AQUACULTURE OF EMERGENT MARINE INVERTEBRATES: ADVANCES IN NUTRITION, REARING TECHNOLOGY AND END-PRODUCT QUALITY

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PUBLISHED IN: Frontiers In Marine Science

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-83250-856-5
DOI 10.3389/978-2-83250-856-5

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AQUACULTURE OF EMERGENT MARINE INVERTEBRATES: ADVANCES IN NUTRITION, REARING TECHNOLOGY AND END-PRODUCT QUALITY

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Citation: Lourenço, S., Valente, L. M. P., eds. (2022). Aquaculture of Emergent Marine Invertebrates: Advances in Nutrition, Rearing Technology and End-Product Quality. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83250-856-5

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frontiers | Frontiers in Marine Science

TYPE Editorial
PUBLISHED 22 December 2022
DOI 10.3389/fmars.2022.1119422

OPEN ACCESS

EDITED AND REVIEWED BY
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SPECIALTY SECTION
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

RECEIVED 08 December 2022
ACCEPTED 12 December 2022
PUBLISHED 22 December 2022

CITATION
Lourenço S and Valente LMP (2022) Editorial: Aquaculture of emergent marine invertebrates: Advances in nutrition, rearing technology and end-product quality.
Front. Mar. Sci. 9:1119422.
doi: 10.3389/fmars.2022.1119422

Editorial: Aquaculture of emergent marine invertebrates: Advances in nutrition, rearing technology and end-product quality

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KEYWORDS

nutrition, physiology, rearing technology, aquafeed, bioactive compounds

Editorial on the Research Topic
Aquaculture of emergent marine invertebrates: Advances in nutrition, rearing technology and end-product quality

The growing demand for nutritious healthy seafood with high safety standards drives the aquaculture future. While farmed species of high trophic level are still highly dependent on fishmeal and fish oil that may limit its production growth, marine invertebrates are often species of low trophic level with high market value and high potential to expand. Sea urchins, sea cucumbers and the european clam (*Ruditapes decussatus*) have been extensively investigated, but other species recently emerged as potential candidates for aquaculture diversification, including gammarid crustaceans, jellyfish, tunicates (ascidians) and limpets.

This Research Topic on "Aquaculture of emergent marine invertebrates - Advances in nutrition, rearing technology and end-product quality" gathers nine research articles and a systematic review dedicated to the physiology, nutrition, and rearing technology of marine invertebrate species emergent for aquaculture. The articles addressed key nutritional requirements of various species; novel feed formulations and their implication in specific physiological functions, including the quality of end-product; the impact of climate change related factors in species survival; as well as production technologies to maintain healthy broodstock and improve larvae and juvenile production.

Sea urchins and sea cucumbers have long been identified as candidates to aquaculture diversification. In their work, Suckling et al. analysed the market potential of *A. punctulata* gonads, a common but still unexplored sea urchin in the western Atlantic Ocean. Various diets were tested and shown to be able to promote gonad size with some individuals exhibiting gonads with acceptable colours for European market. Grosso et al. focused on trophic requirements of different life stages of *Paracentrotus lividus*, to help defining suitable and cost-effective diets maximizing somatic growth and gonadal production depending of life cycle stage. A general growth model covering the full post-metamorphic *P. lividus* life cycle was defined for each dietary condition, evidencing a consequent variation of trophic requirements and food energy allocation with increased size. Complementarily, the work conducted by Zuo et al highlighted that lipid dietary sources affect the reproductive performance and early larvae development in *Strongylocentrotus intermedius*. Fish oil was considered the best lipid source based on growth, reproductive performance, survival rate and quality of larvae. Altogether, these results highlight the need for adopting efficient feeding strategies to promote the reproductive performance and gonad quality of sea urchins.

