neogracile*, *Skeletonema marinoi*, *Thalassiosira* sp. and *Nitzschia* sp. This comparison confirms the interspecific nutritional value variability due to the differences in absolute amounts of TFA, EPA and DHA between algae species (Boelen et al., 2013).

DHA to EPA Ratio

Due to the changes in the absolute value of DHA and EPA, the DHA to EPA ratio is affected. In our study, the DHA to EPA ratio the first 120 h in N-starvation remained relatively stable, 0.75 $\pm$ 0.05. This ratio is similar to the ratio that has been reported before for *Rhodomonas* species (Dunstan et al., 2005; Drillet et al., 2006; Vu et al., 2016). Boelen et al. (2017) reported that DHA to EPA ratio of *Rhodomonas salina* decreased from exponential to stationary and late stationary phase, from 0.72 to 0.56 and 0.46, respectively. In contrast to Boelen et al. (2017) results, our research showed a twofold increase of DHA to EPA ratio of *Rhodomonas* sp. to 1.5 after 192 h in N-starvation. The effect of N-starvation on DHA and EPA can affect the nutritional quality of *Rhodomonas* sp., as it is reported that the nutritional value of microalgae in aquaculture depends not only on the quantity of DHA and EPA but also on the DHA to EPA ratio, which has proved essential for fish larvae development (Rodríguez et al., 1998).

CONCLUSION

This study has illustrated that *Rhodomonas* sp. adapts rapidly to changes in N availability by changes in cell density and volume and biochemical composition and as a result, N availability can strongly enhance the nutritional value of *Rhodomonas* sp. The high PUFA concentration of *Rhodomonas* sp. and the increase of PUFA under N-starvation makes it good nutritional feed for aquaculture animals. This research demonstrates that in the application of *Rhodomonas* sp. as aquaculture feed, the time of

harvest (and the starvation applied) is essential in order to obtain the desired quality as a feed.

In order to benefit from our results on a (semi-)commercial scale cultivation systems of management should be adapted. A cultivation system could be divided into two phases. The first phase enables a steady and high production rate of *Rhodomonas* sp. in optimal cultivation conditions and N-sufficient medium (20 × f/2 medium, 300 μmol photons m^{-2} s^{-1} and 22 °C). The second phase induces N-starvation, where *Rhodomonas* sp. accumulates lipids. In the second phase, there is a critical point for harvesting, and it depends on the purpose of the feed. PUFAs have the highest concentration 120 h after N-starvation, while EPA and DHA present maximum concentration 72 h after N-starvation and DHA to EPA ratio rises above 1 after 144 h in N-starvation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CL contributed to the design and implementation of the research, analysis of the results, and writing of the original draft of the manuscript. JH and KT supervised the project and results, and contributed to the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Marine Science

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Effect of Short-Term Rotifer Enrichment With Marine Phospholipids on Growth, Survival, and Composition of Meager (*Argyrosomus regius*) Larvae

Sirwe Ghaderpour[1] *and Alicia Estevez*[2]*

[1] *Department of Aquaculture, Faculty of Natural Sciences, Urmia University, Urmia, Iran,* [2] *Centro de San Carlos de la Rápita, Institut de Recerca i Tecnologia Agroalimentaries (IRTA), San Carlos de la Rápita, Tarragona, Spain*

Larval rearing and ongrowing of meager have experienced a great advance during the last years and nowadays this species is considered as one of the most important new species in Mediterranean aquaculture. However, larval nutrition in meager still needs some improvements especially regarding live prey enrichment and fatty acid composition. In this study, a trial for larval rearing of meager (*Argyrosomus regius*) using different commercial products for rotifer enrichment (Multigain, Red Pepper and concentrated microalgae) with and without phospholipids (PL), and with a different fatty acid composition, especially regarding DHA, have been used to assess the growth and survival of the larvae and check the effect of PLs in larval growth. Lipid class composition was the same among the larvae whereas % DHA was always higher in larvae fed Multigain and Red Pepper enriched rotifers. The dominant fatty acids in the PL of the larvae and PL of the diets were well-correlated and the results suggested a close relationship between the composition of dominant PL fatty acids in the rotifer and in the larvae, especially in the case of DHA and 16:0 that show a high correlation.

Keywords: survival, growth, marine phospholipids, rotifers (*Brachionus plicatilis*), meager (*Argyrosomus regius*), fatty acid (composition)

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***Correspondence:**
Alicia Estevez
alicia.estevez@irta.es

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INTRODUCTION

Nutrition and feeding during early development is one of the main important issues for marine fish larvae culture, especially for new species (Campoverde and Estévez, 2017). The success of larval rearing is influenced by the live prey selected for first feeding and their nutritional quality, being their content in lipids and their fatty acid profile the most important nutritional factors affecting larval growth and survival (Watanabe, 1993).

Marine lipids are rich in saturated and monounsaturated fatty acids, which are the main source of metabolic energy for the fast developing and growing fish larvae. Polyunsaturated fatty acids (PUFA) must also be provided in the first feeding diets of marine fish larvae, because they are considered essential fatty acids (EFA) since they cannot be biosynthesized. Three long chain PUFA (LC-PUFA), docosahexaenoic acid (DHA, 22:6n−3), eicosapentaenoic acid (EPA, 20:5n−3), and arachidonic acid (ARA, 20:4n−6) play several important roles in vital functions of fish. They are

the main components of membranes and precursors of bioactive molecules such as eicosanoids which have hormone-like activity (Tocher, 2010). DHA also plays an important role during larval development as it is incorporated into nervous and retina tissue (Mourente and Tocher, 1992; Bell et al., 1996), and in the case that DHA is not provided to the larvae, poor growth, high mortality and several behavioral, physiological and morphological alterations have been observed (Lingenfelser et al., 1995; Tocher, 2010).

Phospholipids (PLs) are considered essential for fish larvae (Tocher et al., 2008; Cahu et al., 2009) due to their limited capacity of biosynthesis. Between 2 and 12% must be supplied in the diet for a normal growth and functional development as Tocher et al. (2008) suggested. *Artemia* and rotifers, the live preys used in aquaculture, have high PL content (Olsen, 2004) but their fatty acid composition, that is the absence of LC-PUFA, is not adequate for early larvae and they must be enriched before feeding them. On the other hand, copepods, the live prey that the larvae feed in the wild, are rich in phospholipids and in n−3 PUFA. Although the content of PL per dry weight (DW) of rotifers and *Artemia* cannot be manipulated, the fatty acid composition of PL can be modified in some degree (Guinot et al., 2013; Li et al., 2014; Rogacki et al., 2019). Supplementation of PL is usually carried out using commercial lecithin, either obtained from plants (soybean), chicken eggs, or pure phospholipids. Recently several marine phospholipids derived from krill, with a better fatty acid profile in terms of n−3 PUFA, are also available and have been used for *Artemia* enrichment (Guinot et al., 2013; Rogacki et al., 2019). One of the main problems in enriching the *Artemia* with DHA and PLs is the rapid metabolism of the metanauplii, that retroconvert DHA into EPA (Navarro et al., 1999), and when these LC-PUFA are delivered to *Artemia* as PLs, the metanauplii metabolize them into other lipid classes, particularly triacylglycerols (TAG), metabolizing EFA from PL fraction into the NL fraction (Guinot et al., 2013), being one of the most important handicaps for bioencapsulation of PLs and LC-PUFA in *Artemia*. On the other hand, in the case of rotifers Li et al. (2015) have shown that it is possible to enrich the DHA levels in PLs of rotifers to relatively high levels using short-term enrichment strategies.

Meager, *Argyrosomus regius*, is a new and valuable finfish species with increasing interest in European aquaculture due to its rapid growth during ongrowing and the quality of its meat with muscle low in fat content (Poli et al., 2003; Grigorakis et al., 2011). Protocols regarding spawning induction (Duncan et al., 2012), larval development (Jimenez et al., 2007; Cardeira et al., 2012) and larval rearing (Roo et al., 2010; Vallés and Estevez, 2013, 2015b; Campoverde et al., 2017) have already been published. Some information is also available about the nutritional requirements of the larva, including LC-PUFA, either using commercial enrichment products or microdiets for larval feeding (Vallés and Estevez, 2015a; Campoverde and Estévez, 2017; El Kertaoui et al., 2017). On the other hand, recent studies carried out with cod larvae have shown that DHA is better available to the larvae when it is incorporated into dietary PLs than in NLs (Olsen et al., 2014) for a better growth and organ and skeleton development.

Thus, the objective of the present study was to learn about the effect of different rotifer enrichment diets based on their content in LC-PUFA, and supplemented with or without marine phospholipids, on the growth, survival and fatty acid composition of meager larvae. The capacity of meager larvae to incorporate DHA from dietary PL in the early stages of feeding and growth was also examined.

MATERIALS AND METHODS

Meager Larviculture and Sampling

Newly hatched meager (*Argyrosomus regius*) larvae were obtained from a commercial hatchery and transported by road to the Institut de Recerca i Tecnologia Agroalimentaries (IRTA, San Carles de la Rapita, Spain). Larvae were distributed into 18 40 L mesh-made basckets provided with air-lifts, placed in triplicates in 200 L tanks at a density of 50 larvae L^{-1}. The tanks were connected to a recirculation unit (IRTAmar^{TM}) and water temperature was controlled every day and maintained at 20.0 ± 1.2°C, salinity at 35.82 ± 0.33 ppt, dissolved oxygen at 7.2 ± 1.0 mg L^{-1} whereas pH (7.97 ± 0.06) nitrites (0.02 ± 0.02 mg L^{-1}) and ammonia (0.1 ± 0.05 mg L^{-1}) were checked 2 times per week (Hach Colorimeter DR/890, USA). Photoperiod was kept at 16 h light: 8 h darkness and light intensity was maintained at 500 lux at water surface following the recommendations of Vallés and Estevez (2013). At mouth opening, 2 days post-hatching (dph), larvae started being fed enriched rotifers until 14 dph twice daily (09:00 and 17:00 h) at a density of 10 rotifers mL^{-1}.

Samples of 10 larvae were collected from each bascket at the beginning and at the end of the trial (15 dph) and killed with an overdose of anesthetic MS222 (1,000 mg L^{-1}). Standard length (SL) was measured using a dissecting microscope and an image analyzer (Analysis, SIS Gmbh, Germany). The upper jaw length (Shirota, 1978) was measured in order to analyze larvae species gape size (G_S) and the mouth opening at 90° calculated (Shirota, 1970). The same larvae were then washed on a mesh with distilled water, dry blotted to remove excess water, and pooled onto pre-weighted coverslips. They were then oven-dried at 60°C for 24 h and weighted to determine dry weight (DW) on a Mettler A-20 microbalance (Mettler Toledo, Columbus, OH, USA) to the nearest ±1 μg. Specific growth rate (SGR) was calculated at the end of the trials using the formula:

$$SGR = (\ln Wf - \ln Wi \times 100)/t \ (\% \ day^{-1})$$

Where lnWf = the natural logarithm of the final weight; lnWi = the natural logarithm of the initial weight; and t = time (days) between lnWf and lnWi.

At the end of the experiment, all the larvae remaining in the tanks were anesthetized with MS-222, concentrated in a mesh, and counted to calculate the survival rate. All the larvae from the three replicates were washed with distilled water before freezing at −20°C for later analysis of lipids, lipid class composition, and fatty acids.

Rotifer Enrichment

Rotifers (*Brachionus* sp. Cayman) were cultivated in batch culture in 100 l conical fiberglass tanks at a salinity of 26 ppt, water

temperature of 26°C, and 8 mg l^{-1} dissolved oxygen. The rotifer culture was aerated and daily fed microalgae (*Tetraselmis chuii)* at 4 × 10^5 cell ml^{-1} and yeast (Mauripan, Spain) at 0.7 g/million rotifers. The daily ration of rotifers was calculated after counting the rotifers, carefully harvested and divided into six oxygenated 10 l containers filled with UV filtered seawater and enriched for 12 h with the commercial enrichers Larviva Multigain (MG, a powder product, Biomar, Denmark), Red Pepper (RP, liquid product, Bernaqua, Belgium) and concentrated Nannochloropsis (NC, concentrated microalgae 6.25 × 10^9 cels ml^{-1}, Blueclownfish, Spain) at a density of 500 rotifers ml^{-1} and 28 ± 1°C. The enrichment products were administered to the rotifers in 2 doses of 0.6 g l^{-1} of MG, 2 doses of 0.9 g l^{-1} of RP, and 2 doses of 2.5 g l^{-1} of NC following the recommendations of the products, doses were distributed at the beginning (0 h) and after 6 h enrichment. After 12 h the rotifers were gently filtered and washed using a 40 μm mesh with UV filtered seawater and disinfected by further rinsing for 1 min with freshwater, before feeding to the larvae. The same dose of rotifers, previously counted, enrichments, and treatments were used for the rotifers enriched with phospholipids, but in that case, a short-term enrichment, also known as boosting, was carried out for 3 h adding 0.6 g l^{-1} of LC60 (phospholipids extracted from krill, Phosphotech, France). The rotifers were then concentrated, rinsed, and washed with freshwater before feeding the larvae.

Samples of enriched rotifers for biochemical analyses were collected during the trial (days 2, 8, and 14), the samples were concentrated, washed with distilled water, and freezed at −20°C until analysis. All the analyses were carried out in duplicates.

Rotifers were chosen as the only live prey due to the difficulties in enriching *Artemia* with phospholipids and/or n−3 PUFA. Most studies of DHA enrichment of PL in *Artemia* showed that this fatty acid ended up in other lipid classes, mostly triacylglycerols (TAG), and very little was incorporated into PL, as Guinot et al. (2013), Monroig et al. (2006), and Sargent et al. (1999) have already established. Li and Olsen (2015) using a

TABLE 1 | Total lipid and main lipid class content and fatty acid composition of the commercial products used for rotifer enrichment: Larviva Multigain (MG), Red Pepper (RP), Concentrated Nannochloropsis (NC), and marine phospholipids (LC60).

	Enrichment product			
	MG	**RP**	**NC**	**LC60**
Total lipids (mg g^{-1} DW)	459.4 ±3.8c	438.7 ±1.7b	18.7 ±2.5a	508.8 ±9.3d
Total FA (mg g^{-1} Lipids)	772.2 ±13.5	774.6 ±13.8	532.8 ±15.8	588.2 ±13.9
Total PL (% Total Lipids)[1]	9.3 ± 0.0a	nd	17.0 ± 3.1b	30.7 ± 1.3c
Phosphatidylcholine (PC)	6.1 ±0.1b	nd	4.4 ±0.6a	12.6 ±0.6c
Phosphatidylethanolamine (PE)	0.6 ±0.0a	nd	10.0 ±1.3b	9.3 ±0.1*b*
Total NL (% Total Lipids)[2]	90.8 ± 0.0c	100.0 ± 0.0d	83.0 ± 3.1b	69.3 ± 1.3a
Triacylglycerols (TAG)	76.1 ±0.9*c*	74.4 ±0.7c	8.6 ±0.8b	3.1 ±0.1a
Cholesterol (CHO)	3.0 ±0.1a	4.0 ±0.1a	6.6 ±2.3a	17.3 ±0.5b
Fatty acids (mg g^{-1} Lipids)				
16:0	34.1 ±7.7	230.1 ±1.7	115.8 ±4.5	159.5 ±1.8
Total saturated[3]	315.3 ± 0.0d	243.9 ± 1.7c	133.8 ± 4.5a	185.4 ± 4.4b
16:1	nd	3.8 ±0.8	95.7 ±5.0	2.4 ±1.5
18:1	16.8 ±0.0	24.8 ±0.3	21.4 ±14.9	17.5 ±3.0
Total monounsaturated[4]	16.8 ± 0.0a	28.6 ± 5.1a	139.2 ± 21.2c	53.6 ± 3.5b
18:2n−6	19.7 ±1.2	27.9 ±0.8	21.5 ±3.4	6.2 ±0.0
20:4n−6	nd	5.3 ±0.0	nd	7.5 ±0.3
22:5n−6	102.0 ±1.8	100.0 ±4.2	3.6 ±2.4	nd
Total n–6 PUFA[5]	124.4 ± 0.7c	135.4 ± 4.8d	34.5 ± 7.7b	15.6 ± 0.2a
18:3n−3	8.8 ±0.4	10.6 ±0.3	32.5 ±7.7	nd
20:5n−3	5.8 ±0.2a	18.0 ±2.8ab	172.7 ± 12.0c	81.5 ±0.0b
22:6n−3	299.9 ±4.9b	335.1 ±9.5b	20.2 ±8.9a	251.9 ±4.2b
Total n–3 PUFA[6]	315.7 ± 5.2b	366.8 ± 12.4b	255.3 ± 6.7a	335.2 ± 4.0b
Total PUFA	440.1 ± 5.8c	502.1 ± 17.2d	259.8 ± 1.0a	350.8 ± 2.0b
n−3/ n−6	2.5 ±0.3a	2.7 ±2.4a	7.3 ±0.0ab	21.5 ±5.6b
DHA/EPA	51.6 ±1.1d	18.6 ±1.4c	0.1 ±1.4a	3.1 ±0.4b

[1]*Includes phosphatidylinositol, phosphatidylserine, cardiolipin and phosphatidic acid.*
[2]*Includes free fatty acids, sterol esters, and waxes.*
[3]*14:0, 15:0, 17:0, 18:0, 20:0, 21:0.*
[4]*Includes 14:1, 16:1, 18:1n−7, 20:1, 22:1, 24:1n−9.*
[5]*Includes 18:3n−6.*
[6]*Includes 18:4n−3, 20:3n−3, 22:5n−3.*
Different letters indicate significant differences among the groups (ANOVA, $P < 0.05$).

long-term rotifer enrichment protocol for cod larval feeding obtained high DHA levels in the PL fraction of rotifers showing a better capacity of this live prey to be enriched in PLs.

Commercial enrichment products Multigain and Red Pepper were selected due to its high use in European hatcheries and their particularities, Multigain is a powder product with 90% NL and 10% PL whereas Red Pepper is a liquid composed only by NLs.

Phospholipid enrichment of rotifers was carried out for 3 h to assure enough quantity of DHA in the PL of the rotifers (E. Almansa, pers.com., Mylonas et al., 2016).

Lipid Content, Lipid Class Composition and Fatty Acid Analysis

Total lipids were extracted in chloroform:methanol (2:1, v:v) using the method of Folch et al. (1957) and quantified gravimetrically after evaporation of the solvent under a nitrogen flow followed by vacuum desiccation overnight. Total lipids were stored in chloroform:methanol (2:1, 20 mg ml^{-1}) containing 0.01% butylated hydroxytoluene (BHT) at −20°C prior to analysis. Acid catalyzed transmethylation was carried out using the method of Christie (1982). Methyl esters were extracted twice using isohexane diethyl ether (1:1, v:v), purified on TLC plates (Silica gel 60, VWR, Lutterworth, UK) and analyzed by gas–liquid chromatography on a Thermo TraceGC (Thermo Fisher, Spain) fitted with a BPX70 capillary column (30 m × 0.25 mm id; SGE, UK), using a two-stage thermal gradient from 50°C (injection temperature) to 150°C after ramping at 40°C min^{-1} and holding at 250°C after ramping at 2°Cmin^{-1}. Helium (1.2 ml min^{-1} constant flow rate) as the carrier gas and on-column injection and flame ionization detection at 250°C were used. Peaks of

TABLE 2 | Total lipid and main lipid class content and fatty acid composition of the enriched rotifers used for larval feeding: Larviva Multigain (MG), Red Pepper (RP), Concentrated Nannochloropsis (NC), and marine phospholipids (LC60).

	Enriched rotifer					
	MG	**RP**	**NC**	**MG+LC60**	**RP+LC60**	**NC+LC60**
Total lipids (mg g^{-1} DW)	135.4 ± 4.0c	112.9 ± 27.7b	85.2 ± 11.5a	129.4 ± 10.0b	127.0 ± 2.7b	108.3 ± 23.7b
Total FA (mg g^{-1} Lipids)	705.6 ± 3.7	762.4 ± 2.6	677.0 ± 8.6	751.1 ± 14.2	608.5 ± 1.1	696.3 ± 5.1
Total PL (% Total Lipids)[1]	24.6 ± 0.3a	22.48 ± 0.2a	26.8 ± 1.5a	24.4 ± 0.1a	26.6 ± 2.0a	40.8 ± 1.3b
Phosphatidylcholine (PC)	11.1 ± 0.3	11.28 ± 0.3	11.7 ± 1.2	9.59 ± 0.6	12.0 ± 0.5	12.15 ± 0.6
Phosphatidylethanolamine (PE)	7.0 ± 0.3	4.9 ± 0.2	8.3 ± 0.0	6.8 ± 0.2	6.4 ± 0.3	15.3 ± 0.3
Total NL (% Total Lipids)[2]	75.4 ± 0.3b	77.52 ± 0.2c	73.2 ± 1.5b	75.6 ± 0.1b	73.4 ± 2.0b	59.2 ± 1.3a
Triacylglycerols (TAG)	49.1 ± 1.0f	39.32 ± 0.8d	26.2 ± 0.9b	41.91 ± 1.1e	32.3 ± 0.3c	15.69 ± 0.3a
Cholesterol (CHO)	8.5 ± 0.0a	10.4 ± 0.0b	14.3 ± 0.2c	12.2 ± 0.5b	8.2 ± 0.0a	12.8 ± 0.3b
Fatty acids (mg g^{-1} Lipids)						
16:0	113.7 ± 11.5	134.5 ± 12.1	85.7 ± 6.8	140.2 ± 18.0	121.7 ± 2.6	96.8 ± 13.4
Total saturated[3]	162.3 ± 20.1b	196.1 ± 12.8a	152.1 ± 18.5b	188.0 ± 15.3a	174.6 ± 18.1ab	149.7 ± 10.6b
16:1	12.4 ± 1.2	13.1 ± 1.6	36.8 ± 3.7	10.8 ± 5.1	10.6 ± 1.9	16.0 ± 0.6
18:1n−9	31.7 ± 4.6	37.9 ± 1.2	57.3 ± 9.7	30.6 ± 5.1	34.0 ± 4.2	22.7 ± 6.0
18:1n−7	15.2 ± 4.8	18.5 ± 2.9	29.5 ± 1.7	15.2 ± 6.9	17.3 ± 4.6	17.8 ± 6.6
Total monounsaturated[4]	81.3 ± 13.4b	95.8 ± 1.7b	112.6 ± 14.4a	77.5 ± 10.1b	78.9 ± 15.9b	75.0 ± 16.0b
18:2n−6	56.1 ± 3.6	64.7 ± 3.8	78.9 ± 8.5	60.7 ± 5.3	58.9 ± 9.7	46.9 ± 3.7
20:4n−6	7.9 ± 1.4	8.8 ± 1.4	10.0 ± 2.5	8.1 ± 2.6	8.8 ± 0.1	8.1 ± 2.2
22:5n−6	56.0 ± 10.5	40.5 ± 13.9	6.5 ± 0.4	42.3 ± 10.2	16.2 ± 6.0	16.1 ± 6.0
Total n–6 PUFA[5]	129.5 ± 10.1b	126.7 ± 5.7b	105.5 ± 19.4b	121.3 ± 4.3b	103.7 ± 9.6ab	86.3 ± 5.4a
18:3n−3	51.3 ± 6.3	57.9 ± 7.5	91.1 ± 8.7	54.2 ± 4.7	47.1 ± 8.2	66.0 ± 9.2
20:5n−3	29.6 ± 2.0ab	25.3 ± 1.5a	34.4 ± 8.2ab	39.9 ± 4.6ab	43.7 ± 5.1b	42.4 ± 9.2ab
22:6n−3	193.0 ± 0.2d	145.5 ± 7.7c	20.0 ± 4.9a	199.2 ± 6.6d	151.0 ± 6.9c	105.4 ± 8.1b
Total n–3 PUFA[6]	287.1 ± 14.6c	238.2 ± 6.4b	164.6 ± 2.7a	303.8 ± 8.7c	251.3 ± 6.9b	224.3 ± 3.3b
Total PUFA	416.6 ± 4.6d	364.9 ± 7.0c	270.1 ± 7.6a	425.1 ± 8.3d	355.0 ± 3.5c	310.6 ± 2.1b
Total UK[7]	45.4 ± 3.2	105.6 ± 5.8	142.2 ± 8.7	60.5 ± 3.1		161.0 ± 9.0
n−3/n−6	3.2 ± 0.6b	1.9 ± 0.2a	1.5 ± 0.1a	3.1 ± 0.2b	2.7 ± 0.2b	2.7 ± 0.3b
DHA/EPA	6.5 ± 0.2f	5.8 ± 0.1e	0.6 ± 0.0a	5.0 ± 0.2d	3.5 ± 0.2c	2.5 ± 0.0b

[1]Includes phosphatidylinositol, phosphatidylserine, cardiolipin, and phosphatidic acid.
[2]Includes free fatty acids, sterol esters, and waxes.
[3]14:0, 15:0, 17:0, 18:0, 20:0, 21:0.
[4]Includes 14:1, 16:1, 18:1n−7, 20:1, 22:1, 24:1n−9.
[5]Includes 18:3n−6.
[6]Includes 18:4n−3, 20:3n−3, 22:5n−3.
[7]Includes 16:3 and 16:4 and not identified fatty acids.
Different letters indicate significant differences (ANOVA $P < 0.05$).

each fatty acid were identified by comparison with known standards (Supelco Inc., Spain) and a well-characterized fish oil, and quantified by means of the response factor to the internal standard, 21:0 fatty acid, added prior to transmethylation, using a Chrom-card for Windows (TraceGC, Thermo Fisher, Spain).

Lipid class composition was determined by high-performance thin-layer chromatography (HPTLC). Approximately 10 μg of lipid was applied as a 2 mm streak and the plate developed to two-thirds distance with methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol.), to separate polar lipid classes, and then fully developed with isohexane/diethyl ether/acetic acid (85:15:1, by vol.). Lipid classes were visualized by charring at 160°C for 15 min after spraying with 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid and quantified by densitometry using a GS-800 Densitometer (Bio-Rad Laboratories, Spain) following Olsen and Henderson (1989). The identities of individual lipid classes were confirmed by comparison with authentic standards.

In order to analyze the fatty acid profile of the PL and NL separatedly, an additional HPTLC analysis was carried out to separate the two fractions. The HPTLC plates were developed in the same solvents but in this case the neutral and polar lipids marked, the bands scrapped off and lipids extracted and methylated as described above.

All the analyses were carried out in duplicates.

Statistics

Results in terms of larval DW, SL, mouth size, and survival of the larvae; as well as lipid classes and fatty acid profile of the larvae and the live prey were compared by one way analysis of variance (ANOVA). Percentage values were arcsine transformed and the assumption of homogeneity of variances was checked using the Shapiro–Wilk test. Data was analyzed at a significance level of 0.05. When significant differences were found, the Tukey HSD multiple range test was performed, using SigmaPlot 12.0 software. All data are given as mean values and standard deviations (±SD). A regression analysis was also performed with the results of the fatty acid composition of PL fraction in rotifers and larvae in order to assess the relationship between them. All data were analyzed using SigmaPlot 12.0 (Systat Software Inc., USA).

RESULTS

Tables 1, **2** show the total lipid, lipid classes, and fatty acid composition of the enrichment products (**Table 1**) used for live prey enrichment and the enriched rotifers (**Table 2**). Statistically significant differences in the composition of enrichment products was observed not only in total lipid content, that was significantly lower in NC and higher in LC60, but also in the total amount of PL (significantly higher in LC60), NL (significantly higher in RP), total saturated fatty acids (SAT, significantly lower in NC), monounsaturated fatty acids (MUFA, significantly higher in NC), n−6 PUFA content (lower in NC and LC60), and in the content of EPA (higher in NC and lower in MG) and DHA (lower in NC) as well as in total PUFA (higher in RP and MG) and in the ratios n−3/n−6 (higher in LC60) and DHA/EPA (higher in MG).

The rotifers enriched for 12 h with the commercial enrichers MG, RP and NC show a total lipid content that reflects the content of the enrichment product used. Similarly, the highest amounts of PL were observed in NC group, as in the case of the product used for enrichment. The use of 3 h post-enrichment with marine phospholipids LC60 showed a positive effect and all the enriched rotifers slightly increased their levels of PLs, although not showing statistically significant differences, with the exception of the rotifers enriched with the microalga (NC+LC60) that had a 40.8% of PLs. Rotifers enriched with Red Pepper (RP) showed the highest amounts of NL, reflecting the composition of the commercial product. Statistically significant differences were found in SAT and MUFA, and in both cases, the 3 h post-enrichment with LC60 induced a reduction in their levels, except in MG+LC60 enriched rotifers that showed an increase in SAT. N−6 PUFA content was very similar in all the enriched rotifers and the addition of LC60 induced a reduction due to the lower content of linoleic acid (LA, 18:2n−6). Total n−3 PUFA was always significantly higher in the rotifer enriched with MG, and the addition of LC60 contributed to increase EPA (20:5n−3) and DHA (22:6n−3) content and reduce the alpha linolenic acid (ALA, 18:3n−3). NC enriched rotifers also showed a statistically significant increase in EPA and DHA being 1.25 and 5.3 times higher in NC+LC60 group, respectively.

Larval growth (standard length, dry weight, and SGR results), size of the mouth, and survival rate are presented in **Table 3**. The use of rotifers enriched with LC60 contributed to a significantly

TABLE 3 | Standard length (SL), dry weight (DW), and specific growth (SGR) and survival rates of meager larvae fed enriched rotifers with and without marine phospholipids: Larviva Multigain (MG), Red Pepper (RP), Concentrated Nannochloropsis (NC), and marine phospholipids (LC60).

	SL (mm)	DW (mg)	Mouth size (mm)	SGR (%day^{-1})	Survival (%)
MG	5.56 ± 0.17a	35.59 ± 0.99a	0.37 ± 0.02	3.33	13.07 ± 1.30c
RP	5.83 ± 0.20ab	42.00 ± 0.71*c*	0.39 ± 0.03	3.52	7.27 ± 1.17a
NC	5.61 ± 0.15a	37.7 ± 0.85b	0.34 ± 0.01	3.42	10.5 ± 0.61b
MG+LC60	6.03 ± 0.11b	53.3 ± 0.85e	0.41 ± 0.04	3.76	11.97 ± 0.63bc
RP+LC60	6.02 ± 0.20b	52.24 ± 0.91e	0.37 ± 0.02	3.74	5.35 ± 0.91a
NC+LC60	5.9 ± 0.20ab	46.69 ± 0.97*d*	0.39 ± 0.03	3.67	6.97 ± 1.03*a*
	ANOVA $P < 0.001$	ANOVA $P < 0.001$			ANOVA $P < 0.001$

Different letters indicate significant differences (ANOVA $P < 0.05$).

TABLE 4 | Total lipid and main lipid class content and fatty acid composition of 1 dph and 15 dph meager larvae fed rotifers enriched with Larviva Multigain (MG), Red Pepper (RP), Concentrated Nannochloropsis (NC), and marine phospholipids (LC60).

	Meager Larvae						
	Initial	**MG**	**RP**	**NC**	**MG+LC60**	**RP+LC60**	**NC+LC60**
Total lipids (mg g^{-1} DW)	86.0 ± 36.0	88.7 ± 2.9a	125.5 ± 4.1c	110.0 ± 1.2b	116.8 ± 4.1bc	124.5 ± 4.3c	124.3 ± 1.3c
Total FA (mg g^{-1} Lipids)	674.7 ± 1.5	671.4 ± 5.0	704.7 ± 7.2	649.8 ± 10.5	679.5 ± 5.0	657.7 ± 12.0	669.0 ± 13.3
Total PL (% Total Lipids)	29.4 ± 1.6	47.7 ± 0.1	49.4 ± 1.2	47.9 ± 1.3	47.3 ± 0.7	50.8 ± 0.3	48.4 ± 0.7
Phosphatidylcholine (PC)	14.4 ± 1.4	20.2 ± 0.1b	10.3 ± 0.9a	19.9 ± 0.5bc	21.8 ± 0.7c	21.6 ± 0.1c	19.0 ± 0.7b
Phosphatidylethanolamine (PE)	7.2 ± 0.8	16.4 ± 0.4	16.3 ± 0.8	17.6 ± 0.7	16.7 ± 0.0	17.8 ± 0.4	16.9 ± 0.4
Total NL (% Total Lipids)	65.8 ± 1.6	52.3 ± 0.1	50.6 ± 1.2	52.1 ± 1.3	52.7 ± 0.7	49.2 ± 0.3	51.6 ± 0.7
Triacylglycerols (TAG)	23.5 ± 1.0	4.9 ± 0.3b	3.2 ± 1.0ab	2.2 ± 0.7a	7.3 ± 0.7c	7.3 ± 0.2c	1.9 ± 0.3a
Cholesterol (CHO)	13.1 ± 0.6	28.2 ± 0.8a	33.0 ± 0.7bc	34.3 ± 2.1c	30.1 ± 0.0ab	26.9 ± 0.1a	32.6 ± 0.1bc
Fatty acids (mg g^{-1} Lipids)							
16:0	14.24 ± 5.3	117.9 ± 6.1	100.1 ± 5.2	94.6 ± 5.2	113.1 ± 6.1	87.4 ± 4.4	91.8 ± 5.4
Total saturated[3]	193.6 ± 4.6	211.6 ± 6.3b	186.3 ± 4.3a	190.4 ± 6.7a	199.4 ± 5.7ab	186.2 ± 8.5a	191.9 ± 8.5a
16:1	39.1 ± 4.5	5.1 ± 1.1	7.0 ± 0.3	9.6 ± 0.2	4.4 ± 0.1	7.8 ± 0.1	8.5 ± 0.2
18:1n–9	95.8 ± 3.1	48.0 ± 3.0	51.6 ± 3.2	54.8 ± 30.3	42.3 ± 3.3	49.1 ± 3.3	60.2 ± 4.4
18:1n–7	12.8 ± 7.0	18.0 ± 1.0	19.4 ± 1.1	22.9 ± 1.1	18.3 ± 1.1	20.5 ± 1.1	21.4 ± 0.9
Total monounsaturated[4]	151.8 ± 8.4	80.3 ± 4.4a	88.6 ± 4.1ab	94.7 ± 4.3ab	73.2 ± 4.6a	85.0 ± 4.1ab	99.7 ± 5.3b
18:2n−6	31 ± 6.0	34.2 ± 2.0	38.2 ± 3.0	52.2 ± 3.4	25.8 ± 2.2	50.3 ± 1.3	50.2 ± 3.3
20:4n−6	12.8 ± 1.1	23.1 ± 1.1	23.6 ± 2.1	19.3 ± 1.1	23.1 ± 2.1	18.4 ± 0.9	17.5 ± 0.1
22:5n−6	1.7 ± 0.5	40.9 ± 2.1	40.6 ± 2.1	11.3 ± 1.0	48.3 ± 3.4	17.5 ± 1.0	11.7 ± 0.1
Total n−6 PUFA[5]	59.4 ± 4.3	105.2 ± 6.2	109.2 ± 6.5	95.4 ± 4.5	104.5 ± 6.5	102.6 ± 6.8	93.6 ± 5.2
18:3n–3	8.8 ± 0.1	20.6 ± 1.0	23.7 ± 1.5	44.1 ± 3.0	7.4 ± 0.0	45.0 ± 3.1	42.8 ± .,0
20:5n–3	30.4 ± 3.2	11.5 ± 0.9a	20.1 ± 0.9bc	23.9 ± 1.2d	14.8 ± 0.9b	22.1 ± 1.2cd	23.4 ± 0.4d
22:6n–3	186.9 ± 8.4	233.6 ± 6.1b	231.0 ± 6.8b	122.0 ± 6.6a	243.7 ± 7.2b	237.4 ± 6.5b	127.1 ± 5.4a
Total n−3 PUFA[6]	238.8 ± 9.3	270.4 ± 8.5b	281.6 ± 8.6b	199.1 ± 8.1a	271.3 ± 8.5b	270.6 ± 8.6b	201.4 ± 7.5a
Total PUFA	298.2 ± 9.6	375.5 ± 10.2b	390.8 ± 9.3b	294.5 ± 9.1a	375.8 ± 10.3b	373.2 ± 9.5b	295.0 ± 8.1a
Total UK[7]	31.1 ± 0.7	4.0 ± 0.0	39.0 ± 5.2	70.2 ± 6.7	31.1 ± 2.1	13.3 ± 0.8	82.4 ± 3.5
n−3/n−6	4.0 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	2.1 ± 0.4	2.6 ± 0.1	2.6 ± 0.3	2.2 ± 0.4
DHA/EPA	6.2 ± 0.4	20.3 ± 1.0d	11.5 ± 0.5b	5.1± 0.2a	16.5 ± 0.9c	10.7 ± 0.7b	5.4 ± 1.2a

[1]*Includes phosphatidylinositol, phosphatidylserine, cardiolipin, and phosphatidic acid.*
[2]*Includes free fatty acids, sterol esters, and waxes.*
[3]*14:0, 15:0, 17:0, 18:0, 20:0, 21:0.*
[4]*Includes 14:1, 16:1, 18:1n−7, 20:1, 22:1, 24:1n−9.*
[5]*Includes 18:3n−6.*
[6]*Includes 18:4n−3, 20:3n−3, 22:5n−3.*
[7]*Includes 16:3 and 16:4 and not identified fatty acids.*
Different letters indicate significant differences among the groups (ANOVA, $P < 0.05$).

higher growth in length and dry weight with no significant differences in the size of the mouth and low survival rate in all the groups. This low survival can be a consequence of feeding the larvae only with rotifers, considering that the standard protocol for meager larval rearing includes feeding with *Artemia* nauplii from day 8 post-hatch, the use of only rotifers may have had an effect in larval survival due to cannibalism, although as explained previously the trial was designed to use only rotifers for their easy way to enrich them with phospholipids, not that easy in the case of *Artemia*.

Total lipid and lipid class content and fatty acid composition of the larvae at the end of the trial is shown in **Table 4**. The content of total lipids were significantly higher in 15 dph larvae fed the rotifers boosted with marine phospholipids in contrast with those fed standard enriched rotifers, except for RP group. On the contrary, total fatty acids were very similar between newly hatched and 15 dph larvae fed the different diets ($P > 0.05$). Total PL and NL were clearly different having the newly hatched larvae a higher content of NL probably due to the presence of the yolk sac and lipid droplet. Fatty acid composition of the newly hatched larvae also showed a higher content of MUFA (mainly 18:1n−9) and EPA and a lower content of ARA (20:4n−6) and 22:5n−6 that is quite high in the enrichment products and in the 15 dph larvae fed enriched rotifers. The most important differences found among the 15 dph larvae was the lower amount of lipids in MG group, the significantly higher content of SAT in MG and MG+LC60 larvae, and the significantly higher content in MUFA, EPA and lower content of DHA in larvae fed NC and NC+LC60 enriched rotifers. Consequently, DHA/EPA ratios were significantly lower in NC and NC+LC60 fed larvae and

TABLE 5 | Total PL content and fatty acid profile of the PL fraction of enriched rotifers and meager larvae.

	Enriched rotifer						Meager larvae					
	MG	**RP**	**NC**	**MG+LC60**	**RP+LC60**	**NC+LP60**	**MG**	**RP**	**NC**	**MG+LC60**	**RP+LC60**	**NC+LP60**
Total PL (mg g^{-1} DW)	33.3	33.7	34.8	41.7	39.9	40.2	40.9	58.5	55.6	55.2	61.5	69.1
Total FA (mg g^{-1} Lipids)	446.7	386.7	478.8	568.8	543.7	513.0	554.5	485.3	365.2	625.4	526.2	449.9
Fatty acids (mg g^{-1} Lipids)												
16:0	84.0	89.8	88.2	121.0	112.7	82.3	94.3	76.0	55.7	127.6	83.9	52.8
18:0	22.4	19.9	27.5	21.2	25.0	28.9	85.9	80.1	65.1	92.5	84.3	68.4
Total saturated[1]	117.6	122.1	124.0	154.1	149.0	131.3	186.7	166.5	128.7	224.8	173.9	133.1
16:1	6.9	3.2	27.0	17.7	14.0	14.0	0.0	5.8	3.7	0.0	5.1	13.9
18:1n−9	26.7	25.7	42.7	29.3	28.3	44.1	28.2	31.4	28.5	29.6	31.7	32.4
18:1n−7	24.4	26.5	55.1	46.4	40.7	60,0	39.8	47.9	30.4	49.6	42.3	42.1
Total monounsaturated[2]	75.8	68.1	148.8	108.3	97.8	108.7	74.1	85.1	62.7	86.3	82.1	91.2
18:2n−6	64.8	56.3	78.4	67.8	63.9	57.5	26.4	32.9	36.7	26,0	29.1	40.1
20:4n−6	0.0	0.0	0.0	0.0	5.2	0.0	15.8	13.4	0.0	17.4	15.8	10.5
22:5n−6	14,0	9.3	2.3	13.1	14.6	2.1	32.8	12.4	7.6	34.7	24.9	7.0
Total n−6 PUFA[3]	88.8	75.0	80.7	87.4	90.2	72.4	79.1	58.8	44.2	82,0	69.8	72.4
18:3n−3	34.9	33.3	57.0	34.0	34.1	55.7	0.0	0.0	0.0	4.1	11.0	0.0
20:5n−3	25.8	20.4	33.4	38.9	30.0	36.0	10.2	22.6	18.2	13.4	13.0	19.1
22:6n−3	65.0	36.8	12.6	78.2	68.5	28.7	196.6	138.5	83.3	210.6	152.9	109.2
Total n−3 PUFA[4]	128.8	93.0	107.9	157,0	137.8	125.4	209.6	165.9	106.2	232.3	181.9	132.7
Total PUFA	217.6	168.0	188.6	244.4	228.0	197.8	288.7	224.7	150.4	314.2	251.7	205.1
Total UK[5]	35.7	20.0	17.4	62.0	68.9	75.2	5.0	9.0	23.4	0.1	18.5	20.5

[1] *14:0, 15:0, 17:0, 18:0, 20:0, 21:0.*
[2] *Includes 14:1, 16:1, 18:1n−7, 20:1, 22:1, 24:1n−9.*
[3] *Includes 18:3n−6.*
[4] *Includes 18:4n−3, 20:3n−3, 22:5n−3.*
[5] *Includes 16:3 and 16:4 and not identified fatty acids.*

higher in MG fed groups. Similar amounts of total PL and total NL and in the fatty acid composition were observed in all the groups. A positive, although not significant, correlation ($r = 0.352$) was found between the DHA content in the rotifers and the final DW of the larvae that suggested a possible relationship between both parameters. Thus, to check which could have been the effect of the addition of marine PLs (LC60) in rotifer enrichment on larval growth, a further analysis of the fatty acid composition of PL and NL was carried out separately and the results of the fatty acid profile of PL in enriched rotifers and larvae are presented in **Table 5** and **Figure 1**. Due to the small amount of sample analyzed no replicates and consequently no statistical analysis could be performed. The composition of the dominant fatty acids in PL of the rotifers was reflected in 15 dph larvae which show the highest content of DHA both in the rotifers and in MG fed larvae, and the lowest in NC enriched rotifers and fed larvae, in this case, the addition of marine phospholipids (LC60) contribute to a slight increase in the levels of DHA in MG, RP and NC larvae. On the contrary 16:0 and total SAT was increased in the rotifers enriched with MG and MG+LC60 and the larvae that fed on them, whereas they were reduced in NC+LC60 fed rotifers and larvae. **Figure 1** shows a summary of these results. In MG and MG+LC60 fed larvae an increase of DHA (7%), EPA (31%), ARA (10%), and 18:1 (5%) fatty acids was observed whereas the content in 16:0 showed a 35% increase produced by the addition of LC60. RP and RP+LC60 larvae also showed similar amounts of 18:1n−9, but in this case the addition of marine phospholipids induced a reduction in EPA content (−42.8%), and a higher accumulation of DHA (18.4%), ARA (17.9%), and 16:0 (10.4%) fatty acids. In the case of NC and NC+LC60 larvae all the fatty acids, ARA (100%), DHA (31%), 18:1n−9 (13.7%), EPA (4.9%), and 16:0 (12.7%) increased with the addition of LC60.

The relationship between the content of the dominant fatty acids in the live feed vs. those of the corresponding 15 dph larvae is presented in **Figure 2**. The results suggested a close relationship between the composition of dominant PL fatty acids in the rotifer and in the larvae, especially in the case of DHA and 16:0 that show a high correlation ($r^2 = 0.872$, $P = 0.006$ and $r^2 = 0.546$, $P = 0.093$, respectively).

DISCUSSION

The fatty acid composition of enriched live prey and larvae reflected the profile obtained in the products used for enrichment, as already published in previous reports (Vallés and Estevez, 2015b; Campoverde and Estévez, 2017). There is a clear effect of the DHA level in the live prey on larval fatty acid composition (see **Tables 3**, **4**, regression coefficient $r^2 = 0.79$, $P = 0.062$) and growth (regression coefficient between DHA content in the enriched rotifer and final weight $r = 0.379$, $P = 0.459$) although in this case not as high as expected. DHA levels found in this study,193 mg g^{-1} lipids (27% of total fatty acids)

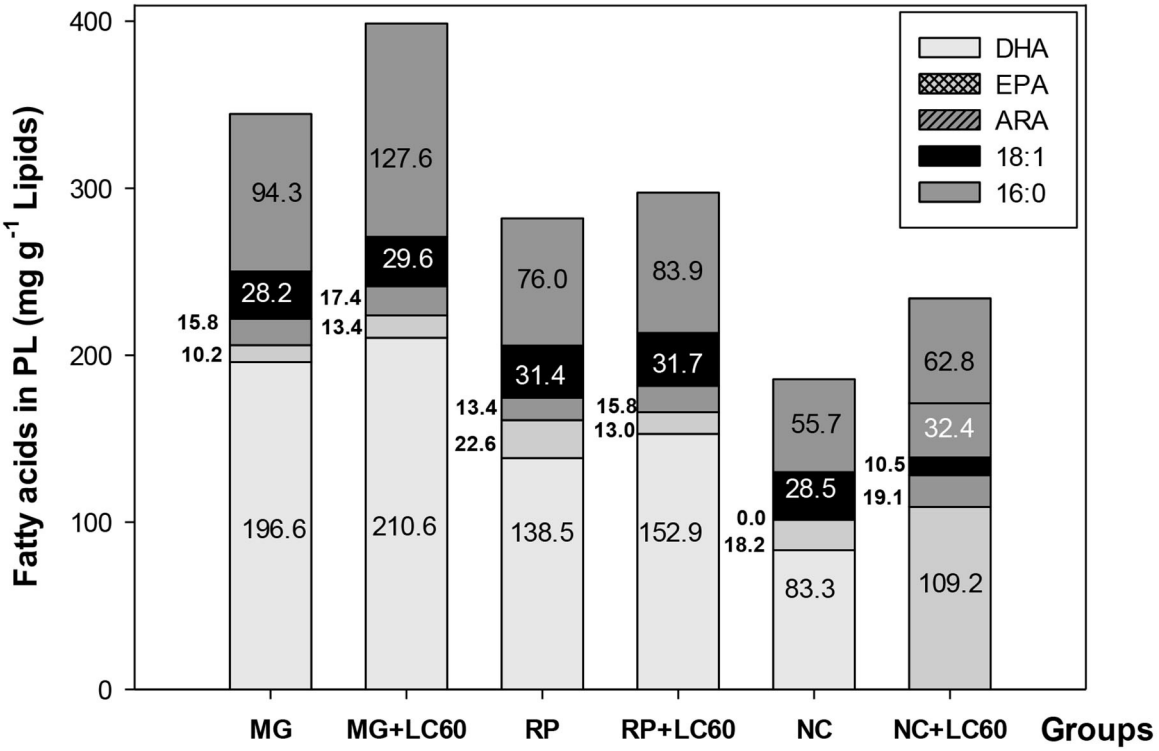

FIGURE 1 | Main fatty acid content (mg) in PL of meager larvae fed different enriched rotifers until 15 dph.

for MG enriched rotifers are similar to those reported by Vallés and Estevez (2015b) using commercial enrichers (24.9% DHA in MG enriched rotifers) and considered the most adequate for the proper growth and development of meager larvae. The authors concluded that DHA levels around 12–15% TFA in live prey, that correspond to around 40–50% of TFA in the enrichment product, might be considered as the required levels for this species. Similar levels of DHA are cited for other marine fish larvae, such as gilthead sea bream (Salhi et al., 1994) and red porgy (Roo et al., 2010), for growth and to prevent skeletal deformations. Similar amounts of DHA were used in the present study, except for diets NC and NC+LC60.

There is a clear effect of DHA content in the rotifers as a result of boosting rotifers with marine phospholipids (LC60) on larval growth. However, no effects on survival rate could be detected mainly due to the use of rotifer as the only live prey during larval rearing, inducing cannibalism of the fast growing larvae on their slow growing counterparts, as already have been observed in this species (Campoverde and Estévez, 2017). Similar results regarding DHA or fatty acid levels having no effect on larval survival have been cited for other species such as cod (Park et al., 2006; Garcia et al., 2008; Copeman and Laurel, 2010) or striped trumpeter (Bransden et al., 2005). These authors observed a clear relationship between the length and dry weight of the larvae at the end of the experiment with dietary fatty acids, whereas survival rate was not influenced by them. Other studies carried out with other species of marine fish larvae showed that high levels of DHA (or n−3 PUFA) reduce larval survival (Planas and Cunha, 1999). In the case of Japanese flounder (*Paralichthys olivaceus*) larvae, Izquierdo et al. (1992) showed that either low or high DHA content in live prey did not affect survival rate, being the larvae significantly larger when fed *Artemia* containing a high percentage of DHA (up to 3.5%). All these results with different species confirmed what Sargent et al. (1999) suggested, that the requirement of dietary DHA levels of marine finfish larvae is species dependent.

Another question addressed in this trial was to study if meager larvae can incorporate DHA into their tissues more efficiently if they are provided by dietary PLs and not TAG in the early stages of first feeding, and if the larvae can grow and perform better. We have found that meager larvae fed on rotifers enriched with different commercial enrichment diets rich or not in PLs showed a positive relationship between the percentage of DHA and 16:0 (or total SAT) in meager larval PL and the same percentage in rotifer PL (**Figure 2**, $r^2 = 0.872$ for DHA and 0.546 for 16:0). A high percentage of DHA in larval tissues and PLs coincided with the higher larval growth rate at 15 dph ($r = 0.352$). Thus, the results indicate that the availability of fatty acids in general and LC-PUFA in particular might be different if they are provided as PLs or TAG, being those provided from PLs more accessible and the main source for larval PL synthesis and growth.

In this study one of the main challenges was to incorporate PLs in the rotifers and manipulate their fatty acid composition.

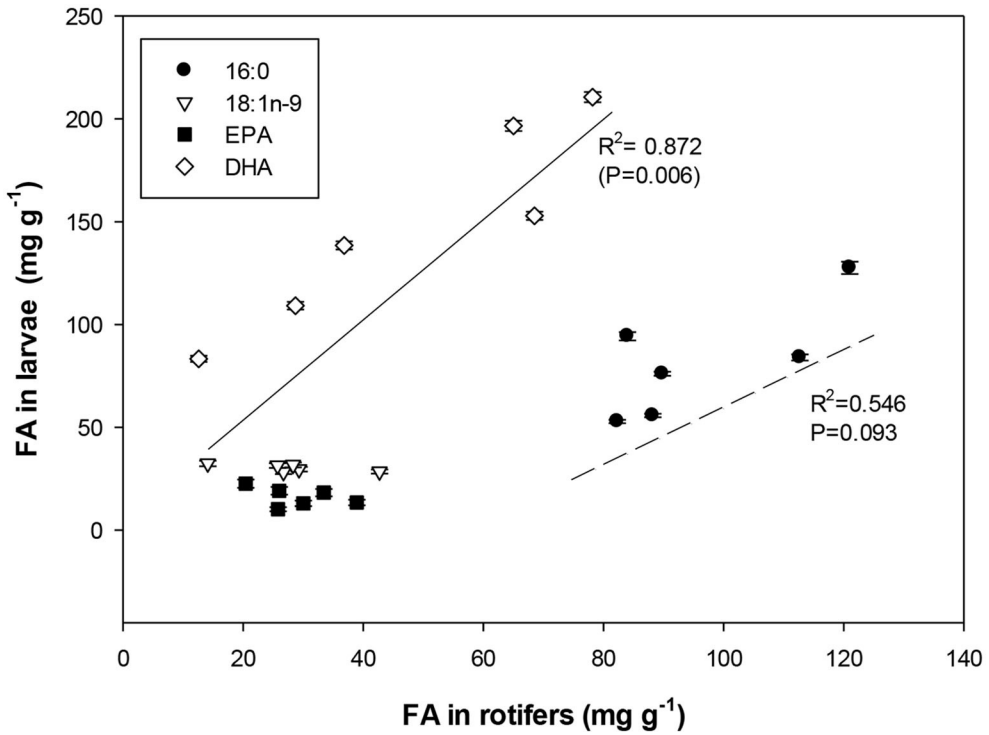

FIGURE 2 | Linear regressions found between the most important fatty acids in PL in 15 dph meager larvae as a function of the respective fatty acid composition in the PL of enriched rotifers.

The use of commercial enrichers for 12 h followed by the addition of marine phospholipids (LC60) using a short-term enrichment of 3 h was effective for that in the case of rotifers, as it has been shown by other authors (Olsen et al., 2014; Li and Olsen, 2015; Mylonas et al., 2016). In the case of *Artemia* a recent paper published by Rogacki et al. (2019) showed that a certain amount of PLs can be obtained using Multigain as enricher and LC60 as the phospholipid source, similarly to our results, and open the possibility of using this PL-enriched *Artemia* in the future.

Olsen et al. (2014) and Li et al. (2014) obtained a level of 9.4% of DHA (in % total fatty acids) in the PL fraction of the rotifers enriched with Marol E and suggested that this low level might be genetically or metabolically constrained. In the present study, DHA was present between 3.3% (NC group) and 13.7% (MG+LC60 group) of total fatty acids in the PLs of the rotifers, indicating that higher levels can be obtained using a different approach, either in the enrichment process or products used. Other result that is very interesting and needs more research is the positive correlation between the content of 16:0 in rotifer PLs and its content in the larvae. Li and Olsen (2015) also found high levels of 16:0 in MG enriched rotifers that may act as a stimulant of PL biosynthesis not only in the rotifers but also in the larvae, as the authors suggested. In the case of humans, it has been shown that 16:0 facilitates the incorporation of dietary DHA into plasma PC (Subbaiah et al., 1993) and the 16:0-DHA-PC is preferably retained in the plasma membrane (Williams et al., 1999). It seems that in meager larvae something similar might occur and need more research.

CONCLUSIONS

Rotifers can be enriched with phospholipids using marine phospholipids derived products (LC60) and short-term enrichment (3 h boosting) procedures especially when the products used for enrichment are deficient in PLs (Red Pepper or concentrated microalgae).

The use of phospholipid enriched rotifers contributed to an increase in growth rate and a relationship was found between the DHA content in the rotifer and the final body weight of the larvae.

A clear relationship was found between the 16:0 and DHA content in the PLs of the rotifers and the larvae. More research is needed to understand the role of 16:0 in PL synthesis and larval growth.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Generalitat de Cataluña.

AUTHOR CONTRIBUTIONS

AE designed the trial, gave instructions about enrichment and sampling, help with the biochemical analyses, and wrote the manuscript. SG performed the trial with larvae, collected the samples, helped in the biochemical analyses, and contributed in writing the manuscript. All authors contributed to the article and approved the submitted version.

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DHA Accumulation in the Polar Lipids of the Euryhaline Copepod *Pseudodiaptomus inopinus* and Its Transfer to Red Sea Bream *Pagrus major* Larvae

*Hideaki Matsui[1], Taku Sasaki[2], Toru Kobari[1,2], Viliame Waqalevu[3], Kazuma Kikuchi[2], Manabu Ishikawa[1,2] and Tomonari Kotani[1,2]**

[1] Faculty of Fisheries, Kagoshima University, Kagoshima, Japan, [2] Graduate School of Fisheries, Kagoshima University, Kagoshima, Japan, [3] EPHE-UPVD-CNRS, USR 3278 CRIOBE BP 1013, PSL Research University, Moorea, French Polynesia

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***Correspondence:**
Tomonari Kotani
kotani@fish.kagoshima-u.ac.jp

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The euryhaline copepod *Pseudodiaptomus inopinus* play important roles in coastal waters as vectors of docosahexanoic (DHA) and eicosapentaenoic (EPA) acids for larval fish. While DHA and EPA in polar lipids (PLs) are more effective for fish larval development than non-polar lipid forms (NLs), there is little knowledge how much these lipids are accumulated in copepods from microalgae and are effective for early development of fish larvae. We report PLs fatty acid profiles of *P. inopinus* fed DHA-poor microalgae and evaluate its significance as a food source for larvae development of *Pagrus major*, compared with DHA-enriched rotifers. Copepods and rotifers were fed a mixed diet of three algal species (*Phaeodactylum tricornutum*, *Tisochrysis lutea*, and *Pavlova lutheri*), in addition of DHA-supplemented Super Fresh Chlorella (SFC) for rotifers. Compared with SFC, the algal mixture had higher EPA but lower DHA. Copepods had higher DHA and EPA in total lipids than rotifers fed each diet. Copepod PLs were specifically enriched with DHA and their contents were higher than both rotifers. On the other hand, PLs EPA contents were comparable between preys, indicating that copepods selectively fortified the PLs. Fish culture experiment showed that larvae fed copepods had higher growth than those fed SFC-enriched rotifers. Principal component analysis for each organism fatty acid composition emphasized trophic modification of DHA by copepods toward larval fish. This study highlighted that *P. inopinus* contribute to enhanced growth of coastal larval fish by efficiently transferring DHA via copepod fatty acid metabolism.

Keywords: coastal waters, copepod, DHA, fish larvae, lipids, microalgae, *Pseudodiaptomus*, rotifer

INTRODUCTION

Based on the critical period hypothesis (Hjort, 1914), food availability during initial feeding periods is known as a crucial factor for early development of fish larvae and its recruitment. As match-mismatch hypothesis (Cushing, 1990) states, prey quantity well contributes to the recruitment in fish stocks, while laboratory experiments, especially those for aquaculture application, has pointed

Abbreviations: ANOVA, Analysis of variance; NLs, Non-polar lipids; PLs, Polar lipids; SFC, Super Fresh Chlorella.

the importance of prey quality (i.e., nutrition) to explain fish stocks. Marine finfish lack the ability to synthesize *n*-3 highly unsaturated fatty acids (*n*-3 HUFAs), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), and therefore must supplement these essential fatty acids from diets for their growth and survival (Tocher, 2003). Indeed, larval fish reared under sufficient amounts of diets further enhance their growth, survival, and development when the diets are rich in *n*-3 HUFA (especially DHA; Watanabe et al., 1989; Nćss et al., 1995; Shields et al., 1999; Evjemo et al., 2003; Karlsen et al., 2015). Larvae inhabiting in the coastal areas, also exhibit higher growth rate when the ambient zooplankton contain higher *n*-3 HUFAs (Paulsen et al., 2014). These facts suggest the necessity to investigate how fatty acids are transferred toward larval fish for further understanding of fish recruitment.

Coastal and estuarine waters are known as a nursery area for various larval species and support their abundance (Borges et al., 2007). The euryhaline calanoid *Pseudodiaptomus inopinus* is a very common copepod species in such ecosystems around East Asia, Russia, and the United States (Sakaguchi and Ueda, 2011). The genus *Pseudodiaptomus* has a tolerance to a broad range of salinity (Beyrend-Dur et al., 2011), and high turbidity (Hwang et al., 2010), so that it is often the most abundant prey for larval fish. This copepod is reported to have high DHA and/or EPA in total lipids (Toledo et al., 1999; Rayner et al., 2015). Thus, *P. inopinus* with rich fatty acids can play important roles for larval development in coastal ecosystems.

On the other hand, in coastal and estuary waters, the dominant primary producers of *n*-3 HUFA are often diatoms (Kleppel, 1993; Trigueros and Orive, 2001; Ansotegui et al., 2003). They lack DHA despite having specifically high EPA (Kates and Volcani, 1966; Volkman et al., 1989). Indeed, DHA was relatively low in spring blooms where diatoms are dominated (Sargent et al., 1985). On the diatom–copepod–larval fish food chain, little is known how *P. inopinus* contributes to early development of larval fish by transferring DHA. To deal with this issue, fatty acid profiles, particularly polar lipids (PLs) of copepods, require further investigation (Olsen et al., 2014). For larval fish, dietary *n*-3 HUFA distributed to the PLs can be more efficiently absorbed than those distributed to the non-polar lipids (NLs; Salhi et al., 1999), resulting in enhanced larval growth and survival (Gisbert et al., 2005; Wold et al., 2009). However, the distribution of DHA and EPA in *P. inopinus* PLs is unknown.

Our objective is to evaluate the fatty acid profiles of PLs in *P. inopinus* cultivated using DHA-poor microalgae and their significance in early development of fish larvae. Rather than copepods, successful larviculture is well established using artificially DHA-enriched *Brachionus* rotifers as the diet (Koven et al., 1990; Castell et al., 2003; Thépot et al., 2016). In this study, we used such rotifers fed on a commercial DHA enrichment diet as a standard for evaluating copepod fatty acids. We also cultured *P. major* larvae with copepods, and compared the performances with those cultured using enriched rotifers. *P. major* is a widely distributed fish in coastal waters of Japan (Shoji et al., 2007), and is of commercial importance to Japan, Korea, and China (Hossain et al., 2016). We then discuss fatty acid trophic transfer in coastal ecosystems, and the significance of copepods on early development of coastal fish larvae.

MATERIALS AND METHODS

Cultivation of Rotifers

The L-type rotifer *Brachionus plicatilis* species complex (Obama strain) was used as control diet for larval fish. As an experimental control, artificially enriched rotifers were prepared by feeding *Chlorella vulgaris* containing DHA (Super Fresh Chlorella V-12, SFC; Chlorella industry, Tokyo, Japan). SFC is produced by heterotrophic cultivation of *C. vulgaris* with fish oil DHA as a carbon source (Hayashi et al., 2001). Following the procedure of rotifer enrichment described by Waqalevu et al. (2018), SFC was fed to rotifers at 46×10^3 cells per rotifer per a day. Seawater for the culture medium was filtered through 100 μm cartridge filters (Micro-Cilia; Roki Techno Co. Ltd., Japan); water temperature was maintained at 25°C and salinity at 17‰. Taurine, an essential amino acid for red sea bream larvae (Chen et al., 2004), was also enriched in rotifers using Aquaplus ET (Marubeni Nisshin Feed Co, Ltd., Japan) following manufacturer instructions (60 mg L^{-1}). The enriched rotifers were prepared daily during larval rearing experiment (for 20 days) and harvested to directly supply to larvae. In addition, for fatty acid analysis, the rotifers were directly harvested from three randomly-selected cultures, washed with distilled water, and then freeze-dried and preserved at −80°C until measurement. Starvation periods to make rotifer digestive tracts empty were not set before harvesting them.

To compare the fatty acid composition of rotifers and copepods fed the same diet, the rotifers were also fed same algal mixture as the copepods. Rotifers were fed the mixture daily, cultivated at 20°C water temperature and salinity 17‰. When rotifer density reached 800–1,000 individuals ml^{-1} they were harvested and preserve for later fatty acid analysis (these rotifers were not used for fish larval diet). Such rotifer samples were prepared in triplicate as SFC-enriched ones.

Cultivation of Microalgae

Copepods were fed on the diatom *Phaeodactylum tricornutum*, with the haptophytes *Pavlova lutheri* and *Tisochrysis lutea* (originated from *Isochrysis* affinis *galbana* Tahiti strain; Bendif et al., 2013) as a mixture. We supplemented the diatom with the two haptophytes for mass production of copepod, since monoalgal diatom is well reported to suppress copepod egg productivity and survival (Støttrup and Jensen, 1990; Schipp et al., 1999; Shields et al., 2005; Ohs et al., 2010). The strains of *P. tricornutum* and *P. lutheri* were supplied by National Research Institute of Aquaculture, Fisheries Research Agency (Mie, Japan); and *T. lutea* strain was from the Graduate School of Fisheries and Environmental Science, Nagasaki University, Japan. Each microalga was incubated in 5 L polycarbonate bottles containing a seawater culture medium at 20°C with continuous 0.5 L min^{-1} aeration and irradiance of 120 μmol photons m^{-2} s^{-1}. Seawater for the microalgae culture medium was filtered through a GF/F filter (Whatman), diluted to salinity 17‰, autoclaved, and enriched with KW21 medium (Daiichi Seimo

Co. Ltd., Japan). Fresh cultures for each species were prepared weekly and maintained for about 1 week. Cells of the three algal species were counted every day using a Thoma hematocytometer (0.1 mm depth; Sunlead Glass Co. Ltd., Tokyo, Japan). Cells were harvested for a diet and fatty acid analysis once they reached the end of logarithmic growth phase or the beginning of stationary phase. Microalgae were directly used as a diet without temporary storage. Three samples of each algal species for fatty acid analysis were twice washed with 0.5 M ammonium formate, and then freeze-dried and preserved at −80°C.

Cultivation of Copepods

Copepods (*P. inopinus*) were collected using a plankton net (mesh 63 μm) from the surface of Yakugachi River (Kagoshima, Japan), in waters of 19.7°C and salinity 11.5‰. Seawater for the copepod culture medium was pumped from Kagoshima Bay (Kagoshima, Japan), filtered through a graded series of 100, 25, and 10 μm pore sized cartridge filters (Micro-Cilia; Roki Techno Co. Ltd., Japan), before being subjected to ultraviolet irradiance (UVF-1000; Iwaki Co. Ltd., Japan). Copepods were cultivated at 20°C, salinity 17‰, and photoperiod of 12L:12D, initially using 5-L plastic beakers or 50-L polycarbonate tanks as a container. As the copepod population grew, the cultures were transferred to larger polycarbonate tanks (100 and 200 L). Aeration was gently performed via a glass tube at 1 ml min^{-1} for <50 L of medium, 3 ml min^{-1} for <100 L of medium, and 6 ml min^{-1} for <200 L of medium. Microalgae were supplied to copepods as a mixture every 2 days to achieve the following dry mass-based concentrations (*Phaeodactylum*, 31 μg ml^{-1}, *Pavlova*, 20 μg ml^{-1}, and *Tisochrysis* 12 μg ml^{-1}, at the ratio of 5:3:2), where cell concentrations were 400×10^3, 200×10^3, and 400×10^3 cells ml^{-1}, respectively. The medium for copepod culture was fully exchanged every 2–3 days. Generations of the copepods were repeatably incubated for a year before the present experiment, and the species was confirmed to be *P. inopinus* from the subsamples collected monthly. Except for the 200-L culture of copepods used as a diet for larval fish, three 50-L culture tanks were prepared for copepod fatty acid analysis. Protocols for copepod harvest, wash, and preservation were the same as those for rotifers.

Larval Rearing

Naturally fertilized and spawned red sea bream eggs were obtained from Ogata Suisan Inc. (Amakusa, Japan). The eggs were placed into black 100-L polyethylene tanks at a stocking density of 2,000 eggs per tank. The number of hatched larvae was estimated using a volumetric method described by Kotani et al. (2017), to be 1,900 individuals in each tank. Rearing tanks (100 L) were maintained under 12L:12D light conditions, with water quality monitored twice daily; pH and dissolved oxygen (DO) were measured by portable pH (D-51; Horiba Co. Ltd., Kyoto, Japan) and DO (FDO925; WTW, Bavaria, Germany) meters. Temperature was maintained at 21.8 ± 1.5°C via a 1 kW titanium heater (Nittokizai Co, Ltd., Saitama, Japan); salinity was maintained at 33 ± 1‰. Rearing tank aeration was initially supplied at 15 ml min^{-1}, and this gradually increased with larval growth. The first water exchange occurred 5 days post hatching (dph), after which water was exchanged at 3.5 ml s^{-1} via a siphoning system.

Larvae were reared until 20 dph following two prey conditions: SFC-enriched rotifers (control), and algal mixture-fed copepods (**Figure 1**). Both preys were sufficiently fed to fish larvae based on the dry mass, twice a day (at 08:00 and 16:00) from 3 dph until 19 dph. On critical days 3 and 4 post-hatching, to achieve a successful first feeding under copepod supply, the number of nauplii fed to fish larvae was increased to that of rotifers (ca. 5 inds ml^{-1}). Larval rearing was terminated at 20 dph morning before feeding. Twenty fish larvae in each treatment were sampled at 0, 5, 10, 15, and 20 dph. All fish larvae surviving in control and copepod treatments at 20 dph morning were sampled, washed with distilled water, and separately pooled as 1 sample to detect their fatty acids. Larvae for fatty acid analysis were freeze-dried and preserved at −80°C until lipid measurement.

Length and Dry Mass of Rotifers and Copepods

Lengths of rotifers and copepods were measured using a micro-ruler under microscopy (SMZ800; Nikon Co, Ltd., Tokyo, Japan) after fixation in acidic Lugol's solution. The length of rotifer lorica (from crown spines to the lower extremity; Fu et al., 1991) was measured. Four categories of copepods were measured: nauplius (stages I–VI), copepodite (stages I–V), and adult male and female (copepodite stage VI). Adult males were distinguished from other stages by their asymmetrically developed first antennae; females were deemed adult when they possessed egg sacs. Body length was measured for nauplii (n = 40), and prosome length for copepodites (n = 30) and adults (n = 20).

Rotifer dry mass was measured by rinsing samples from three cultures with distilled water, freeze-dried, and weighed using a semi-micro balance (readability of 0.01 mg; GH-202; A and D, Japan). Dry mass of individual copepod was estimated from body length (μm) and prosome length (μm) using the equation adapted by Uye et al. (1983), with a factor (0.45) to convert carbon content to dry mass (Omori and Ikeda, 1984):

$$\text{Dry mass of nauplius } (\mu g) = 10^{(2.00 \log (\text{Body-length} \times 1{,}000) - 5.67)}/0.45$$

$$\text{Dry mass of copepodite and adult } (\mu g) = 10^{(3.17 \log (\text{Prosome-length} \times 1{,}000) - 8.63)}/0.45$$

Larval Growth and Gut Contents

Fish standard length (SL) was measured after fixation in 10% formalin-seawater solution. Fifteen larvae sampled 2 h after feeding (5, 10, and 15 dph) were used for gut content analysis. In gut contents, rotifers and copepods at each developmental stage (nauplius, copepodite, and adult) were identified by microscopy (SMZ800; Nikon Co, Ltd., Tokyo, Japan) and counted. Total dry mass (μg $larva^{-1}$) and DHA mass (ng $larva^{-1}$) of ingested preys were calculated from the number of prey items in larval guts

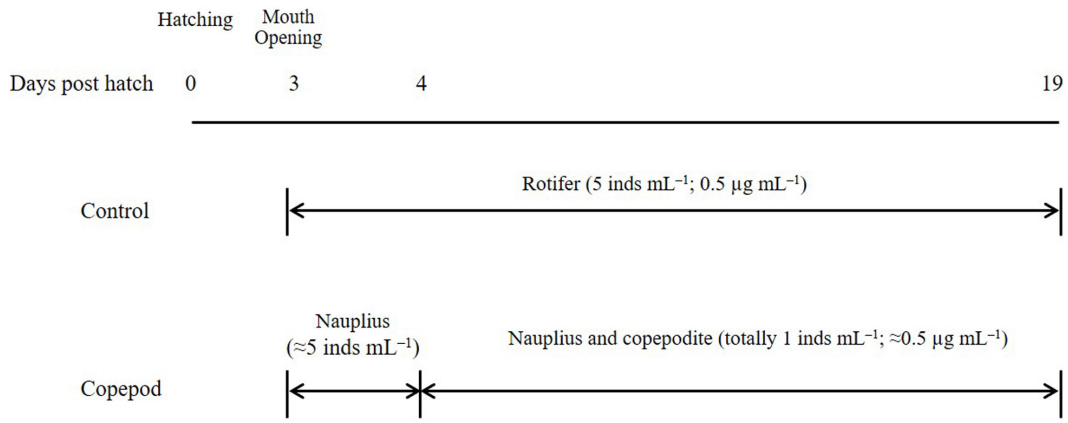

FIGURE 1 | Temporal change in prey items of larval red sea bream *Pagrus major* in experimental treatments (rotifers and copepods). Values in parentheses present densities based on individual number (ind. mL^{-1}) and dry mass ($\mu g\ mL^{-1}$). Rotifers were prepared by feeding docosahexaenoic acid-supplemented Super Fresh Chlorella (SFC); and the copepods fed algal mixture were used. Rotifer: *Brachionus plicatilis* sp. complex. Copepod: *Pseudodiaptomus inopinus*.

multiplied by their dry mass ($\mu g\ ind^{-1}$) and DHA content ($mg\ g^{-1}$).

Fatty Acid Analysis

After homogenization of dried samples, total lipids were extracted following the method of Folch et al. (1957). Extracted lipids were separated into NLs and PLs fractions by column chromatography using a Sep-Pak silica cartridge (Waters, SA, United States; Juaneda and Rocquelin, 1985), with a chloroform-methanol mixture (98:2, v/v) for NLs and methanol for PLs. Methyl-esterification of fatty acids for total lipids, NLs, and PLs, was conducted as described by Matsui et al. (2019). Gas chromatography (GC-2010 Plus; Shimadzu, Kyoto, Japan) equipped with a hydrogen flame ionizing type detector (260°C) and Omegawax capillary column (30 m length, 0.32 mm internal diameter, 0.25 μm film; Supelco, PA, United States) was used for detection of each fatty acid. Each fatty acid species was determined according to the equivalent chain length (ECL), and its proportion was calculated from the peak area. Quantification of fatty acid content was carried out based on the content of non-adecanoic acid (C19:0) analyzed together as an internal standard. Except for microalgae, rotifers, and copepods ($n = 3$), replicates for lipid analysis are not made for larval fish, due to difficulties in mass production of copepods used for later larviculture. Since fatty acid compositions for *Phaeodactylum*, *Tisochrysis*, and *Pavlva* microalgae have already been well reported (Volkman et al., 1989; Reitan et al., 1994a), each species was analyzed once ($n = 1$) and added to this study only as supplementary data for algal mixture.

Statistical Analysis

Statistical analysis was undertaken using Sigma-Plot ver 11.0 software (Systat Software Inc., CA, United States) and JMP version 11.0 software (SAS Institute Inc., NC, United States), $p = 0.05$. The length and dry mass of copepods at different stages were non-parametrically analyzed by Kruskal–Wallis test, followed by Steel–Dwass test, since Shapiro–Wilk's normality test and Bartlett's test both failed. A Student's *t*-test was conducted to compare total lipid fatty acid species of SFC and algal mixtures. Between rotifer fed on SFC and algal mixture, and copepods, the fatty acid proportions and contents for total lipids, NLs, and PLs were compared by one-way analysis of variance (ANOVA), then subjected to a Tukey pairwise multiple comparison *post hoc* test if significant differences were observed. An arcsine transformation was performed for proportions of fatty acids in each lipid before being subjected to a one-way ANOVA or Student's *t*-test. Larval growth rate was compared between feeding treatments (control and copepod) and between rearing times using two-way ANOVA. Total lipid fatty acid proportions of microalgae, rotifers, copepods, and larvae were analyzed using a principal component analysis (PCA) to characterize fatty acid profiles of each trophic level organism.

RESULTS

Length and Dry Mass of Copepod Stages

The length of copepods increased ontogenetically (**Figure 2A**). Nauplii showed similar dry mass but larger body length than those for rotifers ($p < 0.05$; **Figure 2B**). Length and dry mass were significantly higher in copepodites and adults than nauplii ($p < 0.05$). The length and dry mass were larger for adult females than adult males ($p < 0.05$).

Fatty Acid Profiles of Microalgae

Total lipid fatty acid compositions of the SFC and algal mixture (three species) were determined (**Table 1**). SFC had peaks of C16:0, C16:2*n*-4, C18:2*n*-6, and C18:3*n*-3 fatty acids, and DHA, and the C18:2*n*-6 was highly enriched (19.57%). The fatty acid composition of the algal mixture was characterized by peaks of C14:0, C16:1*n*-7, C16:3*n*-4, and C18:4*n*-3 fatty acids, and EPA. These peaks in the algal mixture contributed to total saturated fatty acids (Σ SFA), monounsaturated fatty acids (Σ MUFA), and *n*-3 polyunsaturated fatty acids (Σ *n*-3) being higher than

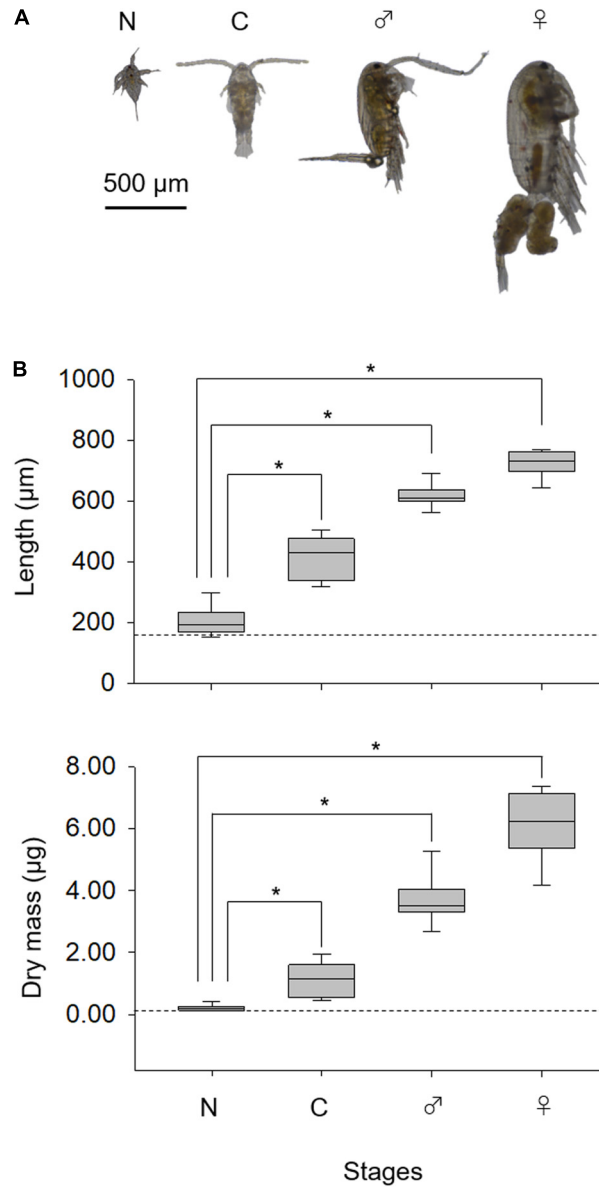

FIGURE 2 | Developmental changes in *Pseudodiaptomus inopinus* **(A)** and the lengths and dry mass **(B)**. Nauplius (N), copepodite (C), adult male (♂), and female (♀). Nauplius length is expressed as body length; and copepodite and adult length are expressed as prosome length. Broken lines depict average rotifer lorica length (upper) and dry mass (lower). For box plots, the central line represents the median value, and the box limits are the 25 and 75% quartiles; whiskers cover 5–95% of the data. Asterisks denote significant differences in values (Steel–Dwass test, $p < 0.05$).

those of SFC ($p < 0.05$). EPA was higher in the algal mixture ($p < 0.05$) because of the high proportion of fatty acids in *P. lutheri* (24.44%) and *P. tricornutum* (23.74%). While the algal mixture also contained DHA, mainly due to the fatty acids supplied from *T. lutea* (11.85%) and *P. lutheri* (9.62%), the DHA was lower than SFC ($p < 0.05$). The sum of the EPA and DHA (*n*-3 HUFAs) were occupied at approximately 20% of total fatty acids in both SFC and algal mixture. However, there was remarkable difference in DHA/EPA ratio between SFC (2.21) and algal mixture (0.35; $p < 0.05$). Arachidonic acid (ARA) was scarce (<1%) in all microalgae.

Fatty Acid Profiles of Rotifers and Copepods

The total lipid fatty acid composition of rotifers fed an algal mixture was compared with that of control diet (rotifers fed SFC; **Table 2**). The fatty acids C16:0, C18:1 (*n*-9 + *n*-7), and C18:2*n*-6 were high in total lipids of rotifers fed SFC. Rotifers fed SFC also exhibited EPA and DHA at 4.66 and 6.40%, respectively, resulting in DHA/EPA ratio of 1.38. When rotifers were fed an algal mixture, the C14:0, C16:1*n*-7, and C18:4*n*-3 fatty acid proportions increased significantly, and tended to have more EPA compared with those fed SFC (although the difference was not significant). On the contrary, the 18:2*n*-6 fatty acid was drastically decreased in rotifers fed an algal mixture ($p < 0.05$). The DHA was almost half as much as that of rotifers fed SFC. The fatty acid compositions of the two rotifer groups (those fed SFC, and those fed an algal mixture) corresponded with fatty acid peaks in SFC and the algal mixture (**Table 1**).

Compared with rotifers fed SFC, copepods had higher proportions of C14:0, C16:1*n*-7, C16:3*n*-4, and C18:4*n*-3 fatty acids, and lower proportions of C16:2*n*-4, C18:2*n*-6, and C18:3*n*-3 ($p < 0.05$; **Table 2**). Trends of such C14–18 fatty acids were consistently observed in comparison between rotifers fed SFC and algal mixture. However, except for EPA and DHA, copepods had lower proportions of C20–22 fatty acids than rotifers fed SFC or the algal mixture ($p < 0.05$). Conversely, EPA and DHA in copepods were specifically higher than rotifers fed SFC ($p < 0.05$). Copepod EPA and DHA accounted for 15.28 and 16.18% of total fatty acids, suggesting that it was a main component of the Σ *n*-3. In particular, copepods had higher DHA, despite being fed an algal mixture which contained lower DHA than SFC (**Table 1**) and decreased DHA in rotifers (**Table 2**). Copepods had a comparable DHA/EPA ratio to rotifers fed SFC ($p > 0.05$). The ratio was higher compared with rotifers fed algal mixture ($p < 0.05$).

The NLs and PLs of fatty acids among rotifers and copepods were further compared to evaluate the distributions of EPA and DHA (**Tables 3**, **4**). Compared with rotifers fed SFC, rotifers fed the algal mixture had NLs containing no significantly different EPA, but a lower DHA. Copepod NLs had a higher proportion of EPA than rotifers fed SFC or the algal mixture ($p < 0.05$; **Table 3**). DHA in NLs of copepods was, however, lower than in rotifers enriched by SFC ($p < 0.05$). Conversely, an extremely high proportion of DHA in copepods was distributed to PLs (27.97%; **Table 4**). DHA in PLs for copepods was five times higher than rotifers fed SFC, and 10 times higher than in those fed the algal mixture ($p < 0.05$). A comparison of dry mass-based contents of their fatty acids (mg g^{-1}) revealed that copepod EPA and DHA were remarkably higher in NLs and PLs, respectively, (**Figure 3**). The PLs-EPA of copepods were comparable to those of rotifers fed both SFC and the algal mixture ($p > 0.05$). A comparison of DHA between the rotifers fed SFC and the algal mixture revealed

TABLE 1 | Total lipids and their fatty acid proportions in docosahexaenoic acid-supplemented Super Fresh Chlorella (SFC), algae *Phaeodactylum* (Pha), *Pavlova* (Pav), and *Tisochrysis* (Tiso), and their mixture (Algal mixture).

	SFC	Algal mixture	Pha	Pav	Tiso
Total lipids (g g^{-1} dry mass)	0.127 ± 0.003	0.194 ± 0.013	0.199	0.182	0.244
Total fatty acids (g g^{-1} dry mass)	0.065 ± 0.001	0.079 ± 0.005	0.102	0.074	0.107
% total fatty acids					
C14:0	2.48 ± 0.06	12.78 ± 0.31*	8.43	16.01	20.29
C16:0	13.59 ± 0.19*	9.75 ± 0.02	9.52	12.24	7.98
C16:1*n*-7	1.76 ± 0.03	14.46 ± 0.42*	20.70	12.10	2.99
C16:2*n*-4	9.15 ± 0.02*	1.25 ± 0.08	1.96	0.46	0.40
C16:3*n*-4	0.42 ± 0.01	3.46 ± 0.08*	5.79	0.95	0.64
C18:0	1.87 ± 0.02	1.52 ± 0.19	2.02	2.13	1.53
C18:1*n*-9 + *n*-7	2.89 ± 0.04*	2.33 ± 0.04	0.65	1.79	9.39
C18:2*n*-6	19.57 ± 0.06*	1.50 ± 0.15	1.02	2.77	1.78
C18:3*n*-3	9.61 ± 0.08*	2.53 ± 0.13	0.87	1.60	6.19
C18:4*n*-3	0.25 ± 0.10	8.57 ± 0.11*	0.51	6.48	24.85
C20:1*n*-9	0.02 ± 0.03	0.61 ± 0.08*	0.08	0.08	2.14
C20:3*n*-6	0.13 ± 0.00	UD.	0.08	UD.	UD.
C20:3*n*-3	UD.	0.01 ± 0.02	0.00	0.04	0.03
ARA	UD.	0.10 ± 0.01	0.05	0.27	0.09
C20:4*n*-3	0.04 ± 0.00	0.03 ± 0.05	0.08	0.08	0.00
EPA	5.77 ± 0.10	17.43 ± 0.61*	23.74	24.44	0.59
C22:5*n*-6	0.10 ± 0.01	0.37 ± 0.02*	UD.	0.43	1.08
C22:5*n*-3	3.42 ± 0.02*	0.02 ± 0.03	UD.	0.03	UD.
DHA	12.74 ± 0.04*	6.05 ± 0.10	1.32	9.62	11.85
n-3 HUFA (EPA + DHA)	18.51 ± 0.12	23.47 ± 0.70*	25.06	34.06	12.44
Σ SFA	17.95 ± 0.24	24.05 ± 0.48*	19.97	30.38	29.79
Σ MUFA	4.67 ± 0.04	17.40 ± 0.32*	21.43	13.97	14.51
Σ *n*-6	19.81 ± 0.05*	1.97 ± 0.18	1.14	3.46	2.95
Σ *n*-3	31.84 ± 0.23	34.63 ± 0.60*	26.52	42.29	43.52
Σ PUFA	61.22 ± 0.18*	41.30 ± 0.62	35.41	47.16	47.50
Others	16.16 ± 0.04	17.25 ± 0.02	23.19	8.49	8.19
DHA/EPA	2.21 ± 0.04*	0.35 ± 0.01	0.06	0.39	20.12

Except for Tiso, Pav, and Pha (n = 1) values, values for the algal mixture and SFC represent means ± standard deviation (n = 3). Asterisks denote significant differences in each fatty acid proportion between SFC and the algal mixture (Student's t-test, $p < 0.05$). Total saturated fatty acids (ΣSFA) present sums of C14:0, C16:0, and C18:0; total monounsaturated fatty acids (ΣMUFA) present sums of C16:1n-7, C18:1n-9 + n-7, and C20:1n-9; and total polyunsaturated fatty acids (ΣPUFA) present sums of C16:2n-4, C16:3n-4, C18:2n-6, C18:3n-3, C18:4n-3, C20:3n-6, C20:3n-3, ARA, C20:4n-3, EPA, C22:5n-6, C22:5n-3, and DHA. UD., undetected.

that they presented comparable PLs-DHA contents ($p > 0.05$) unlike NLs-DHA contents. Accordingly, rotifers fed SFC had higher NLs-DHA, while copepods had higher PLs-DHA.

Larviculture Performance of Red Sea Bream

Fish larvae fed rotifers and copepods exhibited exponential growth curves ($p < 0.05$; **Figure 4A**). SL at 20 dph was greater in fish larvae fed copepods ($p < 0.05$; **Figure 4B**). The growth coefficient of larvae fed copepods was higher than that for rotifer feeding treatment ($p < 0.05$; **Table 5**).

Based on gut contents at 5 dph (**Figures 5A,B**), fish larvae fed rotifers ingested an average of 2.0 ± 1.3 rotifers $larva^{-1}$, while those fed copepods ingested an average of 1.0 ± 1.2 nauplii $larva^{-1}$. Larval ingestion rates increased ontogenetically in both rotifer- and copepod-feeding treatments. Copepodites occurred in the guts of fish larvae from 10 dph. The total dry mass of ingested prey (μg $larva^{-1}$) was estimated from the number of preys in the gut by multiplying each dry mass (**Figure 5C**). Total dry mass ingested did not differ between fish larvae fed rotifers or copepods at 5 dph, although the difference in total dry matter was greater in fish larvae fed copepods at both 10 dph (1.7 ± 1.8 μg $larva^{-1}$) and 15 dph (5.1 ± 3.6 μg $larva^{-1}$; $p < 0.05$). Estimates of the total DHA mass ingested by fish larvae (ng $larva^{-1}$) based on their dry mass-based contents in preys (**Figure 5D**) showed that copepod-fed fish larvae ingested more PLs-DHA at 5 dph (0.77 ± 0.89 ng $larva^{-1}$), 10 dph (5.85 ± 6.20 ng $larva^{-1}$), and 15 dph (17.87 ± 12.47 ng $larva^{-1}$) than fish larvae fed rotifers. The NLs-DHA mass ingested was comparable between fish larvae fed copepods and rotifers irrespective of their ages.

Fatty Acid Profiles of Fish Larvae

Fish larvae fed rotifers and copepods had relatively higher values of Σ *n*-3: 32.45% of total fatty acids in larvae fed rotifers, and 39.39% of total fatty acids in larvae fed copepods (**Table 6**), comprising mainly DHA (larvae fed rotifers, 20.26%; larvae fed

TABLE 2 | Fatty acid composition in total lipids of preys enriched with docosahexaenoic acid-supplemented Super fresh chlorella (SFC), or algal mixture.

	SFC	Algal mixture	
	Rotifer	Rotifer	Copepod
Total lipids (g g^{-1} dry mass)	0.143 ± 0.048	0.108 ± 0.008	0.136 ± 0.030
Total fatty acids (g g^{-1} dry mass)	0.091 ± 0.040	0.042 ± 0.001	0.081 ± 0.041
% total fatty acids			
C14:0	5.69 ± 1.55^{c}	8.78 ± 0.70^{b}	13.15 ± 1.64^{a}
C16:0	15.15 ± 0.22^{a}	14.93 ± 0.81^{a}	12.79 ± 0.83^{b}
C16:1*n*-7	3.82 ± 0.63^{b}	8.30 ± 0.76^{a}	11.13 ± 2.14^{a}
C16:2*n*-4	5.25 ± 1.51^{a}	0.61 ± 0.06^{b}	1.25 ± 0.71^{b}
C16:3*n*-4	0.67 ± 0.05^{b}	1.27 ± 0.36^{ab}	3.47 ± 0.51^{a}
C18:0	2.08 ± 0.29	3.48 ± 0.91	2.83 ± 0.71
C18:1*n*-9 + *n*-7	10.33 ± 0.63^{a}	7.30 ± 0.54^{b}	9.98 ± 1.42^{a}
C18:2*n*-6	20.63 ± 2.43^{a}	3.92 ± 0.37^{b}	1.39 ± 0.36^{b}
C18:3*n*-3	7.37 ± 1.15^{a}	4.34 ± 0.11^{b}	2.92 ± 0.10^{b}
C18:4*n*-3	0.29 ± 0.02^{b}	2.94 ± 0.28^{a}	3.54 ± 1.34^{a}
C20:1*n*-9	1.46 ± 0.05^{b}	2.84 ± 0.17^{a}	0.22 ± 0.09^{c}
C20:3*n*-6	1.97 ± 0.24	0.09 ± 0.15	0.15 ± 0.07
C20:3*n*-3	0.23 ± 0.08^{b}	0.42 ± 0.09^{a}	0.05 ± 0.04^{b}
ARA	1.22 ± 0.15^{a}	0.48 ± 0.11^{b}	0.17 ± 0.03^{c}
C20:4*n*-3	1.56 ± 0.12^{b}	7.91 ± 0.18^{a}	0.21 ± 0.10^{c}
EPA	4.66 ± 0.80^{b}	7.46 ± 1.02^{b}	15.28 ± 2.53^{a}
C22:0	0.21 ± 0.06	0.21 ± 0.20	UD.
C22:1*n*-9	0.36 ± 0.17^{b}	1.08 ± 0.07^{a}	UD.
C22:5*n*-6	0.69 ± 0.13^{a}	0.43 ± 0.06^{b}	0.06 ± 0.06^{c}
C22:5*n*-3	3.51 ± 1.11^{a}	2.99 ± 0.49^{a}	1.07 ± 0.24^{b}
DHA	6.40 ± 1.01^{b}	3.31 ± 0.16^{b}	16.18 ± 5.02^{a}
n-3 HUFA (EPA + DHA)	11.06 ± 1.80^{b}	10.77 ± 1.13^{b}	31.46 ± 5.25^{a}
Σ SFA	23.23 ± 1.50^{b}	27.40 ± 1.90^{ab}	28.88 ± 2.55^{a}
Σ MUFA	15.97 ± 1.13^{b}	19.53 ± 1.17^{ab}	21.33 ± 2.41^{a}
Σ *n*-6	24.52 ± 1.94^{a}	4.91 ± 0.63^{b}	1.78 ± 0.48^{c}
Σ *n*-3	24.01 ± 1.94^{b}	29.37 ± 0.41^{b}	39.24 ± 4.62^{a}
Σ PUFA	54.45 ± 1.68^{a}	36.16 ± 0.98^{c}	45.74 ± 4.76^{b}
Others	6.35 ± 1.05	16.92 ± 0.25	4.05 ± 1.93
DHA/EPA	1.38 ± 0.07^{a}	0.45 ± 0.04^{b}	1.08 ± 0.43^{a}

Values are presented as means ± standard deviation (n = 3). Different superscript letters within a row indicate significant differences between three preys (Tukey test, $p < 0.05$, a > b > c). Total saturated fatty acids (ΣSFA) present sums of C14:0, C16:0, C18:0, and C22:0; total monounsaturated fatty acids (ΣMUFA) present sums of C16:1n-7, C18:1n-9 + n-7, C20:1n-9, and C22:1n-9; and total polyunsaturated fatty acids (ΣPUFA) present sums of C16:2n-4, C16:3n-4, C18:2n-6, C18:3n-3, C18:4n-3, C20:3n-6, C20:3n-3, ARA, C20:4n-3, EPA, C22:5n-6, C22:5n-3, and DHA. UD., undetected.

TABLE 3 | Fatty acid proportions in non-polar lipids of preys enriched with docosahexaenoic acid-supplemented Super Fresh Chlorella (SFC), or algal mixture.

	SFC	Algal mixture	
	Rotifer	Rotifer	Copepod
Proportion (%)			
C14:0	5.44 ± 3.35^{b}	12.89 ± 1.38^{a}	13.66 ± 2.44^{a}
C16:0	12.98 ± 0.83	10.93 ± 1.89	11.73 ± 0.33
C16:1*n*-7	3.73 ± 0.32^{c}	10.61 ± 1.53^{b}	14.49 ± 1.47^{a}
C16:2*n*-4	7.29 ± 1.22^{a}	0.87 ± 0.10^{b}	1.06 ± 0.19^{b}
C16:3*n*-4	0.31 ± 0.18^{c}	1.66 ± 0.57^{b}	5.32 ± 0.56^{a}
C18:0	3.38 ± 1.35	4.43 ± 2.28	3.53 ± 0.10
C18:1*n*-9 + *n*-7	4.70 ± 1.46^{b}	8.25 ± 0.94^{a}	4.95 ± 1.14^{b}
C18:2*n*-6	19.99 ± 1.83^{a}	2.52 ± 0.24^{b}	1.14 ± 0.10^{b}
C18:3*n*-3	7.03 ± 0.69^{a}	3.90 ± 0.28^{b}	2.26 ± 0.37^{c}
C18:4*n*-3	*UD.*	3.58 ± 0.38^{b}	6.18 ± 1.39^{a}
C20:1*n*-9	0.07 ± 0.01	2.25 ± 0.16	0.08 ± 0.01
C20:3*n*-6	0.80 ± 0.03	0.06 ± 0.10	0.42 ± 0.07
C20:3*n*-3	2.11 ± 0.24^{a}	0.30 ± 0.09^{b}	0.15 ± 0.03^{b}
ARA	0.85 ± 0.07^{a}	0.35 ± 0.08^{b}	0.32 ± 0.08^{b}
C20:4*n*-3	0.87 ± 0.07^{b}	4.34 ± 0.30^{a}	0.33 ± 0.04^{c}
EPA	4.52 ± 0.51^{b}	7.88 ± 2.16^{b}	14.37 ± 2.02^{a}
C22:0	0.03 ± 0.04	0.23 ± 0.29	UD.
C22:1*n*-9	0.17 ± 0.02^{b}	0.75 ± 0.07^{a}	UD.
C22:5*n*-6	0.76 ± 0.10^{a}	0.70 ± 0.09^{a}	0.28 ± 0.06^{b}
C22:5*n*-3	3.04 ± 0.32^{a}	1.71 ± 0.15^{b}	0.17 ± 0.03^{c}
DHA	12.14 ± 1.71^{a}	4.64 ± 0.36^{c}	7.49 ± 0.62^{b}
Σ SFA	21.87 ± 5.16	28.49 ± 4.79	28.92 ± 2.04
Σ MUFA	8.67 ± 1.76^{b}	21.86 ± 2.36^{a}	19.52 ± 0.33^{a}
Σ *n*-6	22.40 ± 2.01^{a}	3.63 ± 0.32^{b}	2.18 ± 0.30^{b}
Σ *n*-3	29.70 ± 3.46	26.35 ± 2.39	30.94 ± 4.46
Σ PUFA	59.71 ± 6.46^{a}	32.52 ± 2.96^{b}	39.49 ± 5.45^{b}
Others	9.76 ± 2.96	17.14 ± 1.16	12.06 ± 3.88

Values depict means ± standard deviation (n = 3). Different superscript letters within a row indicate significant differences between three preys (Tukey test, $p < 0.05$, a > b > c). Total saturated fatty acids (ΣSFA) present sums of C14:0, C16:0, C18:0, and C22:0; total monounsaturated fatty acids (ΣMUFA) present sums of C16:1n-7, C18:1n-9 + n-7, C20:1n-9, and C22:1n-9; and total polyunsaturated fatty acids (ΣPUFA) present sums of C16:2n-4, C16:3n-4, C18:2n-6, C18:3n-3, C18:4n-3, C20:3n-6, C20:3n-3, ARA, C20:4n-3, EPA, C22:5n-6, C22:5n-3, and DHA. UD., undetected.

copepods, 27.52%). The DHA/EPA ratios for fish larval total lipids were 4.50 (rotifer-fed fish larvae) and 3.10 (copepod-fed fish larvae)—in both cases about three times higher in larvae than in their food (**Table 3**). When fish larvae total lipids were separated into NLs and PLs, the NLs-DHA of those fed copepods were about four times higher (26.62%) than those fed rotifers (8.74%). Larval PLs were similarly enriched with DHA (those fed rotifers, 22.34%; those fed copepods, 28.26%).

Difference in Fatty Acid Characteristics of Microalgae, Zooplankton, and Fish Larvae

Fatty acid compositions of microalgae, zooplankton, and larval fish were summarized using PCA with the first two PC axes (PC1 and PC2). The PC1 and PC2 explained 66.5% and 20.4% of all variations, respectively. On PC1, the score was correlated with almost all fatty acids (C14:0, C16:1*n*-7, C16:2*n*-4 C16:3*n*-4, C18:2*n*-6, C18:3*n*-3, C18:4*n*-3, EPA, and C22:5*n*-3), but not with C16:0 and DHA. On the other hand, C16:0 and DHA were negatively correlated with the PC2 score.

According to PCA biplot (**Figure 6**), the PC1 distinguished two groups depending on the origin of dietary fatty acids (i.e., SFC or algal mixture). The most negative scores

TABLE 4 | Fatty acid proportions in polar lipids of preys enriched with docosahexaenoic acid-supplemented Super Fresh Chlorella (SFC), or algal mixture.

	SFC	Algal mixture	
	Rotifer	Rotifer	Copepod
Proportion (%)			
C14:0	1.21 ± 0.08^{b}	5.98 ± 0.41^{a}	5.82 ± 0.21^{a}
C16:0	21.19 ± 0.67^{a}	17.73 ± 0.23^{b}	16.93 ± 0.25^{b}
C16:1*n*-7	3.09 ± 0.15^{b}	6.68 ± 0.14^{a}	6.53 ± 0.22^{a}
C16:2*n*-4	2.04 ± 0.04^{a}	0.43 ± 0.01^{c}	1.56 ± 0.07^{b}
C16:3*n*-4	0.47 ± 0.03^{c}	1.00 ± 0.25^{b}	1.45 ± 0.10^{a}
C18:0	0.48 ± 0.04^{c}	2.89 ± 0.03^{a}	0.70 ± 0.08^{b}
C18:1*n*-9 + *n*-7	6.71 ± 0.31^{a}	6.63 ± 0.18^{a}	5.17 ± 0.28^{b}
C18:2*n*-6	23.69 ± 0.49^{a}	4.87 ± 0.52^{b}	0.66 ± 0.11^{c}
C18:3*n*-3	6.64 ± 0.37^{a}	4.64 ± 0.08^{b}	0.79 ± 0.16^{c}
C18:4*n*-3	UD.	2.50 ± 0.29^{a}	1.35 ± 0.22^{b}
C20:1*n*-9	0.13 ± 0.02^{b}	3.26 ± 0.19^{a}	*UD.*
C20:3*n*-6	1.80 ± 0.17^{a}	0.11 ± 0.19^{b}	0.05 ± 0.09^{b}
C20:3*n*-3	1.63 ± 0.07^{a}	0.50 ± 0.09^{b}	0.09 ± 0.07^{c}
ARA	1.53 ± 0.04^{a}	0.56 ± 0.12^{b}	0.25 ± 0.03^{c}
C20:4*n*-3	1.95 ± 0.08^{b}	10.34 ± 0.24^{a}	0.25 ± 0.02^{b}
EPA	5.46 ± 0.12^{c}	7.12 ± 0.41^{b}	14.88 ± 0.56^{a}
C22:0	0.19 ± 0.03	0.20 ± 0.14	UD.
C22:1*n*-9	0.45 ± 0.06^{b}	1.30 ± 0.06^{a}	UD.
C22:5*n*-6	0.81 ± 0.04^{a}	0.24 ± 0.07^{b}	0.20 ± 0.02^{b}
C22:5*n*-3	4.38 ± 0.05	3.86 ± 0.66	0.29 ± 0.01
DHA	4.97 ± 1.02^{b}	2.39 ± 0.17^{c}	27.97 ± 0.82^{a}
Σ SFA	23.08 ± 0.63^{b}	26.80 ± 0.41^{a}	23.44 ± 0.47^{b}
Σ MUFA	10.37 ± 0.38^{c}	17.87 ± 0.17^{a}	11.70 ± 0.49^{b}
Σ *n*-6	27.83 ± 0.47^{a}	5.78 ± 0.82^{b}	1.16 ± 0.17^{c}
Σ *n*-3	25.04 ± 1.32^{c}	31.35 ± 1.27^{b}	46.76 ± 1.80^{a}
Σ PUFA	55.37 ± 1.34^{a}	38.55 ± 1.79^{c}	49.59 ± 1.47^{b}
Others	11.18 ± 1.57	16.78 ± 1.22	8.75 ± 0.73

Values depict means ± standard deviation (n = 3). Different superscript letters within a row indicate significant differences between three preys (Tukey test, $p < 0.05$, a > b > c). Total saturated fatty acids (ΣSFA) present sums of C14:0, C16:0, C18:0, and C22:0; total monounsaturated fatty acids (ΣMUFA) present sums of C16:1n-7, C18:1n-9 + n-7, C20:1n-9, and C22:1n-9; and total polyunsaturated fatty acids (ΣPUFA) present sums of C16:2n-4, C16:3n-4, C18:2n-6, C18:3n-3, C18:4n-3, C20:3n-6, C20:3n-3, ARA, C20:4n-3, EPA, C22:5n-6, C22:5n-3, and DHA. UD., undetected.

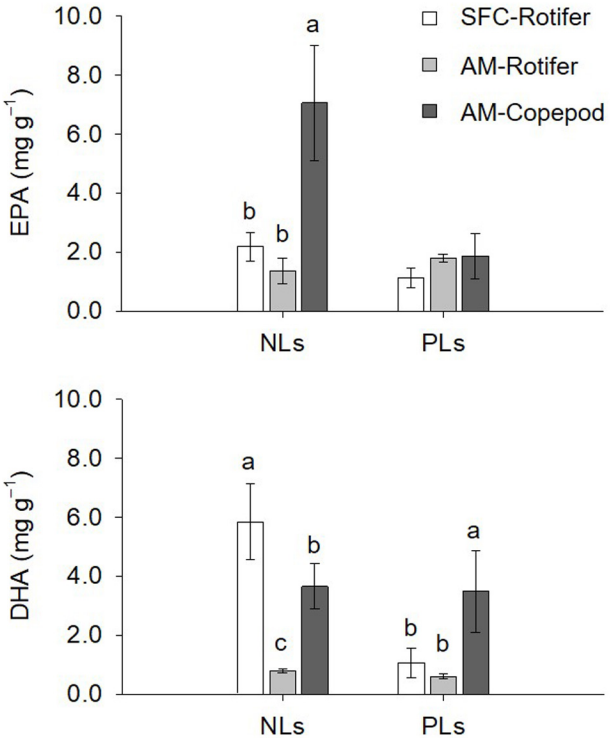

FIGURE 3 | Dry mass-based contents of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) for non-polar lipids (NLs) and polar lipids (PLs) in enriched preys. SFC-Rotifer represents rotifers fed docosahexaenoic acid-supplemented Super Freshwater Chlorella (SFC). AM-Rotifer and AM-Copepod represent rotifers and copepods fed algal mixture, respectively. Values represent means ± standard deviation ($n = 3$). Letters above bars indicate significant differences (Tukey test, $p < 0.05$, a > b > c).

on PC1 were found for SFC and the scores of rotifers fed SFC and larvae fed those rotifers were close to the SFC scores. On the contrary, algal mixture had the most positive score on PC1. The scores of rotifers fed algal mixture were close to zero, and copepods fed algal mixture had positive scores between algal mixture and the rotifers. Larvae fed those copepods had relatively higher score than larvae fed SFC-enriched rotifers, although their scores were commonly negative.

The PC2 mainly separated larvae from the lower trophic level organisms. Irrespective of the diets, larvae were commonly positioned at the most negative scores. On the contrary, SFC and algal mixture had the most positive scores. The scores of rotifers fed each diet little varied within the positive side, unlike those for PC1. Compared with rotifers, copepods modified the PC2 scores of microalgae toward those of larvae.

DISCUSSION

Accumulation of DHA in PLs of Copepods

The result of PCA for total lipid fatty acid proportions of each trophic level revealed the followings; (1) fatty acid compositions of microalgae (algal mixture and SFC) were commonly the most different from those for larval fish, and (2) compared with microalgae, *P. inopinus* and rotifers had closer characteristics of fatty acids toward larvae based on DHA and the others (except for C16:0), respectively, (**Figure 6**). In particular, *P. inopinus* had PLs specifically enriched with DHA despite its diet containing low DHA relative to EPA (**Tables 1**, **4**). Such selection of fatty acids to accumulate in the PLs can support the function of *P. inopinus* as a vector of DHA to larval fish.

Fraser et al. (1989) reported wild-caught *Calanus finmarchicus*, *Pseudocalanus* sp., and *Temora longicornis* copepods to have abundant DHA in PLs (30.9, 31.8, and 31.9%,

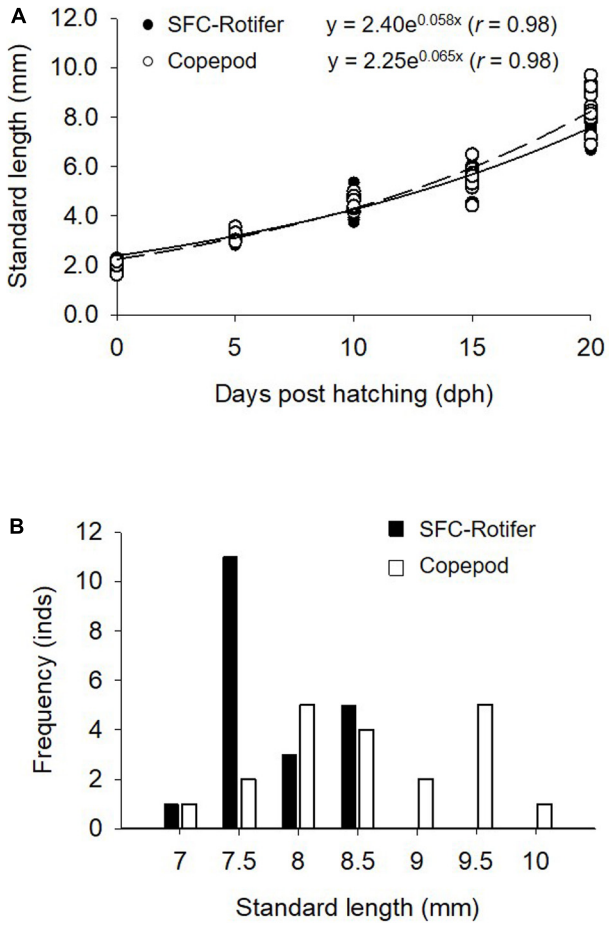

FIGURE 4 | Growth curve for *Pagrus major* larvae reared under different feeding regimes **(A)** and a histogram of larval standard length at the end of rearing periods (20 days post hatching) **(B)**. The curve for larvae fed rotifers (SFC-Rotifer; solid line) and those fed copepods (broken line) is fitted based on data from 20 samples obtained at 0, 5, 10, 15, and 20 dph.

TABLE 5 | The result of two-way analysis of variance (ANOVA) for the exponential growth coefficient of *Pagrus major* larvae reared during 20 days.

	df	SS	MS	*F*	*P*
Feeding treatments	1	8.31	8.31	51.51	<0.05
Reared periods	4	1,528.48	382.12	2,369.04	<0.05
Feeding treatments × Reared periods	4	20.12	5.03	31.19	<0.05
Residue	460	74.20	0.16		

df, degree of freedom; SS, sum of squares; and MS, mean squares.

respectively), which is consistent with our copepod species, *P. inopinus* (**Table 4**). Each copepod contained little DHA in triacylglycerol and wax esters, the main components of NLs (Fraser et al., 1989). It stresses the importance of DHA as a preferential component in the PLs of copepods. PLs, including phospholipids, mainly form lipid bilayer of plasma and organelle membranes in animals (Spector and Yorek, 1985). An abundance of DHA in *C. finmarchicus* eggs and nauplii was reported by Sargent and Falk-Petersen (1988). Egg production and hatching success for *T. longicornis* are enhanced with increased DHA concentration in the diet (Evjemo et al., 2008; Jónasdóttir et al., 2009). Considering specific abundance of DHA in PLs, the DHA in copepods probably functions as a key membrane component in the gonad and embryo during development.

On the other hand, proportions of DHA in PLs of artificially enriched rotifers were lower than in *C. finmarchicus* (Bell et al., 2003; Olsen et al., 2014), which is consistent with our comparison (**Table 4**). Rotifers fed SFC had DHA enriched in NLs but not in PLs (**Figure 3**). There was no significant difference in PLs-DHA of rotifers fed SFC and the algal mixture irrespective of differences in their microalgae DHA (**Table 1**). Thus, rotifers possibly have PLs with relatively limited capacities for DHA (Li et al., 2015). Rotifers are reported to use DHA mainly as an energy source (Rainuzzo et al., 1994), which is a different DHA metabolic strategy to copepods.

In this study, it was unclear how *P. inopinus* could retain high concentrations of DHA in their PLs. In mammals and fish, phospholipase A2 and phospholipid acyltransferase function to modify fatty acid composition belonging to PLs (Sun et al., 1979; Balsinde, 2002; Tocher et al., 2008). Such enzymatic activities can also be investigated in copepods to reveal how DHA is preferentially accumulated in their PLs. In addition, copepods can biosynthesize DHA via fatty acid desaturation and elongation even when they cannot ingest diets including DHA (Monroig et al., 2013; Nielsen et al., 2019; Lee et al., 2020). In fact, DHA is detected in *Pseudodiaptomus annandalei* adults and nauplii fed non-DHA possessing microalga *Tetraselmis chuii* (Rayner et al., 2017). It indicates that both activities (i.e., accumulation of DHA in the PLs, and fatty acid biosynthesis) can support copepods to maintain DHA in their PLs.

Significance of Copepod Fatty Acid Profiles to Fish Larvae

Larvae fed rotifers and copepods had extremely high DHA relative to EPA (at the ratio of 4.50 and 3.10; **Table 6**), indicating the preference of DHA for larvae. In particular, PLs in larval fish fed both copepods and rotifers were commonly enriched with DHA regardless of dietary differences in total lipid DHA (**Table 2**). DHA is highly concentrated in the larval fish brain; it is a key membrane component, and functions in development of the nervous system (Furuita et al., 1998; Masuda et al., 1999). In this case the DHA is often paired with C16:0 (Farkas et al., 2000), explaining why C16:0 was also presentative fatty acid for larvae (**Figure 6**). Because of the limited capacity of *n*-3 HUFA biosynthesis in marine fish (Tocher, 2003), red sea bream might preferentially retain DHA obtained from its diet in its PLs. In this study, although there are no replicates in the fatty acid analysis for larvae, NLs-DHA in larvae fed copepods is quite high and therefore non-negligible (**Table 6**). Dietary DHA is efficiently incorporated into marine finfish larvae when the

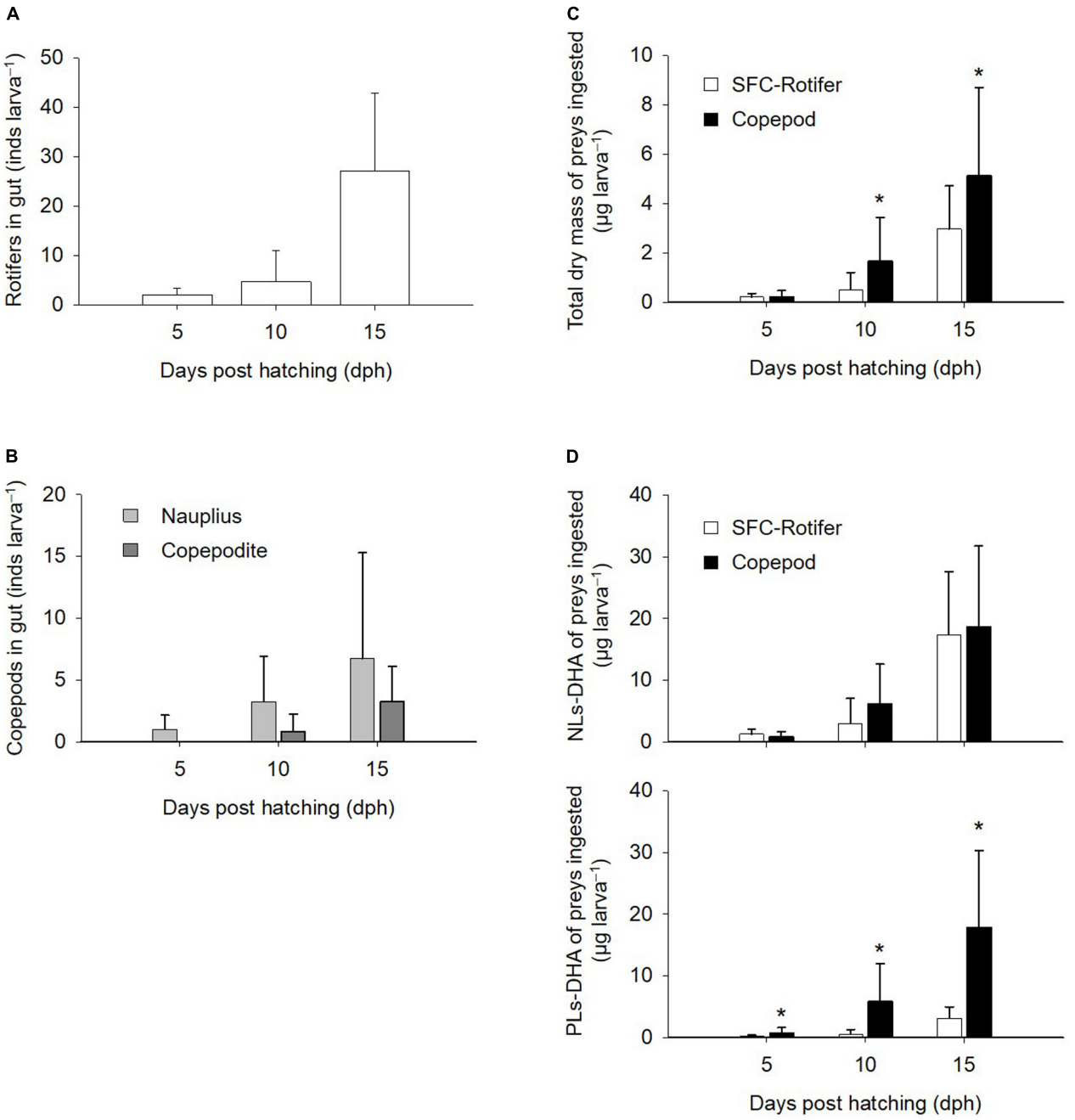

FIGURE 5 | Gut contents of larval *Pagrus major* based on the number of preys **(A,B)**, total dry mass **(C)**, and docosahexaenoic acid (DHA) mass **(D)**. Ingested mass of DHA is presented for non-polar (NLs-DHA) and polar (PLs-DHA) lipid forms. Values represent means ± standard deviation (n = 15). Asterisks depict significant differences in dry and DHA masses between rotifers fed docosahexaenoic acid-supplemented Super Fresh Chlorella treatment (SFC-Rotifer) and copepod treatments (Student's t-test, $p < 0.05$).

fatty acid is formed in the PLs (Salhi et al., 1999; Gisbert et al., 2005; Wold et al., 2009). In fact, *P. inopinus* had high PLs-DHA, while rotifers fed SFC had high DHA in NLs but not in PLs (**Figure 3**). Larvae also ingested considerably more DHA mass from copepod PLs (**Figure 5D**). Our results suggest that accumulation of DHA in copepod PLs functions to efficiently transfer DHA to fish larvae.

In addition to larval DHA, the growth was enhanced by feeding copepods compared with rotifers (**Figure 4**). Our results indicate that the higher growth is achieved by an increase in the ingested copepod mass (mainly copepodite stage) and by more nutrition derived from copepods. Busch et al. (2010) suggest that nutritional status of larvae is a key to explain the differences in growth of larval fish fed

TABLE 6 | Fatty acid composition of total lipids, non-polar lipids (NLs), and polar lipids (PLs) of *Pagrus major* larvae at the end of rearing periods (20 days post hatching).

	Total lipids		NLs		PLs	
	Control	Copepod	Control	Copepod	Control	Copepod
Proportion (%)						
C14:0	1.55	2.46	6.37	7.12	0.48	1.07
C16:0	20.07	22.55	19.11	17.48	20.51	23.98
C16:1 *n*-7	2.10	3.51	3.17	4.09	1.92	3.33
C16:2 *n*-4	0.83	0.66	0.72	0.40	0.84	0.71
C16:3 *n*-4	0.97	1.82	1.03	2.06	0.97	1.83
C18:0	1.52	1.38	0.46	0.38	1.65	1.70
C18:1 *n*-9 + *n*-7	19.28	16.01	23.78	13.74	19.49	21.29
C18:2 *n*-6	10.44	0.77	9.19	1.01	10.78	0.70
C18:3 *n*-3	2.18	0.31	3.38	0.44	1.93	UD.
C18:4 *n*-3	0.10	0.11	0.21	0.22	0.16	0.18
C20:0	0.77	0.21	1.58	0.25	0.55	0.30
C20:1 *n*-9	1.43	0.30	1.14	0.14	1.44	UD.
C20:3 *n*-6	0.88	0.13	0.64	0.31	0.84	0.15
C20:3 *n*-3	0.17	UD.	0.70	UD.	UD.	UD.
ARA	1.31	2.00	1.15	2.79	1.31	1.79
C20:4 *n*-3	0.55	0.28	0.77	0.22	0.55	0.37
EPA	4.50	8.87	4.23	12.78	4.57	7.68
C22:0	0.33	UD.	2.16	UD.	UD.	UD.
C22:1 *n*-9	0.32	UD.	0.25	UD.	UD.	UD.
C22:5 *n*-6	UD.	UD.	1.05	UD.	0.09	UD.
C22:5 *n*-3	4.86	2.30	3.33	2.02	5.19	2.20
DHA	20.26	27.52	8.74	26.62	22.34	28.26
Σ SFA	22.36	24.14	21.15	18.11	22.71	25.98
Σ MUFA	23.13	19.82	28.34	17.97	22.85	24.62
Σ *n*-6	12.63	2.90	12.03	4.11	13.02	2.64
Σ *n*-3	32.45	39.39	20.66	42.30	34.74	38.69
Σ PUFA	46.88	44.77	34.44	48.87	49.57	43.87
Others	6.35	9.02	8.42	8.18	4.94	4.76
DHA/EPA	4.50	3.10	2.07	2.08	4.89	3.68

Total saturated fatty acids (ΣSFA) present sums of C14:0, C16:0, C18:0, C20:0, and C22:0; total monounsaturated fatty acids (ΣMUFA) present sums of C16:1n-7, C18:1n-9 + n-7, C20:1n-9, and C22:1n-9; and total polyunsaturated fatty acids (ΣPUFA) present sums of C16:2n-4, C16:3n-4, C18:2n-6, C18:3n-3, C18:4n-3, C20:3n-6, C20:3n-3, ARA, C20:4n-3, EPA, C22:5n-6, C22:5n-3, and DHA. UD., undetected.

rotifers and copepods. Larval Atlantic cod *Gadus morhua* growth was positively correlated with proportions of DHA (Cutts et al., 2006). Thus, higher DHA in *P. inopinus*-fed larvae (especially in those NLs) can be linked to the enhanced growth.

As the other *n*-3 HUFA, EPA is essential fatty acid to further enhanced growth and survival of larvae (Ganuza et al., 2008; Eryalçın et al., 2013). In fact, an increase in concentrations of dietary *n*-3 HUFA resulted in higher larval growth as long as the DHA/EPA ratio is appropriate (Rodríguez et al., 1998). On the contrary, excess EPA over DHA (i.e., low DHA/EPA ratio) negatively affects larval growth and development (Reitan et al., 1994b; Rodríguez et al., 1997). *P. inopinus*, despite extremely low DHA/EPA in their diet (**Table 1**), increased that ratio of total lipids until larvae predated the preys (**Table 2**). In particular, *P. inopinus* accumulated excess EPA in the NLs (**Figure 4**), which might be consumed as an energy source like NLs DHA for rotifers. They indicate that *P. inopinus* contribute to larval growth by transferring DHA and/or excluding EPA from microalgae.

Roles of *P. inopinus* in Coastal Regions in Transferring *n*-3 HUFA to Larvae

In the debate on fish recruitment, contribution of microalgae fatty acids to early development of larval fish has been discussed (Bell and Sargent, 1996). In marine ecosystems, specific fatty acids in microalgae are sufficiently transferred via mainly copepods to wild fish larvae so that they are used as biomarker for microalgae (Klungsøyr et al., 1989; Rossi et al., 2006). Especially in coastal and estuarine regions like *P. inopinus* habitats, microalgae diatoms are often dominated (Ubertini et al., 2012) and such blooms had higher proportions of EPA than DHA (Sargent et al., 1985). Sediments, which can be fed by the genus *Pseudodiaptomus* copepods (Jacoby and Greenwood, 1988), also include diatoms as a main

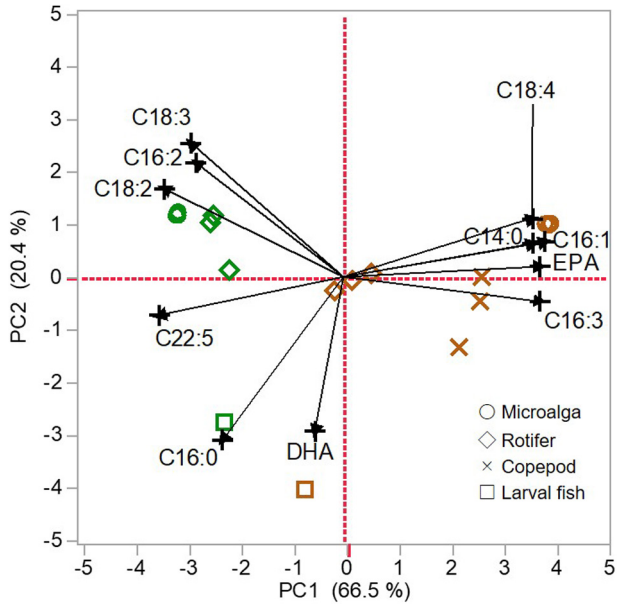

FIGURE 6 | Principal component analysis (PCA) of total lipid fatty acid compositions of microalgae (circle), zooplankton (rotifers, diamond; copepods, cross), and larval fish (square). Green: docosahexaenoic acid-supplemented Super Fresh Chlorella (SFC). Brown: algal mixture. The PCA was calculated using proportions of C14:0, C16:0, C16:1, C16:2, C16:3, C18:2, C18:3, C18:4, EPA, C22:5, and DHA, which were significant differences of one or more percent between SFC and algal mixture. The PC1 scores were correlated with C14:0 (r = 0.913), C16:1 (r = 0.973), C16:2 (r = 0.708), C16:3 (r = 0.944), C18:2 (r = 0.860), C18:3 (r = 0.733), C18:4 (r = 0.909), EPA (r = 0.892), C22:5 (r = 0.892); and the PC2 ones were correlated with C16:0 (r = 0.783) and DHA (r = 0.742).

component (mainly benthic microalgae; Grippo et al., 2010). However, larvae experienced on diatom-fed copepods showed lower growth than those on copepods fed dinoflagellate with higher DHA and lower EPA (St. John et al., 2001). They can hypothesize that fatty acid distribution in coastal microalgae is not suited for larval growth. According to the result of PCA on each fatty acid species for three trophic levels, however, two kinds of microalgae with distinct fatty acid compositions did not regulate larval DHA (**Figure 6**). Compared with rotifers, *P. inopinus* shifted algal fatty acid compositions much more toward DHA peak where larvae were positioned, and furthermore, enhanced larval growth (**Figure 4**). Our results suggest that what feeds on microalgae is more important factors than microalgae to determine larval growth. On the classical food chain in coastal waters, *P. inopinus* can function as a buffer of microalgae fatty acids for enhanced larval recruitment.

CONCLUSION

We artificially recreated a series of trophic levels in the coastal ecosystem using the cultured copepod *P. inopinus*, its prey (DHA-poor algae), and predatory fish larvae. Fatty acid profiles of *P. inopinus* and fish larvae fed these copepods were compared with those fed DHA-enriched rotifers. Copepods upgraded DHA levels compared with the dietary fatty acids and selectively fortify their PLs. Concentrations of PLs-DHA were considerably higher in copepods compared with rotifers. Red sea bream larvae fed copepods had higher trends in NLs-DHA and enhanced the growth performance. We suggest that *P. inopinus* play an important ecological role as a carrier of the essential fatty acid DHA in PLs to coastal finfish larvae.

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.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Experimental Committee, Kagoshima University.

AUTHOR CONTRIBUTIONS

HM, TS, and TKot designed the study and experiments. HM and TS conducted the experiments and collected the data. TKob identified copepod species. HM prepared a draft of the article and revised it with TKob and VW. MI provided the methodologies for GC analysis. HM performed data analyses. TKot supervised the studies. All the authors read and approved the final manuscript.

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Effects of Rotifers Enriched With Different Enhancement Products on Larval Performance and Jaw Deformity of Golden Pompano Larvae *Trachinotus ovatus* (Linnaeus, 1758)

Zhengyi Fu[1,2,3], Rui Yang[1,2,3], Shengjie Zhou[1,2,3], Zhenhua Ma[1,2,3] and Tao Zhang[4]**

[1] Tropical Aquaculture Research and Development Center, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Sanya, China, [2] Key Laboratory of South China Sea Fishery Resources Exploitation and Utilization, Ministry of Agriculture, Guangzhou, China, [3] Sanya Tropical Fisheries Research Institute, Sanya, China, [4] Dalian Tianzheng Industry Co., Ltd., Dalian, China

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Yen-Ju Pan,
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***Correspondence:**
Zhenhua Ma
zhenhua.ma@hotmail.com
Tao Zhang
zht_3000@163.com

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This study evaluated the effects of rotifers enriched with three enhancement products (*Nannochloropsis*, S.presso, and Algamac 3080) on the body fatty acid composition, growth, survival, jaw deformity, and bone development-related gene expression of the golden pompano larvae. The rotifers enriched with *Nannochloropsis* were rich in EPA, and the rotifers enriched with S.presso and Algamac 3080 were rich in docosahexaenoic acid (DHA). The level of DHA in Algamac 3080 is higher than that in S.presso. The first feeding started at 3 DPH, and data were collected at 8 DPH. The results showed that the body fatty acid composition of the larvae was basically the same as that of the feeding rotifers. The specific growth rate of S.presso and Algamac 3080 treatment was significantly higher than the un-enriched treatment ($P < 0.05$). The survival rate of Algamac 3080 treatment was significantly lower than the other treatments ($P < 0.05$), and the jaw deformity rate of S.presso treatment was significantly lower than the *Nannochloropsis* and un-enriched treatment ($P < 0.05$). The expression level of BMP2 and BMP4 in golden pompano larvae were not significantly affected by the enhancement products ($P > 0.05$), and the expression level of RXRα decreased significantly in the S.presso and Algamac 3080 treatment ($P < 0.05$). This study indicates that S.presso was an enhancement product more suitable for rotifers for golden pompano larvae. This study provided reliable reference and guidance for the first feeding of golden pompano larvae and also provided more reference data for the study of the mechanism of diet on larval fish bone deformity.

Keywords: enrichment, larvae rearing, growth, survival, deformity, fatty acids, gene expression

INTRODUCTION

In the artificial breeding of marine fish, the choice of feed during first feeding period is particularly important, because they are very vulnerable during this period (Hamre et al., 2013). The critical period for the transformation of endogenous nutrition into exogenous nutrition is also critical for the development of bones and systems of larval fish (Koedijk et al., 2010). Unsuitable feed can lead to nutrient deficiency and starvation, resulting in growth retardation, deformity, and even death (Rice et al., 1987; Gisbert et al., 2004; Waqalevu et al., 2019). Rotifers and *Artemia nauplii* are widely used in aquaculture due to the advantages of suitable size and easy cultivation (Kotani et al., 2009; Ma et al., 2018). In particular, *Brachionus* is usually selected as the weaning food for marine finfish (Kobayashi et al., 2008; Kotani et al., 2009).

Furthermore, some studies have revealed that rotifers lack essential nutrients, so they must be enriched before feeding them to fish larvae (Hamre, 2016; Kotani, 2017). Common enriched methods include the use of fresh microalgae or commercial enhancement products, but because microalgae are difficult to operate and cultivate, commercial enhancement products are more widely used in enrichment process (Eryalcin, 2018). In the enriching of live feeds, polyunsaturated fatty acids (PUFA) such as arachidonic acid (ARA, 20:4 n-6), eicosapentaenoic acid (EPA, 20:3 n-3), and docosahexaenoic acid (DHA, 22:6 n-3) are usually the nutrient components that people pay more attention to. They play an important role in neurological and visual development, and stress resistance (Hernández-Cruz et al., 2015). Recent studies have also confirmed that they, especially DHA, are associated with bone and cartilage development in fish and provide a contribution to the prevention of bone deformities (Roo et al., 2009; Izquierdo et al., 2013).

In addition to focusing on growth and survival of larval fish in evaluating the effects of different enriched rotifers, bone deformities are also particularly important. Especially in the early stage, the development of the jaw will affect eating, growth, etc.; and bone deformities will become a potential that affects the later growth and survival rate (Cobcroft et al., 2001; Fraser and de Nys, 2005; Ferraresso et al., 2010). In golden pompano, some osteogenic marker genes have been studied in depth. Among them, bone morphogenetic protein (BMP) and retinoid X receptor (RXR) have been found to be significantly related to bone deformities (Yang et al., 2015; Ma et al., 2018). As a growth factor, BMPs play an important role in morphogenesis during embryonic development, and it is also an osteoinductive factor with osteogenic properties (Nishimura et al., 2012; Marques et al., 2016). RXRs can work with other genes such as IGF, BMP, Hox, or shh in morphogenesis and are related to bone deformation and survival (Cahu et al., 2009). In addition, RXRs can also be used as a transcription factor to regulate gene expression in various biological processes (Zhao et al., 2000; Shulman et al., 2004).

Golden pompano is one of the important economic fishes in the southern coastal areas of China and is popular in consumers for its tender and delicious meat (Ma et al., 2016, 2018). The artificial breeding technology of golden pompano is relatively mature, but the low survival rate and high deformity rate during the first feeding of larval fish are still technical bottlenecks. It is more effective and realistic to optimize the nutrition enriched method of rotifers to solve this problem so we selected an instant algae and two commercial enrichment products and unenhanced rotifers for comparison to explore a reasonable and convenient way of enriching rotifers and provide reference for the optimization of the details of the artificial breeding process of golden pompano.

MATERIALS AND METHODS

Eggs and Larval Fish Rearing

Fertilized eggs of golden pompano were obtained from Guanghui Aquaculture Hatchery, Hainan Province, China, and were transported to Tropical Aquaculture Research and Development Center, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Xincun Town and hatched in 500-L fiberglass incubators at 26°C. On 3 days post-hatching (DPH), larvae were stocked into 12 larval rearing tanks (1000-L) at a density of 40 fish L^{-1}. Rearing tanks were supplied with filtered seawater and conducted in indoor flow-through culture system with a daily exchange rate of 200% tank volume. Two air stones were used in each tank to keep the dissolved oxygen level close to saturation. During the experimental period, the water quality parameters were measured daily and maintained at ammonia nitrogen < 0.1 mg L^{-1}, nitrite nitrogen < 0.02 mg L^{-1}, pH 7.9–8.1, salinity 33‰, and dissolved oxygen > 7.0 mg L^{-1}.

Cultivation of Rotifers

This experiment included four dietary treatments with three replicates each, including (1) instant microalgal paste at 8×10^6 cell mL^{-1} (*Nannochloropsis* sp., Qingdao Hong Bang Biological Technology Co., Ltd, Qingdao, China), (2) S.presso (Selco S.presso®, INVE Aquaculture) at 350 mg L^{-1}, (3) Algamac 3080® (Aquafauna, United States) at 200 mg L^{-1}, and (4) Rotifers without enrichment served as control. The pure cultures of a strain of *Brachionus plicatilis* (L-type) with a typical lorica length of about 160 μm were supplied by Haiyou Jiayin Biotechnology Co., Ltd, Qionghai, China. Rotifers were fed with S.parkle® (INVE Aquaculture) at a density of 500 rotifers ml^{-1}. The condition for rotifer culture was set at 24.3 ± 0.7°C, > 5.8 mg dissolved oxygen L^{-1}, pH 7.95–8.11, and 37.5 g L^{-1} salinity. Prior to enrichment, the rotifers collected from the culture tank were rinsed with filtered seawater on a 100-μm mesh screen. During enrichment, the density of rotifers increased to 1,000 ml^{-1}. After enrichment for 12 h, the rotifers were harvested on a 100-μm mesh screen and fed to the fish larvae. Starting from 3 DPH, the density of rotifers was kept at 15 ml^{-1} in the larval fish rearing tank. The instant microalgae (Nanno 3600 paste, Reed Mariculture, United States) were also added into all the larval fish tanks at a density of about 1×10^6 cell ml^{-1} to create a green color background.

Jaw Deformity

A total of 50 fish larvae were randomly collected from each rearing tank to examine the incidence of deformity. The fish were

anesthetized by overdosing of Aqui-S (AQUI-S, New Zealand) and fixed in 4% paraformaldehyde. Jaw deformity was assessed by observing under a stereo microscope (Olympus SZ40, Japan) using the criteria described by Cobcroft and Battaglene (2009). The appearance of the jaws of each larvae was rated on a scale of 0–3 according to the jaw malformation index (Cobcroft et al., 2004) modified for golden pompano larvae. A score of 0 indicated a normal jaw (**Figure 1A**). A score of 0.5 indicated very minor malformation e.g., slightly short lower jaw (**Figure 1B**), that would not be considered malformation from a commercial perspective. The larvae were defined as malformed when the jaw score reached 1, 2, or 3: Score 1, minor variation from normal structure, some resistance to closing mouth, e.g., snub nose (**Figure 1C**); Score 2, intermediate where some elements are abnormal in shape or position although limited movement occurs to open and close the mouth, e.g., ventral transposition of the glossohyal (**Figure 1D**); Score 3, severe malformation where jaw elements have abnormal shape or are in abnormal positions, and do not move to close the mouth, e.g., severe bending Meckel's cartilage (**Figure 1E**).

Fatty Acids Analysis

After enrichment, four million rotifers from each treatment in three replicates were collected and preserved in liquid nitrogen until analysis. On 8 DPH, 0.5 g fish larvae (wet weight) in five replicates were sampled for fatty acid analyses. All rotifer and fish samples were pre-washed using an ammonium formate solution (0.5 M) to remove salt, and paper tower was used to remove extra water before preservation in liquid nitrogen. The fatty acids were analyzed at South China Sea Fisheries Research Institute, China, following the method described by Ma and Qin (2014).

Gene Expression Analysis

The fish larvae were sampled on 8 DPH. Total RNA was extracted using TRIzol (Invitrogen, United States). RNA integrity was verified by agarose gel electrophoresis. RNA concentration was measured by spectrophotometry (Bioteke Corporation Co., Ltd., China) at 260 nm, and the purity was determined at the OD 260/280 ratio and agarose gel electrophoresis. The RNA was immediately used for cDNA synthesis. Subsequently, reverse transcription was performed on 1 μg of total RNA using TransScript-Uni One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgen Biotech Co., Ltd., China). The synthesized cDNA samples were stored at −20°C until further use.

The primers of BMP4, BMP2, RXRα, and EF1α (**Table 1**) were previously designed and validated by Yang et al. (2015) and Ma et al. (2018). In quantitative real-time PCR, EF1α was used as the internal reference and amplified. The reaction conditions were as follows: initial denaturation at 95°C for 15 min, 40 cycles of denaturing at 95°C for 10 s, annealing at 58°C for 20 s, and extension at 72°C for 30 s. For each test, three replicates were performed in this study. No template control was included with each assay to verify that PCR master mixes were free of contamination. Dissociation curves were employed to ensure that only one single PCR product was amplified in each gene reaction. After verification of PCR efficiency to be 95–105%, the relative gene expression was calculated using the ΔCT (comparative threshold cycles) (ΔCT = CT of target gene–CT of EF1α, ΔΔCT = sample CT – ΔCT of calibrator sample).

Calculations and Statistical Analysis

Specific growth rate (SGR) was calculated as: SGR = 100 × [ln (W_f) – ln (W_i)]/ΔT, where W_f was the final body weight, W_i was the initial body weight, and ΔT was the experimental duration.

The data were expressed as the mean ± standard deviation (SD). Statistical analyses were carried out by PASW Statistics (version 18). Comparisons between different groups were conducted by Tukey's test, and significant difference was set at $P < 0.05$. All percentage data were transformed using square root to satisfy the assumptions of ANOVA.

RESULTS

Fatty Acid Composition in Rotifers and Fish Larvae

The specific fatty acid composition in rotifers significantly varied between treatments (**Table 2**). The amount of EPA (20:5n-3) in the rotifers enriched with *Nannochloropsis* (9.05%) was significantly higher than other treatments ($P < 0.05$). The EPA was not significantly different between the treatments of S.pressa, Algamac 3080, and un-enriched ($P > 0.05$). The amount of DHA (22:6n-3) in the rotifers enriched with Algamac 3080 (30.12%) was significantly higher than other treatments ($P < 0.05$). The DHA in S.pressa treatment was significantly higher than that in *Nannochloropsis* treatment, while un-enriched treatment was not significantly different from them ($P > 0.05$). The DHA/EPA of all treatments showed significantly different ($P < 0.05$), and the order from large to small was: Algamac 3080 (9.53), S.presso (4.36), Un-enriched (1.98), and *Nannochloropsis* (0.43). The EPA/ARA in S.presso treatment (2.69) and Algamac 3080 treatment (1.47) were significantly lower than un-enriched treatment (3.81, $P < 0.05$).

The specific fatty acid composition in fish larvae significantly varied between treatments (**Table 3**). The trend of EPA (20:5n-3) amount in fish larvae was consistent with rotifers, the amount of EPA in the fish larvae of *Nannochloropsis* treatment (8.09%) was significantly higher than other treatments ($P < 0.05$). The trend of DHA (22:6n-3) amount in fish larvae was not completely consistent with rotifers. The amount of EPA in Algamac 3080 treatment fish larvae was no significant difference with S.presso treatment ($P > 0.05$), but it was still significantly higher than the other two treatments ($P < 0.05$). The DHA/EPA of all treatments showed significantly different ($P < 0.05$) and had the same trend as rotifers. The EPA/ARA of all treatments showed significantly different ($P < 0.05$), and the order from large to small was: Un-enriched (3.33), *Nannochloropsis* (2.33), S.presso (1.28), and Algamac 3080 (0.69).

Larval Fish Growth and Survival

SGR showed significant difference between un-enriched and commercial enrichment products treatments ($P < 0.05$,

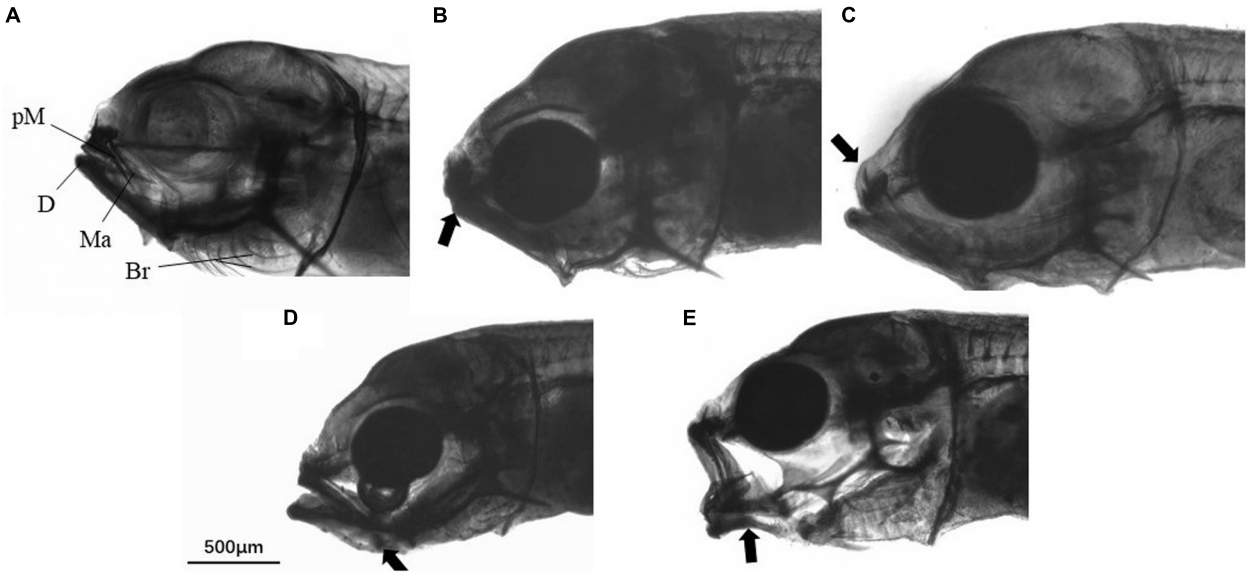

FIGURE 1 | Golden pompano larvae with different jaw malformation index scores. **(A)** Score 0, **(B)** score 0.5, **(C)** score 1, **(D)** score 2, and **(E)** score 3. Abbreviations: pM, Premaxilla; Ma, maxilla; D, dentary; Br, branchiostegal rays. The arrow points to the location of the deformity.

TABLE 1 | Primers of bone morphogenetic protein 2 (BMP2), bone morphogenetic protein 4 (BMP4), retinoid X receptor α (RXRα), and extension factor 1α (EF1α) genes in golden pompano used in qPCR.

Gene Abbreviation	Sequence (5′–3′)	Amplicon size (bp)
BMP2-Qf	CAGGCAGCCACTCCGCAAAC	146
BMP2-qR	TCCCCGTGGCAGTAAAAGG	
BMP4-qF	GTGAACAACAACATTCCCAAGG	126
BMP4-qR	GCAGCCCTCCACTACCATTT	
RXRα-qF	ACAGAGACCTACATTGAGAC	174
RXRα-qR	GTCATCCTTCTACGAGCA	
EF1α-qF	CCCCTTGGTCGTTTTGCC	101
EF1α-Qr	GCCTTGGTTGTCTTTCCGCTA	

Figure 2). The highest SGR were observed in the two commercial enrichment products (S.presso and Algemac 3080), and there was no significant difference between them ($P > 0.05$).

The survival rate of *Nannochloropsis* treatment was significantly higher than the un-enriched treatment ($P < 0.05$, **Figure 3**). Among the two commercial enrichment products treatments, there was no significant difference in survival rate between S.presso and Algamac 3080 treatment ($P > 0.05$), and the survival rate of Algamac 3080 treatment was significantly lower than all treatments ($P < 0.05$).

Jaw Deformity

Jaw deformity rate showed no significant difference between *Nannochloropsis* and un-enriched treatment ($P > 0.05$, **Figure 4**), and the rate of S.presso treatment was significantly lower than *Nannochloropsis* and un-enriched treatment ($P < 0.05$). There was no significant difference in jaw deformity rate between Algamac 3080 treatment and all treatments ($P > 0.05$).

Relative Expression Level of Bone Development Related Genes

The expression level of BMP2 showed no significant difference between treatments ($P > 0.05$, **Figure 5**). The expression level of BMP4 showed no significant difference between the three enriched treatments and un-enriched treatment ($P > 0.05$), and S.presso treatment was significantly higher than *Nannochloropsis* treatment ($P < 0.05$). The RXRα expression level of the three enriched treatments was significantly lower than the un-enriched treatment ($P < 0.05$), and there was no significant difference between the two commercial enrichment products treatments ($P > 0.05$); meanwhile, they were significantly lower than the *Nannochloropsis* treatment ($P < 0.05$).

DISCUSSION

Effects of Feeding Rotifers Enriched With Different Enhancement Products on Larval Performance

The initial feeding food must reach a level close to the nutritional requirements of the larval fish to be fully utilized (Conceição et al., 2010; Lall and Dumas, 2015). Generally, the utilization of food can be roughly estimated by observing and comparing the body composition of fish (Belal, 2005; Faulk et al., 2005). Rotifers enriched with *Nannochloropsis* were characterized by high EPA, while rotifers enriched with

TABLE 2 | Fatty acid composition (% of total fatty acids) of rotifers.

Fatty acid	Un-enriched	*Nannochloropsis*	S.presso	Algamac 3080
20:4n-6 (ARA)	1.36 ± 0.75^{a}	2.05 ± 0.13^{ab}	1.57 ± 0.23^{a}	2.15 ± 0.23^{b}
20:5n-3 (EPA)	5.18 ± 1.87^{a}	9.05 ± 0.32^{b}	4.22 ± 0.24^{a}	3.16 ± 0.37^{a}
22:6n-3 (DHA)	10.23 ± 6.98^{ab}	3.93 ± 0.11^{a}	18.42 ± 0.65^{b}	30.12 ± 4.15^{c}
Total Sats	21.37 ± 4.21^{a}	22.45 ± 2.13^{a}	25.36 ± 0.91^{a}	22.13 ± 2.39^{a}
Total Monos	44.12 ± 17.22^{c}	36.29 ± 2.39^{c}	28.89 ± 2.35^{b}	$19.14 \pm 5.32a$
Total n-9	7.49 ± 2.16^{c}	6.87 ± 0.61^{c}	5.79 ± 0.39^{b}	3.91 ± 0.83^{a}
Total n-7	37.52 ± 16.74^{b}	32.66 ± 2.43^{b}	25.81 ± 2.11^{b}	13.57 ± 2.44^{a}
Total Poly Unsats	27.82 ± 14.18^{a}	31.79 ± 2.12^{ab}	43.21 ± 3.19^{bc}	58.21 ± 5.23^{c}
Total n-6	9.13 ± 2.64^{bc}	9.93 ± 0.78^{bc}	12.76 ± 0.41^{c}	4.22 ± 0.19^{a}
Total n-4	0.71 ± 0.46^{a}	1.55 ± 0.06^{b}	0.57 ± 0.07^{a}	0.39 ± 0.07^{a}
Total n-3	19.89 ± 11.01^{a}	22.18 ± 1.53^{a}	32.46 ± 2.45^{a}	51.38 ± 7.53^{b}
DHA/EPA	1.98 ± 0.76^{b}	0.43 ± 0.04^{a}	4.36 ± 0.23^{c}	9.53 ± 1.12^{d}
EPA/ARA	3.81 ± 1.48^{c}	4.41 ± 0.25^{bc}	2.69 ± 0.05^{ab}	1.47 ± 0.29^{a}

Different letters represent significant differences at $P < 0.05$.

TABLE 3 | Fatty acid composition (% of total fatty acids) in 8 days post-hatching (8-DPH) fish fed enriched and un-enriched rotifers.

Fatty acid	Un-enriched	*Nannochloropsis*	S.presso	Algamac 3080
20:4n-6 (ARA)	1.56 ± 0.23^{a}	3.47 ± 0.21^{c}	2.89 ± 0.15^{b}	3.51 ± 0.21^{c}
20:5n-3 (EPA)	5.21 ± 0.28^{c}	8.09 ± 0.51^{d}	3.69 ± 0.38^{b}	2.43 ± 0.36^{a}
22:6n-3 (DHA)	19.58 ± 0.38^{b}	16.79 ± 0.83^{a}	29.21 ± 0.94^{c}	30.05 ± 1.87^{c}
Total Sats	23.38 ± 0.21^{a}	31.49 ± 0.57^{b}	30.22 ± 0.71^{b}	31.39 ± 2.31^{b}
Total Monos	20.48 ± 2.31^{b}	20.67 ± 0.97^{b}	17.63 ± 2.32^{a}	15.97 ± 0.23^{a}
Total n-9	4.63 ± 0.13^{a}	5.72 ± 0.49^{b}	4.33 ± 0.33^{a}	4.28 ± 0.23^{a}
Total n-7	16.55 ± 1.74^{c}	15.32 ± 0.79^{c}	13.01 ± 1.02^{b}	10.87 ± 0.49^{a}
Total Poly Unsats	41.45 ± 1.24^{a}	39.32 ± 2.63^{a}	43.61 ± 2.74^{a}	40.32 ± 0.97^{a}
Total n-6	4.57 ± 0.05^{a}	10.23 ± 0.21^{c}	9.48 ± 0.12^{c}	6.59 ± 0.075^{b}
Total n-4	0.44 ± 0.02^{a}	0.53 ± 0.15^{a}	0.51 ± 0.22^{a}	0.61 ± 0.22^{a}
Total n-3	37.23 ± 0.18^{d}	29.55 ± 0.71^{a}	30.43 ± 0.89^{b}	34.52 ± 1.02^{c}
DHA/EPA	3.76 ± 0.15^{b}	2.08 ± 0.22^{a}	7.92 ± 0.41^{c}	12.37 ± 1.35^{d}
EPA/ARA	3.33 ± 0.21^{d}	2.33 ± 0.19^{c}	1.28 ± 0.23^{b}	0.69 ± 0.17^{a}

Different letters represent significant differences at $P < 0.05$.

S.presso had a high DHA state. Judging from the fatty acid composition of golden pompano larvae, such levels of EPA and DHA could be absorbed and utilized. The Algamac 3080-enhanced rotifers had a higher DHA level than S.presso, but its utilization effect in golden pompano larvae did not seem to match the DHA content of rotifers. The optimal diet of young fish should be similar to the content of yolk sac (Barroso et al., 2013; Hauville et al., 2016), but unfortunately there have been no reports on the nutritional composition of yolk sac of golden pompanos. In the artificial breeding eggs of fat snook (*Centropomus parallelus*) (Barroso et al., 2013) observed a DHA:EPA:ARA ratio of 11.4:2.4:1.0, which is similar to the rotifers' body composition in the S.presso treatment.

In this study, increasing the DHA in the food had a significant positive effect on the growth of golden pompano larvae. Similar results were found in California halibut *Paralichthys californicus* larvae (Vizcaíno-Ochoa et al., 2010). Not only DHA, HUFA n-3 (including DHA, EPA, α-linolenic acid) promotes the normal growth and development of larvae (Rodrìguez et al., 1998; Hamre and Harboe, 2008). HUFA n-3 is also an important component of cell membrane and plays a very important physiological function in organisms (Roo et al., 2019). However, in Algamac 3080 treatment which had the highest DHA content of rotifers, the SGR of golden pompano larvae was limited. This phenomenon showed that the growth promotion effect of DHA is only established within a certain limit, and the similar phenomenon was found in the Gilthead Seabream *Sparus aurata* (Rodriguez et al., 1994).

Excessive DHA supplementation reduced the survival of golden pompano larvae, the survival rate of Algamac 3080 treatment was only about half of un-enriched treatment. However, it is generally believed that DHA is helpful to improve the survival of larvae fish. In seawater carnivorous fish such as Atlantic Cod *Gadus Morhua* and black sea bass

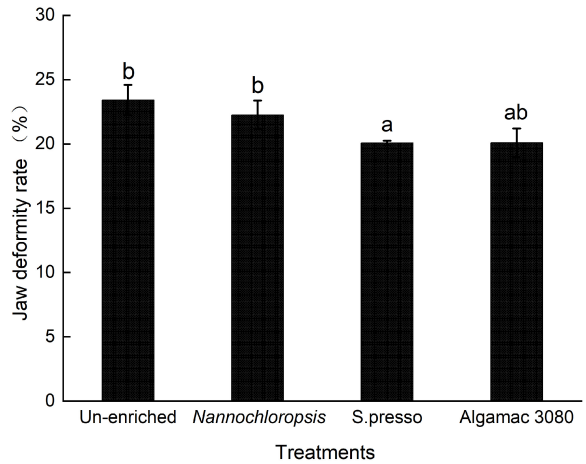

FIGURE 2 | Specific growth rate of golden pompano larvae in four treatments.

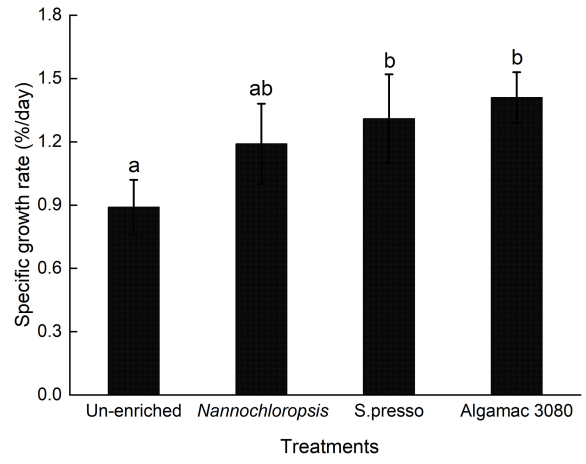

FIGURE 4 | Jaw deformity rate of golden pompano larvae in four enrichment treatments.

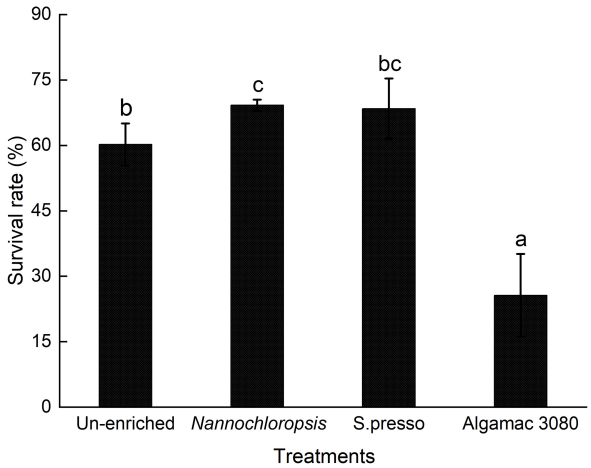

FIGURE 3 | Final survival rate of golden pompano larvae in four treatments.

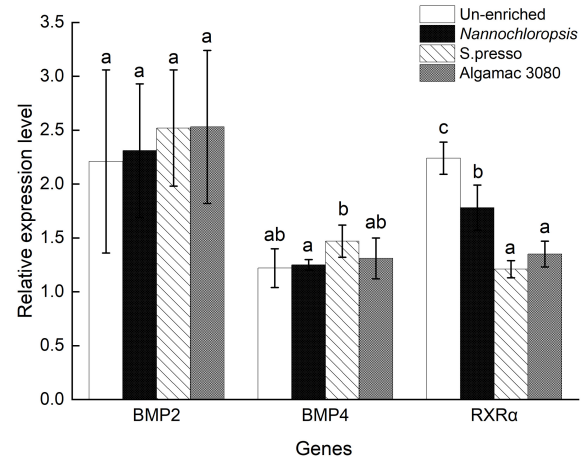

FIGURE 5 | Relative expression level of bone morphogenetic protein 2 (BMP2), bone morphogenetic protein 4 (BMP4), and retinoid X receptor α (RXRα) genes of golden pompano larvae in four enrichment treatments.

Centropristis striata, DHA improvement in an appropriate range is helpful to improve the survival rate of larvae (Park et al., 2006; Rezek et al., 2010). In other fish such as Senegal sole *Solea senegalensis* and California halibut *Paralichthys californicus*, DHA has no significant effect on larval survival (Villalta et al., 2005; Vizcaíno-Ochoa et al., 2010). Some scholars believe that excessive DHA will aggravate lipid peroxidation and cause pathological tissue damage in larval fish, which may lead to death (Betancor et al., 2011). This view may be one of the hypotheses that led to the results of this study, but further research is needed to prove it. In addition, studies have shown that the larvae demand for HUFA N-3 is not only reflected in the number of individual components, but also related to the DHA/EPA ratio (Rodríguez et al., 1997). Seeking a balanced ratio is also the key point to explore the appropriate first feeding to improve survival for larval fish.

Effects of Feeding Rotifers Enriched With Different Enhancement Products on Jaw Deformity

Increased levels of DHA, or DHA/EPA, seemed to contribute to a decrease in the jaw deformity rate of larvae, similar patterns have been observed in larval longfin yellowtail *Seriola rivoliana*, but the specific role of PUFA n-3 in the bone formation mechanism is still unclear (Cobcroft et al., 2012). However, the effect of reducing jaw deformity rate may be limited to S.presso-level DHA or DNA/EPA, because the survival rate of golden pompano larvae in Algamac 3080 treatment was too low, and the jaw deformity rate does not differ from that of the un-enriched treatment, which may be due to the death from deformity. It is a pity that we did not investigate the cause of death of larval fish in this study, which

can provide reference for more rigorous experimental design in similar studies in the future.

First feeding of different enhancement products had a significant effect on the expression of RXRα. From the results, it is not difficult to find that the jaw deformity rate and the expression level of RXRα had same trend. RXRα and α isoform of RAR can form active dimers that cause apoptosis, which is speculated to be a potential cause of bone deformities especially in the head of larval fish (Egea et al., 2001; Villeneuve et al., 2005; Zambonino Infante and Cahu, 2007; Ferraresso et al., 2010; Yang et al., 2015). Studies have shown that, in addition to the metamorphosis period, the expression of RXRα in fish will be upregulated under stress conditions (Cobcroft et al., 2012; Roo et al., 2019). The stress events in this study may be caused by the lack of nutrients in the un-enriched and *Nannochloropsis* treatment, which is similar to Yang's result (Yang et al., 2015). However, some studies have found that DHA can stimulate the expression of RXRα (Cahu et al., 2009), contrary to the results of this study, it may be that the lack of other nutrients such as vitamins in the un-enriched and *Nannochloropsis* treatment of rotifers led to stronger transcription stimulation (Zambonino Infante and Cahu, 2007; Mazurais et al., 2008; Darias et al., 2012). The BMP4 and retinoic acid pathways could produce a synergistic response and aggravate cell apoptosis (Villeneuve et al., 2005). In this study, no consistent changes in BMP4 with the jaw deformity rate were observed. It may not be the main cause of the jaw deformity.

In summary, the use of different enrichment products had caused significant differences in the fatty acid composition of rotifers; had a synergistic effect on the fatty acid composition of golden pompano larvae; and also caused different degrees of growth, survival, and jaw deformity. S.presso and Algamac 3080 treatment had more advantages in growth of golden pompano larvae, Algamac 3080 treatment exposed considerable disadvantages in survival. S.presso treatment had the lowest jaw deformity rate. Therefore, S.presso was a more suitable enrichment product for enriching rotifers for the first feeding of larval golden pompano. In addition, this study also found that BMP2 and BMP4 were not sensitive to changes in different enrichment product. On the contrary, RXRα had an opposite trend to the jaw deformity rate.

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.

ETHICS STATEMENT

The animal study was reviewed and approved by The Animal Care and Use Committee of South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences.

AUTHOR CONTRIBUTIONS

ZM and TZ conceptualized the study. SZ was responsible for the experimental operation. ZF, RY, and SZ were in charge of the field sampling. ZF and RY conducted the sample determination. ZF prepared and wrote the original draft. ZM and TZ reviewed, edited, and wrote the manuscript. All authors read and approved the final manuscript.

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Palm Oil-Based Enriched Diets for the Rotifer, *Brachionus plicatilis*, Improved the Growth of Asian Seabass (*Lates calcarifer*) Larvae

*Nik Siti Zaimah Safiin, Saleem Mustafa, Fui Fui Ching and Rossita Shapawi**

Borneo Marine Research Institute, Universiti Malaysia Sabah, Kota Kinabalu, Malaysia

Different types and inclusion levels of palm oil were incorporated in the enriched diets of L-type rotifer, *Brachionus plicatilis*, and fed to Asian seabass (*Lates calcarifer*) larvae. The dietary fish oil was replaced with either 50 or 75% of crude palm oil, CPO (CPO50, CPO75) and refined bleached deodorized palm olein, RPO (RPO50, RPO75). The enriched diet containing 100% fish oil (FO100) was used as the experimental control. Triplicate groups of the fish larvae of initial length 2.72 ± 0.14 mm were fed with enriched rotifer for 15 days. In general, palm oil-based enriched diets performed better than the control diet (FO100). Specifically, final mean body weight (31.3 ± 9.2 mg), final mean total length (11.5 ± 1.6 mm), SGR (29.0 ± 1.4%/day) and WG (7,769.4 ± 1,510.8%) of Asian seabass larvae fed RPO75 were significantly higher ($P < 0.05$) compared to those fed the other palm oil-based diet and FO100. The rotifer enriched with palm oil significantly affected the body proximate composition and fatty acid profiles of the fed larvae. The present study suggests that RPO and CPO can be considered as a good alternative dietary lipid for enrichment of rotifer to positively influence the nutritional requirements of the Asian seabass larvae and support their survival and growth.

Keywords: ***Brachionus plicatilis*, palm oil, fish oil replacement, live feed, Asian seabass**

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Edited by:
Sami Souissi,
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Reviewed by:
Yun-Zhang Sun,
Jimei University, China
Ghasem Rashidian,
Tarbiat Modares University, Iran

****Correspondence:***
Rossita Shapawi
rossita@ums.edu.my

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INTRODUCTION

Asian seabass, *Lates calcarifer*, is a tropical marine fish that is widely farmed throughout Asia. Despite being an established aquaculture species, many obstacles remain in using an acceptable live feed in the first feeding of the larval rearing phase. Currently, rotifer (primarily the species, *Brachionus plicatilis*) is widely used as the first feed for Asian seabass larvae mainly because it is relatively easy to culture, highly digestible, rich in protein, and has been used as a vector for delivering compounds of diverse nutritional value to larval fish (Skalliaa and Robin, 2004; Das et al., 2012; Dey et al., 2015). It is well-documented that live feed should be enriched prior to providing it to the larvae, with many studies focusing on the benefits of live feed enrichment using highly unsaturated fatty acids, HUFA (Rainuzzo et al., 1994; Sargent et al., 1999; Benítez-Santana et al., 2007, Tocher, 2010, Radhakrishnan et al., 2020) such as docosahexaenoic (DHA, 22:6n−3), eicosapentaenoic acid (EPA, 20:5n−3), and arachidonic acid (ARA, 20:4n−6) as the main energy sources and to ensure proper larval development (Rainuzzo et al., 1997; Sargent et al., 1999; Benítez-Santana et al., 2007, Tocher, 2010). Major dietary lipid source used in larviculture is fish-oil based emulsion through live feed enrichment (Rainuzzo et al., 1997). With the growth of aquaculture

industry the demand of fish oil for fish nutrition has increased (Alder et al., 2008; Nichols et al., 2010) resulting in shortages and threatening the sustainability of wild prey fish populations (Wassef et al., 2007). Fish oil substitution is, therefore, crucial to reducing the pressure on marine ecosystem while at the same time supporting the aquaculture industry (Tacon and Metian, 2008; Naylor et al., 2009; Alhazzaa, 2012). Developing aquafeeds by replacing fish oil and meal is an important area of research to pursue for reducing the pressure on forage fish. This will be among the major steps toward securing the sustainability of fed finfish aquaculture (Hua et al., 2019; Turchini et al., 2019).

Recent years have witnessed a heightened research interest in replacing fish oil with alternative lipid sources, mainly of plant origin. Palm oil is one of the vegetable oils that has received increasing attention in aquaculture feed research. It is abundantly available in several Asian countries. Apart from supplying dietary fat, palm oil is also a rich source of vitamin E, carotenoids, tocotrienols and natural antioxidants, and has a much cheaper price than the fish oil (Nasaretnam and Muhammad, 1993; Ng et al., 2007; Han et al., 2012; Singh et al., 2012). However, research on the use of palm oil as an alternative dietary fat source mostly involved juvenile and larger fish (Miller et al., 2008; Turchini et al., 2009; Torstensen and Tocher, 2010; Shapawi et al., 2011). In our earlier study based on juvenile stage of the Asian seabass (∼5 g), the specimens fed diet based on RPO gained significantly higher ($P < 0.05$) weight than the other fish groups that were offered diets based on fish oil, soybean oil and canola oil at the end of feeding trials (Shapawi et al., 2011). Therefore, research is needed to investigate the potential of the abundantly available palm oil in the larval nutrition of Asian seabass to curtail the mortality and boost health and stamina of the most vulnerable phase of life of the fish. There is a glaring paucity of data on the application of edible plant-based oil enriched rotifer (Poh-Leong et al., 2012; Dhaneesh and Kumar, 2016; Campoverde and Eztevez, 2017), whereas the use of artemia has received a great deal of attention in nutrition of fish larvae (Villalta et al., 2007; Arulvasu and Munuswamy, 2009; Hafezieh et al., 2010). The present study reports the results of experimental trials carried out for testing the effectiveness of the two different types of palm oils (CPO and RPO) as the lipid source in the enriched diets for the rotifer on Asian seabass larvae. To the best of our knowledge this is the first report on the replacement of fish oil with palm oil in the enriched diets for rotifers in the Asian seabass larvae. Other attempts to use palm oil as a substitute of fish oil have focused on juvenile and grow-out stages. For example, Wan et al. (2013) demonstrated that complete dietary fish oil replacement with either palm oil or lipid from poultry waste in barramundi (*Lates calcarifer*) juveniles (∼3.6 g initial body weight) did not affect growth or hepato-somatic index. In our previous study on humpback grouper (*Cromileptes altivelis*) juveniles of about 10 g initial body weight, CPO and RPO were shown to have successfully replaced fish oil at 50% replacement level in a fish meal-based formulated diets without any detrimental effects on growth and survival (Shapawi et al., 2008). Similarly, growth performance of hybrid grouper (*Epinephelus fuscoguttatus* x *E. lancelatus*) juveniles (∼11 g initial body weight) fed CPO and RPO at 50% replacement level was slightly better than in those fed fish oil-based diet (Yong et al., 2019). Bell et al. (2002) have demonstrated that feeding diets containing palm oil (25 to 100% of added oil) to Atlantic Salmon (*Salmo salar*) juveniles (∼55 g) had no significant effect on growth rate or feed conversion ratio, compared to fish fed 100% marine fish oil.

MATERIALS AND METHODS

Enriched Diet Preparation

A 2 × 3 factorial design using two types of palm oil (CPO and RPO) and three different levels (0, 50, and 75%) was applied to substitute fish oil in the enrichment diets for the rotifer. Fish oil-based enriched diet (FO100) was used as a control treatment. The oil emulsion was prepared by mixing 0.5 g of soy-lecithin and 10 g of egg albumin (Watanabe et al., 1989). Other ingredients included 2.1 g vitamin premix and 1.0 g mineral premix. The emulsion weighing 100 g was used for respective concentrations (50%, 75% and 100%) of either palm oil or fish oil in addition to 35.4% filtered seawater as shown in **Table 1**. The oil emulsion was homogenized using IKA homogenizer (T-25 basic Ultra Turrax) at 14,000 rpm/minute.

Rotifer Culture and Enrichment Protocol

The L-type rotifer was obtained from Fish Hatchery of Borneo Marine Research Institute (BMRI), Universiti Malaysia Sabah (UMS). The rotifers were cultured in 150 L conical fiber reinforced plastic (FRP) tank with the seawater volume of 100L at the density of 600 rotifers/ml. The salinity and temperature of water were maintained at 30 ppt and 28.59 ± 0.16°C, respectively. Individual rotifer tanks were well-aerated (800 ml/second). Rotifers were enriched using fortified diets in a concentration of 0.5 g/L for 24 h. After this treatment, the enriched rotifers were harvested by using a 60 μm—nylon mesh screen, washed in de-chlorinated seawater and then used for larval feeding at the density of 20 individual/ml. A sample was stored for subsequent chemical analysis.

Proximate and Fatty Acid Analysis

The enriched diets, rotifer and larval whole-body proximate composition were analyzed by methods suggested by the Association of Official Analytical Chemists (AOAC, 1997). The enrichment diets and enriched rotifer were subjected to lipid extraction by using chloroform: methanol (1:1, v/v) following Bligh and Dyer (1959) method. The lipid extract was then fractionated by a short column filled with silica gel 60 F254 (Merck, Darmstadt, Germany) with a mesh size of 0.063–0.2 mm in a hexane: ethyl acetate solvent system (9:1, v/v). Methylation of the extract was carried out inside a reaction vessel for 2 h using sodium methylate. The extract was purified using the silica gel column system before the fatty acid methyl esters were analyzed in a gas chromatograph (Shimadzu GC-2010, Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionization detector and an auto injector. The esters were separated using a capillary column (60 m × 0.25 mm ID; BPX70 column, SGE Australia Pty. Ltd., Ringwood, Vic., Australia) and chromatograph peaks were identified by comparing retention

TABLE 1 | Ingredients and proximate composition of different enrichment diets (g/100 g).

Ingredients	Enrichment diets				
	FO100	**CPO50**	**CPO75**	**RPO50**	**RPO75**
Fish oil	50.0	25.0	15.0	25.0	15.0
[a] RBDPO	/	/	/	25.0	35.0
[b] CPO	/	25.0	35.0	/	/
[c] Vitamin premix	2.1	2.1	2.1	2.1	2.1
[d] Mineral premix	1.0	1.0	1.0	1.0	1.0
[e] Soy-lecithin	0.5	0.5	0.5	0.5	0.5
[f] Egg albumin	10.0	10.0	10.0	10.0	10.0
Filtered sea water	36.4	36.4	36.4	36.4	36.4
Total	100.0	100.0	100.0	100.0	100.0
Component	**Proximate composition (%DM)**				
	FO100	**CPO50**	**CPO75**	**RPO50**	**RPO75**
Moisture	39.6 ± 0.1	40.7 ± 0.	41.4 ± 0.2	41.4 ± 0.2	39.0 ± 0.1
Lipid	50.3 ± 2.8	51.5 ± 1.3	50.7 ± 1.0	51.3 ± 0.9	51.5 ± 2.2
Protein	1.6 ± 0.2	1.9 ± 0.3	1.7 ± 0.1	1.5 ± 0.4	1.8 ± 0.1
Ash	4.2 ± 0.4	4.5 ± 0.2	4.6 ± 0.5	4.1 ± 0.2	4.2 ± 0.3

[a,b] Sawit Kinabalu Sdn. Bhd. (Lumadan Palm Oil Mill, Beaufort, Sabah).

[c] Vitamin premix (Dexchem Industries Sdn. Bhd.), contains (kg-1 dry weight): ascorbic acid 45 g; inositol 5 g; choline chloride 75 g; niacin 4.5 g; riboflavin 1 g; pyridoxine HCl 1 g; thiamine HCl 0.92 g; dicalciumpantothenate 3 g; retinyl acetate 0.6 g; vitamin D3 0.08 g; menadione 1.67 g; dialpha tocopherol acetate 8 g; d- Biotin 0.02 g; folic acid 0.09 g; vitamin B12 0.001 g; cellulose.

[d] Mineral premix (Dexchem Industries Sdn. Bhd.), contains (kg-1 dry weight):calcium phosphate monobasic 270.98 g; calcium lactate 327 g; ferrous sulphate 25 g; magnesium sulphate 132 g; potassium chloride 50 g; potassium iodide 0.15 g; copper sulphate 0.785 g; manganese oxide 0.8 g; cobalt carbonate 1g; zinc oxide 3 g; sodium selenite 0.011 g; calcium carbonate 129.27 g.

[e] Xi'an YuenSun Biological Technology Co., Ltd. (China Manufacturer).

[f] STF Agriculture Sdn. Bhd.

TABLE 2 | Growth performance of Asian seabass larvae fed different types of enriched rotifers.

Enriched diet (Palm oil type/level)	Parameter			
	Final BW (mg)	**Final TL (mm)**	**SGR (% mg day^{-1})**	**WG (%)**
One-way ANOVA				
FO100	9.4 ± 8.8^{c}	7.3 ± 0.8^{d}	21.0 ± 0.5^{c}	2,233.3 ± 180.9^{c}
CPO50	23.0 ± 10.0^{b}	10.3 ± 1.7^{b}	26.9 ± 1.2^{ab}	5,656.3 ± 1,033.8^{b}
CPO75	17.3 ± 8.9^{bc}	9.5 ± 1.5^{c}	24.8 ± 1.8^{b}	4,110.4 ± 1,073.1^{bc}
RPO50	11.1 ± 3.8^{c}	8.7 ± 1.3^{c}	21.7 ± 2.0^{c}	2,587.5 ± 795.6^{c}
RPO75	31.3 ± 9.2^{a}	11.5 ± 1.6^{a}	29.0 ± 1.4^{a}	7,769.4 ± 1,510.8^{a}
Two-way ANOVA (*p*-value)				
Palm oil type	0.663	0.624	0.621	0.662
Palm oil level	0.000	0.000	0.000	0.000
Oil type ×Oil level	0.000	0.000	0.000	0.000

Initial body weight = 0.4 ± 0.1 mg; Initial total length = 2.7 ± 0.1 mm.

Mean values with same superscripts in the same column are not significantly different between the treatments (Duncan's multiple range test, P > 0.05).

times with known standards (Supelco™ 37 Component FAME mix, Supelco Inc., Bellefonte, USA).

Experimental Design and Feeding Trial

Asian seabass larvae were obtained from the Fish Hatchery of BMRI (UMS). A total of 54,000 newly hatched larvae with an initial weight and length of 0.44 ± 0.11 mg and 2.72 ± 0.14 mm, respectively were observed for general health condition and held in the tanks for experimental trials. Triplicate groups of larvae were randomly distributed into 18 conical FRP tanks (150 liters) with initial stocking density of 30 individual per liter. The experimental tanks were randomly arranged in triplicate and

TABLE 3 | Survival of Asian seabass larvae fed different types of enriched rotifers.

Enriched diet (Palm oil type/level)	Survival (dAH)		
	5	10	15
One-way ANOVA			
FO100	88.7 ± 3.1	76.7 ± 8.3	54.5 ± 2.0^{b}
CPO50	91.3 ± 4.2	81.0 ± 1.0	77.4 ± 9.4^{a}
CPO75	89.3 ± 3.1	74.7 ± 8.1	56.0 ± 4.5^{b}
RPO50	91.3 ± 4.2	76.0 ± 2.0	61.2 ± 8.7^{b}
RPO75	90.3 ± 4.9	82.7 ± 4.5	67.8 ± 12.3^{ab}
Two-way ANOVA (*p*-value)			
Palm oil type	0.856	0.737	0.694
Palm oil level	0.498	0.827	0.018
Oil type × oil level	0.966	0.226	0.024

Mean values with same superscripts in the same column are not significantly different between the treatments (Duncan's multiple range test, P > 0.05).

in closed system, and supplied with continuous mild aeration (250 ml/second). Starting from 36 h after hatching (hAH), the respective batches of enriched rotifer were fed to the fish larvae at the density of 20 individual/ml. *Nannochloropsis oculata* was also provided in all the tanks at concentration of 0.5×10^6 cell/ml. There was no cleaning for up to 5 days to reduce the disturbance to the larvae. After 5 days of rearing, water was routinely changed (5%) to avoid accumulation of metabolic wastes. The density of enriched rotifer was monitored under a profile projector (Mitutoyo, PJ-A3000, Japan) twice daily at 0830 am and 1400 pm. The physiochemical parameters of water temperature, dissolved oxygen and pH throughout the experiment were 29.31 ± 0.80°C, 5.54 ± 0.11mg/L and 8.12 ± 0.04, respectively. The experimental duration was 15 days.

Performance Evaluation

Triplicate pooled sample of initial stock and 5 days after hatching (dAH)-larvae ($n = 100$) and 10 dAH and 15 dAH-larvae ($n = 50$) were measured using an analytical balance. At every 5 days interval up to 15 dAH, 10 larvae ($n = 10$) from each treatment were sampled, anesthetised with Transmore (alpha-methylquinoline) (Nika Trading, Puchong, Malaysia) and measured under a profile projector (Mitutoyo, PJ-A3000, Japan) for morphometric observations on total length (TL), standard length (SL), body height (BH), muscle height (MH), head length (HL), head depth (HD), gut height (GH), and eye diameter (ED). The larvae were examined under a light microscope (Nikon, Eclipse E600, Japan) for rotifer ingestion and gut observation. The condition factor and ratio of total length to body weight (CF) (Chatain, 1994) were determined at the end of the experiment.

Other parameters were calculated using the following formulas:

Specific growth rate (SGR, %/d) = [ln final weight, mg ln initial weight, mg)days] × 100

Survival (%) = [(Initial fish number final fish number) Initial fish number] × 100

Condition factor (CF) = [fish weight(total length)] × 100

Total length/ body weight (TL/W) ratio = [(total length, mm)(body weight, mg)]

Weight gain, WG (%) = [(final body weight, g − initial body weight, g)initial body weight, g] × 100.

Statistical Analysis

IBM Statistical Package of Social Sciences Version 23.0 was used for all the statistical analyses. All the data in percentage were arcsine transformed before the analysis. The quantitative data was subjected to one-way analysis of variance (ANOVA) to determine the mean differences among the treatments at 0.05 significance level. The homogeneity of variance was tested using Levene's test and multiple comparisons among treatments were presented using a Duncan's multiple range test. Effects of oil types and levels, and their interactions were determined by two-way ANOVA.

RESULTS

Growth Performance

Data on larval growth performance is presented in **Table 2**. Final mean body weight (31.3 ± 9.2 mg), final mean total length (11.5 ± 1.6 mm), SGR (29.0 ± 1.4%/day) and WG (7,769.4 ± 1,510.8%) of Asian seabass larvae fed RPO75 were significantly higher ($P < 0.05$) compared to those fed the other palm oil-based diet and FO100. The palm oil inclusion level significantly affected these parameters at the end of experiment. **Table 3** and **Figure 1** show the survival rate and variation (%) of the larvae in the 15 day experimental period. The different batches of enriched rotifer did not show any significant effect on 5 and 10 dAH. However, at the end of the experiment (15 dAH), the survival was significantly affected by palm oil level. CPO50 yielded higher survival (77.4 ± 9.4%) compared to FO100 (54.5 ± 2.0%) and RPO-based treatments (61.2 ± 8.7–67.8 ± 12.3%).

Morphometric Variables and Body Condition

The morphometric variables and body indices of Asian seabass at the end of the experiment are presented in **Table 4**. The morphometric value of each parameter was affected by palm oil level. RPO75 shows significantly higher ($P < 0.05$) SL, MH, ED (10.0 ± 1.0, 2.6 ± 0.5 and 1.0 ± 0.1, respectively). The lowest ($P < 0.05$) value of HL, HD and ED (2.5 ± 0.2, 1.9 ± 0.2 and 0.7 ± 0.1, respectively) was observed in fish fed FO100 compared to the other treatments. SL, BH, MH, GH (6.4 ± 0.8, 2.1 ± 0.2, 1.6 ± 0.1, 0.4 ± 0.1, respectively of FO100 treatment were significantly lower than the other ones, except RPO50. Meanwhile, the condition factor was not significantly affected by palm oil type and level. However, T:W was significantly influenced by oil type only. TL:W was significantly ($P < 0.05$) higher in FO100 and RPO50 compared to the other treatments.

Proximate Composition of Enriched Rotifer and Larval Fish

Table 5 shows the proximate composition of enriched rotifer. The proximate composition of enriched rotifer was not significantly affected by palm oil type and level except for protein

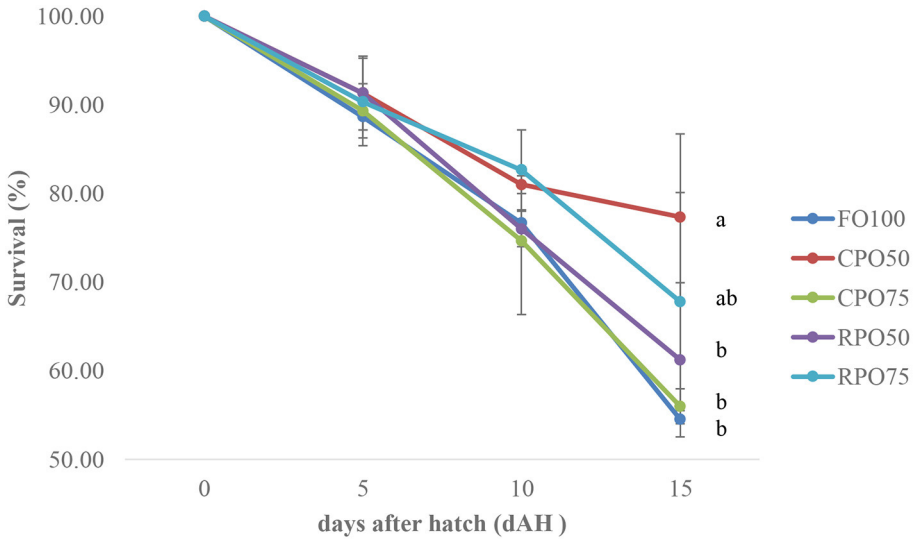

FIGURE 1 | Survival variation (%) of Asian seabass larvae fed enriched rotifer. Mean with same superscripts in the same row are not significantly different ($P > 0.05$) from each treatment (Mean ± SD).