Li et al. investigated the long-term effects of historic diets on the digestive enzyme activities in regenerated intestines of *Apostichopus japonicus* after evisceration (characteristic behaviour of sea cucumber in stressful situations). The results showed that diets provided in earlier stages of sea cucumbers life cycle can affect their digestive physiology and can persist in regenerated intestines producing long-term effects on the growth and metabolism.

The study conducted by Rato et al. evaluated the combined effect of temperature and salinity on mortality and feeding behaviour of European calm, *Ruditapes decussatus*, showing that abrupt reductions in salinity and sharp increases in temperature leaded to high mortality. Juvenile clams were shown to be more sensitive to the increase of temperature in a less saline environment, resisting more to extremely high temperatures under more saline conditions. These results are particularly important under climate change scenarios that predict extreme events of high temperatures and heavy rainfall in the south of Europe, that may compromise the recruitment of European clam.

Castejón et al presented a methodological article with major advances in limpets' larval culture, settlement, and juvenile growth. This research work introduces, adapted and optimized methods to produce, at experimental scale, the native species of the Macaronesia region, *Patella aspera* and *Patella candei*, two limpet species with relevant cultural, gastronomic, and economic importance.

Ribes-Navarro et al. explored the combined effects of diet and environmental factors, on survival, growth, and LC-PUFA content of *Gammarus locusta*. The effects of temperature on the gammarid fatty acids were not evident, with diet being the main modulator of the profiles. The results suggest an ability of *G. locusta* for LC-PUFA biosynthesis (trophic upgrading) and/or retention, making this species a promising source of high-value ingredients for aquafeeds.

Ballesteros and colleagues have focused on the production potential of jellyfish for a variety of applications including biomedical, cosmetic, and pharmacological industries. Ballesteros et al. designed and set up an effective rearing system to produce the early planktonic stages of *Pelagia noctiluca* in both flow-though and closed systems. Moreover, Ballesteros et al. defined optimal feeding conditions for *P. noctiluca* culture. They have guaranteed the culture durability by obtaining a third generation of the species and validated the use captive-bred specimens to produce the toxins of biotechnological interest. Both studies highly contributed to the improvement of breeding techniques, nutrition and production in aquaculture of the most important jellyfish in the Mediterranean Sea.

Finally, a thorough systematic revision was conducted by Marques et al on the potential contribution of tunicates as extractive species, further exploring its potential as sources of nutrients and bioactive compounds for aquafeed. The review showed that ascidians present high filtration and fast growth rates, performing well under an integrated multitrophic aquaculture system framework. They also hold great potential for aquafeed formulations and dietary supplements as sources of n-3 LC PUFA.

Overall, the ten articles included in the Research Topic in Aquaculture of emergent marine invertebrates mirror the high diversity of species candidate for aquaculture; the technological challenges behind their full-life cycle production; the need to identify species nutritional requirements, and to understand the diversity of species physiologic responses to climate change scenarios. But foremost, this collection of manuscripts shows that emergent marine invertebrates have the potential to expand the aquaculture sector and produce high-value products for gastronomic niche markets. The wide range of species considered can also contribute with valuable novel raw materials for the aquafeed industry, but also for a variety of biotechnological, pharmacological and nutraceutical industries.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Acknowledgments

SL acknowledges Fundação para a Ciência e Tecnologia (FCT), through the strategic project LA/P/0069/2020 granted to the Associate Laboratory ARNET and UIDB/04292/2020 and

UIDP/04292/2020 awarded to the MARE-Marine and Environmental Sciences Centre.

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.

Publisher's note

frontiers in Marine Science

ORIGINAL RESEARCH
published: 24 February 2022
doi: 10.3389/fmars.2022.843612

Persistence of Digestive Enzyme Activities of Sea Cucumber (*Apostichopus japonicus*): Evidence From Diet Switch After Evisceration

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OPEN ACCESS

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Specialty section:
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

Received: *26 December 2021*
Accepted: *07 February 2022*
Published: *24 February 2022*

Citation:
Li X, Gao Q, Tian Y, Mei Y, Xu Y, Cao Y, Dong S, Zhou Y, Sun Y and Ju W (2022) Persistence of Digestive Enzyme Activities of Sea Cucumber (Apostichopus japonicus): Evidence From Diet Switch After Evisceration. Front. Mar. Sci. 9:843612. doi: 10.3389/fmars.2022.843612

A two-stage diet-switch experiment was conducted to examine the hypothesis that the changes in digestive enzyme activities of sea cucumber (*Apostichopus japonicus*) induced by historic diets might persist in the regenerated intestines. In stage I, *A. japonicus* were treated with two different diets for 56 days, including diet A with 11% crude protein, 1% crude lipid, and 40% carbohydrate, and diet B with 18% crude protein, 2% crude lipid, and 35% carbohydrate. In stage II, each treatment was subjected to evisceration with 0.35M KCl or not (eviscerated and non-eviscerated groups), half of which were then switched to different diets from diet A to B or vice versa for 112 days. The persistence of digestive enzyme activities was evaluated by measuring the changes in digestive enzyme activities before and after evisceration. In stage I, diets B and A increased trypsin and amylase activities, respectively. In stage II, the higher trypsin activities were observed in eviscerated and non-eviscerated *A. japonicus* that had consumed diet B in stage I. The higher amylase activities were observed only in eviscerated *A. japonicus* that had consumed diet A in stage I. It indicated that the historic diets showed long-term effects on the digestion of *A. japonicus*, which led to the persistence of changes in both trypsin and amylase activities in intestines, especially in the regenerated intestines. In addition, the specific growth rates (SGRs) and metabolic rates (MRs) of *A. japonicus* were affected by the long-term effects of historic diets. Meanwhile, the relationships between enzymic activities, SGRs, and MRs were observed in *A. japonicus*, indicating that the historic diets could produce long-term effects on the growth and metabolism of *A. japonicus* through their long-term effects of historic diets on digestive enzyme activities. In conclusion, the present study showed that the changes in digestive enzyme activities induced by different diets in stage I could persist in the intestines and regenerated intestines, leading to long-term effects of historic diets on the growth and metabolism of *A. japonicus*.

Keywords: trypsin activity, amylase activity, specific growth rate, metabolic rate, carbon stable isotope

INTRODUCTION

The stimulus of historic nutrition can induce temporary or long-term changes in digestive ability and subsequent growth potential and metabolic status in later life of organisms (Patel and Srinivasan, 2002; Vera et al., 2017). This phenomenon originating from mammals is termed nutritional programming (Symonds et al., 2009). In recent decades, nutritional programming has been widely studied and reported in numerous aquaculture species (Fang et al., 2014; Gong et al., 2015; Moghadam et al., 2015; Balasubramanian et al., 2016). For instance, early feeding diets enriched in plant-based lipids negatively influenced the growth and survival of gilthead seabream (*Sparus aurata*) in later life (Turkmen et al., 2015). An acute glucose stimulus during the first feeding period induced the long-term repression of weight gains and disturbed the gluconeogenesis regulation of Siberian sturgeon (*Acipenser baerii*) (Gong et al., 2015). Fang et al. (2014) reported that the activities of amylase were significantly enhanced in adult zebrafish (*Danio rerio*) fed a high-carbohydrate diet than those fed a low-carbohydrate diet in the yolk-sac larval stage, indicating the long-term effects of early high-carbohydrate diets on carbohydrate digestion of zebrafish. Similar observations were also detected in Atlantic salmon (*Salmo salar*) (Vera et al., 2017) and rainbow trout (*Oncorhynchus mykiss*) (Balasubramanian et al., 2016).