TABLE 4 | Morphometric variables and body indices of Asian seabass larvae fed different types of enriched rotifers.

Enriched diet (Palm oil type/level)	Parameters								
	SL	BH	MH	HL	HD	GH	ED	K	TL:W
One-way ANOVA									
FO100	6.4 ± 0.8^{c}	2.1 ± 0.2^{d}	1.6 ± 0.1^{c}	2.5 ± 0.2^{d}	1.9 ± 0.2^{d}	0.4 ± 0.1^{c}	0.7 ± 0.1^{c}	3.4 ± 0.4	0.8 ± 0.3^{bc}
CPO50	8.9 ± 1.3^{b}	3.2 ± 0.4^{ab}	2.1 ± 0.5^{b}	3.5 ± 0.6^{ab}	2.5 ± 0.3^{ab}	0.6 ± 0.2^{b}	0.9 ± 0.1^{b}	3.2 ± 0.4	0.5 ± 0.1^{a}
CPO75	8.1 ± 1.3^{b}	3.0 ± 0.4^{b}	2.0 ± 0.3^{bc}	3.2 ± 0.5^{bc}	2.3 ± 0.3^{bc}	0.7 ± 0.1^{ab}	0.8 ± 0.1^{b}	3.0 ± 0.4	0.6 ± 0.2^{ab}
RPO50	7.8 ± 1.0^{c}	2.6 ± 0.5^{d}	2.0 ± 0.4^{bc}	3.0 ± 0.4^{c}	2.2 ± 0.3^{c}	0.5 ± 0.1^{c}	0.8 ± 0.1^{b}	2.9 ± 0.2	0.9 ± 0.2^{c}
RPO75	10.0 ± 1.0^{a}	3.5 ± 0.5^{a}	2.6 ± 0.5^{a}	3.9 ± 0.5^{a}	2.8 ± 0.2^{a}	0.8 ± 0.2^{a}	1.0 ± 0.1^{a}	3.1 ± 0.3	0.4 ± 0.1^{a}
Two-way ANOVA (*p*-value)									
Palm oil type	0.949	0.658	0.125	0.702	0.427	0.511	0.037	0.597	0.015
Palm oil level	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.048	0.138
Oil type × oil level	0.000	0.001	0.007	0.001	0.001	0.013	0.005	0.534	0.142

Means with same superscripts in the same column are not significant differences between treatment (Duncan's multiple range test, P > 0.05).

content that was markedly influenced by palm oil type ($P = 0.005$). RPO50-enriched rotifer contained significantly ($P < 0.05$) higher protein (55.2 ± 0.3%) compared to the other treatments. Meanwhile, the whole-body proximate composition of Asian seabass was not significantly affected by the enriched diet. Fish fed RPO-enriched diet resulted in slightly higher protein and lipid contents compared to the other treatments as shown in **Table 6**.

Fatty Acid Composition of Enriched diets, Rotifer, and Larval Fish

In general, the replacement of fish oil with palm oil (CPO and RPO) increased the C:16 (palmitic acid), C18:1n9 (oleic acid) and reduced total PUFA and n-3HUFA, especially DHA content of the enriched diets. The total saturates, monoenes and total PUFA were affected by palm oil level and n-3 and n-3/n-6 were affected by the palm oil type and level (**Table 7**). The total saturated fatty acids in palm oil-based enriched rotifer were significantly ($P < 0.05$) higher than in the control diet, ranging from 39.0 ± 0.1% to 40.9 ± 0.1% (**Table 8**). The replacement of fish oil with dietary palm oil had significantly affected the level of saturates and monoenes of the enriched rotifers. The total PUFA, n-3 PUFA, n-3/n-6, EPA and DHA levels were significantly higher in FO100 than in other groups. The total fatty acids of enriched rotifer were significantly affected by palm oil type and level. **Table 9** shows the whole body fatty acid composition of Asian seabass at the end of experiment. Total fatty acids of Asian seabass body composition yielded the same pattern as in the enriched diet and rotifer. The palm oil-based diets contained significantly ($P < 0.05$) higher level of C:16 and C18:1n9 compared to FO100. Overall, the total fatty acids of palm oil enriched diets, rotifers and Asian seabass larvae were significantly affected by the palm oil type and level except total PUFA and n-3 which showed no significant effect by the palm oil type.

TABLE 5 | Proximate composition (% dry weight basis) of enriched rotifer.

Enriched diets (Palm oil type/level)	Parameters			
	Moisture	Protein	Lipid	Ash
FO100	89.3 ± 0.4	51.3 ± 1.6^{ab}	24.4 ± 3.7	6.4 ± 0.6
CPO50	89.9 ± 0.3	49.8 ± 5.5^{ab}	22.6 ± 4.0	6.3 ± 1.0
CPO75	89.8 ± 0.1	45.9 ± 0.3^{b}	20.4 ± 1.9	5.8 ± 0.4
RPO50	89.7 ± 0.3	55.2 ± 0.3^{a}	21.7 ± 2.6	6.2 ± 0.7
RPO75	89.9 ± 0.1	53.6 ± 2.7^{a}	23.3 ± 3.9	6.3 ± 0.7
Two-way ANOVA				
Palm oil type	0.879	0.005	0.686	0.669
Palm oil level	0.878	0.252	0.358	0.680
Palm oil type × oil level	0.764	0.072	0.624	0.751

Means with same superscripts in the same column are not significant differences between treatment (Duncan's multiple range test, P > 0.05).

TABLE 6 | Proximate composition (% wet weight basis) of Asian seabass larvae.

Enriched diets (Palm oil type/level)	Parameters			
	Moisture	Protein	Lipid	Ash
One-way ANOVA				
FO100	78.7 ± 0.3	13.6 ± 1.0	3.1 ± 0.2	1.0 ± 0.1
CPO50	77.9 ± 1.5	13.6 ± 0.7	3.3 ± 0.3	1.4 ± 0.3
CPO75	77.5 ± 3.3	13.8 ± 1.1	3.1 ± 0.6	1.2 ± 0.2
RPO50	74.2 ± 4.7	14.9 ± 0.8	3.6 ± 0.6	1.1 ± 0.1
RPO75	74.4 ± 3.0	14.7 ± 0.7	3.6 ± 0.2	1.3 ± 0.2
Two-way ANOVA				
Palm oil type	0.428	0.045	0.153	0.102
Palm oil level	0.526	0.007	0.212	0.190
Palm oil type × oil level	0.833	0.228	0.503	0.472

Means with same superscripts in the same column are not significant differences between treatment (Duncan's multiple range test, P > 0.05).

DISCUSSION

Lipid is the main energy source for marine fish, especially in the early stage of its life. Short-term enrichment of rotifer using fish oil emulsion results in lipid-encapsulated rotifers rich in EPA and DHA. Generally, this is a nutritionally stable composition and makes the diet palatable which is important especially for fish larvae (Dhert et al., 2001). In the present study, Asian seabass larvae achieved faster growth by ingesting the palm-oil enriched rotifer, indicating the ability of the larvae to utilize the available lipid for energy consumption and growth. Fish fed palm oil-based enriched rotifer showed highest growth performance compared to control diet. It is well-documented that palm oil is characterized by high level of ß-carotene and is a rich source of antioxidants. These components are known to be excellent natural free radical acceptors and may exert beneficial effects on growth when fish are fed high levels of palm oil (Lim et al., 2001; Ng et al., 2003). A study by Jalali et al. (2008) emphasized that supplementation of antioxidants is necessary when high levels of n-3 PUFA are incorporated in larval diets to avoid adverse effects and to improve larval performance on beluga (*Huso huso)* larvae. Investigations conducted by Tocher et al. (2003) have shown that supplementation of α-tocopherol which is present in palm oil improved the growth and reduced the lipid peroxidation in sea bream and turbot (*Psetta maxima*) tissues. Betancor et al. (2013) observed that the elevation of dietary vitamin E levels in the palm oil markedly reduced the incidence of these symptoms and increased the tissue content of several PUFA, and improved the growth in European sea bass. Espe et al. (2007) stated that feeding lower level of fish oil than plant oil improved n-3 HUFA retention efficiency. However, inclusion of high level of fish oil in the enriched diet resulted in decline in growth of Asian seabass larvae, mainly due to the poor retention efficiency of fatty acids (Bell et al., 2002; Espe et al., 2007). Lipid oxidation impairs the nutritional value of animal products due to high proportion of unsaturated fatty acids. This is consistent with the findings reported by Jalali et al. (2010) and Babalola et al. (2013). Studies on the use of palm oil in enriching the live feed, particularly rotifer, are limited and the information is scarce on marine fish larvae. Poh-Leong et al. (2012) used palm oil mill effluent (POME)- enriched bacteria as food for rotifer and fed it to marble goby, *Oxyeoleotris marmorata*. The study reported that the POME significantly enhanced the rotifer production, and improved growth and survival. Concordant findings were also obtained on other plant oil-based enriched live foods for different species (Smith et al., 2004; Menoyo et al., 2007; Huanng et al., 2008; Hafezieh et al., 2010; Tehrani et al., 2014; Kazemi et al., 2016).

The morphological features (phenotypic characters), CF and TL/W ratio that are normally used as indicators of nutritional status of fish larvae showed that the Asian seabass larvae were developing normally based on the consistent pattern in all the treatment groups. During the larval phase, survival appeared to be strongly influenced by the energy status of the larvae. Thus, the high levels of fatty acids in the live prey are essential to satisfy the elevated energy demand and to promote growth (Dhaneesh and Kumar, 2016). Current study shows that the survival is affected by palm oil level and fatty acid composition particularly DHA and EPA. Rich amounts of these substances in the control treatment significantly reduced the survival. A study conducted by Planas and Cunha (1999) also suggested that higher levels of DHA or n-3 HUFA reduced the survival of marine fish larvae. Izquierdo et al. (1992) demonstrated that in the larvae of Japanese, *P. olivaceus*, DHA content in Artemia metanauplii did not affect the survival rate but influenced the size when the larvae were fed with artemia containing high level of DHA. Thus, the requirement of dietary DHA level of marine finfish larvae is species dependant as highlighted by Sargent et al. (1999).

The fatty acid composition of enriched rotifer and Asian seabass larvae reflected the fatty acid profile in the enriched diets. Earlier studies have demonstrated that fatty acid composition of enriched rotifer is a reflection of its enrichment and, therefore, it depends on the culture condition and presence of nutrients (Kotani et al., 2013; Hamre, 2016). Specifically, the fish oil replacement at 50% with CPO and 75% with RPO performed

TABLE 7 | Fatty acid composition (% of total fatty acids) of enriched diets.

[1]Fatty acid (One-way ANOVA)	Enriched diets				
	FO100	**CPO50**	**CPO75**	**RPO50**	**RPO75**
C14:0	3.6 ± 0.0^{a}	3.3 ± 0.0^{b}	2.5 ± 0.1^{c}	3.3 ± 0.1^{b}	1.7 ± 0.0^{d}
C14:1	0.8 ± 0.0^{a}	0.5 ± 0.0^{b}	0.5 ± 0.0^{b}	0.7 ± 0.0^{a}	0.6 ± 0.1^{b}
C15:0	0.5 ± 0.0^{a}	0.4 ± 0.0^{ab}	0.3 ± 0.0^{b}	0.3 ± 0.2^{b}	0.3 ± 0.1^{b}
C16:0	12.7 ± 0.1^{d}	24.8 ± 0.5^{a}	15.4 ± 0.1^{c}	24.3 ± 0.5^{a}	16.9 ± 0.1^{b}
C16:1	9.7 ± 0.2^{a}	5.3 ± 0.1^{b}	4.7 ± 0.1^{c}	4.4 ± 0.1^{d}	2.9 ± 0.1^{e}
C17:0	0.4 ± 0.0^{b}	0.4 ± 0.0^{b}	0.3 ± 0.0^{c}	0.4 ± 0.0^{a}	0.4 ± 0.0^{b}
C18:0	2.6 ± 0.0^{d}	5.1 ± 0.1^{a}	2.5 ± 0.1^{d}	4.2 ± 0.1^{b}	2.6 ± 0.1^{d}
C18:1n9	15.6 ± 0.0^{e}	34.5 ± 0.2^{c}	43.6 ± 0.2^{a}	41.3 ± 0.9^{b}	41.3 ± 0.4^{b}
C18:2n9	5.6 ± 0.02^{a}	0.2 ± 0.0^{d}	0.2 ± 0.0^{d}	0.3 ± 0.0^{cd}	0.5 ± 0.0^{b}
C18:2n6	3.5 ± 0.1^{e}	3.2 ± 0.0^{f}	10.6 ± 0.1^{a}	3.8 ± 0.1^{d}	9.7 ± 0.1^{b}
C18:3n3	7.4 ± 0.1^{d}	nd	0.4 ± 0.5^{c}	0.2 ± 0.0^{c}	0.6 ± 0.1^{bc}
C20:1	2.6 ± 0.4^{a}	1.9 ± 0.1^{bc}	1.9 ± 0.0^{bc}	2.1 ± 0.0^{b}	1.5 ± 0.0^{c}
C20:3n6	0.3 ± 0.0^{b}	0.8 ± 0.1^{a}	nd	0.9 ± 0.1^{a}	nd
C20:4n6	2.6 ± 0.2^{a}	0.3 ± 0.1^{c}	0.5 ± 0.0^{d}	0.4 ± 0.1^{d}	0.9 ± 0.0^{c}
C22:1n9	1.9 ± 0.1^{a}	0.7 ± 0.0^{d}	1.4 ± 0.0^{b}	0.9 ± 0.0^{c}	0.7 ± 0.0^{d}
C20:5n3	15.7 ± 0.1^{a}	7.9 ± 0.4^{b}	7.0 ± 0.1^{c}	5.1 ± 0.4^{d}	7.9 ± 0.3^{b}
C22:6n3	20.4 ± 0.2^{b}	10.3 ± 0.6^{c}	7.4 ± 0.1^{d}	6.4 ± 0.3^{d}	10.6 ± 0.0^{c}

[2] Enriched diets (Palm oil type/level)	Parameters					
	Total saturates	**Total monoenes**	**Total PUFA**	**n-3**	**n-3/n-6**	**DHA/EPA**
One-way ANOVA						
FO100	19.9 ± 0.2^{d}	30.8 ± 0.6^{f}	49.3 ± 0.4^{b}	37.1 ± 0.3^{b}	5.6 ± 0.1^{a}	1.3 ± 0.0^{ab}
CPO50	34.2 ± 0.5^{a}	43.0 ± 0.4^{d}	22.8 ± 0.9^{e}	18.4 ± 1.0^{c}	4.4 ± 0.3^{b}	1.3 ± 0.0^{ab}
CPO75	21.4 ± 0.3^{c}	52.2 ± 0.3^{a}	26.4 ± 0.5^{d}	15.0 ± 0.4^{d}	1.3 ± 0.0^{e}	1.1 ± 0.0^{c}
RPO50	33.0 ± 0.7^{b}	49.4 ± 1.0^{b}	17.6 ± 0.5^{f}	12.0 ± 0.4^{e}	2.3 ± 0.1^{c}	1.3 ± 0.1^{b}
RPO75	22.4 ± 0.1^{c}	47.2 ± 0.3^{c}	30.4 ± 0.3^{c}	19.3 ± 0.4^{c}	1.8 ± 0.1^{d}	1.3 ± 0.0^{ab}
Two-way ANOVA (*p*-value)						
Palm oil type	0.757	0.112	0.125	0.012	0.000	0.007
Palm oil level	0.000	0.000	0.000	0.000	0.000	0.025
Oil type × oil level	0.001	0.000	0.000	0.000	0.000	0.000

[1]Minor fatty acids (<0.2% from total fatty acids) were not included in the table.
Means with same superscripts in the same [1]row and [2]column are not significant differences between treatment (Duncan's multiple range test, $P > 0.05$).

better than the other palm oil treatments and fish oil-based enriched diet in terms of the level of EPA and DHA that were significantly reduced in the rotifer fed palm oil-based enriched diets (EPA ranged from 5.12 to 7.93% and DHA ranged from 6.43 to 10.61%). It is evident from these findings that the n3:n9 ratio and variable fatty acid composition, particularly palmitic (C16:0) and oleic acid (C18:1n9), in the enriched rotifer promoted better growth of Asian seabass. Analysis of the growth performance of the palm oil-enriched rotifer in the present study suggested that the essential fatty acids in the enriched rotifer were sufficient to support the normal growth of the larvae. This investigation also revealed that the RPO75, CPO50, and RPO50 containing n3:n9 were 1.1, 1.4, and 2.1, respectively were effective and were within the recommended ratio of 0.58–2.24:1 as suggested by Watanabe et al. (1989) and Williams and Barlow (1999). However, fish fed RPO75 enriched rotifer showed a significant correlation between the significantly high growth performance and DHA:EPA ratio (1.4) compared to other palm-oil based treatments. Current study further showed that high dietary DHA level did not result in any significant growth benefit to the larvae. However, the nutritional requirements for DHA and EPA have been found to be both species-specific as well as specific to the developmental stage of the larvae (Copeman et al., 2002; Villalta et al., 2005).

The fatty acid composition of the diets was clearly reflected in the enriched rotifer and body fatty acid composition of Asian seabass larvae. However, n-3 HUFA level in fish body fed RPO75 and CPO50 showed inconsistent profile where n-3 HUFA levels were higher (9.9 and 11.4%, respectively) which contrasted with the supplied enriched rotifer (6.5 and 8.1%, respectively). Findings of Tocher et al. (2003) and Mohd-Yusof

TABLE 8 | Fatty acid composition (percentage of total fatty acids) of enriched rotifers.

[1]Fatty acid (One-way ANOVA)	Enriched diets				
	FO100	**CPO50**	**CPO75**	**RPO50**	**RPO75**
C14:0	3.4 ± 0.1^{b}	2.8 ± 0.0^{d}	4.1 ± 0.1^{a}	3.1 ± 0.1^{c}	1.8 ± 0.0^{e}
C15:0	0.5 ± 0.0^{b}	0.2 ± 0.0^{d}	0.3 ± 0.0^{c}	0.2 ± 0.0^{d}	0.2 ± 0.0^{d}
C16:0	23.4 ± 0.3^{d}	32.0 ± 0.0^{b}	29.8 ± 0.0^{c}	32.1 ± 0.2^{b}	34.1 ± 0.0^{a}
C16:1	7.7 ± 0.8^{a}	2.9 ± 0.0^{d}	4.7 ± 0.1^{b}	3.6 ± 0.2^{c}	1.9 ± 0.0^{e}
C17:0	0.4 ± 0.0^{b}	0.2 ± 0.0^{c}	0.2 ± 0.0^{c}	0.2 ± 0.0^{d}	0.2 ± 0.0^{cd}
C18:0	4.6 ± 0.1^{b}	4.7 ± 0.0^{b}	4.7 ± 0.0^{b}	4.8 ± 0.0^{b}	4.79 ± 0.0^{b}
C18:1n9	24.3 ± 0.2^{f}	42.1 ± 0.1^{b}	34.9 ± 0.3^{d}	39.9 ± 0.3^{c}	46.7 ± 0.1^{a}
C18:2n6	2.9 ± 0.1^{d}	5.5 ± 0.1^{a}	4.4 ± 0.1^{b}	4.3 ± 0.4^{b}	5.8 ± 0.1^{a}
C20:0	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}
C20:4n6	0.9 ± 0.0^{b}	0.3 ± 0.0^{d}	0.5 ± 0.0^{c}	0.3 ± 0.0^{d}	0.3 ± 0.0^{d}
C22:1n9	4.0 ± 0.3^{a}	1.2 ± 0.0^{bc}	2.1 ± 0.1^{b}	1.4 ± 0.0^{bc}	0.6 ± 0.0^{c}
C20:5n3	7.7 ± 0.1^{ab}	4.9 ± 0.0^{d}	8.4 ± 0.1^{a}	6.0 ± 0.1^{c}	2.7 ± 0.0^{e}
C22:6n3	10.2 ± 0.3^{b}	3.1 ± 0.0^{e}	5.6 ± 0.1^{c}	3.9 ± 0.1^{d}	3.8 ± 0.1^{d}

[2] Enriched diets (Palm oil type/level)	Parameters					
	Total saturates	**Total monoenes**	**Total PUFA**	**n-3**	**n-3/n-6**	**DHA/EPA**
(One-way ANOVA)						
FO100	31.6 ± 0.2^{d}	36.0 ± 1.2^{d}	21.7 ± 0.5^{a}	17.9 ± 0.4^{a}	4.7 ± 0.0^{a}	1.3 ± 0.0^{a}
CPO50	39.8 ± 0.1^{b}	46.2 ± 0.2^{b}	13.8 ± 0.2^{cd}	8.1 ± 0.1^{e}	1.4 ± 0.0^{d}	0.6 ± 0.0^{b}
CPO75	39.0 ± 0.1^{c}	41.7 ± 0.5^{c}	18.8 ± 0.3^{b}	14.0 ± 0.2^{b}	2.9 ± 0.0^{b}	0.7 ± 0.0^{b}
RPO50	40.2 ± 0.3^{b}	44.9 ± 0.5^{b}	14.5 ± 0.6^{c}	9.9 ± 0.2^{c}	2.1 ± 0.2^{c}	0.7 ± 0.0^{b}
RPO75	40.9 ± 0.1^{a}	49.2 ± 0.2^{a}	12.6 ± 0.2^{d}	6.5 ± 0.1^{d}	1.1 ± 1.4^{e}	1.4 ± 0.6^{a}
Two-way ANOVA (*p*-value)						
Palm oil type	0.000	0.000	0.000	0.000	0.000	0.000
Palm oil level	0.000	0.000	0.000	0.000	0.000	0.000
Oil type × oil level	0.000	0.000	0.000	0.000	0.000	0.000

[1] Minor fatty acids (<0.2% from total fatty acids) were not included in the table.
Means with same superscripts in the same [1]row and [2]column are not significant differences between treatment (Duncan's multiple range test, $P > 0.05$).

et al. (2010) have revealed that Asian seabass was able to maintain levels of DHA above the dietary level and attributed it to the selective retention of these nutrients in the palm oil linked to the conversion of EPA to DHA. This study demonstrated that the nutritional value of rotifer was not influenced by the enrichment diets except the protein level. RPO-enriched rotifer showed higher protein level compared to the other treatments. The mean protein level in rotifers varied in the range of 45.9–55.2%. Generally, protein level in rotifers are relatively stable at ∼34–52% as reported by Caric et al. (1993) and Hamre et al. (2013); Hamre (2016). Slight changes in lipid level in rotifers have been observed to affect the dry matter protein content (Srivastava et al., 2006). Variable protein level in rotifers in the present study may have been influenced by palm oil type that affects the ingestion rate of rotifer with 24 h of the enrichment process. The acceptability of rotifer might be due to the preference of available food as a consequence of the response of rotifers upon encountering the supplied feed (Wallace, 1980; Hotos, 2003). Evidently, the enriched diet did not significantly affect the whole-body proximate composition which suggested that Asian seabass larvae efficiently utilized the enriched rotifer to support survival and growth.

CONCLUSION

Palm oil in the form of CPO and RPO can be successfully used as a dietary lipid source in the enriched diets for rotifer and is able to support good survival and growth of Asian seabass larvae. Replacing 75% fish oil with RPO performed better than the other palm oil-based enriched diets and the control. A successful replacement of fish oil with palm oil in the enriched diets for rotifer will be able to reduce larval rearing production cost of Asian seabass. It is also a significant finding in larval nutrition research that can contribute to sparing the natural populations of forage fish from unsustainable exploitation. Rotifer represents a suitable live prey for enrichment with the ingredients that positively influence the nutritional requirements of the early larval stages of the fish widely used in aquaculture. Further research toward optimizing the alternative sources

TABLE 9 | Fatty acid composition (% of total fatty acids) of Asian seabass.

[1]Fatty acid (One-way ANOVA)	Enriched diets				
	FO-100	**CPO50**	**CPO75**	**RPO50**	**RPO75**
C14:0	0.9 ± 0.0^{a}	0.9 ± 0.1^{a}	0.7 ± 0.0^{b}	0.8 ± 0.0^{a}	0.7 ± 0.1^{b}
C15:0	0.5 ± 0.0^{ab}	0.5 ± 0.0^{ab}	0.6 ± 0.0^{a}	0.5 ± 0.0^{ab}	0.5 ± 0.0^{b}
C16:0	26.5 ± 1.3^{c}	32.2 ± 0.0^{b}	33.3 ± 0.0^{ab}	30.8 ± 0.8^{a}	32.4 ± 0.9^{b}
C16:1	4.0 ± 0.0^{a}	4.0 ± 0.1^{a}	3.2 ± 0.2^{b}	3.6 ± 0.1^{a}	3.0 ± 0.0^{b}
C17:0	1.9 ± 0.1^{a}	1.7 ± 0.0^{b}	1.7 ± 0.0^{b}	1.4 ± 0.1^{a}	1.4 ± 0.01^{c}
C18:0	4.0 ± 0.0^{b}	5.5 ± 0.1^{a}	5.5 ± 1.0^{a}	4.2 ± 0.4^{b}	4.3 ± 0.4^{b}
C18:1n9	18.3 ± 0.0^{c}	20.3 ± 0.0^{b}	20.4 ± 0.1^{b}	21.6 ± 0.6^{c}	21.5 ± 0.6^{a}
C18:2n9	4.9 ± 0.1^{a}	0.9 ± 0.7^{b}	0.2 ± 0.0^{c}	0.2 ± 0.0^{a}	nd
C18:2n6	1.0 ± 0.1^{d}	1.6 ± 0.0^{b}	1.3 ± 0.1^{c}	2.0 ± 0.1^{d}	1.8 ± 0.0^{a}
C20:1	0.6 ± 0.0^{b}	0.7 ± 0.0^{a}	0.8 ± 0.0^{a}	0.7 ± 0.0^{b}	0.7 ± 0.0^{a}
C20:4n6	2.9 ± 0.0^{c}	3.5 ± 0.0^{a}	2.4 ± 0.0^{d}	2.9 ± 0.1^{c}	3.1 ± 0.1^{b}
C20:5n3	3.9 ± 0.0^{b}	4.3 ± 0.0^{a}	2.6 ± 0.0^{d}	3.9 ± 0.1^{b}	3.8 ± 0.1^{c}
C22:6n3	10.1 ± 0.3^{a}	7.0 ± 0.3^{b}	5.0 ± 0.8^{cd}	4.6 ± 0.3^{d}	6.0 ± 0.7^{c}

[2]Fatty acid (One-way ANOVA)	Parameters					
	Total saturates	**Total monoenes**	**Total PUFA**	**n-3**	**n-3/n-6**	**DHA/EPA**
FO100	33.9 ± 1.2^{d}	23.5 ± 0.0^{c}	23.6 ± 0.4^{a}	14.1 ± 0.3^{a}	3.1 ± 0.0^{a}	2.6 ± 0.1^{a}
CPO50	40.8 ± 0.1^{ab}	25.8 ± 0.0^{a}	17.9 ± 0.6^{b}	11.4 ± 0.3^{b}	2.1 ± 0.1^{b}	1.6 ± 0.1^{c}
CPO75	41.9 ± 1.0^{a}	25.0 ± 0.3^{b}	12.2 ± 0.7^{d}	7.8 ± 0.8^{d}	1.8 ± 0.2^{b}	1.9 ± 0.3^{b}
RPO50	37.7 ± 1.4^{c}	26.6 ± 0.6^{a}	14.9 ± 0.6^{c}	8.7 ± 0.3^{d}	1.4 ± 0.1^{c}	1.2 ± 0.1^{d}
RPO75	39.2 ± 1.5^{bc}	25.9 ± 0.7^{a}	15.3 ± 0.5^{c}	9.9 ± 0.6^{c}	1.8 ± 0.1^{b}	1.6 ± 0.2^{c}
Two-way ANOVA (*p*-value)						
Palm oil type	0.005	0.010	0.937	0.291	0.003	0.006
Palm oil level	0.000	0.000	0.000	0.000	0.000	0.000
Oil type × oil level	0.084	0.132	0.000	0.000	0.001	0.099

[1]Minor fatty acids (<0.2% from total fatty acids) were not included in the table.
Means with same superscripts in the same [1]row and [2]column are not significant differences between treatment (Duncan's multiple range test, $P > 0.05$).

of protein and lipid from plants for live feeds will help in shaping the aquaculture industry consistent with the criteria of sustainable development.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study was conducted following the Researcher's guidelines on code of practice for the care and use of animals for scientific purposes (Universiti Malaysia Sabah).

AUTHOR CONTRIBUTIONS

NS conducted the larval rearing experiment, raw data collection and analysis, and draft writing. RS leader of research project, designed the experimental layout, analysis of results, and manuscript writing. SM manuscript reviewing and improving presentation of data. FC involved in larval rearing design and execution. All authors contributed to the article and approved the submitted version.

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frontiers in Marine Science

REVIEW
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Essential Fatty Acid Requirements in Tropical and Cold-Water Marine Fish Larvae and Juveniles

Sahar Chauffour Mejri[1], Réjean Tremblay[2], Céline Audet[2], Paul S. Wills[1] and Marty Riche[1]*

[1]Harbor Branch Oceanographic Institute, Florida Atlantic University, Fort Pierce, FL, United States, [2]Institut des Sciences de la Mer, Université du Québec à Rimouski, Rimouski, QC, Canada

To improve survival at early developmental stages (larvae and juveniles) of captive fish species, essential nutrients [i.e., essential fatty acids (EFA)] need to be identified. The physiological needs are likely to be different among species, particularly among those using different thermal habitats, because lipids are largely used to maintain cell membrane integrity (homeoviscous adaptation) in fishes. This review paper will focus on currently published research and the main results from our laboratories regarding optimum qualitative EFA requirements during larval and early juvenile stages in a warm-water marine species, the Florida pompano (*Trachinotus carolinus*), and a cold-water marine species, the winter flounder (*Pseudopleuronectes americanus*). To identify the qualitative optimal EFA requirements, we calculated the ratio of certain fatty acids (FA) in larval or early juvenile tissues to total FA present in the diet. This ratio indicates whether a specific FA from prey is selectively incorporated by larvae and juveniles. Overall, we found that young larvae from both cold- and warm-water species have greater demands for n-3 and n-6 highly unsaturated fatty acids (HUFA) than do larvae at weaning stages. However, the qualitative EFA requirements of the cold-water species at all early developmental stages were higher than those of the warm-water species. Enriched rotifer diets provided satisfactory amounts of omega 3 and omega 6 in Florida pompano, with small selective retention for docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (ARA), suggesting a potential minor diet deficiency in these EFA. There were higher deficiencies in the cold-water species fed enriched rotifers, as demonstrated by the higher selective retentions of all EFA (DHA, EPA, and ARA), with the exception of larvae fed with copepods. The physiological needs in EFA for juvenile development seemed to be better met for both species when they were fed micro pellets. From the beginning of settlement and in young juveniles, qualitative values of 12% DHA, 10% EPA, 5% ARA, and 40% PUFA of total FA seem to be required for winter flounder juvenile development. In Florida pompano, these requirements could be met until larger juvenile stages, with 15% DHA, 3% EPA, 2% ARA, 2% DPA, and total PUFA below 30% of total FA. This review was done to aid future research aiming to develop nutritionally balanced microdiets or live-prey enrichment diets to satisfy the physiological requirements of captive tropical and cold-water marine fish species.

Keywords: warm-water fish, cold-water fish, essential fatty acids, larvae, juveniles, diet

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Edited by:
Sami Souissi,
Lille University of Science and Technology, France

Reviewed by:
Alicia Estevez Garcia,
Institut de Recerca i Technologia Agroalimentàries, Spain
Zhenhua Ma,
South China Sea Fisheries Research Institute (CAFS), China

****Correspondence:***
Réjean Tremblay
rejean_tremblay@uqar.ca

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INTRODUCTION

Lipid energy is transferred from phytoplankton to fish *via* zooplankton. A good indication of the energetic importance of lipids for fish was demonstrated by Yaragina et al. (2000), who showed that total lipid energy is a predictor of reproductive potential in fish stocks. During digestion, ingested lipids containing fatty acid chains of more than 14 carbons are hydrolyzed to yield free fatty acids, monoacylglycerols, and – in the case of phospholipids (PL) – lysophospholipids. These products pass through the mucosal wall of the small intestine, reform into triglycerides, and are transported to tissues by chylomicrons in the blood (Budge et al., 2006). Small-chain fatty acids (<14°C) are transported directly to the liver to be oxidized. Thus, long-chain fatty acids can be preserved during the digestive process and selectively retained by fish to sustain later development.

Aquaculture development of new marine fish species is challenged by a critical knowledge gap concerning the nutritional needs at larval and early juvenile stages. The optimization of feeding conditions and the nutritional quality of starter diets, which are generally at least partially made up of enriched live prey, directly influence the success of these early stages as estimated by growth and survival surveys. Lipids are a major nutritional constituent and are the main source of metabolic energy sustaining the rapid development of early stages (Glencross, 2009). Lipids provide at least two-thirds more energy per gram than proteins or carbohydrates (Parrish, 2013). Of the different lipid classes, neutral lipids, including wax esters, sterols, free fatty acids, and triglycerides have a rapid turnover and can satisfy short-term energy needs (Budge et al., 2006), while the β-oxidation of saturated fatty acids (SFA) releases energy more efficiently than polyunsaturated fatty acids (PUFA; Langdon and Waldock, 1981).

Despite years of research, lipids remain the least well-understood nutrient (Glencross, 2009; Parrish, 2013). Some fatty acids are considered as essential fatty acids (EFA) for marine fish development because they cannot be biosynthesized to support normal development (Glencross, 2009). EFA are involved in different physiological functions, including reproduction, immunity, ion balance regulation, muscle contraction, cell adhesion, vascular tone, buoyancy control, and brain and eye development, and thus directly affect the growth and survival of marine animals (Glencross, 2009; Pond and Tarling, 2011; Gurr et al., 2016). These EFA are mainly from two related families (n-3 and n-6) and from three long-chain PUFA: docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3), and arachidonic acid (ARA, 20:4n-6), which have long been considered crucial. DHA and EPA have important roles in the maintenance of membrane fluidity while ARA and EPA are precursors of bioactive eicosanoids (Tocher, 2010). During larval development, DHA is preferentially incorporated into nervous and retina tissue (Izquierdo et al., 2000; Villalta et al., 2008), and growth anomalies and high mortality are observed when DHA is insufficient (Tocher, 2010). Other dietary FA, including linoleic acid (LA; 18:2n-6) and α-linolenic acid (LNA; 18:3n-3), are stored in muscle tissue to meet physiological needs and are also related to fish growth and survival to some extent (Jardine et al., 2020). These FA are precursors of DHA, EPA, and ARA, but as already pointed out; the activities of the specific enzymes (desaturase and elongase) responsible for their biosynthesis are limited in most marine species.

Artemia and rotifers contain high levels of PL, which are considered valuable for fish nutrition (Tocher et al., 2008), and they are the main live prey used in aquaculture. However, their low levels of EFA are not adequate for early life stages, so they must be enriched before being used as prey (Øie et al., 2011). In contrast, copepods, which are natural fish prey in the wild, are rich in phospholipids and EFA, but their laboratory production is complex and challenging (Drillet and Lombard, 2015). Although the PL content per dry weight (DW) in rotifers and *Artemia* cannot be manipulated, their fatty acid composition can be modified with the use of enrichments (Castell et al., 2003; Monroig et al., 2003; Seychelles et al., 2009; Hawkyard et al., 2015).

One interesting method used to determine the prey's nutritional quality in terms of EFA is the ratio of specific fatty acids in larval or early juvenile tissues to the total fatty acids present in the prey [the fish to diet (FD) ratio]. This FD ratio indicates whether a specific prey fatty acid is selectively incorporated by larvae and juveniles (Copeman et al., 2002; Pernet and Tremblay, 2004; Gendron et al., 2013; Martinez-Silva et al., 2018). If the relative proportion of a specific fatty acid in larvae or juveniles to their diet is equal to or below 1, then the specific requirement for this fatty acid could be considered as satisfied. In contrast, if the relative proportion is higher than 1, then we would presume that this FA is selectively incorporated by larvae or juveniles, which may indicate a potential dietary deficiency.

The objective of this review paper is to present published research regarding live-prey diets used in early developmental stages of Florida pompano (*Trachinotus carolinus*) and winter flounder (*Pseudopleuronectes americanus*), two contrasting models from tropical and temperate coastal habitats, respectively, to determine whether their ontogenic EFA needs are comparable. Both species are commercially important and contribute to the economies of their distribution range, compelling the aquaculture industry to search for more effective rearing methods. Our aim is to examine whether FD ratios can give information about differing needs through the first stages of development and how these needs are comparable between tropical and cold-temperate species. To estimate potential dietary deficiencies in EFA, we used the FD ratio as an optimization tool. The FD ratio as well as compositions of fatty acid that are considered to be essential or of special importance for larval and juvenile development [18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3, 22:6n-3, total n-3, total n-6, SFA, monounsaturated fatty acids (MUFA), and PUFA] are presented and compared between relatively similar ontogenic stages in the two species considered (**Table 1**).

The lipid composition of larvae and/or juveniles has been presented in detail for Florida pompano (Cavalin and Weirich, 2009; Hauville et al., 2014, 2016; Rombenso et al., 2016, 2017;

TABLE 1 | Age or mass of larvae and juveniles of the two species compared in this review.

Ontogenic stage	Florida pompano	Winter flounder
Mouth opening	No data available	4 DPH
Live prey feeding	9 DPH	15–22 DPH
Weaning	17 DPH	26 DPH
Beginning of settlement		38 DPH
Early settled juveniles		30 DPS
0+ juveniles		60 and 90 DPS
Juveniles	Around 40 g	

Florida pompano (T. carolinus): larval rearing at 26–27°C; winter flounder (P. americanus): larval rearing at 10°C. DPH, days post-hatch; DPS, days post-settlement.

Jackson et al., 2020) and for winter flounder (Mercier et al., 2004; Seychelles et al., 2011, 2013; Vagner et al., 2013; Bélanger et al., 2018; Martinez-Silva et al., 2018). While these studies examined different developmental stages, we have merged the results for the purpose of comparison.

NUTRITIONAL RATIOS AND FATTY ACID COMPOSITION

FD Ratio in Larvae

Florida pompano larvae at the live-prey feeding period-fed several enriched rotifer diets [*Isochrysis* sp. (ISO), *Pavlova* sp. (PAV), Protein Selco Plus (PS+), Ori-Green (OG), Protein highly unsaturated fatty acid (HUFA; PH), and AlgaMac 3050 (AM)] showed specific needs for DHA, EPA, and ARA, with selective retention of these three EFA (**Figure 1**). The FD ratio for EPA was relatively close to one in larvae fed the different enriched diets, particularly for the AM- and OG-enriched diets, showing accumulations of up to 7% of total FA (**Table 2**). Exceptions were noted for ISO-enriched diets, which showed ratios near 1.5. DHA requirements seemed to be satisfied by most diets, with an average of 15% of total FA (**Table 2**) and the best FD ratio for DHA being observed in larvae fed diets enriched with PAV and PH (ratio = 1.053 and 1.056, respectively). This suggests that these diets satisfied most physiological needs of the larvae at this developmental stage. All diets showed FD ratios above one for ARA, suggesting that these enrichments did not satisfy the requirements for this PUFA (**Figure 1**). The maximal ARA accumulation observed at the live-prey feeding stage was 3.5% of total FA (**Table 2**), with FD ratios between 1.26 and 1.89 depending on the diet. LA and LNA were not selectively retained by larvae at this stage, with ratios < 1 and low relative percentages of LNA (0.6% of total FA; **Table 2**). However, two diets (PAV and AM) seemed to better supply the LA and LNA requirements, with FD ratios equal to one (**Figure 1**). Overall, total omega 3 (n-3) and omega 6 (n-6) FD ratios were close to one for all diets (**Figure 1**), suggesting that the different enriched rotifer diets provided satisfactory amounts of n-3 and n-6 PUFA for larvae at the live-feeding stage.

At the weaning period [17 days post hatch (DPH)], Florida pompano larvae were fed different microdiets (Gemma, Otohime, and LR803) and generally showed FD ratios close to one.

TABLE 2 | Relative percentages of fatty acids in Florida pompano (*T. carolinus*) larvae at live prey feeding and weaning (9 and 17 DPH, respectively; Cavalin and Weirich, 2009; Hauville et al., 2014).

	Live prey feeding	Weaning
18: 2 n-6 (LA)	6.58 ± 2.53	16.32 ± 10.45
18: 3 n-3 (LNA)	0.59 ± 0.28	2.22 ± 0.99
20: 4 n-6 (ARA)	3.55 ± 0.41	0.47 ± 0.15
20: 5 n-3 (EPA)	7.28 ± 1.22	5.20 ± 2.65
22:6 n-3 (DHA)	15.06 ± 4.59	8.57 ± 2.29
Total n-3	28.64 ± 2.31	–
Total n-6	14.80 ± 1.70	–
SFA	–	29.99 ± 1.87
MUFA	–	23.80 ± 3.87
PUFA	–	39.29 ± 6.37

Data represent mean ± SD from all diet treatments at 9 and 17 DPH. LA, linoleic acid; LNA, linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; and PUFA, polyunsaturated fatty acids.

One exception was noted: EPA ratios were between 1.47 and 1.86, indicating selective retention for this EFA and potential diet deficiencies. Among the three microdiets, Otohime micro pellets followed by LR803 seemed to be the most appropriate; they fulfilled the physiological fatty acid requirements of larvae at the weaning stage, with a PUFA ratio equal to 1.01 for both diets (**Figure 1**).

In winter flounder larvae, overall larval contents in neutral EPA and DHA and in polar DHA were higher at the mouth-opening stage, reflecting the maternal nutritional contribution (yolk sac; **Table 3**). Overall, EPA and DHA contents were usually higher in polar than in neutral lipids, as reflected by the total n-3 value (**Table 3**). Once larvae started feeding, FD ratios for the neutral lipids showed that the three EFA (ARA, DHA, and EPA) were immediately retained (FD values much greater than one), regardless of the diet [rotifers enriched with Selco or days' post settlement (DPS)]. For most diets, the high retention levels of EFA in the neutral fraction were maintained throughout development, but larvae fed rotifers enriched with Sparkle or copepods showed ratios near or below one (**Figure 2**). This may be related to crucial biological needs, since EFA are important for physiological functions. Indeed, FD ratios for polar lipid fractions were mostly greater than one whatever the diet or the larval stage of development, except for larvae fed copepods at the live-prey feeding stage (**Figure 2**). For each EFA, the use of copepods as live prey decreased the FD ratio of polar lipids in larvae relative to diet content, but the ratio was slightly greater than one for ARA and EPA. In these larvae, overall LNA contents were usually very low. This is not surprising since *Artemia* were not included in the larval diets (**Table 3**), and *Artemia* are usually characterized by high LNA levels (Rocha et al., 2017). The requirements for omega n-6 seemed to be met regardless of the diet, as indicated by their elimination in neutral lipids and their incorporation into polar lipids at percentages lower than those found in the different diets (**Figure 2**). However, the sum of omega n-3 showed ratios suggesting potential feed deficiency (>1), particularly in the polar fraction, indicating that larval needs were not met except when copepods were used as live prey.

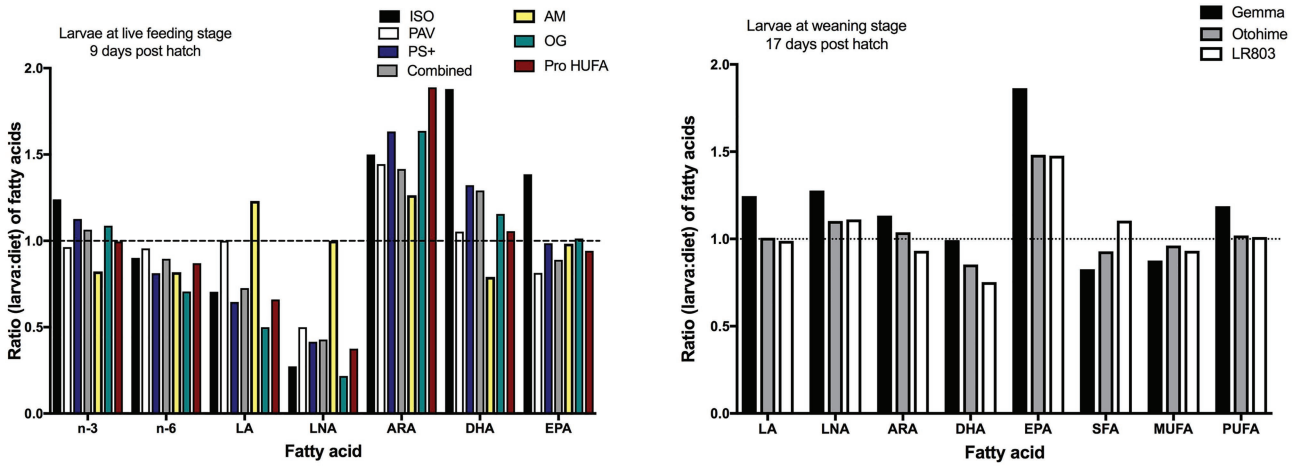

FIGURE 1 | Ratio (larva: diet) of fatty acids in Florida pompano (*Trachinotus carolinus*) larvae at the live feeding [9 days post hatch (DPH)] and the weaning period (17 DPH). Larvae at 9 DPH were fed several enriched rotifer diets: two microalga concentrates [*Isochrysis* sp. (ISO) and *Pavlova* sp. (PAV)], a semi-moist paste [Protein Selco Plus (PS+)], a combination of the three (Combined), and three dry enrichment diets [AlgaMac 3050 (AM), Ori-Green (OG), and Protein highly unsaturated fatty acids (HUFA; PH); Cavalin and Weirich, 2009]. Larvae at 17 DPH were fed three microdiets [Gemma, Otohime, and a reference diet (LR803); Hauville et al., 2014]. The dashed lines (ratio = 1) indicate equal amounts of fatty acids in the larvae and in the diet. LA, linoleic acid; LNA, linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; and PUFA, polyunsaturated fatty acids.

FD Ratio in Juveniles

Only a few papers (Rombenso et al., 2016, 2017; Jackson et al., 2020) have discussed the physiological needs of Florida pompano juveniles in terms of fatty acids. In these studies, docosapentaenoic acid (DPA) was also observed in juvenile tissues. We have included it in this review since it has been identified as a potential EFA (Parrish et al., 2007). Rombenso et al. (2016) assessed the fatty acid composition in different tissues of Florida pompano juveniles (43.4 ± 0.2 g) following fish oil replacement (**Table 4**; **Figure 3**). Juveniles were fed six different diets for 8 weeks; the diets contained menhaden fish oil, 25:75 blends of fish oil and standard soybean oil, MUFA-enriched soybean oil, SFA-enriched soybean oil, palm oil, or poultry fat. ARA was selectively retained in the brain irrespective of the diet that was provided (FD ratio ≥ 1.35), suggesting a physiological need for this EFA in this tissue. However, the same FA was present at satisfactory levels in the eye, with FD ratios not exceeding 1.14 for all diets (**Figure 3**). The same tendency was observed in the liver except for juveniles fed the palm diet (ratio = 1.57). ARA ratios were equal to zero in the muscle tissue of all juveniles, suggesting that ARA is not required in muscle tissue at this stage and is probably not used for energy purposes (**Figure 3**). On the other hand, DHA and DPA seemed to have been strongly retained in all tissues, with higher selective retention in the brain and eye. FD ratios for DHA were > 2.2 in the eyes of juveniles fed the SFA soy and MUFA soy diets. The FD ratio in brain tissue was as high as 3.28 for the same diets. Higher DPA retention levels were also observed in the eye, with FD ratios ≥ 2.22 in juveniles fed all diets, suggesting the importance of this FA in eye development. FD ratios for EPA were ≤ 0.5 in all tissues for all diet treatments, which suggests that diets with EPA levels below 3% of total FA (**Table 4**) fulfilled EFA needs at this developmental stage. Other PUFA, such as LA and LNA, showed different trends in different tissues. FD ratios for LA were < 1 in all tissues except for muscle tissue in juveniles fed the fish diet; these had an FD ratio equal to 1, suggesting that this FA might be used for energy purposes. There is little or no physiological need for LNA, as evidenced by the lack of retention in any tissue (ratio < 0) combined with levels below 2% in muscle and eye and 0% in liver and brain (**Table 4**). We suggest that LNA was used mostly as an energy substrate. FD ratios for SFA, MUFA, and PUFA were all close to one in each studied tissue (**Figure 3**).

Rombenso et al. (2017) also evaluated the requirements for EPA and DHA in Florida pompano juveniles (41.0 ± 0.5 g) by feeding them for 8 weeks with diets containing fish oil, beef tallow, or beef tallow partially or fully supplemented with EPA, DHA, or both. It was interesting to see that ARA was more balanced in the brain tissue for fish fed beef tallow supplemented with EPA and DHA (FD ratio = 1.14), while this same fatty acid was more balanced in the eye tissue with all diets, but not with the non-supplemented beef tallow diet (**Figure 4**). ARA was highly retained in liver and muscle tissue except for two diets (fish oil only and the fully supplemented beef diet), where FD ratios were equal to 0.92. DHA and DPA were still highly retained in all tissues. However, DHA and DPA FD ratios were 1.1 and 1.28, respectively, in the brain of juveniles fed the fully supplemented beef diet (**Figure 4**), highlighting these specific needs in the brain tissue. The FD ratio for DHA was 1.04 in the eye tissue of juveniles fed the fully supplemented beef diet, which might suggest that this diet best fulfilled the DHA requirements of Florida pompano juveniles. EPA ratios in all tissues were comparable to a previous

TABLE 3 | Relative percentages of neutral and polar fatty acids in winter flounder larvae (*P. americanus*) at mouth opening, live-prey feeding, and weaning (4; 15–16, 22; and 26 DPH, respectively; Seychelles et al., 2011; Martinez-Silva et al., 2018).

	Larvae			
	Mouth opening	**Live-prey feeding**		**Weaning**
Neutral fatty acid	4 DPH	15–16 DPH	22 DPH	26 DPH
18: 2 n-6 (LA)	4.55 ± 0.78	3.16 ± 1.27	1.39 ± 0.06	5.30 ± 0.42
18: 3 n-3 (LNA)	0	0.20 ± 0.34	0.34 ± 0.12	0
20: 4 n-6 (ARA)	2.45 ± 0.07	2.32 ± 1.18	1.29 ± 0.30	3.55 ± 0.35
20: 5 n-3 (EPA)	9.7 ± 1.1	6.5 ± 3.2	2.9 ± 0.8	3.6 ± 1.3
22:6 n-3 (DHA)	20.25 ± 0.35	7.86 ± 4.81	2.73 ± 0.81	7.30 ± 2.12
Total n-3	31.80 ± 1.13	17.96 ± 9.63	8.19 ± 1.85	19.75 ± 3.32
Total n-6	7.10 ± 0.85	5.82 ± 1.83	3.26 ± 0.23	9.15 ± 0.92
SFA	29.60 ± 3.90	65.44 ± 13.63	74.41 ± 16.00	24.44 ± 0.59
MUFA	27.43 ± 3.51	22.57 ± 9.02	13.2 ± 8.0	36.83 ± 8.86
PUFA	42.97 ± 1.59	11.99 ± 5.92	12.38 ± 8.34	38.73 ± 8.28
Polar fatty acid				
18: 2 n-6 (LA)	3.50 ± 0.00	4.15 ± 0.66	2.30 ± 0.20	4.90 ± 0.71
18: 3 n-3 (LNA)	0	0	0.40 ± 0.01	0
20: 4 n-6 (ARA)	3.55 ± 0.07	5.50 ± 1.68	2.90 ± 0.01	6.10 ± 0.57
20: 5 n-3 (EPA)	11.55 ± 0.21	8.75 ± 4.92	17.26 ± 0.16	9.55 ± 0.49
22:6 n-3 (DHA)	22.90 ± 0.57	13.75 ± 3.48	6.72 ± 0.27	12.35 ± 4.74
Total n-3	35.85 ± 0.78	27.09 ± 1.60	27.16 ± 0.48	28.1 ± 4.9
Total n-6	7.30 ± 0.14	10.15 ± 2.28	6.00 ± 0.23	11.35 ± 0.21
SFA	34.33 ± 0.78	43.48 ± 11.42	52.45 ± 14.50	29.97 ± 1.76
MUFA	20.03 ± 0.76	20.96 ± 3.76	13.75 ± 2.76	23.77 ± 6.23
PUFA	45.57 ± 1.07	35.56 ± 9.95	33.81 ± 12.60	46.23 ± 4.71

Data represent mean ± SD from all diet treatments. LA, linoleic acid; LNA, linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; and PUFA, polyunsaturated fatty acids.

study conducted by the same authors (Rombenso et al., 2016); FD ratios $\leq$ 0.5. LNA were only found in eye tissue and were zero in all other tissues (**Figure 4**), confirming that there was no specific physiological need for this FA at this developmental stage. FD ratios for LA were close to one in muscle, liver, and eye tissues for all diets tested, indicating an adequate supply of this FA. LA was not selectively retained in the brain tissue, which confirms that it is not required for brain development/function. In all tissues examined, FD ratios for SFA, MUFA, and PUFA were close to one (**Figure 4**), suggesting that most diets tested, except the beef diet, were suitable fatty acid sources for Florida pompano juveniles. Juveniles fed the beef-only diet showed selective PUFA retention in almost all tissues, suggesting that this diet did not provide enough omega n-3 and PUFA to allow proper juvenile development. Thus, diets for juvenile Florida pompano development should include around 2% ARA, 3% EPA, 2% DPA, and 15% DHA, for a total PUFA below 30%, to sustain development.

Winter flounder juveniles showed low EFA accumulation in their neutral lipids until 60 DPS (**Table 5**), and no active retention in lipid reserves was observed except for ARA. Before 60 DPS, all EFA seemed to be directly incorporated into polar lipids, possibly in response to vital physiological requirements. Following settlement, DHA and EPA requirements seemed to be adequately met with the Gemma micro diet and diets of rotifers enriched with commercial Selco DHA protein or with INVE Selco sparkle in older fish, since the FD ratios of these EFA in the lipid polar fraction were below one (**Figure 5**). However, the FD ratio for ARA in the polar fraction was much greater than one in all diets (**Figure 5**), suggesting inappropriate enrichment for this FA. As juveniles grew, the relative content of fatty acids increased except for ARA, which remained close to values observed at the early juvenile stage (**Table 5**). Indeed, almost all FD ratios were below one in young juveniles (60 and 90 DPS), indicating that needs were met regardless of the diet, even though ARA was still actively retained (**Figure 5**). Thus, these results suggest that diets with 10% EPA, 12% DHA, more than 5% ARA, and a total of 40% PUFA could be adequate to sustain proper development in winter flounder juveniles.

QUALITATIVE ESSENTIAL FATTY ACID REQUIREMENTS FOR EARLY STAGES

The qualitative requirements for EFA in Florida pompano larvae were the highest during the live-feeding period, when the relative percentages of DHA, EPA, and ARA represented 16, 7, and 4% of total fatty acids, respectively (**Figure 6**). These findings are comparable to those obtained, at 8 DPH, in a closely related species; the golden pompano (*Trachinotus ovatus*). The authors suggested that at least 18% DHA, 5% EPA, and 3.5% ARA of total FAs are needed for the larvae to achieve optimal development and reduce deformities (Fu et al., 2021). During the weaning period, the requirements for DHA and ARA decreased by 50 and 75% from the live-feeding period, respectively (7 and 1% of total FA for DHA and ARA, respectively), while EPA percentages did not change (7% of

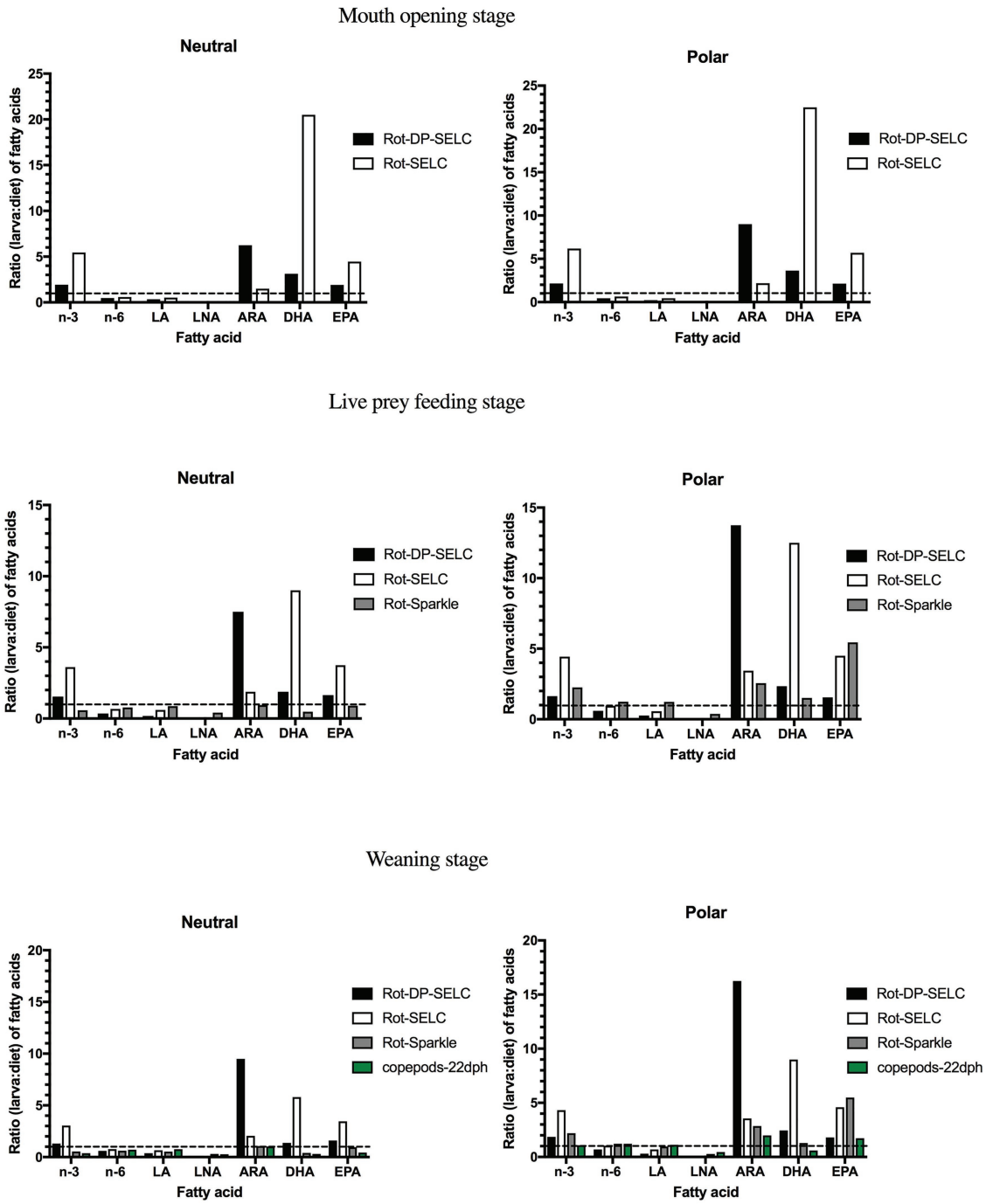

FIGURE 2 | Ratio (larva: diet) of neutral and polar fatty acids in winter flounder (*Pseudopleuronectes americanus*) larvae at mouth opening (4 DPH), live feeding (15–16 DPH), and beginning of weaning (26 DPH). Larvae had been fed several enriched rotifer diets: Selco 3000 (SELC), DHA Protein Selco (DP-SELC), and sparkle-INVE (Sparkle). Larvae at 22 DPH were fed copepods (Seychelles et al., 2011; Martinez-Silva et al., 2018). The dashed lines (ratio = 1) indicate equal amounts of fatty acids in larvae and in the diet. LA, linoleic acid; LNA, linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; and EPA, eicosapentaenoic acid.

total FA; **Figure 6**). The relative percentages of LA and LNA increased over time, from 3 to 5% of total FA for LA and from 0 to 1% of total FA for LNA. By comparison golden pompano larvae that were fed several enriched *Artemia nauplii* diets from 11 to 27 DPH, showed a slightly lower requirement for DHA (5% of total FAs), but relatively similar requirements for EPA, ARA, LA, and LNA (6.7, 1.3, 3, and 1% of total FAs, respectively; Ma et al., 2016).

TABLE 4 | Relative percentages of fatty acids in juvenile Florida pompano (*T. carolinus*; 43.4 ± 0.2 g) muscle, liver, eye, and brain tissues.

Fatty acid	Muscle	Liver	Eye	Brain
18: 2 n-6 (LA)	7.41 ± 4.19	5.25 ± 2.80	7.18 ± 3.95	1.74 ± 1.22
18: 3 n-3 (LNA)	1.27 ± 0.57	–	0.76 ± 0.18	–
20: 4 n-6 (ARA)	1.09 ± 0.17	1.93 ± 0.94	0.91 ± 0.19	1.48 ± 0.16
20: 5 n-3 (EPA)	3.21 ± 1.19	1.46 ± 1.07	3.44 ± 1.63	1.59 ± 0.30
22:5 (DPA)	1.71 ± 0.60	1.69 ± 0.82	2.47 ± 0.67	1.29 ± 0.27
22:6 n-3 (DHA)	10.80 ± 3.09	15.22 ± 5.14	11.43 ± 1.96	14.98 ± 2.68
SFA	38.09 ± 2.77	40.33 ± 2.54	38.82 ± 2.76	47.84 ± 2.07
MUFA	35.34 ± 4.22	31.91 ± 6.80	33.36 ± 5.21	30.61 ± 1.53
PUFA	26.52 ± 4.16	27.69 ± 7.01	27.80 ± 4.79	21.54 ± 3.01

Juveniles were fed six diets for 8 weeks. Diets contained menhaden fish oil or 25:75 blends of fish oil and standard soybean oil, MUFA-enriched soybean oil, SFA-enriched soybean oil, palm oil, or poultry fat. Data represent mean ± SD from all diet treatments. LA, linoleic acid; LNA, linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; and PUFA, polyunsaturated fatty acids.

The same tendency was observed for winter flounder larvae, with decreased EFA requirements over time. However, EFA requirements at all winter flounder larva stages were higher than for Florida pompano larvae. The relative percentages of DHA, EPA, and ARA were 23, 12, and 4% of total FA at the mouth-opening stage (**Figure 6**). The relative percentage of DHA decreased by 75% at the live-prey feeding and weaning stages (8 and 7% of total FAs, respectively; **Figure 6**), while the relative percentages of EPA decreased slightly to 9% of total FA at the live-prey feeding stage and increased to 17% of total FA at the weaning stage (i.e., 26 DPH). The relative percentages of ARA decreased by 1% at both the live-prey feeding and weaning stages. LA requirements were the same during the first days of life and decreased by half (2% of total FA) at the weaning stage (**Figure 6**).

Overall, winter flounder larvae had higher requirements for omega 3 (n-3) FA at the mouth-opening stage (36% of total FA) than at the live-prey feeding and weaning stages (28–27% of total FA), while requirements for omega 6 (n-6) seemed to increase over time (7% of total FA at the mouth-opening stage vs. 11–12% of total FA at the live-prey feeding and weaning stages).

Studies on other marine finfish species have shown that the dietary inclusion of n-3 HUFA (i.e., DHA and EPA) and n-6 HUFA (i.e., ARA) improved larval growth, development, and metamorphosis. When examining additional nutritional studies on warm-water and cold-water marine finfish species, we found that EFA requirements during larval development from first feeding to weaning were similar, with few exceptions, to the results reported here. Indeed, common snook (*Centropomus indecimalis*) larvae at the rotifer-feeding stage (live-prey feeding) showed the same qualitative requirements for EFA as Florida pompano (15, 7, and 3% of total FA for DHA, EPA, and ARA, respectively; Hauville et al., 2016). Red drum (*Sciaenops ocellatus*) larvae at the weaning period (18 DPH) were found to have slightly lower requirements for DHA and EPA (4 and 5% of total FA, respectively) and similar requirements for ARA (1% of total FA) compared to Florida pompano larvae at the same developmental stage (Brinkmeyer and Holt, 1998).

These requirements seemed to be lower in warm-water marine fish at the weaning period, particularly for DHA and ARA, suggesting that the EFA requirements are higher in rapidly growing larvae at the live-feeding stage. In addition, the ratio of DHA to EPA is greater at the live-feeding period (2.2) than during the weaning period (1.0; **Figure 6**), which is similar to what was observed in red drum larvae as well as larvae from four other species of marine finfish (Watanabe and Kiron, 1995; Brinkmeyer and Holt, 1998). It has been suggested that the high DHA to EPA ratios at the live-feeding stage play a role in stress resistance. The high levels of DHA observed during early larval development for both species reflect the importance of this EFA in larval structural development and in neural and visual functions (Bell et al., 1995a,b; Sargent et al., 1999).

The qualitative requirements for DHA and ARA appeared to be higher in larvae of species that undergo metamorphosis. DHA and ARA values reported in southern flounder (*Paralichthys lethostigma*) and Atlantic halibut (*Hippoglossus hippoglossus*) larvae at 15 DPH (live-prey feeding period) were 17 and 5% of total FA, respectively (Hamre and Harboe, 2008; Alam et al., 2015; Oberg and Fuiman, 2015; Hamre et al., 2020), which is more than twice the percentage of DHA and five times the percentage of ARA found in Florida pompano larvae at the same developmental stage. These differences strongly suggest greater requirements for DHA and ARA during the physiologically demanding metamorphosis process. Hamre et al. (2020) suggested that FA needed to have at least 13% DHA for normal pigmentation in Atlantic halibut, highlighting the effect of DHA both on eicosanoid production and the development of vision and nervous tissue (Denkins et al., 2005; Roman et al., 2007; Hamre et al., 2020).

An adequate EPA to ARA ratio is important to achieve normal pigmentation and complete eye migration in flatfish species; this has been reported for turbot (*Scophthalmus maximus*), Atlantic halibut, Senegalese sole (*Solea senegalensis*), and yellowtail flounder (*Limanda ferruginea*; McEvoy et al., 1998; Estévez et al., 1999; Copeman et al., 2002; Villalta et al., 2005). The EPA to ARA ratio in winter flounder was 3.0 during early larval development (4 and 15 DPH) and close to 6.0 at the weaning period (22–26 DPH; **Figure 6**). For Florida pompano, the highest ratio (ratio = 7.0) was reported at the weaning period, but other than that, the ratio did not exceed two. A previous study on turbot larvae has concluded that it is important to consider the ratio of DHA to EPA and found that it is positively correlated with pigmentation success (Rainuzzo et al., 1994).

At the juvenile stage, the qualitative requirement for DHA was similar between the two species (12% of total FA). However, 0+ juvenile winter flounder were much smaller than juvenile Florida pompano (~ 1 vs. 42 g), thus their DHA requirement might be higher for the same stage of development (**Figure 6**). The different DHA requirements can also be coupled with water temperatures of the different habitats, with cold water fish species generally having higher DHA levels than more temperate species (Hamre et al., 2020). In general, poikilotherm fish counteract the lower temperature effect to maintain their

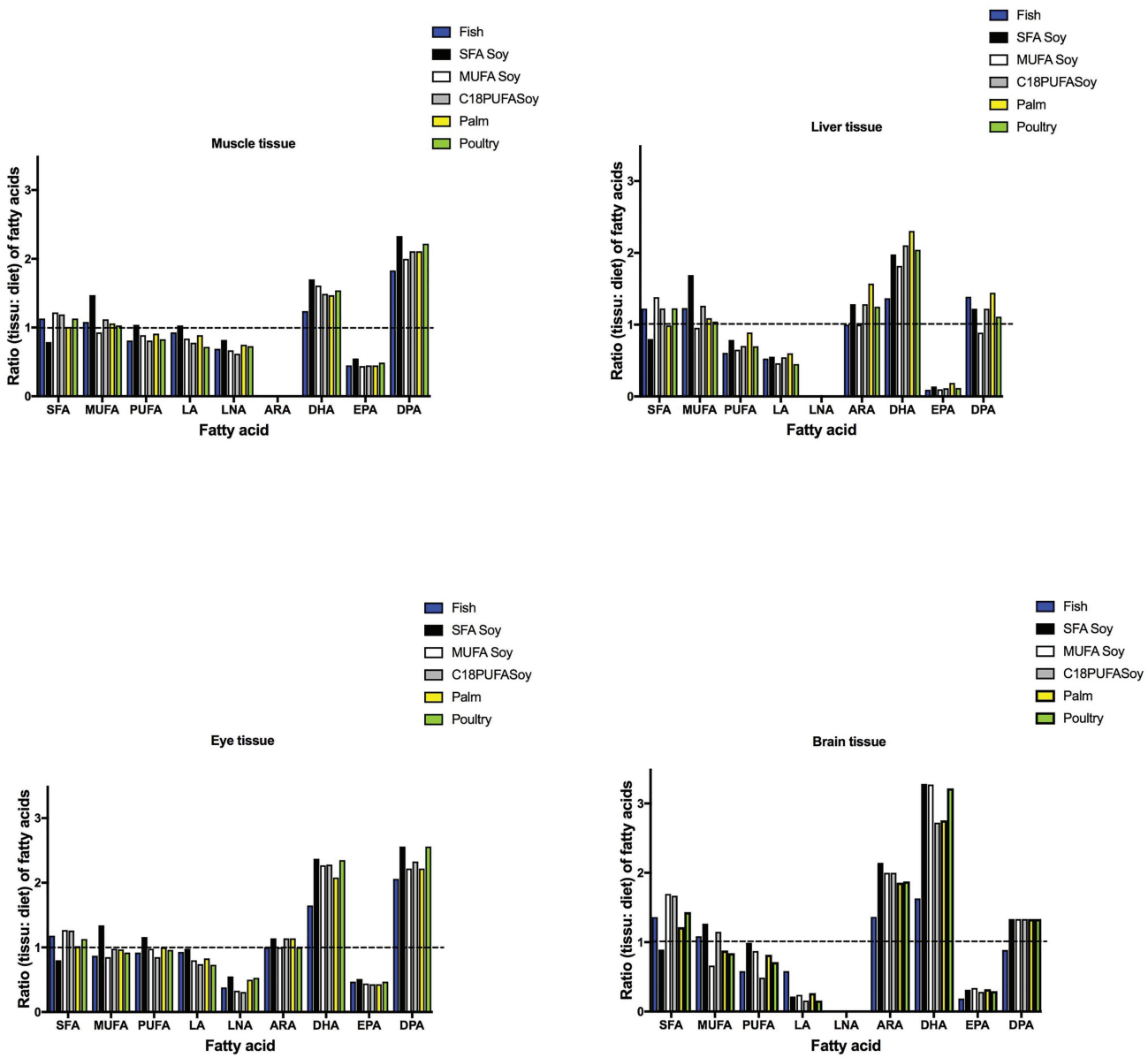

FIGURE 3 | Ratio (tissue: diet) of fatty acids in juvenile Florida pompano (*T. carolinus*) muscle, liver, eye, and brain tissue (44 g average weight). Diet treatments consisted of a diet containing menhaden fish oil (Fish), SFA-enriched soybean oil (SFA Soy), MUFA-enriched soybean oil (MUFA Soy), 25:75 blends of fish oil and standard soybean oil (C18 PUFA Soy), palm oil (Palm), or poultry fat (Poultry; Rombenso et al., 2016). The dashed lines (ratio = 1) indicate equal amounts of fatty acids in the juveniles and in the diet. LA, linoleic acid; LNA, linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; and PUFA, polyunsaturated fatty acids.

metabolism by remodeling fatty acids in the membrane. In cold conditions, the integration of PUFA, particularly DHA, in phospholipids maintains membrane fluidity and metabolic rate, a process known as homeoviscous adaptation (Hazel, 1995). Thus, cold-water species should require more DHA to maintain their physiological function, as observed in this comparison between winter flounder and Florida pompano. EPA and ARA requirements seemed to be higher in winter flounder, ranging from 6% at the early settlement stage to 11% at the 0+ juvenile stage for EPA and from 3 to 4% for ARA for the same stages, respectively (EPA: ARA ratio is approximately 2.5; **Figure 6**). Hamre et al. (2020) showed that Atlantic halibut juveniles would need a combination of at least 13% DHA of total FA and a higher ratio of EPA to ARA (ratio = 3.5) to promote growth and survival of young juveniles.

One of the most interesting differences between the two species was the presence of LA and LNA in Florida pompano larvae and juveniles at all stages, while LNA was absent in winter flounder. Jackson et al. (2020) recently published data suggesting that Florida pompano juveniles have some

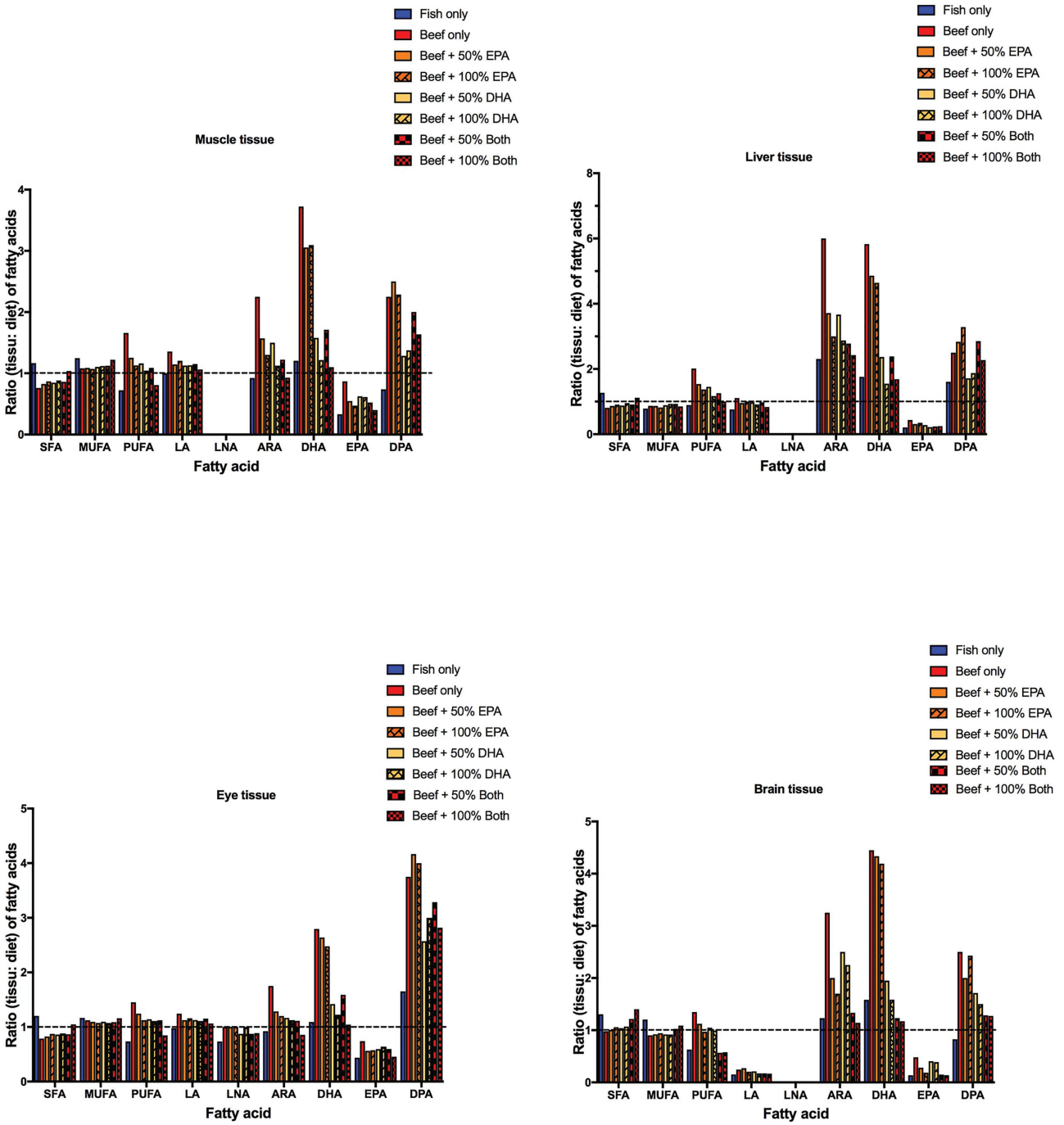

FIGURE 4 | Ratio (tissue: diet) of fatty acid in juvenile Florida pompano (*T. carolinus*) muscle, liver, eye, and brain tissues (41 g average weight). Diet treatments contained fish oil, beef tallow, or beef tallow partially or fully supplemented with EPA, DHA, or both (Rombenso et al., 2017). The dashed lines (ratio = 1) indicate equal amounts of fatty acids in juveniles and in the diet. LA, linoleic acid; LNA, linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; and PUFA, polyunsaturated fatty acids.

capacity to elongate and desaturate fatty acids from C18 precursors (i.e., LA and LNA) and may be able to survive on diets containing C18 PUFA. However, the authors strongly recommend that juveniles be directly provided with HUFA to perform optimally (Jackson et al., 2020). Recent studies concluded that golden pompano juveniles (ranging in sizes from 8 to 50 g), a species belonging to the same family as the Florida pompano, might have low capacity to biosynthesize HUFA (Liu et al., 2018; Li et al., 2020; Wang et al., 2020). Wang et al. (2020) have found that golden pompano juveniles may have the capability of converting EPA to DHA but lack the Δ5 desaturation activity, required

TABLE 5 | Relative percentages of neutral and polar fatty acids in winter flounder (*P. americanus*) juveniles at the beginning of settlement (38 DPH), 30 DPS, and at the 0+ juvenile stage (60 and 90 DPS).

	Juveniles				Reference
	Settlement	Early settled juveniles	0+juveniles		
Neutral fatty acid	38 DPH	30 DPS	60 DPS	90 DPS	
18: 2 n-6 (LA)	5.40 ± 0.00	5.55 ± 0.07	7.27	7.60	38 DPH and 30 DPS: Seychelles et al., 2011, and Bélanger et al., 2018
18: 3 n-3 (LNA)	0	0	0	0	60 and 90 DPS: Bélanger et al., 2018
20: 4 n-6 (ARA)	1.65 ± 0.92	1.10 ± 0.14	3.15	1.53	
20: 5 n-3 (EPA)	2.50 ± 0.0	2.40 ± 0.14	9.01	6.90	
22:6 n-3 (DHA)	1.00 ± 0.00	1.00 ± 0.00	10.57	6.80	
Total n-3	4.10 ± 0.14	3.70 ± 0.00	21.01	14.44	
Total n-6	7.05 ± 0.92	6.65 ± 0.07	16.27	15.68	
SFA				28.00	Bélanger et al., 2018
MUFA				34.05	
PUFA				34.95	
Polar fatty acid					
18: 2 n-6 (LA)	7.20 ± 0.28	7.85 ± 0.07	5.19	7.02	38 DPH and 30 DPS: Seychelles et al., 2011, and Bélanger et al., 2018
18: 3 n-3 (LNA)	0	0	0	0	60 and 90 DPS: Bélanger et al., 2018
20: 4 n-6 (ARA)	3.30 ± 0.00	3.00 ± 0.00	4.60	4.03	
20: 5 n-3 (EPA)	6.50 ± 0.00	6.00 ± 0.00	10.88	10.88	
22:6 n-3 (DHA)	3.75 ± 1.77	2.50 ± 0.71	12.53	10.81	
Total n-3	12.45 ± 1.63	6.95 ± 3.61	23.99	22.56	
Total n-6	10.65 ± 0.35	14.0 ± 4.24	13.99	15.79	
SFA				33.35	Bélanger et al., 2018
MUFA				23.55	
PUFA				41.60	

Data represent mean ± SD from all diet treatments. LA, linoleic acid; LNA, linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; and PUFA, polyunsaturated fatty acids.

to convert 20:3 n-6 and 20:4 n-3 to ARA and EPA, respectively, suggesting incomplete HUFA biosynthesis ability.

It is hypothesized that trophic level is a better predictor of a requirement for C18 PUFA vs. HUFA rather than the species' thermal or salinity preference (Jackson et al., 2020; Trushenski and Rombenso, 2020). It is interesting to note that Florida pompano and winter flounder, despite their contrasting thermal environments, both belong to the same trophic level (Florida pompano trophic level = 3.5, Froese and Pauly, 2016; winter flounder trophic level = 3.6 ± 0.1, Murdy et al., 1997), which might explain some of the similarities in EFA requirements observed here. The validation of this hypothesis would change our way of investigating requirements for fatty acids in fish species and thus allow standardization of feed formulations for different developmental stages.

CONCLUSION

Essential fatty acid requirements vary qualitatively and quantitatively during fish ontogeny, with the larval and juvenile stages being arguably the most critical periods. In this review, we give a comprehensive synthesis of these requirements, highlighting the similarities and differences between two species occupying contrasting environments: a warm-water tropical species (Florida pompano) and a cold-water flatfish species (winter flounder). In general, we found that the young larvae of both species are characterized by greater requirements for n-3 and n-6 HUFA compared to larvae at the live-feeding and weaning stages as well as juveniles. Florida pompano larvae at the live-feeding stage require more DHA, EPA, and ARA than larvae at the weaning stage, with minimal levels probably around 15, 7, and 3.5% of total FA, respectively, for young larvae (i.e., 9 DPH) and 9, 5, and 0.5% of total FA for larvae at the weaning stage. Nevertheless, none of the diets seemed to fully satisfy the EFA needs for both larval stages. Studies on the EFA requirements during early larval development in winter flounder suggest that the physiological needs of larvae were not met for all the rotifer-enriched diets tested. Ratios of DHA, EPA, and ARA in the polar lipid fraction related to diet were systematically well over 1.0, indicating strong retention. Ratios ≤ 1 were only obtained when larvae at the weaning stage were fed copepods, suggesting a minimal qualitative requirement of 12% DHA, 10% EPA, and 6% ARA of total FA at this stage, and up to 20% DHA in younger larvae. During metamorphosis, winter flounder – like many flatfish species – has specific EFA requirements necessary to achieve correct pigmentation and eye migration. Thus, an early supply of dietary DHA, EPA, and ARA emphasizing the importance of dietary DHA: EPA: ARA ratios is essential for successful pigmentation and eye migration. Concerning juvenile development, the qualitative EFA requirements seem to be similar in both species, with slightly higher needs for EPA and ARA in winter flounder. Diets containing around 15% DHA, 3% EPA, 2% DPA, and 2% ARA, for a total PUFA below 30% of total FA, seem to be appropriate for Florida pompano

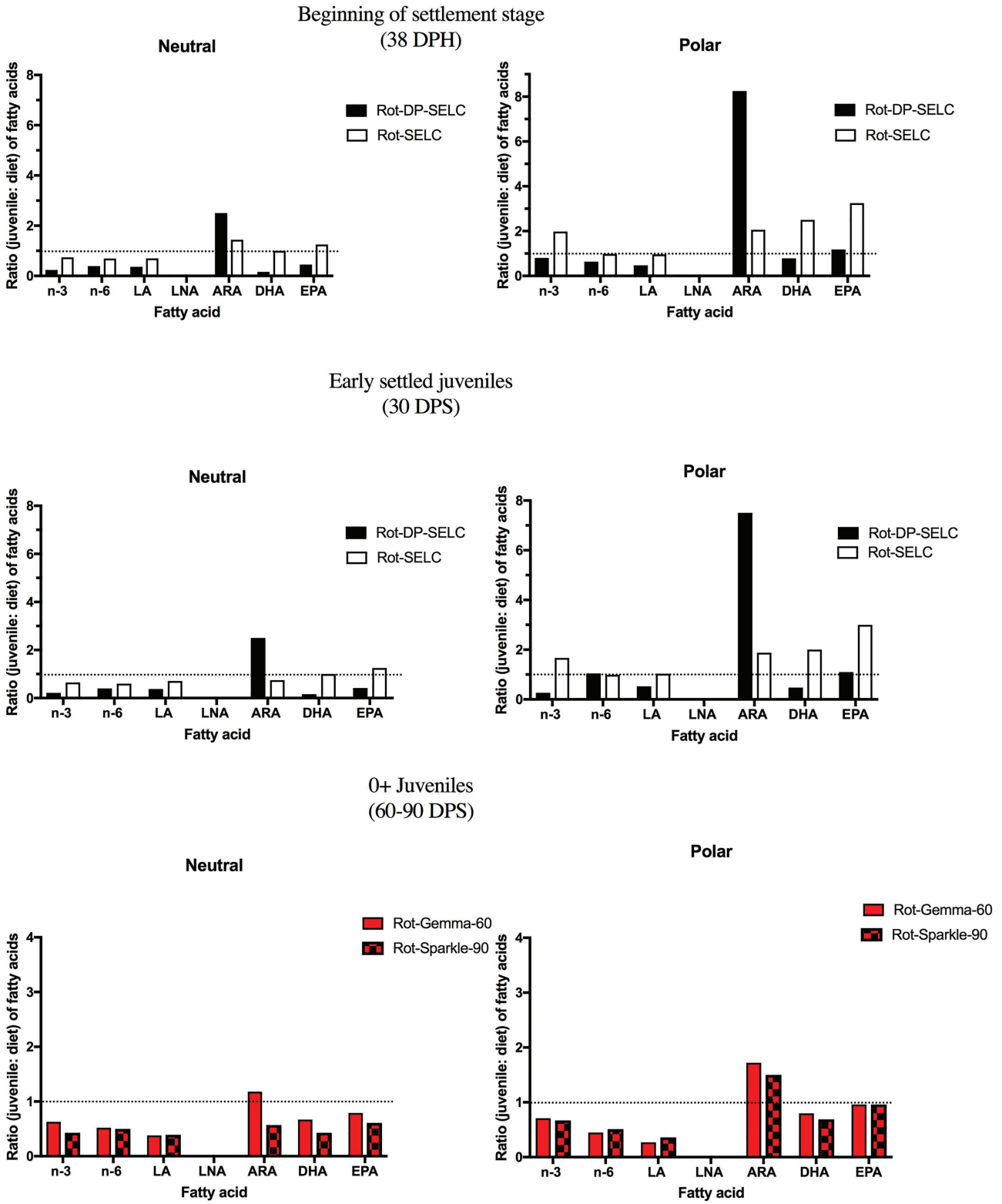

FIGURE 5 | Ratio of neutral or polar fatty acids in winter flounder (*P. americanus*) juveniles relative to diet total fatty acids contents (juvenile: diet) at the beginning of settlement (38 DPH), early settled juveniles [30 days post settlement (DPS)], and 0+ juveniles (60–90 DPS) fed several enriched rotifer diets: Selco 3000 (Rot-SELC), DHA Protein Selco (DP-SELC), sparkle-INVE (Rot-Sparkle), and Gemma microdiet (Rot+Gemma). The dashed lines (ratio = 1) indicate equal amounts of fatty acids in the juveniles and in the diet. LA, linoleic acid; LNA, linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; and EPA, eicosapentaenoic acid.

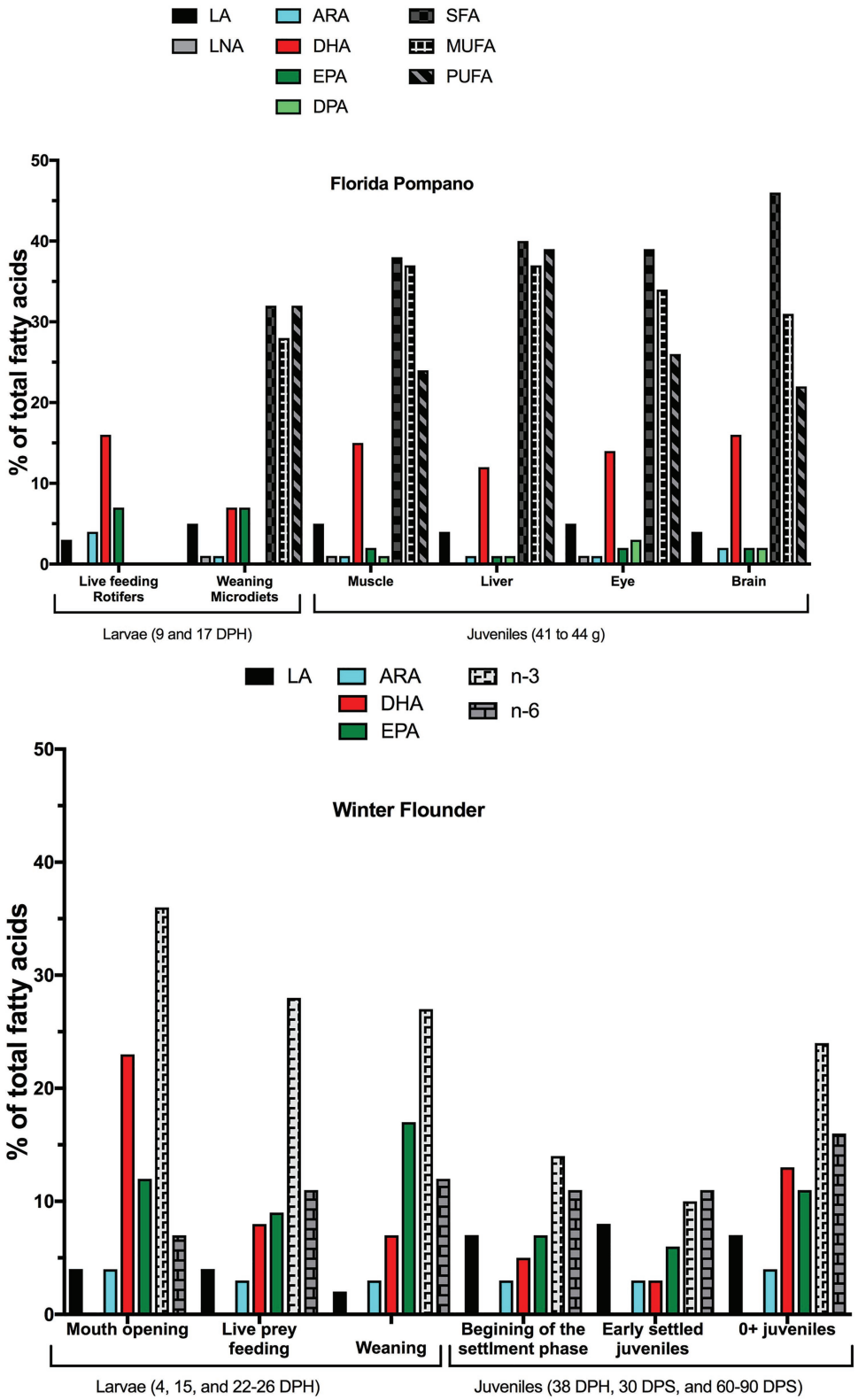

FIGURE 6 | Optimum relative percentages of essential fatty acids (EFA) in Florida pompano (*T. carolinus*) larvae and juveniles (top) and winter flounder (*P. americanus*) larvae and juveniles (bottom). We determined the optimum qualitative requirements (percentages) from data that had larva to diet or juvenile to diet ratios closest to one. DPH, days post hatch; DPS, days post settlement; LA, linoleic acid; LNA, linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; and PUFA, polyunsaturated fatty acids.

development, while diets with 12% DHA, 10% EPA, and 5% ARA, for a total of 40% PUFA, could be better for juvenile winter flounder development.