So far, studies about the long-term effects induced by historic diets remain limited in aquatic invertebrates, especially for those with the regeneration ability. Sea cucumber (*Apostichopus japonicus*) is considered an economically important species (Yang et al., 2015). It has been widely cultured along the coastal regions of several Asian countries for centuries, including China, Japan, Korea, and Russia, because of its high nutritional and medicinal value (Xia et al., 2015a). In 2011, the annual production of *A. japonicus* in the world was more than 138,000 tons, more than 90% of which originated from aquaculture in China. Until 2021, *A. japonicas* in China achieved nearly 200,000 tons of annual production (Ministry of Agriculture and Rural Affairs of People's Republic of China, 2011, 2021). With the expansion of *A. japonicus* cultivation, more artificial diets were applied in commercial aquaculture to improve their production (Ying et al., 2009; Liao et al., 2015b). Because the detritus of macroalgae and sea mud in the sediment are the main food sources for *A. japonicus*, sea mud and powdered macroalgae are usually used as the main component of artificial diets (Ying et al., 2010; Sudong et al., 2012). Besides, Xia et al. (2015b) found that *A. japonicus* fed a diet with fish meal showed the best growth performance, indicating the important effect of animal-source protein on *A. japonicus*. The optimum protein level of diet fed to *A. japonicus* was 18–24%; hence, fish meal is used as the dietary component to improve the protein level of artificial diets (Zhu et al., 2005; Liao et al., 2015a). Numerous studies reported that the digestive physiology of *A. japonicus* would be affected by different diets, suggesting the flexibility of digestive physiology (Liao et al., 2015b; Wen et al., 2016b). In addition, other fascinating biological behaviors were observed in *A. japonicus*, such as autolysis, aestivation, evisceration, and regeneration (Wang et al., 2015; Ru et al., 2019). Evisceration involves various complex physiological processes and results in the expulsion of the digestive tracts and other viscera (García-Arrarás et al., 1998; Ding et al., 2019). Following evisceration, the new intestine is regenerated from the residual coelomic epithelial cells through the processes of dedifferentiation, migration, redifferentiation, and division (Leibson, 1992; García-Arrarás and Greenberg, 2001; Zang et al., 2012). The persistence of the digestive physiology for the regenerated intestines of *A. japonicus* after evisceration, that is, whether the digestive ability of the regenerated intestine is affected by the historic digestive status before evisceration, remains unclear.

Digestion is one of the crucial metabolic processes in animals because it determines the availability of nutrient requirements (Gisbert et al., 2009). The whole digestive process primarily relies on the types and activities of digestive enzymes (Sveinsdóttir et al., 2006; Nazemroaya et al., 2015). Trypsin is an important serine alkaline protease responsible for protein digestion (Mir et al., 2018). Previous studies examined the trypsin activities to assess the digestion ability of aquatic organisms under different nutritional conditions, such as European sea bass (*Dicentrarchus labrax*) (Parma et al., 2019), olive flounder (*Paralichthys olivaceus*) (Bae et al., 2020), rainbow trout (Kasiga et al., 2020), and sea cucumber (Bai et al., 2015; Wen et al., 2016b). In addition, amylase and lipase are crucial digestive enzymes that can reveal the absorptive capability for dietary carbohydrates and lipids, respectively (Hidalgo et al., 1999; Liao et al., 2015b; Xia et al., 2015a). Previous studies demonstrated that the digestive enzymes, including trypsin, amylase, and lipase, could respond to different diets, which is termed as enzymatic plasticity (Sabat et al., 1999; Liu and Wang, 2007). For example, Bolasina et al. (2006) reported that the changes in diets led to subsequent variations in the trypsin and lipase activities during the ontogenetic development of Japanese flounder (*P. olivaceus*). European sea bass consuming a microparticulate diet showed higher trypsin and amylase activities compared with those consuming natural prey (Infante Zambonino and Cahu, 1994). The adjustment of enzymatic plasticity is crucial for organisms to maintain an appropriate level of digestive ability in response to different environmental conditions and physiological statuses (Fu et al., 2006; Garland, 2011; Kasiga et al., 2020).

Carbon stable isotope analysis is routinely employed to quantify food assimilation, providing details of time-integrated food (Kürten et al., 2013; Vander Zanden et al., 2015; Wen et al., 2016a). The turnover of stable isotope in organisms is generally driven by both growth and metabolism (Macavoy et al., 2006; Tarboush et al., 2006). With known growth performance in terms of specific growth rate (SGR), the metabolic rate (MR) can be estimated based on the simulation of isotopic turnover curves, and the metabolic status of the organism is thus evaluated with stable isotope analysis (Buchheister and Latour, 2010; Antonio and Richoux, 2016). The objectives of the present study were to investigate the long-term effects of historic diets on the digestive enzyme activities of regenerated intestines of *A. japonicus* after evisceration and assess the persistence of digestive physiology and subsequent effects on the growth and metabolism of *A. japonicus* by means of diet switch and stable isotope analysis.

TABLE 1 | Ingredients and nutritional composition of experimental diets fed to *A. japonicus*.

	Diet types	
	Diet A	**Diet B**
Diet ingredients (dry matter %)		
Fish meal [a]	0	10
Sea mud [b]	20	20
Sargassum thunbergii[c]	80	70
Nutritional composition (dry matter %)		
Crude proteins	10.88 ± 0.31^a	17.67 ± 0.51^b
Crude lipids	1.07 ± 0.07^a	1.65 ± 0.12^b
Carbohydrate	40.41 ± 1.25^b	34.55 ± 0.72^a
Ash	47.64 ± 1.14^b	46.13 ± 0.77^a

Data are presented as mean ± SD (n = 5). Different letters in the same row indicate significant differences between diet A and diet B ($P < 0.05$).

[a] Fish meal: crude protein 78.75%, crude lipid 8.25%, and carbohydrate 8.05%.

[b] Sea mud was burned in the Muffle furnace at 500 °C for 6 h to remove organic, after which only ash was in the sea mud.

[c] Sargassum thunbergii: crude protein 13.55 %, crude lipid 1.24 %, and carbohydrate 49.74%.

MATERIALS AND METHODS

Diet Preparation

Numerous studies reported that diets consisting of sea mud, fish meal, and macroalgae, including *S. thunbergii*, *Ulva lactuca*, and *Gracilaria lemaneiformis*, could meet the nutritional requirements and lead to the rapid growth of *A. japonicus* (Ying et al., 2009; Sun et al., 2012; Wen et al., 2016a). Meanwhile, two different types of diets were formulated based on previous studies for the following feed experiment, to avoid the effects of dietary additives on the digestive enzyme activities (Shi et al., 2015; Wen et al., 2016a), and identified as diet A and diet B. Diet A comprised sea mud and *S. thunbergii.* Sea mud was collected from the intertidal zone (Qingdao, Shandong province, China), and *S. thunbergii* was obtained from Xiaoheishan Island (Yantai, Shandong province, China). Besides these contents, commercial fish meal (Qingdao Seven Good Biological Technology Co., Ltd.) was added to diets denoted as diet B. The ingredients of these diets were ground, sieved through a 0.25-mm mesh, mixed adequately, slightly watered, stirred, and extruded into pellets (diameter = 1.8 mm) using a feed processing machine (Tairun Equipment, Jining, China). The pelleted diets were dried at 60°C for 48 h and stored at –20°C for the feeding experiment. The concentrations of nitrogen and carbon elements in diets were measured using an elemental analyzer (Vario EL III; Elementar Co., Germany), and the content of crude protein was estimated as nitrogen concentration multiplied by 6.25. The crude lipid content was analyzed using the Soxhlet extraction method with petroleum ether (Luque De Castro and Priego-Capote, 2010). For ash content measurement, dried diets were combusted at 500°C for 6 h in a muffle furnace (Nabertherm, Germany). Carbohydrate = 100 - crude protein - crude lipid – ash (Seo et al., 2011; Xia et al., 2015a; Hassaan et al., 2018). The crude protein, lipid, and carbohydrate contents of diet A versus diet B were 10.88 vs. 17.67%, 1.07 vs. 1.65%, and 40.41 vs. 34.55%, respectively. The detailed ingredients and nutritional composition of these diets are shown in **Table 1**.