Identifying the EFA needs in marine fish larvae is particularly difficult because of the fishes' small size and often poorly developed digestive system. The complexity and time-consuming effort of determining precise microdiets led to the use of live feeds, such as rotifers and *Artemia*, but these are often inadequate for marine larvae and require enrichment in HUFA. HUFA enrichments of live feeds do not provide sufficient DHA and adequately balanced levels of HUFA (Tocher, 2010), thus it is necessary to continue work toward developing nutritionally balanced microdiets that satisfy the physiological needs of marine fish larvae. In addition, the growing aquaculture industry – by pressing the need for marine fish meal and oils to be replaced with plant-derived products – has reintroduced the need to identify the precise qualitative and quantitative EFA requirements for marine fish larvae and juveniles. There must be detailed knowledge of the molecular and biochemical bases of HUFA requirements and metabolism: the physiological needs of a fish species to achieve optimal growth and stay healthy are different from the requirements to maintain nutritional quality, which leads to health benefits for human consumers.

AUTHOR CONTRIBUTIONS

SM and RT have developed the idea and all authors contributed to this review. All authors contributed to the article and approved the submitted version.

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Pre-breeding Diets in the Seahorse *Hippocampus reidi*: How Do They Affect Fatty Acid Profiles, Energetic Status and Histological Features in Newborn?

Miquel Planas[1], Ike Olivotto[2], María Jesús González[3], Rosaria Laurà[4], Carlo Angeletti[5], Adolfo Amici[5] and Matteo Zarantoniello[2]*

[1] Department of Ecology and Marine Resources, Instituto de Investigaciones Marinas, Vigo, Spain, [2] Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona, Italy, [3] Marine Products Chemistry Department, Instituto de Investigaciones Marinas, Vigo, Spain, [4] Dipartimento di Scienze Veterinarie, Università di Messina, Messina, Italy, [5] Dipartimento di Scienze Cliniche Specialistiche ed Odontostomatologiche, Università Politecnica delle Marche, Ancona, Italy

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***Correspondence:**
Miquel Planas
mplanas@iim.csic.es

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Seahorses (*Hippocampus* spp.) are exceptional marine species considering their reproductive patterns and other features. Due to the iconic characteristics of these fishes, aquarium trade, and research efforts have increased in the last years. Consequently, novel rearing techniques have been developed; however, there is a need for improvements on a series of issues, namely reproduction success enhancement. The tropical species *Hippocampus reidi* is the most traded seahorse but many aspects of breeding and its impact on the quality of neonates are still poorly understood. In the present study, we assessed the effects of two pre-breeding diets on newborn quality and viability considering biochemical characteristics, energetic status, and ultrastructural aspects of muscular tissue. During the whole pre-breeding season (5 months), the breeders were fed on one of the following diets: M0 (adult non-enriched *Artemia*) and M5 (adult non-enriched *Artemia* + mysidaceans). From the onset of the reproduction period, all breeders were fed for 6 months on diet M5. Breeding success and energetic status (ATP, total adenylic nucleotides, AEC, and NAD) of newborns resulted considerably enhanced in treatment M5. However, initial differences in neonates quality did not affect further newborn performance (survival and growth until day 7 after male's pouch release) while gaining access to high-quality preys (copepods). Besides, morphological alterations in muscle tissue were not observed. The reproduction in the species followed a capital–income continuum pattern characterized by an initial mixed capital-income period (until 70–100 days since the onset of the breeding season) followed by an income breeding period with progressive exhaustion of body reserves, especially in M0-newborns. Interestingly, the effects of pre-breeding diets were also noticed in the second half of the breeding period. Our results seemed to indicate that the requirements in essential fatty acids in *H. reidi* are lower than in other seahorse

species (e.g., *Hippocampus guttulatus*). Globally, the results achieved revealed that high-quality pre-breeding diets enhanced reproduction success and would likely result advantageous to improve newborn endurance in conditions of moderate starvation or sub-optimal feeding.

Keywords: Syngnathidae, seahorses, *Hippocampus reidi*, breeder's diet, newborn, fatty acids, energy charge, histology

INTRODUCTION

Seahorses (*Hippocampus* spp.) are flagship fishes submitted to over-exploitation in nature due to their use in several human activities, habitat loss, and the aquarium and curio trade, compromising their conservation (Vincent, 1996; Foster and Vincent, 2004; Martin-Smith et al., 2004; Vincent et al., 2011). In recent years, concern over how to reduce the exploitation of wild populations has been increasing in parallel to rising efforts to provide reliable tools for their rearing in *ex situ* facilities (Koldewey and Martin-Smith, 2010; Olivotto et al., 2011b; Cohen et al., 2016). Breeding in captivity can partially replace the capture of wild seahorses and reduce the pressure over natural populations.

There are more than thirty seahorse species but only a few are currently reared with certain success in captivity. The tropical fish *Hippocampus reidi* Ginsburg, 1933 is one of the seahorse species most in demand for the marine aquarium trade, and one of the most promising for industrial cultivation. The geographic distribution of the species ranges from Florida to Rio de Janeiro (Brazil) and Gulf of Mexico (Kuiter, 2013). Adults measure up to 26 cm in TL and inhabit shallow inshore to moderately deep water (15–55 m) (Rosa et al., 2002).

A singular characteristic of seahorses is reproduction, in which females provide most nutrients to eggs whereas males take care and also supply nutrients to embryos developing from eggs transferred by females to their brood pouches (Carcupino et al., 2002; Skalkos et al., 2020; Wittington and Friesen, 2020). Mating and parturition are highly synchronized with egg maturation so that the release of eggs and newborn occur almost simultaneously and at similar time intervals, which vary depending on species, temperature, and breeder's diet (Lin et al., 2006, 2007; Planas et al., 2010; Otero-Ferrer et al., 2020). In seahorses, the assessment of nutritional and energetic requirements in seahorse breeders is lacking. Only a few studies have focused on the impact of breeder's diet on newborn quality and further viability. Most of them were carried out in *H. guttulatus*, *H. hippocampus*, and *H. reidi* by comparing diets differing in biochemical profiles [namely fatty acids (FAs)] and nutritional quality (e.g., *Artemia*, mysidaceans, and amphipods as prey) (Woods and Valentino, 2003; Murugan et al., 2009; Otero-Ferrer et al., 2012, 2016, 2020; Palma et al., 2012; Saavedra et al., 2014; Saavedra et al., 2015; Planas et al., 2020b).

The effect of breeder's diet in fish have been usually focused on the impact of FAs and the resulting quality of fry (Bromage, 1995; Brooks et al., 1997; Izquierdo et al., 2001; Fernández-Palacios et al., 2011; Migaud et al., 2013). In seahorses, most studies have focused on the effects of diets provided during the breeding season (Woods and Valentino, 2003; Binh and Serrano, 2012; Otero-Ferrer et al., 2012, 2020; Palma et al., 2012, 2017; Planas et al., 2020b) or shortly before reproduction (Otero-Ferrer et al., 2016). In a previous study on *H. guttulatus*, we assessed isotopically the assimilation by breeders of three diets furnished during the whole pre-breeding period and their incorporation into newborns (Planas, 2021). We demonstrated that the species follows a capital-income breeding strategy ((Williams et al., 2017). The optimization of seahorse breeding also should consider how newborn development is affected by pre-breeding dietary regimes. For that, newborn characteristics and initial viability must be properly addressed. Condition indices, energy allocation, and metabolic scope in early ontogeny aim to evaluate the physiological status of fish (Cunha et al., 2003, 2007; Di Pane et al., 2019). Adenylic energy charge (AEC) was proposed as an indicator of the energy potentially available for cellular metabolism (Atkinson, 1977; Dickson and Franz, 1980; Harms, 1992). To our knowledge, the unique study available focused on adenylic nucleotides and energetic status in fish larvae was carried out in clownfish (Olivotto et al., 2011a).

In the present study, we assessed the effects of two dietary treatments on the reproduction of the seahorse *H. reidi* by analyzing (1) breeding success and general newborn characteristics (FA profiles, endogenous nucleosides, and AEC), and (2) determining the breeding pattern of the species. To our knowledge, this is the first multiapproach study on the effect of long-term pre-breeding diets on reproductive performance and newborn energetic status in seahorses.

MATERIALS AND METHODS

Bioethics

Animal handling and sampling were conducted in compliance with all bioethics standards on animal experimentation of the Spanish Government (Real Decreto 1201/2005, 10th October) and the Regional Government Xunta de Galicia (REGA ES360570202001/15/FUN/BIOL.AN/MPO01).

Seahorse Maintenance

Adult seahorses *H. reidi* Ginsburg, 1933 were kept in captivity at the facilities of Instituto de Investigaciones Marinas (IIM-CSIC) (Vigo, Spain) for at least two generations, or donated by l'Oceanogràfic aquarium (Valencia, Spain). The fish were maintained in 320 L aquaria (Planas et al., 2008) in a semi-closed system with a 10–14% daily renewal of seawater. Seawater temperature was maintained constant at 26 ± 0.5°C, and a natural-like photoperiod regime was applied (16L:8D). Water

quality was checked periodically for NO_2, NO_3, and NH_4/NH_3 content (0 mg L^{-1}). Salinity and pH levels were 37 ± 2 and 8.0 ± 0.2, respectively. Wastes and uneaten food were removed daily by siphoning the bottom of aquaria.

The fish were routinely fed twice daily on diets comprising enriched adult *Artemia* (Iberfrost, Spain; 40–70 *Artemia* seahorse^{-1} dose^{-1}) and frozen mysidaceans *Neomysis* sp. (Ocean Nutrition, Spain). Adult *Artemia* was produced in 100 L units at 26–28°C with gentle aeration and constant light on a mixture consisting of live microalgae *Phaeodactylum tricornutum* and *Isochrysis galbana*, dried *Spirulina*, and subsequently long-time enrichment for at least 3 days (from day 16 onward) on the commercial product Red Pepper (Bernaqua, Belgium) (Planas et al., 2017).

Experimental Design

Pre-breeding (Period 1)

Two groups of seahorses (groups M0 and M5; eight females and six males per group) were randomly selected from the seahorse broodstock and kept separate for 5 months (from November 1, 2017 to March 31, 2018) in two 320 L and maintained under the same chemical-physical conditions reported above. For each group, males and females were kept physically separate during the whole period.

The breeders were fed (*ad libitum*) twice daily according to the following feeding scheme (**Figure 1**):

1. Group M0: adult non-enriched *Artemia* (Iberfrost, Spain).
2. Group M5: adult non-enriched *Artemia* and frozen mysidaceans (*Neomysis* sp.; Ocean Nutrition, United States) (1:1).

Prey lengths were 5–7 mm for *Artemia* and about 1–1.5 cm for mysidaceans.

Adult non-enriched *Artemia* was produced as reported above but not enriched with Red Pepper.

The mixed diet used in group M5 was selected considering that it provides the best trophic enrichment factors in syngnathids (Planas et al., 2020a). The lipid content and FAs profile of prey are provided in **Table 1**.

Mean weight (DW) and standard length (SL) of breeders at the onset of Period 1 did not differ significantly across groups (K-W test, $n = 32$, $p = 0.951$ for DW and 0.878 for SL). Mean DW and SL were 6.17 ± 2.07 g and 14.15 ± 1.53 cm in M0, and 6.76 ± 3.09 g and 14.43 ± 2.22 cm in M5, respectively. At the end of the pre-breeding period, mean DW (9.18 ± 2.69 g in M0; 10.99 ± 2.96 g in M5), SL (16.03 ± 1.42 cm in M0; 17.01 ± 1.60 cm in M5) and G (daily weight-specific growth rates) (0.355 ± 0.196% day^{-1} in M0; 0.434 ± 0.221% day^{-1} in M0) were similar across groups (K-W test, $n = 32$; $p = 0.242$ for DW, 0.140 for SL, and 0.124 for G).

Breeding (Period 2)

Males and females from the same experimental treatment were regrouped on April 1, 2018 and maintained until September 30, 2018 under the same conditions reported above for Period 1, except for the diet. During this period, both experimental groups were fed twice daily on a mixture of non-enriched adult *Artemia* and frozen mysidaceans *Neomysis* sp. (1:1).

Seahorses were monitored several times a day to check for newborn releases from male's pouch. Samples of freshly released newborn were immediately collected for further analyses (electronic microscopy, total lipids, FAs, endogenous nucleotides, and energy charge calculation) and weight/size determination. Sampled juveniles were euthanized with Tricaine MS-222 (0.1 mg L^{-1}, Sigma-Aldrich). For length and weight measurements, the juveniles were transferred to Petri dishes, photographed, and weighed individually on a Sartorius microbalance MC210P (±0.01 mg). Curved SL were measured (SL = head + trunk + curved tail) from digital photographs using image processing software (NIS, Nikon).

After sampling, the remaining released juveniles were carefully collected, counted, and transferred (2–5 juveniles L^{-1}) to 30 L pseudo-Kreisel aquaria connected to a semi-opened

TABLE 1 | Total lipids (% dry weight) and fatty acids (relative percentage) in prey ($n = 2$) for seahorse breeders.

Fatty acids (%)	*Artemia*	Mysidacea
14:0	1.43 ± 0.09	3.41 ± 0.02
15:0	0.46 ± 0.05	0.84 ± 0.03
16:0	17.92 ± 0.92	21.63 ± 0.15
16:1n-7	8.31 ± 0.17	9.06 ± 0.04
17:0	3.22 ± 0.10	1.23 ± 0.03
17:1	0.06 ± 0.01	0.12 ± 0.09
18:0	11.44 ± 0.56	2.65 ± 0.04
18:1n-7	10.39 ± 3.98	4.11 ± 0.00
18:1n-9t	6.97 ± 0.50	5.99 ± 0.05
18:2n-6c	12.55 ± 0.68	1.17 ± 0.01
18:3n-3	0.86 ± 0.04	1.31 ± 0.01
18:3n-6c	6.36 ± 0.36	0.22 ± 0.00
18:4n-3	0.15 ± 0.03	0.18 ± 0.02
20:0	0.64 ± 0.01	0.20 ± 0.03
20:1n-9	0.30 ± 0.01	0.84 ± 0.01
20:2n-6	0.31 ± 0.00	0.52 ± 0.02
20:3n-6	0.66 ± 0.06	0.27 ± 0.00
20:4n-3	0.24 ± 0.00	0.35 ± 0.02
20:4n-6 (AA)	1.45 ± 0.05	2.33 ± 0.01
20:5n-3 (EPA)	15.43 ± 0.76	22.19 ± 0.04
22:4n-6	0.12 ± 0.01	0.28 ± 0.01
22:5n-3	0.00 ± 0.00	0.43 ± 0.08
22:5n-6	0.00 ± 0.00	0.83 ± 0.05
22:6n-3 (DHA)	0.51 ± 0.01	19.69 ± 0.03
24:0	0.20 ± 0.02	0.15 ± 0.01
Saturated	35.3	30.1
Monounsaturated	26.0	20.1
Polyunsaturated	38.7	49.8
n-3	17.2	44.2
n-6	21.5	5.6
n-3 HUFA	16.2	42.6
DHA/EPA	0.03	0.89
DHA/ARA	0.35	8.45
n-3/n-6	0.80	7.87

Sample size: 10–20 mg dry weight.

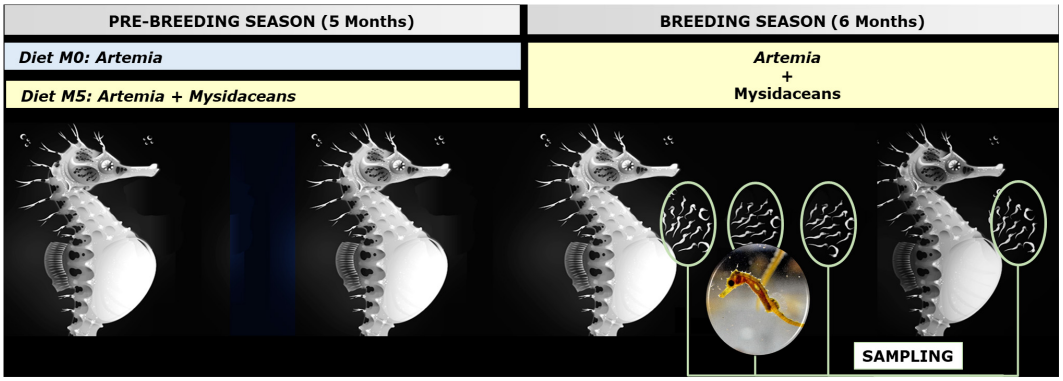

FIGURE 1 | General scheme of the experimental framework (*Hippocampus reidi* breeders fed on diets M0 and M5) and sampling (dorsal fin and newborn). Seahorse illustration by Sergio Picazo Ferro (https://sergiopicazo.com/).

recirculation system (Blanco et al., 2014). The newborns were reared until day 7 (7 DAR) after male's pouch release (DAR) at 26°C under a constant 14L:10D photoperiod. Further details on the rearing system were reported by Planas et al. (2012).

From 0 to 5 DAR (days after male's pouch release), newborn seahorses were fed on two daily doses of *Acartia tonsa* (0.67 copepods mL^{-1} $dose^{-1}$), whereas one daily dose of *A. tonsa* (0.67 copepods mL^{-1} $dose^{-1}$) and one daily dose of *Artemia* nauplii (1 *Artemia* mL^{-1} $dose^{-1}$) were supplied on 6–7 DAR.

Dead seahorses were daily removed (8:00 a.m. and 3:00 p.m.) and counted. Final survivals of juveniles were recorded at 7 DAR, corresponding to complete mortality in unfed seahorses (personal observation).

Microalgae and live prey (copepods and *Artemia* nauplii) were cultured as reported by Planas et al. (2020b).

Sample Analysis

Total Lipids and Fatty Acids

Total lipids from live prey and 0 DAR juveniles (10–20 mg dry weight per sample; n = 30–60) were extracted according to Bligh and Dyer (1959). Aliquots of total lipid extracts with known lipid content were centrifuged, resuspended with 0.5 M ammonium formate solution, freeze-dried and stored at −80°C. Total lipid content was quantified gravimetrically (Herbes and Allen, 1983). FA composition in total lipids was analyzed by gas-chromatography (GC) according to Christie (1982). Lipids were transmethylated (Lepage and Roy, 1986) and FA analyzed by GC (Perkin Elmer, GC-FID Clarus 500 gas chromatograph) (Planas et al., 2010).

Total C and N

Elemental composition (total C and N) were analyzed in dried newborn seahorses ($n > 5$; pooled individuals). The samples were measured by continuous flow isotope ratio mass spectrometry using a FlashEA1112 elemental analyzer (Thermo Finnigan, Italy) coupled to a Delta Plus mass spectrometer (FinniganMat, Bremen, Germany) through a Conflo II interface. Further details are provided by Planas (2021).

NAD, ATP, ADP, and AMP Assay and Energy Charge Calculation

Whole seahorses, in five replicates each, were anesthetized and rapidly collected, frozen, and grounded in liquid nitrogen, weighed in aliquots, and stored at −80°C and processed according to Olivotto et al. (2011a).

All data in this work are referred to as grams of N_2-powder (five fresh weight tissues). Tissue N_2-powders (20–200 mg aliquots) were added with 6 mL g^{-1} of 0.4 M $HClO_4$ containing 50 mM cAMP (300 nmol g^{-1}) as internal standard and thawed by vortexing. The perchloric extracts were sonicated (30 s at 50 watts with 0.5-s pulses) and centrifuged (16.000*g*, 10 min at 4°C). The supernatants were neutralized by adding 0.29 vol of 1 M K_2CO_3 and centrifuged again to remove the precipitated $KClO_4$. The clear supernatant, referred as to neutralized extract, was used for subsequent analyses of metabolites.

The endogenous nucleotides and the internal standard cAMP were quantified by UV C18-HPLC analysis, both under reverse-phase and ion-pairing conditions. For details see Mori et al. (2014). The column temperature was 8°C and the eluate absorbance was monitored by a diode-array detector (Shimadzu SPD-M10A). For quantitation, the integrated peak areas, expressed by the Shimadzu LC solution v1.24 software used as mAU s^{-1}, were first converted into mAU × ml by appropriate factoring, then into nanomoles dividing by the appropriate extinction coefficient at 260 nm (18.6 mM^{-1} cm^{-1} for NAD; 15.4 mM^{-1} cm^{-1} for ATP, AMP, and cAMP, respectively). The extraction yield calculated from cAMP recovery was 95% in all samples.

For the AEC evaluation, the following equation was used (Atkinson and Walton, 1967):

$$AEC = [ATP + {}^{1}/_{2}ADP]/[ATP + ADP + AMP]$$

Transmission Electron Microscopy

Abdominal portions of newborns (three newborns per dietary treatment) were processed for Durcupan ACM (Fluka) resin embedding as follows: pieces fixed in 2.5% glutaraldehyde in 0.2 M phosphate-buffered saline (pH 7.4), for 2 h at 4°C, were washed repeatedly in the same buffer and postfixed in

1% osmium tetroxide in 0.2 M phosphate-buffered saline for 1 h at 4°C. Then, the tissues were dehydrated with increasing alcohol concentrations. The dehydrated pieces were embedded in Durcupan ACM (Squadrito et al., 2017). Finally, the sections were obtained with a Reichert Jung Ultracut E. Semi-thin sections (1 μm) were stained with toluidine blue and examined with a light microscope. Ultrathin sections (740 Å) were obtained from selected areas of the semi-thin sections, stained with uranyl acetate and lead citrate, and examined and photographed with a transmission electron microscope (JEOL JEM 1400 Flash) (Viña et al., 2014).

Data Analysis

Statistical analyses were conducted in R v.3.6.3 (R Core Team, 2020a). Variability across treatments was examined by non-parametric Kruskal–Wallis test for independent samples (Kassambara, 2020a). When significant, the means were compared by Wilcoxon range test (Pgirmess v1.6.9 package of R) (Giraudoux et al., 2018) or Dunnet's test (Kassambara, 2020a). Correlations (Pearson's correlation coefficients) and hierarchical clustering linkage (complete method) of variables and samples for newborn batches were performed with factoextra v1.0.7 (Kassambara, 2020b) for computational analysis and igraph v1.2.6 (Csardi and Nepusz, 2006) for plotting. Additionally, principal component analyses (PCA) were performed to summarize and visualize the information newborn datasets. For that, we used factoMineR v2.3 (Husson et al., 2020), factoextra v1.0.7 (Kassambara, 2020b), and corrplot v0.8.4 (Wei et al., 2017) packages in R. The data values were standardized (mean = 0; SD = 1) for clustering and PCA. Data normality was checked with the Shapiro–Wilk normality test (stats v3.6.2 R package) (R Core Team, 2020b). Significance levels were set at $p < 0.05$ and results are presented as mean ± standard deviation. Graphics were constructed using ggplot2 v3.3.0 (Wickham et al., 2020) and lattice v0.20-41 (Sarkar et al., 2020) packages in R.

The present study was carried out on an endangered/threatened species. Bioethics referred to these organisms imply the use of a number of specimens as much reduced as possible (strictly the necessary). Besides this, the availability of endangered species (particularly breeders) and large aquaria is certainly limited. For that reason, only one aquarium was used for each dietary treatment. Consequently, the breeders were considered pseudoreplicates and the aquaria were the basic experimental units. There is literature available that justify the use of pseudoreplicates in some circumstances and recommends that pseudoreplication not be invoked when reviewing research manuscripts and research proposals (see Planas, 2021).

RESULTS

Breeding Success

The total number of newborn batches produced in treatments M0 and M5 were 11 and 22, respectively, whereas total newborn production was 4,397 in M0 and 7,298 in M5 (**Figure 2**). The average batch size in treatments M0 (400 ± 182 newborns) and M5 (332 ± 155 newborns) were similar (K-W test, $n = 36$, $p = 0.321$). Survivals of juveniles at 7 DAR did not differ similarly across dietary treatments (K-W test, $n = 33$, $p < 0.219$), ranging from 86 ± 7% in group M5 to 88 ± 10% in group M0 (**Figure 2**).

Breeders from group M5 produced larger newborn both in SL (K-W test, $n = 36$, $p = 0.002$) and DW (K-W test, $n = 36$, $p < 0.001$) (**Figure 2**). Mean weights of newborns were 0.22 ± 0.03 mg in M0 and 0.28 ± 0.04 mg in M5, whereas mean SL were 8.0 ± 0.3 mm in M0 and 8.6 ± 0.6 mm in M5.

Total Lipids and Fatty Acids

Overall, total lipids content in newborns ranged from 14.6 ± 1.7 to 14.4 ± 1.6% dry weight and did not differ among treatments (K-W test, $n = 33$, $p = 0.942$) but decreased continuously in group M5, especially during the first half of the breeding season (**Figure 3**).

Regarding FA groups across dietary treatments, the relative content (percentage of total FA) in saturates (37.4–37.7%), monoenes (20.6–23.7%) and polyenes (38.9–41.7%) was similar (K-W test, $n = 33$; $p = 0.643$, 0.417 and 0.153, respectively) (**Figure 3**). Total n-3 HUFA accounted for 28.4–29.6% (K-W test, $n = 33$, $p = 0.942$).

DHA/EPA (range: 3.33–3.36), DHA/AA (4.31–4.70) and DHA/DPA (4.73–4.91) values gradually increased during the breeding season but average ratios for the whole period did not differ among treatments (K-W test, $n = 33$, $p = 0.817$, 0.113 and 0.589, respectively) (**Figure 3**).

Statistical mean comparisons for individual FAs (area percentage) for the whole Period 1 only revealed significant differences in 16:0 (K-W test, $n = 33$, $p = 0.021$), 18:0 ($p = 0.025$), 18:3n-3 ($p = 0.045$), 20:2n-6 ($p = 0.049$), and 22:4n-6 ($p < 0.001$) (**Figure 4**).

Nucleotides and Energy Charge

Figure 5 reports the progress of endogenous nucleotide (AMP, ADP, ATP, and NAD) content and AEC in newborn seahorses along the breeding season. With regards to both AMP and ADP content, no significant differences were detected between M0 (5.49 ± 3.42 and 8.33 ± 3.52 pmol/μg of proteins for AMP and ADP, respectively) and M5 (5.60 ± 3.08 and 8.63 ± 2.75 pmol/μg of proteins for AMP and ADP, respectively) groups. Differently, M0-group showed significantly ($n = 19$; $p < 0.05$) lower values of ATP (13.26 ± 5.51 pmol/μg of proteins) and NAD (2.33 ± 0.81 pmol/μg of proteins) content as compared to M5-group (30.16 ± 10.41 and 3.35 ± 0.82 pmol/μg of proteins for ATP and NAD, respectively). Finally, M0-group (0.62 ± 0.14) evidenced a significantly ($p = 0.001$) lower AEC compared to M5-group (0.78 ± 0.05).

ATP/ADP ratios in M5 and M0 treatments (3.69 ± 1.20 and 1.82 ± 0.83, respectively) were significantly different ($n = 17$; $p = 0.005$).

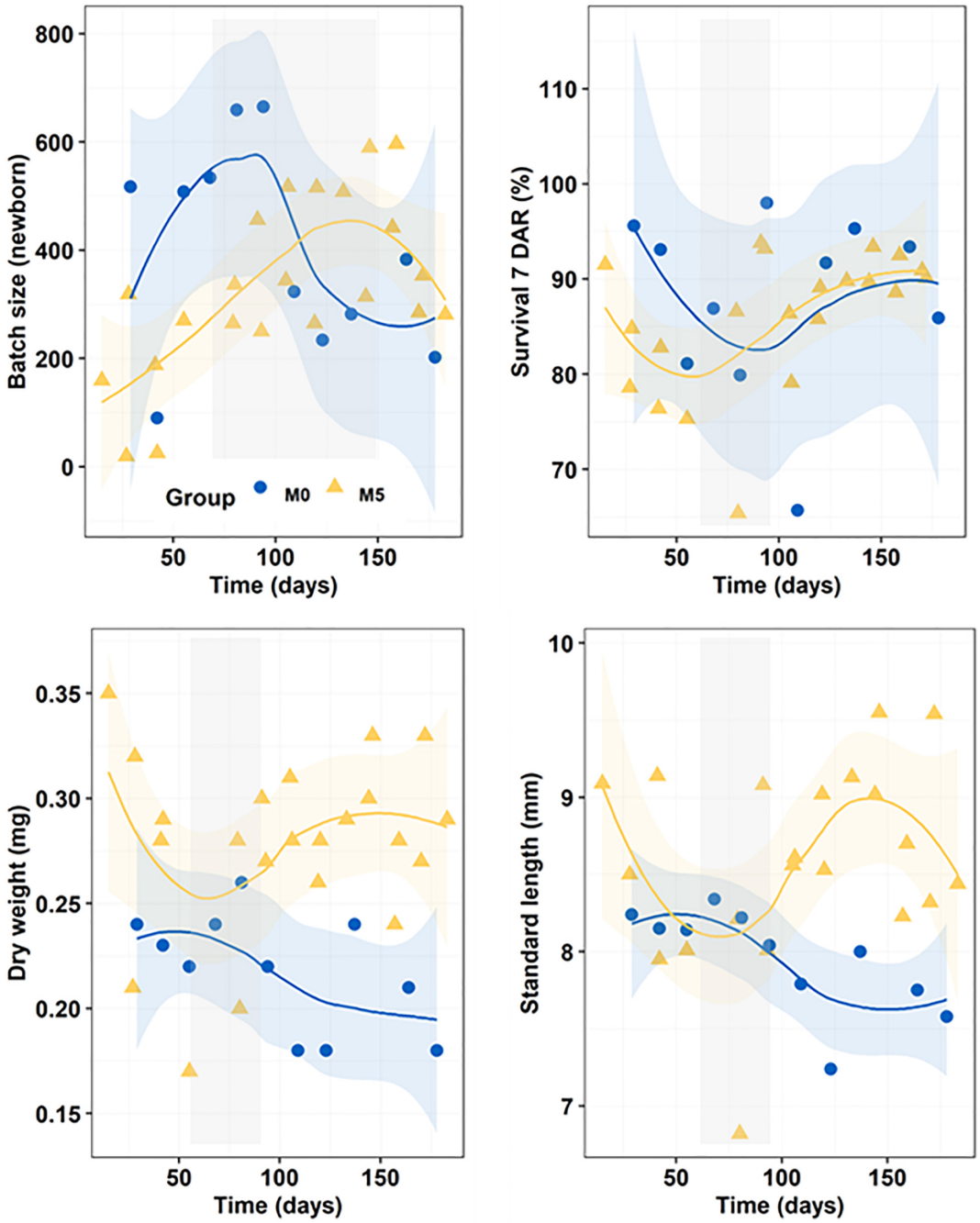

FIGURE 2 | Progressive changes (smoothed trends with 95% confidence intervals) in batch size (newborn), survival at 7 DAR (%), dry weight (DW; g), and standard length (SL; mm) along the breeding period in newborn released by seahorses from groups M0 and M5. Gray shadowed bars show inflection periods indicating a remarkable change (knee) on the smoothed trend.

Pooled adenylic nucleotides were significantly correlated with total C (df = 15, $r = 0.587$, $p = 0.01$), ADP ($r = 0.641$, $p = 0.006$), and especially ATP ($r = 0.925$, $p < 0.0001$) and NAD ($r = 0.630$, $p < 0.007$). Furthermore, NAD was also positively correlated with total C ($r = 0.544$, $p = 0.024$), AMP ($r = 0.541$, $p = 0.025$), ADP ($r = 0.693$, $p < 0.002$) and ATP ($r = 0.630$, $p = 0.007$). Also, AEC was positively correlated with ATP ($r = 0.640$, $p = 0.006$) and ATP/ADP ratio ($r = 0.722$, $p = 0.001$), and negatively correlated with AMP ($r = -0.556$, $p = 0.020$).

Changes With Breeding Season Progress

Average values for the whole breeding period in the variables assessed in newborns did not differ significantly in most cases. However, the smoothed trends showed in **Figures 2–6** revealed

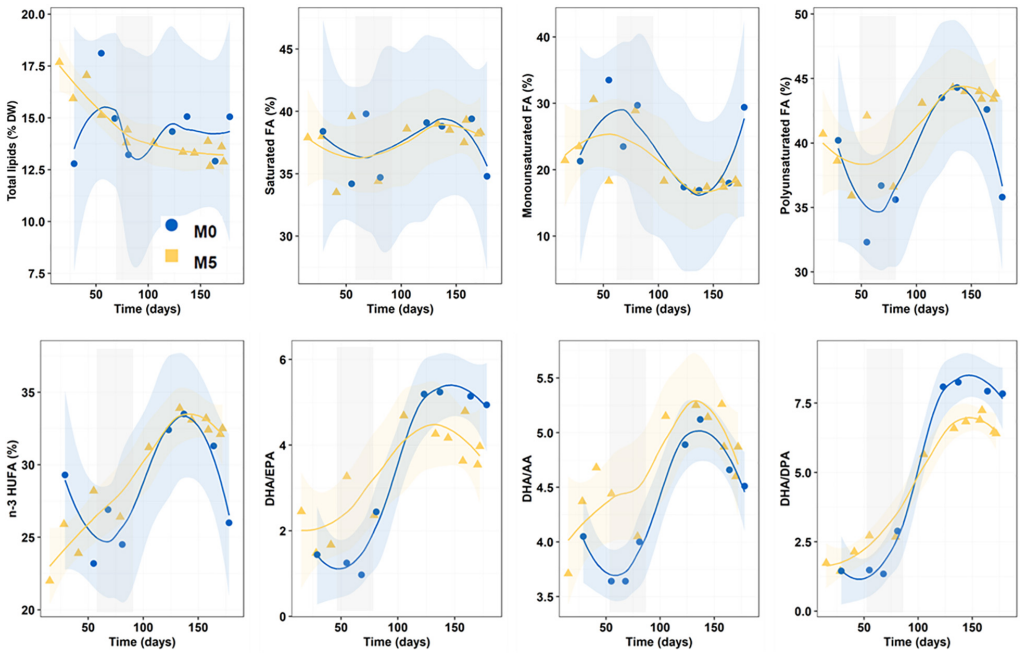

FIGURE 3 | Progressive changes (smoothed trends with 95% confidence intervals) in total lipids (dry weight percentage), fatty acid series (percentage of total FA) and fatty acid ratios along the breeding period in newborn released by seahorses from groups M0 and M5. Gray shadowed bars show inflection periods indicating a remarkable change (knee) on the smoothed trend. AA, EPA, DPA, and DHA: 20:4n-6, 20:5n-3, 22:5n-3, and 22:6n-3, respectively.

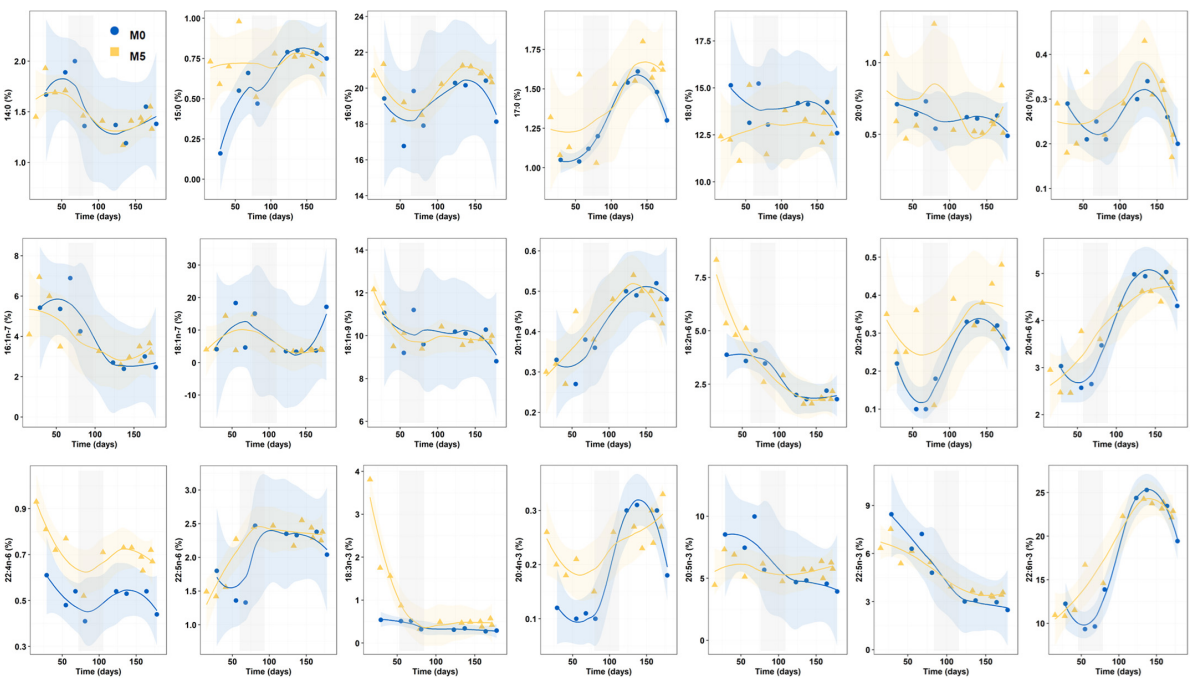

FIGURE 4 | Progressive changes (smoothed trends with 95% confidence intervals) for fatty acids (percentage of total FA) along the breeding period in newborn released by seahorses from groups M0 and M5. Gray shadowed bars show inflection periods indicating a remarkable change on the smoothed trend.

that the diverse characteristics of the batches released differed along the breeding season. Accordingly, the characteristics of batches released in the first part of the season (Period 1) differed in most cases from those released later (Period 2). Hence, a comparison between both periods was more informative than the analyses performed over the entire breeding period.

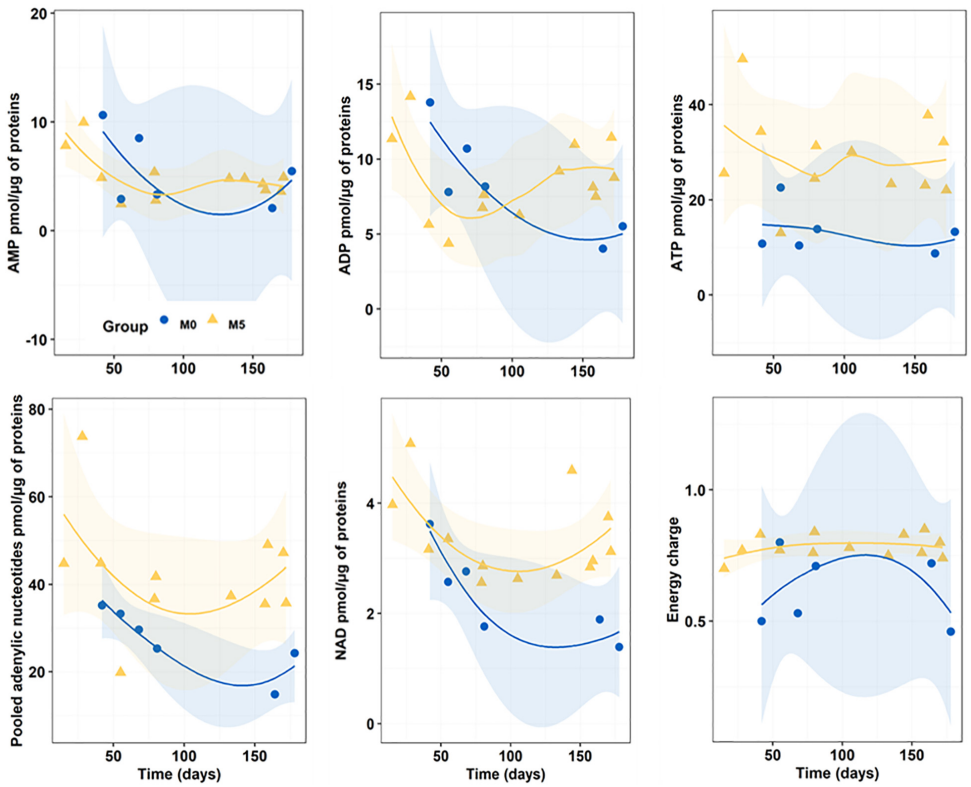

FIGURE 5 | Progressive changes (smoothed trends with 95% confidence intervals) in AMP, ADP, ATP, total adenylic nucleotides, and NAD content (pmol/µg of proteins), and in adenylic energy charge (AEC) along the breeding period in newborn released by seahorses from groups M0 and M5.

An intermediate phase, tentatively ranging from day 70 to day 100 (see gray bars in **Figures 2–4**), would indicate a transitional change between stages 1 and 3. The comparative analyses revealed that DW and SL in newborn released on stage 3 were lower in M0 than in M5 batches (K-W test, $n = 33$, $p < 0.01$). However, survivals at 7 DAR did not differ with treatments nor stages (K-W test, $n = 33$, $p = 0.370$). Concerning total lipids and FAs, the most significant differences occurred between stages 1–2 and 3, whereas MO and M5 batches released in stage 1 showed similar profiles and wide ranges for most FA. Interestingly, DHA (22:6n-3), AA (20:4n-6), and 11-eicosenoic acid (20:1n-9) contents in M0 and M5 batches from stage 1 were significantly lower than those in stage 3 ($n = 20$, $p = 0.002$).

About AMP, ADP, ATP, NAD, and AEC across groups M0 and M5, no statistically significant differences were revealed by the comparisons carried out on newborn batches produced on the first and second periods of the breeding season.

Additional data and results on statistical tests performed on newborn characteristics, lipid and FAs content, endogenous nucleotides, AEC and NAD are provided in **Supplementary Figures 1–4**. Data on the content in total C, total N and protein are shown in **Supplementary Figure 5**.

Transmission Electron Microscopy

Ultrathin sections of abdominal portions of newborn seahorses were studied by transmission electron microscopy (TEM) to analyze the ultrastructural aspects of muscular tissue. Longitudinal sections from groups M0 and M5 showed the sarcoplasm filled with myofibrils oriented parallel to the long axis of the cell. The sarcomeres displayed the characteristic Z lines, light isotrope bands I, dark anisotrope bands A, and central H-bands bisected by M lines. That sarcoplasmatic reticule appeared regularly spaced. The sarcotubules formed a continuous system around and between individual myofibrils. Sarcoplasmic reticulum cisternae in contact with invaginations of the sarcolemma, T tubules, where they formed the sarcoplasmatic triad have, also, been observed. These were located at the Z line level of sarcomeres (**Figures 6a,b**, Upper).

The oblique (**Figures 6a,b**, Middle) and transverse sections (**Figures 6a,b**, Lower) from groups M0 and M5 of a striated muscle through the A and I bands showed that the myofibrils sectioned at various levels through the sarcomeres revealed the regular different arrays of filaments. The myofibrils were represented by closely packed irregular polygonal profiles and this hexagonal organization determined by the arrangement of the thick and thin of the myofilaments was clearly visible. These are separated by a small amount of sarcoplasm containing rows of mitochondria regularly organized among the sarcomeres. The nuclei of fiber muscles, myonucleus, were located in the peripheral layer of the fiber beneath the sarcolemma. Numerous satellite cells, identified by their location inside the basal lamina and outside the sarcolemma, have been seen. They were

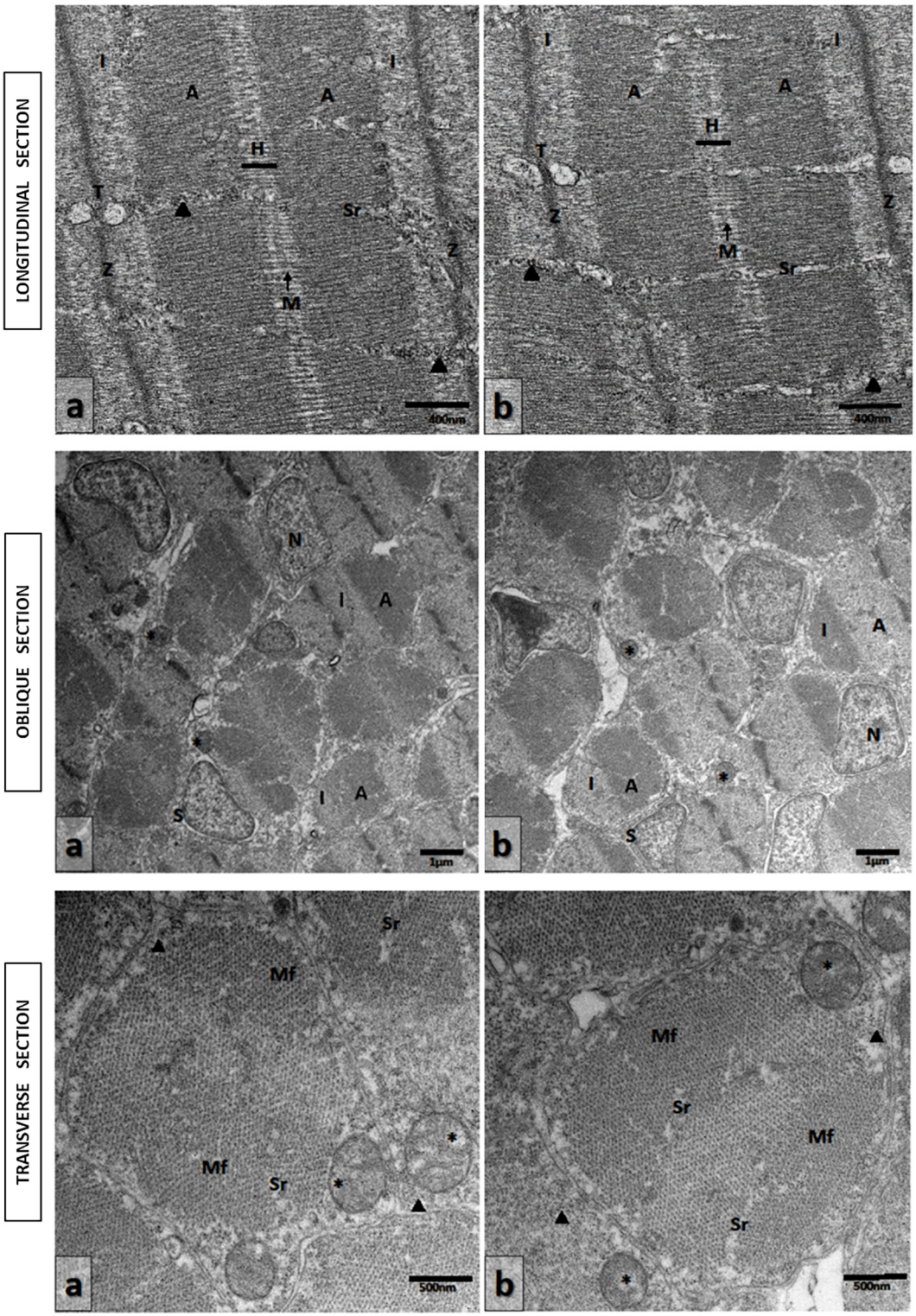

FIGURE 6 | Transmission electron micrographs of skeletal muscle in newborn seahorses from groups (**a**) M5 and (**b**) M0. *Upper*: Longitudinal sections. Sarcomeres with characteristic Z lines, I bands, A bands, and central H-bands bisected by M lines. Triad (T) located at the Z line level. Sarcoplasmic reticulum (Sr). Glycogen granules (arrowheads). Scale bars: 400 nm. *Middle*: Oblique sections at various levels through the sarcomeres. Light isotrope band (I); dark anisotrope band (A); myonucleus (N); mitochondria (asterisks); muscle satellite cell (S). Scale bars: 1 µm. *Lower*: Transverse sections. Myofibrils (Mf); mitochondria (asterisks). Sarcoplasmic reticule (Sr). Glycogen granules (arrowheads). Scale bars: 500 nm.

characterized by a very little cytoplasm around the nucleus, devoid of myofilaments and few small mitochondria. Glycogen granules were also observed (**Figures 6a,b**, Upper and Lower).

Global Assessment

The PCA performed on datasets (see variables included in **Figure 7**) revealed that factors 1 and 2 explained 61.4% of total data variability. Newborn batches released on the first half of the breeding season were displayed on the left side of the plot, whereas samples released on the second half were closer to each other and located on the right side. Factor 1 was positively associated with time (days elapsed since the onset of the breeding period), 17:0, 20:1n-9, 20:4 n-6, 22:6n-3, DHA/EPA, and DHA/DPA, and negatively related with 16:1n-7 and 22:5n-3. The plot also showed a higher similarity between samples collected on the second half of the breeding period (batches M0-2 and M5-2) compared to those on the first half of that period (M0-1 and M5-1). Factors 3 and 4 accounted for 18.2% of total variability, which was mostly explained by the content in 18:0 (M0 < M5).

%

With regards to PCA results on endogenous nucleotides and energy charge in newborn seahorses (**Figure 7**), Factor 1 (36.8%) was positively associated with NAD, ATP, and newborn weight, whereas factor 2 (21.7%) was positively related to time and survival at 7 DAR, and negatively associated with lipid content. The plot showed clear discrimination among treatments, with lower endogenous nucleotide levels in treatment M0, particularly in M0-2. The main finding in factors 3 (12.9%) and 4 (9%) biplot was the higher energy charge in newborn from treatment M5 during the whole breeding season.

DISCUSSION

Diets and Breeding Success

The results achieved on the assessment of effects of dietary treatments on the pre-breeding season in the tropical seahorse *H. reidi* denoted significant improvements in size, weight, endogenous nucleotide content and AEC in newborn, as well as several changes in the biochemical composition (FA profiles). The use of *Artemia* as the unique prey (treatment M0) resulted in lower initial fitness in newborns as compared to those fed on the mixed diet including mysidaceans (treatment M5). Since some aspects of the influence of the diet on reproductive potential and success in *H. reidi* have been assessed previously (Planas, 2021), this discussion will be mostly focused on biochemical characteristics, energetic status and tissue ultrastructure in newborns.

The experimental diets were efficiently incorporated into the breeder's tissues as assessed by stable isotopes (Planas, 2021). Hence, the results achieved would rely mainly on the biochemical characteristics of the diets provided during the pre-breeding season. The negative effect of low-quality diets (i.e., non-enriched adult *Artemia*) on fish breeding has been demonstrated in many fish species (Izquierdo et al., 2000, 2001; Piccinetti et al., 2012). Providing a mixed diet including a potential high-quality prey (i.e., mysidaceans, amphipods, or carideans) or supplementary enrichment (Murugan et al., 2009; Otero-Ferrer et al., 2012; Saavedra et al., 2014) would certainly enhance breeding success as reported in *H. guttulatus* (Palma et al., 2012, 2017; Planas et al., 2020b). Neither average lipid content nor initial survivals for the whole reproduction season resulted significantly affected by the type of pre-breeding diet offered to breeders. However, overall breeding performance (spawning events and total newborn produced), and neonates size and weight were markedly enhanced in breeders fed on the mixed diet (M5-group).

We did not observe premature egg clutch dropping nor premature newborn release in breeders fed exclusively on *Artemia*, which is in agreement with previous findings in *H. guttulatus* (Planas et al., 2020b) but not with those in *H. hippocampus* (Otero-Ferrer et al., 2012; Saavedra et al., 2015) or *Hippocampus kuda* (Saavedra et al., 2014). Therefore, the quality of breeder's diet during the pre-reproduction period should not affect postnatal viability if offspring are subsequently fed on high-quality prey such as copepods. However, it is known that the quality of male's diet offered from 1 month before conception may result in large neonates with low viability (Otero-Ferrer et al., 2016). An effect of diet quality in males has also been reported on other syngnathids (Sagebakken et al., 2017). Our findings might not be extrapolated to other syngnathid species as the early performance of newborns would likely depend upon specific characteristics such as the balance between brood size, newborn size, and survival (Planas et al., 2020b), and on parental size and age (Dzyuba et al., 2006; Faleiro et al., 2016). In that respect, our breeder's groups were similar in average weight but there were within-group differences in ranges for both size and age. Since those characteristics seem to affect further juvenile performances, it would have been interesting to analyze both growth and survival until more advanced developmental stages and to identify parents involved in each bath produced as suggested by the results obtained by Dzyuba et al. (2006). Unfortunately, parental identities for each batch produced could not be identified in the present study.

Fatty Acids Profiles

The importance of essential FAs, namely n-3 HUFA, to marine and freshwater fish larvae has been studied extensively (Sargent et al., 1999; Izquierdo et al., 2000; Kanazawa, 2003; Olivotto et al., 2010; Tocher, 2010; Zarantoniello et al., 2018), but only a few studies have been published in eggs or newborn seahorses. FA profiles in seahorse eggs, neonates and also adults largely differ depending on species, temperature and breeder's diet (Chang and Southgate, 2001; Lin et al., 2008; Faleiro and Narciso, 2010; Otero-Ferrer et al., 2010, 2012; Planas et al., 2010, 2020b; Saavedra et al., 2014, 2015; Buen-Ursue et al., 2015), hence, comparative analyses are difficult to assess. Despite the large differences in the proportion of dietary FA groups, average relative proportions in saturates, monoenes, polyenes, and n-3 HUFA in newborn seahorses were rather similar across our dietary treatments. That finding indicates that the production of eggs/neonates in *H. reidi* is accomplished following a conservative plan for global FA groups, essential FAs (arachidonic and docosahexaenoic acids) and FA ratios.

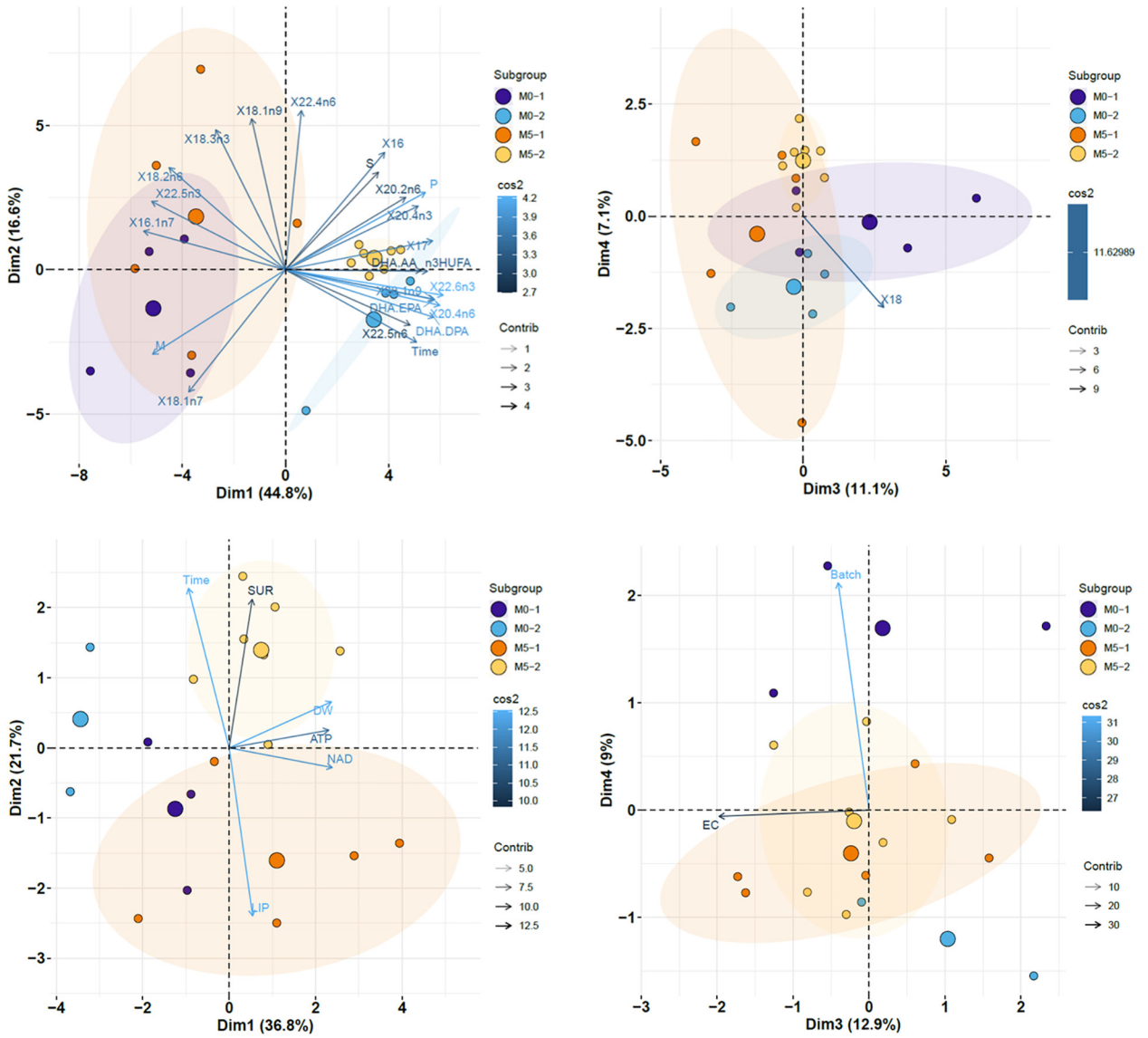

FIGURE 7 | Factor score plots for the principal component analysis (PCA) on newborn seahorses released by breeders in groups M0 and M5, considering batches released on the first (M0-1 and M5-1; 0–100 DAR) and second (M0-2 and M5-2; >100 DAR) half of the breeding season. Only the most contributing variables ($cos^2 > 0.6$) are shown. Ellipses correspond to 95% confidence intervals. Original variable names were automatically modified by R software. Variables in upper PCA plots: days elapsed since the onset of the breeding period (Time), SL, DW, survival at 7 DAR, total lipids, saturated FA (S), monoenes (M), polyunsaturated FA (P), n-3 HUFA, n-3 FA, n-6 FA, n-3/n-6 (n3.n6), DHA/EPA, DHA/AA, and individual FA. saturated FA (S), monoenes (M), polyenes (P), n-3 HUFA, DHA/EPA, DHA/AA, DHA/DPA, and individual FA. Variables in lower PCA plots: Time, SL, DW, survival at 7 DAR, AMP, ADP, ATP, NAD, and AEC (Adenylic energy charge).

Interestingly, the shapes of the patterns followed by those FAs and ratios in *H. reidi* resembled those in *H. guttulatus* if time lags and differences in values are not considered (**Figure 8** and **Supplementary Figure 6**). Conversely to AA and DHA, EPA values in both species along the second phase of the breeding season followed different patterns. These three FA, especially DHA and AA, are essential biochemical compounds in marine fish larvae, being highly dominant within the total pool of FA (Rainuzzo, 1993; Rainuzzo et al., 1997; Sargent et al., 1999). DHA is the main FA in both vitellogenin and developing embryos of marine fishes (Sargent et al., 1999; Tocher, 2003). However, their stores in young seahorses may be partially used as fuel, especially EPA, which is consumed at a higher rate (Faleiro and Narciso, 2010). Differences in EPA catabolism compared to AA and DHA were likely reflected in differences in the patterns of change discussed above. When comparing *H. reidi* and *H. guttulatus*, time lags observed for certain FA and ratios (e.g., AA, DHA/EPA, and DHA/DPA) might be due to species-specific differences on inter-batch intervals and temperature thresholds. The ratio DHA/EPA and the content in AA seem to be higher in species from warmer water temperatures (Dey et al., 1993; Ogata et al., 2004; Ibáñez, 2018) but the validity of those assumptions would

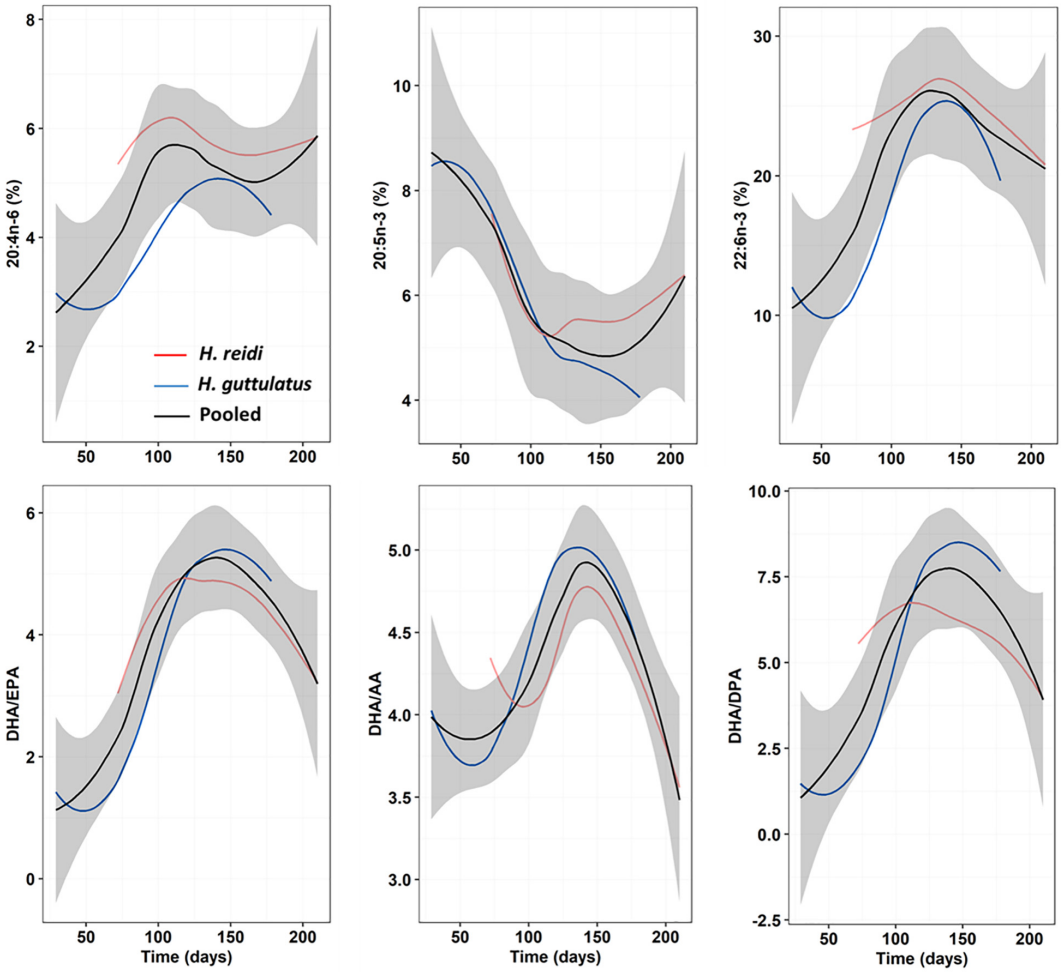

FIGURE 8 | Progressive changes (smoothed trends) in essential FA (AA, EPA, and DHA) and FA ratios (DHA/EPA, DHA/AA, and DHA/DPA) along the breeding period in newborn released by *H. reidi* (red line; present study) from group M0. For comparison purposes, the same data are provided for *H. guttulatus* breeders (blue line; Planas et al., 2020b) submitted to a similar dietary treatment. The black curve shows the loess fitting with the confidence interval (95%) in the gray shading for pooled M0-samples from both species.

likely depend on a series of factors other than temperature (e.g., breeder's diet).

The interest of analyzing by TEM the ultrastructure of muscular tissue in newborns was due to a previous study in which newborn of *H. guttulatus* released by breeders fed on *Artemia* during the reproduction season showed the presence of ultrastructural muscle alterations (Planas et al., 2020b), likely due to deficiencies in DHA (Cong-cong et al., 2019). The observations by TEM have not detected morphological alterations nor differences as for both analyzed groups M0 and M5, suggesting that both administered diets ensured the regular development of muscular tissue.

Adenylic Energy Charge

The energy status of an organism is fully charged when it consists of ATP alone (adenylate charge = 1.0). A charge value of 0.5 indicates that only ADP is present, whereas the energy charge (AEC) is zero when all adenine nucleotide pool is in form of AMP (system fully discharged) (De la Fuente et al., 2014). The AEC might be a relatively conservative property and changes in AEC are always accompanied if not preceded by changes in the quantity of adenylate nucleotides (Atkinson, 1968). Commonly, AEC values in unstressed organisms are above 0.80 (Ivanovici, 1980; Vetter and Hodson, 1982), so that AEC was proposed as an indicator of the energy potentially available for cellular metabolism (Atkinson, 1977; Dickson and Franz, 1980). Interestingly, ATP concentration, total adenylate concentration, and AEC might be useful indices of environmental impact (i.e., stress) (Vetter and Hodson, 1982; Dehn and Schirf, 1986; Methling et al., 2017) and condition factor in fishes (Giesy, 1988). However, AEC level did not always reflect the growth of organisms, as reported by Harms (1992) in sub-optimally fed larvae of the crab *Hyas araneus*, in which pooled adenylic nucleotides were found to be correlated with the content in total C and protein, reflecting differences in the experimental diets. In our study, pooled adenylic nucleotides were correlated with

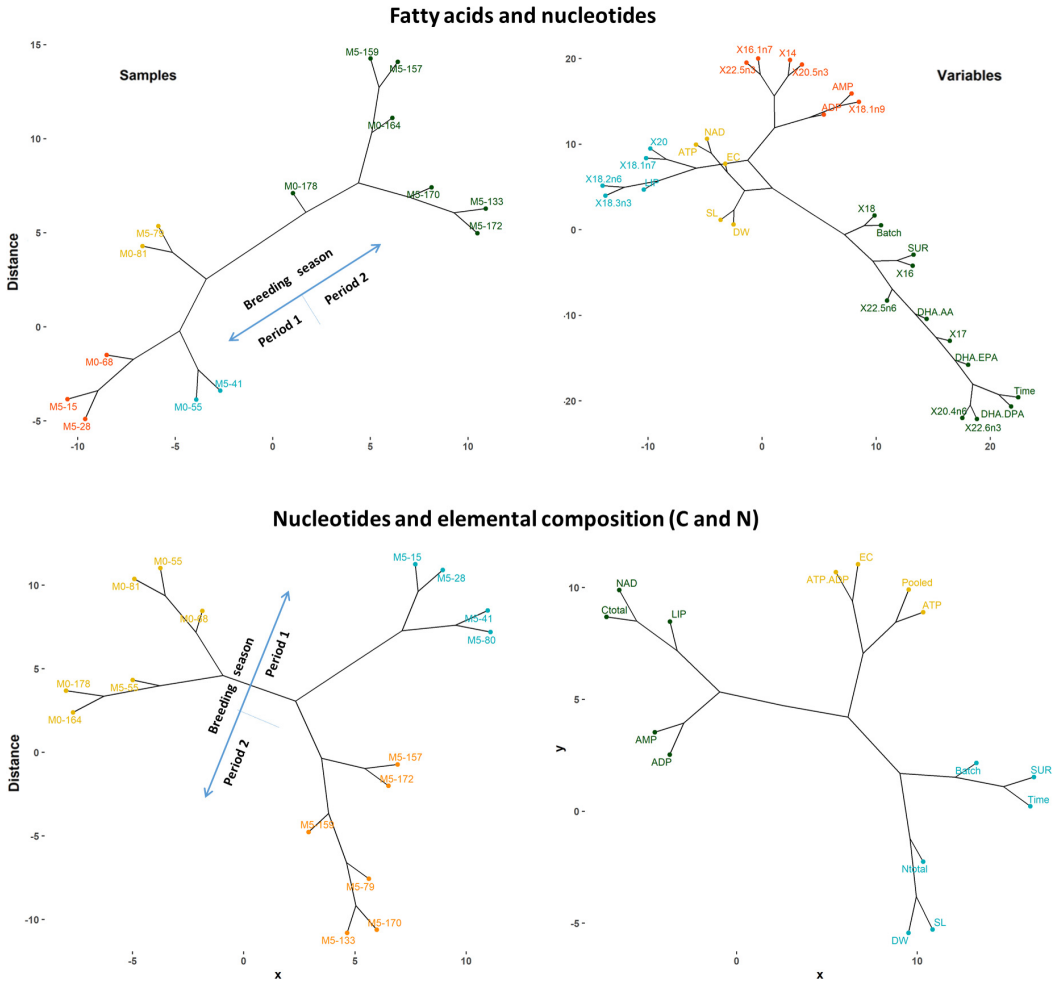

FIGURE 9 | Hierarchical cluster plot (complete linkage, Pearson method) for samples (*left*) and variables (*right*) analyzed for fatty acids, nucleotides, and elemental composition (C and N) in newborn batches. Original variable names were automatically modified by R software. Sample identifiers Mx-y indicate experimental group (Mx) and days elapsed since the onset of the breeding period (y). Variables: days elapsed since the onset of the breeding period (Time), batch size (Batch), length (SL), dry weight (DW), survival at 7 DAR (SUR), AMP, ADP, ATP, Pooled (AMP + ADP + ATP), NAD, adenylate energy charge (EC), total C, total N, total lipids (LIP), fatty acids (>1% percentage), DHA/EPA, DHA/AA, and DHA/DPA.

total C, ATP, and NAD but not with total N (protein content: 65.9 ± 1.4 and 65.1 ± 0.8% in M5 and M0 newborn, respectively). Hence, increases in ATP were reflected in increases in AEC values and total adenylate concentration rather than in declines in ADP or AMP (Vetter and Hodson, 1982). The pool of free amino acids from yolk proteolysis increases in demersal fish eggs during ripening (Craik and Harvey, 1984; Greeley Calder and Wallace et al., 1986). In many teleosts, the pool constitutes the main energy source at that stage, during embryogenesis, and in newly hatched larvae throughout the stage of endogenous nutrition (mostly in larvae lacking oil globules) (Rønnestad et al., 1999). However, lipids are an important initial fuel store in many early developing fish larvae, especially in lecithotrophic larvae with oil globules (Rainuzzo et al., 1997). Our results on nucleotides and AEC in newborn seahorses seem to confirm those findings.

Total adenylic nucleotides pools (mostly reflected in ATP content) and AEC values in batches from M5-treatment were quite stable along the breeding season and similar between batches, approaching 0.8 for AEC (0.78 ± 0.05). In treatment M0, the values were lower (0.62 ± 0.14 for AEC) and much more variable, suggesting a worse energetic fitness in newborn. The findings support the statement of Atkinson (1968), who pointed out that the AEC might be a relatively conservative property and that changes in AEC are always preceded or accompanied by changes in the level of adenylate nucleotides. The differences achieved between treatments for AEC and total adenylic nucleotides pools reflected the food conditions of parents during the pre-breeding period.

The initial ontogeny of fishes is characterized by low assimilation efficiencies, very high growth rates and high energy-consumption for hunting and swimming. The first complete energy allocation budget (discriminating costs of maintenance, growth, and activity) available for early larval stages of marine teleost fish species was carried out by Cunha et al. (2007) in

turbot (*Psetta maxima*) larvae. Those authors reported a daily weight-specific growth rate of 29.6% day^{-1}, and suggested a trade-off between fast growth and viability in the larvae. With growth rates of up to about 25% day^{-1}, the energy demands for growth represent a large fraction of the energy available (Pedersen, 1997). The ATP/ADP ratio is a robust readout of the cellular energy status and its value should remain high in order to maximize the energy status and cell metabolism. Conversely, energy depletion with an accumulation of AMP would deplete the cell's stores of ATP and ADP leading to cell death. Considering the higher AEC values in M5-newborn compared to M0-newborn (3.69 ± 1.20 and 1.82 ± 0.83, respectively), a greater amount of energy would be available in the former for growth and cell metabolism. The values of ATP/ADP ratio in newborn seahorses support that statement, showing that suboptimal feeding conditions in breeders were reflected in the nucleotide pool and energy charge of newborn. In this context, higher initial growth rates would be expected in M5-newborns. However, neither survivals nor specific growth rates until day 7 differed significantly (SGR: 17.7 ± 7.4 and 20.8 ± 4.5% day^{-1} in M5- and M0-juveniles, respectively) (data not provided). Those growth rates were high but markedly lower than in larvae of other fish species, likely due to low digestion and assimilation efficiencies in early developing seahorses (Novelli et al., 2015; Ofelio et al., 2018; Valladares and Planas, 2021). Seahorse newborn from treatment M0 enhanced their AEC values along the first stage of the breeding season, suggesting a progressive energetic improvement. Increasing AEC values in newborn might facilitate a rapid resumption of synthetic activity when accessing high-quality food (Harms, 1992) such as copepods (Olivotto et al., 2008; Palma et al., 2017; Randazzo et al., 2018).

NAD plays an important role as energy and signal transducer in cellular metabolism and can be synthesized from NMN (nicotinamide mononucleotide) and ATP. Overall, ATP, total adenylic nucleotides and endogenous NAD in seahorse newborns were highly correlated, and their levels in group M5 were about 2. 3-, 1. 7-, and 1.4-fold higher than in newborns from group M0. Studies on this subject in marine fish larvae are extremely scarce but our findings agree with those reported in developing clownfish (*Amphiprion ocellaris*) larvae by Olivotto et al. (2011a). In that study, the authors suggested that the enhancement in HUFA content in preys may improve larval development through the presence of better-structured mitochondria, and higher energy status and ATP synthesis compared to the control. Consequently, growth performance and survivals enhanced in clownfish larvae supplemented with HUFA and the larval phase was shortened, resulting in better larval condition to undertake metamorphosis.

In seahorse neonates, yolk reserves are lacking or almost exhausted by the time of male's pouch release (Novelli et al., 2015; Ofelio et al., 2018). Hence, the quantity of lipid of energy reserves would be particularly important for the newborns to withstand starvation or low-quality prey supply, especially in species lacking yolk sac at hatching (e.g., seahorses). However, the presence of ontogenetic shifting in the energy allocation strategy (growth vs storage) demonstrated in other fish larvae (Cunha et al., 2007; Olivotto et al., 2011a; Di Pane et al., 2019) is unknown in seahorses, deserving further investigation.

Breeding Strategy

The pattern of change throughout the breeding season was investigated to identify and assess the effects with time of pre-breeding feeding on the variables analyzed (see **Figures 2–5** and **Supplementary Figure 6**) and the timing of energy allocation to reproduction. The detection of knee-phases and their onset on general patterns for each variable were used to determine the breeding strategy followed by *H. reidi*. In a previous study, we applied a similar assessment to the temperate seahorse *H. guttulatus*, whose breeders fed on different diets (but similar to those used in the present study) during the breeding season (Planas et al., 2020b). Our results in *H. reidi* were highly similar to those achieved in *H. guttulatus*. Both species displayed a mixed capital-income strategy, which implies a progressive decrease in newborn quality along the breeding season (McBride et al., 2015; Griffen, 2018). Due to species-specific characteristics concerning temperature and inter-brood intervals, the knee-phase in *H. reidi* occurred about 40–50 days earlier than in the temperature species. Inter-brood intervals are about 14–16 days at 25–27°C in *H. reidi* (Otero-Ferrer et al., 2016) and 27–35 days at 18–20°C in *H. guttulatus* (Planas et al., 2010).

In most cases, knee-periods identified in general trends for *H. reidi* were located within the period comprising days 70 and 100 since the onset of the breeding season, depending on the variable and treatment considered. Hence, samples of newborn released before day 100 were notably different to those subsequently released, as confirmed by the PCS analysis and the hierarchical cluster analysis provided in **Figure 9**. The similarity dendrograms also showed high differences among groups from both dietary treatments, particularly for those released before day 100. Accordingly, three stages can be recognized along the whole breeding period:

1. Period 1 (mixed capital-income sources): this stage would extend until days 70–100 since the onset of the breeding season. Most energy and nutritional resources allocated to eggs/newborn originated from the diet provided before the onset of the breeding period (capital breeding). However, the importance of income breeding (sources gained to the experimental diet) increases gradually.
2. Inflection period (income sources): this is a transitory period shown by a knee on smoothed curves. Considering the whole datasets, the duration of this stage would be about 1 month, indicating the end of the capital-income breeding period and the use of nutritional resources from the breeder's diet.
3. Period 2 (income sources and exhaustion of body reserves): globally, the similarity between dietary treatments increased compared to samples released in Period 1. This statement is especially true for FAs profiles. Conversely, differences for total adenylic nucleotides and NAD were maximal by the end of this period. The drop observed

in those products and in survivals from group M0 was noticeable. These findings together with the values for DHA and DHA/EPA in late batches suggest different levels of body resources exhaustion, particularly notorious in M0-newborn as the result of limitation in the availability of nutrients for egg/newborn formation. In this period, brood size and newborn size, and weight were considerably lower in treatment M0.

Breeding patterns are a conditional reproductive strategy since patterns along the capital–income continuum may shift with ontogeny or environmental conditions (McBride et al., 2015; Williams et al., 2017). Shiftings on the acquisition of nutritional and energy supplies for newborns along the breeding season were reported in *H. guttulatus* (Planas et al., 2020b) and validated for *H. reidi* in the present study. Shifting patterns, timing and effective duration of feeding treatments are crucial factors to understand breeding success in synchronous species such as seahorses. The duration of the improvement in eggs or fry quality is a pivotal aspect that must be precisely determined. However, the data available are rather contradictory. Some characteristics in newborn resulted notably enhanced in most M5-batches along the whole reproductive period compared to those from treatment M0. This finding agrees with our previous study on *H. guttulatus*. However, the effect of enhanced diets provided to *H. kuda* females on egg size was limited to the first spawning (Saavedra et al., 2014). From a nutritional and energetical point of view, the roles played by seahorse males and females in the production of eggs and neonates remain unclear, even though the contribution of males (and the effect of their diets) seems to be important in nutritional supply (Skalkos et al., 2020) and a key determinant in newborn quality (Otero-Ferrer et al., 2020).

CONCLUSION

The results achieved in this study in *H. reidi* demonstrate the importance of feeding breeders on a high-quality diet (M5-treatment) during the pre-breeding period. The improved diet, including *Artemia* and mysidaceans during the whole breeding season, resulted in more reproductive events, and larger size and higher energetic status (ATP, total adenylic nucleotides, AEC, and NAD) in neonates. Initially, the effect of dietary treatments on n-3 and n-6 HUFA, especially on DHA, were lower than expected but noticed in FA ratios. This finding suggests (but not demonstrates) lower requirements in n-3 HUFA than other seahorse species. The species displayed a mixed capital-income breeding continuum, as previously demonstrated in *H. guttulatus*, in which the effects of the pre-reproductive diet were mainly noticed on the first 2–3 months of the breeding season. Even though the further newborn performance was not jeopardized when the juveniles gained access to high-quality prey, the effect of parental diets should deserve further investigation under low quality or availability of food to seahorse juveniles.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories (http://dx.doi.org/10.17632/n6zt5zw22w.1).

ETHICS STATEMENT

The animal study was reviewed and approved by the Xunta de Galicia (REGA ES360570202001/15/FUN/BIOL.AN/MPO01).

AUTHOR CONTRIBUTIONS

MP: funding acquisition, project administration, conceptualization, methodology, fatty acids and other analyses, data curation, formal analysis, visualization, writing—original draft preparation, and writing—review and editing. IO: formal analysis, data curation, visualization, and writing—review and editing. MG: methodology, fatty acids analysis, and data curation. RL: methodology, TEM analysis, formal analysis, visualization, and writing—review and editing. CA and AA: methodology, nucleotides analysis, data curation, and writing—review and editing. MZ: methodology, nucleotides analysis, data curation, formal analysis, and writing—review and editing. 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.2021.688058/full#supplementary-material

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Effects of Temperature and Salinity on Egg Production, Hatching, and Mortality Rates in *Acartia ohtsukai* (Copepoda, Calanoida)

Seo Yeol Choi[1], Eun Hye Lee[2], Ho Young Soh[1,2] and Min-Chul Jang[3]**

[1] Department of Ocean Integrated Science, Chonnam National University, Yeosu, South Korea, [2] Department of Environmental Oceanography, Chonnam National University, Yeosu, South Korea, [3] Ballast Water Research Center, Korea Institute of Ocean Science and Technology, Geoje, South Korea

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***Correspondence:**
Ho Young Soh
hysoh@chonnam.ac.kr
Min-Chul Jang
mcjang@kiost.ac.kr

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The calanoid copepod *Acartia ohtsukai* predominates the estuarine and coastal waters of East Asia during summer. Its occurrence characteristics confer it with good potential as live prey for fish larvae through mass culture. To investigate the effect of temperature and salinity combinations on its egg production rate (EPR), hatching success (HS), and mortality rate, experiments were undertaken and repeated three times for combinations of five temperatures (10, 15, 20, 25, and 30°C) and seven salinities (10, 15, 20, 25, 27, 30, and 33 psu). EPR and HS were highest at temperatures of 25 and 30°C, respectively, with a salinity of 27 psu. Mortality rate was highest at 10°C in almost all salinity gradients, whereas it was lower at water temperature and salinity ranges of 20–30°C and 20–30 psu, respectively. These findings indicate that *A. ohtsukai* can inhabit wide ranges of water temperatures and salinities, and that the optimized condition for mass culture is a combination of water temperature of 25°C and salinity of 27 psu.

Keywords: Acartiidae, intensive culture, reproduction, Yeoja Bay, egg production rates

INTRODUCTION

In the aquaculture industry, *Artemia* spp. and rotifers have widely been used as prey for fish larvae (Hoff and Snell, 1999; Chesney, 2005), although fish larvae prefer copepod nauplii to comprise more than 50% of the stomach contents (Støttrup, 2000). However, a lack of variation in sizes and nutritional contents has led to the search for alternative prey sources. Copepods, which connect primary producers such as phytoplankton and higher consumers in the marine food web, have been considered as excellent live feeds owing to the size variation in their developmental stages as well as nutritional sufficiency in terms of fatty acids, free amino acids, and other essential micronutrients (Sargent and Falk-Petersen, 1988; McEvoy et al., 1998; Støttrup, 2000, 2003; van der Meeren et al., 2008). In particular, since copepods mainly contain essential *n*–3 highly unsaturated fatty acids (HUFA), docosahexaenoic acid (22: 6n–3; DHA), eicosapentaenoic acid (20: 5n–3; EPA), and phospholipid *n*–6 HUFA arachidonic acid (20: 4n–6; ARA), it is critical for marine fish larvae to feed on them to ensure adequate growth and development (Gapasin and Duray, 2001; Bell et al., 2003). Additionally, marine fish larvae fed with copepods instead of *Artemia* spp. and rotifers have advantages such as higher survival, better pigmentation, and more robust growth (Naess et al., 1995; Støttrup and Norsker, 1997; Wilcox et al., 2006).

Several studies on the mass cultivation of copepods have been undertaken to investigate their commercial use as live prey for fish larvae (**Table 1**), but only a few species have successfully been reared for mass cultivation for aquaculture because there is very little information available on the physiological processes and population dynamics of the best candidates (van der Meeren and Naas, 1997; Støttrup, 2000, 2003). Of the calanoid copepods, *Acartia tsuensis*, *Acartia tonsa*, and *Eurytemora affinis* have been considered for use in mass cultivation (Ban, 1994; Takahashi and Ohno, 1996; Peck and Holste, 2006). The egg production rate (EPR), hatching rate, survival/mortality, and growth of nauplii and copepodites of these species are affected by various environmental factors such as water temperature, salinity, pH, and quantity and quality of food (Chinnery and Williams, 2004; Holste and Peck, 2005; Peck and Holste, 2006).

In particular, temperature and salinity are the two most important environmental variables that affect the growth and egg production of marine copepods (Miller and Marcus, 1994; Peck and Holste, 2006). Rhyne et al. (2009) found that temperature had a significant effect on nauplii production and survival in *Pseudodiaptomus pelagicus*. Ban (1994) showed that temperature altered egg production and adult size in *E. affinis*. Previous studies on *Acartia* species indicated that salinity was not completely responsible for the hatching success (HS) of eggs (Chinnery and Williams, 2004; Dutz and Christensen, 2018; Wilson et al., 2021). Dutz and Christensen (2018) showed that there were no significant differences in the HS of eggs in the brackish water species *Acartia longiremis* at different salinities (4, 5, 6, 7.7, and 16 psu). The netric species *Acartia fancetti*, which inhabits hypersaline areas, showed no significant differences in the HS of eggs at different salinities (30, 40, and 50 psu), except at 60 psu. However, Holste and Peck (2005) showed that the HS of eggs produced by the copepod *A. tonsa* significantly decreased under a salinity of less than 15 psu. Variations in salinity were found to lead to different rates of population growth and egg hatching in the tropical copepod *Acartia sinjiensis* (Milione and Zeng, 2008). Notably, Chinnery and Williams (2004) reported that salinity treatment altered egg hatching rates and nauplii survival in four *Acartia* species. Several studies have also indicated an interactive effect of salinity and temperature (Bradley, 1986; Nagaraj, 1988). Such discrepancies in results indicate high levels of phenotypic plasticity among copepod species in terms of the salinity effect (Wilson et al., 2021). Thus, assessing the effects of temperature and salinity on calanoid copepods can provide information for their utilization as live prey in aquaculture.

Acartia ohtsukai, which occurs in the estuarine and coastal waters of Korea, China, Vietnam, and Japan (Razouls et al., 2005-2020), is also an excellent candidate live prey species for aquaculture, because it is found in high densities owing to its adaptability to rapid changes in temperature and salinity (Choi et al., 2019; Lee et al., 2020). *A. ohtsukai* dominates in summer and autumn in Yeoja Bay, comprising more than 60% of the zooplankton community in the inner bays in September (Lee, 2019). The aim of the present study was to understand the combined effect of temperature and salinity on egg production, HS, and mortality in *A. ohtsukai*, with the ultimate aim of assessing its use in mass cultivation.

MATERIALS AND METHODS

Sampling and Rearing for the Experiments

The present experiments used *A. ohtsukai* from a single population. *A. ohtsukai* individuals were collected using a conical net (mesh size 200 μm, mouth opening size 45 cm) from Sangjin Port (34°48′43″ N, 127°24′22″ E) in the northern part of Yeoja Bay, South Korea on August 9, 2019. At the sampling site, the water temperature was 29°C and the salinity was 26 psu. The stock culture was stored in 20 L carboys filled with 1 μm filtered seawater at a salinity of 25 ± 1 psu and a temperature of 30 ± 1°C, and gentle aeration was provided through a 1 ml serological pipette at the bottom of the carboy. Water quality was maintained by replacing 100% of the culture water once every 5 days. The photoperiod was maintained at 12L–12D (12 h light:12 h dark) and a light intensity of 120 lux. Temperature and salinity were measured daily during the experiments (WM-32EP, DDK-TOA CO., Japan). For culture maintenance and experiments, daily rations of marine microalgae *Isochrysis galbana* (4.5 μm diameter) and *Tetraselmis suecica* (8 μm diameter) were provided, and a mixed diet of 40,000 cells/ml and 3,000 cells/ml (Milione and Zeng, 2007), respectively, was fed. Microalgae *I. galbana* (Haptophyceae) and *T. suecica* have been used in previous diet experiments of the temperate copepod *A. tonsa* (Støttrup et al., 1986; Feinberg and Dam, 1998). Microalgae cultures were grown in a 2 L roller bottle under a fluorescent light regime of 12L–12D with an intensity of 2500 lux. Microalgae *I. galbana* and *T. suecica* were grown in f/2 medium (Guillard and Ryther, 1962). The culture conditions were kept stable until the end of the experiments.

Egg Production Rate

Experiments were conducted to investigate the EPR of *A. ohtsukai* in relation to a wide range of culture temperatures and salinities. The daily EPR of *A. ohtsukai* was quantified at seven salinities (10, 15, 20, 25, 27, 30, and 33 ± 1 psu) and five temperatures (10, 15, 20, 25, and 30 ± 1°C). In previous studies, the critical points of water temperature and salinity of *A. ohtsukai* have been reported to be 10–30°C and 4–30 psu, respectively (Youn and Choi, 2008; Park et al., 2015). Since eggs incubated for 48 h at low salinity could burst and be erroneously identified as hatching eggs, the low salinity treatment was not used (Holste et al., 2004). Copepods used in the experiments were gradually adapted to various water temperatures and salinities via increments of 5°C and 2 psu every 6 h until the required water temperature and salinity level were reached. Three replicates were set up for each treatment for egg production experiments. Twenty-one replicate sets per temperature were used, with a total of 105 sets of *A. ohtsukai* adults and 200 ml beakers with 2 females and 2 males, respectively. A U-shaped 100 μm mesh was attached to the bottom of each of the 200 ml beakers to prevent egg cannibalism and facilitate egg collection. After approximately 24 h of incubation, all the contents of the replicate bottles were drained into a 40 μm mesh and the

TABLE 1 | The table provides information on copepod culture.

Species	Temperature, salinity	Light regime and food	Region	References
Acartia grani	19°C, 38 ppt	12L/12D, *Rhodomonas salina*	Barcelona Harbor (Spain)	Drillet et al., 2011*
Acartia sinjiensis	27–30°C, 30–35 ppt	18L/6D, *Tetraselmis chuii* and T-iso	Townsville Chanel (Australia)	Drillet et al., 2011*
Acartia southwelli	25–30°C, 15–20 ppt	12L:12D, *Isochrysis galbana*	Pingtung (Taiwan)	Drillet et al., 2011*
Acartia tonsa	17°C, 30 ppt	Dim light, *Rhodomonas salina*	Øresund (Denmark)	Drillet et al., 2011*
Amphiascoides atopus	24.5–30.7°C, 30–33‰	12L:12D, cultured phytoplankton	United States	Drillet et al., 2011*
Eurytemora affinis	10–15°C, 15 ppt	12L:12D, *Rhodomonas marina*	River Seine Estuary (France)	Drillet et al., 2011*
Eurytemora affinis	10–15°C, 15 ppt	12L:12D, *Rhodomonas marina*	Gironde Estuary (France)	Drillet et al., 2011*
Eurytemora affinis	10–15°C, 15 ppt	12L:12D, *Rhodomonas marina*	Loire Estuary (France)	Drillet et al., 2011*
Eurytemora affinis	10–15°C, 15 ppt	12L:12D, *Rhodomonas marina*	Baie de l'Isle Verte/St Laurent Estuary (Canada)	Drillet et al., 2011*
Oithona davisae	20°C, 30 ppt	natural light, *Oxhyrrhis*	Barcelona Harbor (Spain NW Mediterranean)	Drillet et al., 2011*
Acartia tonsa	18 and 23°C, 25 to 30 psu	12L/12D, *Rhodomonas* sp.	Charlottenlund (Denmark) Kiel Bay (Germany)	Peck and Holste, 2006
Acartia sinjiensis	10–34°C, 10–50 psu	12L:12D, *Tetraselmis chuii* and T-iso	Townsville Chanel (Australia)	Milione and Zeng, 2008
Acartia tsuensis	24.5–30.7°C, 30–33‰	12L:12D, *Nannochloropsis oculuta*	Momoshima (Japan)	Ohno et al., 1990
Apocyclops royi	28°C, 0–35 psu	12L:12D, *Isochrysis galbana*	(Taiwan)	Pan et al., 2016
Pseudodiaptomus incisus	26, 30, and 34°C, 0–40 ppt	12L:12D, *Isochrysis galbana*	Cam Ranh Bay (Vietnam)	Nguyen et al., 2020
Oithona rigida	26–30°C, 28–34‰	natural light, *Chlorella marina*	Vellar Estuary (India)	Santhanam and Perumal, 2012
Parvocalanus crassirostris	26°C	16L:8D, T-iso	Townsville (Australia)	Alajmi and Zeng, 2015

*Review in Drillet et al. (2011).

numbers of eggs that remained on the mesh were determined and recorded. All samples were counted and recorded under a stereo microscope at ×20–40 magnification (Nikon SMZ 745; Nikon, Japan).