Diet-Switch Experiment

A total of 300 *A. japonicus* juveniles were obtained from Tianheng aquaculture company (Qingdao, Shandong province, China) with an average body weight of 8.80 ± 0.21 g. These individuals were acclimated to the experimental conditions for 2 weeks. Then, the feeding experiment was conducted for two different stages (stage I and stage II). In stage I, all *A. japonicus* were transferred to 2 tanks [2 m × 1.5 m × 0.8 m (L × W × H)], resulting in 150 individuals in each tank. They were classified into two groups, which were fed diet A (group A) and diet B (group B) for 56 days, respectively. Three individuals in each group were randomly sampled and quickly dissected for intestines and body walls after freeze anesthesia at six time points, including days 0, 7, 14, 28, 42, and 56, during the period of the feeding experiment in stage I. The intestines and body walls were immediately stored at −80°C until assayed for digestive enzyme activities and the carbon stable isotope. All *A. japonicus* were starved for 24 h prior to sampling.

Further, 120 individuals in each tank were transferred to 12 glass aquariums [70 cm × 40 cm × 40 cm (L × W × H)] for a further 112-day feeding experiment (stage II). These aquariums from each tank were classified into two groups (six aquariums in each group), which were then subjected to evisceration by the intra-coelomic injection of 0.35M KCl (eviscerated group) or not (non-eviscerated group). The eviscerated *A. japonicus* from groups A and B were named as groups AE and BE while the non-eviscerated individuals from groups A and B were named as groups AN and BN, respectively. Each non-eviscerated and eviscerated group was further divided into two groups (three aquariums in each group), of which one was fed the same diets as in stage I and the other was treated with diets switching from diet A to B or vice versa. Group AN, BN, AE, and BE treated with diet switch or not were renamed as ANA, ANB, BNA, BNB, AEA, AEB, BEA, and BEB, respectively (**Figure 1**).

Finally, three individuals in each non-eviscerated group, including groups ANA, ANB, BNA, and BNB, were sampled on days 7, 14, 28, 49, 70, 91, and 112 in stage II. Because of 21-day intestine regeneration, the sample collections in eviscerated groups (AEA, AEB, BEA, and BEB) were conducted only on days 21, 28, 35, 49, 70, 91, and 112. The sampling tissues and method in stage II were the same as those in stage I. In addition, the initial and final weights of *A. japonicus* were measured in both stages I and II for the following calculation of the body weight gain (BWG), SGR, and growth rate constant (k).

During the whole experiment, *A. japonicus* were fed once a day at 15:00 with a daily ration of 5% wet body weight, based on which the individuals were under the apparent satiation. The residual feed and feces were cleaned by siphoning every day. The temperature was constantly kept at 16.5 ± 0.5°C, the salinity varied between 29 and 31 ppt, the levels of dissolved oxygen were greater than 6.0 mg/ L, and a normal photoperiod 14:10 h (L/D) was applied. The water environmental conditions were kept relatively constant for each aquarium during the experiment.

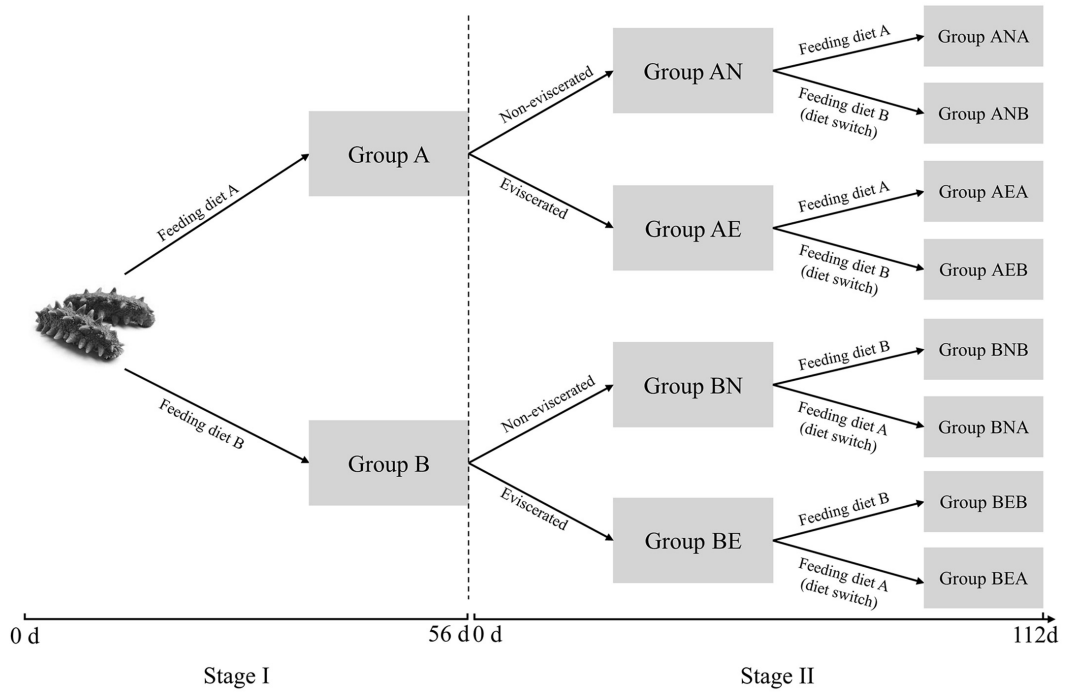

FIGURE 1 | Schematic presentation of diet-switch experiment in *A. japonicus*. The whole experiment was carried out for 168 days and classified into two stages. In stage I, *A. japonicus* were randomly assigned to group A and group B, which were fed diet A and diet B for 56 days, respectively. In stage II, each group was subjected to evisceration or not (eviscerated and non-eviscerated), and switched to different diets from diet A to B or vice versa for 112 days.

Determination of Enzymatic Activity

The intestinal tissues were homogenized for 2 min in a pre-cooled homogenization buffer using a tissue grinder (JXFSTPRP-CL-24; ShangHaiJingXin Co., China). The supernatants were obtained after centrifugation at 20,000*g* for 25 min at 4°C, pipetted into clean centrifuge tubes, and immediately stored at 4°C until analysis (less than 12 h).

The protein concentration, trypsin activities, amylase activities, and lipase activities in the intestines of *A. japonicus* were determined using the standard kits (A080-2, C016, A045-4, and A054-2-1) from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China) (Xionggao et al., 2014; Liao et al., 2015b; Ye et al., 2019). The experimental procedures were conducted following the manufacturer's protocols. Both the trypsin and amylase activities were tested using a spectrophotometer (UV5100, METASH, Shanghai, China). The protein concentrations and lipase activities were determined using a microplate reader (Synergy Mx, Bio Tek, VT, United States). The specific trypsin and amylase activities were expressed as trypsin and amylase unit per mg protein, respectively. The specific lipase activity was expressed as lipase unit per g protein.

Growth Performance

The BWG (%) of *A. japonicus* was calculated as (Liao et al., 2015b):

$$\mathrm{BWG}\,(\%) \;=\; \frac{\mathrm{FBW}-\mathrm{IBW}}{\mathrm{IBW}} \;\times\; 100$$

where IBW and FBW are the initial body weight and final body weight, respectively.