Hatching Success

To investigate the effect of temperature and salinity on HS, the eggs spawned at the bottom of the beaker during the EPR experiments were collected by siphoning. After these eggs were rinsed with filtered seawater and randomly distributed with 20 eggs per well in six-well cell culture plates, HS was observed at 12 h intervals for 48 h. Each of the six-well cell culture plates used parafilm to minimize contaminants and evaporation. The HS (%) was calculated by dividing the number of eggs hatched after 48 h by the number of original eggs. The water temperature and salinity gradients for measuring HS were the same as those used for EPR.

Mortality Rate

To investigate the effect of temperature and salinity on mortality rate, healthy adult *A. ohtsukai* females (n = 10) were placed in 200 ml beakers with seven salinities (10, 15, 20, 25, 27, 30, and 33 ± 1 psu), and the beakers were incubated in a Multi-Room Incubator (WIM-RL4, DAIHAN Scientific Co., Wonju, South Korea) set to five temperatures (10, 15, 20, 25, and 30 ± 1°C). Females were observed three to five times (5–8 h intervals) a day under a stereo microscope (Nikon SMZ 745; Nikon, Japan) for 10 days or until death. *A. ohtsukai* individuals were transferred from a 200 ml beaker with water prepared once every 3 days to a beaker with the same conditions to minimize manipulation stress. Other experimental conditions were maintained according to the stock culture protocol. The mortality rate of the *A. ohtsukai* females was calculated as follows:

$$\textit{Mortality rate}\ (\%) = (\textit{total dead adults}/\textit{total surviving adults}) \times 100 \quad (1)$$

Statistical Analysis

Data from all experiments were analyzed by two-way analysis of variance (ANOVA) without replication. When a significant difference ($p < 0.05$) was found, it was tested using the Tukey's multiple comparison test. All statistical analysis was performed using the SPSS program version 20.0 (SPSS Inc., Chicago, IL, United States). Standard errors for EPR, HS, and mortality are presented to show the variation within each treatment.

RESULTS

Egg Production Rate

Egg production rates of *A. ohtsukai* were significantly different at various water temperature and salinity combinations (**Table 2**). The EPR of *A. ohtsukai* ranged from 1.90 ± 0.40 to 11.10 ± 1.20 eggs f^{-1} d^{-1} (eggs per female per day) depending on the water temperature and was highest at 25°C and lowest at 10°C (**Figure 1**). An exceptionally high EPR of more than 11 eggs f^{-1} d^{-1} was observed at 20–30°C. However, the EPR at 10 and 15°C was lower than 5 eggs f^{-1} d^{-1}. In addition, egg productivity differed for different water temperature and salinity combinations (**Figures 2A–E**). For the 10 and 15°C treatments, eggs were not laid at specific salinities of 10, 15, and 33, and at 10 and 33 psu, respectively (**Figures 2A,B**). For the 20–30°C range, eggs were produced in all salinity ranges (**Figures 2C–E**). In particular, at 25 and 30°C, a high EPR was observed at 25–30

TABLE 2 | Two-way ANOVA without replication to test the effect of egg production rate (EPR), hatching success (HS), and mortality on water temperature and salinity.

Variable	Source	df	*f*	*p*
EPR	Water temperature	4	7.94	0.00
	Salinity	6	3.06	0.02
	Error	24		
HS	Water temperature	4	21.86	0.00
	Salinity	6	3.03	0.02
	Error	24		
Mortality	Water temperature	4	37.01	0.00
	Salinity	6	14.09	0.00
	Error	24		

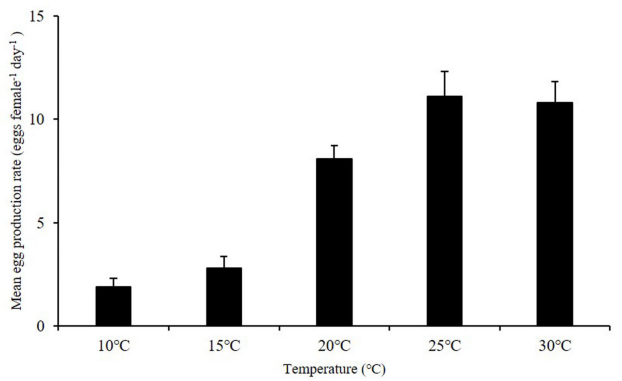

FIGURE 1 | Egg production rate (EPR) of adult female *Acartia ohtsukai* at each temperature. For each treatment, the mean 24-h EPR at seven salinities (10, 15, 20, 25, 27, 30, and 33 psu) is shown. Data are presented as mean ± standard error (SE). Different letters above the bars indicate significant differences ($p < 0.05$).

and 15–27 psu, respectively. There was also a clear decline in EPR as salinity increased above 27 psu at water temperatures of 10 and 15°C.

Hatching Success

The average HS (%) of *A. ohtsukai* increased as the water temperature increased ($p < 0.01$) (**Table 2** and **Figure 3A**). The average HS was lowest at 10°C (0.2 ± 0.4%) and highest at 30°C (42.1 ± 8.7%). In terms of the water temperature and salinity, the HS was 1.1 ± 0.1% at 10°C and 25 psu, and in other salinity conditions, hatching was not observed (**Figure 3B**). At 15°C, the HS was highest at 20 psu (10.7 ± 0.8%), and no hatching was observed at 10, 15, 27, and 33 psu. At 20°C, the HS was highest at 27.3 ± 1.2% at 10 psu. At 25°C, the HS was highest (37.2 ± 2.0%) at 10 psu, and 0% at 33 psu. At 30°C, the HS was about 37% or more between 10 and 30 psu, and no hatching was observed at 33 psu.

Mortality

During the experiment, the mortality of adult females was about 18% at temperatures above 20°C, and above 39% at lower temperatures (10 and 15°C) ($p < 0.01$) (**Table 2** and **Figure 4A**). The average mortality was lowest at 25°C (16.6 ± 11.4%) and highest at 10°C (42.6 ± 8.4%). The mortality was over 42% at 10°C, and the lowest mortality was observed at 27 psu (32 ± 1.4%) (**Figure 4B**). In the 15°C treatment, the mortality was highest at 15 psu, with a value of 45.0 ± 0.8%, whereas in the 20°C treatment, the mortality was highest at 10 psu, with a value of 35 ± 2.1%. In the 25°C treatment, the mortality was highest at 10 psu, with a value of 34 ± 1.9%, whereas in the 30°C treatment, the mortality was highest at 33 psu, with a value of 34 ± 1.9%.

DISCUSSION

Copepods egg production, hatching rate, survival are affected by several factors, including water temperature, salinity, food quantity and quality, and photoperiod (Peterson et al., 1991; Miralto et al., 2003; Shin et al., 2003). In particular, water temperature and salinity are important factors affecting not only the temporal and spatial distribution of copepods, but also egg production, HS, and mortality rates (Miller and Marcus, 1994; Peck et al., 2015). However, these depend on the different physiological responses of each species at various temperatures and salinities. Chinnery and Williams (2004) showed that the HS of *Acartia clausi* and *A. tonsa* was highest at 20°C and 33.3 psu, and at 20°C and 15.5 psu, respectively, whereas that of *Acartia bifilosa* was significantly higher at 10°C than at 20°C, with no significant influence of salinity. Further, they showed that the HS of *Acartia discaudata* did not differ significantly with temperature (5, 10, and 20°C) and salinity (15.5, 20.6, 25.1, and 33.3 psu). They also reported that nauplii of four *Acartia* species (*A. discaudata*, *A. clausi*, *A. tonsa*, and *A. bifilosa*) had improved survival and showed faster development as temperature increased from 5 to 20°C. Ohs et al. (2010) reported that the optimal salinity range for achieving maximum nauplii production in *P. pelagicus* was 15–25 g/L, and the percentage of ovigerous females peaked at 20 g/L and decreased in proportion of ovigerous females at salinities above or below this value. Milione and Zeng (2008) found that *A. sinjiensis* had the highest HS at 34°C, but this did not differ significantly when cultured at 25, 30, and 34°C. In addition, the hatching rate was highest at 30 psu, but there were no significant differences at other salinity concentrations. Further, they suggested that when the temperature rises above a certain level, the positive effect on egg production and hatching rates decreases (Milione and Zeng, 2008). Although *A. tsuensis* can develop normally from egg to adult within the temperature range of 17.5–30°C, optimal growth and minimum mortality were confirmed to occur at about 25°C (Takahashi and Ohno, 1996).

The results of this study on the effect of temperature on egg production and egg HS in *A. ohtsukai* are consistent with those of previous studies. With regard to the effect of temperature, the EPR of *A. ohtsukai* increased at 20°C in comparison to that at 25°C, but it slightly decreased at 30°C, while it was lower at 10 and 15°C. In particular, the EPR at 30°C was three times higher than that at 10–15°C (**Figure 1**). On the other hand, the combined effect of temperature and salinity was highest at 25°C and 27 psu (**Figure 2**). The HS increased as

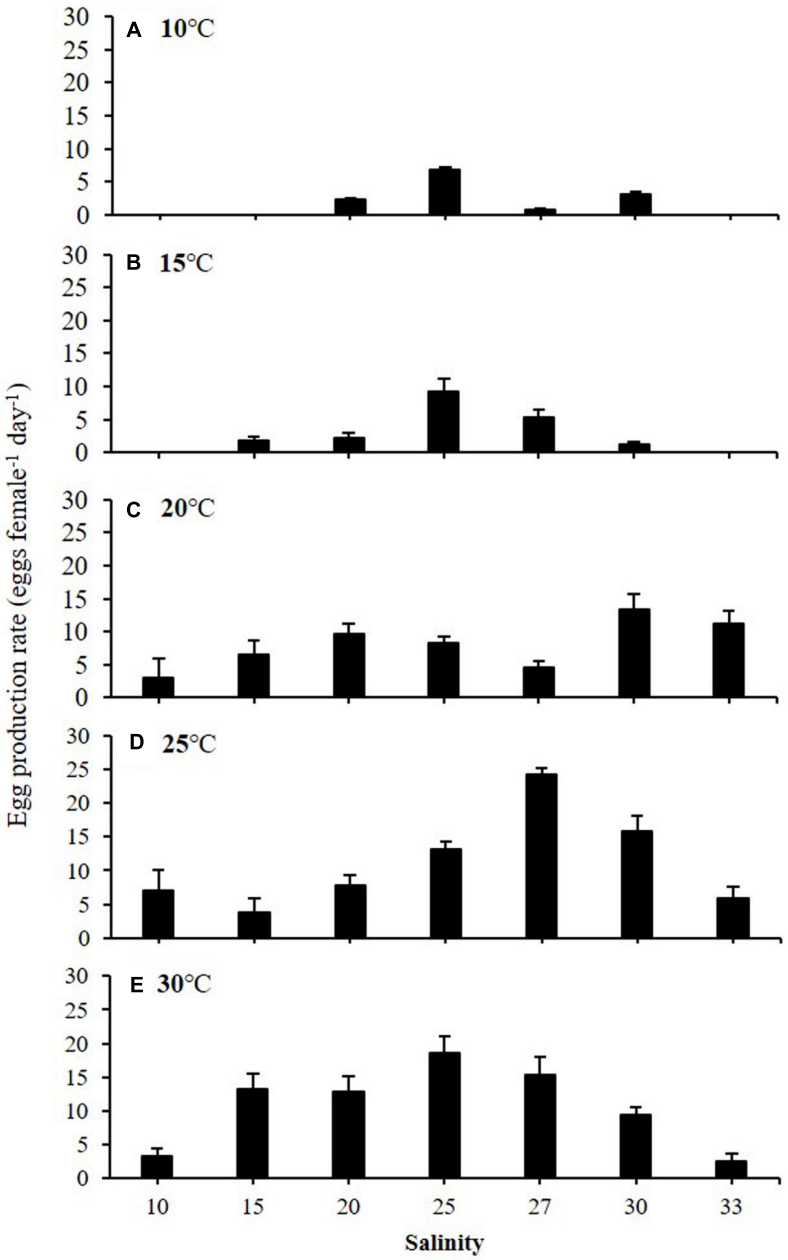

FIGURE 2 | Egg production rate (EPR) of adult female *Acartia ohtsukai* at each temperature and salinity. **(A)** 10°C, **(B)** 15°C, **(C)** 20°C, **(D)** 25°C, and **(E)** 30°C. Data are presented as mean ± standard error (SE). Different letters above the bars indicate significant differences ($p < 0.05$).

the water temperature increased and was highest at 30°C in all salinity gradients except at 33 psu (**Figure 3**). The present study shows that *A. ohtsukai* had different ranges of EPR and HS under various water temperature and salinity conditions. The egg production at water temperatures of 10 and 15°C occurred only in a specific salinity range (15–30 psu). When the water temperature reached 20°C or higher, the EPR was expanded to a wider range, ranging from 10 to 33 salinity. The species showed very low HS at 10 and 15°C, and relatively high hatching rates above 20°C in a wide salinity range. The highest HS was shown at 30°C, and hatching was not observed at a salinity of 33 psu. In other words, at temperatures below 20°C, *A. ohtsukai* showed low EPR and HS in a narrow salinity range, whereas above 20°C, it showed high EPR and HS in a wide salinity range. Therefore, the EPR and HS of *A. ohtsukai* can be expected to vary depending on the salinity range based on the water temperature of 20°C. The interaction between water temperature and salinity is particularly important in determining the range of physiological tolerance of copepods (Holste and Peck, 2005; Peck et al., 2015).

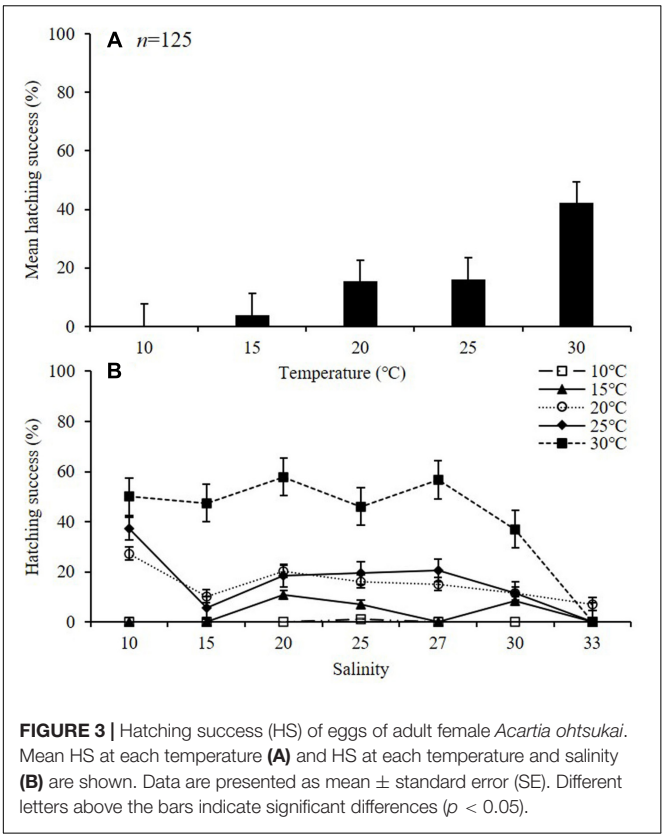

FIGURE 3 | Hatching success (HS) of eggs of adult female *Acartia ohtsukai*. Mean HS at each temperature **(A)** and HS at each temperature and salinity **(B)** are shown. Data are presented as mean ± standard error (SE). Different letters above the bars indicate significant differences ($p < 0.05$).

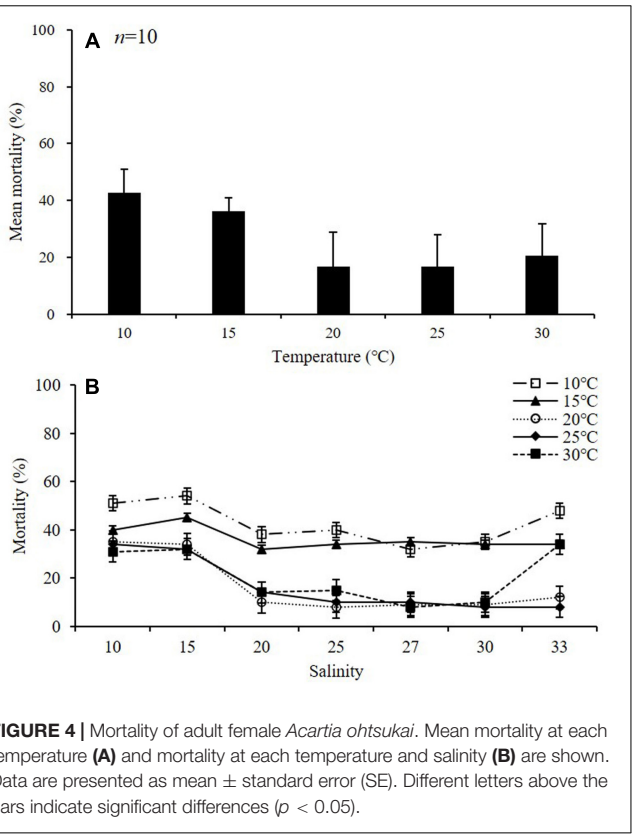

FIGURE 4 | Mortality of adult female *Acartia ohtsukai*. Mean mortality at each temperature **(A)** and mortality at each temperature and salinity **(B)** are shown. Data are presented as mean ± standard error (SE). Different letters above the bars indicate significant differences ($p < 0.05$).

The results of this study show that as the temperature decreases, the HS (%) markedly decreases, indicating that more than 90% of the eggs do not hatch within 48 h at temperatures of 10 and 15°C. It is speculated that this may be due to differences in the proportions of the different types of eggs that are produced based on different factors (water temperature, salinity, and food quality and quantity). In many species of the family Acartiidae, each single female can simultaneously produce subitaneous and diapause eggs (Onoue et al., 2004), and eggs could be morphologically distinguished depending on the species (Belmonte, 1992, 1998). The previous studies conducted in Gamak Bay have shown that *A. ohtsukai* produces normal, subitaneous, and resting eggs (Choi et al., 2019). Normal and subitaneous eggs hatch rapidly within a few days after spawning, whereas diapause egg hatch after a certain period (the refractory phase) of time (Uye, 1980; Marcus, 1996). The observed HS (%) during 48 h used in this study may not be sufficient time for resting eggs (Marcus, 1996; Peck and Holste, 2006). No difference was identified in the shape of hatching eggs and non-hatching eggs in this study (magnification ×400, Nikon ECLIPSE 80i; Nikon, Japan). Recent studies have shown that it could be difficult to recognize differences in egg types in the family Acartiidae (Belmonte and Rubino, 2019; Choi et al., 2021).

Mortality rates provide basic information on the tolerance of organisms to environmental conditions (Pörtner and Peck, 2010). In this study, *A. ohtsukai* showed high mortality rates in all salinities at 10 and 15°C, whereas at 20–30°C it showed a low mortality rate in the range of salinity of 20–33 psu. On the other hand, Støttrup et al. (1986) reported that the daily copepod mortality rate of *A. tonsa* was constant at 5% in the optimal salinity gradient, but it increased by about 50% or more in high salinity with values above 25 psu (Medina and Barata, 2004). It is not practical to accommodate more than 50% mortality of live prey in mass production (Jepsen et al., 2015). The mortality of *A. ohtsukai* observed for 10 days showed a low rate of less than 25% on average at all water temperatures and salinities except at salinity values of 10 and 15 psu at a water temperature of 10°C. Further, extensive salinity changes, except for a high salinity value of 33 psu, did not significantly affect HS in *A. ohtsukai* over 48 h. However, in the low temperature (10 and 15°C) experiment, no eggs were produced at salinities of 10, 15, and 33 psu. These results showed that the productivity was affected by salinity in the experiment at low temperatures, while it was affected by temperature when the values were above 20°C. Therefore, the optimal range for the survival of *A. ohtsukai* based on the combined effect of water temperature and salinity is estimated to be approximately 20–30°C temperature and 20–33 psu salinity. The physiological properties of *A. ohtsukai* suggest that the species can be an excellent candidate for mass cultivation as a prey of fish larvae, and that it may persist in the water column under various water temperature and salinity conditions.

Some recent studies suggest an unclear role of salinity on biological traits of the genus *Acartia*. Castro-Longoria (2003) found that the EPR of *Acartia* species (*Acartia margalefi*,

A. discaudata, *A. clausi*, and *A. tonsa*) were not significantly affected by a salinity range of 15–33 psu. **Figure 2** shows that the EPR decreased as the salinity increased above 27 psu at water temperatures of 10 and 15°C. However, in contrast to water temperature, the effect of salinity on EPR in *A. ohtsukai* was less consistent in the present study. For example, Peck and Holste (2006) found that *A. tonsa* had the highest EPR at a salinity value of 15 psu among a salinity range of 5–30 psu, whereas EPR gradually decreased in salinity above 20 psu. In addition, an optimal EPR of *Acartia japonica* was observed at a salinity value of 15 psu and a tendency of decrease in EPR at salinity values above 20 psu among a salinity range of 5–30 psu was shown (Wilson et al., 2021).

Several calanoid species living in estuarine waters have been reported to be able to withstand a wide range of salinity conditions (Uye, 1982). Støttrup (2000) showed that although salinity can affect productivity, these species can survive in less than suboptimal conditions. Although adults can withstand a relatively wide range of water temperature and salinity, the nauplius stage that is used as live feed for larvae of several fish species, including red snapper (*Lutjanus campechanus*), mangrove jack (*Lutjanus argentimaculatus*), and grouper (*Epinephelus coioides*) (Schipp et al., 1999; Lee et al., 2010), is sensitive in terms of salinity tolerance (Lance, 1964; Chinnery and Williams, 2004). In particular, in the case of the nauplius stage, exposure to adverse environments at this sensitive stage leads to developmental delay, ecdysis, or death (Tester and Turner, 1991; Devreker et al., 2004). Due to the difference in mortality rates according to developmental stages, it was difficult to estimate the mortality rate of nauplii according to water temperature and salinity in this study. Therefore, it is necessary to understand the effects on the population of *A. ohtsukai* according to environmental changes by investigating the mortality rate in future developmental stages. Although this study has not been undertaken on nauplii and copepodites according to their developmental stages, we have provided information on the optimum water temperature and salinity conditions of *A. ohtsukai* that can be applied to mass culture.

CONCLUSION

Acartia ohtsukai could survive in a relatively wide range of temperature and salinity conditions, but it was confirmed that temperature significantly affected EPR and HS. Salinity is considered to also have a significant effect on EPR and HS of *A. ohtsukai*, but to affect mortality rate to a lesser degree. To optimize the intensive culture of *A. ohtsukai*, we recommend the following: in order to produce and cultivate a high rate of eggs, the culture should be maintained at a temperature of 25°C and salinity of 27 psu. The immediate use as live feed for fish larvae is considered to be efficient when hatching occurs at 30°C. This demonstrates that the plasticity of *A. ohtsukai* enables it to adapt to changes in water temperature and salinity and that this species can be used as an efficient live feed in the cultivation of larvae of various fishes.

DATA AVAILABILITY STATEMENT

These data generated from this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

SYC conceived and designed the experiments, performed the experiments, analyzed the data, prepared the figures and tables, authored, reviewed drafts of the manuscript, and approved the final draft. EHL analyzed the data, authored, reviewed drafts of the manuscript, and approved the final draft. HYS and M-CJ conceived and designed the experiments, performed the experiments, analyzed the data, authored, reviewed drafts of the manuscript, and approved the final draft. All authors contributed to the article and approved the submitted version.

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Microalgal Diet Influences the Nutritive Quality and Reproductive Investment of the Cyclopoid Copepod *Paracyclopina nana*

Paul Dayras[1], Capucine Bialais[1], Irina Sadovskaya[2], Min-Chul Lee[3], Jae-Seong Lee[3] and Sami Souissi[1]**

[1] UMR 8187, CNRS, Laboratoire d'Océanologie et de Géosciences, Station Marine de Wimereux, University of Lille, University of Littoral Côte d'Opale, Lille, France, [2] UMR 1158 BioEcoAgro, TERRA Viollette, USC Anses, INRAE, Yncréa, University of Lille, University of Littoral Côte d'Opale, University of Artois, University of Picardie Jules Verne, University of Liège, Boulogne-sur-Mer, France, [3] Department of Biological Sciences, College of Science, Sungkyunkwan University, Suwon, South Korea

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Hans Uwe Dahms, Kaohsiung Medical University, Taiwan

***Correspondence:**
Sami Souissi
sami.souissi@univ-lille.fr
Jae-Seong Lee
jslee2@skku.edu

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Copepods represent an interesting alternative or a complement live food to brine shrimps and rotifers commonly used in aquaculture. They constitute the natural prey of many fish species and therefore do not require a potential nutritional enrichment. But an optimization of the microalgal diets used to feed copepods is essential to improve their mass culture. This study examined the effects of seven microalgal diets, namely single-species diets of *Rhodomonas salina* (R), *Tisochrysis lutea* (T), and *Pavlova lutheri* (=*Diacronema lutheri*) (P), two-species diets (R + T, T + P, and R + P), and a three-species diet (R + T + P), on the fatty acid and monosaccharide composition of the cyclopoid copepod *Paracyclopina nana* as well as its reproductive investment. Experiments were run during 15 days in 10-L beakers; starting with nauplii collected from a large 300-L batch culture. Copepods fatty acid contents were studied, particularly the relative amounts of docosahexaenoic acid (DHA) and eicosa-pentaenoic acid (EPA). The R + T, R, and T diets induced the highest total fatty acid amount in copepods. R + T and R also generated the lowest DHA/EPA ratios in copepods due to high EPA contents. The highest value of total monosaccharides was found in copepods fed with R + T + P. Diets R + T and R induced the greatest prosome volumes and clutch volumes in ovigerous females. Both prosome volume and clutch volume in *P. nana* ovigerous females were correlated to the individual EPA amount. The results demonstrated that all diets including *R. salina* enhanced the productivity of *P. nana* in mass culture, particularly when combined with *T. lutea*. *R. salina,* and *T. lutea* induced complementary fatty acid and monosaccharide profiles, confirming that R + T represents the best microalgae combination for productive culture of *P. nana*. Conversely, *P. lutheri* did not enhance the nutritional profile nor the fecundity of *P. nana* in the culture. This study is the first to demonstrate that *R. salina* is a suitable microalga for productive mass culture of *P. nana* for use as live food in aquaculture.

Keywords: copepod, fatty acid composition, live prey, microalgae, monosaccharide composition, *Paracyclopina nana*, reproductive investment

INTRODUCTION

Copepods are the primary component of zooplankton and have a key role as the trophic link between phytoplankton and secondary consumers in marine ecosystems (Breteler et al., 1990; Mauchline, 1998; Støttrup, 2000). They serve as an important food source for numerous fish and crustacean larvae (Sun and Fleeger, 1995; Turner, 2004). Commercial fish species are bred in aquaculture on diets comprising brine shrimps and rotifers, as these organisms are easy to grow in large volumes at relatively low cost (Støttrup and McEvoy, 2008; Dhont et al., 2013). However, brine shrimp and rotifers are not the natural prey species of many of the fish species grown in aquaculture, and do not always induce optimal fish larval growth because of their inadequate size or fatty acid content, therefore nutritional enrichment is sometimes required (Fernández-Reiriz et al., 1993; Thinh et al., 1999; Sorgeloos et al., 2001; Lubzens et al., 2003). Mass culture of several marine copepod species for use as live feed for marine fish cultures has been explored by several research groups (Ohno et al., 1990; Støttrup and Norsker, 1997; Payne and Rippingale, 2000; Van der Meeren et al., 2014; Blanda et al., 2017). Copepods have been found to have superior nutritional value to that of commonly used live prey such as brine shrimp and rotifers, and to enhance the growth and development of many fish species of commercial or ornamental interest such as the Atlantic halibut, the Pink snapper or the Yellowtail clownfish (McEvoy et al., 1998; Shields et al., 1999; Payne and Rippingale, 2000; Payne et al., 2001; Olivotto et al., 2008). More recent studies confirmed that the use of small nauplii of copepods such as the calanoid *Parvocalanus crassirostris* in breeding of new ornamental fishes with very small mouth is required (Anzeer et al., 2019; Burgess et al., 2020).

Copepods have a high content of highly unsaturated fatty acids (HUFAs) such as docosahexaenoic acid (DHA) and eicosa-pentaenoic acid (EPA), which are particularly important nutrients for the development of fish larvae (Sargent et al., 1997; McEvoy et al., 1998; Pan et al., 2014; Mejri et al., 2021) thus, copepod species with a high lipid concentration and high fecundity are desirable from an aquaculture perspective. Copepod productivity can be measured by female fecundity and fatty acid content (Souissi et al., 2016; Lee et al., 2017), which is related to the amount of carbon that the copepods ingest. Monosaccharides, are the only type of carbohydrates that are rarely stored by crustaceans, but carbohydrates are also no less important than lipids because they are rapidly metabolized after ingestion for many vital functions and thus are key contributors to biological performance (Cuzon et al., 2000; Hohnke and Scheer, 2012). It is therefore essential to supply copepod mass cultures with optimal microalgal diets to induce maximal fecundity, larval development, and nutritional storage (Pan et al., 2014). Optimization of diets for copepod cultures has been investigated previously, but many questions remain to be explored. The appropriate microalgal diet has been investigated for copepods in the orders Harpacticoida (Pinto et al., 2001; Arndt and Sommer, 2014), Calanoida (Milione et al., 2007; Camus et al., 2009; Ohs et al., 2010; Jeyaraj and Santhanam, 2012; Pan et al., 2014; Siqwepu et al., 2017), and Cyclopoida (Lee et al., 2006; Rasdi and Qin, 2016; Pan et al., 2018). Results have shown that mixed algal diets are more effective than monoalgal diets. However, these previous studies did not use the same species combinations of microalga and cannot be generalized to all copepod species. Several studies have noted the beneficial effects of red microalgae from the genus *Rhodomonas* on female fecundity (Ohs et al., 2010; Siqwepu et al., 2017). The biochemical composition of *Rhodomonas* sp. is well characterized, especially the fatty acid profiles of species in this genus (Latsos et al., 2020). Renaud et al. (1999) found that lipids accounted for 18.7% of the dry weight of *Rhodomonas* sp. and that most of this lipid content comprised polyunsaturated fatty acids (65.8%). However, the carbohydrate profile of *Rhodomonas* has not been well characterized. Another micro-algae genus widely used in aquaculture is *Isochrysis.* The lipid content of *Isochrysis* sp. can reach 23.4% of dry weight (Renaud et al., 1999). The carbohydrate content of *Tisochrysis lutea*, a species in the genus *Isochrysis*, accounts for 23% of this organism's dry weight and has been reported to be a complex mixture of polysaccharides such as glucose, galactose, mannose, xylose, arabinose, xylose, and rhamnose in various proportions, all of which are essential for copepod metabolism (Chu et al., 1982; Gnouma et al., 2017). The marine phytoflagellate *Pavlova* sp. (=*Diacronema* sp.) is also of great interest as it is known to be able to synthesize DHA and EPA in large amounts (Rehberg-Haas, 2014). Patil et al. (2007) stated that *Pavlova* sp. comprises around 13 mg/g of DHA and 18 mg/g of EPA and that these values can even be enhanced by accordingly adjusting its culture conditions.

The brackish water cyclopoid copepod *Paracyclopina nana* Smirnov 1935 has strong potential for use in the aquaculture industry (Lee et al., 2006, 2013; Ki et al., 2009). Native to the bays and estuaries of Eastern Asia (Japan, Taiwan, South Korea), it has a small size at adult stage (600 μm on average), high tolerance of salinity (from 5 to 30 ppt), endures culture at high densities (up to 20 ind./mL), and grows under a wide range of temperatures (from 15 to 30°C) (Lee et al., 2012b, 2017). Its small first naupliar stage (<80 μm) is also important when breeding fish species that have tiny first feeding larval stages. Recent studies conducted on *P. nana* attempted to establish the optimal diet for its growth in aquaculture (Min et al., 2006; Lee et al., 2012a). However, these studies focused on algal monoculture, while studies carried out on other copepod species demonstrated that microalgae quality and diversity can enhance copepods productivity (Pan et al., 2014). It is therefore essential to optimize copepod culture conditions by selecting the right combination of microalgae.

The aim of this study was therefore to examine which of a variety of different microalgal diets had optimal nutritive quality and maximized reproductive investment of *P. nana* populations grown in mass culture.

MATERIALS AND METHODS

The section "Materials and Methods" used in this study are the same as those described in Dayras et al. (2020), as the results presented here were derived from the same set of experiments. Methods and materials are therefore summarized below and the reader is invited to refer to Dayras et al. (2020) for further details.

On the contrary the volume measurement of copepod females as well as the nutritional profile of microalgae and copepods are only presented in this paper and therefore their methods were detailed.

Microalgae Species Selection

Three species of microalgae were individually grown in the laboratory using standardized methods: *Rhodomonas salina* (RCC20), *T. lutea* (RCC1349), and *Pavlova lutheri* (=*Diacronema lutheri*) (RCC1537), which were obtained from the Roscoff Culture Collection of Marine Microalgae[1]. *R. salina* and *T. lutea* are currently widely used in zooplankton cultures for their nutritional value and *P. lutheri* has a desirable fatty acid profile (DHA, EPA) (Coutteau, 1996; Brown, 2002). These three species belong to separate genera and families of microalgae. As a result, their physiology, and therefore their physico-chemical composition and nutritional quality, are assumed to be different (Brown, 2002).

To establish which species and combinations of microalgae species are the most beneficial to *P. nana* maintained in culture in terms of population growth and individual nutritional value, it is essential to construct microalgal diets where the species are combined in equivalent proportions relative to their chemical composition (Pan et al., 2014). Within the different defined diets, each microalgal species should provide equivalent amounts of carbon to test only the effect of the species.

We used carbon as a proxy to establish equivalent ratios among the three microalgae species (Pan et al., 2014). CHN analysis was performed using an elemental analyzer (FLASH 2000 Series CHNS/O Analyzer, Thermo Fisher Scientific, Waltham, MA, United States) to assess the basic chemical profiles of each of these three microalgae cultures and deduce their respective cellular carbon concentrations. All different possible microalgal diets of the combined species were then constructed so that the total feeding volume remained the same. Diets were provided for a period of 15 days to copepods starting from early naupliar stages.

Establishment of Microalgae Diets

CHN Analysis, Microalgal Cell Density, and Carbon Cellular Amount

Prior to experiments, CHN analysis was performed on samples of each of the three microalgal cultures at their exponential phase to determine the carbon content of each species. Microalgae culture conditions in the laboratory were standardized to growth in natural seawater salinity, a constant temperature of 18°C, and a 12-h light:12-h dark photoperiod. These culture conditions were used before CHN analysis and were maintained throughout the experiment. Microalgal cultures in exponential phase were used to feed copepods. To estimate the carbon content of single algal cells, the number of cells present in a 1-mL culture of each microalgal species was counted in five replicates. The average amount of carbon in each species in this volume was then divided by the total number of cells present in this volume. Further details can be found in Dayras et al. (2020).

[1]http://roscoff-culture-collection.org/

Differences in carbon concentrations in each microalgal species compared with those reported in the literature are likely due to culture conditions (temperature, light, and salinity), which can affect carbon content (Renaud et al., 1995; Hu, 2004; Pal et al., 2011). For this reason, we used the results from our CHN analyses as reference values in this study.

Microalgal Diet Combinations and Relative Proportions of Microalgae

Given three species of microalgae [*R. salina* (R), *T. lutea* (T), and *P. lutheri* (P)], seven different diets combinations are possible:

- Three single-species diets: R, T, and P.
- Three two-species diets: R + T, T + P, and R + P.
- One three-species diet: R + T + P.

Because of variability in cellular carbon concentration and cell density in microalgae cultures, we used the volume of microalgal solution as a proxy for carbon concentration in our study. The total required volume of each diet for daily feeding was deduced from the standard volume of microalgae used to feed permanent copepod cultures maintained in the laboratory, and corresponded to a volume of 9.1 mL of microalgal culture per day for each large beaker of 10 L. This volume of algal cells corresponds to a carbon amount that is not limiting for *P. nana* (Lee et al., 2012a). To create the combination diets, volume ratios of each of the microalgal species that contained the same proportional amount of carbon in the same total volume of 9.1 mL were deduced based on the results of CHN analysis so that species parameter is the only parameter being tested.

Microalgal Cultures and Initial Sampling

Microalgae were grown in batch cultures in six different autoclaved 2 L flasks following the protocol of Sadovskaya et al. (2014). Specific culture conditions used are described in detail in Dayras et al. (2020). Three additional 2 L flasks were set up with the exact same protocol, one for each species, and were used exclusively for fatty acid and carbohydrate analyses. When exponential phase growth was reached by each of the three microalgal species, 1 mL samples were obtained and fixed with Lugol's solution to count cells. The entire remaining volume of culture of each species was then centrifuged and lyophilized for further fatty acid and carbohydrate analyses.

Copepod Culture and Initial Sampling

Paracyclopina nana used in current experiments was originally obtained from J-SL from Sungkyunkwan University of South Korea in 2015. Copepods were grown in the laboratory in a 300-L cylindrical acrylic tank. Specific culture conditions and the collecting protocol are described in Dayras et al. (2020). Adult stages were removed and half the amount of nauplii present in the tank were kept for experiments.

Seven 10-L beakers containing 8 L of culture medium were set up, one for each of the diet combination being evaluated. The use of a single replicate of these large beakers was justified by our preliminary observations of low variability between replicate *P. nana* cultures. Each beaker was filled with 8 L of 15 ppt

water and placed at a constant temperature of 18°C with a 12-h light:12-h dark photoperiod. The 1.2 L aliquot of concentrated nauplii was divided in 12× 100-mL aliquots. Seven of these aliquots were diluted in one of the seven beakers used in the experiments; two were individually filtered to remove any water, concentrated in cryotubes, and stored at −80°C for both fatty acid and carbohydrate analyses; and the remaining three aliquots of 100 mL were used for other analyses.

Experiment Monitoring

Daily Feeding With Microalgal Diets

Each beaker of copepods was fed daily with a total volume of 9.1 mL of each combination of microalgal diet containing relative proportions of the different microalgal species as described in Dayras et al. (2020).

Daily and Final Sampling

Each day, 1 mL of each microalgal culture was sampled to determine the exact daily amount of carbon provided after cellular counting. Corresponding data can be found in Dayras et al. (2020).

At the end of the experiment (15 days), two volumes of 500 mL were obtained from each of the seven copepod beakers. These 500-mL samples were individually filtered to remove water, and copepods were collected and concentrated in cryotubes and stored at −80°C for both fatty acid and carbohydrate analyses. This sampling was supplemented by sorting 20 ovigerous females for morphological measurements (prosome length and width) and fertility assessment (number and size of eggs per ovigerous female).

Ovigerous Female Morphological Measurements

The 20 fixed ovigerous females obtained from each diet condition were individually photographed and studied under an inverted microscope (model Olympus IX71, Tokyo, Japan) with 10× magnification coupled to a ToupCam camera (model UCMOS05100KPA, Zhejiang, China) connected to a computer using ToupView software from ToupTek Photonics (version 3.7). For each single ovigerous female, a photo of the whole body was taken for measurements. Egg sacs were then manually detached and the eggs were photographed.

Morphological measurements were assessed from photographs using ImageJ software (version 1.48v) as described in Souissi et al. (2016). Prosome length and width and egg diameter were manually measured for each ovigerous female. Prosome volume was then calculated using the formula used to calculate the volume of an ellipsoid, namely $V = \frac{4}{3}ab^2$ where a corresponds to half the body length and b corresponds to half the body width.

Fatty Acid Analysis

To investigate variations in internal fatty acid quantity induced by the different tested diets, fatty acid methyl esters (FAMEs) of copepods and microalgae samples stored at −80°C were prepared by direct transesterification reactions. We used the method detailed in Pan et al. (2018). Five milligrams of lyophilized copepods and microalgal pellets (n = 1 and n = 3, respectively) were evaluated. Extracted FAMEs were stored at −20°C until gas chromatography (GC) analysis, which was performed using a Trace GC ULTRA system (Thermo Fisher Scientific, Waltham, MA, United States) equipped with a capillary column NMTR-5MS (30 m × 0.25 mm) and FID detector using a temperature gradient of 170°C (3 min) → 250°C at a heating rate of 5°C/min. Peaks were identified with appropriate standards and samples previously analyzed by GC-MS (Pan et al., 2018). Copepod values were converted and quantified using the internal standard C17:0 quantity introduced in each sample (10 μg) and directly expressed in μg/mg DW (μg per mg of dry weight). The fatty acid amount was then estimated relative to the fatty acid quantity measured in a control sample (T0) at the beginning of the experiment. For microalgae, the number of cells in 1 mL triplicates was counted and converted to the initial tested volume of 1700 mL for each species, then extrapolated for 5 mg and a mean value was calculated. Results were then also expressed in μg/mg DW.

Carbohydrate Analysis

Internal carbohydrate quantities of the different copepod and microalgae samples were also measured. Monosaccharide analysis of cells was performed as described previously (Sadovskaya et al., 2014). After lyophilization of all samples, whole copepod samples and 5 mg of microalgal pellets in triplicate were used (n = 1 and n = 3, respectively), after recording sample weights. Samples were defatted by extraction with CHCl–MeOH, 2:1 and 1:2 (v/v), and then air-dried. Residue was hydrolyzed with 4 M TFA (110°C, 3 h) in the presence of a known amount of myo-inositol, the internal standard. Fifty microliters of myo-inositol concentrated to 180 μg/100 μL (corresponding to 90 μg) was used for microalgae samples and 50 μL of myo-inositol concentrated to 9 μg/100 μL (corresponding to 4.5 μg) was used for copepod samples. The acid was evaporated with nitrogen, and released monosaccharides were reduced with $NaBH_4$ overnight. Excess $NaBH_4$ was removed with 10% AcOH in MeOH and the solution was evaporated. The procedure was repeated twice more with 10% AcOH in MeOH and twice more with MeOH to remove borates. Alditols were then acetylated with 0.4 mL of Ac_2O–0.4 mL pyridine mixture for 1 h at 100°C. Reagents were evaporated with toluene, residue was dissolved in chloroform, washed twice with water, filtered through a cotton filter, and concentrated prior to GC analysis. GC was performed using a Trace GC ULTRA system (Thermo Fisher Scientific, Waltham, MA, United States) equipped with a capillary column NMTR-5MS (30 m × 0.25 mm) and FID detector using a temperature gradient of 170°C (3 min) → 250°C at a heating rate of 5°C/min. Results were converted to μg/mg DW from the initial standard myo-inositol quantity introduced into each sample and mean values were then calculated.

Statistical Analysis

To test the effect of diet and potential correlations between the three parameters of prosome volume, clutch volume, and EPA amount in ovigerous females, Pearson's chi-squared test was used

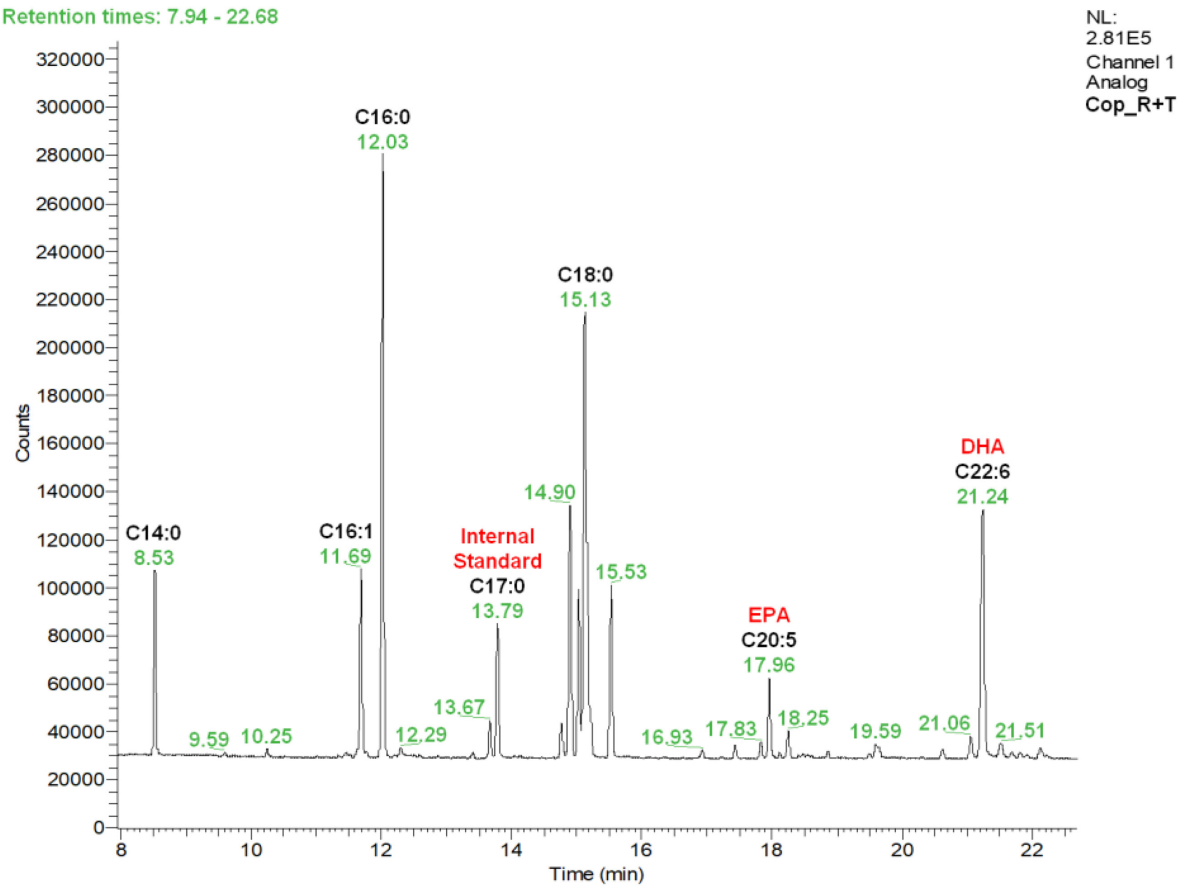

FIGURE 1 | Fatty acid profile obtained by gas chromatography (GC) in *Paracyclopina nana* copepods fed with the microalgal diet R + T. Respective rentention times for each peak appear in green. Peaks corresponding to the identified fatty acids and the internal standard are annotated in bold black. R, *Rhodomonas salina;* T, *Tisochrysis lutea*.

and applied to each combination of two parameters. All statistical tests were conducted at the 95% confidence level. To test the effect of all treatments on female volume and clutch volume, we performed an ANOVA and MULTICOMPARE tests. These analyses were performed using the Matlab Software (Mathworks Inc., Version, 7.5).

RESULTS

Effects of Microalgal Diet on Fatty Acid Composition of *P. nana*

A typical chromatogram illustrating the fatty acid profiles obtained by gas chromatography (GC) in copepod samples can be seen in **Figure 1**, corresponding to the copepods fed with the R + T diet. Predominant fatty acids in *P. nana* were the C14:0, C16:1, C16:0, C18:0, C20:5 (EPA), and C22:6 (DHA), and their detailed amounts in all microalgae and copepods samples are presented in **Table 1**. *Tisochrysis lutea* (T) had the highest total fatty acid content of the three species evaluated (85.38 ± 4.36 μg/mg DW), whereas, *R. salina* (R) had the lowest total fatty acid content (66.38 ± 7.22 μg/mg DW) and also the lowest amount of DHA (5.04 ± 0.62 μg/mg DW). Both of these microalgae contained particularly high amounts of C18:0 (22.35 ± 2.54 μg/mg DW for R and 23.62 ± 1.25 μg/mg DW for T), the double what P contained (11.22 ± 0.66 μg/mg DW of C18:0). *T. lutea* contained the highest DHA amount (8.31 ± 0.56 μg/mg DW) but the lowest EPA amount (0.69 ± 0.24 μg/mg DW) among the three tested microalgae species, resulting in the highest DHA/EPA ratio (12.1). *P. lutheri* (P) presented high levels for each individual fatty acid except C18:0 compared to the other two species and particularly concerning C16:1 and C16:0 (12.68 ± 2.02 μg/mg DW and 12.52 ± 1.90 μg/mg DW, respectively). *P. lutheri* especially contained a much higher EPA amount (25.43 ± 1.27 μg/mg DW) and a relatively high DHA amount (7. 68 ± 0.84 μg/mg DW), resulting in the lowest DHA/EPA ratio among the three species (0.30).

Copepods fed on diets R + T (291.1 μg/mg DW), R (251.7 μg/mg DW), and T (246.2 μg/mg DW) for 15 days had the highest amounts of fatty acids; these values correspond to increase ratios of 12.77, 11.04, and 10.80, respectively. Diets R and T both engendered high levels of C16:0 (10.72 μg/mg DW and 10.98 μg/mg DW, respectively) and the highest C18:0 levels (19.13 μg/mg DW and 17.57 μg/mg DW, respectively)

TABLE 1 | Total fatty acid amounts and respective EPA and DHA contents in each copepod and microalgae lyophilized sample.

Microalgae samples	Total FA amount (μg), *n* = 3	FA ratio	μg total FA/mg DW, *n* = 3
R. salina	331.9 ± 36.09	C17x331.9	66.38 ± 7.22
T. lutea	426.9 ± 21.80	C17x426.9	85.38 ± 4.36
P. lutheri	375.5 ± 70.16	C17x375.5	75.10 ± 14.03

Microalgae samples	μg C14:0/mg DW, *n* = 3	μg C16:1/mg DW, *n* = 3	μg C16:0/mg DW, *n* = 3	μg C18:0/mg DW, *n* = 3	μg EPA/mg DW, *n* = 3	μg DHA/mg DW, *n* = 3	DHA/EPA ratio
R. salina	3.34 ± 0.29	1.46 ± 0.30	7.93 ± 0.89	22.35 ± 2.54	8.06 ± 0.90	5.04 ± 0.62	0.63
T. lutea	10.69 ± 1.36	6.85 ± 0.41	10.69 ± 0.37	23.62 ± 1.25	0.69 ± 0.24	8.31 ± 0.56	12.1
P. lutheri	8.08 ± 1.20	12.68 ± 2.02	12.52 ± 1.90	11.22 ± 0.66	25.43 ± 1.27	7.68 ± 0.84	0.30

Copepod samples	Total FA amount (μg)	FA increase ratio	μg total FA/mg DW
T0 (control)	22.8	T0	4.56
R	251.7	T0x11.04	50.34
T	246.2	T0x10.80	49.24
P	157.6	T0x6.91	31.52
R + T	291.1	T0x12.77	58.22
T + P	182.3	T0x8.0	36.46
R + P	159.2	T0x6.98	31.84
R + T + P	168.5	T0x7.39	33.70

Copepod samples	μg C14:0/mg DW	μg C16:1/mg DW	μg C16:0/mg DW	μg C18:0/mg DW	μg EPA/mg DW	μg DHA/mg DW	DHA/EPA ratio
T0 (control)	0.277	0.728	0.899	1.33	0.106	0.404	3.81
R	3.07	5.86	10.72	19.13	2.66	5.57	2.09
T	3.10	7.96	10.98	17.57	2.08	5.37	2.58
P	1.67	5.98	7.23	10.76	1.36	3.08	2.26
R + T	3.07	18.55	15.44	12.75	3.19	3.40	1.07
T + P	2.07	4.16	7.70	14.78	1.04	4.38	4.21
R + P	1.83	2.41	6.40	12.84	1.28	4.25	3.32
R + T + P	2.13	6.18	7.89	11.11	1.40	3.04	2.17

in copepods. The highest amounts of C16:1 (18.55 μg/mg DW) and C16:0 (15.44 μg/mg DW) in copepods have been induced by the R + T diet. Diets that induced the smallest fatty acid increases in copepods were R + T + P (168.5 μg/mg DW), R + P (159.2 μg/mg DW), and P (157.6 μg/mg DW), corresponding to increase ratios of 7.39, 6.98 and 6.91, respectively. Overall, the R + P and R + T + P diets engendered lower levels of each fatty acid than the other diets, and particularly the P diet which led to copepods containing some of the lowest levels of each individual studied fatty acid. R + T also induced the highest EPA amount of 3.19 μg in copepods among all seven tested diets, and the lowest DHA/EPA ratio (1.07). The highest DHA amounts were detected in copepods fed the R and T diets at 5.57 μg/mg DW and 5.37 μg/mg DW, respectively. A high level of C18:0 (14.78 μg/mg DW) and also the highest DHA/EPA ratio were found in copepods fed the T + P diet due to the low amount of EPA this diet induced (1.04 μg/mg DW).

Effects of Microalgal Diet on the Monosaccharide Composition of *P. nana*

Detailed amounts of seven specific monosaccharides in microalgae and copepods samples are shown in **Table 2**. *Rhodomonas salina* (R) had the highest total monosaccharide content of the three species (46.60 ± 4.71 μg/mg DW), whereas, *T. lutea* (T) had the lowest total monosaccharide content (28.28 ± 9.20 μg/mg DW). R had a higher amount of glucose than the other two species (36.46 ± 3.31 μg/mg DW) and was also the only species in which fucose was detected (1.23 ± 0.17 μg/mg DW). T contained the highest amounts of mannose (3.35 ± 1.03 μg/mg DW), galactose (7.55 ± 2.82 μg/mg DW), arabinose (9.55 ± 2.53 μg/mg DW), and xylose (2.66 ± 1.22 μg/mg DW). P contained the smallest amounts of each of the different monosaccharides evaluated with the exception of ribose (3.96 ± 1.50 μg/mg DW).

The R + T + P diet induced the highest total monosaccharide amount in copepods after 15 days of feeding among the seven tested diets at 40.69 μg/mg DW. T + P (22.89 μg/mg DW) and R + T (15.09 μg/mg DW) diets also resulted in a high total monosaccharide content in copepods. Fucose was only detected in copepods fed these three diets : R + T + P, T + P, R + T). R + T + P and T + P diets both led to relatively high amounts of each of the studied monosaccharides in comparison with the five other diets, with the monosaccharide quantities generally highest for the R + T + P diet with the exception of ribose, which was present in the highest quantity in copepods fed the

TABLE 2 | Total monosaccharide amounts and respective amounts of seven specific monosaccharides for each copepod and microalgae lyophilized sample.

Microalgae samples	μg total MonoS/mg DW, *n* = 3	μg Glc/mg DW, *n* = 3	μg Man/mg DW, *n* = 3	μg Gal/mg DW, *n* = 3	μg Rib/mg DW, *n* = 3	μg Fuc/mg DW, *n* = 3	μg Ara/mg DW, *n* = 3	μg Xyl/mg DW, *n* = 3
R. *salina*	46.60 ± 4.71	36.46 ± 3.31	1.86 ± 0.15	2.95 ± 0.34	2.17 ± 0.32	1.23 ± 0.17	0.87 ± 0.41	1.07 ± 0.24
T. *lutea*	28.28 ± 9.20	3.15 ± 1.32	3.35 ± 1.03	7.55 ± 2.82	2.01 ± 0.42	–	9.55 ± 2.53	2.66 ± 1.22
P. *lutheri*	32.67 ± 3.97	19.21 ± 0.51	2.17 ± 0.34	3.26 ± 0.51	3.96 ± 1.50	–	1.65 ± 0.48	2.42 ± 0.68
Copepod samples	**μg total MonoS/mg DW**	**μg Glc/mg DW**	**μg Man/mg DW**	**μg Gal/mg DW**	**μg Rib/mg DW**	**μg Fuc/mg DW**	**μg Ara/mg DW**	**μg Xyl/mg DW**
T0 (control)	4.08	0.29	0.43	0.40	2.22	0.12	0.32	0.30
R	6.28	1.65	0.55	0.86	1.88	–	0.58	0.76
T	–	–	–	–	–	–	–	–
P	3.83	0.74	0.49	0.95	0.67	–	0.46	0.53
R + T	15.09	1.85	1.56	1.39	7.70	0.43	1.11	1.05
T + P	22.89	5.19	3.46	5.18	2.61	0.49	2.75	3.22
R + P	–	–	–	–	–	–	–	–
R + T + P	40.69	5.43	4.79	8.81	7.33	0.45	7.41	6.45

Cells containing a "–" correspond to values for which the detection did not work due to an insufficient biomass quantity.

R + T diet (7.70 μg/mg DW). Diet P was associated with the lowest total monosaccharide amount in copepods (3.83 μg/mg DW) in comparison with all treatments, which was even less than the initial amount present in the T0 copepod sample (4.08 μg/mg DW). Furthermore, diet P was associated with the lowest amount of monosaccharides among the seven tested diets except for galactose (0.95 μg/mg DW).

Effects of Microalgal Diet on the Reproductive Investment of *P. nana* Ovigerous Females

Ovigerous Female Prosome Volume and Clutch Volume

The distribution of ovigerous females prosome volumes after 15 days of receiving one of the seven diets is shown in **Figure 2A**. R + T and R diets induced the greatest prosome volumes in ovigerous females (R + T: $8.40 \times 10^{-3} \pm 0.66 \times 10^{-3}$ mm^3, R: $7.82 \times 10^{-3} \pm 0.82 \times 10^{-3}$ mm^3), whereas, the R + P and T diets were associated with the smallest prosome volumes (R + P: $7.37 \times 10^{-3} \pm 1.16 \times 10^{-3}$ mm^3, T: $6.82 \times 10^{-3} \pm 0.93 \times 10^{-3}$ mm^3). The average clutch volume of these same ovigerous females (**Figure 2B**) was also the greatest for copepods fed the R + T, R, and R + T + P diets (R + T: $2.59 \times 10^{-3} \pm 0.55 \times 10^{-3}$ mm^3, R: $2.36 \times 10^{-3} \pm 0.37 \times 10^{-3}$ mm^3, R + T + P: $2.27 \times 10^{-3} \pm 0.47 \times 10^{-3}$ mm^3), whereas, the P diet resulted in the smallest clutch volume ($1.09 \times 10^{-3} \pm 0.31 \times 10^{-3}$ mm^3).

A plot of clutch volume versus prosome volume for each microalgal diet confirmed a linear relationship between female clutch volume and prosome volume (**Figure 3**). In particular, the R + T diet resulted in the highest values for both parameters and a linear relationship between ovigerous females clutch volume and prosome volume (y = 0.9163x - 5.069) with a significant correlation coefficient ($R^2 = 0.5841$, $p < 0.01$). Both of these morphological parameters were also evaluated with regard to the EPA data; clutch volume and prosome volume were each plotted against EPA amount for each microalgal diet (**Figures 4**, **5**, respectively). As can be seen from these plots, R + T and R diets yielded the highest values. A linear relationship was found between ovigerous female clutch volume and EPA amount (y = 0.4781x + 0.9374), with a significant correlation coefficient ($R^2 = 0.446$, $p < 0.01$), as well as between ovigerous female prosome volume and EPA amount (y = 0.387x + 6.8058), with a significant correlation coefficient ($R^2 = 0.4202$, $p < 0.01$). The other tested relationships, in particular DHA amount or total fatty acid amount, did not show any significant correlations with clutch volume or prosome volume.

Representative photographs of the morphology of the different *P. nana* ovigerous females fed each diet are presented in **Figure 6**. R-fed and R + T-fed ovigerous females visually looked the biggest and had the biggest egg sacs. Females fed the R + T + P diet and the R + P diet had the next largest egg sacs and body sizes. The smallest ovigerous females with the smallest egg sacs were those that received the T + P diet, the T diet and finally, the P diet.

DISCUSSION

Effects of Microalgal Diet on the Fatty Acid Composition of *P. nana*

The fatty acid profile of copepods is directly linked to their diet and its fatty acid composition, as these nutrients are stored in the copepod body as lipid droplets (Lee et al., 2017). It is therefore essential to investigate both copepod and diet fatty acid composition to determine the optimal diet for copepod aquaculture (Van der Meeren et al., 2008) and to gain insight into the trophic ecology of copepods in marine ecosystems (El-Sabaawi et al., 2009).

In the present study, copepods fed the R + T, R, and T diets had the highest final fatty acid amounts. Diets R and T both induced particularly high C18:0 amounts in copepods and this is consistent with the C18:0 amounts in both microalga *R. salina* and *T. lutea* that were originally really high. Diets

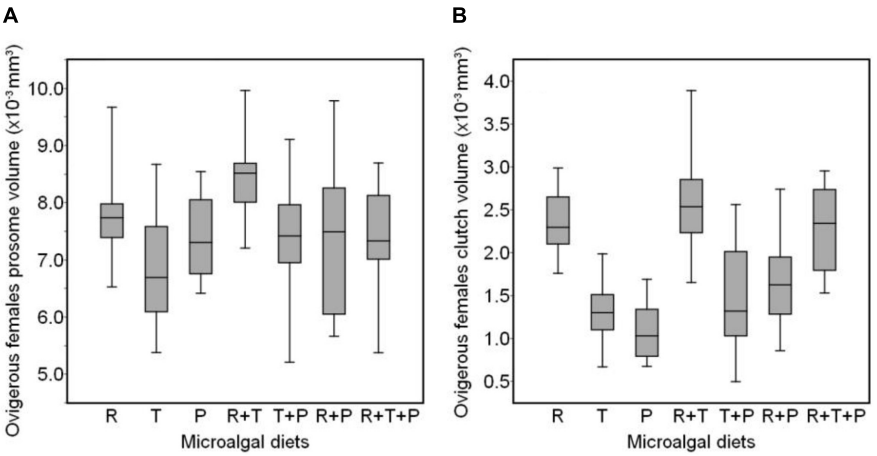

FIGURE 2 | Boxplot showing the distribution of prosome volume **(A)** and clutch volume **(B)** of *Paracyclopina nana* ovigerous females fed on different microalgal diets. Shaded boxes represent the first and third quartiles with the middle bar indicating the median. The end of the whiskers extends from the hinge to the lowest and highest value within a 1.5 inter-quartile range. R, *Rhodomonas salina;* T, *Tisochrysis lutea;* P, *Pavlova lutheri.*

associated with the lowest fatty acids amounts in copepods were the R + T + P, R + P, and P diets, confirming that *P. lutheri* is not the best microalgal prey species for *P. nana*. However, *P. lutheri* contained a large amount of fatty acids while *R. salina* contained the lowest amount of fatty acids among the three microalgal species evaluated. This implies that the fatty acid profile of copepods is not determined by the relative quantity of fatty acids in their diet, but rather the nature and quality of dietary fatty acids. This is supported by the DHA/EPA ratio results we obtained. The highest DHA/EPA ratio was found in *T. lutea* due to the very low amount of EPA present in this species, but the T diet yielded some of the highest DHA and EPA amounts in *P. nana* individuals. This implies that copepods have the ability to bio-convert EPA to DHA. Desvilettes et al. (1997) previously reported that *P. nana* could convert high amounts of α-linolenic acid (ALA) from *Tetraselmis suecica* to EPA and DHA when fed on it. In our study, another conversion effect is observed in *P. nana* as the individuals fed R + T present the highest amounts of C16:1 and C16:0, whereas, our results showed that both *R. salina* and *T. lutea* contain lower amounts of these two fatty acids than *P. lutheri*. Lee et al. (2020) also showed that the fatty acid contents of *P. nana* individuals were affected by the fatty acid composition of their microalgal diet. Results from food selectivity experiments of different microalgae species demonstrated that *P. nana* fed *Nannochloropsis oculata* contained long-chain saturated fatty acids (C20:0 and C22:0), although this microalgae did not contain any C20:0 and C22:0 fatty acids, indicating that *P. nana* can biosynthesize saturated fatty acids. Thus, cyclopoid copepods can incorporate shorter chain fatty acids from microalgae into EPA and DHA. Nielsen et al. (2019) investigated the potential ability of the cyclopoid copepod *Apocyclops royi* to biosynthesize polyunsaturated fatty acids (PUFA) through feeding experiments using PUFA-poor *Dunaliella tertiolecta* and PUFA-rich *Isochrysis galbana*. Results indicated that the copepods always contained high contents of DHA, and no significant differences in absolute DHA content were detected between treatments, even when copepods were starved of DHA for two generations. Gene expression analysis revealed significantly higher expression of two desaturases in copepods fed PUFA-poor microalgae compared to copepods fed PUFA-rich microalgae. This suggested active PUFA biosynthesis and DHA production capability in *A. royi* fed low-PUFA diets, confirming the idea that cyclopoid copepods have the ability to bio-convert dietary essential fatty acids into complex storage fatty acids such as EPA and DHA.

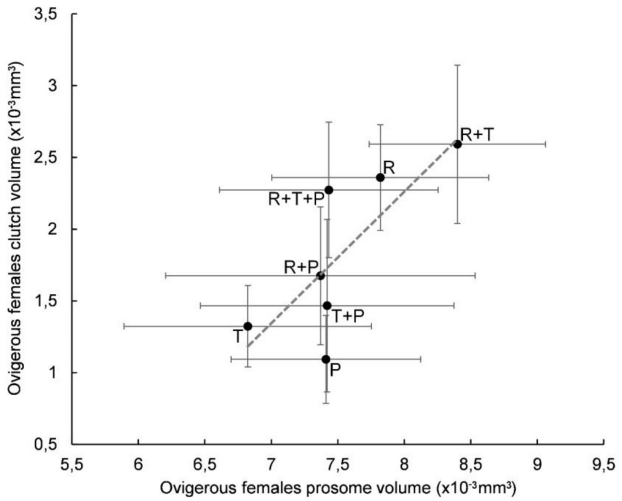

FIGURE 3 | Relationship between average prosome volume and clutch volume in *Paracyclopina nana* ovigerous females for each microalgal diet. Error bars show the standard deviations obtained from $n = 20$ females. Dashed line corresponds to the linear regression (EV = 0.9163 X PV – 5.069, $R^2 = 0.5841$, $p < 0.01$). Pearson's test was performed to determine the significance of the coefficient of determination R^2. R: *Rhodomonas salina*, T: *Tisochrysis lutea*, P: *Pavlova lutheri.*

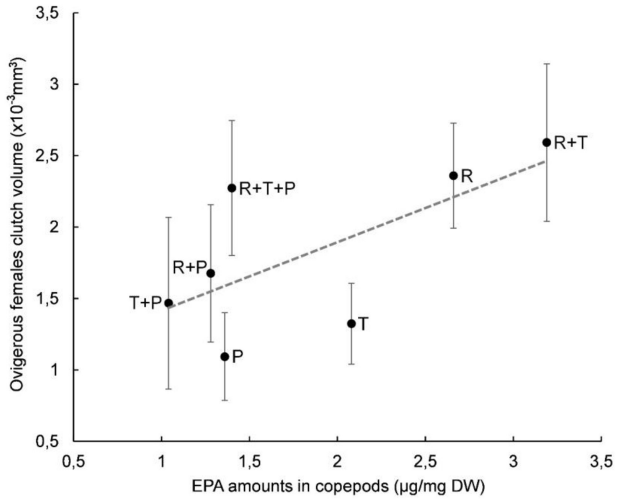

FIGURE 4 | Relationship between average clutch volume and EPA amount in *Paracyclopina nana* ovigerous females for each microalgal diet. Error bars show the standard deviations obtained from $n = 20$ females. Dashed line corresponds to the linear regression (EV = 0.4781 X PV + 0.9374, $R^2 = 0.446$, $p < 0.01$). Pearson's test was performed to determine the significance of the coefficient of determination R^2. R, *Rhodomonas salina*; T, *Tisochrysis lutea*; P, *Pavlova lutheri.*

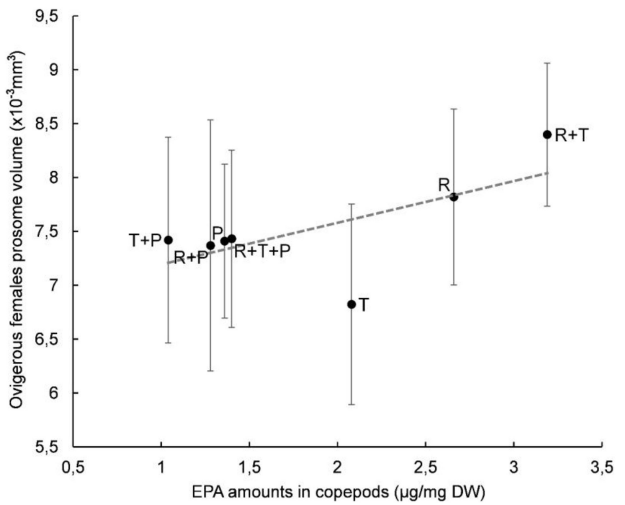

FIGURE 5 | Relationship between average prosome volume and EPA amount in *Paracyclopina nana* ovigerous females for each microalgal diet. Error bars show the standard deviations obtained from $n = 20$ females. Dashed line corresponds to the linear regression (EV = 0.387 X PV + 6.8058, $R^2 = 0.4202$, $p < 0.01$). Pearson's test was performed to determine the significance of the coefficient of determination R^2. R, *Rhodomonas salina*; T, *Tisochrysis lutea*; P, *Pavlova lutheri.*

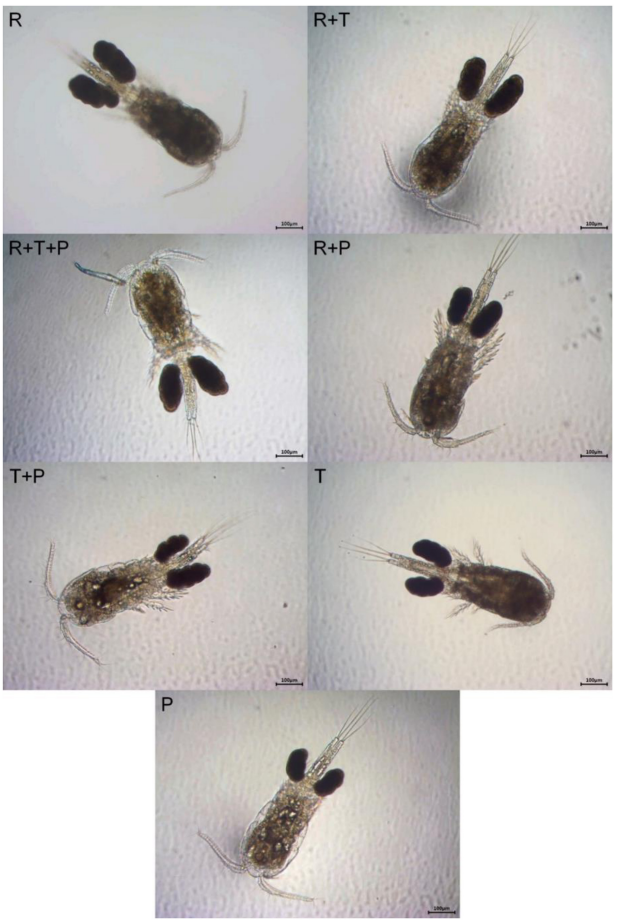

FIGURE 6 | Visual comparison of the body size and the size of the egg sacs of *Paracyclopina nana* ovigerous females fed on different microalgal diets. Additional supporting data on ovigerous females respective prosome length and clutch size can be found in Dayras et al. (2020). R, *Rhodomonas salina*; T, *Tisochrysis lutea*; P, *Pavlova lutheri.*

In our study, the lowest DHA/EPA ratio was found in *P. lutheri* because of its high EPA amount, but copepods fed the P diet did not have elevated amounts of EPA and in fact had some of the lowest DHA and EPA amounts among all tested diets. Although *P. lutheri* contains potential precursor fatty acids for DHA synthesis, these precursors may not be efficiently absorbed by copepods. A similar phenomenon was observed for the other fatty acids studied since the copepods fed with the P diet presented some of the lowest levels of each individual fatty acid while *P. lutheri* is the microalga containing the highest amounts of C16: 1 and C16: 0 among the three microalgae studied. This may be related to the fact that *P. lutheri* is the smallest microalgal species among the three tested here (4–6 µm) (Kamiyama and Arima, 2001; Rehberg-Haas, 2014). It is more than twice as small as *R. salina*, which measures 12.9 × 7.7 µm (average coefficient of variation 19.4%) (Renaud et al., 1999; Schipp et al., 1999). This likely makes *P. lutheri* the most expensive of the three microalgal species to graze on for *P. nana*, or *P. nana* may not have the adaptions required to digest this microalgal species. The genus *Pavlova* is also known to be a very fragile one. Most common strains of *Pavlova* are known to be sensitive against shear force and high temperature (>28°C). They appear to be very sensitive to downstreaming processes and have a very short shelf-life, and for this reason it has not been possible to produce *Pavlova* sp. in an industrial scale so far (Rehberg-Haas, 2014). This fragility may

also justify why *P. lutheri* was not the most performant microalga of our experiment.

In our work, *R. salina* had average DHA and EPA amounts, but its diet combination with *T. lutea* (R + T) led to the lowest DHA/EPA ratio in copepods, with a record EPA amount, and its combination with *P. lutheri* (R + P) led to the highest DHA/EPA ratio in copepods, with a high DHA amount. The R diet alone resulted in a record DHA amount and one of the highest EPA amounts in *P. nana*. Many studies have pointed out the crucial roles of dietary DHA and EPA in fish development (Rainuzzo et al., 1997; Sargent et al., 1999; Izquierdo et al., 2000; Mejri et al., 2021). These particular fatty acids help maintain the function of visual and neural cells as well as cell membrane structure. Therefore, a diet deficient in DHA and EPA could lead to delayed growth and increased mortality in fish larvae (Mejri et al., 2021). DHA is of greater importance than EPA in marine fish as the former is present in high concentrations in fish larval tissue and promotes development to a greater extent than EPA. The relative proportion of DHA to EPA in larval diets is an important component to consider, and the optimal ratio of DHA to EPA has been established to be 2:1 (Sargent et al., 1997). In the present study, the ratio of DHA to EPA in *P. nana* was always higher than 2:1 among the different tested microalgal diets, except for R + T (1:1). Particularly high DHA-to-EPA ratios were observed in copepods fed the T + P and R + P diets, probably because of the contributions of *P. lutheri*, which had by far the highest amount of EPA that was then converted to DHA.

Comparison of microalgae and copepods fatty acid profiles confirmed that *P. nana* has fatty acid conversion capability. Based on our results, R + T and R diets appear optimal for culture and enrichment of *P. nana* as these diets induced record amounts of DHA and EPA in this copepod species and globally increased its fatty acid content. Diets T + P and R + P are preferable diets to feed *P. nana* cultured for use in aquaculture if the aim is to obtain copepods with the highest DHA/EPA ratio.

Effects of Microalgal Diet on the Monosaccharide Composition of *P. nana*

Carbohydrates in aquatic animals have been mostly studied in fish species to investigate the ability of fish to use dietary carbohydrates; few studies have been performed on crustaceans and even fewer on copepods. Most research has focused on the carbohydrate metabolism of shrimp and crab species because of their use as high quality protein sources in human diets (Wang et al., 2016). Carbohydrates in copepods are poorly characterized and previous investigations provided conflicting results, likely because carbohydrate content in copepods varies widely according to study conditions and various intrinsic factors (Kleppel, 1993). Carbohydrates are rarely stored in crustaceans, but are rather rapidly metabolized for several vital functions (Cuzon et al., 2000).

The three tested microalgal species had different monosaccharide profiles. *R. salina* had the highest total monosaccharide content per milligram, with almost one third more than the two others species. However, the individual amounts of monosaccharides were always the lowest in this species except for glucose, which was present at a high concentration in *R. salina*, which positively correlated with the high total monosaccharide content of this microalgal species. *R. salina* therefore seems to be a glucose-specialized microalga. Conversely, *T. lutea* had the lowest total monosaccharide content, with 10-fold less glucose per milligram than *R. salina*, but generally had the highest individual amounts of each of the individual monosaccharides evaluated. *P. lutheri* had an intermediate monosaccharide profile characterized by a moderately glucose content and lower concentrations of the individual monosaccharides than *T. lutea*.

When monosaccharide content in copepods was evaluated, copepods fed the R + T + P diet combination had the highest total monosaccharide content and also, in general, the highest amounts of each individual monosaccharide. These results suggest an additive effect of each microalgal species on monosaccharide richness; the higher the amounts of various monosaccharides in the diet, the greater the concentration of monosaccharides in *P. nana*. Both T + P and R + T diets induced high monosaccharide concentrations in *P. nana*. Diets that yielded the lowest total monosaccharide contents in copepods were the single-species diets, often resulting in individual monosaccharide quantities less than 1 μg/mg. Diet P was the least efficient diet in term of monosaccharide content in *P. nana*, which is consistent with the very low concentrations of monosaccharides found in this microalgal species.

Comparing both fatty acids and monosaccharides profiles in microalgae (Appendix A), *R. salina* had the highest monosaccharide content but lowest fatty acid content. Inversely, *T. lutea* had the lowest monosaccharides content but the highest fatty acid content. The same phenomenon was observed in copepods fed these diets. R + T + P induced the highest monosaccharide concentration in copepods but a low fatty acid content, whereas, R + T and R diets resulted in the highest fatty acid concentration in copepods but not monosaccharide content. Our results therefore suggest that, for fish larvae rearing purposes, it is preferable to use a varied diet comprising several species of microalgae; we recommend the R+T diet as the best compromise diet for productive culture of *P. nana*.

Effects of Microalgal Diet on Reproductive Investment by *P. nana* Ovigerous Females

The three diets of R + T, R, and R + T + P gave the best results in terms of clutch volume and ovigerous female prosome volume in *P. nana*. We also demonstrated that these three diets were associated with the highest egg numbers per ovigerous female. As shown in **Figure 3**, diets R + T, R, and R + T + P induced both large females with bulky prosomes and a large clutch size. The photographs shown in **Figure 6** also confirm that it is the ovigerous females fed with the diets R, R + T, and R + T + P which ended to look the biggest and obtained the largest egg sacs. These observations are also consistent with the conclusions from the prosome length and clutch size analyses which can be found in Dayras et al. (2020). In our present study, these three diets were associated with the lowest DHA/EPA ratios in copepods.

A low DHA/EPA ratio and high EPA content therefore appear to be linked to increased fecundity in *P. nana* copepod females, both in terms of number of eggs and morphological quality of eggs and females. Copepods fed the R + T and R diets had the highest EPA amounts and also the highest total fatty acid amounts. Ovigerous females rich in fatty acids and particularly in EPA appeared to be the largest and most fertile females.

The P diet was associated with the lowest clutch volumes and lowest egg numbers in ovigerous females. P-fed females were also the smallest and carrying the smallest egg sacs. All the diets in which *P. lutheri* was involved were the lowest ones in terms of EPA content in copepods, confirming again its poor performances in the culture of *P. nana*.

Ovigerous females prosome volume and clutch volume seem to be not only appear to be influenced in the same way by EPA richness and to be correlated with each other. We demonstrated a significant linear relationship between prosome volume and clutch volume in *P. nana* ovigerous females and between each one of these two parameters and EPA amount. Thus, EPA affects the fecundity of *P. nana*. Arendt et al. (2005) found a positive relationship between the EPA amount ingested by the copepod *Temora longicornis* and its egg production rate. It appears that the higher the EPA content of the copepod, the higher its egg production. Previous studies have shown that copepod fecundity may be influenced by the DHA and EPA content of their diets (Støttrup and Jensen, 1990; Kleppel et al., 2005). Similar to the findings of Pan et al. (2018) for *Apocyclops royi*, our results indicate that dietary DHA levels did not have a large effect on *P. nana* fecundity, and may rather play an indirect role. Consistent with Lee et al. (2006) findings, *P. nana* appears to be able to synthesize DHA from short-chain fatty acids. This ability would allow this copepod species to remain reproductively active even in the absence of DHA in its diet.

CONCLUSION

Overall, our experimental results indicate that *R. salina* is the diet that induces the highest productivity of *P. nana* in culture, especially when combined with *T. lutea*. A mixed diet composed of *R. salina* and *T. lutea* induced the highest total fatty acid content in *P. nana*, a good monosaccharide profile, and the greatest prosome and clutch volumes in ovigerous females. Conversely, *P. lutheri* does not appear to be an effective microalgal prey species for *P. nana* raised for aquaculture purposes. This study is the first to explore the effects of different diets on the fatty acid and monosaccharide profile of *P. nana*, and to demonstrate that *R. salina* is a suitable microalgal species for productive and effective mass culture of this copepod species for use as a live prey in aquaculture.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SS and J-SL conceived the experiment, helped in drafting the manuscript and approved the current version. PD conducted the expertiments and drafted the manuscript. CB contributed to all experiments and commented on the manuscript. IS realized fatty acid and monosaccharides analyses and commented on the manuscript. M-CL contributed to writting of the manuscript and commented on the manuscript in earlier version. All authors contributed to the article and approved the submitted version.

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APPENDIX

Appendix 1 | Comparison of fatty acid and monosaccharide profiles in the microalgal and copepod samples.

Microalgae samples	**μg total FA/mg DW, *n* = 3**	**μg total MonoS/mg DW, *n* = 3**
R. salina	66.38 ± 7.22	46.60 ± 4.71
T. lutea	85.38 ± 4.36	28.28 ± 9.20
P. lutheri	75.10 ± 14.03	32.67 ± 3.97
Copepod samples	**μg total FA/mg DW**	**μg total MonoS/mg DW**
T0 (control)	4.56	4.08
R	50.34	6.28
T	49.24	–
P	31.52	3.83
R + T	58.22	15.09
T + P	36.46	22.89
R + P	31.84	–
R + T + P	33.70	40.69

Cells containing a "–" correspond to values for which the detection did not work due to an insufficient biomass quantity.

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Effects of Epibiotic Diatoms on the Productivity of the Calanoid Copepod *Acartia tonsa* (Dana) in Intensive Aquaculture Systems

Yen-Ju Pan[1,2], Wei-Lung Wang[3], Jiang-Shiou Hwang[2,4,5] and Sami Souissi[6]**

[1] Department of Aquaculture, National Taiwan Ocean University, Keelung, Taiwan, [2] Center of Excellence for the Oceans, National Taiwan Ocean University, Keelung, Taiwan, [3] Department of Biology, National Changhua University of Education, Changhua City, Taiwan, [4] Institute of Marine Biology, National Taiwan Ocean University, Keelung, Taiwan, [5] Center of Excellence for Ocean Engineering, National Taiwan Ocean University, Keelung, Taiwan, [6] Université de Lille, CNRS, Université du Littoral Côte d'Opale, UMR 8187 LOG, Laboratoire d'Océanologie et de Géosciences, Station Marine de Wimereux, Lille, France

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***Correspondence:**
Jiang-Shiou Hwang
jshwang@mail.ntou.edu.tw
Sami Souissi
sami.souissi@univ-lille1.fr

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We evaluated here the effects of the epibiotic diatom *Tabularia* sp. on the productivity of the calanoid copepod *Acartia tonsa* (Dana) for assessing their risk on copepod intensive aquaculture industry for the provision of live feed. In the first experiment, uninfested and intensively infested females were cultivated individually for the assessment of egg production. Intensively infested females appeared to have a significantly lower egg production (5.0–9.0 eggs/female/d) than uninfested females (22.0–26.0 eggs/female/d) during 5 consecutive days. In the second experiment, effects of culture densities on diatom epibiosis were investigated in 9 L cultures at three different densities (200, 400, and 600 ind. L^{-1}). Another culture at higher volume (250 L) and lowest density (200 ind. L^{-1}) was also carried out to test the effect of culture volume on diatom epibiosis. The infestation rate (%), infestation intensity (ratio of surface diatom coverage levels, classified as levels 0–3) and daily egg harvest rate (number of harvested eggs per day per liter) were evaluated among the four culture populations. The copepods had higher infestation rate (53.69–60.14%) and intensity rate (high ratios at level 2 and 3) when the densities were increased from 200 ind./L to 400 and 600 ind./L. Although egg harvest increased with increasing culture density, it seemed that the diatom-infested *A. tonsa* population reach a saturated egg production when the density was higher than 400 ind./L. Nevertheless, the differences of culture volumes (250 and 9 L) appeared to be not to have any effect when the copepods were cultivated at the same density (200 ind./L). This study reveals for the first time that the epibiosis of the diatom *Tabularia* sp. reduces the individual egg production, and egg harvest rate in high-density culture of the copepod *A. tonsa*. Our findings implicate that diatom epibiosis should be avoid in copepod intensive culture systems.

Keywords: epibiotic diatom infestation, copepod, egg production, density-dependent effects, culture volume

INTRODUCTION

Copepods represent important trophic linkages in marine food webs (Støttrup, 2000; Hwang et al., 2004; Turner, 2004). They provide nutritional benefits (van der Meeren et al., 2008; Rayner et al., 2015; Pan et al., 2018) and great palatability (Chesney, 2005; Højgaard et al., 2017) for feeding aquatic larvae. Based on the emerging developments of intensive culture techniques, different copepod species are meanwhile cultivated and used as live feed in marine larviculture (Lee et al., 2005; Drillet et al., 2011; Blanda et al., 2015; Hansen, 2017). The indoor intensive culture system could be consistently maintained at optimal culture conditions facilitating higher copepod productivity than extensive outdoor culture systems. Furthermore, they should be managed sustainably and cost-effective (Abate et al., 2016). Laboratory studies on the optimal culture conditions of several copepod species have been accomplished, and resulting evidence suggests that these can be upgraded to mass culture systems. However, a complete removal of microorganisms that provide potential health risks to copepods in intensive culture systems remains challenging (Paerl and Tucker, 1995; Petkeviciute et al., 2015; Rurangwa and Verdegem, 2015).

Copepods are common hosts of epibiotic microorganisms such as bacteria, protists and microalgae (Carman and Dobbs, 1997; Utz and Costs, 2005; Mantha et al., 2013; Burris and Dam, 2014; Romano et al., 2021). The effects of epibiotic ciliates and bacteria were the focus point of several studies (Nagasawa, 1987; Puckett and Carman, 2002; Bickel et al., 2012; Souissi et al., 2013; Burris and Dam, 2014; Jones et al., 2016). Their infestations are considered to be adverse for copepod reproduction. Epibionts are mostly found on the surface of crustacean zooplankton during their growth phase which results in the dispersion of epibionts to a new basibiont (de Souza Santos et al., 2020). The constitution of the substratum dictates the number of species that can establish themselves on the basibiont to the point of bringing about a high level of adaptability in epibiotic communities. Locating a suitable substratum is of utmost importance to an epibiont (Purushothaman et al., 2021).

Recent studies have shown host preference among epibionts (Silver-Gorges et al., 2021). Epibionts were found on planktonic crustaceans in eutrophic water conditions, but the relation between the physical parameters of water and epibiosis have not yet been studied in detail (Nayak et al., 2021). Ecologically, epibiosis is an important phenomenon as it is a direct reflection of the level of pollution since absence or presence of certain epibiont-basibiont associations can be used for bioindication (Purushothaman et al., 2021). Ciliate epibionts are frequently found on crustacean species, such as copepods (Souissi et al., 2013; Burris and Dam, 2014).

However, occurrences of microalga epibiosis on copepods were mostly documented with particular focus on epibiont morphology, phylogeny, and distribution (Carman and Dobbs, 1997; Fernandes and Calixto-Feres, 2012; Li et al., 2014; Gómez et al., 2018; Nayak et al., 2021). The impacts of algal epibiosis on copepod reproduction were rarely investigated as yet. Hakimzadeh and Bradley (1990) and Petkeviciute et al. (2015) noted higher expressions of stress-related proteins and genes in the algal-infested calanoid copepods *Eurytemora affinis* (Poppe) and *Acartia tonsa*. A field study showed that the algal infestation seemed not to affect copepod survival and egg production (Møhlenberg and Kaas, 1990). Nevertheless, the impact of epibiotic microalgae on copepod productivity remains unclear and should be further investigated.

The calanoid copepod *A. tonsa* has been considered as a suitable live prey in several larviculture studies (Wilcox et al., 2006; Øie et al., 2017; Vanacor-Barroso et al., 2017). Intensive mass cultures of *A. tonsa* has been established in a pre-industrial culture facility affiliated to the University of Lille, France since late 2014. In December 2015, epibiotic diatom infestations occurred in *A. tonsa* cultures calling for an investigation of its effects on copepod mass culture. Two independent experiments were performed to verify the following questions: (1) the effects of epibiotic diatoms on the egg production of individual copepods, (2) the effects of culture volume and copepod density on the epibiotic diatom infestation rate, infestation intensity and copepod egg harvest rate in the mass cultures. Our study aimed to assess the consequences of diatom epibiosis on the reproduction and mass culture productivity of *A. tonsa*.

MATERIALS AND METHODS

Microalgae and Copepod Stock Cultures

Copepod and microalgae cultures were maintained in an indoor and pre-industrial culture facility programmed at 18 ± 1°C and 12L:12D light:dark cycle. The culture line of microalga *Rhodomonas baltica* (RCC350) as copepod food was obtained from the Roscoff Culture Collection, France. Batch cultures were maintained in 10 L flasks with treated natural seawater (1-μm-filtered, UV-radiated, chlorine-sterilized, at salinity 34) enriched with Walne's medium (Walne, 1970). The algae were used for feeding the copepods at exponential growth phase (2–3 days after inoculation), and the cultures were re-inoculated every 6–7 days. The copepod *A. tonsa* culture strain (DFH.AT1) was obtained from Roskilde University, Denmark, and reared in 250 L culture columns containing the treated seawater where the microalgae *R. baltica* was added as feed (2×10^4 cells mL^{-1}). The water of the batch cultures was completely replaced every 2 weeks.

Light Microscopy, Scanning Electron Microscopy Examinations, and Terminology of Epibiotic Diatoms

Copepods infested by epibiotic diatom were randomly collected from the stock cultures. Alive samples were observed and photographed under an inverted light microscope (IX71, OLYMPUS, Tokyo, Japan). Finally, the copepods were fixed with 4% buffered glutaraldehyde for SEM analysis. For SEM preparation, individual copepods were dehydrated in an ethanol gradient (70% → 85% → 95% → 100% → 100%), and were transferred to aluminum stubs. Then a drop of hexamethyldisilazane (HMDS) was added for critical point drying. The stubs were sputter-coated with Gold-Paladium (E1010, Hitachi Ltd., Tokyo, Japan), and observed using a Hitachi

TM3000 SEM (Hitachi Ltd., Tokyo, Japan) at an accelerated voltage of 20 KV.

Colonies of epibiotic diatoms were collected and lyophilized, then treated with $KMnO_4$ and HCl to eliminate organic matter. The acid-washed diatom valves were conserved in Milli-Q filtered water. Fifty microliters of the specimen were placed and air-dried on an aluminum stub, then as for the copepods sputter-coated with the Gold-Paladium. Specimens were observed using a Hitachi S-4800 field emission scanning electron microscope (Hitachi Ltd., Tokyo, Japan). The morphometric measurements of the diatoms were performed using imagery software Image J (v 1.41, National Institutes of Health, United States).

Experimental Design

Individual Experiment

To evaluate the impact of diatom epibiosis on the egg production of *A. tonsa* at individual scale, 6 uninfested and 6 intensively infested (> 70% epibiotic diatom coverage) adult females were sorted from the 250 L culture columns and cultured individually in 6-well culture plates containing treated natural seawater (10 mL/well) and *R. baltica* were added as feed. The environmental conditions were maintained as aforementioned (section "Microalgae and Copepod Stock Cultures"), and the culture medium was replaced every day. The daily egg production was documented during 5 consecutive days using a stereomicroscope (SZX9, OLYMPUS, Tokyo, Japan).

Population Experiment

The copepods collected from the same batch culture were divided into 4 separate culture populations to immediately initiate the experiment after a volumetric density estimation. The volumes and densities of the 4 populations (one culture each) were designed as follows: (A) 200 individuals L^{-1}, 250 L; (B) 200 individuals L^{-1}, 9 L culture; (C) 400 individuals L^{-1}, 9 L culture; (D) 600 individuals L^{-1}, 9 L culture. Population B was designated as the positive control, which examined the effects of culture volume and copepod density when the results were compared to population A, and populations C, D, respectively.

The culture conditions of the 4 populations were maintained as aforementioned (section "Microalgae and Copepod Stock Cultures"). Daily egg production was documented in all populations during 5 consecutive days as analytical replicates. After 15 min of no aeration, the eggs were siphoned from the bottom and isolated by sieving through 120 μm (to retain the copepods) and 70 μm meshes (to collect eggs). The copepods and the water were returned to the cultures, and the number of eggs was volumetrically estimated under the stereomicroscope. At the 5th day (endpoint), all the copepods were collected and volumetrically counted to estimate the final density. Around 200 each adult male and female were randomly sorted from the population, then fixed in 4% formaldehyde for further analysis. The copepod specimens were examined under inverted microscope (IX71, Olympus, Japan), and the infestation rate (%) was calculated as: number of infested copepods/total number of collected copepods. Subsequently, all the infested copepods were visually classified under the microscope into four levels of diatom exoskeleton coverage (Møhlenberg and Kaas, 1990). Meaning of the different coverages levels as level 0: 0% coverage; level 1: < 10% coverage; level 2: 10–50% coverage; level 3: > 50% coverage (**Figure 1**). Infestation intensity (ratio% of different coverage levels) was calculated as: number of copepods at various infestation levels/total number of infested copepods.

Data Analysis

Statistical analysis was carried out using the SPSS program (Version 17.0). In the individual experiment, Student's *t*-test was used to compare the mean values of the egg production number between uninfested and intensively infested females ($n = 6$ each) on a daily basis. In the population experiment, the daily egg harvest number (daily egg production per liter) was estimated during 5 consecutive days in each treatment. We first confirmed the absence of interaction between treatment (population) × time (replicate) by using a repeated measure ANOVA. Then, a one-way ANOVA test was applied to the average data of daily egg production per liter obtained over 5 days in each population. Once the significant differences were detected among populations ($p < 0.05$), Tukey's multiple comparison test was used to analyze specific differences between pairs of populations.

RESULTS

Light Microscopy and Scanning Electron Microscopy Examinations of Epibiotic Diatoms

LM pictures of infested copepods and the epibiotic diatoms are shown in **Figure 2**. The mono-species diatom colonies adhered to the *A. tonsa* exoskeleton without apparent preference for micro-locations on the exoskeleton (**Figures 2A,B**). The diatom could have 2–3 segmented chloroplast plates (**Figures 2C,D**) or a large chloroplast plate (**Figure 2F**) in the silicate valve. In accordance to LM examinations, the SEM pictures indicated a mono-specific diatom infestation on the exoskeleton of *A. tonsa* (**Figure 3A**). Detailed SEM pictures (**Figure 3B**) indicate that the diatoms attached to the copepods used a mucilaginous pad. The linear-lanceolate valves (**Figures 3C,D**) of the diatom measured ($n = 15$) 32.4 ± 6.2 μm in apical axis and 3.4 ± 0.3 μm in transapical axis, and the striae (10.3 ± 1.1 per 10 μm) were distributed symmetrically bilateral on the non-raphe valve with broad axial area (**Figures 3E,G**). Apexes were rounded but not capitate, and carried one rimoportula at each polar nodule (**Figures 3F,H**). A literature review was facilitated for diatom species identification based on their morphological characteristics by Snoeijs (1992); Kaczmarska et al. (2009), Totti et al. (2009); Suzuki et al. (2015), Cao et al. (2018), and Gómez et al. (2020), and the description of the genus *Tabularia* reported by Williams and Round (1986). The diatom was identified as *Tabularia* sp.

Individual Culture Experiment

The individual daily egg production declined significantly ($p < 0.01$) when the copepods were intensively infested with epibiotic diatoms (**Figure 4**) during 5 consecutive days.

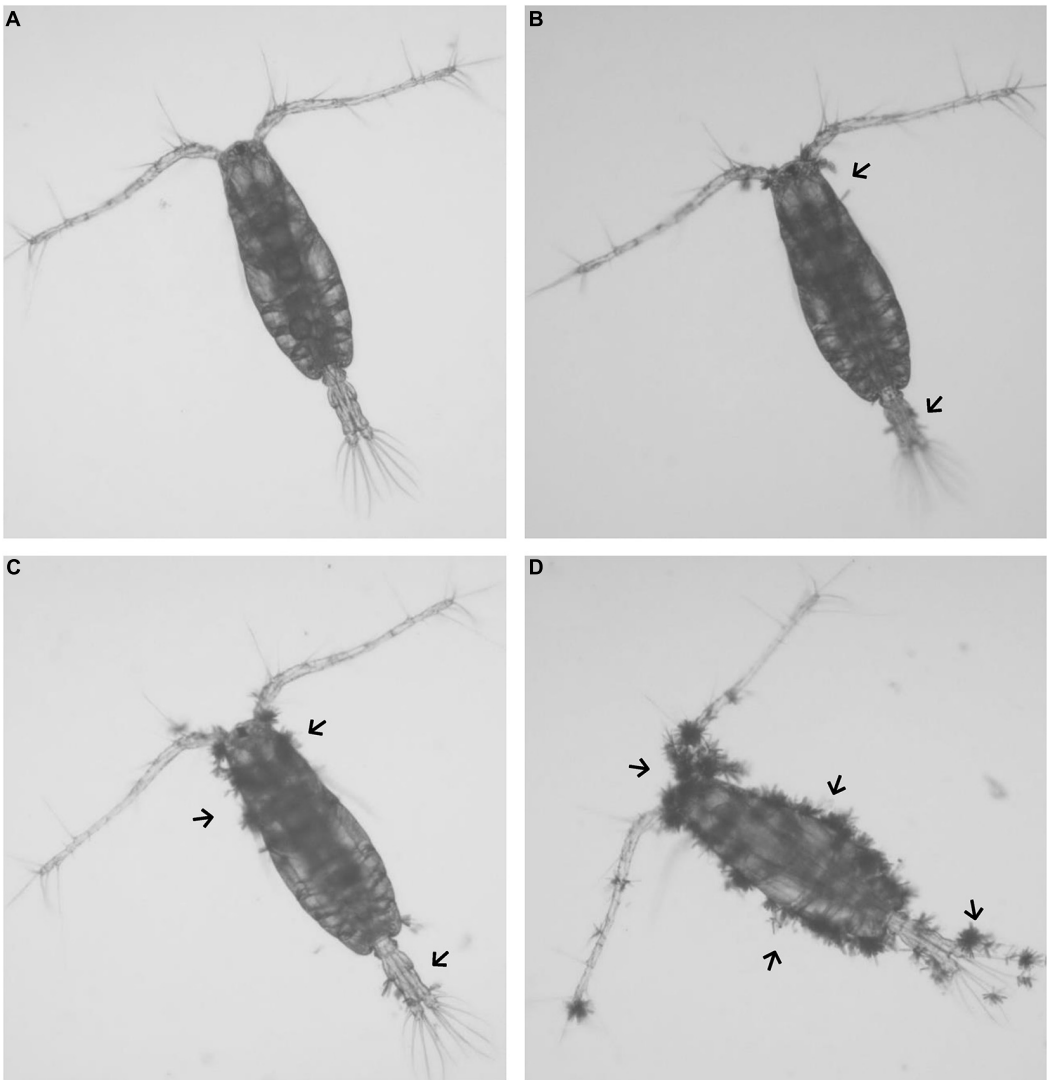

FIGURE 1 | Illustration of different epibiont infestation levels on copepod *A. tonsa*. **(A)** Level 0: 0% coverage; **(B)** level 1: < 10% coverage; **(C)** level 2: 10–50% coverage; **(D)** level 3: > 50% coverage.

The average egg production of uninfested females ranged from 22.0–26.0 eggs/female/day, whereas this was reduced to a range of 5.0–9.0 eggs/female/day in intensively infested individuals.

Population Culture Experiment

Figure 5 shows that the daily egg production rate (eggs/L/day) averaged from the data obtained during five consecutive days. A significantly lowest egg production (496.4 ± 51.4) was found in the population A (250 L, 200 ind./L). The populations C (9 L, 400 ind./L) and D (9 L, 600 ind./L) had the top two highest egg production (3078.52 ± 524.86 and 3227.56 ± 596.81 eggs/day/L, respectively) among populations, yet the two populations were not statistical different to group B (9 L, 200 ind./L). At the same population density (200 ind./L), populations A (250 L) and B (9 L) had similar levels of infestation ratio and intensity, different in males and females (**Tables 1**, **2**). On the other hand, both male and female infestation rate were higher when the population density increased from 200 (population B) to 400 (population C) and 600 (population D). Notably, female copepods had higher epibiotic rates (40.9–73.2%) and intensity (higher ratios in level 2 and 3) compared to males. All populations remained at similar density after the 5-day cultivation, except a remarkable decline was found in population D (**Table 1**).

DISCUSSION

Pennate diatoms belonging to the genus *Tabularia* are common components in marine benthic communities (Snoeijs, 1992). Their epibiotic associations are documented in benthic organisms such as bryozoans (Wuchter et al., 2003) and marine

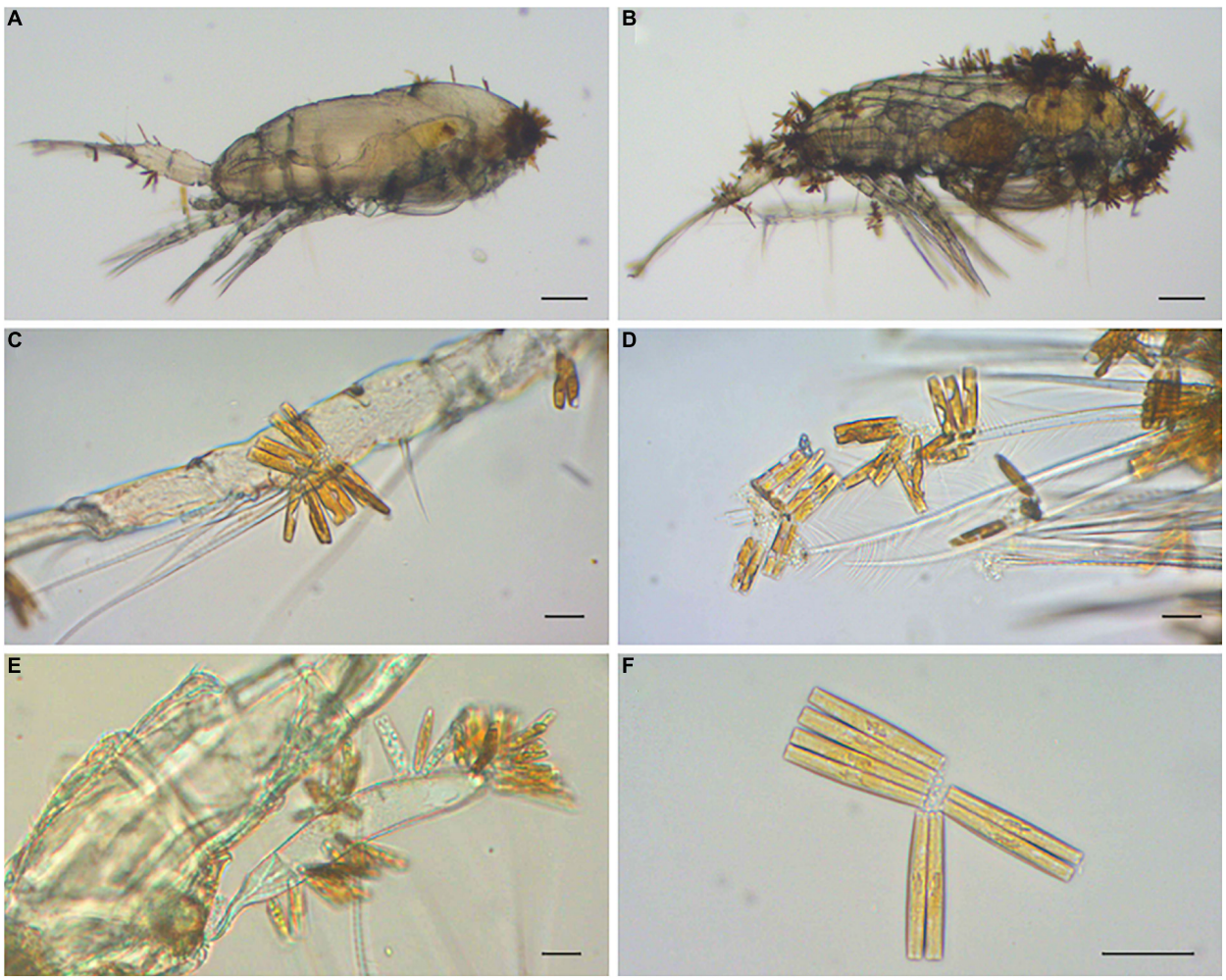

FIGURE 2 | The copepod *A. tonsa* infested with the epibiotic diatom *Tabularia* sp. **(A)** adult male **(B)** adult female **(C)** infestation on an antennule **(D)** infestation on caudal setae **(E)** infestation on a spermatophore attached on female **(F)** diatom colony isolated from copepods. Scale bars: **(A,B)**: 150 μm; **(C–F)**: 20 μm.

macroalgae (Totti et al., 2009). The occurrence of *Tabularia* epibiosis on copepods has been reported recently in the English Channel (Gómez et al., 2020), where it is close to the copepod culture unit in the present study. Although equipped with a well-established filtration system (sand filter, UV, and bio-filtration), the origin of the diatom contamination was likely from the inlet of natural sea water to the copepod culture environment. The epibiotic association of *Tabularia* with the planktonic copepod *A. tonsa* in aquaculture environment is reported here for the first time. Microalgal epibiosis is considered as an ecological strategy to access higher light exposure and nutritional replenishment provided by their mobile host swimming in the water column (Totti et al., 2010). In addition, the epibiotic microalgae may have higher chances to absorb the excretion released from their host as nutrients (Wahl et al., 2012). Based on the aforementioned statements, the survival and mobility of the hosts are crucially benefitting epibiotic diatoms. Thus, the infestation of epibiotic diatoms seem not to be lethal in an acute sense. Based on the analysis by light microscopy and SEM (**Figures 2**, **3**), the reported epibiosis was a mono-specific event. This observation suggests that the diatom *Tabularia* sp. could outcompete other epibionts during biofouling at certain circumstances. The main object of the present work was to investigate the productivity of copepods in the events of epibiont infestation for aquaculture propose. Despite the fact that the diatom was identified as *Tabularia* sp. based on the morphological features examined under light and scanning electron microscopy, and a deep literature review, it should be noted that diatom molecular taxonomy or phylogeny is not the main focus in our work.

Ikeda (1977) noted a similar metabolic ratio of the diatom-infested and uninfested copepod *Calanus plumchrus*. However, the negative impacts of epibionts on the swimming behavior of their zooplankton hosts were confirmed (McAllen and Scott, 2000; Souissi et al., 2013; Burris and Dam, 2014). The authors suggested that the zooplankton hosts need to expend additional energy to cope with the extra burden and water drag caused by the epibiotic assemblages. Especially, the burden effect could be pronounced in the case of diatom epibiosis, where heavy

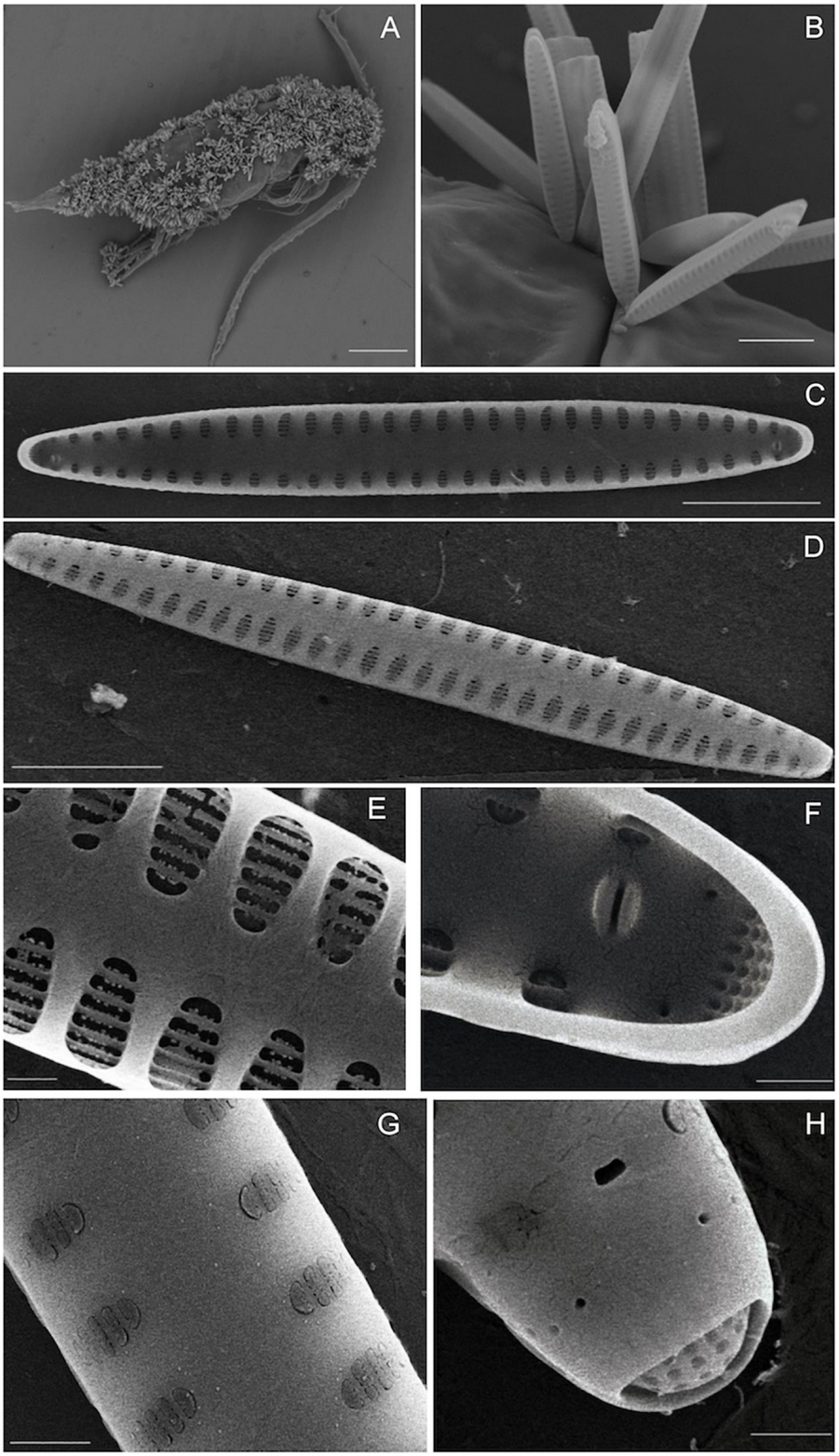

FIGURE 3 | SEM image of the infested copepod *A. tonsa* and the epibiotic diatom *Tabularia* sp. **(A)** Infested *A. tonsa* female. **(B)** Colony of epibiotic diatom *Tabularia* sp. attached on copepod exoskeleton. **(C)** Interior view of entire valve. **(D)** External view of entire valve. **(E)** Interior view of striae structure. **(F)** Interior apex of valve showing details of rimoportula and ocellulimbus. **(G)** External view of striae structure. **(H)** External apex of valve showing details of rimoportula aperture and ocellulimbus opening. Scale bars: **(A)** 250 μm; **(B)** 15 μm; **(C,D)**: 5 μm; **(E–H)** 1 μm.

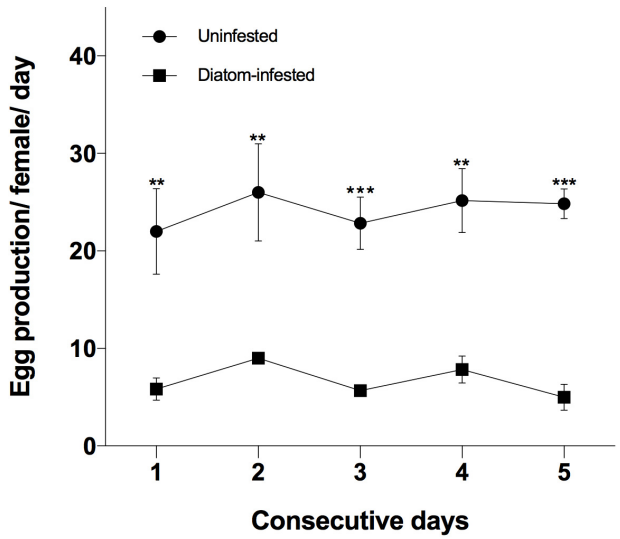

FIGURE 4 | Egg production per female per day of the uninfested and diatom-infested copepod *A. tonsa* during 5 consecutive days. Data are presented as average ± standard error ($n = 6$), where $^{**}p < 0.01$, $^{***}p < 0.001$.

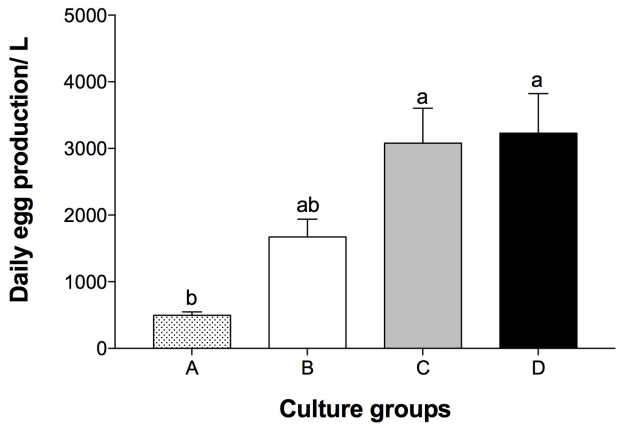

FIGURE 5 | Daily *A. tonsa* egg production per liter in the culture populations at different volumes and population densities. A: 200 ind./L in 250 L, B: 200 ind./L in 9 L, C: 400 ind./L in 9 L, D: 600 ind./L in 9 L. The data were obtained during 5 consecutive days, and presented as average ± standard error. The letters (a and b) above bars indicate significant differences ($p < 0.05$) identified by Tukey's *post hoc* test.

TABLE 1 | Infestation rate (%) of adult male and female *A. tonsa* in different culture populations.

Culture populations		Final density on the 5th day	Infestation rate		
			% total female	% total male	% total individual
A	(200 ind./L in 250 L)	220 ind./L	43.40	26.83	36.17
B	(200 ind./L in 9 L)	201 ind./L	40.89	30.47	35.32
C	(400 ind./L in 9 L)	435 ind./L	73.24	48.05	60.14
D	(600 ind./L in 9 L)	427 ind./L	56.28	51.29	53.69

silicate valves can provide a remarkable weight burden to their host (Purushothaman et al., 2021). Based on the results obtained in individual experiments, all diatom-infested *A. tonsa* females survived during the 5 experimental days yet produced a significantly lower quantity of eggs. This coincided with reduced egg production found in a previous study of *A. tonsa* females infested with ciliate epibionts (Burris and Dam, 2014). It is worthy to note that the age of copepods examined in the individual experiment of the present study, and Burris and Dam's work were not controlled. Although we attempted to investigate the relationship between host age and diatom epibiosis by carrying out an extended experiment, it was challenging to artificially induce the diatom adherence on the copepods. Indeed, the egg production decline with increasing age of copepod (Pan et al., 2014; Rodríguez-Graña and Calliari, 2020). If the diatom epibiont increased accumulatively on the copepod across age, it is highly possible that the age could be a co-factor with diatom epibiosis reducing copepod egg production. The combined effect of age and epibiosis on the decline of egg production, if it could occur in our study, should be amplified with increasing copepod age, because the hosts become older and the epibionts accumulate more. Although the constant egg production rates were found in our 5-day individual experiment, it should be noted that the effects of age and epibiosis on the change of *A. tonsa* egg production may occur gradually in a time-scale of weeks. Therefore, an extended monitoring is required to verify the combined effect of age and epibiosis on the egg production of the copepod.

To better understand the risk of diatom epibiosis for *A. tonsa* aquaculture, we assessed diatom epibiosis and copepod egg productivity in culture populations with three densities and two volumes. The infestation rate and intensity were not different in the 9 and 250 L populations when maintained at lowest density (200 ind./L). This finding suggests that the impact of culture volume is minor with respect to diatom epibiosis. On the other hand, the diatom infestation rate and intensity of adult *A. tonsa* were higher in the populations with higher copepod densities (400 and 600 ind./L). Likewise, the density-dependent epibiont prevalence was compared with some Cladocera and copepod species in lake and pond waters (Barea-Arco et al., 2001; de Souza Santos et al., 2020). In the aquaculture environment, as a closed water system similar to lakes or ponds, the higher density of *A. tonsa* could facilitate higher encounter rates and the possibility of epibiont transmission between copepods (Burris and Dam, 2014).

The density of copepods in culture is a crucial parameter affecting copepod productivity (Jepsen et al., 2007; Mahjoub et al., 2014; Rayner et al., 2017). Jepsen et al. (2007) demonstrated that egg production (eggs L^{-1} d^{-1}) increased with increasing copepod density from 100 to 600 ind. L^{-1} using the same *A. tonsa* strain (DFH-ATI). Due to the different methods of egg collection, the egg harvest per liter in the present study was lower than the result of Jepsen et al. (2007). Nevertheless, the designated densities (200–600 ind. L^{-1}) in our study were

TABLE 2 | Infestation intensity: ratio (%) of four levels of epibiont surface coverage of *A. tonsa* in different culture populations.

Culture populations		Level 0			Level 1			Level 2			Level 3		
		% total female	% total male	% total individual	% total female	% total male	% total individual	% total female	% total male	% total individual	% total female	% total male	% total individual
A	(200 ind./L in 250 L)	56.60	73.17	63.83	26.42	19.51	23.40	15.09	7.32	11.70	1.89	0.00	1.06
B	(200 ind./L in 9 L)	59.11	69.53	64.68	24.14	26.18	25.23	11.82	3.43	7.34	4.93	0.86	2.75
C	(400 ind./L in 9 L)	26.76	51.95	39.86	57.28	44.16	50.45	10.80	3.03	6.76	5.16	0.87	2.93
D	(600 ind./L in 9 L)	43.72	48.71	46.31	23.72	39.66	31.99	13.95	9.91	11.86	18.60	1.72	9.84

below the limiting density threshold for *A. tonsa* egg production. Instead of increasing with higher copepod density, similar egg production levels (3000 eggs L^{-1} d^{-1}) were measured in the culture populations C (400 ind. L^{-1}) and D (600 ind. L^{-1}). This finding implicates that the diatom-infested *A. tonsa* population, if under the same culture conditions and population origin, may reach the saturating egg production capacity at lower population density. The notable decrease of population was measured in the population D (**Table 1**), which had the highest ratio of infested copepods at the greatest diatom coverage (level 3). The higher intensity of infestation could be correlated to the higher encounter rate under the crowded conditions. As aforementioned, the diatom infestation is physiologically stressful to copepods, and it not only reduces individual egg production but also increases copepod mortality. Since the heavily infested copepods may die earlier, it could be expected that the infestation rate was slightly lower in population D than in population C. Overall, our findings clearly indicate the remarkable reduction of egg harvest rate in *A. tonsa* mass culture due to high diatom infestation intensity and copepod mortality.

Regardless of culture densities and volumes, females of *A. tonsa* had higher infestation rate than males in all culture populations (**Table 1**). The higher ratio of surface coverage level 2 and 3 was found in infested females (**Table 2**), which indicates that the female *A. tonsa* (body length: 1068.36 ± 56.20 μm) can carry more diatoms on their body surface than the male (body length: 931.98 ± 18.22 μm) does. This discovery coincided with the "habitat patch size effects" hypothesis stating that large-sized host provides larger targets for epibiont adherence (de Souza Santos et al., 2020). Host behavior could also strikingly impact the host-epibiont relationship. Ramos-Rivera et al. (2021) stated that the injured host had slower swimming speed and stayed more frequently at the water surface, which facilitated a greater opportunity for epibiont adherence. It's been noted that *A. tonsa* females have significantly lower swimming speeds and tended to aggregate around the tank bottom (Buskey et al., 2002; Kiørboe and Bagøien, 2005), where the benthic diatom *Tabularia* sp. abundantly colonized. This behavior pattern may lead to a higher encounter frequency and period to the benthic *Tabularia* in the culture environment, and eventually cause a higher epibiont infestation rate and intensity on female *A. tonsa*. On the other hand, female *A. tonsa* is known to live longer than male (Rodríguez-Graña et al., 2010). The greater longevity could be another explanation of female's higher epibiotic infestation if the epibiont really increase accumulatively with copepod age.

Diatom biofouling has been extensively studied in the context of anti-fouling coating or substance applications (Molino et al., 2009; Al-Naamani et al., 2017; Wanka et al., 2018). The mechanism of diatom fouling on zooplankton was rarely studied. This may be due to the difficulty of investigating diatom life cycles and their complex interactions with their host and environmental factors. Consequently, we attempted to expose uninfested *A. tonsa* individuals at the same-age (i.e., hatched and grow out from the same batch of eggs) to the prior isolated *Tabularia* cells. However, no infestation was documented during a 14-day period. Based on our observation, the diatom cells changed their cell morphology when it was cultivated independently. This preliminary finding suggests on physiological modifications of *Tabularia* sp. between the free-living and epibiotic phases of its life cycle. Furthermore, Mantha et al. (2013) stated that the deterioration of water quality affects the exoskeleton of copepods. In their study this was an outcome of high epibiotic and ectoparasitic infestation. Although the analysis of water quality was excluded in the present work, higher accumulation of copepod excretions could be expected in the high-density culture populations. Under such conditions epibiosis could be triggered either by signals related to the weakened exoskeleton of copepods or simply by the chemistry of the ambient waters. Further studies are required to verify possible abiotic or biotic stimuli that trigger the settlement of the diatom, which provide implications for aquaculturists to monitor and prevent the prevalence of epibiotic diatoms on copepod mass production. Moreover, the removal protocol of epibiotic diatom using algicidal substances and bacteria (Kitaguchi et al., 2001) could be developed for epibiotic diatom control.

CONCLUSION

In conclusion, our study demonstrated the adverse impact of the diatom epibiont on the productivity of the copepod *A. tonsa*. The diatom-infested copepods decreased their egg production for about 70% at the individual basis, and they reached the saturating egg production capacity and higher mortality at lower population density. These findings implicate the risk of diatom epibiosis causing economic losses for the copepod aquaculture industry. This also means that the presence of epibionts on copepods should be regularly monitored in copepod intensive culture systems to avoid negative developments such as increased mortality and any decrease of growth and egg production.

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

SS and J-SH conceived the original idea of this study. Y-JP designed and conducted the experiments and SEM analysis. W-LW performed the diatom morphology examination and species identification. Y-JP, SS, W-LW, and J-SH wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Effects of Food Concentration and Photoperiod on Egg Production, Female Life Expectancy and Population Dynamics of the Paracalanid Copepod, *Bestiolina amoyensis*

Lin Wang[1,2†], Shuhong Wang[1,2†], Chaoshu Zeng[3], Yuyue Wang[1,2] and Chunxiao Zeng[1,2]*

[1] Fisheries College, Jimei University, Xiamen, China, [2] Ornamental Aquarium Engineering Research Centre in Universities of Fujian Province, Xiamen, China, [3] Centre for Sustainable Tropical Fisheries and Aquaculture, College of Science and Engineering, James Cook University, Townsville, QLD, Australia

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***Correspondence:**
Shuhong Wang
shwang@jmu.edu.cn

† These authors have contributed equally to this work and share first authorship

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The paracalanid copepod, *Bestiolina amoyensis*, is a widely distributed species occurring in subtropical inshore waters across the Pacific Ocean. Its small size, herbivorous feeding habit, and high adaptability make the species one of the most promising candidates as potential live feed for hatchery larval rearing. This study investigated effects of different feeding density of microalgae *Isochrysis* spp. (1×10^5, 2×10^5, 3×10^5, 4×10^5, and 5×10^5 cells ml^{-1}) and photoperiod (8L:16D, 12L:12D, and 16L:8D) on productivity-related parameters, including egg production, female life expectancy and population dynamics of *B. amoyensis*. Results showed that total egg output over female lifespan, final population size and intrinsic rate of population of 12L:12D photoperiod treatments were always the highest among three photoperiod conditions, especially at the food concentration of 4×10^5 cells ml^{-1}, indicating *B. amoyensis* had high reproductive performance and the population was in a more stable status. The number of nauplii from 4×10^5 cells ml^{-1} algal concentration treatment accounted for 75% of the population, and the ratio of females to males approaching 1:1 when photoperiod was 12L:12D; female life expectancy was 10.5 ± 0.6 days. In conclusion, our results showed that *Isochrysis* spp. is a suitable feed for *B. amoyensis* with an optimal concentration at 4×10^5 cells mL^{-1}; the optimal photoperiod for *B. amoyensis* rearing is 12L:12D. The relatively long reproductive lifespan and high intrinsic population increase rate make *B. amoyensis* a good candidate to develop culture techniques for hatchery larval rearing.

Keywords: copepod, sex ratio, productivity, live prey, algal feeding density

INTRODUCTION

Providing palatable nutritional food of appropriate size to marine larvae at different development stages is one of the most challenging tasks in marine hatcheries. For some larvae with small mouth gapes, the traditional live prey of rotifers (mean length 135 μm of SS-type rotifer *Brachionus rotundiformis*) and *Artemia* nauplii (typically between 400–500 μm) could be too large to be

ingested by them (Fielder et al., 2000; Assavaaree et al., 2003; Gopakumar and Santhosi, 2009; Wullur et al., 2009; Conceição et al., 2010; Lindley et al., 2011; Hagiwara et al., 2014), and lacking of appropriate live prey to feed such larvae has been a crucial technical bottleneck. Copepods are the natural food for most marine larvae and nauplii of many copepods are less than 100 μm in size, which can meet the first feeding requirement of even small mouth- gaped fish larvae (McKinnon et al., 2003; Camus and Zeng, 2010; Bradley et al., 2013; Hill et al., 2020). Moreover, copepods typically develop through six naupliar and six copepodite stages with each stage of different size, making them suitable prey for fish larvae with different sizes of mouth gapes, as well as at different developmental stages (Schipp et al., 1999).

Several paracalanid species, including *Parvocalanus crassirostris* (Alajmi and Zeng, 2015) and *Bestiolina similis* (McKinnon et al., 2003; Camus et al., 2009; VanderLugt et al., 2009; Camus and Zeng, 2010), have been recommended as good candidates as larvae prey for fish larvae with small mouth gape. *B. amoyensis,* a species belonging to the same genus of *B. similis,* has been reported to co-exists with *B. similis* in the tropical coastal and estuary waters (McKinnon et al., 2003). The species distributes widely in Pacific Ocean, especially in western Pacific Ocean, and it is a dominant species during autumn (August – November) in subtropical Xiamen Harbor and Dongshan Bay of China (Yang, 2007; Zheng, 2009). However, no attempts have been made to culture the species as the live prey for feeding fish larvae previously.

Comparing to the traditional live prey, the main problem of copepods as live feed for larval culture is their difficulty for intensive culture and/or low culture productivity. Therefore, optimizing the culture conditions has been the focus of previous research. Many factors could affect copepod culture reproductivity and past research has been focused on temperature, salinity, and both quality and quantity of their food (e.g., Holste and Peck, 2006; Camus et al., 2009; Camus and Zeng, 2010; Nogueira et al., 2017; Nguyen et al., 2020; Choi et al., 2021; Dayras et al., 2021), while the effects of photoperiod is less studied despite photoperiod has been shown to significantly affect culture productivity of two *Acartia* species, i.e., *Acartia tonsa* (Peck and Holste, 2006) and *Acartia sinjiensis* (Camus and Zeng, 2008). Photoperiod is well known as one of the most important environmental factors regulating reproductive activity of aquatic animals, including copepods, and it has been reported to influence number of eggs produced by females, as well as the type (i.e., subitaneous vs. diapause eggs) of eggs produced (Marcus, 1982; Peck and Holste, 2006; Camus, 2012; Fereidouni et al., 2015). Furthermore, the physiological "clock" of aquatic animals allows them to response to changes in light condition, which could affect their feeding and reproductive activity (Miliou, 1992). For example, marine zooplankton, especially copepods, are known to undertake diurnal vertical migration (Haney, 1988) with a typical feeding pattern showing higher ingestion rate during night (Fuller, 1937; Petipa, 1958; Dagg and Grill, 1980; Stearns, 1986; Saito and Taguchi, 1996; Calbet et al., 1999), and some calaniod copepods, such as *P. crassirostris* (Sun, 2008) and Calanus (Harding et al., 1951; Zhang, 2003), spawn occurs more frequently at night. Hence, the goal of the current study was to evaluate the effects of photoperiod and microalgae feeding concentration and their interaction effects on culture productivity of *B. amoyensis*, seeking to answer the questions such as is there an optimum photoperiod regime for the cultivation of *B. amoyensis*? Whether there exist interactions between algae feeding concentration and photoperiod? For instance, whether longer darkness hours leading to enhanced egg production may require more abundant food supply to sustain higher productivity?

MATERIALS AND METHODS

Microalgal Culture

The microalgae *Isochrysis* spp. was cultivated in a temperature-controlled room. Several 70 L cylindrical acrylic cylinders filled with 0.01 μm filtered, UV irradiated, and Chlorine treated seawater (salinity 28 psu) were used for the algae culture. All cultures were maintained at 25 ± 1°C with vigorous aeration and f/2 medium was used for the cultures (Guillard and Ryther, 1962). The photoperiod was set as constant light (24 h light) with a light intensity of approximately 4000–5000 Lux. The algal cultures in its exponential growth phase were used for feeding copepods.

Sampling and Isolation of Copepods

Bestiolina amoyensis were initially obtained from a plankton tow performed in coastal water of Jimei District, Xiamen City, Fujian Province, China, on May 20th, 2019. The plankton sample was immediately transported back to a laboratory at Jimei University for isolation of *B. amoyensis*.

To isolate *B. amoyensis*, healthy copepods were firstly attracted to a light source, they were then transferred by a pipette to several petri dishes, where they were sorted according to their morphological characteristics and movement mode. The *B. amoyensis* identified (Li and Huang, 1984; Lian et al., 2018) were gently pipetted individually to wells of a 12-well plate filled with autoclaved seawater, wells were then carefully checked to make sure there was no contamination of other animals. Female *B. amoyensis*, identified by their swollen genital segments, were further sorted and individually cultured in 12-well cell culture plates. The culture volume of each well was 5 ml with *Isochrysis* spp. added at 1×10^5 cells ml^{-1}. Plates were incubated in a unit with light illustrated from down under. The light intensity was 500–800 Lux and photoperiod was set as 12 h L:12 h D.

Bestiolina amoyensis Stock Culture

Following separation, *B. amoyensis* cultures were gradually scaled up and eventually kept in several 300 L tanks filled with 0.01 μm filtered seawater and with gentle aeration. The salinity was 28 ± 1 psu and temperature was maintained at 26 ± 1°C. Light intensity was ∼ 500 Lux with a light: dark cycle of 12 h:12 h. Approximately 30% of the culture water was exchanged daily by gentle siphoning with a 5 cm diameter hollow cylindrical isolation device fitted to the front of the siphon tube, the bottom of which was covered with 48 μm mesh to prevent removal of copepods and reduce damage. *B. amoyensis* was

fed daily with microalgae *Isochrysis* spp. at a concentration of 1×10^5 cells ml^{-1}. Culture vessels were totally drained every 15 days for cleaning when adult copepods were caught with 500 μm special nets and juveniles with 75 μm nets, and the juveniles were transferred to culture carboys cleaned and sterilized with chlorine.

Experimental Design and Setup

A series of experiments were carried out to assess the influence of food concentration and photoperiod on the following parameters related to *B. amoyensis* culture productivity: (1) egg production, (2) adult female life expectancy, and (3) population growth over a 12 days culture period.

Production of Adult Copepods for the Experiment

For all experiments, mature *B. amoyensis* were retrieved with a special 500 μm sieve from stock culture and immediately transferred to a 2.5 L container with the bottom covered by a 75 μm mesh, the container was suspended in a 5 L transparent vessel containing 4 L of 0.01 μm filtered seawater. This design facilitated the separation of adult copepods and eggs produced by the females. After leaving the copepods to spawn for 6 h, each 2.5 L container containing the adult copepods was transferred to a new 5 L container, but the eggs produced by them were kept in the original 5 L container for incubation until hatching. The newly hatched nauplii were cultured for 3 days in the 5 L container before being transferred to an 18 L container for further cultivation until reaching the copepodite V stage (C_V). The culture conditions and feeding were initially the same as those described for stock culture (i. e. 26 ± 1°C; 28 ± 1 psu) and the concentration of feeding algae was 1×10^5 cells mL^{-1}. The last stage copepodites were then transferred to 30 ml Petri dishes and were monitored for molting daily with a microscope until they all developed to adults and ready for the following experiments. Adult *B. amoyensis* were transferred to a 1 L container containing filtered seawater and acclimated for 24 h to eliminate potential residual effects under the previous culture condition. The mixed population of both males and females during acclimation ensured that copepods used for the experiment were fertilized before being introduced into the Petri dishes for experiment.

Egg Production and Adult Female Life Expectancy Experiment

Following the acclimation, pairs of adult *B. amoyensis* that were actively swimming and with intact appendages were selected from the 1L beaker and carefully transferred to each of a 30 mL Petri dishes containing 20 mL seawater to monitor daily egg production. In the case that a male died during the experiment, a new male was introduced (Hall and Burns, 2001). If a female had not produced any eggs by the 3rd days of the experiment, a new female was introduced. Cultures were maintained in a constant temperature incubator and light intensity was set as ∼ 500 Lux.

This experiment used 5 × 3 factorial design with five food concentrations (1×10^5, 2×10^5, 3×10^5, 4×10^5 and 5×10^5 cells ml^{-1}) and 3 photoperiod conditions (8 h:16 h, 12 h:12 h, and 16 h:8 h), hence a total of 15 treatments. With 12 replicate pairs of adult copepods per treatment, a total of 180 Petri dishes were set up. The copepods were starved for 24 h prior to the formal commencement of the trial. Microalgae in exponential growth phase were use in present experiment and five samples were measured daily using an automated cell counter (Count-star IA1000, Shanghai Ruiyu Biotech Co., Ltd.) to maintain the designated concentrations of the experiment. Constant temperature (26°C) incubators were used to set up three photoperiod conditions and the light intensity was ∼ 500 Lux. During the experiment, each pair of copepods were removed from the Petri dish and transferred to new one with fresh seawater and microalgae supplied at the designated concentration every 24 h. The egg production was monitored and recorded using a Leica DMi8 microscope. Any deaths over the 24 h period was also recorded. Experiment was continued until all copepods in a treatment had died, and the mean life expectancy of adult *B. amoyensis* in the treatment was obtained by averaging individual lifespans all 12 replicates of the treatment. The daily egg production and the total egg production over female lifespan for each treatment was calculated by averaging the data obtained from 12 replicates (Camus and Zeng, 2010).

Population Growth Experiment

The same 5 × 3 factorial design of 5 food concentrations and 3 photoperiod conditions was used for the population growth experiment, which lasted for 12 days. There were three replicates per treatment. Adult copepods were produced as described in the section "Production of Adult Copepods for the Experiment." After acclimation, 12 *B. amoyensis* adults (4 males and 8 females) were introduced into each of a series of 45 × 1 L beakers (each 1 L beaker as a replicate) to start the experiment. Cultures were maintained in an incubator with constant temperature (∼ 26°C), salinity 28 psu, and a light intensity of ∼ 500 Lux over 12 days. Approximately 30% of the culture water was exchanged daily by gently siphoning using the cylindrical isolation device described above.

After 12 days of culture, the contents of each beaker were emptied onto a 48 μm sieve, all eggs, nauplii, copepodites and adults retained on the sieve were subsequently rinsed into Petri dishes, and 4–5% formalin was added to fix them for later counting. Eggs, nauplii, copepodites and adults of *B. amoyensis* were counted in each replicate and the final population size averaged from three replicates. Intrinsic rate (r) of population growth was then calculated for each treatment using the following formulation:

$$r = \frac{\ln \frac{N_0}{N_1}}{t}$$

where N_0 = population number at the beginning of the experiment, N_1 = population number at the end of the experiment while t (days) is the duration of the experiment (Fenchel, 1974). Meanwhile, adults were sexed to yield the sex ratio of the population.

Data Analysis

Data are presented as mean ± standard error (SE). A statistical probability of $p < 0.05$ was accepted as significant in all tests. The effects of food concentration and photoperiod and

their interaction on egg production, female life expectancy and population dynamics were analyzed using two-way ANOVA. All data were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) prior to analysis of ANOVA. When significant interaction between the two factors of food concentration and photoperiod was found, the Tukey's multiple comparisons test was performed to determine specific significant differences among treatments. If the assumption of normality was not met, the correlation between two variables was tested by the nonparametric Scheirer-Ray-Hare test (daily egg production data).

RESULTS

Daily Egg Production

Figure 1 shows average daily egg production of *B. amoyensis* when cultured under various combinations of microalgae concentrations and photoperiods. Statistical analysis showed that there was an interaction effect between food concentration and photoperiod on daily egg production ($p < 0.001$). However, there was no significant effects among different photoperiods at all 5 diet concentrations ($p > 0.05$, **Figure 1**). On the other hand, the average daily egg production of the diet concentration treatment of 1×10^5 and 2×10^5 cells ml^{-1} were significantly lower than that of 3×10^5, 4×10^5, and 5×10^5 cells ml^{-1} treatment ($p < 0.01$) under all 3 photoperiod regimes (**Figure 1**).

In term of total egg production over female lifespan, both food concentration ($p < 0.001$) and photoperiod ($p < 0.001$) had a main effect, and there was also a significant interaction effect between food concentration and photoperiod on the total egg production ($p < 0.05$, two-way ANOVA). Under all 3 photoperiods tested, the total egg production over female lifespan increased continuously with increasing food concentration, and reached a peak at 4×10^5 cells ml^{-1} ($p < 0.05$), however, the trend reversed as microalgae concentration further increased to 5×10^5 cells ml^{-1} ($p < 0.05$, **Figure 2**). Indeed, copepod from the 4×10^5 cells ml^{-1} treatment produced the highest number of eggs under all 3 photoperiod conditions, which was 2.3 times higher than the lowest value of the 1×10^5 cells ml^{-1} treatment (**Figure 2**). Similarly, photoperiod also had a significant effect on the total egg production of female at various food concentrations ($p < 0.001$): At microalgae concentration of 3×10^5, 4×10^5, and 5×10^5 cells ml^{-1}, the highest egg production was all found under the photoperiod of 12 h L:12hD, followed by the photoperiod of 8 h L: 16 h D, while the 16 h L:8 h D photoperiod always had the lowest egg production (**Figure 2**). Overall, the highest total egg production over female lifespan was found under the combination of 4×10^5 cells ml^{-1} microalgae and 12 h L:12 h D photoperiod, which reached 139.7 $\pm$ 4.4 eggs female^{-1} (**Figure 2**).

Adult Female Life Expectancy

Both food concentration and photoperiod significantly impacted female lifespan of *B. amoyensis*, and there was a significant interaction between the two factors ($p < 0.01$, **Figure 3**). The female life expectancy of 12L:12D photoperiod was not significantly different from that of other 2 photoperiod groups ($p > 0.05$) in 1×10^5 cells mL^{-1} group, while that of 16L:8D photoperiod group (11 $\pm$ 0.8 d) was significantly higher than that of the 8L:16D photoperiod group (8 $\pm$ 1.4 d) ($p < 0.01$). In 3×10^5cells mL^{-1} group, the female life expectancy of the 12L:12D photoperiod group (10 $\pm$ 2.1 d) was significantly higher than that of the other 2 photoperiod groups ($p < 0.05$), and the difference between 8L:16D photoperiod and 16L:8D photoperiod groups was not significant ($p > 0.05$). When food concentrations were 2×10^5cells mL^{-1}, 4×10^5cells mL^{-1} and 5×10^5cells mL^{-1}, the female life expectancy from 3 photoperiod groups did not differ significantly ($p > 0.05$, **Figure 3**).

There was no statistical difference among different food concentrations under 8L: 16D and 12L: 12D photoperiods for female life expectancy ($p > 0.05$, **Figure 3**). Under the photoperiod regime of 16L:8D, the female lifespan of the 1×10^5 cells mL^{-1} diet treatment was not significantly different from that of the 2×10^5 cells mL^{-1}, and 4×10^5 cells mL^{-1} diet treatments ($p > 0.05$), but significantly higher than those of the 3×10^5 cells mL^{-1}, and 5×10^5 cells mL^{-1} food concentrations ($p < 0.01$, **Figure 3**). At the same time, the female life expectancy of the 2×10^5 cells mL^{-1} treatment was significantly higher than that of the 5×10^5 cells mL^{-1} treatment ($p < 0.05$) although both of them were not significantly different from that of the 3×10^5 cells mL^{-1}, and 4×10^5 cells mL^{-1} treatments ($p > 0.05$, **Figure 3**).

Population Growth and Composition

The average final population sizes of *B. amoyensis* after 12 days of culture under different combinations of food concentrations and photoperiods are presented in two categories: "All Stages Included", which included eggs, and "All Post-Egg-Stages", which excluded eggs (**Table 1**). For "All Stages Included" final population, statistical analysis showed that the final population sizes were significantly affected by both food concentration ($p < 0.01$) and photoperiod ($p < 0.01$), and there was an interaction effect between food concentration and photoperiod ($p < 0.001$).

Of the 3 photoperiods tested, at a same algal concentration, the 12 h L:12 h D photoperiod always produced the largest and significantly bigger final population than those of the other two photoperiods ($p < 0.01$, **Figure 4**). Meanwhile, under both 8 h L:16 h D and 12 h L:12 h D photoperiods, population size increased with increasing food concentration, and reached a peak at 4×10^5 cells ml^{-1} (4386.7 $\pm$ 112 and 7979.7 $\pm$ 172.2 for photoperiod 8 h L:16 h D and 12 h L:12 h D, respectively) before dropped when food concentration further increased to 5×10^5 cells ml^{-1} (**Figure 4**). At photoperiod regime of 16 h L:8 h D, population size similarly increased significantly with increasing algal concentration but the maximum population size was observed at microalgae concentration of 3×10^5 cells ml^{-1} as population decreased significantly as algal concentration further increased to 4×10^5 and 5×10^5 cells ml^{-1}, respectively (**Figure 4**). It is worth noting that levels when compared to the other two photoperiods, at all food concentrations the final population of the photoperiod 12 h L:12 h D remained at relatively high levels (**Figure 4**).

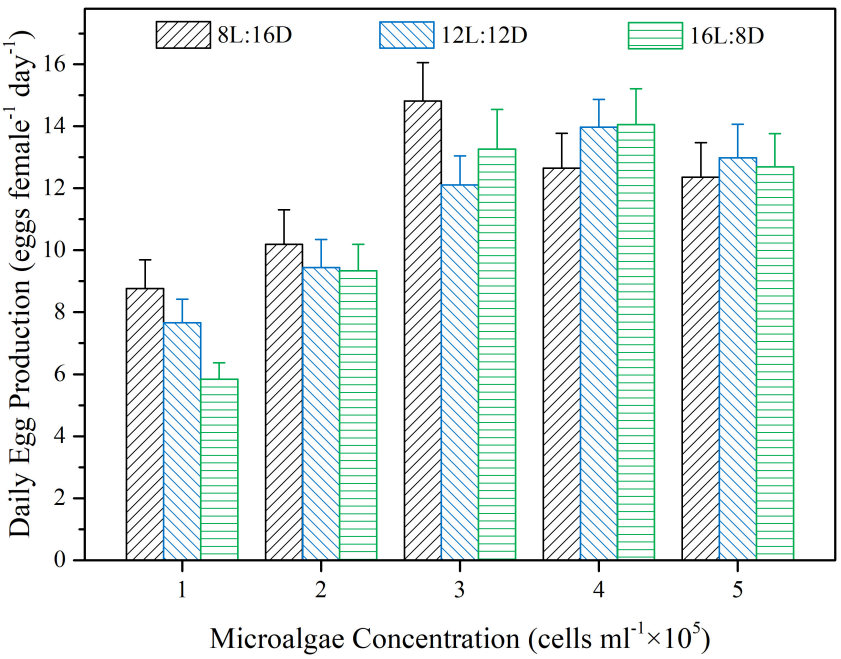

FIGURE 1 | Effects of different concentrations of the microalgal diet and photoperiods on average daily egg production of *Bestiolina amoyensis* (*N* = 12). Data is presented as mean ± standard error (SE).

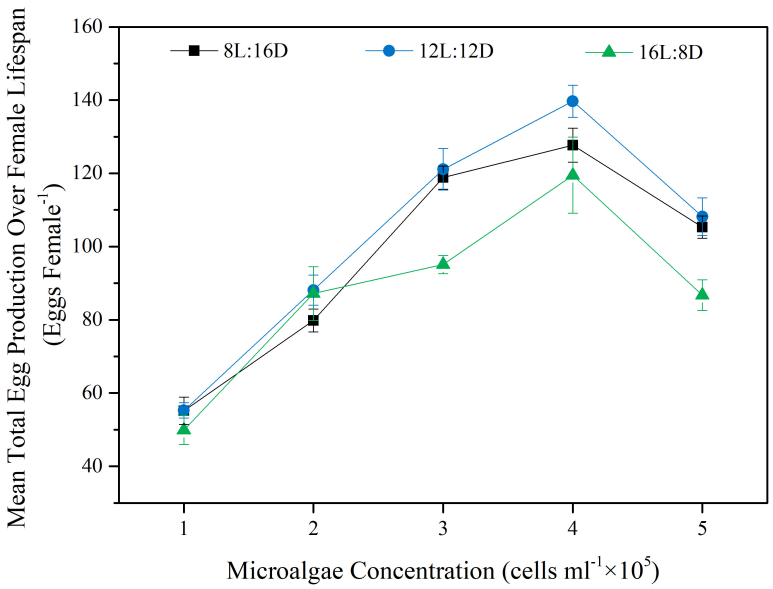

FIGURE 2 | Effects of different concentrations of the microalgal diet and photoperiods on total egg production over female lifespan of *B. amoyensis* (*N* = 12). Data is presented as mean ± SE.

Figure 4 shows final population composition of *B. amoyensis* (eggs, nauplii, copepodites, female and male adults) at the end of the experiment. The maximum amount of nauplii and copepodite was reached under photoperiod of 8L:16D in all diet treatments (**Figure 4**, **Table 1**). Under photoperiod 12 h L:12 h D and 16 h L:8 h D, nauplii stage accounted for more than 50% of the population size. In particular, under the culture condition of photoperiod 12 h L:12 h D and 4 × 10^5 cells ml^{-1} algal feeding concentration, nauplii accounted for 75% of the total population (**Figure 4**).

For the female to male sex ratio, under photoperiod 8 h L:16 h D, it ranged from 1.5:1 of 3 × 10^5 cells ml^{-1} food concentration

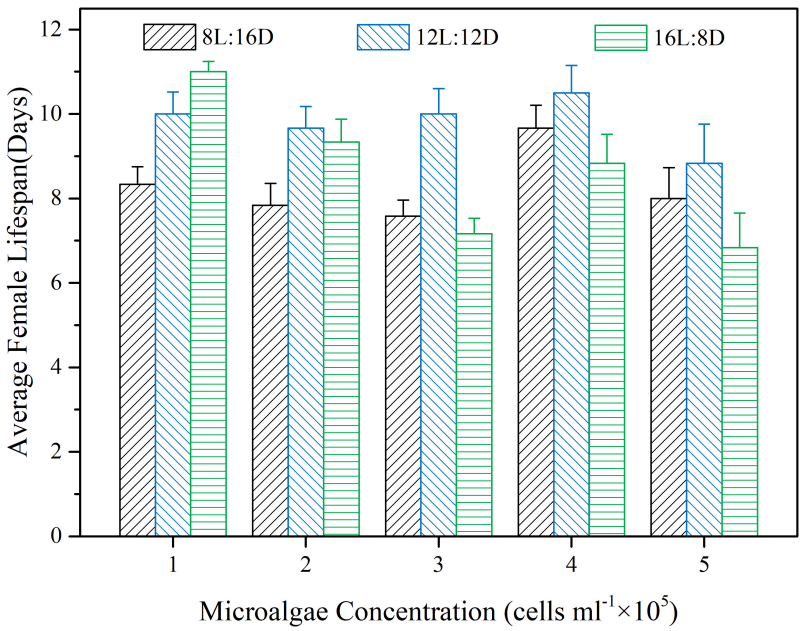

FIGURE 3 | Effects of different concentrations of the microalgal diet and photoperiods on female lifespan of *B. amoyensis* (N = 12). Data is presented as mean ± SE.

TABLE 1 | Final population size and intrinsic rate (r) of *B. amoyensis* cultured over a 12 days period fed on different microalgal diets under three photoperiod regimes.

Photoperiod regime	Microalgal concentration (cells mL^{-1})	Total number of final population – All stages included	*r* – All stages included	Total number of final population – All Post-Egg-Stages	r – All Post-Egg-Stages	Sex ratio Female:Male
8L:16D	1×10^5	1684.3 ± 54.9^{c}	0.40 ± 0.02^{d}	1474.3 ± 49.1^{c}	0.39 ± 0.03^{d}	(2.23 ± 0.03):1
	2×10^5	2275.0 ± 41.1^{c}	0.43 ± 0.02^{c}	2061 ± 32.8^{bc}	0.42 ± 0.03^{c}	(1.88 ± 0.08):1
	3×10^5	3334.7 ± 132.6^{b}	0.46 ± 0.03^{b}	2861.3 ± 145.3^{ab}	0.44 ± 0.02^{b}	(1.53 ± 0.02):1
	4×10^5	4386.7 ± 112^{a}	0.48 ± 0.01^{a}	3746.3 ± 137.3^{a}	0.47 ± 0.02^{a}	(1.70 ± 0.01):1
	5×10^5	3345.3 ± 113.5^{ab}	0.46 ± 0.02^{b}	2827.3 ± 114.3^{ab}	0.44 ± 0.03^{b}	(1.77 ± 0.07):1
12L:12D	1×10^5	6130.7 ± 100.5^{B}	0.51 ± 0.03^{B}	5110.0 ± 107.3^{B}	0.50 ± 0.03^{B}	(1.76 ± 0.07):1
	2×10^5	6329.7 ± 189.8^{B}	0.51 ± 0.02^{BC}	5665.0 ± 212.5^{B}	0.51 ± 0.02^{AB}	(1.58 ± 0.02):1
	3×10^5	7597.0 ± 283.8^{A}	0.52 ± 0.02^{AC}	5877.2 ± 240^{AB}	0.50 ± 0.02^{AB}	(1.62 ± 0.04):1
	4×10^5	7979.7 ± 172.2^{A}	0.53 ± 0.02^{A}	6839.7 ± 160.5^{A}	0.52 ± 0.02^{A}	(1.06 ± 0.01):1
	5×10^5	6085.7 ± 118^{B}	0.51 ± 0.03^{B}	4708.3 ± 119.4^{AB}	0.49 ± 0.04^{B}	(1.83 ± 0.02):1
16L:8D	1×10^5	2866.0 ± 17.5^{J}	0.44 ± 0.02^{K}	2170.3 ± 9.2^{J}	0.42 ± 0.03^{J}	(2.0 ± 0.06): 1
	2×10^5	3269.7 ± 39.2^{J}	0.45 ± 0.02^{JK}	2704.7 ± 32.7^{IJ}	0.44 ± 0.03^{IJ}	(1.54 ± 0.02):1
	3×10^5	6224.4 ± 69.3^{H}	0.51 ± 0.02^{H}	5247.7 ± 89.9^{H}	0.49 ± 0.03^{H}	(1.51 ± 0.07):1
	4×10^5	4815.3 ± 11.7^{I}	0.49 ± 0.03^{I}	3250.3 ± 6.7^{I}	0.45 ± 0.04^{I}	(1.09 ± 0.05):1
	5×10^5	3435.0 ± 9.5^{J}	0.46 ± 0.04^{J}	2388.6 ± 26^{IJ}	0.43 ± 0.05^{J}	(1.50 ± 0.02):1

Data are presented as Mean ± SE; different superscript letters in a same column indicate significant differences ($p < 0.05$) among different diet concentrations under the same photoperiod regime.

to 2.2:1 of 1×10^5 cells ml^{-1} food concentration (**Table 1**). Under both photoperiods 12 h L:12 h D and 16 h L:8 h D, the lowest female to male sex ratio was both 1.1:1 and from the 4×10^5 cells ml^{-1} food concentration treatment. For other food concentration treatments, the sex ratio ranged from 1.6:1 to 1.8 and 1.5:1 to 2.0:1 under photoperiod 12 h L:12 h D and 16 h L:8 h D, respectively (**Table 1**).

It is worth noting that both food concentration and photoperiod had a significantly effect on the intrinsic population increase rates(r) of *B. amoyensis* ($p < 0.001$). The intrinsic rates under photoperiod 12 h L:12 h D was significantly higher than that of other 2 photoperiods ($p < 0.01$) at all 5 food concentrations. The intrinsic rates of 8L:16D photoperiod treatment was significantly lower than that of other 2 photoperiods in 1×10^5 cells ml^{-1} to 3×10^5 cells ml^{-1} food concentrations ($p < 0.001$), while at 4×10^5 cells ml^{-1} and 5×10^5 cells ml^{-1} food concentrations, the intrinsic population increase rates(r) of 8L:16D photoperiod group were

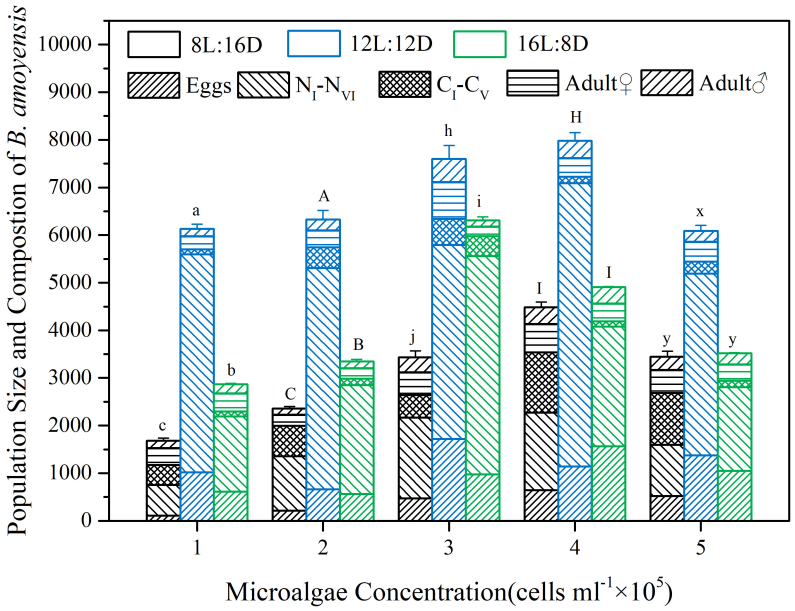

FIGURE 4 | Effects of different concentrations of the microalgal diet and photoperiod regimes on population size and population structure of *B. amoyensis* over 12 days of cultivation ($N = 3$). Data is presented as mean ± SE. Different letters on the top of bars indicate significant differences of different photoperiod treatments at the same microalgae feeding concentration ($p < 0.05$).

not significantly different from that of 16L:8D photoperiod (**Table 1**). When all stages were included, the intrinsic rates of 12 h L:12 h D photoperiod ranged from 0.51 to 0.53, the highest *r* value was reached at food concentration of 4×10^5 cells ml^{-1}, which was significantly higher than other food concentration treatments except the 3×10^5 cells ml^{-1} treatment. On the other hand, the intrinsic rates of under photoperiods 8 h L:16 h D and 16 h L:8 h D were ≤0.48 (4×10^5 cells ml^{-1}) and 0.51 (3×10^5 cells ml^{-1}), respectively (**Table 1**). Similar trend was shown when eggs were excluded with the intrinsic rates ranged from 0.39 to 0.47, 0.49 to 0.52, and 0.42 to 0.49 for photoperiod of 8 h L:16 h D, 12 h L:12 h D, and 16 h L:8 h D, respectively (**Table 1**).

DISCUSSION

Reproductive ability of copepods is affected by the nutritional condition of mature females (Castro-Longoria, 2003), and food quantity is an important factor that can significantly affect female condition. For example, the egg production has been reported to typically increase with the food concentration, reach a maximum level at a certain concentration (Runge, 1984; Hirche et al., 1997; Camus, 2012), but tend to decrease when the food concentration exceeds a threshold level (Zhao et al., 2014). Therefore, determining the optimal microalgae feeding quantity is crucial for optimizing the culture protocol of *B. amoyensis*.

Most of paracalanid copepods are herbivorous (Paffenhöfer, 1984) although a few of them have been reported as could be carnivorous (Suzuki et al., 1999). Similar to its congener *B. similis* (Vineetha et al., 2018; Siddique et al., 2021), we found *B. amoyensis* as herbivorous, feeding mainly on phytoplankton. In the present study, the microalgae *Isochrysis* spp., one of the most commonly used marine unicellular algae for feeding calanoid copepods (Payne and Rippingale, 2000; Vengadeshperumal et al., 2010; Santhanam et al., 2013; El-Tohamy et al., 2021), was confirmed could fulfill the growth and reproduction needs of *B. amoyensis*. *Isochrysis* spp. is known for their high nutritional value, such as rich in polyunsaturated fatty acids (PUFAs), e.g., DHA and EPA (Sukenik and Wahnon, 1991; Gouveia et al., 2008) and grows well in mass cultures, either indoors or outdoors (Kaplan et al., 1986; Fidalgo et al., 1998). When at exponential phase of growth, the flagellated microalgae are not easily accumulated at the bottom like some other microalgae species, such a feature benefited the present experiments: the concentrations of *Isochrysis* spp. largely remained at designated levels during the 24 h period between daily water changes as confirmed by intermittent sampling of culture waters from replicates to estimate the algal concentrations from different treatments (see **Supplementary Table 1**).

Copepods productivity has commonly been indicated by daily egg production of females (Klepper et al., 1998). However, Camus and Zeng (2010) have adopted total egg production over female lifespan to estimate female productivity, and noted that this method is more accurate since it takes female life expectancy and reproductivity changes during lifespan into account. In this study, we have measured both daily egg production and egg production over female lifespan to provide a comprehensive information on egg production of *B. amoyensis* under different culture conditions. Our results showed that under all 3 photoperiods used, average daily egg production of *B. amoyensis* was significantly higher when they were fed *Isochrysis* spp. at a concentration ≥3×10^5 cells ml^{-1} when

compared to that at $\leq 2 \times 10^5$ cells ml^{-1}. In the case of total egg production over female lifespan, under all photoperiods used, the lowest total egg production was found in the lowest algal concentration of 1×10^5 cells mL^{-1}, and total egg production increased by the algal concentration, reaching a maximum level at 4×10^5 cells mL^{-1} before decreased with further increase of food concentration. Similar results were also reported for other copepod species, such as *Calanus pacificus* (Runge, 1984), *Calanus finmarchicus* (Hirche et al., 1997), and *Tisbe furcate* (Zhao et al., 2014). Such a result likely can be explained by that when food concentration is low, copepods need to move actively to catch more algae and with increase movement frequency of feeding appendage to enhance feeding (Paffenhöfer and Lewis, 1990), which increases energy expenditure. As the result, less energy can be channeled for egg production. Indeed, it has been observed that in some calanoids, clearance rate, i.e., the water volume sweeps clear by a copepod per unit time, increased with decreasing food concentration (Corner et al., 1972). Clearly, clearance rate increase is associated with higher energy expenditure. With higher food concentrations, copepods could obtain enough food with a lower clearance rate, which reduces energy consumption while still meeting the energy demands for both maintenance and reproduction (Zhao et al., 2014). However, when the food concentration is too high, it was found that microalgae could adhere to the feeding appendages and body surface of copepods, which could impair their feeding and motility, leading to lower food ingestion, and subsequent lower egg output and shorter female life expectancy. While Garrido et al. (2013) found that ingestion rate of calanoid copepod *Centropages chierchiae* increased linearly with the range of food concentrations they used (6.4–393.8 $\mu C\ L^{-1}$), but out results suggest that the ingestion rate of *B. amoyensis* does not increase infinitely with food concentration and a threshold exists, beyond which the clearance rate would not further increase, or even decrease.

Female life expectancy is one of the factors that could affect how much a female contributes to the total pool of eggs produced in the culture, and hence culture population growth. When food concentrations were $\geq 3 \times 10^5$ cells mL^{-1}, adult females cultured under 16 h L:8 h D photoperiod had a shorter life expectancy than other two photoperiods. The prolonged light phase likely kept *B. amoyensis* active for longer, and the higher metabolic rate may result in shorter female lifespan. Similarly, *A. sinjiensis* cultured under constant light had a relative shorter life expectancy than other photoperiods (Camus and Zeng, 2008).

The results of population growth experiment showed that feeding on *Isochrysis* spp. alone, *B. amoyensis* could significantly expanded its population at all concentrations tested, ranging from 1×10^5 to 5×10^5 cells ml^{-1} and under different photoperiods. Interestingly, at all 5 algal feeding concentrations, *B. amoyensis* cultured under 12 h L:12 h D photoperiod consistently had the maximum and significantly bigger population sizes at the end of the experiment than other two photoperiods, indicating the important of photoperiod in regulating population growth. A possible explanation of such a result is that 12 h L:12 h D mimics typical photoperiod of Xiamen region during autumn when *B. amoyensis* reportedly is a dominant copepod species in Xiamen Harbor of China (Yang, 2007) where *B. amoyensis* used in this study were collected from. Indeed, the photoperiod of Xiamen region in spring and autumn ranges from 12 h L:12 h D, hence *B. amoyensis* may have adapted to such a photoperiod condition for maximum production. Indeed, under 12 h L:12 h D photoperiod, female *B. amoyensis* typically had the longest life expectancy among 3 photoperiods except at 1×10^5 cells ml^{-1} algal concentration, which was also not significant different from the longest life expectancy under 16 h L:8 h D. This should also lead to higher overall egg production while suggesting that 12 h L:12 h D is in fact optimal photoperiod for *B. amoyensis*.

The relative numbers of nauplii and copepodites in a culture population typically indicate the status of the culture (VanderLugt et al., 2009). VanderLugt and Lenz (2009) pointed out that populations dominated by nauplii typically indicate high reproductive rates of females. The maximum numbers and proportion of nauplii in the final populations of the population growth experiment were also found from the 12 h L:12 h D photoperiod, and at all 5 algae concentrations exceeding 50% of the total population. In particular, the number of nauplii of the 4×10^5 cells mL^{-1} treatment achieved 75% (**Figure 3**), indicating that *B. amoyensis* had a high reproductive rate under 12 h L:12 h D photoperiod and when fed *Isochrysis* spp. at 4×10^5 cells mL^{-1}.

Sex-determining in copepods are related to the population size and sex ratio (Mauchline, 1998; Voordouw and Anholt, 2002). In planktonic calanoid copepods, sex may also be controlled by environmental factors (Mauchline, 1998). The female-biased adult sex ratios had been reported in many species, including *Acrocalanus* sp., *Parvocalanus* sp., *Paracalanus indicus*, *B. similis* (McKinnon and Duggan, 2001; McKinnon et al., 2005; Duggan et al., 2008; Gusmão and McKinnon, 2009), *Paracalanus nanus* (McKinnon and Duggan, 2001; Gusmão and McKinnon, 2009), and *Paracalanus aculeatus* (McKinnon and Duggan, 2001; McKinnon et al., 2005; Gusmão and McKinnon, 2009). The sex ratios of *Calanus* spp. vary seasonally, and the food quality and quantity could also significantly affect sex ratio. For example, more males could be observed during the phytoplankton boom season comparing to the pre-phytoplankton boom season (Irigoien et al., 2000). Similarly, there were more male *B. amoyensis* found in the populations of 4×10^5 cells mL^{-1} treatments under 12 h L:12 h D photoperiod with the sex ratio approaching 1:1, indicating the culture population was at a stable status (Fisher, 1930) under the culture condition.

CONCLUSION

The results of this study showed that *Isochrysis* spp. is a suitable diet for *B. amoyensis* culture as the population expanded significantly when it is fed to the copepod at concentrations ranging from 1×10^5 to 5×10^5 cells ml^{-1}, and under photoperiods of 8 h L:16 h D, 12 h L:12 h D and 16 h L:8 h D. However, summarizing the outcomes of a range productivity

related parameters measured in *B. amoyensis*, including egg production, female life expectancy, and population growth and composition, the optimum *Isochrysis* spp. feeding concentration and photoperiod for the culture productivity of the species was determined to be 4×10^5 cells mL^{-1} and 12 h L:12 h D, respectively.

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.

AUTHOR CONTRIBUTIONS

SW and LW conceived the original idea of this study and designed the experiments. LW conducted the experiments and collected the data. LW, SW, and CsZ performed the data analyses. LW wrote the draft of the article and revised it with SW and CsZ. YW and CxZ attended the field works and performed part of data collection. SW supervised the research. 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.2021.788744/full#supplementary-material

Supplementary Table 1 | Concentrations of *Isochrysis* spp. from five different treatment groups after 24 h of incubation with the experimental *Bestiolina amoyensis*.

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Effects of Microalgal Food Quantity on Several Productivity-Related Parameters of the Calanoid Copepod *Bestiolina similis* (Calanoida: Paracalanidae)

Thomas Camus[1]†, Lucrezia Rolla[1], Jufeng Jiang[2]* and Chaoshu Zeng[1]*

[1] Marine Ornamental Captive Breeding Research Group, College of Science and Engineering, James Cook University, Townsville, QLD, Australia, [2] Tianjin Ornamental Fish Technology and Engineering Centre, Tianjin Fisheries Research Institute, Tianjin, China

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***Correspondence:**
Thomas Camus
thomas.camus@ifremer.fr
Jufeng Jiang
jufengjiang@163.com

†Present address:
Thomas Camus, EIO UMR24, UPF/IRD/ILM/Ifremer, Labex CORAIL, Unité RMPF, Centre Océanologique du Pacifique, Vairao, French Polynesia

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The optimization of copepod feeding protocol is paramount to improve culture productivity and to maintain favorable water quality parameters overtime, as well as saving operational costs by preventing the production of unnecessary quantities of microalgae. The influence of microalgal feeding concentration on major parameters related to culture productivity of the calanoid copepod *Bestiolina similis* (Paracalanidae) was investigated in a series of laboratory experiments. *B. similis* was fed eight different concentrations (0, 150, 300, 600, 900, 1,200, 1,500 and 1,800 μgC l^{-1}) of a mixed microalgal diet consisting of Tahitian strain of *Isochrysis* species, *Pavalova* 50 and *Tetraselmis chuii* at 1:1:1 carbon ratio. The results indicate that female daily and cumulative egg production over lifespan, egg hatching rate, naupliar and copepodite survival and development, adult female life expectancy, population growth and fecal pellet production rate (FPPR) were all significantly affected by microalgae feeding ration. Conversely, no significant influence could be established between microalgae food concentration and egg diameter or adult sex ratio. Feeding rations as low as 150 μgC l^{-1} led to lower egg hatching rates, survival and development, adult female life expectancy and population growth compared with the higher microalgae rations tested. Feeding concentration $\leq$ 900 μgC l^{-1} significantly limited female daily egg and fecal pellet production rate, as well as their cumulative egg production over lifespan, when compared to a level of 900 μgC l^{-1}. *Bestiolina similis* fed with 1,200 μgC l^{-1} significantly improved female egg and fecal pellet production when compared to the lower treatments and was responsible for the highest female lifespan egg production and population growth observed among all treatments. Feeding rations as high as 1,500 μgC l^{-1} and 1,800 μgC l^{-1} did not lead to significant improvement in any of the parameters measured. This is likely due to a saturation effect at high food concentration which is known to decrease calanoid copepods feeding efficiency. Finally, *B. similis* FPPR, used as a proxy for ingestion, was found to saturate at a microalgae concentration of 783.4 μgC l^{-1} using a non-linear Michael-Menton (2 parameters), indicating that CVI female ingestion did not increase significantly above this concentration. Based on the above results it is recommended that *B. similis*

cultures should be fed at a concentration of 1,200 μgC l^{-1}, and not above, as rations > 1,200 μgC l^{-1} will not significantly improve any of the productivity-related parameters observed in this study. Feeding rations should never be below 783.40 μgC l^{-1} as this is the threshold level below which adult female ingestion rates become limiting.

Keywords: copepod, food concentration, copepod culture, calanoid copepod, Paracalanidae, egg production, population growth, adult lifespan

INTRODUCTION

Marine copepods are the most abundant metazoans throughout the world's ocean (Boxshall and Halsey, 2004) and constitute the majority of plankton biomass in the epipelagic zone (Bunker and Hirst, 2004). In the wild, copepods mediate energy flow between primary producers and secondary consumers (Frost, 1972; Xu and Wang, 2001) and their naupliar stages often make up fifty percent or more of the stomach contents of early fish larvae (Sampey et al., 2007). Several commercially important planktivorous fish also rely on adult copepods for their nutrition, commonly making up as much as 39% of their stomach content (e.g., *Pampus argenteus*, Dadzie et al., 2000). The ubiquitous occurrence of copepods in the marine environments and their importance as natural prey items for fish larvae has prompted an increasing interest in culturing them as live feeds for marine hatcheries (Shansudin et al., 1997; O'Bryen and Lee, 2005).

Calanoid copepods are known to provide a range of crucial benefits to a variety of commercial and ornamental fish species when compared to traditional live feeds such as *Artemia* and rotifers (Payne and Rippingale, 2001; Drillet et al., 2006; Conceição et al., 2010). Because of their excellent track record in significantly improving the health and fitness of cultured species, calanoids are considered as the solution for larvae that cannot be reared on traditional live feeds (Marcus and Murray, 2001; O'Bryen and Lee, 2005). For example, various marine ornamental and commercial fish species, including green mandarin fish *Synchiropus splendidus* (Zeng et al., 2018), longsnout seahorse *Hippocampus reidi* (Shubert et al., 2016), cloudy damsel *Dacyllus carneus* (Anzeer et al., 2019) and Atlantic bluefin tuna *Thunnus thynnus* (Yúfera et al., 2014), have been successfully reared with significantly improved survival and growth using copepods as the first-feed. Yet, despite gaining more interest in recent years due to their obvious advantages as larval live prey over traditional live feeds, knowledge on copepod performance in intensive cultures remains limited (Abate et al., 2014; Rasdi and Qin, 2016; Jepsen et al., 2021). This under-utilization is mainly attributed to their relatively low productivity in intensive culture (Støttrup, 2000), which in turn could be partially attributed to a lack of research in the field. For instance, most of the research efforts are focused on a handful of primarily coastal calanoid species and even after several decades of study, it was estimated that fewer than 4% of marine planktonic calanoid species have had their fecundity measured (Bunker and Hirst, 2004; Marcus et al., 2004).

Although salinity and temperature are important culture parameters (Alajmi et al., 2014), existing literature suggests that copepod productivity is mainly dependent upon food quality and quantity, within the metabolic constraints set by temperature (Uye, 1981; Kleppel, 1992; Dam et al., 1994; Koski and Kuosa, 1999; Guisande et al., 2000; Tirelli and Mayzaud, 2005; Ismar et al., 2008; Pan et al., 2014; Nogueira et al., 2019).

While an optimal microalgal diet consisting of the tri-algal diet T-Iso+Tet+Pav was determined to be the optimal diet for *B. similis* (Camus et al., 2009; Camus and Zeng, 2010), it is also of paramount importance to evaluate the influence of microalgal quantity on its culture productivity. Hence, this study was set out to investigate the effects of various concentration of this optimal microalgal diet on major productivity-related parameters of *B. similis* with the objective to provide guidelines for optimal feeding ration for *B. similis* under intensive culture conditions.

Bestiolina similis is a small (early nauplii < 100 μm; adults < 700 μm) pelagic calanoid copepod belonging to the family Paracalanidae. It is considered a preferred prey item for larvae of several families of tropical fish because of its small size and excellent nutritional profile (McKinnon et al., 2003; Sampey et al., 2007). Paracalanoid copepods are widely distributed in tropical and temperate waters and frequently dominate copepod communities in surface waters (McKinnon and Duggan, 2001; Boxshall and Halsey, 2004). Over the past decade, several studies have been conducted to investigate various aspects of its culture methods (e.g., VanderLugt and Lenz, 2008; Camus et al., 2009; VanderLugt et al., 2009; Camus and Zeng, 2010).

MATERIALS AND METHODS

Microalgae Culture

A microalgal diet composed of three species of the Tahitian strain of *Isochrysis* species ("T-iso," class Prymnesiophyceae; CS-177), *Pavalova* 50 ("Pav," class Prymnesiophyceae; CS-50) and *Tetraselmis chuii* ("Tet," class Prasinophyceae; CS-176) was previously found to be the optimal diet for intensive culture of *B. similis* (Camus et al., 2009; Camus and Zeng, 2010). These microalgae species are used in marine hatcheries throughout the world and are relatively easy to maintain. For the present study, the starter cultures of the three microalgae were obtained from the Commonwealth Scientific and Industrial Research Organization (CSIRO) Microalgae Supply Service, Hobart, Tasmania, Australia. The starter cultures were gradually scaled up and finally cultured in several 20-l polycarbonate carboys filled with 1 μm filtered, autoclaved and UV irradiated seawater (salinity 30 ± 1) inside a temperature-controlled room (25 ± 1°C). The light was provided by six rows of four fluorescent tubes and photoperiod was set at 12 h light:12 h dark with a light

intensity of approximately 5,000 lx as measured by a MC-88 light meter (TPS Pty Ltd., Australia). The microalgae were cultured using f/2 medium (Guillard and Ryther, 1962) with vigorous aeration (0.2 μm filtered air).

Bestiolina similis Stock Culture

Bestiolina similis were collected by a zooplankton tow at the mouth of the Ross River, Townsville, Queensland, Australia. The plankton samples were brought back to the Marine and Aquaculture Research Facility Unit (MARFU) at James Cook University, Australia, within an hour of collection. Upon arrival at the laboratory, *B. similis* were isolated in a temperature-controlled room (27 ± 1°C) and cultured in a salinity of 30 ± 1, 1 μm filtered seawater. Stock cultures of *B. similis* were gradually scaled up and inoculated into several 20 l plastic carboys with gentle aeration. Depending on water quality parameters, between 30 and 50 % of culture water was exchanged every other day using a siphon with a 25 μm mesh attached to the end to prevent the removal of any copepods. The stock cultures were entirely drained through a 150 μm sieve every 10 to 15 days to remove any build-up of detritus. The 150 μm sieve retained adult and copepodites, but eggs and nauplii were mostly lost. The carboys were then cleaned and sterilized with chlorine before the cultures were restarted (Camus et al., 2009; Camus and Zeng, 2010). *Bestiolina similis* cultures were fed daily with a trialgal diet consisting of T-iso+Pav+Tet offered at an equal ratio of biomass based on carbon concentrations (i.e., 1:1:1 carbon ratio), which were calculated for each species according to Strathmann (1967). The trialgal diet was fed to *B. similis* at ∼ 1,500 μgC l^{-1}, a carbon concentration known to saturate calanoid copepod feeding (Kiørboe et al., 1985).

General Procedure

All experiments were conducted at MARFU, James Cook University, Queensland, Australia. Throughout all experiments, water temperature was maintained at 27 ± 1 °C and salinity at 30 ± 1 while the light regime was set at 12L:12D. Observations and counting of eggs, nauplii, copepodites and adults were made using a Sedgewick–Rafter counter and a Leica CME optical microscope (model TN-PSE30, Wetzlar, Germany).

The experimental microalgae concentrations were as follow: 1,800, 1,500, 1,200, 900, 600, 300,150, and 0 μgC l^{-1}. They were chosen to reflect a wide variety of food conditions, ranging from limiting to saturating, as concentrations of 300 μgC l^{-1} and lower are generally characterized as limiting for calanoid copepods (Koski and Klein Breteler, 2003), while 1,500 μgC l^{-1} and above are known to satiate *A. tonsa,* a similar sized planktonic copepod (Kiørboe et al., 1985). To achieve experimental microalgal concentrations, cell concentrations (cells/ml) of each microalgal species were first determined using a FlowCAM^{TM} particle analyser, before being converted to absolute carbon concentration (μgC l^{-1}) based on McKinnon et al. (2003). Microalgal cultures were subsequently combined in a 1:1:1 carbon ratio and diluted using filtered sea water to make for each of the experimental food concentrations.

A pre-conditioning period of at least one generation was ensured for each experiment. Pelagic copepods are known to acclimate to their food condition on a time scale of hours to days (Mayzaud and Poulet, 1978), and a pre-conditioning period of at least one generation was hence more than sufficient to eliminate any potential residual effect from previous feeding history.

Three experiments were conducted to test for the influences of microalgae concentration on major parameters related to *B. similis* productivity in culture, i.e., (1) daily egg production rate (EPR; egg $female^{-1}$ day^{-1}), egg diameter (μm), egg hatching rate (%), and fecal pellet production rate (FPPR; fecal $pellet.female^{-1}.day^{-1}$); (2) naupliar and copepodite survival (%), median development time from eggs to nauplii, copepodites and adult females (days), adult female life expectancy (days) and cumulative egg production over total female lifespan (eggs $female^{-1}$); (3) population growth and sex ratio.

Daily Egg and Fecal Pellet Production Experiment

Following a pre-conditioning period to a specific feeding concentration, adult females with ripe ovaries were randomly selected and individually incubated in 60 ml containers filled with 50 ml of fresh sea water and microalgal food added at the designated concentrations except for the controls, in which females were starved for 48 h and incubated in filtered sea water without microalgal food (i.e., food ration = 0 μgC l^{-1}). Each container was labeled according to its experimental food concentrations, sealed and mounted on a plankton wheel (rotation rate = 60 cycles/h). Six replicates were set up daily for each treatment. Female egg production rate (EPR) and fecal pellet production rate (FPPR) were assessed after 24 h. In the cases of finding a dead female after 24 h, the replicate was discarded and its egg and fecal pellet production data were not taken into account. New replicates containers were set up daily, with fresh sea water and a new female pre-conditioned to the particular experimental food concentration. The experiment was run for 9 consecutive days. Due to variation in female survival under different food concentrations, an uneven number of replicates was obtained daily for egg production and fecal pellet production data for each treatment. However, with the replacement of invalid replicates, data from a total of 33 valid replicates (n = 33) were eventually achieved for each treatment.

In addition to determine daily egg and fecal pellet production, the diameters of all eggs produced on the final day of the experiment (6 replicates/treatment) were measured with a Leica CME optical microscope (model TN-PSE30). After measurement, the eggs were then incubated for hatching rates calculations. Egg hatching rate (%) was estimated by calculating the difference between the initial number of eggs and the number of unhatched eggs observed after 48 and 96 h of incubation.

$$EHS\ (\%) = \frac{[(\text{No. of eggs introduced initially} - \text{No. of unhatched eggs}) * 100]}{\text{No. of eggs introduced initially}}$$

Nauplii and Copepodites Survival and Development, Adult Female Life Expectancy and Cumulative Lifespan Egg Production Experiment

Groups of 15 sexually mature females were randomly selected and incubated inside containers filled with 500 ml of fresh sea

water and fed one of the 8 designated microalgal concentration. Two males were also added to each container to ensure that female fecundity would not be affected by the absence of male. All containers were labeled with diet concentration treatment before being sealed and mounted on a plankton wheel (rotation rate = 60 cycles/h). After 24 h, all adult copepods were removed so that only eggs and newly hatched nauplii (< 24 h) remained in each of the containers. All newly hatched nauplii were then recorded before gently transferring them using a broad mouth pipette to a new 500 ml container filled with fresh sea water and with the same experimental food concentration. These new containers were labeled to allow identification of the microalgae concentration treatment and the date of the nauplii hatching. Nauplii were then reared in these containers with microalgal food concentration checked daily and adjusted to the designated experimental food concentration accordingly. Following the same procedure, new containers were set up daily for nauplii hatching during the next few days from egg produced by the females. Daily, newly hatched nauplii were set up in separated containers until all eggs had hatched and no more nauplii were found. This allowed for exact hatching date of each nauplius to be precisely recorded, allowing precise data collection about development duration. A total of 4 replicates, each containing at least 20 nauplii were conducted for each of the eight experimental food concentrations tested.

Nauplii development was closely monitored and as they started to develop into copepodites, new containers were similarly set up daily for the newly appearing copepodites. As a result, every copepodites appearing on the same day were cultured in the same container to allow precise recording of their median development duration. The same procedure was applied for the newly appeared adult females in order to allow for the estimation of median development time from egg to CVI adult.

Average naupliar survival was calculated by dividing the total number of nauplii that molted successfully into copepodites by the initial number of nauplii for each replicate and averaged for each algal concentration treatment. Average copepodite survival was similarly calculated by dividing the total number of copepodites that molted successfully to become adults by the initial number of copepodites for each treatment.

Bestiolina similis median development time (MDT) from eggs nauplii/copepodites/adult females is defined as the time when 50% of the eggs had hatched as nauplii or when 50% of population had molted to become copepodites or adult females. Median development duration was calculated using the following formula, (Peterson and Painting, 1990):

$$MDT(nauplii/copepodites/adult\ females) = \frac{\sum_{n}^{n+1} N(\text{development stage})_n{}^{*} n}{\sum N(\text{development stage})}$$

Where N is the number of nauplii, copepodites or adult females found on a given day n.

Adult female life expectancy and their cumulative lifespan egg production were determined as follow: upon noticing the appearance of a mature females (CVI) in a treatment, they were individually transferred to 500 ml containers with the same microalgal concentration. One adult male (CVI) preconditioned to the same microalgal food concentration was also added to ensure that female egg production was not limited by an absence of fertilization. The containers were then labeled, sealed and placed on a plankton wheel (rotation rate = 60 cycles h^{-1}). Every 24 h, each copepod pair was gently transferred to a new container filled with fresh sea water and the same microalgal concentration. The seawater in the original container was drained onto a mesh for counting the number of eggs produced by the females over the past 24 h period. Egg output was determined daily for each female until its death, at which point lifespan and cumulative lifespan egg production was calculated. During the experiment, any dead males found were replaced by a new male preconditioned to the same experimental food concentration.

Population Growth and Sex Ratio Experiment

For the population growth experiment, 7 preconditioned, sexually mature females and two males were introduced to a 500 ml container and cultured under one of the eight microalgae concentration (four replicates/treatment). All experimental containers were then labeled, sealed and mounted on a plankton wheel (rotation rate = 60 cycles h^{-1}) for a duration of 14 days, which allowed time for a second generation to be produced (Camus et al., 2009). The microalgal concentration in each container was maintained daily by adding an appropriate quantity of the trialgal diet to the container, while the build-up of detritus was gently removed using a siphon with a 25 μm mesh sieve attached to its end to prevent the removal of any life stage of *B. similis*. After 14 days, all replicates were drained through a 25 μm sieve and all retained eggs, nauplii, copepodites and adults were fixed using a 10% buffered formalin fixative for later counting and sexing of all adults. The intrinsic rate of population increase r was then calculated for each treatment using the formulation:

$$r = \frac{\ln(\frac{N_0}{N_1})}{t}$$

Where N_0 = population number at the beginning of the experiment, N_1 = population number at the end of the experiment while t (days) is the duration of the experiment (Fenchel, 1974).

Data Collection and Analysis

Data are presented as mean ± standard error (SE). Egg production rate, fecal pellet production rate, egg hatching rate, female proportion, female live expectancy, female total egg production, population growth and median development data were confirmed to meet the parametric test assumptions (i.e., balanced study design, normally distributed, homogeneity of variance) and were analyzed using one-way ANOVA. When a significant difference ($p < 0.05$) was detected, the Tukey's multiple comparisons test was used to determine specific differences among treatments ($p < 0.05$). Egg diameter size data did not meet the parametric test assumptions and a Kruskal–Wallis test was used for statistical analysis. If a significant difference ($p < 0.05$) was detected, a multiple comparison of mean ranks was used to determine specific differences

among treatments ($p < 0.05$). Data on egg hatching rate data were log transformed and pooled across all replicates for each treatment before being analyzed for significant difference between treatments, using the Chi square test. All statistical analyses were conducted using Statistica^{TM} version 8.

The correlation between fecal pellet production rates and microalgal diet concentrations was assessed using nonlinear regression analysis in SigmaPlot^{TM} (version 11). The non-linear Michael-Menton equation (2 parameters; Holling, 1959) was used to describe the relationship between female fecal pellet production rate (fecal pellets female^{-1} day^{-1}) and microalgae concentration (μgC l^{-1}):

$$y = \frac{a * x}{c + x}$$

where y is the fecal pellet production rate and x is the microalgae concentration, a is the maximum rate of fecal pellet production and c is the half saturation rate (microalgae concentration that produce 50% of the highest *y* value).

RESULTS

Daily Egg and Faecal Pellet Production, Egg Size and Hatching Success

Microalgal concentration had a significant effect ($p < 0.05$) on egg production rate (EPR), fecal pellet production rate (FPPR) as well as 48 and 96 h egg hatching rate (EHR) (**Table 1**). However, average egg diameter was not significantly affected ($p > 0.05$). *B. similis* fed at 1,500 μgC l^{-1} exhibited the highest EPR (22.6 $\pm$ 1.4 eggs female^{-1} day^{-1}), significantly higher than all other treatments except for the 1,800 and 1,200 μgC l^{-1} treatments (18.6 $\pm$ 1.4 and 20.1 $\pm$ 1.2 eggs female^{-1} day^{-1}, respectively). Results also showed that *B. similis* fecundity was significantly limited when the daily food ration was below 1,200 μgC l^{-1} (**Table 1**). When the unfed control is excluded from the data analysis, the lowest EPR (2.2 $\pm$ 0.2 eggs female^{-1} day^{-1}) was found at the lowest food concentration (150 μgC l^{-1}), significantly lower than all other treatments except for 300 μgC l^{-1} (4.4 $\pm$ 0.5 eggs female^{-1} day^{-1}).

Bestiolina similis fecal pellet production rate was also significantly influenced by microalgae concentration (**Table 1**). The highest FPPR was found at 1,500 μgC l^{-1} (206.4 $\pm$ 11.5 fecal pellets female^{-1} day^{-1}), not significantly different from those of the 1,800 and 1,200 μgC l^{-1} treatments (182.8 $\pm$ 10.1 and 197.9 $\pm$ 10.1 fecal pellets female^{-1} day^{-1}) (**Table 1**). Conversely, relatively low FPPR were found when *B. similis* was reared using a 150 μgC l^{-1} food ration (80 $\pm$ 6 fecal pellets female^{-1} day^{-1}), significantly different from all other treatments except the 300 μgC l^{-1} treatment (106 $\pm$ 6 fecal pellets female^{-1} day^{-1}, indicating a limiting of ingestion for *B. similis* females when reared using such low food concentrations. Lastly, the FPPR observed in the control was negligible (**Table 1**).

In order to determine with precision, the threshold above which *B. similis* ingestion rate starts to saturate, CVI female FPPR was plotted as a function of microalgae concentration (**Figure 1** and see **Table 2** for details). *Bestiolina similis* FPPR was found to saturate at a microalgal concentration of 783.40 μgC l^{-1} (**Figure 1**).

Bestiolina similis 48- and 96-h egg hatching rate (EHR) were both significantly influenced ($p < 0.05$) by microalgal concentration. Eggs produced at the 150 μgC l^{-1} treatment had the lowest 96-h EHR, significantly different from the higher concentrations tested (**Table 1**). Microalgae rations of 300 μgC l^{-1} and above did not produced significantly different EHS from one another, with 48 h EHR > 83% and 96 h EHR > 89% for food concentrations contained between 300 and 1,800 μgC l^{-1}.

Naupliar and Copepodite Survival and Development, Adult Female Life Expectancy and Lifespan Cumulated Egg Production

Naupliar survival was relatively high ($\geq$85%) for microalgae concentrations higher than 300 μgC l^{-1} and no significant difference was detected among treatments ($p > 0.05$; **Table 3**). However, a significantly lower survival of 65% ($p < 0.05$) was recorded when a food concentration as low as 150 μgC l^{-1} was fed to *B. similis*. As for nauplii, copepodites survival was also reasonably high ($\geq$80%) when reared at concentrations $\geq$ 600 μgC l^{-1}, with no significant difference detected between treatments. However, a significant decrease of survival to 52 $\pm$ 5% was observed when food ration was decreased to 300, μgC l^{-1}. Further reduction of microalgae concentration to 150 μgC l^{-1} led to further decreased in copepodite to only 39 $\pm$ 3%. It is interesting to note that copepodite survival was consistently lower than their naupliar counterparts under similar microalgal ration (**Table 3**).

The median development time (MDT) from egg to nauplius, to copepodite and to adult female were all significantly affected ($p < 0.05$) by microalgal concentration (**Table 3**). Copepodites started to appear on day 3 and the MDT from eggs to copepodites was $\leq$ 3.86 days with no significant difference among treatments when microalgae rations was $\geq$ 300 μgC l^{-1}. However, when microalgae concentration decreased to 150 μgC l^{-1}, the MDT increased significantly to 4.52 $\pm$ 0.12 day (**Table 3**). CVI sexually mature adults started to appear on day 5 of the trial and MDT from eggs to adults was shorter than 6 days when food concentration was $\geq$ 600 μgC l^{-1}. The MDT from eggs to CVI adults was significantly increased to 7.46 $\pm$ 0.10 days at the lowest food ration of 150 μgC l^{-1} (**Table 3**).

Eggs produced at the two lowest microalgae concentrations tested (150 and 300 μgC l^{-1}) had the longest egg incubations times (2.94 $\pm$ 0.02 and 2.35 $\pm$ 0.01 days, respectively) significantly longer ($p < 0.05$) than the rest of the treatments, while the 150 μgC l^{-1} treatment was significant longer than the 300 μgC l^{-1} treatment (**Table 3**). There was no significant different difference in egg incubation time among the other treatments (**Table 3**).

Figure 2 represents the average life expectancy of *B. similis* CVI females from different microalgal feeding concentrations. The CVI females had the longest life expectancy after reaching sexual maturity (6.9–7.1 days) when they were fed at a concentration between 300–900 μgC l^{-1}, while those from

TABLE 1 | *B. similis* CVI female daily egg production rate (EPR) and fecal pellets production rate (FPPR), egg diameter, 48 h and 96 h egg hatching rates (EHR) when fed different concentrations of a trialgal diet (T-Iso+Tet+Pav) at a 1:1:1 carbon ratio.

Food concentration (μgC l^{-1})	EPR (eggs female^{-1} day^{-1})	FPPR (fecal pellets female^{-1} day^{-1})	Egg diameter (μm)	48 h EHR (%)	96 h EHR (%)
1800	18.6 ± 1.4 ^{a}	182.8 ± 10.2 ^{A}	81.64 ± 0.93	83.0 ± 2.0^{fg}	90.7 ± 1.7 ^{F}
1500	22.6 ± 1.4 ^{a}	206.4 ± 11.5 ^{A}	83.48 ± 1.05	85.2 ± 2.1 ^{f}	93.7 ± 1.0 ^{F}
1200	20.1 ± 1.2 ^{a}	197.9 ± 10.1 ^{A}	83.89 ± 0.85	85.5 ± 2.4 ^{fg}	89.3 ± 1.2 ^{F}
900	12.7 ± 0.8 ^{b}	140.0 ± 6.7 ^{B}	84.16 ± 0.74	86.8 ± 1.9 ^{f}	92.8 ± 1.0 ^{F}
600	6.9 ± 0.6 ^{c}	125.5 ± 6.0 ^{B}	87.04 ± 1.23	84.3 ± 1.7 ^{fg}	90.7 ± 1.2 ^{F}
300	4.4 ± 0.5 ^{cd}	106.1 ± 5.9 ^{BC}	85.94 ± 1.35	83.3 ± 2.1 ^{fg}	90.7 ± 0.7 ^{F}
150	2.2 ± 0.2 ^{d}	80.3 ± 5.9 ^{C}	84.08 ± 1.08	76.0 ± 1.8 ^{g}	83.3 ± 1.0 ^{G}
0	(0.3 ± 0.2)	(13.8 ± 1.3)	n/a	n/a	n/a

Different superscript letters indicate significant difference within a column ($p < 0.05$). Data are presented as mean ± standard errors. Values in blanket were not included for statistical analysis due to few replicates retrieved. n/a indicates that not enough data were obtained to calculate reliable average.

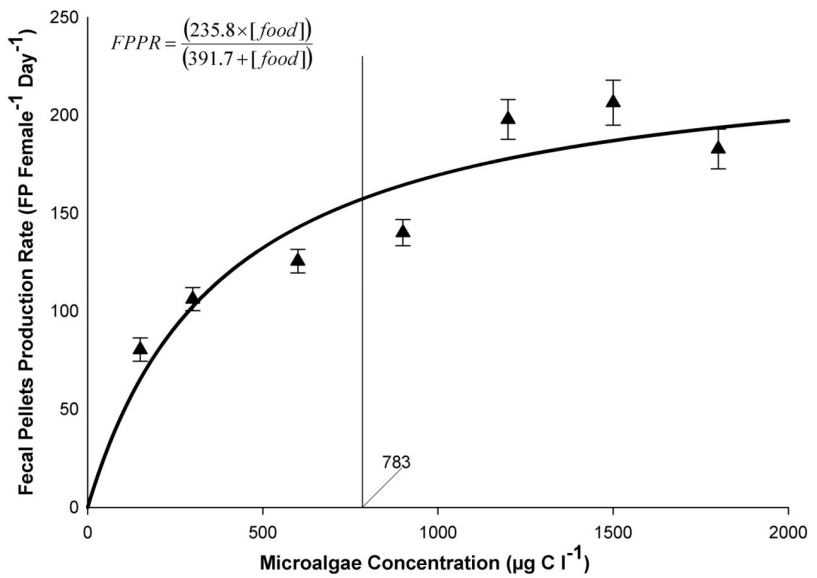

FIGURE 1 | Relationship between fecal pellet production rates and concentration of a mixed trialgal diet (T-Iso+Tet+Pav at a 1:1:1 carbon ratio) in the calanoid copepod *B. similis*. Each data point represents average ± standard error. The vertical line is the carbon concentration to which fecal pellet production saturates. Curves were fitted using SigmaPlot^{TM} (v11.0) using an iterative process.

TABLE 2 | Summary of the model parameters and results for *B. similis* CVI female.

Dependent variable	Function	Equation	a	b	c	Saturation point (μgC l^{-1})	R^2	R^2 adj	p
Female FPPR	Michaelis-Menton, two parameters	y = (a*x)/(b+x)	235.82	391.70	n/a	783.40	0.86	0.83	<0.03

The nonlinear Michaelis-Menton equation was used to describe the relationship between microalgae concentration (μgC l^{-1}) and fecal pellet production rate (fecal pellets female^{-1} day^{-1}). "R^2" is the coefficient of determination, "R^2 adj" is the adjusted coefficient of determination and "p" is the significance level of the fit.

higher concentration treatments (between 1,200 and −1,800 μgC l^{-1}) had a shorter life expectancy (6.0–6.5 days), although the differences between all above treatments were not statistically significant ($p > 0.05$). The shortest average female lifespan (4.7 ± 0.8 days) was recorded at the lowest food ration treatment of 150 μgC l^{-1}, which was significantly shorter than the 300, 600, and 900 μgC l^{-1} treatments but not significantly different ($p < 0.05$) from the 1,200, 1,400 and 1,800 μgC l^{-1} treatments (**Figure 2**).

Microalgal feeding concentration had a similarly significant influence of cumulative egg output over the lifespan of *B. similis* females ($p > 0.05$; **Figure 3**) with the higher total egg productions found at algal concentrations of 1,200, 1,500, and 1,800 μgC l^{-1} (140.6 ± 10.4; 130.6 ± 10.0 and 132.3 ± 8.6 eggs female^{-1}, respectively), significantly higher than those found at lower concentrations from 150 to 900 μgC l^{-1} treatments ($p > 0.05$). Among these lower concentrations, the 600 and 900 μgC l^{-1} treatments produced intermediate lifespan global egg output

TABLE 3 | Average survival of nauplii and copepodites and median development time (MDT) from egg to copepodite and adult stage of the *Bestiolina similis* fed different concentrations of a trialgal diet T-Iso+Tet+Pav with a 1:1:1 carbon ratio.

Microalgal concentration ($\mu gC\ l^{-1}$)	MDT from eggs to nauplii (days)	Naupliar survival (%)	MDT from egg to copepodite (days)	Copepodite survival (%)	MDT from egg to CVI adult (days)
1800	2.14 ± 0.04^{a}	95 ± 2^{d}	3.80 ± 0.08^{f}	92 ± 2^{h}	5.93 ± 0.07^{k}
1500	2.16 ± 0.01^{a}	94 ± 2^{d}	3.78 ± 0.08^{f}	90 ± 2^{h}	5.94 ± 0.08^{k}
1200	2.16 ± 0.04^{a}	94 ± 3^{d}	3.80 ± 0.13^{f}	91 ± 3^{h}	5.96 ± 0.10^{k}
900	2.17 ± 0.03^{a}	92 ± 5^{d}	3.74 ± 0.04^{f}	81 ± 2^{h}	5.91 ± 0.05^{k}
600	2.14 ± 0.01^{a}	96 ± 3^{d}	3.86 ± 0.06^{f}	80 ± 2^{h}	6.00 ± 0.05^{k}
300	2.35 ± 0.01^{b}	85 ± 1^{d}	3.83 ± 0.08^{f}	52 ± 5^{i}	6.18 ± 0.08^{k}
150	2.94 ± 0.02^{c}	65 ± 3^{e}	4.52 ± 0.12^{g}	39 ± 3^{j}	7.46 ± 0.10^{l}

Data are presented as average +/− standard errors. Different superscript letters within the same column indicate significant differences. The unfed control is not included as development was not observed controls as development was not observed beyond the second nauplius stage (NII) due to starvation.

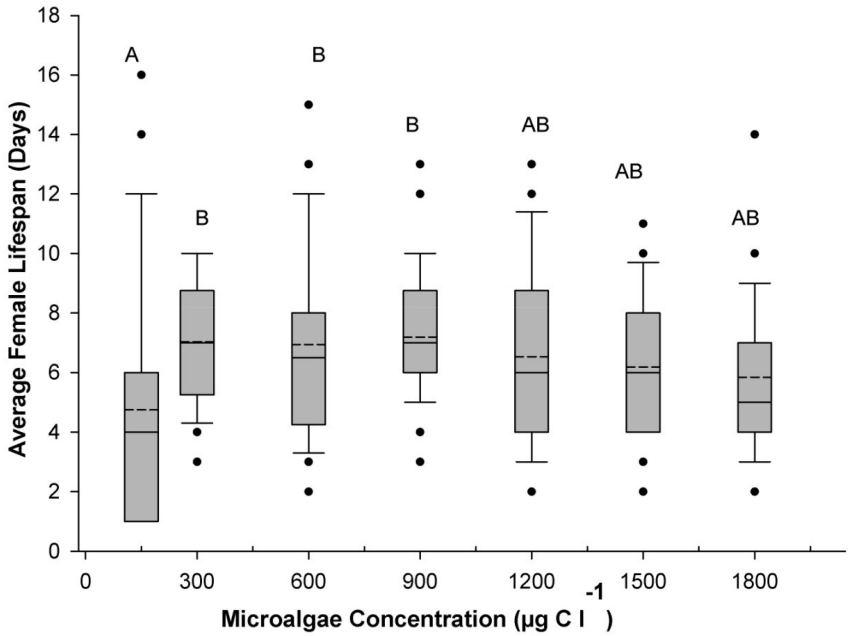

FIGURE 2 | Average *B. similis* female lifespan as a function of concentration of a trialgal diet T-Iso+Tet+Pav at a 1:1:1 carbon ratio. The boundary of the box closest to zero indicates the 25th percentile and the boundary farthest from zero indicates the 75th percentile. Vertical error bars indicate the 90th and 10th percentiles. Average are indicated by the dashed line while median values are indicated with the full line. Black circles indicate outliers. Different letters on top of the box plots indicate significant differences ($p < 0.05$) between the treatments.

(79.7 ± 6.4 and 100.8 ± 7.3 eggs female^{-1}, respectively), which were significantly higher than the 150 and 300 $\mu gC\ l^{-1}$ ($p < 0.05$). The lowest total egg output recorded was 38.3 ± 3.7 eggs female^{-1} in the 150 $\mu gC\ l^{-1}$ treatment although it was not significantly different from that of the 300 $\mu gC\ l^{-1}$ treatment ($p > 0.05$; **Figure 3**).

Population Growth and Composition

After 14 days of culture, population increase of *B. similis* was significantly ($p < 0.05$) affected by microalgae concentration, whether or not unhatched eggs are considered in the final count (**Table 4**). Copepods reared using a 1,200 $\mu gC\ l^{-1}$ food ration had the highest intrinsic rate of population increase (0.27 ± 0.01) although it was not significantly different from the other treatments except for the 150 $\mu gC\ l^{-1}$ treatment, which was the only treatment to produce a negative intrinsic rate of population increase (-0.03 ± 0.03) (**Table 4**). At the end of the experiments, sex ratio of adults in the final populations was consistently heavily skewed toward females (i.e., between 80 and 94% of adults were females) in all treatments and no significant effect of algal feeding concentrations on sex ratio was detected ($p > 0.05$) (**Table 4**).

DISCUSSION

Microalgal concentration is long established as one of the key factors affecting copepod productivity (Klein Breteler and Gonzalez, 1982, 1988; Klein Breteler et al., 1995;

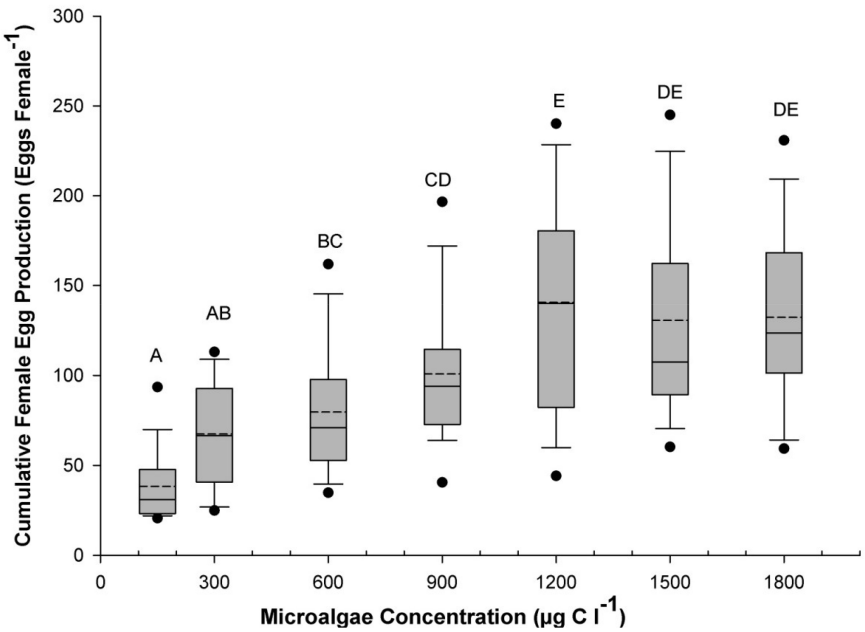

FIGURE 3 | Average total egg production over *B. similis* female lifespan as a function of concentration of a trialgal diet T-Iso+Tet+Pav. The boundary of the box closest to zero indicates the 25th percentile and the boundary farthest from zero indicates the 75th percentile. Vertical error bars indicate the 90th and 10th percentiles. Average are indicated by the dashed line while median values are indicated with the full line. Dark circles indicate outliers. Different letters on top of the box plots indicate significant differences ($p < 0.05$) between the treatments.

TABLE 4 | Final population and intrinsic rate (r) of population increase of *B. similis* cultured over a 14 days period fed on different concentration of a trialgal diet T-Iso+Tet+Pav at a 1:1:1 carbon ratio.

Microalgal concentration (µgC l^{-1})	Intrinsic rate of population increase	Final population number when all stages are included	Final population number of all post-egg-stages	Final female proportion (%)
1800	0.25 ± 0.01 a	323.5 ± 45.88	243.5 ± 35.32	0.91 ± 0.03
1500	0.25 ± 0.01 a	297 ± 26.89	254.75 ± 23.47	0.92 ± 0.01
1200	0.27 ± 0.01 a	433.25 ± 76.88	345 ± 71.51	0.94 ± 0.04
900	0.25 ± 0.01 a	327.25 ± 40.31	241.5 ± 38.21	0.87 ± 0.03
600	0.23 ± 0.01 a	237.5 ± 27.02	179.5 ± 28.00	0.83 ± 0.01
300	0.21 ± 0.02 a	182.5 ± 49.11	162 ± 49.48	0.80 ± 0.02
150	−0.03 ± 0.03 b	7.75 ± 3.77	7.25 ± 3.28	(0.95 ± 0.04)

Different superscripts within the same column indicate significant difference. Values in blanket were not included for statistical analysis due to few replicates retrieved. No enough data were retrieved from the controls as development was not observed beyond the second nauplius stage (NII) due to starvation.

Tirelli and Mayzaud, 2005) and over the past decade many studies have reporting effects of food quality (or algal species) on copepod culture productivity (e.g., Milione and Zeng, 2007; Camus et al., 2009; Camus and Zeng, 2010; Pan et al., 2014; Alajmi and Zeng, 2015; Rasdi and Qin, 2018; Nogueira et al., 2019; Dayras et al., 2020). However, investigations on effects of food quantity (or algal concentration) are far fewer, particularly studies considering copepods as live prey in aquaculture settings. Results from an ecological study conducted in Hawaii reported that *B. similis* naupliar stages selected strongly against prey particles in the 2–5 µm range and a total ingestion rate of 25.4–73.8 ng C nauplius^{-1} h^{-1} (Jungbluth et al., 2017). However, this study is focused exclusively on *B. similis* naupliar stages and levels of food concentrations reflecting those found in the natural environment. In an effort to improve the culture protocol of *Bestiolina. similis*, a promising live prey for marine hatcheries (McKinnon et al., 2003), the present study was designed to determine the effects of eight different feeding concentrations (0 to 1,800 µgC l^{-1}) of a pre-determined optimized trialgal diet (Camus et al., 2009; Camus and Zeng, 2010) on several major productivity-related parameters on CVI adult B. similis.

The optimization of microalgal feeding rations is of paramount importance in a commercial copepod culture setting for several reasons: to ensure that the culture productivity is not limited by microalgae quantity; to limit the accumulation of excess uneaten microalgae that can potentially negatively impact water quality parameters overtime and to save significant amount of time and effort culturing unneeded additional algal biomass,

ultimately cutting down operational costs and saving precious worker's time. Several field studies have demonstrated that the ingestion rates of pelagic copepods were seldom saturated in the wild, even during events of phytoplankton blooms (Mayzaud and Poulet, 1978; Ayukai, 1987; Liang et al., 1994; Hirst and Lampitt, 1998), making it quite difficult to accurately determine their feeding saturation rates *in situ*. Conversely, food conditions can be precisely monitored in laboratory settings and saturations of copepod ingestion rates have commonly been measured (Corner et al., 1972; Frost, 1972; Uye, 1981; Tirelli and Mayzaud, 2005; Gusmão and McKinnon, 2009a). Experiment conducted under laboratory setting therefore helps to understand the full spectrum of copepod responses to food quantity, including higher, limiting food concentrations (Støttrup and Jensen, 1990).

Calanoid copepods are known to contain mostly lipids, such as triacylglycerols, that only reflect recent nutritional conditions rather than prolongated feeding history (Koski and Kuosa, 1999). The negligible amount of eggs and fecal pellets production observed for *B. similis* in the unfed control (0 μgC l^{-1}) confirmed this hypothesis, as no residual effect from previous feeding history was measured.

The lowest microalgal concentration tested (150 μgC l^{-1}) produced the lowest egg production, egg hatching rate, naupliar and copepodite survival and development, adult female life expectancy as well as a negative population growth over a 14 days period of culture. This is consistent with reports of generation time increasing with decreasing food quantity, as reported in previously studies on calanoid copepods (Arnott et al., 1986; Ban, 1994). Higher algal feeding concentration $\geq$ 300 μgC l^{-1} significantly improved several productivity related parameters, such as egg hatching rate, naupliar and copepodite survival and development, and was capable of supporting positive population growth over 14 days. However, microalgal concentrations $\leq$ 900 μgC l^{-1} were still limiting regarding daily egg and fecal pellet production, as well as female cumulative egg production over lifespan, when compared to microalgae concentrations $>$ 900 μgC l^{-1}. These results suggest that microalgal feeding concentrations of 900 μgC l^{-1} and below are still somehow limiting to *B. similis* productivity.

Bestiolina similis cultured using a microalgal concentration of 1,200 μgC l^{-1} experienced the highest cumulative egg production over female lifespan and the highest population growth among all tested concentrations. Increasing this ration further to 1,500 or even 1,800 μgC l^{-1} did not produce any significant improvement in any of the productivity-related parameters measured, suggesting a stagnation in feeding efficiency for feeding rations $>$ 1,200 μgC l^{-1}. Such a result can probably be explained by "dome-shaped" functional responses proposed by Jeschke et al. (2004), who suggested that a decrease in consumption rate by filter feeders may occur at very high food abundance due to confusion, clogging of feeding appendages and/or accumulation of toxic substances produced by excessively high food concentrations (Jeschke et al., 2004). These mechanisms could be responsible in diminishing calanoid copepods ingestion rate during episodes of excessive food abundance and are not always taken into consideration when modeling deposit/filter feeders' functional responses in natural settings. Indications of decreasing productivity at higher food concentrations were observed in the current experiment, as the 1,800 μgC l^{-1} treatment produced lower daily egg and fecal pellet production, decreased female life expectancy and cumulative egg production over lifespan, as well as population growth when compared to the 1,200 μgC l^{-1} treatment. To conduct additional treatments at food concentration $>$ 1,800 μgC l^{-1} could have provided a more complete representation of *B. similis* dome-shaped response due to saturation of feeding under excessive food rations.

Although no significant difference in sex ratio was detected among the microalgae concentrations tested, *B. similis* sex ratio was always highly skewed toward females for all treatments (80–94% adults were females). While sex-determination mechanisms in calanoid copepods remain largely unknown, this highly skewed sex ratios could be explained by the intersexuality mechanism postulated by Gusmão and McKinnon (2009b) in which under certain environmental conditions, a sex change occurs during the late copepodite development. Copepod population in culture strongly skewed toward females are a significant advantage in hatchery setting, as they will produce more eggs at the population level, providing there is no limitation in fertilization due to a low abundance of males.

A 14 days population growth experiment provided positive intrinsic rates of population increase for all microalgae concentrations tested, with the exception for the 150 μgC l^{-1} treatment. Such a low food concentration should hence be avoided in *B. similis* culture as it was too low to support any growth in population over a 14 days period. Interestingly, food concentration ranging from 300 to 1,800 μgC l^{-1} did not produce a significantly different final intrinsic rates of population increase.

Individual CVI females were used to allow data collection for female life expectancy and cumulative egg production over lifespan. Past studies have reported contradicting results on effects of crowding on copepod egg production. For example, Zhang et al. (2015) demonstrated that high stocking density (40–160 individuals l^{-1}) depressed daily egg production of the temperate species *Acartia tonsa* when fed algae *Rhinomonas reticulate* at $\geq$ 500 μgC l^{-1}. Nevertheless, the same authors concluded that egg production was mainly limited by the quantity of food rather than crowding. In *Acartia sinjiensis*, it was, however, found that within a broad range of stocking density (125–2,000 individuals l^{-1}), average daily egg output per female was not significantly affected by this parameter (Camus and Zeng, 2009). The present study did not test for density effect and it is hence unknown whether or not *B. similis* egg production is affected by crowding, which warrants further research.

Food concentration is known to affect the physical characteristics of copepod fecal pellets (Besiktepe and Dam, 2002), the production rates of which are commonly used as a proxy of ingestion rate (Ayukai, 1987; Besiktepe and Dam, 2002). Ingestion rate is not simply related to food quantity but rather to the combined interactions of food quality and quantity with ingestion, gut transit time and assimilation efficiency (Mitra and Flynn, 2007). Gut residence time tend to increase during episodes of high food concentration, producing large

and densely packed fecal pellets (Dagg and Walser, 1986). On the other hand, decrease in gut residence time during episodes of low food concentrations is believe to save the energy cost of ingestion, as copepods are unable to extract much from the ingested materials, resulting in the production of smaller, less dense and more fragile fecal pellets (Mitra and Flynn, 2007). This was confirmed in the present study as visual inspection of the fecal pellets revealed that smaller, less dense pellets of inconsistent shape were produced at food rations of 150 and 300 μgC l^{-1}, whereas comparatively larger and denser fecal pellets of consistent shape were found at food rations of 600 μgC l^{-1} and above. Nonetheless, there are indications that this relationship between food concentration and gut transit time might be species-specific, as other studies reported a decrease in gut transit time associated with increasing concentration of certain food types such as diatoms (Tirelli and Mayzaud, 2005).

Results from this study suggest that marine hatcheries should pay closer attention to improving microalgal feeding for the intensive cultivation of calanoid copepods. Implementing an optimal microalgal feeding ration that will ensure maximum culture productivity, without saturating copepod feeding capacity, provides numerous advantages to a copepod culture, including improving water quality parameters overtime, while saving time and money. In the case of *B. similis,* a trialgal diet of T-Iso+Tet+Pav at 1:1:1 carbon ratio should be fed at a concentration of 1,200 μgC l^{-1} and not above, as rations $>$ 1,200 μgC l^{-1} will not provide any significant improvement in productivity. On the other hand, feeding rations should never be allowed to drop below 783.40 μgC l^{-1} as this is the threshold level below which adult female ingestion rates become limiting.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

TC and CZ: conception and design of the study, provision of study materials, and analysis and interpretation of the data. TC: acquisition of data, drafting the manuscript, and final approval of the manuscript. TC, CZ, LR, and JJ: critical review of the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Marine Science

MINI REVIEW
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Recent Trends in Live Feeds for Marine Larviculture: A Mini Review

Yen-Ju Pan[1,2], Hans-Uwe Dahms[3,4,5], Jiang-Shiou Hwang[2,6,7] and Sami Souissi[8]*

[1] Department of Aquaculture, National Taiwan Ocean University, Keelung, Taiwan, [2] Center of Excellence for the Oceans, National Taiwan Ocean University, Keelung, Taiwan, [3] Department of Biomedical Science and Environmental Biology, Kaohsiung Medical University, Kaohsiung, Taiwan, [4] Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Kaohsiung, Taiwan, [5] Research Center for Environmental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, [6] Institute of Marine Biology, National Taiwan Ocean University, Keelung, Taiwan, [7] Center of Excellence for Ocean Engineering, National Taiwan Ocean University, Keelung, Taiwan, [8] Université de Lille, CNRS, Université du Littoral Côte d'Opale, IRD, UMR 8187 LOG, Laboratoire d'Océanologie et de Géosciences, Station Marine de Wimereux, Lille, France

In marine larviculture, farmed larvae mainly rely on the alimentation of a group of small-sized phytoplankton and zooplankton referred to as live feed. Under the diversifying demands of human consumption and ornamental aquarium industry, new species of live feed and their innovative production methods are essential focuses for sustainable larviculture of many emerging fish and invertebrate species. The selection of proper live feed for larval feeding is based on several parameters, such as size, morphology, nutritional value, stock density, and growth rate. This review aims to highlight the biological characteristics, production approach, common larviculture applications as well as recent innovations in the aquaculture technology of live feed organisms (microalgae, ciliated protists, rotifer, *Artemia*, copepod, and others).

Keywords: marine larviculture, live feed, microalgae, ciliate, rotifer, *Artemia*, copepod

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****Correspondence:***
Yen-Ju Pan
panyj@mail.ntou.edu.tw

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INTRODUCTION

The percentage of world aquaculture production over the total fishery resource has increased from 14.6% in 1986 to 46% in 2018 (FAO, 2020). Although aquaculture is a fast-growing industry, one of the bottlenecks is proper rearing of the early life stages of many farmed fish and invertebrate species (Hu et al., 2018; Gallardo et al., 2022). The significant difficulty is the first feeding at larval weaning stage. When larvae deplete yolk reserves and experience transition from endogenous to exogenous feeding, they do not benefit from a well-developed gastrointestinal tract to efficiently digest the formulated diets (Infante and Cahu, 2001; Yúfera and Darias, 2007). The young larvae have limited capacity of predation (detection and capture) due to its immature jaw, muscle, and optical developments (Hu et al., 2018). Moreover, the specific larval feeding behaviors and nutritional requirements should be considered when selecting suitable first feeding ingredients to achieve a successful larviculture production (Rønnestad et al., 2013; Mejri et al., 2021). Contrary to formulated diets, motile and viable phytoplankton and zooplankton provide more bioavailable nutrients and trigger higher predatory responses, and have been recognized as promising exogenous nutrients for marine larvae (Conceição et al., 2010; Nielsen et al., 2017; Kandathil Radhakrishnan et al., 2020). These dietary planktons could live with the farmed larvae in the rearing system, and be ingested by the larvae whenever desire, are thus referred as live feeds.

Most emerging species in marine aquaculture and aquarium industries have a sensitive and small-mouthed larval stage, and their larviculture are very challenging due to a lack of appropriate first feeding protocols. It is of a crucial interest to enhance diversification and innovation within live

feed production programs to advance the fast-growing marine larviculture industry. Consequently, the aquaculture technologies of live feed productions are a focus point worldwide (Hansen and Møller, 2021). Here we review recent trends of live feed production at laboratory and industrial scales and discuss challenges and perspectives of its applications.

MICROALGAE

Microalgae plays a fundamental role in aquatic food webs by converting solar energy into bioavailable organic compounds and trophic resources. These micro-sized autotrophs are sustainable food item for aquaculture (Hemaiswarya et al., 2011), and are used as live feeds for several marine organisms such as bivalves (Tahir and Ransangan, 2021; Hassan et al., 2022), zooplankton (Pan et al., 2018; Dayras et al., 2021), larvae of crustacean (Sharawy et al., 2020; Sandeep et al., 2021), and echinoderm (Militz et al., 2018; Gomes et al., 2021). In marine hatcheries, the usage of microalgae could be categorized in three scenarios: (i) direct diet to provide nutrients to early developmental stages (Camus et al., 2021; Dayras et al., 2021); (ii) natural enrichment ingredients to zooplankton live feed organisms (Fu et al., 2021); (iii) water conditioners: microalgae are added to create "green water" which conditions water quality, reduces bacterial loads, increases visual contrast, and prey detection (Basford et al., 2021). Based on a variety of microalgal characteristics (**Table 1**) several aspects should be considered in applications: (i) cell size: that should be compatible to the ingestion capacities of the larvae; (ii) cell structure: property of cell walls or skeletons (e.g., cellulose, SiO_2, or $CaCO_3$) could affect the efficiency of ingestion and digestion; (iii) nutritional profile: content (actual amount) and composition (percentage) of various bioactive nutrients should be taken into account according to the nutritional requirements of their consumers (Borowitzka, 2013; Pan et al., 2018; Dayras et al., 2021). In general, the production of marine microalgal Chlorophytes (e.g., *Nannochloropsis* sp. and *Tetraselmis* sp.) can easily be sustained at high cell concentration and wide environmental conditions. Nevertheless, the thick cellulose cell wall and nutritional deficiency [i.e., low docosahexaenoic acid (DHA), 22: 6n-3, DHA or eicosapentaenoic acid (EPA), 20: 5n-3, EPA] hinder their applicability as live feed for some phytoplankton feeders (Pan et al., 2014). On the contrary, haptophyte and cryptophyte species (e.g., *Isochrysis* sp., *Tisochrysis* sp., and *Rhodomonas* sp.) provide superior nutritional values and higher digestibility due to their balanced polyunsaturated fatty acid (PUFA) profiles and soft cell structures (Latsos et al., 2020; Mai et al., 2021). Unfortunately, those microalgal species are relatively fragile and sensitive toward environmental stressors (e.g., temperature, salinity, and pH variations), and require more time and experienced labor to achieve successful productions. Recent studies focused on how to technically enhance their cell densities by manipulating the culture environments at automated regulations. In the past decade, photobioreactors (PBR) have been developed to produce microalgal biomass for biodiesel production (Peter et al., 2022). Currently many programs of biomass production are used to extract bioactive compounds with an increasing use of diverse systems such as mesh ultra-thin layer, tubular glass, plastic bag, and flat-plate PBRs (Sandmann et al., 2021; Tayebati et al., 2021; Wurm and Sandmann, 2021). Although the PBR might increase production cost, these well programmed systems could realize extremely high cell density for aquaculture purposes (Vu et al., 2019; Tibbetts et al., 2020; Leal et al., 2021). Biotechnology has opened new avenues for microalgal applications, where strain selection including non-genetic as well as genetic modifications facilitate beneficial bioactive compounds (e.g., anti-pathogenic, anti-oxidant, etc.) for farmed aquatic larvae (Kiataramgul et al., 2020). Yet the biosecurity of transgenic microalgae should be carefully evaluated before their large-scale utilization.

CILIATED PROTISTS

Ciliates are a group of single-celled protist, which commonly exist in marine environments worldwide. Some ciliate species are pathogenic for fish, because they experience partially or completely their life cycle in or on the host (Jahangiri et al., 2021). Another group of ciliates appear to be planktonic and they have a potential as live feed in marine hatcheries (Wan-Mohtar et al., 2021). Culture techniques for *Euplotes* sp. and *Fabre* sp. have been developed in recent studies (**Table 1**). Ciliates could rapidly increase their populations by fission when fed on baker's yeast, fermented fish meal, and photosynthetic bacteria (de Freitas Côrtes et al., 2013; Balamuralir, 2020; Teiba et al., 2020). The production of these fast-growing protists does not necessarily rely on a microalgal diet, which greatly enhance the feasibility and convenience for culture maintenance. Most importantly, ciliates are known for their tiny cell size (20–60 μm), which is particularly favorable for small-mouthed larvae (Hill et al., 2020). Indeed, ciliate-based diets have been acknowledged to successfully sustain larvae rearing of several marine ornamental or edible fish species (Nagano et al., 2000; Rhodes and Phelps, 2008; Madhu and Madhu, 2014; Leu et al., 2015). On the other hand, the use of bacteriovorous ciliates for pathogen removal has recently emerged. Lin et al. (2020) noted the remarkable increase of survival rate (approx. 60%) in pathogen challenge trials of grouper larvae when the water containing rich *Vibrio campbellii* was prefiltered by the ciliate *Strombidium* sp.

ROTIFERS

Rotifers are a group of multicellular microorganisms making up a phylum Rotifera. Since the 1970s, species and strains of the genus *Brachionus* have been used as live feed for the first feeding of marine larvae during 3–10 days post hatching (dph) (Lubzens et al., 2001). Although the taxonomy of *Brachionus plicatilis* and *Brachionus rotundiformis* complex remains inconclusive, they are normally referred as SS, S, and L type rotifer based on their size. Rotifers are highly demanded in the current larviculture industry due to the following reasons: (1) reasonable size spectrum (100–250 μm) and slow cruising swimming pattern for first feeding of commercially important fish species (e.g., sea bream and sea

TABLE 1 | Characteristics of different live feed organisms used in marine larviculture.

		Size range as live feed	General culture conditions	Common nutrient/diet	Applications as live feeds	Key nutritional advantages (% total FA or AA)	References
Microalgae	*Isochrysis* sp.	3–6 μm	15–30°C, SNS	Walne's, f/2 medium, agricultural fertilizers	Diet for copepod, rotifer, larvae of bivalves, echinoderms, and phytoplanktivorous fish	1–9% EPA; 8.1–12.5% DHA	Pan et al., 2018; Balakrishnan and Shanmugam, 2021; Shekarabi et al., 2021
	Tisochrysis sp.	3–7.5 μm	18–30°C, SNS			0.6–0.8% EPA; 10–11% DHA	Tato and Beiras, 2019; Dayras et al., 2021; Mai et al., 2021
	Pavlova sp.	4–6 μm	23–20°C, SNS			17.8–33.9% EPA; 3.6–10.2% DHA	Rehberg-Haas et al., 2015; Yang et al., 2020; Dayras et al., 2021; Hassan et al., 2022
	Nannochloropsis sp.	2–4 μm	26–30°C, SNS			26.2–35.2% EPA; 0–0.52% DHA	Pan et al., 2018; Yang et al., 2020
	Tetraselmis sp.	13–15 μm	26–30°C, SNS			4.2–5.2% EPA; 23.6–27.9% ALA	Pan et al., 2018; Lee et al., 2021
	Rhodomonas sp.	7–14 μm	15–25°C, SNS			8–15.8% EPA; 6–8.8% DHA	Latsos et al., 2020; Oostlander et al., 2020; Dayras et al., 2021
Rotifer	*Brachionus* sp.	90–320 μm	25°C, 15–35 ppt	Microalgae (fresh cells, lipolyzed powder or concentrated paste)	First-feeding (2–10 dph) of larval fish and crustacean	Nutritional profile could be manipulated by enrichment	Snell et al., 2019
	Colurella sp.	48–99 μm	22–28°C, 15–34 ppt				Chigbu and Suchar, 2006; Madhu et al., 2016
	Proales sp.	82.7 ± 10.9 μm	25°C, 2–25 ppt				Wullur et al., 2011; Hagiwara et al., 2014
Ciliated Protist	*Euplotes* sp.	60–110 μm	25–32°C, 20–30 ppt	Baker yeast, fermented fish diet, and microalgae	First-feeding (2–10 dph) of small-mouthed larval fish	Nutritional profile could be manipulated by enrichment	Tarangkoon et al., 2018; da Annunciação et al., 2020
	Metacylis sp.	37–50 μm	30°C, 33 ppt				Lee and Choi, 2016
Artemia	*Artemia* sp.	Newly-hatched: 400–500 μm; Enriched: 500–700 μm	28°C, 25–33 ppt	No feeding: nauplii used after hatch or enrichment (fish oil, fish soluble emulsions)	Fish or crustacean larvae at second-stage feeding (>10 dph)	Nutritional profile could be manipulated by enrichment	Figueiredo et al., 2009
Copepod	*Pseudodiaptomus annandalei*/ *P. inopinus*	150–1,100 μm/ 200–800 μm	25–30°C, 15–20 ppt/20°C, 17 ppt	Live microalgae cell (*Isochrysis, Rhodomonas/Phaeodactylum, Pavlova, Tisochrysis*, and *Chlorella*)	Several developmental stages (size range: 60–1,200 μm) for larval fish and crustaceans at different feeding stages	2.9–12.8% EPA, 12.6–57% DHA/ 0.6–24.4% EPA, 1.3–12.7% DHA	Golez et al., 2004; Rayner et al., 2015; Matsui et al., 2021; Nielsen et al., 2021
	Acartia bilobata/ *A. tonsa*	100–1,100 μm/ 100–1,200 μm	25–30°C, 15–20 ppt/ 17–23°C, 27–34 ppt	Live microalgae cell (*Isochrysis/Rhodomonas*)		ND/ 16.5% EPA, 7.9% EPA	Drillet et al., 2008; Pan et al., 2014; Chi et al., 2018; Torres et al., 2022
	Apocyclops royi	100–1,000 μm	25–30°C, 15–20 ppt	Live microalgae (*Isochrysis, Rhodomonas*, and *Dunaliella*), baker yeast		1.8–13.4% EPA; 4–35.3% DHA	Chang and Lei, 1993; Pan et al., 2018; Nielsen et al., 2021
	Parvocalanus crassirostris	60–400 μm	21–27°C, 20–36 ppt	Live microalgae (*Tisochrysis, Isochrysis, Rhodomonas, Tetraselmis,* and *Heterocapsa*)		2.8–5.4% EPA; 6.1–22.3% DHA	McKinnon et al., 2003; Alajmi, 2015; Kline and Laidley, 2015; Jackson and Lenz, 2016
	Paracyclopina nana	70–600 μm	18°C, 15 ppt	Live microalgae (*Tisochrysis, Rhodomonas,* and *Pavlova*)		2.3–5.5% EPA; 8.9–13.3% DHA	Lee et al., 2006; Dayras et al., 2021
	Bestiolina similis/ *B. amoyensis*	70–560 μm/ <100–<1,000 μm	26–28°C, 29–31 ppt/ 24–26°C, 28 ppt	Live microalgae (*Isochrysis, Pavlova, Rhodomonas,* and *Tetraselmis/Isochrysis*)		0.6% EPA; 2.5% DHA/ ND	McKinnon et al., 2003; Lian et al., 2018; Camus et al., 2021
Other live feeds	Moon jellyfish *Aurelia aurita*	5 ± 1 cm	22°C, NS	*Artemia* nauplii/wild-captured zooplankton	Lobster phyllosoma larvae, Juveniles of silver pomfret, and threadsail filefish	9.88–17.5% EPA; 1.3–1.8% DHA, 0.8–14.5% glycine	Liu et al., 2015; Wakabayashi et al., 2016b
	Flame jellyfish *Rhopilema esculentum*	2 ± 0.5 cm	22°C, NS			7.8% EPA; 1.36% glycine	Liu et al., 2015
	Fungal-like protists *Schizochytrium* sp.	9–14 μm	30°C, FW	Glucose solution	Diet or enrichment products for copepod, rotifer, and Artemia	40–54% DHA	Ramos-Vega et al., 2018; Guo et al., 2020
	Oyster fertilized egg or trochophore	50-70 μm	27.5-29°C, 35 ppt	No feeding: trochophore used after fertilization	First-feeding (2–10 dph) of small-mouthed larval fish	2.2–5.4% EPA; 2–3.3% DHA	Hur et al., 2008; Basford et al., 2019

FA, fatty acid; AA, amino acid; SNS, sterilized natural seawater; NS, natural seawater; FW, fresh water; EPA, eicosapentaenoic acid (20: 5n3); DHA, docosahexaenoic acid (22: 6n3); ALA, α-Linolenic acid; dph, days post hatching; ND, no data.

bass) (Conceição et al., 2010); (2) parthenogenetic reproduction facilitates high duplication rates (Fu et al., 2021); (3) capacity of tolerating high population densities and environmental variation (Suantika et al., 2003); (4) vector of nutrients or medicine delivery for fish larvae (Eryalcin, 2018; Fu et al., 2021; Safiin et al., 2021). In common batch culture systems, the density of *Brachionus* rotifer peaks during 4–7 day-post-inoculation, then the partial harvest and water exchange are carried out until subsequent inoculation (Sales et al., 2019). A semi-continuous recirculating aquaculture systems (RAS) has been developed to sustain superintensive rotifer cultures (>5,000 ind./mL) for periodic harvest (Suantika et al., 2000; Suantika et al., 2003). The maintenance of superintensive culture, however, increase the cost of rotifer production due to the equipment requirements of a recirculating aquaculture system (Suantika et al., 2003). Besides environmental control, antioxidants could be fed to rotifers to further improve their stress resistance in high density cultures with deteriorating water quality (Gao et al., 2021). The nutritional enrichment of rotifers is necessary due to the lack of many essential fatty acids for fish larvae at the first feeding stage (Ferreira et al., 2018; Ghaderpour and Estevez, 2020). Several enrichment products and protocols have been developed and evaluated to enhance larval growth and survival by improving ω3 highly unsaturated fatty acid (HUFA) content, and a high DHA/EPA ratio in rotifers (Abu-Rezq et al., 2002). Recently, cultures of very tiny rotifer species (<100 μm), such as *Proales similis* and *Colurella adriatica*, have been established (**Table 1**) and used particularly for the first feeding of small-mouthed larvae of marine ornamental species (Hagiwara et al., 2014; Madhu et al., 2016; Rebolledo et al., 2021).

ARTEMIA

Artemia is a genus of aquatic crustaceans in the class Branchiopoda, which dominates in hypersaline habitats (e.g., inland salt lakes). During dry seasons, *Artemia* starts to produce floating resting eggs (aka cysts) due to extreme hypersaline stress. The cysts are collected and processed (purification and dehydration), then canned in dark and cold conditions for further storage and distribution. Although *Artemia* are not naturally accessible food items for most marine or brackish larvae, they are extensively used in larviculture industry due to the following reasons: (i) durable cysts and manipulable hatching: obtain nauplii at desirable timepoints for larval feeding; (ii) size suitability: first naupliar stage of various *Artemia* species is ranging 400–500 μm offering preferable size for second-staged larval feeding (7–14 dph); (iii) vector of nutrients or medicine delivery systems (enrichment needed before use) (Eryalcin, 2018). *Artemia franciscana* (**Table 1**) is one of the most utilized species due to its smaller body size and first-ranked annual production (1,000–2,000 tons) from the Great Salt Lake of Utah, United States. Whereas the production from hypersaline lakes in West Siberia, Russia and Kazakhstan, and salt works at Bohai Bay, China are ranked second or third cyst production areas of *Artemia parthenogenetica* and *A. franciscana*, respectively (Litvinenko et al., 2015). Other production areas, such as Brazil (Camara, 2020), Vietnam (Le et al., 2019), Iran (Manaffar et al., 2020), and Tunisia (Sellami et al., 2020), also contribute certain amounts of cyst production. Due to the high market demand, Artemia Reference Centers have been established at Ghent University, Belgium in 1978, and at Tianjin University of Science and Technology, China in 2018 to promote applications of Artemia globally. Climate change and pollution have significant impacts on the harvest yield of cysts and consequently the price (Guong and Hoa, 2012; Santos et al., 2018; Van Stappen et al., 2020). Proper managements of culture conditions in salt work production (especially in Bohai Bay, China and Mekong Delta, Vietnam) should be addressed to stabilize both cyst and salt production, which might encourage a better socio-economic perspective for Artemia farming and their global supply (Manaffar et al., 2020).

COPEPODS

Planktonic copepods are naturally accessible and preferable live feeds for fish or invertebrate larvae in the marine environment and are used as live feeds in aquaculture hatcheries (Drillet et al., 2011; Santhanam et al., 2019; Fernández-Ojeda et al., 2021). Species from the orders Calanoida, Cyclopoida, and Harpacticoida are commonly selected and cultivated for larval feedings. Copepods provide wide windows of prey size (60–1,500 μm) due to their species diversity and 12 developmental stages (six nauplii, five copepodites, and adult). Their jerky swimming pattern attracts a higher predatory response of fish larvae (Burbano et al., 2020). Remarkably, the nutritional advantages (great contents of ω3 HUFA) make these zooplankters favorable for larviculture even without an additional enrichment process (Matsui et al., 2021). In Taiwan and Vietnam, copepods are commonly harvested from outdoor earthen ponds after fertilization (Su et al., 2005; Blanda et al., 2015; GrØnning et al., 2019). Outdoor combined-species cultures might be feasible and cost effective, but the concerns of unstable production, species composition, and risks in pathogenic transmission have hindered the applications of copepods (Chang et al., 2011; Blanda et al., 2017). On the other hand, mono-species indoor copepod cultures of various species were established at either laboratory or intensive scales (**Table 1**), which facilitate copepod biomass of economic feasibility and biosecurity for larviculture industry (Abate et al., 2016; Santhosh et al., 2018). Particularly, the success in "micro-sized" copepod production (i.e., adult < 1 mm and nauplii < 80 μm, such as in *Parvocalanus* sp., *Bestiolina* sp., and *Paracyclopina* sp.) have opened bright avenues for the larviculture of marine ornamental fish (Kline and Laidley, 2015; Callan et al., 2018; Zeng et al., 2018; Dayras et al., 2021; Wang L. et al., 2021), which are considered as challenging but necessary for trade and conservation demands. Instead of maintaining the culture, resting eggs and cryopreservation are alternative approaches to obtain alive copepods (Kaviyarasan and Santhanam, 2019; Pan et al., 2020; Wilson et al., 2021). Although the cold stored production of a specific copepod species (*Acartia tonsa*) seems to be applicable and commercialized, induction and storage protocol of various dormant copepod species and stages

should be further optimized to universally apply their novelties by the industry.

OTHER LIVE FEEDS

Heterotrophic *Schizochytrium* sp., *Halophytophthora* sp., and *Salispina* sp. (**Table 1**) are a group of unicell or filamentous microorganisms containing great amounts of PUFAs (Estudillo-del Castillo et al., 2009; Su et al., 2021). The spray-dried powder of these microorganisms implicates great potential as alternative or supplementary diets to microalgae for the feeding and enrichment of zooplanktonic live feeds (Eryalçın, 2019). Besides holoplankton, some sessile marine organisms could be used as live feeds at their early developmental stages of planktonic life forms. Fertilized eggs and trochophore of bivalves, such as oyster (*Crassostrea* sp.) and blue mussel (*Mytilus* sp.), could be obtained by strip spawning (Scarpa, 2002; Turan and Kling, 2018). They are of a suitable size (40–60 μm) and great ω3 HUFAs contents, thus particularly supportive for the first-feeding of small-mouthed fish such as grouper and other reef species (Liao et al., 2001; Basford et al., 2019). Planktonic barnacle nauplii (100–150 μm) are also considered as potential live feeds (López et al., 2010; Basford et al., 2019). Cladocera species (e.g., *Daphnia* sp., *Moina* sp., and *Ceriodaphnia* sp.) could be cultivated with low cost using aquaculture biofloc technology and fermented animal wastes (da Silva Campos et al., 2020; Rasdi et al., 2020; Turcihan et al., 2022), and serve as live feeds for many freshwater fish larvae such as tilapia (Herawati et al., 2015), catfish (Vu and Huynh, 2020), and ornamentals like Betta fish (Kwon et al., 2013) and freshwater angelfish (Farhadian et al., 2014). Notably, studies have also indicated the feasibility of using water flea as live feed in marine larviculture of fish (Kamrunnahar et al., 2019) and shrimp (Mona et al., 2017). Jellyfish are used as live feed for the phyllosoma larvae of lobster (Palinuridae and Scyllaridae) (Goldstein and Nelson, 2011; Wakabayashi et al., 2012, 2016a), Threadsail filefish (Miyajima et al., 2011), and silver pomfret juveniles (Wang Q. et al., 2021).

DISCUSSION AND FUTURE PERSPECTIVES

Despite their wide applications in marine larviculture, the widely used live feeds (*Brachionus* rotifers and *Artemia*) show several limitations. The diversification and establishment of new live feed culture (especially micro-sized copepod and rotifer species) is promoting research programs and industrial applications. Production of dormant live feed (e.g., copepod resting eggs) is an ongoing program, and this is expected to pave the road for the marine larviculture industry. Future programs should target both indoor and outdoor aquaculture systems using appropriate RAS techniques with artificial intelligence (AI) technology to optimize both prey and larval culture performances. Developing technology and management of both virus free and bacterial free live feed for larviculture. Transferring scientific technology of live feed from academic achievements to stakeholders such as the aquaculture industry and farmers. Both scientists and farmers should work closely together to ensure the upscaling of pilot studies and maintain a required feedback cycle between industrial needs and their declination as scientific research challenges.

AUTHOR CONTRIBUTIONS

Y-JP wrote the first draft of the manuscript. H-UD, J-SH, and SS contributed to manuscript revision with Y-JP. All authors contributed to conception, design of the mini review, and approved the submitted version.

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An Individual-Based Model to Quantify the Effect of Salinity on the Production of *Apocyclops royi* (Cyclopoida, Copepoda)

Mizuki Yoshino[1], *Yen-Ju Pan*[2,3], *Sami Souissi*[4] *and Gaël Dur*[5*]

[1] Department of Geoscience, Faculty of Science, Shizuoka University, Shizuoka, Japan, [2] Department of Aquaculture, National Taiwan Ocean University, Keelung, Taiwan, [3] Center of Excellence for the Oceans, National Taiwan Ocean University, Keelung, Taiwan, [4] Université de Lille, CNRS, Univ. Littoral Côte d'Opale, IRD, UMR 8187, LOG, Laboratoire d'Océanologie et de Géosciences, Station Marine de Wimereux, Lille, France, [5] Creative Science Unit (Geoscience), Faculty of Science, Shizuoka University, Shizuoka, Japan

In this study, an individual-based model (IBM) was established and applied to simulate the effects of salinity variations on the productivity of a promising live feed cyclopoid copepod *Apocyclops royi* for aquaculture applications. The model integrates the effect of salinity on the different reproductive traits and temperature on female longevity. To calibrate the model developed on the Mobidyc platform, we collected data from previous literature and conducted complementary experiments. The model outputs on total nauplii production match the experimental results. Both showed a progressive increase in nauplii production from 0 up to 21 PSU, beyond which the production decreases. There were no significant differences between the estimated nauplii production and the observed ones for most salinity conditions. We then used the model to estimate the egg and nauplii production of a population initiated with 1,000 females along a salinity gradient from 0 to 39 PSU during 20-d cultivation. Around the optimal salinity of 21 PSU, the egg and nauplii production peaked at 1.8×10^5 eggs and 1.39×10^5 nauplii, respectively. A deviation of 7 PSU from the optimal salinity range would lead to a loss of 22 to 25% in egg and nauplii production. The results indicate that implementing the IBM into a life-cycle model provides useful tool for managing the risks of salinity variation on the copepod productivity in aquaculture conditions.

Keywords: copepod culture, production estimation, reproduction, individual-based model (IBM), salinity

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***Correspondence:**
Gaël Dur
dur.gael@shizuoka.ac.jp

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INTRODUCTION

Fishing activities have increased concomitantly with the rapidly increasing human population. However, the natural productivity of marine products is limited, and overfishing has kept on increasing over the years, hovering around 30% of fish stocks overexploited this last decade (FAO, 2020). Aquaculture can solve part of those issues and is an effective way to increase seafood supply (Costello et al., 2020). In aquaculture, fish are fed a variety of food to enable them to live and grow. Those foods depend on the developmental stage of the fish. Employing suitable feed in each

developmental stage enables aquaculture to produce high-quality fish (Das et al., 2012). In early larval stages, it is common to use Rotifer and Artemia as live feed because they are easy to culture, but copepod nauplii are known to be superior live-feed (Støttrup, 2000; Støttrup, 2003; Conceição et al., 2010; Drillet et al., 2011). Copepods are the natural preys of larvae of many marine species and their nutritional profile is superior to artemia and rotifers (Shields et al., 1999; Payne et al., 2001; Rajkumar and Kumaraguru Vasagam, 2006; van der Meeren et al., 2008; Piccinetti et al., 2014). Several species of copepods could biosynthesize and accumulate polyunsaturated fatty acids (Bell et al., 2003; McKinnon et al., 2003; Dayras et al., 2021; Monroig et al., 2022; see Nielsen et al., 2020 for an exhaustive list of species and references therein), which are important nutrients for the development of fish larvae (Mejri et al., 2021). Additionally, the small size exhibited by the copepod larvae stage makes them the recommended live feed for several marine fishes (Lee et al., 2010; Mitsuzawa et al., 2017; Anzeer et al., 2019; Burgess et al., 2020). The swimming behavior of some copepod species could trigger the urge to forage in small-mouthed larval fish (Buskey, 2005; Mahjoub et al., 2011). All these characteristics have led to increasing interest and effort in the mass cultivation of copepod to serve as a live feed.

Copepods are used extensively in the aquaculture industries of some countries, such as Taiwan (Blanda et al., 2017), China (Zeng et al., 2018), Vietnam (Grønning et al., 2019; Nguyen et al., 2020), Australia (Payne et al., 2001; Camus et al., 2021), and others (van der Meeren et al., 2014; Magouz et al., 2021). Outdoor copepod production is a practical industry in Taiwan (Blanda et al., 2015; Blanda et al., 2017). However, one of the bottlenecks in outdoor copepod culture is the difficulty to maintain stable production, as copepod productivities are under the influences of many drivers, such as temperature (Devreker et al., 2009; Devreker et al., 2012; Liu et al., 2015), food quantity and quality (Pan et al., 2014; Rasdi and Qin, 2018; Tordesillas et al., 2018; Dayras et al., 2020; Aganesova, 2021), stocking density (Alajmi and Zeng, 2014), photoperiod (Camus and Zeng, 2008). In addition, the majority of the outdoor copepod cultures are in subtropical and tropical regions, where sudden rainfall and storm often occur and induce salinity variation. Although brackish copepod could survive at a wide salinity range, salinity can significantly affect the reproductive traits of these tiny crustaceans (Devreker et al., 2009; Devreker et al., 2012; Pan et al., 2016; Nguyen et al., 2020). The response to salinity is linked to the osmotic stress that limits the energy available for other basic physiological functions of copepods (Chen et al., 2006), and it could reduce dramatically their production. Environmental monitoring and on-site management (e.g., introduction of seawater or freshwater for salinity adjustment of the pond water) are both crucial for sustaining a stable copepod production. Although the effects of salinity on copepods have been extensively studied, these experimental results are limited to specific reproductive traits among salinity intervals. For aquaculture management, it would be best to have at one disposal a tool that integrates the effects on the different reproductive traits for estimating the global copepod productivity under a salinity gradient.

Focusing on the individual active components of a system, Individual-Based Models (IBMs) have been more and more proving their efficiency in defining optimal conditions for diverse marine organisms including, shellfish (Nunes et al., 2003), Euphrasia (Dorman et al., 2015), zebrafish (Beaudouin et al., 2015). Individual-based models can provide managerial solutions for the production of well-described species and reduce the cost induced by the classical trial and error method. To our knowledge, only one model has been developed for improving copepod cultivation (Drillet and Lombard, 2015). This model is nevertheless not an IBM and focuses on a free-spawner. To our knowledge, there is no IBM that aims to improve egg-bearing copepod culture.

Among the copepods explored for mass culture, calanoid copepods represent the majority (Rasdi and Qin, 2016). However, Cyclopoid copepods have great potential as live feed due to their small size, high tolerance to salinity and temperature variations, and ability to withstand high densities (Lee et al., 2012; Pan et al., 2016; Lee et al., 2017; Jepsen et al., 2021). Recent research efforts have been made to identify the best external conditions leading to optimize cyclopoid mass production (Lee et al., 2013; Nielsen et al., 2021; Dayras et al., 2021). The key bottleneck for this egg-bearing copepod is the fecundity and nauplii production as we cannot harvest their eggs as it is done for free-spawners like *Acartia tonsa* or *A. bilobata* (Drillet et al., 2014; Pan et al., 2021).

Apocyclops royi is a euryhaline cyclopoid copepod cultured commercially as live feed in the Taiwanese aquaculture industry (Su et al., 2005; Ajiboye et al., 2011). This species has a wide range of tolerance of salinity and temperature, facilitating the establishment of mass culture (Lee et al., 2005). *Apocyclops royi* can also survive on a low polyunsaturated fatty acid diet (Blanda et al., 2015; Nielsen et al., 2020), hence reducing the cost of the copepod diet. Like many cyclopoid copepods, *A. royi* reproduce sexually. The male uses its antennules to hold on to the female and transfers spermatophores to the female genital opening. The female could store the sperm and use it to fertilize many clutches from one insemination. The eggs are spawned simultaneously in the egg sac and the same-clutch nauplii hatch almost synchronously (Liu et al., 2007; Marten and Reid, 2007). The durations of interclutch time and embryogenic development are important reproductive traits to estimate copepod overall productivity in aquaculture, and they vary with environmental conditions (Liu et al., 2007; Devreker et al., 2009). Several experiments have investigated the effect of salinity on the reproductive traits of *A. royi* (Lee et al., 2005; Pan et al., 2016; Van Someren Gréve et al., 2020), providing useful information for building an IBM for this species.

In this study, we integrated the different effects of salinity on the reproductive traits of *A. royi* using an IBM developed on the MOBIDYC platform (Ginot et al., 2002) that simulates the reproductive cycle of egg-bearing copepods (Dur et al., 2009). The model description follows the Overview, Design Concepts,

and Details protocol proposed by Grimm et al. (2006; Grimm et al., 2010; Grimm et al., 2020). The experimental results from studies on the effect of salinity on *A. royi* (Pan et al., 2016; Van Someren Gréve et al., 2020) and from complementary experiments on the reproductive cycle conducted for this study were used for model calibration. Following the calibration, we used the individual-based model to quantify the effect of salinity on the production of a cohort of 1000 A. *royi* females and to determine the optimal salinity for reproduction.

MATERIALS AND METHODS

Purpose and Patterns

The model's higher-level purpose was to determine the optimal salinity for the recruitment of the egg-carrying copepod. More specifically, the model was designed to quantify the effect of salinity on reproductive traits and success in a copepod cohort of 1000 individual females. The model was calibrated for the copepod *A. royi*. Based on laboratory observations, we evaluated our model by its ability to reproduce two patterns:

At the individual level: A decrease in interclutch duration with increasing salinity up to a maximum value beyond which the interclutch duration increases, a decrease in mortality with increasing salinity up to a limit; an increase in clutch size with increasing salinity until reaching a threshold beyond which the clutch size decline slowly.

At the cohort level: An overall increase in egg and offspring production until an optimal salinity beyond which the recruitment slows down and even stops.

Entities, State Variables, and Scales

Spatial Scale

The model was not spatially explicit. The agents (i.e. *A. royi* female) were not located in geometrical space.

Temporal Scale

The simulation ran with a time step fixed at three hours and lasted 20 days, which is the maximum life span of an *A. royi* female at 28°C (unpublished data)

Entities

The model included two kinds of entity predefined in Mobidyc: located agents and non-located agents (Ginot et al., 2002).

The two located agents represent 1) an *A. royi* individual of the adult female stage, and 2) an individual of the first naupliar stage. As *A. royi* is an egg carrier, reproduction is relatively complex and involves a multi-phase cycle (Dur et al., 2009). Adult females of *A. royi* have been credited with several state variables, called attributes in Mobidyc (**Table 1**). The nauplii are represented in the model only to confirm the hatching success being taken into consideration. The nauplii of a clutch hatch synchronously and die at the second time step following the hatching after being counted. The state variable of nauplii is, therefore "age" only. While the assumption of synchronous hatching does not reflect the biology of copepod, it does not affect the outcome of the present model. Asynchronous hatching does not affect *A. royi* adult females reproductive process but the nauplii development, which is not considered in the present model. Moreover, according to our observations, *A. royi* nauplii of a given clutch hatch within a couple of hours. The time step of the model is three hours. Hence, the hatching can be considered synchronous.

The non-located agents of the platform Mobidyc were used to design the model and collect required data from the simulation (Ginot et al., 2002). The first three non-located agents were used to store global variables, reducing the number of attributes carried by the located agents (**Table 2**). ParamDev contained the parameter values of the normal distribution used to introduce individual variability on a female's life span. ParamSurv reflected the survival probability of the females and how it varied based on the salinity. ParamRepro stored all parameters of the normal distribution used to introduce individual variability on the clutch size (CS),

TABLE 1 | State variables used to represent the located agent of *Apocyclops royi* adult female and *A. royi* first naupliar stages (N1N3).

Agent	State variables	Definition	Unit	Dynamics/ Statics	Type	Range
A. royi female	life	State the agent is alive (life = 1) or dead (life = 0)	None	Dynamic	Binary	0 or 1
	age	Age accumulated by individual	[days]	Dynamic	Time	0 – 15.5
	agedie	Age when the agent dies	[days]	Dynamic	Time	0 – 15.5
	longevity	Life span of an adult female	[days]	Static	Time	10.755 – 17.814*
	ovigerous	Ovigerous status of the female: ovigerous (ovigerous = 1) or non-ovigerous (ovigerous = 0)	None	Dynamic	Binary	0 or 1
	latencyTime	Required time between hatching and spawning a new clutch	[days]	Dynamic	Time	0.126 - 2.519*
	latence	Time accumulated after the last hatching event	[days]	Dynamic	Time	0 - 2.5193
	EmbryonicDT	Required time between producing spawning and hatching	[days]	Dynamic	Time	0.126 - 5.558*
	devembryo	Time accumulated after the last spawn event	[days]	Dynamic	Time	0 - 5.577
	clutchsize	Numbers of eggs in one clutch	[eggs]	Dynamic	Real number	11.79 - 23.75*
	offspring	Number of successfully hatched nauplii from the clutch.	[Nauplii]	Dynamic	Real number	9.0 - 18.0
A. royi N1N3	age	Age cumulated by an individual	[days]	Dynamic	Time	0 – 0.25

**State variable for which individual variability or interclutch variability was implemented in the model c.f. Submodel section for the details.*

TABLE 2 | Attributes of non-located agents facilitating the computation of the reproductive cycle of the Adult female copepod *Apocyclop royi (ParamX)*, the environment (*Envi*), and the one used to extract and save the output data (*Scientist*).

Agent	Attributes	Definition	Unit	Dynamics/ Statics	Range
Envi	$Time^{sv}$	The simulation time	[days]	Dynamic	0 - 15
	$Temperature^{P}$	Temperature of environment	[°C]	Static	28
	$Salinity^{sv}$	Salinity of environment	[PSU]	Dynamic	0 - 39
	$deadcount^{sv}$	Tell when 24 hours have passed.	[day]	Dynamic	0 - 1
ParmDev	$mdAdf^{P}$	Mean lifespan of *A. royi* adult females at 28°C	[days]	Static	14.457
ParamRepro	$Hatchingsuccess^{P}$	Hatching success of a clutch	[%]	Static	74.5
	$meanLT^{sv}$	Mean latency time between hatching and spawning of a new clutch at a given salinity	[days]	Dynamic	0.1791 - 2.5193*
	$meanEDT^{sv}$	Mean embryonic development time of egg at a given salinity	[days]	Dynamic	1.0097 - 5.5577*
	$meanCS^{sv}$	Mean size of an egg clutch at a given salinity	[eggs]	Dynamic	11.79 - 23.75
ParamSurv	$pSurvAdf^{sv}$	Survival rate of the adult females	[%/day]	Dynamic	1.7488 - 18.4891*
Scientist	lCS^{mo}	List of all clutch sizes produced by an individual agent at each time step	[eggs]	Dynamic	11.79 - 23.75
	lLT^{mo}	List of all latency time experienced by an individual agent at each times step	[days]	Dynamic	0.1791 - 2.5193
	$lEDT^{mo}$	List of all embryonic development time experienced by an individual agent	[days]	Dynamic	1.0097-5.5577
	$lN1N3^{mo}$	List of all nauplii number produced after individual agent hatching event at each time step	[nauplii]	Dynamic	12.0 - 24.0
	$lagedie^{mo}$	List of all ages of death of individual agent at each time step	[days]	Dynamic	0 – 15.5

data obtained through experiments conducted for this study (c.f.* ***Supplementary Material**).*
State variables, constant parameters and model output are identified with the subscripts ^{sv}, ^{P}, and ^{mo} respectively.

embryonic development time (EDT), and latency time (LT). ParamRepro also stored the value of the hatching success (HS), which was set constant at 75% according to our lab observations (reanalyzed data of the results presented in Pan et al., 2016). Two additional non-located agents were used to represent the environment and the experimenter. The environment agent (Envi) refers to the overall environment, including temperature (fixed at 28°C) and salinity. The experimenter agent (Scientist) recorded the age of the female at the time of death and the number of eggs and nauplii produced throughout the simulation.

Process Overview and Scheduling

Summary

1. The non-located agent Envi executes its "GrowOlder" submodel, with increments for its state variable "time" of three hours.
2. The non-located agent ParamDev executes its "mdAdf" which defines the mean lifespan of *A.royi* female under 28°C.
3. The non-located agent ParamRepro executes its "SalEffect" submodel, which computes the value of mean CS, mean EDT, and mean LT in relation to the salinity.
4. The non-located agent ParamSurv executes its "DefProbSurv" submodel, which calculates the survival probability of the adult females, pSurvAdf, in relation to salinity.
5. The adult female agents each execute different tasks simulating the "reproductive cycle". A schematic representation of the reproductive cycle and the flowchart of the submodel scheduling are provided in **Figures 1**, **2**. The located female agents execute their submodels in the following order:

a. "Survive", which defines whether the agent will live or die every 24 hours.

b. "DefImpTime", which defines the *longevity* of the female when the female age is 0 and the *latencyTime* to produce the subsequent clutch every time the female is non-ovigerous and has a *latence* of 0.

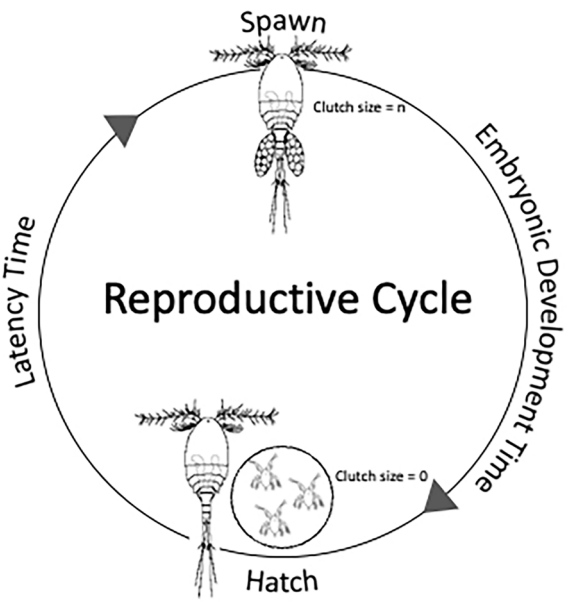

FIGURE 1 | Schematic representation of the reproductive cycle of the cyclopoida copepod *Apocyclops royi*. Females enter this cycle after molting from the last juvenile stage C5 and perform this cycle until their death defined by their survival probability and longevity. The cycle integrates two main processes: the production (spawning) of a clutch of size n (Clutch size = n) and the hatching of the eggs. The time between hatching and spawning is defined as Latency Time, and between hatching and spawning as Embryonic Development Time.

c. "TimeCumul", which increments the female *age*, *latence*, and *devembryo* by one-time step (i.e. three hours).

d. "Spawn", which sets the *ovigerous* status to 1, calculates a new value of clutch size and *embryoDT* and resets the value of

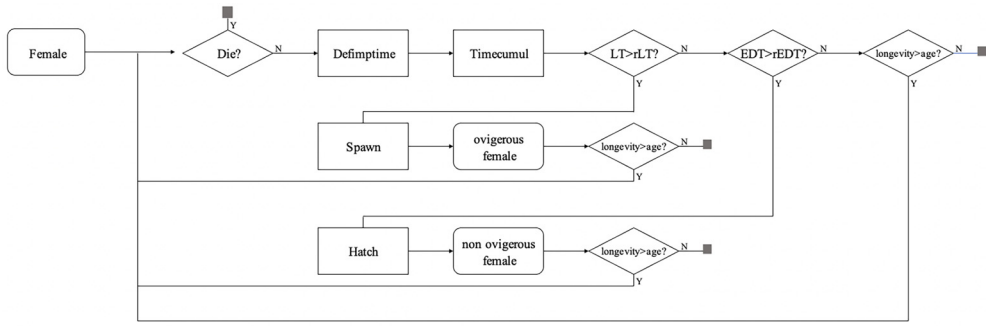

FIGURE 2 | Flow chart representing the scheduling of the model processes used to define the reproductive cycle of the copepod *Apocyclops royi*. The conditional tasks "Spawn" and "Hatch" respectively require the latency time (LT) and Embryonic Development Time (EDT) to reach a threshold value (rLT and rEDT) for their execution. The details on each task are presented in **Table 3**.

latence and *devembryo* when the proper conditions for spawning are met (cf submodel section).

e. "Hatch", which creates the nauplius 1 agents and resets to zero the attributes *ovigerous*, *devembryo*, *latence*, and *clutchsize*.

f. "Die", which kills the individual when *age* exceed the *longevity*

6. The *Naupliar 1* agents only survive until there are counted.

7. The non-located *Scientist* (a) save the value of the clutch size and age of death of the female, and (b) counts the number of nauplii produced and kills them.

Rationale

The Envi is scheduled first because the subsequent *ParamSurv* action depends on salinity. In the female agent, the submodel *DefImpTime* occurs before *TimeCumul* to allow the computation of the required *latencyTime* just after the hatching process (i.e. *Reproduction*) and the *latencyTime* of the first clutch. *EggProduct* has to occur before *Reproduce* to allow the proper condition and timing for hatching. Finally, *Scientist* actions take place at the end of the time step.

Design Concepts

Basic principles – In this model, the salinity affected the survival rate of the female, the latency time, the embryonic development time, and the number of eggs per clutch (clutch size, CS). To determine the effects, we used empirical relationships that are described hereafter (see *Submodel*, below).

Emergence – The key outcomes of the model are the total production and cumulated production of eggs and offspring by a female cohort. All results emerge from the effect of salinity on the survival rate and reproductive traits.

Adaptation – Adaptation is not implemented in the model

Objectives – Objectives measures are not implemented in the model

Learning – Learning is not implemented in the model

Prediction – The model includes no prediction process

Fitness – Individual fitness is determined by a female's survival rate, longevity, clutch size (CS), latency time (LT), embryonic development time (EDT), and hatching success (HS). Although the consequences of variation in the fitness at a higher level can be quantified, the present study did not address this question.

Sensing – The state variable "ovigerous" defines the proper time to perform appropriate reproductive tasks, e.g. spawning and hatching.

Interactions – Interactions between individuals were not introduced in the present model. We assumed that the time required for a male (not represented in the model) to anchor its spermatophore to a female is included in the latency time. Neither the limitation due to mating nor the failure of egg fertilization was considered here.

Stochasticity – Survival was interpreted as a random variable; a survival rate of 95% means an individual will die only if the generated random number belonging to a uniform probability density between [0,1] is larger than 0.95. Stochasticity is used to assign each individual female a life span (see the *Submodel* section). The LT, EDT, and CS are also stochastic as their values were drawn randomly from predefined distributions (see the *Submodel* section) after the hatching process (for LT) and the spawning process (for EDT and CS) of each clutch produced.

Collectives – The model includes no collectives.

Observation – The non-located *Scientist* (a) save the value of the clutch size and age of death of the female, and (b) counts the number of nauplii produced and kills them. This procedure allowed total egg and offspring production to be described, but also investigate the distribution of different reproductive traits (cf *Emergence*).

Initialization

We first confirm the proper implementation of the model by running a set of simulations with conditions similar to those of the experiment conducted by Pan et al. (2016), i.e., 8 females, 8 salinities (0 to 35), four replicates, 14 days. We then tested 40 salinity conditions, with salinity ranging from 0 to 39. For this second set of simulations, at initialization, the female cohort consisted of 1000 newly molted adult females (i.e. *age* = 0), and the simulations last 20 days. This number of individuals was selected after visualization of the stabilization of the variability of the output variables (**Supplementary Figure 1**). For each condition, the simulated experiments were repeated 15 times (15 replicates) to consider the output variability produced by the same set of parameters and initial conditions (**Supplementary Figure 2**).

Input

The temperature was fixed at 28°C, and salinity values were constant for each simulation experiment. The model did not use input data to represent time-varying processes.

Submodels

Reproductive Cycle of a Female

To simulate the reproductive cycle of the females, we slightly adapt the model of Dur et al. (2009) to the case of *A. royi*. Compared to the model of Dur et al. (2009), the individual variability was not represented by a gamma distribution but by a Gaussian distribution. We, therefore, modified the tasks *DefImpTime* and *Spawn*. **Table 3** presents the different tasks used to simulate the reproductive cycle of *A. royi* and their respective pseudocode. For the rationales behind these tasks, we report the reader to the paper of Dur et al. (2009).

TABLE 3 | Settings of the tasks used to develop the reproductive cycle of an adult female of the copepod *Apocyclops royi*.

Agent task	Type	Setting
Survive	Mathscript	IfTrue [(my_life = 1) and (envi_deadcount > = 1)] rand = randomReal (0, 1) state = ParamSurv_pSurvAdf/100 - rand endIfTrue IfTrue state < 0 my_life = 0 (The agent is dead) endIfTrue
DefimpTime	Define task	IfTrue (my_life = 1) IfTrue (my_age = 0) my_longevity = randomNormal (ParamDev_mdAdf, 1, 5) my_latencyTime = randomNormal (ParamRepro_meanLT,1,5) endIfTrue IfTrue [(my_ovigerous = 0) and (my_latence = 0)] my_latencyTime = randomNormal (paramRepro_meanLT,1,5) endIfTrue
TimeCumul	Mathscript	my_age = Simulator_timestep + my_age my_devembryo = Simulator_timestep + my_devembryo my_latence= Simulator_timestep + my_latence
Spawn	Mathscript	IfTrue [(my_life = 1) and (my_latence > = my_latencyTime)] my_ovigerous = 1 my_clutchsize = randomGamma (ParamRepro_alCS, ParamRepro_btCS) my_offspring = (ParamRepro_hatchingsuccess/100) × my_clutchsize my_offspring = round (my_offspring) my_devembryo = 0 my_latence = 0 my_latenceTime = 999 my_EmbryoDT = randomNormal (ParamRepro_meanEDT,1,5) endIfTrue
Hatch	Predefine	If (my_devenbryo >=my_EmbryoDT) and (my_ovigerous=1) and (my_life=1)) Then my_offspring will reproduce in nauplii. my_ovigerous = 0 my_EmbryoDT=999 my_devembryo=0 my_latence=0 my_clutcsize=0 my_offspring=0
Die	Math script	IfTrue [(my_life = 1) and (my_age > = my_longevity)] The agent is dead

Effect of Salinity on *A. royi* Reproductive Traits

To implement the effect of salinity on *A. royi* reproductive traits into the model, we collected information from published data (Pan et al., 2016; Van Someren Gréve et al., 2020) and conducted some experiments for the traits with no available data (i.e., EDT). Details on the experimental procedures and the results used for calibration are presented in supplementary materials (**Supplementary Text 1**). We subsequently proceeded to the calibration by testing different functions types to express the effect of salinity on those reproductive traits.

Latency Time and Embryonic Development Time.

Preliminary analysis of the experimental results revealed a pattern similar to a temperature-dependent development, i.e. LT and EDT decreased with increasing salinity. Consequently, we tested different commonly used equations to define the effect of temperature on the development of arthropods (Quinn, 2017 – **Supplementary Table 1**). Among the five function types tested, that of Yamakawa and Matsuda (1997) exhibited the best fit for both LT and EDT (**Figure 3**).

The effect of salinity (S) on the mean latency time (meanLT, in days) was expressed as:

$$meanLT = 2704(S + 1.254)^{-1.04}(44.81 - S)^{-1.98}$$

and the effect of salinity (S) on the mean embryonic development time (meanEDT, in days) as:

$$meanEDT = 5342(S + 1.973)^{-1.04}(50 - S)^{-1.575}$$

Both mean values were computed by the non-located agent ParamRepro and stored into its attribute meanLT and meanEDT (**Table 2**).

Clutch Size

For the effect of salinity on the clutch size (CS), we slightly modified the mathematical expression of Parker (1974) to fit the experimental data of Pan et al. (2016). The experimental CS data of Pan et al., 2016 revealed that within the viability range of *A. royi* there is an optimal salinity for the clutch size. And the equation of Parker is one of the many equations proposed to reproduce the effect of an optimal temperature on different process. We replaced the temperature variables in Parker equation by salinity variables, and added a constant value (c):

$$\begin{cases} CS = \left(\frac{S}{S_o}\mathcal{X}^u\right)^{\theta} + c \\ \mathcal{X} = \frac{S_m - S}{S_m - S_o} \\ u = \frac{S_m - S_o}{S_o} \end{cases}$$

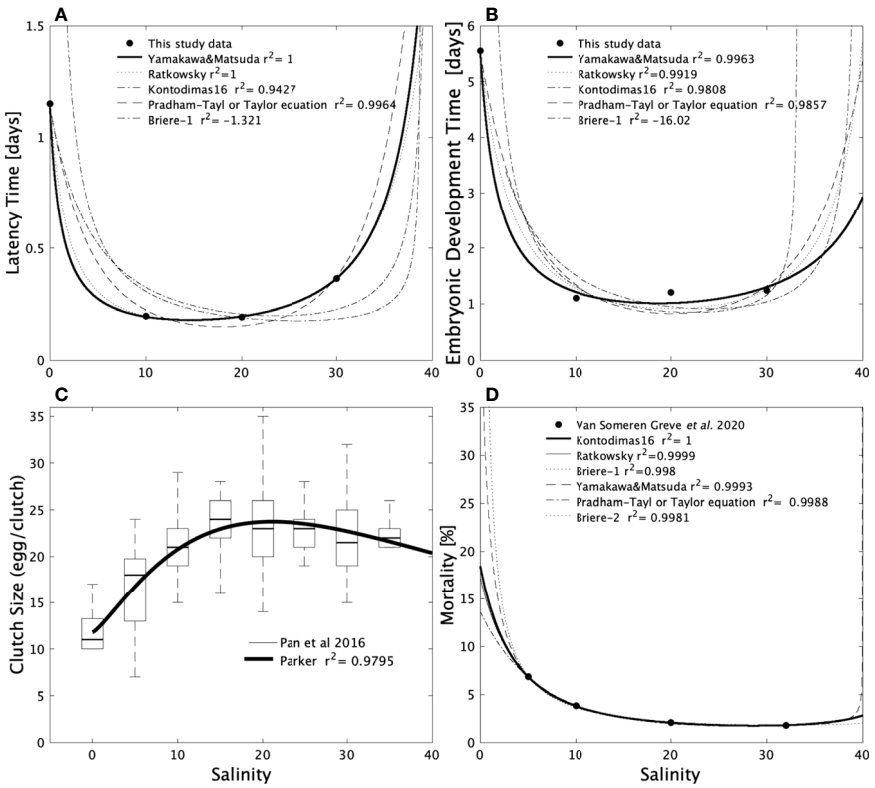

FIGURE 3 | Relationship between salinity and the Latency Time **(A)** Embryonic Development Time **(B)**, the Clutch Size **(C)**, and the mortality **(D)**. The black continuous lines represent the function implemented in the model to represent the effect of salinity. Details on the functions plotted here are provided in **Supplementary Table 1**.

where S_m: is the upper limit of salinity beyond which the female does not produce egg ($CS(S_m) = 0$), S_o is the optimal salinity for spawning ($CS\ (S_o)$ = CSmax, and θ is a parameter of the function shape.

With such an equation, we obtained a good fit on the experimental data of Pan et al., 2016 (**Figure 3C**).

Survival

Among the six tested equations, that of Kontodimas-16 provided the "best" fit to represent the effect of salinity (S) on mortality data obtained in the lab (c.f. **Supplementary Material**). This function is characterized by a min and a max value that can be associated with the range of tolerance of *A. royi* (**Table S1**; **Figure 3D**).

$$Mortality(\%/day) = \frac{1}{2.592 \times 10^{-5}\,(S+6.714)^2(46.29-S)}$$

In the model, we used survival rate instead of mortality. Hence the probability for a female to survive one day of simulation at salinity S was 100 – Mortality.

The survival (or death) of the female was tested every day (i.e. when Envi_deadcount ≥1). For a high value of survival (e.g. ParamSurv_Adf = 0.95), the probability of drawing a random number larger than ParamSurv_Adf is low; consequently, the individual has a larger probability of survival than dying every day.

Adult Female Longevity

In the present model, we considered the mean longevity of females to be independent of salinity as shown by Lee et al. (2005). The simulations were conducted for a constant temperature of 28°C in accordance with the temperature used for the experiment (Text S1). To define the mean longevity of *A. royi* female at 28°C, we first fit five common equations used to describe the effect of temperature on development on the data obtained by Lee et al. (2005). The best fit was obtained with the Bělehraídek equation (**Supplementary Figure 3**) which gave mean longevity (ParamDev_mdAdf) of 14.457 days at 28°C. Individuals with 100% daily survival rate reach their maximum age (longevity) defined at the first timestep of the simulation by DefImptime (**Table 3**).

Individual and Interclutch Variabilities

Individual variability was considered for the female longevity and was modeled with a normal density function of variance equal to one and the mean value of ParamDev_mdAdf. The task DefImpTime was affected by this task.

DefImpTime was also used to implement interclutch variability on the latency time. Similar to the longevity, the latency time for each clutch was randomly sorted from a gaussian distribution with a variance equal to one and a mean value of ParamRepro_meanLT.

The task Spawn was used to take into consideration the interclutch variability in EDT and CS (**Table 3**). The number of eggs produced by the female at each spawning event is randomly selected in a gamma distribution defined by a shape parameter (α) and the scale parameter (β). The values of were determined using the method described in Souissi (2006) to separate the contribution of salinity from the intrinsic variability (α kept constant and β multiplied by ParamRepro_meanCS which depends on salinity). The number of offspring that will hatch from each clutch represents a part of the randomly sorted CS (i.e. HS x CS). The EDT required for every single clutch was also defined by the task Spawn, and sorted from a gaussian distribution of variance equal to one and mean value equal to ParamRepro_meanEDT.

Statistical Analysis

To confirm the proper implementation of the model, we compared the results obtained by Pan et al. (2016) with the model outputs for the same conditions. The Kolmogorov-Smirnov test confirmed the heteroscedasticity of the data. We, therefore, used the Wilcoxon rank-sum test (aka Wilcoxon T-test, WT) to compare the model results to the experimental results for each tested condition. All statistical tests were conducted at the 95% confidence level. These analyses were performed using the Matlab software (Mathworks Inc., Version, 7.5)

RESULTS

Model Results Compared With Experimental Data

The total nauplii production estimated with the model and experimentally exhibit a similar response to increasing salinity (**Figure 4**), with an increase in production up to salinity 20 and a decrease beyond. The model results were not significantly different from the experimental results for salinities 10 to 25 (WT, $p>0.05$). However, the model outputs were significantly higher for salinities 5, 30, and 35 (WT, $p<0.05$). At salinity 0, the simulated total nauplii production was significantly lower than the one observed experimentally (WT, $p<0.05$).

Effect of Salinity on Individual Endpoints

The numerical outputs provided a dynamical explanation for the effect of salinity on the total production of *A. royi* (**Figure 5**). Due to the implemented effect of salinity on survival, female longevity was strongly affected by low salinities, decreasing below 10 days for salinities lower than 7. Consequently, the number of clutches a female can produce during its lifetime was reduced at low salinities. The number of clutches increased with increasing salinities, reaching a maximum of about 8 clutches between salinities 15 and 25, and steadily decreasing for higher salinities. This decrease observed at higher salinity was not related to female longevity which stayed relatively constant. Instead, the decrease in clutch number at higher salinity is related to the effect of salinity on the LT and EDT, which became longer when the salinity exceeds 10 for LT and 20 for EDT (**Figure 3**). The daily egg production, fecundity, and total offspring followed a trend similar to that of clutch number.

Analysis of the distribution of parameter values "confirmed" the importance of female longevity (**Figure 6**). For all parameters besides clutch size, the distribution shifted toward zero as the salinity tends to 0. The span of the distribution of clutch number expanded when salinity is between 8 and 32. Conversely, the span of the clutch size distribution stayed constant for salinities

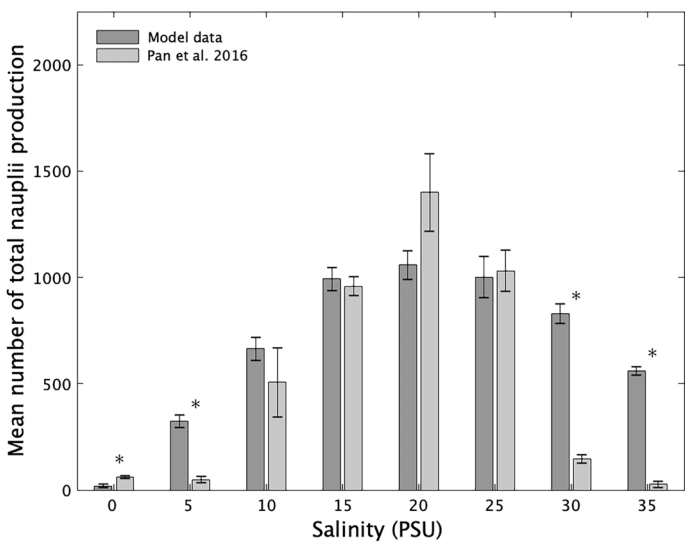

FIGURE 4 | Comparison of experimental (in grey) and simulation (dark grey) results of the total number of nauplii produced by 8 females of *A. royi* at eight salinities. Vertical bars represent the mean values over four replicates and the error bars represent the standard error. Asterisks indicate a significant difference between experimental and simulation results.

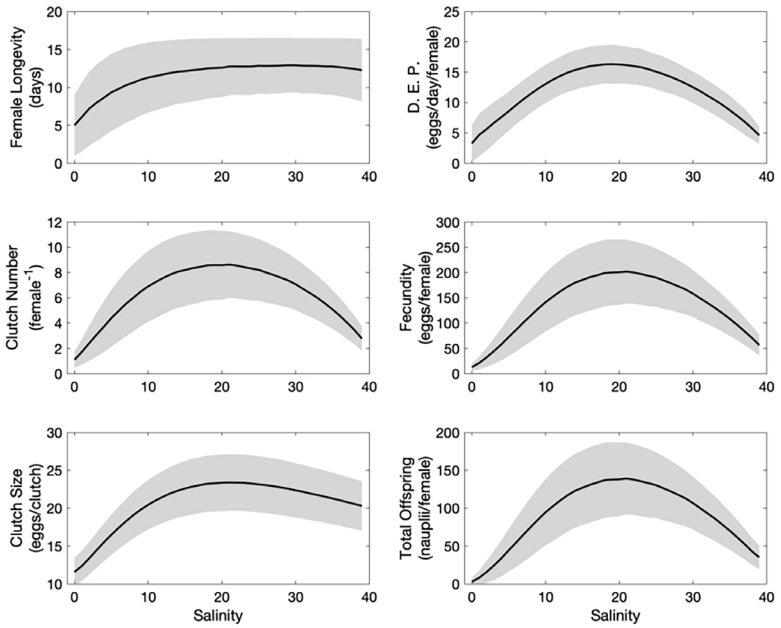

FIGURE 5 | Average (black line) and standard deviation (gray area) of different reproductive traits (Left: female longevity, clutch number, clutch size) and the resulting individual production (right: daily egg production, total egg production, total offspring production) for 15,000 females (15 runs of 1000) exposed to different salinities.

higher than 8. The fecundity and total offspring distributions center slid slightly toward the higher class, with a tail toward the lower classes as salinity increased from 0 to 20 and exhibited a reverse pattern for salinities higher than 24.

Salinity Effect on Cohort Endpoints

At the cohort level, the model results showed the importance of the interclutch duration on the production (**Figure 7**). At low and high salinities, the time between the burst of production is quite long resulting in few eggs and nauplii production. Females can only produce two and three clutches at salinity 0 and 39, respectively. At salinities 3 and 39, the cumulated production of both eggs and nauplii is quite similar, but the dynamics are different. At salinity 3, two peaks of production appeared within the first 6 days of simulation, and they are followed by relatively low but constant production. As salinity increased, so did the regularity of production, leading to quasi-linear increases in the total production for salinities between 7 to 31. The dynamics of the egg and nauplii production were quite similar for salinities between 15 to 23.

At the end of the 15 days of simulation, the highest total egg and nauplii productions were obtained at 21 PSU (1.85×10^5eggs,1.39×10^5 nauplii) and the lowest at 0 PSU (1.11×10^4 eggs, 8.23×10^3 nauplii) (**Figure 8**). The total egg and nauplii production decreased the further it goes out of the optimal range, which is related to the individual behavior (**Figure 5**). Between 19 and 22 PSU, the egg cumulated production exceeded $1.8\text{x}10^5$. And for salinities between 18 and 23, nauplii cumulated production exceeded $1.35\text{x}10^5$. Between 7 and 31 PSU, the egg and nauplii production is growing steadily, but outside this range, it is unstable and not increasing uniformly (**Figure 7** bottom graphs). The difference between egg production and nauplii production is related to the constant hatching success (74.5%, data from Pan et al., 2016).

DISCUSSION

Copepod is a great live feed for aquaculture (Shields et al., 1999; Støttrup, 2000; Payne et al., 2001; Rajkumar and Kumaraguru Vasagam, 2006; van der Meeren et al., 2008; Piccinetti et al., 2014), but its application has yet to reach the practicality and cost-effectiveness required for the global aquaculture hatcheries due to unstable productivity. The present study developed a modeling tool to identify the risk of salinity variations on the mass culture of the cyclopoid copepod *Apocyclops royi*. Here, we focused on the effects on reproduction by first defining dose-response relationships based on the results of laboratory experiments on females. We then implemented those relationships in an IBM representing the reproductive cycle of egg-carrying copepods (Dur et al., 2009). The model represents a new approach to define the optimal salinity to induce maximal fecundity of *A. royi* and provides a mechanistic understanding of the effect of salinity on production.

Production of *A. royi*

Salinity influences the ecological and biological response of copepod and has been investigated extensively during the past

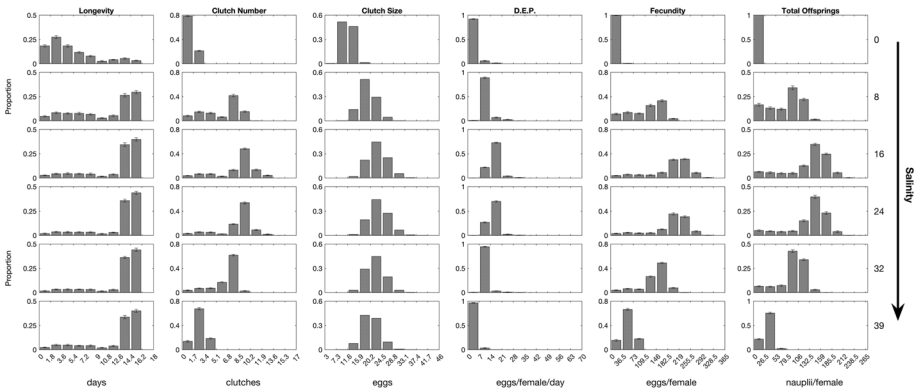

FIGURE 6 | Probability density function of the individual endpoints for 1000 females exposed to different salinities. The histogram represents the average proportion per class over the 15 replicate simulations and the error bars represent the standard deviation.

decades. Chen et al. (2006) determined the effect of salinity of *Pseudodiaptomus pelagicus*, for 10 days with individual adult pairs to define the optimal salinity culture. In terms of total nauplii production, under 15g/L observed the highest value (131.2 ± 67.6). For *Acartia* species, egg production patterns were more irregular, but some species produced fewer eggs at the lowest salinity tested (Castro-Longoria, 2003). Castro-Longoria (2003) demonstrated that hatching success increased with increasing salinity and was maximal at 25 PSU of *Acartia tonsa* (Copepoda: Calanoida). In this study, the model output (**Figure 7**) suggested an optimal salinity for *A. royi* reproduction at 21 PSU and 0 PSU as the adverse salinity condition. Pan et al. (2016) conducted a population experiment to find the optimal salinity with eight different salinity conditions (0, 5, 10, 15, 20, 25, 30, and 35) with the same species. In their result, the highest mean number of the total populations was observed at 20 PSU and low at extreme salinity treatments (0,5,30, and 35). In terms of optimal and suboptimal conditions, these two results matched. However, the mean number of total populations cultivated at salinities higher than 30 was low in the experiment compared to our model results in terms of offspring production. This discrepancy may be due to the female mortality rate implemented in the model. The mortality data were obtained from Van Someren Gréve et al. (2020). Before testing the 24hour mortality of adult female copepod *A. royi*, Van Someren Gréve et al. (2020) acclimated the copepods to 32 PSU for at least 111 days. Because we used data of copepods that were adapted to high salinity conditions, the model results did not show a strong decrease in productivity at high salinity.

The negative effect of high salinity on female longevity and the clutch size is not as strong as for lower salinity conditions (**Figure 4**). The decrease in total production at salinities higher than 23 is however similar to that at lower salinities. Under high salinity conditions, the decrease in production is mainly due to extended interclutch duration. High salinity negatively affects LT and EDT (**Figure 3**). The lengthened LT and EDT lead to fewer spawning events, and a subsequent decrease in production. These results support the claim that, for egg-carrying copepods, it is important to take into consideration the effect of environmental factors on the time component to avoid misleading egg production rate estimation (Lloyd, 2006; Dur et al., 2009).

In order to achieve the goal of producing a large number of eggs, we documented that the salinity should be carefully maintained in a given range of salinities. A high production of ~18.10^4 eggs was predicted with the model for salinities between 18 and 23. Except in the rare case of equipment failure, salinity can be easily maintained in batch culture within indoor facilities. But outdoor cultures can exhibit significant salinity fluctuations (i.e., 8-15PSU, Blanda et al., 2015) and possibly affect considerably the production of the culture species. According to our model, a deviation of 7 PSU from the optimal range of salinity would lead to a loss of 22 to 25% in *A. royi*. This finding provides implications for risk control in copepod aquaculture, where the water exchange or management should be necessarily performed to avoid production loss.

Model Potential and Current Limitations

This version of the model allows already some valuable applications for aquaculture. In the case of outdoor ponds, the model can help to estimate the production in case of sudden rainfall and improve harvest management. In the case of indoor culture, the model can be used as a tool for salinity management to increase production. Nevertheless, several limitations of the current version should be highlighted.

Although several studies have investigated the effect of salinity on the reproductive capacity of copepods, none has provided functional relationship between environmental salinity and embryonic development time or latency time. The effect of salinity on the survival rate of the calanoid copepod *Pseudodiaptomus annandalei* was expressed by a two-order polynomial fit (Chen et al., 2006). Contrarily to *P. annandalei*, *A. royi* did not exhibit a decrease in survival at salinity 0. We therefore tested different equations to fit our data. There is no clear consensus as to what is the "best' type of function to apply

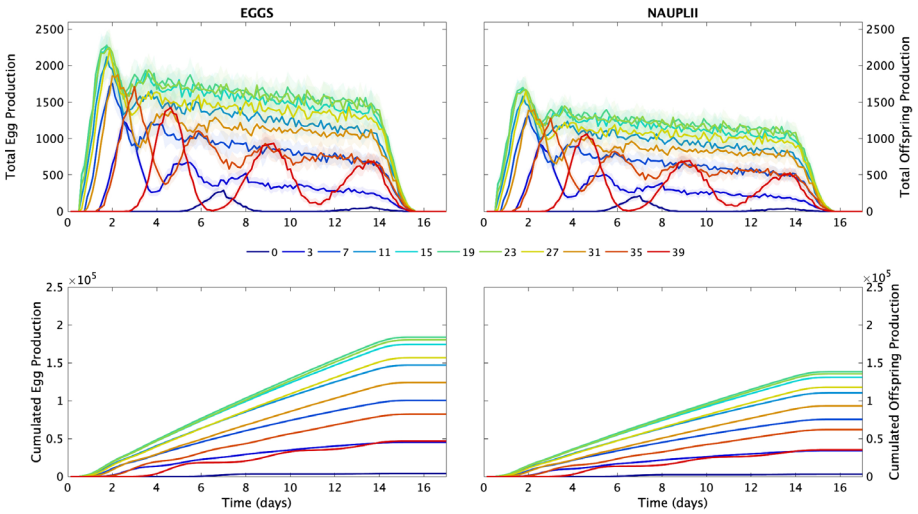

FIGURE 7 | Total production (Top) and cumulative production (bottom) of eggs (left) and offspring (right) by 1000 females over 20 days at different salinity conditions (PSU). All individuals died before the end of the simulation. The x-axis was therefore limited to 17 days. Lines represent the average over the 15 replicate simulations. The shaded area is the standard deviation.

to salinity response data. Here, we tested temperature-dependent equations to fit our data as they presented similar types of response, i.e. faster development and lower mortality at optimal condition and longer development and higher mortality when the environmental condition goes further than this optimal. All equations commonly used to represent temperature-dependent relationship applied well to our study by fitting the relationship between salinity and the reproductive traits.

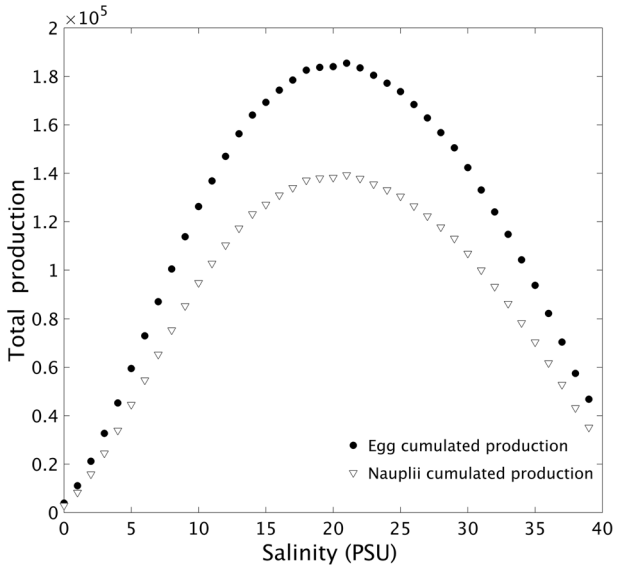

FIGURE 8 | Relation between the cumulated mean production of eggs and nauplii by 1000 females after 20 days and the salinity condition (PSU).

Euryhaline copepod has the ability to adapt relatively quickly to change in salinity after some acclimatation period (Hansen et al., 2022). This ability might result in changes in shape and position of the functional relationship with salinity after acclimation. In the present model, we did not consider any acclimation period and only focussed on the productivity of females at constant salinity. We therefore did not implement any adaptative process in the model. However, with appropriate data such implementation should be conducted for future version of the model.

Individual variability is important for population persistence and stability under stressful conditions (Morozov et al., 2013; Bi and Liu, 2017). In the present model, the individual variability in female longevity and interclutch variability in LT and EDT are represented by normal distributions, the parameters of which were constant. However, previous observations revealed an increase of the individual variability in the developmental duration of copepod in relation to non-optimal temperature (Souissi et al., 1997; Jiménez-Melero et al., 2005). The calibration of gamma distributions to represent individual variability is possible (Dur et al., 2009; Dur et al., 2013) but requires an important number of observations at the individual scale (Devreker et al., 2012; Liu et al., 2015).

Several studies showed that salinity affects the HS brackish copepods whether they carry their eggs (Nguyen et al., 2020) or release them (Holste and Peck, 2006; Choi et al., 2021). However, this relation between salinity and hatching success seems to be species-specific. For instance, for the egg-bearing copepod *Eurytemora affinis*, there was no effect of salinity on the hatching success which is always above 90% (Devreker et al., 2012). In the present model, we considered a constant HS of 74.5% based on the observation in the laboratory (data from Pan et al., 2016) that showed no effect of salinity on HS. While

the eggs are tolerant to salinity changes, the nauplius stages could respond differently to salinity stressors. When exposed to adverse salinities, the nauplii suffer from retarded development, molting failure and even decreasing survival rate (Devreker et al., 2004). Besides the effect of salinity, nauplii might also be subject to cannibalism. Drillet and Lombard (2015) build a simple development model based on the well-known life-history traits of *Acartia tonsa* to define the optimal density condition with the effect of cannibalism in production. In the present study, cannibalism in *A. royi* is not considered as the model focuses only on reproduction. Considering the importance of the naupliar stages for population maintenance (Nagaraj, 1988), the effect of salinity and other factors, such as cannibalism, on nauplii life traits should be considered in the future development of the model which will simulate the entire life cycle of *A. royi*.

In the present model, some individuals died before their eggs hatched (i.e., they spawn but died before the EDT was completed). Other egg-bearing copepods, such as *Eurytemora carolleeae* could drop the egg sacs and allow the nauplii to be born independently (Nowakowski and Sługocki, 2021). In this study, the model is based on the hypothesis that *A. royi*'s egg sac does not detach and cannot survive if the female dies. However, if *A. royi* exhibit abilities similar to that of *E. carolleeae*, the productive capacity of *A. royi* would be even more than the present estimation.

Our model simulated the case of 1000 females, which if it is considered as 1000 ind/L can represent a high density. The present model did not consider any crowding effect. There are some contradictory results concerning the effect of high density on copepods. In *Acartia tonsa*, high-density can have a negative effect on adult survival, and fecundity (Drillet et al., 2014; Franco et al., 2017). However, the same species did not show any stress in terms of behavior, respiration, and gene expression when exposed to high culture densities (Nilsson et al., 2017). Similarly, the copepod *Acartia steueri* mortality and fecundity are independent of density due to a reallocation of its energy from the metabolic to the reproductive process (Takayama et al., 2020). Further research about the response of *A. royi* to high densities is necessary to decide the need for implementing any effect of high-density on its reproductive traits.

In outdoor culture, the temperature is another important parameter that exhibits some variation (Blanda et al., 2017) and influences considerably the production of egg-carrying copepods (Dur et al., 2009; Nguyen et al., 2020). The present model does consider only the effect of temperature variation on longevity. In the present model, the effect of temperature on LT, EDT and CS is not considered despite its relevance because of a lack of data. However, with proper laboratory data, this effect can easily be implemented in the model (Dur et al., 2009). Additionally, in practice, the model can investigate the effect of temperature change on production. For example, we can consider the hypothetical case where the salinity is maintained at 21, which is theoretically the optimal condition (**Figure 7**), and estimate the production at the minimum and maximum temperature observed in outdoor aquaculture pond in Taiwan (26.1, and 32°C, Blanda et al., 2017). The total egg production increased by 4.27% at 26.1°C and decreased by 10.1% at 32°C compared to 28°C. In the case of total nauplii production, the model predicted an increase of 9.97% at 26.1°C and a decrease of 10.1% at 32°C (**Figure 9**). One should note that this result does not consider the effect of temperature on other reproductive processes, such as clutch size and interclutch variability. The next improvement of the current model must focus on this aspect, considering the increase in the appearance of extreme temperatures due to global warming. It will be interesting to integer both the negative effect of high temperature on fecundity and nauplii production (Nguyen et al., 2020) and the positive effect of cold temperature on productivity (Pan et al., 2017).

Although the Mobidyc platform was developed two decades ago (Ginot et al., 2002), it contributed to promoting and democratizing multi-agent systems to con-computer experts. Its development has been recently accelerated with the new RE : Mobidyc project (Oda et al., 2021). The recent development in collaboration with computer scientists will lead to a future version accessible to a wide range of users including the aquaculture field.

CONCLUSION

Compared to experimental investigation, our model presents the ability to integrate the effect of different environmental parameters on all reproductive traits as well as individual variability. On top of that, the model has the potential to aggregate fine-scale knowledge and investigate coarser-scale phenomena. Based on the present version of the model, other environmental factors such as temperature, food quality, and quantity could potentially be incorporated pending the availability of data.

In this study, we only focused on the reproductive traits of adult females of copepod. The goal of this model is to determine the optimal condition for reproduction and estimate the loss and gain of change in salinity. Ultimately, we are aiming to create a model that represents the entire life cycle of *A. royi*. The reproductive process constitutes a necessary step in the development of a complete population dynamics model (Prestidge, 1995; Souissi et al., 2005). In addition to the previously proposed improvements, the perspective of the model is to be included within an already developed population dynamics model integrating all development stages (Dur et al., 2013). This population dynamics model will be an important tool for establishing and maintaining copepod mass culture through the determination of optimal culture conditions for growth and reproduction, estimation of the production, and defining appropriate harvest time.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

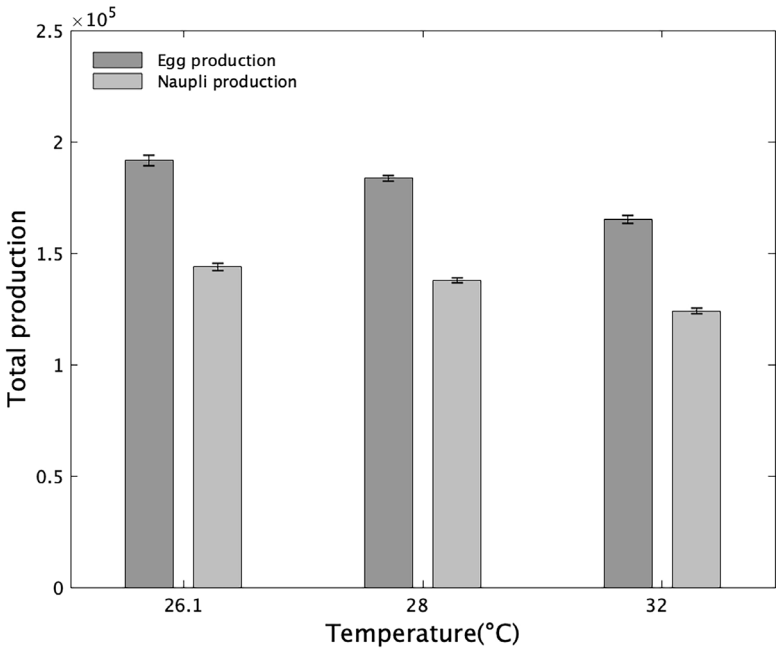

FIGURE 9 | Cumulated total egg and nauplii production of 1000 females over a period of 20 days at three constant temperatures. Salinity was fixed at 21 PSU. Vertical bars represent the mean values over fifteen replicates and the error bars represent the standard error.

AUTHOR CONTRIBUTIONS

MY conducted the model calibration, run the model and the output analyses, and draft the manuscript. Y-JP conceived and run the complementary experiments, commented on the manuscript. GD conceived the model and the complementary experiments, helped in writing the manuscript. SS contributed to writing the manuscript, commented earlier version of the manuscript. All authors contributed to the article and approve 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.2022.863244/full#supplementary-material

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EDITED BY
Yen-Ju Pan,
National Taiwan Ocean University, Taiwan

REVIEWED BY
Rafael Campos-Ramos,
Centro de Investigación Biológica del Noroeste (CIBNOR), Mexico
Paulo Gavaia,
University of Algarve, Portugal

*CORRESPONDENCE
Tu A. Vo
tu.a.vo@ntnu.no

†PRESENT ADDRESS
Trina F. Galloway,
AquaGen AS, Trondheim, Norway

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Effect of diet on molecular relationships between Atlantic cod larval muscle growth dynamics, metabolism, and antioxidant defense system

Tu A. Vo[1]*, Trina F. Galloway[2†], Augustine Arukwe[1], Rolf B. Edvardsen[3], Kristin Hamre[3], Ørjan Karlsen[3], Ivar Rønnestad[4] and Elin Kjørsvik[1]

[1]Department of Biology, Norwegian University of Science and Technology, Trondheim, Norway, [2]Department of Marine Resources Technology, SINTEF Fisheries and Aquaculture, Trondheim, Norway, [3]Feed and Nutrition, Institute of Marine Research, Bergen, Norway, [4]Department of Biological Science, University of Bergen, Bergen, Norway

We studied molecular effects (RNAseq and qPCR) of first feeding prey types (copepods or rotifers/*Artemia*) on skeletal muscle myogenesis and growth dynamics (proliferation, differentiation), metabolism (glycolysis, gluconeogenesis, oxidative phosphorylation), and antioxidant defense system (production/regulation of reactive oxygen species (ROS) in cod (*Gadus morhua*) larval skeletal muscle. Larval somatic growth rates were significantly higher in copepod fed larvae, although shifts in gene expressions related to muscle growth dynamics between hypertrophy and hyperplasia and generation and regulation of ROS mostly occurred around 5-, 10-, and 15-mm standard length (SL) for both groups. Gene expression for cell membrane proteins (such as *nox1* and *igf1r*) peaked at 7 mm SL in all larvae, corresponding with increased ROS expressions in cod muscle during the exponential stratified hyperplasia phase from 7 mm SL. Expression for muscle differentiation (*mef2a*) occurred continuously (strongest from 10 mm SL). Expressions for muscle proliferation (*pcna*) and hydrogen peroxide (H_2O_2) generation (*sod1* and *sod2*) occurred in the 5 - 15 mm SL range, peaking at 10 mm SL in all larvae. A downregulation of *sod1* and *sod2* in skeletal muscle from 15 mm SL indicated the first response of the defense antioxidant system. Gene expressions related to glucose metabolism (*slc2A11*, *pfk*, *fpb2*, *ldha*) was 3 - 10 times higher in copepod-fed larvae than in rotifer/*Artemia*-fed larvae between 7 – 10 mm (live prey period). Copepods move faster than rotifers, and cod larvae will also gradually increase their active swimming periods, due to less viscous forces. Active swimming during the strongest muscle stratified hyperplasia phase (7 – 10 mm SL) could promote a better delivery and transport across the muscle membrane and intracellular flux through glycolysis and oxidative phosphorylation and would contribute to the observed earlier and more effective glucose metabolism in the larvae fed copepods. We suggest that active swimming is an important factor promoting cod larval muscle growth, especially during the strongest muscle hyperplasia phase between 7 and 10 mm

SL. The rapid movements of copepods and better nutritional composition could play important roles in stabilizing ROS levels, promoting high swimming activities and enhancing long-term muscle growth in cod.

KEYWORDS

Atlantic cod larvae, larval nutrition, muscle hypertrophy and hyperplasia, larval metabolism, antioxidant defense system, muscle growth and differentiation, *Gadus morhua* (L.), larval growth

Introduction

Marine pelagic fish larvae such as the Atlantic cod (*Gadus morhua*) larvae hatch at a small size (a few mm standard length; SL) and with a rather immature developmental stage, and they need to develop the most important functions for survival before the small yolk-sac is exhausted (Blaxter, 1988). The larval feeding success is dependent on their functional anatomical and physiological development, and they need to grow fast to obtain a wider selection of prey and to avoid predation (Lasker, 1981).

Zooplankton such as copepods are generally superior to rotifers and *Artemia* as prey for pelagic marine fish larvae (Shields et al., 1999; Støttrup, 2000; Evjemo et al., 2003; Boglione et al., 2013; Hamre et al., 2013; Rønnestad et al., 2013). Rotifers and *Artemia* are the dominant live prey cultivated for feeding of fish larvae, and they are relatively easy to cultivate in large scale production, in contrast to copepods. Enriched rotifers and *Artemia* are not nutritionally optimal for cod larval development, and significantly better growth and development is obtained by using zooplankton/copepods during the whole live-feed period (Kvenseth and Øiestad, 1984; Kjørsvik et al., 1991; Imsland et al., 2006; Van der Meeren et al., 2008; Busch et al., 2010; Koedjik et al., 2010; Hamre et al., 2013; Mæhre et al., 2013; Karlsen et al., 2015). Copepods as live feed have also given positive long term effects on growth in cod (Imsland et al., 2006; Karlsen et al., 2015), and even copepod feeding for shorter periods during the earliest feeding stages has shown positive effects on the juvenile size (Øie et al., 2015). Copepods have a well-balanced composition of proteins, free amino acids and lipids, including significant amounts of n-3 fatty acids in the phospholipids (Shields et al., 1999; Evjemo et al., 2003; Drillet et al., 2006; Van der Meeren et al., 2008; Olsen et al., 2014; Karlsen et al., 2015). Changing the first-feeding diets from rotifers to copepods have shown promising results in several aquatic species (Conceição et al., 2010). However, the mechanisms leading to this prolonged growth advantage are yet unknown.

Skeletal muscle is the largest and most rapid growing tissue in fish larvae (Osse et al., 1997; Johnston et al., 2011). The two main mechanisms for growth of skeletal muscle in vertebrates are hypertrophy (increase in muscle size) and hyperplasia (increase in the number of muscle cells) (Weatherley et al., 1988). Generally, hypertrophy occurs all the time throughout most of a fish' life, whereas stratified hyperplasia occurs in distinct phases (Rowlerson et al., 1995; Johnston et al., 1998). In mammals, hyperplasia also occurs in two major waves, but hyperplasia stops shortly after birth (Kelly and Zacks, 1969; Denetclaw et al., 1997; Gros et al., 2004; Rossi and Messina, 2014). The primary muscle fibers in fish are formed from embryonic myoblasts (called embryonic muscle fibers). In pelagic fish larvae, new muscle fibers are formed between the red and white muscle layers (stratified hyperplasia) from the start of exogenous feeding, and from metamorphosis new fibers are formed between the existing white muscle fibers in the whole myotome (mosaic hyperplasia) (Weatherley et al., 1988; Veggetti et al., 1990; Rowlerson et al., 1995).

In cod larvae, stratified hyperplasia occurs in the 5 – 15 mm SL larvae and is the predominant muscle growth mechanism in the 7 – 10 mm SL cod larvae (Vo et al., 2016) during the live prey feeding period. After hatching teleost muscle hyperplasia requires the proliferation of muscle precursor cells and a common feature of many proliferating cells is aerobic glycolysis, a phenomenon termed "the Warburg effect" (Vander Heiden et al., 2009). A major function of aerobic glycolysis is to support macromolecular synthesis of DNA, RNA, proteins, and lipids by providing precursors for these chemical constituents (Lunt and Vander Heiden, 2011). However, glycolysis is inefficient in generating adenosine triphosphate (ATP) and the yield is only 2 ATP/glucose, whereas oxidative phosphorylation can generate up to 36 ATP/glucose (Lehninger et al., 1993). Oxidative phosphorylation in mitochondria (aerobic energy) is therefore the main contributor of ATP in most cells, including in proliferating cells (Lunt and Vander Heiden, 2011). Therefore,

Abbreviations: *casp3*, caspase 3; cat, catalase, Dph, days post-hatch; DWF, deep white muscle fibers; *gpx1*, glutathione peroxidase 1, H_2O_2, hydrogen peroxide, MH, mosaic hyperplasia; O_2^-, superoxide, SH, stratified hyperplasia; SL, larval standard length; sods, superoxide dismutases.

investigating the molecular effects of live prey types on glycolysis, gluconeogenesis and oxidative phosphorylation processes in relation to muscle growth phases may shed new light on the underlying cellular mechanisms behind the variation in larval growth rates caused by live prey types.

The fundamental events in myogenesis (the specification, proliferation, differentiation and fusion of myogenic precursor cells (MPCs)) are regulated by myogenic regulatory factors (i.e., *myod*, *Myf5*, *Myogenin*, *Mrf4*) (Johnston, 2006; Rossi and Messina, 2014), but it also depends on external signaling factors, released by the surrounding tissues (Valente et al., 2013; Rossi and Messina, 2014). The insulin-like growth factor (IGF) system is one of the central signaling pathways of hypertrophic growth in fish larvae and regulates protein synthesis, breakdown and replacement (Johnston et al., 2011; Valente et al., 2013; García de la Serrana et al., 2014). However, the specific signaling factors and pathways that control the temporal muscle hyperplastic growth phases remain unclear in vertebrates (Valente et al., 2013; Rossi and Messina, 2014).

A rapid growth to a larger size is also beneficial for the larvae's ability to swim faster, which in small pelagic larvae is dependent on the viscous forces of water relative to their body size. Skeletal muscle is the main organ for locomotion, which probably needs to develop continuously to adapt to these perceived changes in water viscosity relative to their increasing body size. Cod larvae hatch at around 3 – 4 mm SL, they start with an anguilliform swimming behavior, characterized by undulations throughout the body, and they gradually shift to a carangiform swimming mode, with movements mainly near the tail (Weihs, 1980; Webb and Weihs, 1986; Osse and van den Boogart et al., 2004; Müller et al., 2008; Voesenek et al., 2018). Their swimming activity/capability increase exponentially from around 10 mm SL (Clark et al., 2005; Peck et al., 2006; Leis et al., 2007), which is associated with a shift from muscle hyperplasia to muscle hypertrophy (Vo et al., 2016). The "adult type" mosaic hyperplasia occurs from around 20 mm SL (Vo et al., 2016).

The period of stratified hyperplasia (7 – 10 mm SL) is associated with a shift to a more oxidized redox state during cod larval development (Hamre et al., 2014). The cellular redox state is known as a key determinant of cell fate (cell survival, proliferation, differentiation, and apoptosis) in multicellular organism development (Kamata and Hirata, 1999; Matsuzawa and Ichijo, 2008). The change in redox state in a biological system occurs commonly in response to a change in the level of reactive oxidative species (ROS), including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH·) (Urao and Ushio-Fukai, 2013; Holmström and Finkel, 2014; Schieber and Chandel, 2014; Chandel et al., 2016). ROS have long been considered to evoke damage or oxidative stress to tissues, but especially H_2O_2 is now also considered as a secondary messenger when induced in small amounts. ROS act as signaling molecules to initiate biological processes and to maintain physiological functions in cells; so-called ROS signaling – redox biology (Urao and Ushio-Fukai, 2013; Holmström and Finkel, 2014; Schieber and Chandel, 2014; Jones and Sies, 2015; Chandel et al., 2016). ROS are by-products of aerobic metabolism (Halliwell, 1991), which is the main metabolism of red muscle, while white muscle function changes from predominantly aerobic to anaerobic metabolism during climax-metamorphosis in fish larvae (El-Fiky et al., 1987). During intense contractile activity, muscle mitochondria generate a high ROS flow, which may trigger different signaling pathways leading to diverging responses, from adaptation to cell death (see reviews by Jackson, 2005; Barbieri and Sestili, 2012). Therefore, our hypothesis is that the viscous force of seawater and the ROS level in muscle tissues, represent external and internal drivers that generate the correlation between muscle growth dynamics and body size growth in cod larvae.

The cod larvae in this experiment were fed either harvested zooplankton (mainly copepods) or enriched rotifers and *Artemia* as described by Karlsen et al. (2015). Larvae fed copepods had a much higher growth rate (Karlsen et al., 2015), and Penglase et al. (2015) found that the rotifer fed cod larvae developed a more reduced redox potential than copepod fed larvae. Even at the late juvenile stage (28.5 to 31.5 cm SL range, 15 months), the body size was still significantly larger in the cod where the larvae were fed copepods compared to those fed rotifers/*Artemia*. The largest dietary growth effect was thus observed especially after weaning to a formulated diet, and our hypothesis is that the predominant stratified hyperplastic growth phase in larval cod muscle is a window for modulating the growth rate in larvae and that it may lead to a long-lasting improved growth potential.

Our aim in this study was to investigate the effect of larval live feed (copepods or rotifers and *Artemia*) on the molecular relationships between somatic growth and the skeletal muscle growth dynamics, metabolism, and antioxidant defense system in developing Atlantic cod larvae from the experiment of Karlsen et al. (2015). We focused on whole body analyses (RNAseq) for gene expressions of myogenic markers (proliferation, differentiation) and target genes and receptors of metabolism (glycolysis, gluconeogenesis, oxidative phosphorylation). For more specific data from skeletal muscle tissue samples, we used qPCR analyses for gene expressions of skeletal muscle myogenesis, and antioxidant defense system (production/regulation of reactive oxygen species (ROS)). The target genes that were used in the molecular analyses are listed in Table 1.

Materials and methods

Experimental design and larval sampling

The cod larvae and juveniles were reared in accordance with the Norwegian Animal Welfare Act of 20th December 1974, No. 73, Sections 20– 22, amended 19th June 2009. Fertilized eggs were obtained from coastal Atlantic cod from

TABLE 1 Target genes and receptors selected for evaluation of possible effects from live prey type in relation to myogenesis, glycolysis, gluconeogenesis and oxidative phosphorylation in cod larvae.

Area	Genes	Function	References
Myogenesis	*pax7*	myogenic progenitor cells (MPC)	Johnston, 2006; Rossi and Messina, 2014
	myod	activated MPCs	
	pcna	cell proliferation	
	mef2	differentiation muscle cells	Garcia de la Serrana et al., 2014
	igf1	regulator myoblast proliferation	Valente et al., 2013
	igf1-r	transmembrane receptor	
Metabolism	*pfkl* – liver isoform of phosphofructokinase	glyolytic enzyme	Knox et al., 1980; Koster et al., 1980; Dunaway et al., 1988
	pfkm - muscle isoform of phosphofructokinase		
	fbp2 - Fructose diphosphatase	gluconeogenic enzyme	Rogatzki et al., 2015
	ldhA - Lactate dehydrogenase	glycolysis	
	slc2a11 - muscle-specific	fructose transport facilitator	Zhao and Keating, 2007
	ppar-α - nuclear receptor	lipid metabolism	Lefebyre et al., 2006
Antioxidant defense system	*sod1 / sod2*	convert O_2^- to H_2O_2	Brand, 2010
	gpx1 - glutathioneperoxidase	Enzymatic antioxidant defense	Fridovich, 1997
	cat - catalase	Enzymatic antioxidant defense	
	p38	Inhibit cell division	Igea and Nebreda, 2015
	caspase	Mediator apoptosis	Lakhani et al. 2006
	nox1	Encoding the enzyme NADPH oxidase 1	Lambeth, 2004

western Norway, which were held at the Institute of Marine Research (IMR), Austevoll Research Station. Details for the main cod larval feeding experiment related to larval growth and survival, experimental conditions, prey types, prey quantities, prey and larval sampling, and biochemical composition are described in Van der Meeren et al. (2014) and in Karlsen et al. (2015).

In summary, the cod larvae were stocked in four 500-litre tanks from hatching to 4 days post-hatch (dph), then they were divided into six tanks at 50000 cod larvae/tank. Larvae in three tanks were fed harvested copepods from a marine pond system (Copepod-fed larvae), while larvae in the other three tanks were fed cultivated and enriched rotifers followed by enriched *Artemia* nauplii (Rotifer/*Artemia*-fed larvae). Weaning started with formulated diets from around 15 mm standard length (SL) and continued until around 50 mm SL (juvenile stage) (see Figure 1).

Whole larvae were sampled at 4 dph (the control point before the start of exogenous feeding), and 2 hours after morning feeding on other sampling days. Due to the expected increased differences in larval growth between treatments, the larvae and early juveniles were sampled according to specific developmental stages and mean standard lengths, as described in Karlsen et al. (2015) and in Table 2.

All larvae were sampled randomly from the experimental tanks. The sampled larvae were euthanized in tricaine methane sulfonate (MS-222, Argent Chemical Laboratories Inc., Redmond, WA, USA), and rinsed in distilled water for a few seconds before further fixation and analytical treatments.

RNA sequencing

Samples of whole larvae were stored in RNA later (Ambion, Austin, Texas, USA). A pooled sample of larvae was used at stage 0

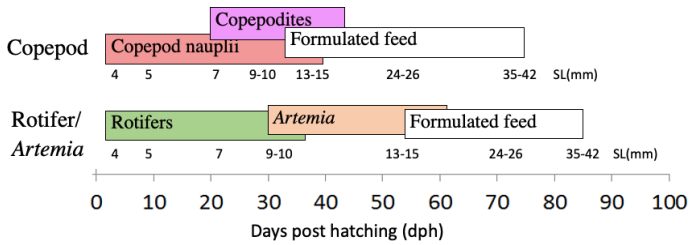

FIGURE 1
Experimental setup. Cod larvae were fed either copepods (nauplius and copepodite stages) or rotifers and *Artemia*, then weaned onto a commercial formulated diet from around 15 mm size. Cod larvae were sampled at corresponding larval body sizes at around 4, 5, 7, 9-10, 24-26, and 35-42 mm SL in the two treatments.

TABLE 2 Overview of larval developmental stages and mean sizes for RNA-seq and qRT-PCR at different sampling points.

Develop-mentalstage	Meta-morphic stage	Diets	Copepod-fed larvae			Rotifer-fed larvae		
			Day post-hatch	Mean larval sizes (SL - mm)		Day post-hatch	Mean larval sizes(SL - mm)	
				RNA-seq	qPCR		RNA-seq	qPCR
0	Pre-	Before feeding	4	4.5 ± 0.2	4.5	4	4.5 ± 0.2	4.5
1	Pre-	Copepods *vs.* Rotifers	11	5.1 ± 0.3	5.2	11	5.2 ± 0.4	5.2
2	Pro-	Copepods *vs.* Rotifers	22	7.0 ± 0.7	7	22	6.9 ± 0.6	7
3	Pro-	Copepods *vs.* Rotifers	29	10.1 ± 0.9	10	31	8.5 ± 0.7	10
4	Climax-	Copepods *vs. Artemia*	37	12.9 ± 1.4	15	54	14.8 ± 2.6	15
5	Post climax-	Commercial formulated diet	53	23.7 ± 3.1	25	71	26.5 ± 3.2	25
6	Juvenile	Commercial formulated diet	74	–	40	85	–	40

Developmental stages, and mean size of larval samples for RNA-seq are as described in Karlsen et al., 2015. From each treatment, pooled larval samples were used for RNAseq analysis, and four larvae close to the mean SL (± 0.1 mm) for each stage were dissected for muscle qPCR-analysis.

(control point, 4 dph), and total RNA from pooled samples of five whole larvae in each triplicate were used for sequencing in later stages.

The details of the RNA extraction, quantification, and sequencing analysis were described in Penglase et al. (2015), and the mean larval sizes at each sampling points are shown in Table 2. The gene expression data by RNA-seq were from 4 – 30 mm range, i.e., only larval stages. The raw data of the cod larvae from this experiment can be found at The Sequence Read Archive at NCBI (Accession ID: PRJNA277848). The reads were normalized by the total number of mapped sequences. Thereafter, they were expressed as fold change compared to day 4 post-hatch larval samples (4 mm/control samples) and were presented as a function of larval body size (SL) and of white muscle growth dynamics as described in Vo et al. (2016).

Quantitative real-time PCR (qRT_PCR)

For more specific analyses of development in the larval muscle tissue, messenger RNA (mRNA) expression of genes encoding for myogenic markers (*pcna*, *pax7*, *myod1*, *myodh1*) and antioxidant enzyme genes (*sod1*, *sod 2*, *cat*, *gpx1*, *nox1*, and *p38*) were analyzed by quantitative real-time (RT) PCR (see Table 3 for details). The larvae were snap frozen in liquid N_2, freeze dried and stored at -80°C before further treatment. To quantify the gene expression levels in the larval muscle tissues, head and gut were dissected (mostly muscle and skeleton remaining) from sampled larvae that were of approximately similar size for each stage and treatment (stages 0 – 5, up to mean size of 40 mm SL).

TABLE 3 Primer pair sequences, amplicon size for genes used for real time PCR.

Target gene	5′-3′ primer sequence		Amplicon size
	Forward	Reverse	
Pax7	CGTGTTGAGGGCCCGGTTTGGCA	CCTCGTCTGTGCGGTTGCCTTTA	131
Pcna	GATGGGTTTGACTCCTACCG	CGAGCGTGTCAGCATTGTCT	126
Myod1	ACGCGTTTGAGACACTGAAG	AGTGTCCTCCTGTCCTCCAC	112
Myod1h1	CGCTGAAGAGGAGCACCCTGATG	TCCTGCTGGTTGAGCGAGGAGAC	121
Sod1	CATGGCTTCCACGTCCATG	CGTTTCCCAGGTCTCCAACAT	133
Sod2	ATGTGGCCTCCTCCATTGAA	GCATCACGCCACCTATGTCA	129
Gpx1	CCAAATATGGACGGCATAGGA	CAAACGCTACAGCCGGAACT	101
p38	AGTCTCTGCTTCCAGTCGGT	CTCGACAGCTTCTTCACTGC	132
nox1	GCCTATATGATTGGCCTGATGAC	GCTGTGCTGAGTGGGTCGTA	107
cat	GCCAAGTTGTTTGAGCACGTT	CTGGGATCACGCACCGTATC	101

(Annealing temperature conditions at 60°C).

At each stage, each treatment had three to four replicates of 1-6 larvae. All samples were homogenized in Trizol reagent according to the manufacturer's protocol. The purity and integrity of the RNA samples were verified by NanoDrop ND-1000 Spectrophotometer (Nano- Drop Technologies), OD260 nm/OD280 nm ratio for all samples ranged between 1.96 and 2.10 and formaldehyde agarose gel electrophoresis. Complementary DNA (cDNA) for the quantitative PCR (qPCR) reactions were generated from 1 µg total RNA using a combination of random hexamer and poly-T primers from iScript cDNA Synthesis Kit, as described by the manufacturer (Bio-Rad). The expressions of individual gene targets were analyzed using Mx3000 real-time PCR system (Stratagene, La Jolla, CA). Each 25-µL DNA amplification reaction contained 12.5 µL of iTAQTMSYBR Green Supermix with ROX (Bio-Rad), 5 µL of diluted cDNA (1:6), and 200 nM of both forward and reverse primer. The three-step real-time PCR program included an enzyme activation step at 95°C (3min) and 40 cycles at 95°C (15s), 55–60°C (30s), and 72°C (20s).

PCR reaction efficiency (E) for each gene assay was determined using serial dilutions of plasmid containing the amplicon of interest and showed a stable expression pattern between larval pools. To confirm amplification specificity, the PCR products from each primer pair were subjected to melting curve analysis during qPCR assays. Cycle threshold (Ct) values obtained were converted into mRNA copy number using standard plots of Ct versus log copy number. The standard plots were generated for each target sequence using known amounts of plasmid containing the amplicon of interest, as previously described, and validated (Arukwe, 2006; Kortner et al., 2011). Data obtained from cDNA amplification were log transformed, averaged, and thereafter expressed as fold change of day 4 post-hatch larvae (control sample).

Statistical analysis

Statistics were performed with SPSS 18.0 for Windows. Normality and homogeneity of variances were tested with the Shapiro–Wilk W-test and Levene's test, respectively. Significant differences between different larval stages were determined using One-way ANOVA followed by Tukey's Multiple Comparison Test. To find the combined differences between diets and larval stages of the two dietary treatments, a two-way ANOVA was used, using larval stages as repeated measurement. The level of statistical significance was set at $p < 0.05$.

Results

Growth and survival

The cod larvae from both groups had similar growth rates up to 22 dph (mean 7 mm SL, stage 2). In the size range 7 - 15 mm SL (stages 2 – 4), the specific growth rate of the Copepod-fed cod larvae increased to about two times higher (15.5% day^{-1}) than for the Rotifer/*Artemia*-fed larvae (8.6% day^{-1}). After weaning to the same commercial formulated diet from around 15 mm SL (stage 4/5), they had similar specific growth rates (about 12% day^{-1}) until the end of the experiment at around 49 mm SL (stage 6, the juvenile stage). With regards to time, larvae in the copepod-fed group reached a mean SL of 10 mm at about 30 dph, while larvae in the rotifer/*Artemia*-fed group reached 10 mm SL at around 40 dph. Cod larvae reached a mean SL of 49 mm at 73- and 84 dph in the copepod-fed and in the rotifer/*Artemia*-fed group, respectively.

Gene expression patterns (RNAseq) of whole cod larvae

The cod larvae had significantly more upregulated *pcna* expression at 7 – 10 mm SL (stage 2 – 3), and from 15 - 25 mm SL (stage 5) expression levels were no longer different from those in unfed yolk sac larvae (stage 0) in both larval groups as shown in Figure 2A.

The myogenic precursor cell (MPC) population (expression of *pax7*) was significantly upregulated in the 4 - 10 mm SL range (stages 0 – 3), then expression values decreased steadily in the 15 - 25 mm SL range, with no significant differences between the nutritional treatments (Figure 2B).

Further, the expression pattern of *myod1* was more similar to *mef2a* expression (Figures 2C, E) while *myod2* in Copepod-fed larvae showed a comparable expression pattern to *pcna* (Figures 2A, D),

Expression of *pcna* and *myod2* were significantly more upregulated in the Copepod-fed larvae at 7 - 10 mm SL (Figures 2A, D). At 15 mm SL, the expressions of *pcna, pax7*, and *myod2* (Figures 2A, B, D) were significantly higher in the Copepod-fed larvae than in the rotifer/*Artemia*-fed larvae.

Expression of *casp3* approximately doubled between 4 and 10 mm SL in both groups. From then the *casp3* gene in rotifer fed larvae was rapidly upregulated to six fold-change. This rapid upregulation of *casp3* only occurred in Copepod-fed larvae from 15 mm SL onwards (Figure 2F).

Igf1 was continuously and significantly upregulated from 4 to 30 mm SL, while *igf1r* expression increased significantly at 5 mm SL and was stable thereafter. No significant differences in the expression of *igf1* and *igf1r* were found between the two groups at any given body size (Figure 3).

The expression pattern of *pfkl* was relatively stable whereas *pfkm* continuously increased in both groups in the 7 - 30 mm SL size range (Figures 4A, B). The expression of *fbp2* and *ldhA* were continuously upregulated from 7 to 30 mm SL (Figures 4C, D). The expression of *slc2a11* was stable in the 4 – 15 mm SL range, and then increased significantly in the 15 - 30 mm SL range (Figure 4E), and expression values were significantly higher in

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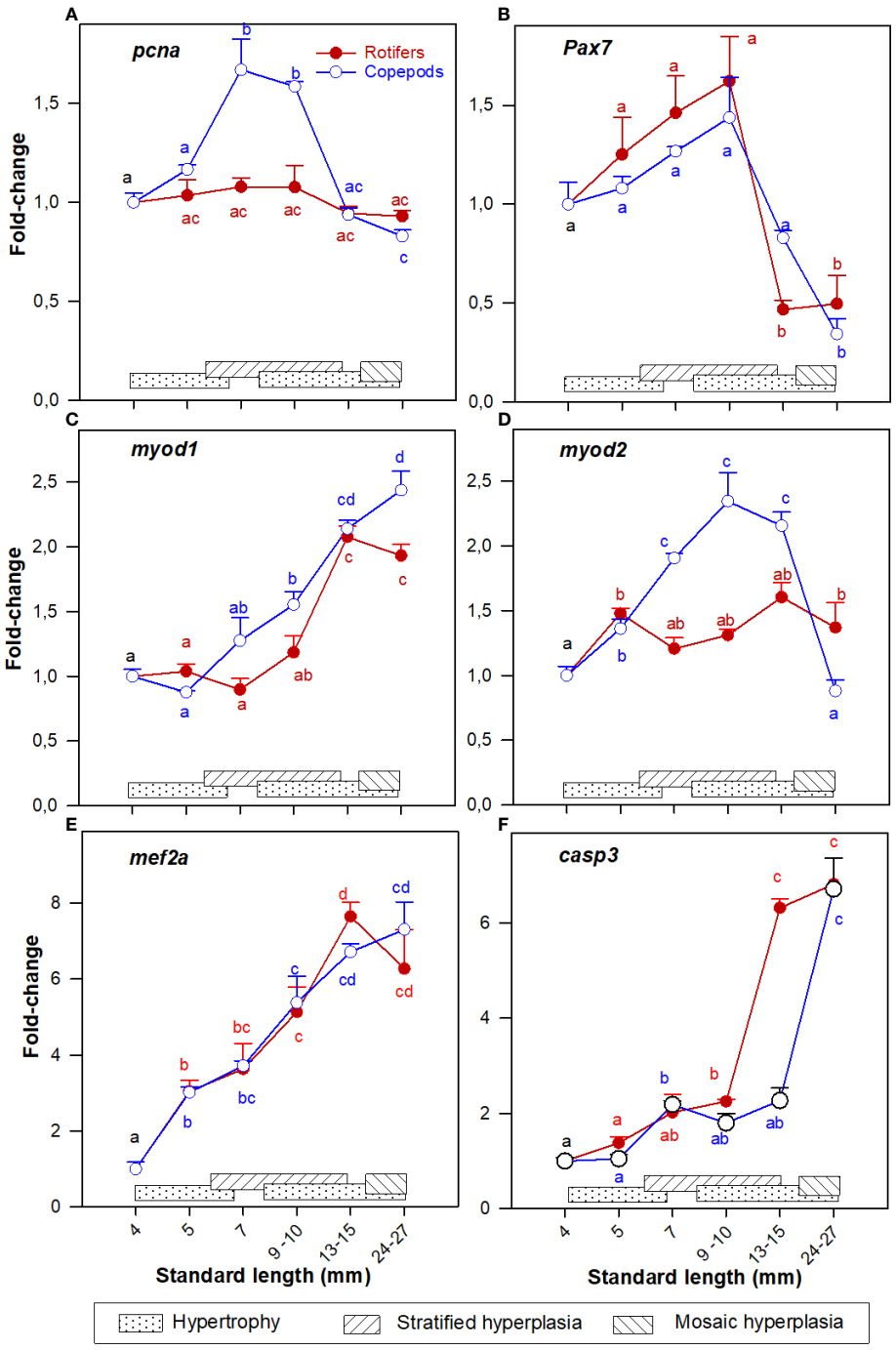

FIGURE 2
Gene expression (RNA-seq) of **(A)** *pcna*; **(B)** *pax7*; **(C)** *myod1*; **(D)** *myod2*; **(E)** *mef2a*; and **(F)** *casp3* in whole-body Atlantic cod larvae fed rotifers/*Artemia* (red line) or Copepods (blue line) in relation to muscle growth dynamics. Muscle growth dynamics (hypertrophy and stratified and mosaic hyperplasia) in relation to body size is indicated by horizontal bars (data from Vo et al., 2016). Data are given as mean (n=3 [n= 4 at 4 dph] ± SEM) and values are expressed as fold change compared to day 4 post-hatch. Different letters indicate significant differences between the larval stages and treatments ($P < 0.05$).

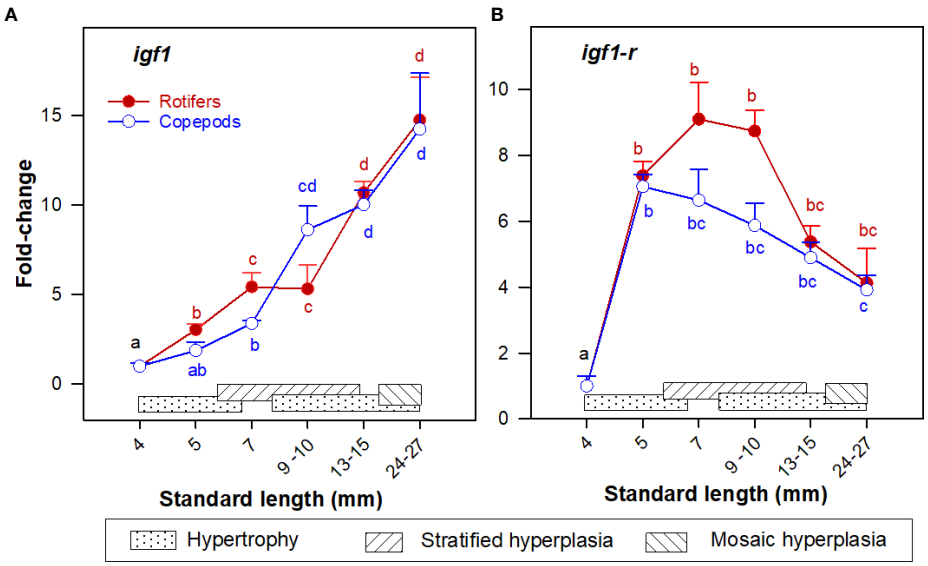

FIGURE 3
Gene expression (RNA-seq) of **(A)** *igf1;* and **(B)** *igf1r* in whole-body Atlantic cod larvae fed rotifers/*Artemia* (red line) or copepods (blue line) in relation to muscle growth dynamics. Muscle growth dynamics (hypertrophy and stratified and mosaic hyperplasia) in relation to body size are indicated by horizontal bars (data from Vo et al., 2016). Data are given as mean (n=3 [n= 4 at 4 dph] ± SEM) and values are expressed as fold change compared to day 4 post-hatch. Different letters indicate significant differences between the larval stages and treatments ($P < 0.05$).

Copepod-fed larvae in all stages after exogenous feeding started. The expression of *ppar-α* increased significantly in the 5-7 mm SL range, then decreased during development and the lowest levels in both groups were in the 15 – 30 mm SL range (Figure 4F).

At 7 and 10 mm SL the expressions of *pfkm, fbp2* and *ldhA* (Figures 4B–D) were significantly higher in the Copepod-fed larvae. At 7, 10, and 15 mm SL the expression of *slc2a11* (Figure 4E) was about 5 to 10 times higher in Copepod-fed larvae. At 10 mm SL, the expression of *ppar-α* (Figure 4F) was significantly higher in rotifer/*Artemia*-fed larvae than in the Copepod-fed larvae.

Gene expression patterns (qPCR) in skeletal muscle tissue

The cod larvae had upregulated *pcna* expression about 10 – 30 times at 7 – 10 mm SL (stage 2 – 3), and from 15 - 25 mm SL (stage 5) expression levels were no longer different from those in unfed yolk sac larvae (stage 0) in both larval groups, as shown in Figure 5A. The myogenic precursor cell (MPC) population (expression of *pax7*) continuously increased in the 4 - 10 mm SL range (stages 0 – 3), then values decreased steadily in the 10 - 40 mm SL range (Figure 5B). *Myod1* and *myodh1* expressions in both cod larval groups increased in the 4 – 15 mm SL range, then slightly decreased and remained stable in the 25 – 40 mm SL range (Figures 5C, D).

At 10 mm SL, the expression of *pcna* was more upregulated in the Copepod-fed larvae, and at 15 mm SL the expressions of *pcna, pax7, myod1* and *myodh1* (Figures 5A–D) were significantly higher in the Copepod-fed larvae than in the rotifer/*Artemia*-fed larvae.

In both larval groups the expression patterns of *sod1* and *sod2* continuously increased in the 4 – 10 mm SL range, peaking at 10 mm SL (stage 3) and then decreased 15 - 40 mm SL (Stages 4- 6) (Figures 6A, B). The expression pattern of *cat* decreased in both groups in the 4 - 15 mm SL range and increased somewhat in the 25 - 40 mm SL range (Figure 6C). Further, *gpx1* expression pattern was relatively low and stable in the 4 – 15 mm SL range, and then increased about 10 – 20 folds at 25- and 40-mm SL (Figure 6D), although no significant difference between the groups was observed at 40 mm SL. The expression pattern of *nox1* was stable at 4 – 5 mm SL, peaking at 7 mm SL, and decreasing thereafter from 10 to 40 mm SL (Figure 6E). Expression pattern of *p38* increased continuously from 4 to 10 mm SL, then decreased from 10 to 40 mm SL in larvae from both feeding groups (Figure 6F). Also, at 15 mm SL, the expression of *p38* was significantly higher in Copepod-fed larvae (Figure 6F). In the largest larvae around 40 mm SL, no significant differences were found for any of the ROS-related genes between the larval groups.

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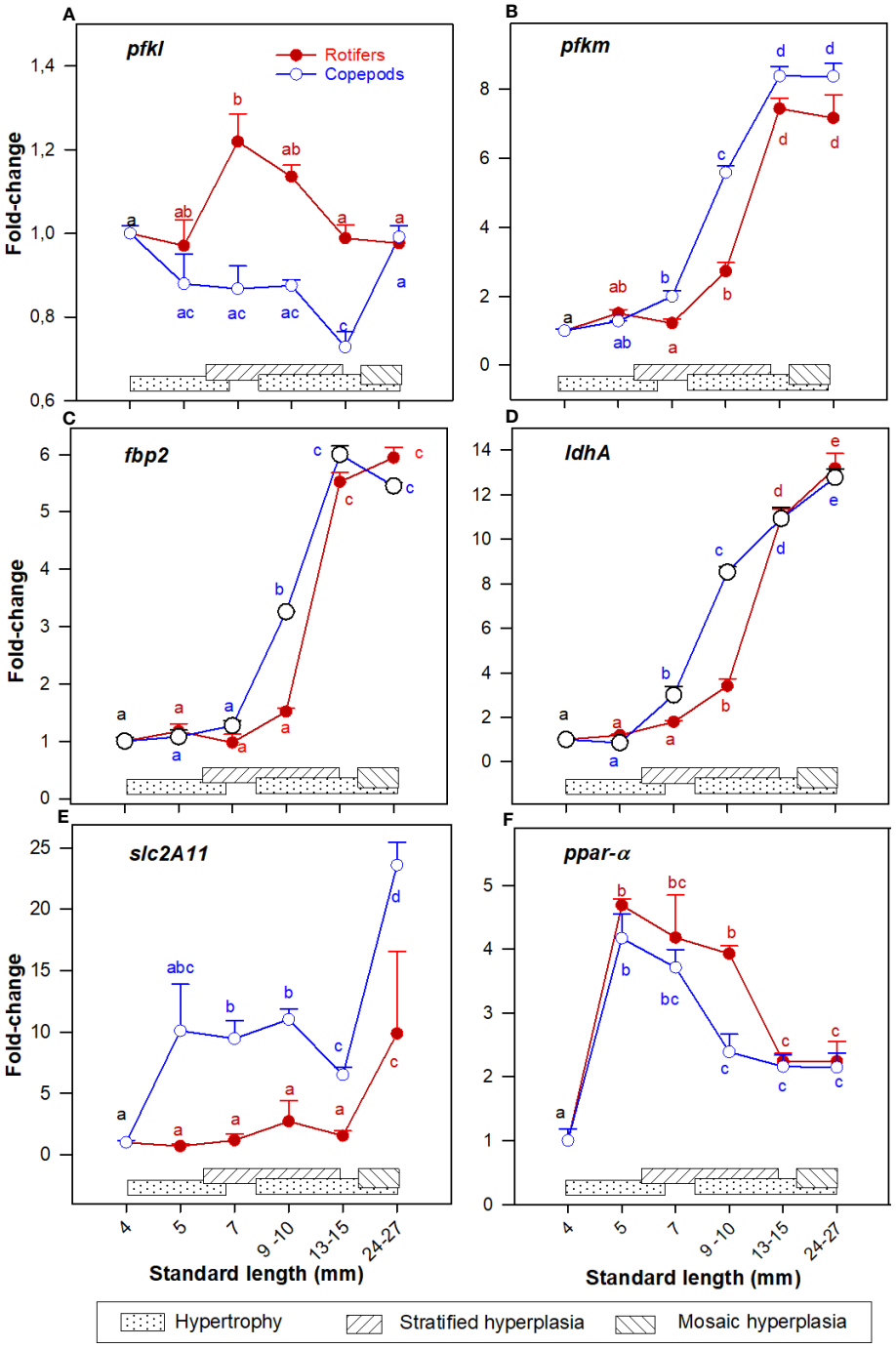

FIGURE 4
Gene expression (RNA-seq) of **(A)** *pfkl*; **(B)** *pfkm*; **(C)** *fbp2*; **(D)** *ldhA*; **(E)** *slc2a11*; and **(F)** *ppar-a* in whole-body Atlantic cod larvae fed rotifers/*Artemia* (red line) or Copepods (blue line) in relation to muscle growth dynamics: Muscle growth dynamics (hypertrophy and stratified and mosaic hyperplasia) in relation to body size are indicated by horizontal bars (data from Vo et al., 2016). Data are given as mean (n=3 [n= 4 at 4 dph] ± SEM) and values are expressed as fold change compared to day 4 post-hatch. Different letters indicate significant differences between the larval stages and treatments ($P < 0.05$).

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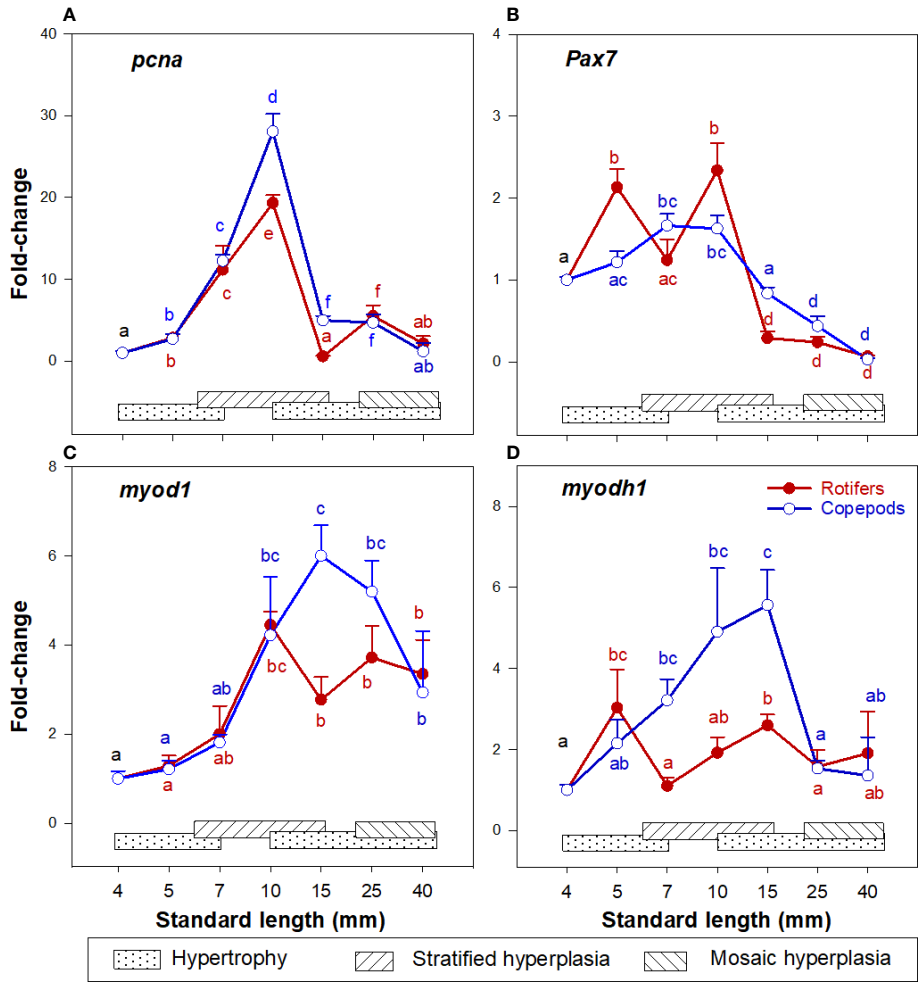

FIGURE 5
Gene expression (qPCR) of **(A)** pcna; **(B)** pax7; **(C)** myod1; and **(D)** myodh1 in skeletal muscle tissue of Atlantic cod larvae fed rotifers/Artemia (red line) or Copepods (blue line) (quantitative real-time PCR (qPCR) in relation to muscle growth dynamics: Muscle growth dynamics (hypertrophy and stratified and mosaic hyperplasia) in relation to body size is indicated by horizontal bars (data from Vo et al., 2016). Data are given as mean (n= 4 ± SEM) and values are expressed as fold change compared to day 4 post-hatch. Different letters indicate significant differences between the larval stages and treatments ($P < 0.05$).

Discussion

Identifying key factors promoting growth in Atlantic cod larvae

Cod larvae fed copepods had a much higher growth rate than those fed rotifers/*Artemia*, and the dietary effects should be due to the prey's composition of protein, lipids, as well as micronutrients. The level of protein is about two times higher in copepods than in rotifers and *Artemia*, and is one of the key factors generating the growth variations between the two larval groups (Karlsen et al., 2015). Finn et al. (2002) found that in cod larvae fed copepods the contribution of protein to energy fuel was at a high level in the 4 – 7 mm SL range and started declining beyond 7 mm SL.

Another major factor related to the larval growth difference in this experiment, is the dietary long-chained polyunsaturated fatty acids (PUFAs). Marine larvae have high requirements of PUFAs (Izquierdo et al., 2000), and lipid composition in the copepods is very different from that of enriched rotifers and *Artemia*. The essential dietary highly unsaturated n-3 fatty acids (HUFAs) docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA) are incorporated in the phospholipids (PL) of the copepods, whereas they are included in the triacyl-glycerides (TAG) in enriched rotifers and *Artemia* (Evjemo et al., 2003; Van der Meeren et al., 2008). Marine fish larvae can utilize the

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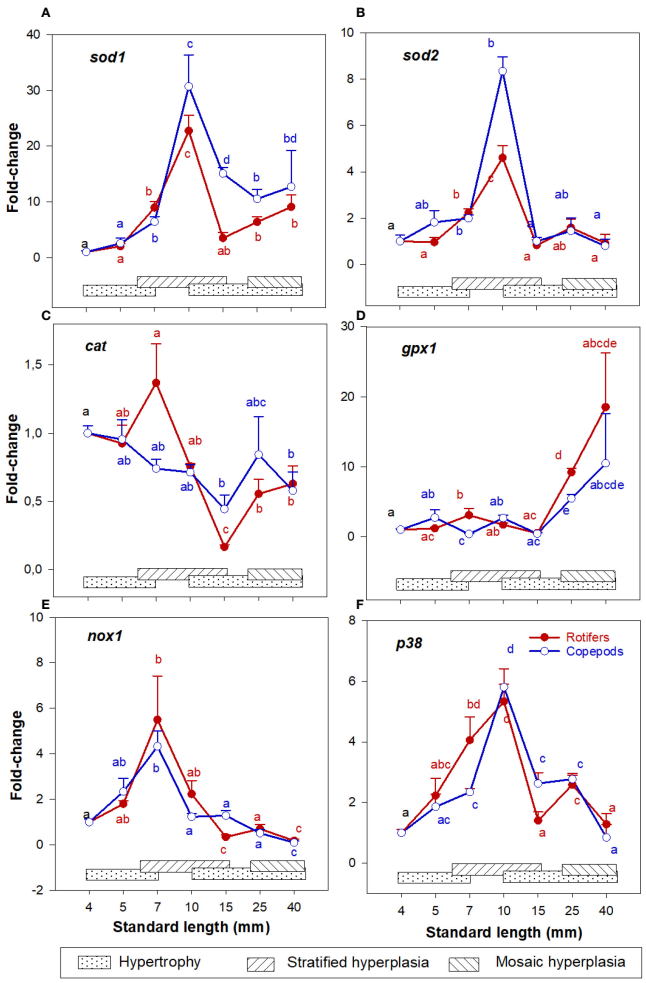

FIGURE 6
Gene expression (qPCR) of **(A)** *sod1*; **(B)** *sod2*; **(C)** *cat*; **(D)** *gpx1*; **(E)** *nox1*; and **(F)** *p38* in skeletal muscle tissue of Atlantic cod larvae fed rotifers/*Artemia* (red line) or Copepods (blue line) in relation to muscle growth dynamics. Muscle growth dynamics (hypertrophy and stratified and mosaic hyperplasia) in relation to body size is indicated by horizontal bars (data from Vo et al., 2016). Data are given as mean (n= 4 ± SEM) and values are expressed as fold change compared to day 4 post-hatch. Different letters indicate significant differences between the larval stages and treatments ($P < 0.05$).

dietary PUFAs more efficiently for growth and normal development if they are incorporated in the PL than in the TAG (Gisbert et al., 2005; Kjørsvik et al., 2009; Wold et al., 2009; Olsen et al., 2014). In marine larvae, these PL requirements seem to decline towards the juvenile stage (Tocher et al., 2008; Cahu et al., 2009).

Ppar-α is a key nuclear receptor for regulation of liver and skeletal muscle lipid metabolism and glucose homeostasis (Lefebyre et al., 2006). However, *ppar-α* is also one of the key transcriptional regulators of intermediary metabolism during fasting in mammals. It induces hepatic fatty acid oxidation, regulates hepatic glucose production, and affects amino acid metabolism (Kersten, 2014). In our experiment, larval expression of *ppar-α* had a similar developmental pattern in larvae from both treatments. It was rapidly upregulated between 4- and 5-mm SL (4 – 11 dph), which is the short period of first exogenous feeding, when some yolk reserves are still present ("mixed feeding period") and larval digestion switches from endogenous to exogenous nutrients (Hoehne-Reitan and Kjørsvik, 2004). It was significantly more upregulated in 5-10 mm SL rotifer-fed larvae, and it was similar between the groups from 15 mm SL, when stratified hyperplasia was ending and all larvae were weaned to the same formulated diet.

Ppar-α in the cod larvae was rapidly upregulated when their yolk nutrients were diminishing, and they had to start exogenous

feeding. The higher expression level of ppar-α in the rotifer fed larvae during the live feed period was thus most likely due to suboptimal nutrition, especially during the period with slowest growth. Both sea bass (Vagner et al., 2009) and Siberian sturgeon larvae (Luo et al., 2019) demonstrated lower growth and upregulated mRNA levels of ppar-α and other genes involved in fatty acid transport during feeding with low levels of n-3 HUFAs, and differences in *ppar-α* levels disappeared in older larvae and juveniles, just as we found in this experiment.

The largest growth variation between our two larval groups was found from 7 to 10 mm SL (stage 2-3), which is also the strongest phase of stratified muscle hyperplasia (Vo et al., 2016). Our present results demonstrate that the gene expression of glycolysis, gluconeogenesis, oxidative phosphorylation and lactate flux were significantly higher in Copepod-fed larvae than in rotifer/*Artemia*-fed larvae in this size range, and this could result in higher enzyme activities in the Copepod-fed larvae. The glycolytic enzyme activity of white muscle tissue increases with body size (Somero and Childress, 1980; Sullivan and Somero, 1983), with a high rate of lactate production in the white muscle during maximal burst-type activity (anaerobic power) (Goolish, 1989). Fish larvae rely almost entirely on aerobic power until climax metamorphosis (Wieser, 1995), which occurs from around 10 mm SL in cod larvae (Vo et al., 2016). A higher ratio of dietary carbohydrate in diets promotes the rates of glycolysis and gluconeogenesis in gilthead seabream (*Sparus aurata*) (Metón et al., 1999) and in zebrafish, (*Danio rerio*) (Rocha et al., 2015). Carbohydrate content ranges from 0.2 to 5.1% of dry weight in copepods (Corner and O'Hara, 1986) and from 10 to 27% in rotifers (Støttrup and McEvoy, 2003), and fish do not seem to utilize carbohydrates effectively (Wilson, 1994; Moon, 2001).

Proliferating cells rely on aerobic glycolysis with a high rate of lactate generation (about 90% of total glucose consumption) for macromolecular synthesis, but they need a high energy supply from oxidative phosphorylation (Warburg, 1956; Vander Heiden et al., 2009; Lunt and Vander Heiden, 2011) . In general, lactate is generated in muscle, then circulated to the liver, where lactate is converted back to glucose by gluconeogenesis, called the Cori cycle (Cori and Cori, 1929). Lactate is now known as the primary production for spatially linking glycolysis to oxidative phosphorylation (Rogatzki et al., 2015), as it is a major carbon source to generate ATP *via* oxidative phosphorylation in mitochondria in most tissues in mice and humans (Faubert et al., 2017; Hui et al., 2017). In the 7 – 10 mm SL range (stage 2-3) of cod larvae, a high density of mitochondria was observed not only in red muscle fibers but also in new white muscle fibers (Vo et al., 2016). The number of new white muscle fibers account for more than 90% of the total number of muscle fibers in the 7 – 10 mm SL range (Vo et al., 2016). At this stage, lactate generated by proliferating cells thus has a high potential to go through oxidative phosphorylation in red and new muscle fibers.

However, Chauton et al. (2015) found that the content of lactate was higher in the rotifer/*Artemia*-fed larvae than in the Copepod-fed larvae from around 9 - 25 mm SL. Cod larvae fed rotifers/*Artemia* also had a significantly higher glycogen store in the liver than larvae fed copepods at around 6 -7 mm SL (Kjørsvik, pers. comm.). These data suggest that lactate and glucose sources were highly available, but larvae were unable to convert it all to energy for growth when they were fed rotifers/*Artemia*. Our present results (*ldhA*, *pfk*, *slc2a11*) also showed that the glucose metabolism was triggered, and that it contributed to the energy needed from around 7 mm SL in cod larvae fed copepods, whereas this occurred only from 10 mm SL in larvae fed rotifers/*Artemia*.

We found no differences between the larval groups in the transcript levels of *igf1* and *igf1r* in the present study, similar to Katan et al. (2016) who did not find differences in hormones and hormone receptors (*igf1*, *igf2*, growth hormone (*gh*), *gh1r*, *gh2r* and myostatin (*mstn*)) between cod larvae fed natural zooplankton or rotifers and *Artemia*. However, the expression of *slc2a11* (muscle-specific fructose transport facilitators) was about 5 to 10 times higher in Copepod-fed larvae than in rotifer/*Artemia*-fed larvae in the 5 – 15 mm SL range (the live prey feeding period). Exercise is now known as an important factor for promoting glucose metabolism (by up to 50-fold) by increasing the delivery and transport across the muscle membrane and intracellular flux in humans (Sylow et al., 2017). Cod larvae need to use higher swimming speeds (intense burst) for capturing fast swimming prey (copepods) than for slow swimming prey (rotifer or protozoan), and there are less escape responses when cod larvae attack slow-swimming prey rather than the fast prey organisms (Hunt von Herbing and Gallager, 2000). Moreover, at around 10 mm SL, the expression of *sod2* in skeletal muscle was significantly higher in the Copepod-fed larvae than in the rotifer/*Artemia*-fed larvae. *Sod2* is responsible for rapidly converting superoxide, which is generated by mitochondria during muscle contraction to hydrogen peroxide (H_2O_2) (Fridovich, 1997). Cod larvae thus probably need to use a higher swimming speed and spend more time for hunting copepods than for rotifers and *Artemia*. In other words, the duration of active swimming of cod larvae would be higher in those fed copepods than in those fed rotifers/*Artemia*. This probably led to a better delivery and transport across the muscle membrane, and to intracellular flux through glycolysis and oxidative phosphorylation. We therefore propose that active swimming may be a key factor for enhancing muscle growth, in terms of utilizing the available energy sources for growth.

A high level of taurine in skeletal muscle is vital for excitation-contraction coupling, organelle structure and regulation of skeletal muscle energy metabolism during exercise (Ito et al., 2014). In the present experiment, taurine and zinc were at least ten times higher in copepods than in rotifers and *Artemia* (Karlsen et al., 2015), which could affect the

swimming capacity of the cod larvae. Taurine deficiencies result in lipid accumulation, mitochondrial damage, and oxidative stress (Militante and Lombardini, 2004; Espe et al., 2012; Jong et al., 2012; Espe and Holen, 2013; Jong et al., 2021),. The low levels of zinc in rotifers and *Artemia* could have negative effects on the enzymatic activity of *sod1*. Enzymatic activity of *sod1* depends on the presence of Cu and Zinc for proper protein folding and stability (Fukai and Ushio-Fukai, 2011; Marreiro et al., 2017). In larvae from our experiment, this would probably lead to accumulation of superoxide, more associated with oxidative stress than with redox signaling, from around 7 mm SL in rotifer/*Artemia*-fed larvae. Rise et al. (2015) also found that the redox system in cod larvae fed rotifers/*Artemia* was affected by a low level of selenium, which plays an important role in stabilizing the function of *sod1* (Rükgauer et al., 2001). Copepods generally contain much higher levels of minerals than cultivated rotifers and *Artemia* sp. (Mæhre et al., 2013). Therefore, high concentrations of taurine and minerals in larval diets may be crucial for achieving high muscle contractions, with subsequent and stabilized ROS levels and improved growth in cod larvae; especially in the 4 – 10 mm SL range when the most intense stratified hyperplastic muscle growth is occurring.

External and internal drivers in cod larval muscle growth dynamics

We previously found that muscle growth dynamics (relationship between hypertrophy and hyperplasia) is strongly related to body size in Atlantic cod larvae (Vo et al., 2016). Although the molecular developmental patterns between larval groups were similar, the myogenic genes (*pcna*, *pax7*, *myod*) showed a stronger upregulation in Copepod-fed larvae.

Muscle differentiation (*mef2a* expression) always occurred, the *mef2a* expression was increasing throughout development, and no difference was observed between the treatments. Hypertrophy is the predominant early growth mechanism in cod larval muscle from 4 to 7 mm SL (Vo et al., 2016), which requires enhanced synthesis of muscle protein and muscle membranes (Valente et al., 2013). Muscle proliferation (stratified hyperplasia), as shown by *pcna* expression, occurred in the 5 - 15 mm SL range (stage 1-4), peaking at 10 mm SL, which is similar to our observations from histology of cod larvae from other experiments (Vo et al., 2016). Muscle proliferation would also increase the number of mitochondria in the muscle, which was indirectly confirmed as we found a peak in ROS-signaling and hydrogen peroxide (H2O2) generation (*sod1* and *sod2* expressions) in larvae around 10 mm SL. In aerobic organisms, superoxide is often produced by mitochondria and NADPH oxidases (NOXs family) (Fridovich, 1997; Brand, 2010). Whereas ROS production by mitochondria is more related to intracellular signaling, ROS from the NOXs family are cell membrane proteins that can be signaling intracellularly and extracellularly (Brown and Griendling, 2009; Finkel, 2011). The extracellular ROS from the NOXs family regulate gene expression, and proliferation, differentiation, and migration of cells in mammals (Lambeth, 2004; Bedard and Krause, 2007; Brown and Griendling, 2009). The gene expression of cell membrane proteins such as *nox1* and *igf1r* peaked at 7 mm SL (stage 2) in both cod larval groups. Therefore, the NOXs family might be an important source of ROS, leading to the increasing ROS levels we observed in cod muscle at the onset of stratified hyperplasia growth around 7 mm SL (see also Penglase et al., 2015).

The highest expressions of *sod1*, *sod2*, and *pcna* in the skeletal muscle tissue (qPCR) were found around 10 mm SL (stage 3) and their expressions were dramatically decreased from 15 mm length (stage 4) in both cod larval groups. At around 10 mm length, cod larvae change from a viscous to a transitional swimming mode (Peck et al., 2006), and the larvae increase their swimming activity exponentially (Peck et al., 2006; Stanley et al., 2012). Hence, decreasing values thereafter of *sod1* and *sod2* in skeletal muscle in cod larvae in both larval groups, might be the first response of the defense antioxidant system for controlling the levels of hydrogen peroxide (H_2O_2). However, this could probably result in accumulation of superoxide (O_2^-), which is more associated with oxidative stress than redox signaling (Fridovich, 1997). The expression patterns of *cat* and *gpx1* were relatively stable in the 4 – 15 mm SL range, and *gpx1* increased significantly at 25 mm SL (stage 5) in both larval groups. Penglase et al. (2015) also found that the redox potential (the 2GSH/GSSG concentrations) were significantly higher in the 5 - 10 mm SL range than in the 15 – 25 mm SL range in larvae from the present experiment. Significant changes in these ratios are usually a sign of oxidative stress, and causing toxicity rather than signaling (Murphy, 2011). Moreover, the expressions of *p38* (muscle, qPCR) and *casp3* (whole body, RNAseq) increased to the highest level at 10 and 15 – 25 mm SL, respectively. The cells will respond to a low oxidative stimulus of hydrogen peroxide (H_2O_2) by activating *p38* to inhibit cell proliferation (Iwasa et al., 2003) and by expressing *casp3* before or at the stage of lost cell viability (apoptosis) (Porter and Jänicke, 1999). Overall, from around 10 mm SL, ROS levels in skeletal muscle cells probably reached a lower stress level due to an expanded duration of active swimming of the cod larvae. In response, *p38* and *casp3* would inhibit the MPC proliferation or even commit to cell death programming in muscle at around 10 - 15 mm SL, leading to a decrease in and/or an ending of the stratified hyperplastic phase in skeletal muscle.

The expression of *p38*, *cat*, and *gpx1* in skeletal muscle was higher in the rotifer/*Artemia*-fed larvae than in the Copepod-fed larvae at 7 mm SL. Penglase et al. (2015) also found that from 5 to 7 mm SL the redox potential decreased significantly in rotifer-fed cod larvae but was stable in Copepod-fed larvae. At low oxidative stress, *p38* is often selectively activated for mitotic arrest without apoptosis induction (Kurata, 2000), and the

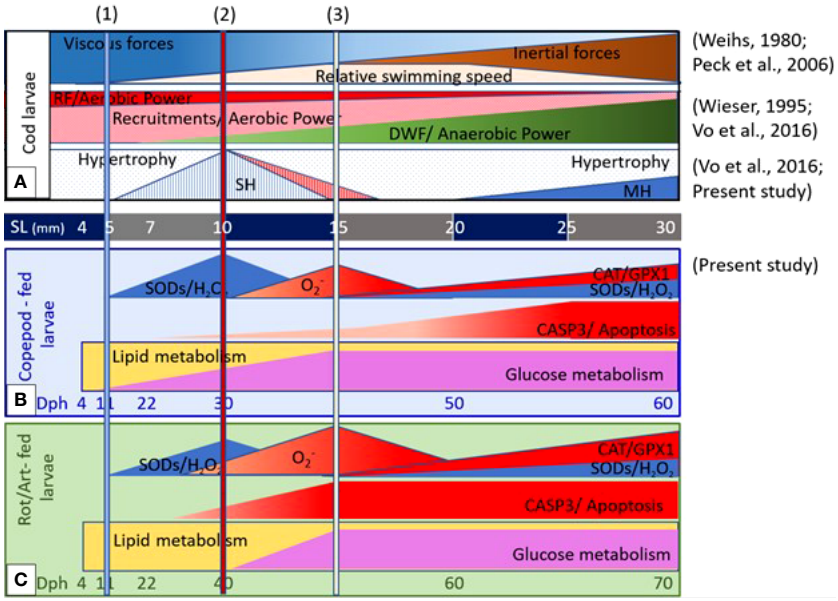

FIGURE 7
Proposed correlation between muscle development and growth dynamics, external and internal drivers, and energy metabolisms in relation to live prey types during ontogeny of cod larvae. **(A)** Changes in viscous and inertial forces and relative swimming speed (Weihs, 1980; Peck et al., 2006), and the relationship between aerobic and anaerobic power in different muscle fiber types (Wieser, 1995), and muscle growth dynamics between hypertrophy and hyperplasia (Vo et al., 2016; the present study) develop as a function of body size. Live prey types affected the levels of ROS generation and regulation and the shift in metabolisms from lipid and glucose in **(B)** cod larvae fed copepods and in **(C)** cod larvae fed rotifers and *Artemia* (the present study). The columns at 5-, 10-, and 15 mm standard length (SL) indicate when cod larvae start a viscous swimming mode, the transitional swimming mode, and the inertial swimming mode (1, 2 and 3) (Weihs, 1980; and Peck et al., 2006).

10.3389/fmars.2022.814022

prolonged mitotic arrest could trigger partial activation of apoptosis (Orth et al., 2012). To grow from 7 to 10 mm SL, rotifer-fed larvae needed 10 days more than Copepod-fed larvae in this experiment. Moreover, the expression of *casp3* (regulating apoptosis and inhibiting proliferation) reached the highest level at a smaller size in rotifer-fed larvae (from around 15 to 25 mm SL) than in Copepod-fed larvae (from around 25 mm SL). At around 15 mm SL (at the end of stratified muscle hyperplasia), the number of MPCs (seen by the expression of p*ax7*, *pcna*, *myod1* and *myodh2*) was significantly higher in the Copepod-fed larvae than in the rotifer/*Artemia*-fed larvae. This corresponds well with our observation that at a given body size, mosaic hyperplasia in the early and late juvenile stages were stronger in Copepod-fed larvae (Vo et al., 2016). Therefore, the prolonged proliferation at a low stress level could reduce the generation of MPC numbers during the stratified hyperplasia phase, which would be the underlying mechanism of the observed long-term effects of live prey types on muscle growth potential in cod larvae.

Conclusion

The proposed correlation between muscle development and growth dynamics, external and internal drivers and energy metabolism in cod larvae fed different live prey types are summarized in Figure 7. Although the growth rates of cod larvae were significantly different between the two larval groups, shifts in gene expressions related to muscle growth dynamics between hypertrophy and hyperplasia and the generation and regulation of ROS mostly occurred around 5, 10, and 15 mm SL. These body sizes coincided with when cod larvae start the viscous, transitional, and inertial swimming modes, reflecting the relative changes between viscous and inertial forces of seawater on the swimming capabilities of the cod larvae. We suggest that the viscous forces of seawater and ROS levels in muscle tissue might be the external and internal drivers for shifts in muscle growth dynamics in cod larvae around 5, 10, 15 mm SL.

Promoted active swimming during the strongest muscle proliferation phase (stratified hyperplasia) could enhance the glycolysis and oxidative phosphorylation flux between muscle cells (proliferating red and white muscle fibers) for energy production. In turn, a high-energy flux can promote the proliferation in muscle. We therefore suggest that active swimming is probably an important factor for promoting cod larval muscle growth, especially during the strongest hyperplasia phase between 7 and 10 mm SL. However, increasing swimming activities may also lead to increasing ROS levels in muscle tissue, which represent a double-edged sword between redox signaling and oxidative stress. High concentrations of n-3 HUFAs, minerals and taurine and fast swimming prey would probably play important roles in stabilizing ROS levels and promoting high swimming activities, as well as good growth and development of the skeletal muscle in cod larvae.

We found that the stratified hyperplasia in fish larvae is a good model for investigating the details regarding the regulation of ROS signaling. Further studies on the specific ROS signaling pathway in the stratified hyperplasia phase, and the roles of ROS and swimming activity in driving the development of different muscle fiber types should also be investigated further to achieve a better understanding of fish larval muscle growth dynamics and the long-term effects on juvenile muscle development.

Data availability statement

The datasets presented in this study are presented in the supplementary file, and the overall data from the sequencing are deposited in the depository https://www.ncbi.nlm.nih.gov/, accession number PRJNA277848.

Ethics statement

Ethical review and approval was not required for the animal study because it was carried out within the Norwegian animal welfare act guidelines, in accordance with the Animal Welfare Act of 20th December 1974, amended 19th June 2009, at a facility with permission to conduct experiments on fish (code 93) provided by the Norwegian Animal Research Authority (FDU, www.fdu.no). The start-feeding trial was assumed to be a nutrition trial not expected to harm the animals, and no specific permit was required under the guidelines.

Author contributions

All authors developed the experimental concept, contributed to manuscript revisions and approved the submitted version. TV developed the muscle study concept, analyses and she prepared the manuscript. RE and AA were involved in the molecular analyses. TG and EK developed the concept and approach and contributed to the preparation and editing of the manuscript. ØK, KH, and IR performed the experiment.

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Conflict of interest

Author TG is now employed by the company AquaGen AS.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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.2022.814022/full#supplementary-material

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