The SGR of *A. japonicus* was calculated as (Giri et al., 2013):

$$\mathrm{SGR}\;(\%\ \text{initial body weight/day}) \;=\; (\ln \mathrm{W_t} - \ln \mathrm{W_0})\ /\mathrm{t} \;\times\; 100$$

where W_t and W_0 are the final and initial weights of individuals, respectively, and t is the duration of the experiment.

Carbon Stable Isotope Analysis

The diets and body walls of *A. japonicus* were freeze-dried for 48 h to constant weight and homogenized. A stable isotope mass spectrometer (EA-IRMS, Thermo Finnigan MAT Delta-plus) was used to determine the ratio of carbon isotope ($\delta^{13}C$) with the accuracy of $\pm$ 0.1‰.

The ratio of carbon stable isotope is generally defined as follows (Peterson and Fry, 1987):

$$\delta^{13}\mathrm{C} \;=\; [(R_{sample}/R_{standard}) \;-\; 1] \;\times\; 1000‰$$

where R is the ratio of ^{13}C and ^{12}C.

The stable isotope turnover model is defined as follows (Hesslein et al., 1993):

$$C_t \;=\; C_f \;+\; (C_0 - C_f)e^{-\,(k\,+\,m)t}$$

where C_t represents the carbon stable isotope ratio of biological tissue on day t. C_f represents the ratio of stable isotope when diets

and tissue turnover reach a balance after dietary transformation. C_0 represents the carbon stable isotope ratio of the initial biological tissue. k is the growth rate constant, and m is the metabolism rate constant. $k+m$ is the isotopic turnover rate.

The growth rate constant (k) was calculated as follows (Buchheister and Latour, 2010):

$$k\,(\mathrm{d}^{-1}) = (\ln W_f - \ln W_i)/t$$

where W_f and W_i represent the final and initial weights of *A. japonicus,* respectively, and t is the duration of the experiment in days.

The isotopic turnover rate ($k+m$) was obtained by fitting the exponential model to match the observed isotopic data, and the metabolism rate constant (m) was thus obtained by means of $k+m$ minus k (Winter et al., 2019).

Statistical Analysis

Significant differences in nutritional composition between diet A and diet B were determined using the Student's *t*- test. For stage I, the differences in the digestive enzyme activities between groups fed different diet types and between sampling times were compared with a two-way analysis of variance (ANOVA) and the differences in the FW, BWG, SGRs, and MRs at the end of stage I between different diet types were compared with the Student's *t*- test. For stage II, a four-way ANOVA was applied to explore the effects of diet in stage I, diet in stage II, evisceration, and sampling time on the digestive enzyme activities, while a three-way ANOVA was used to compare the differences in FW, BWG, SGRs, and MRs at the end of stage II between diet in stage I, diet in stage II and evisceration. For ANOVA, if the interaction of main factors was detected, a one-way ANOVA or the Student's *t*- test was conducted to examine the effects of one main factor at the specific level of the other main factor(s). The factors detected to be significant by ANOVAs were further analyzed using a Bonferroni multiple comparison procedure, which adjusted the observed significance level by multiplying it by the number of comparisons being made (Zar, 1999; Norusis, 2008). Pearson correlation analysis was used to examine the relationships of SGRs and MRs to the digestive enzyme activities. A P value of 0.05 was used as the significance level. Before statistical analyses, raw data were diagnosed for the normality of distribution and homogeneity of variance with the Kolmogorov–Smirnov test and Levene's test, respectively. The statistical analysis was conducted using IBM SPSS Statistics (Version 25) and Origin (Version 9.1) software.

RESULTS

Enzymatic Activities

The digestive enzyme activities of *A. japonicus* fed different diets in stage I are presented in **Figure 2**. Similar trends of trypsin activities of *A. japonicus* were observed in groups A and B (**Figure 2A**). Specifically, the trypsin activities of *A. japonicus* in groups A and B first increased, reaching the peak on day 42 and then decreased on day 56. *A. japonicus* fed diet B showed significantly higher trypsin activities than those fed diet A in stage I ($P < 0.05$). The amylase activities

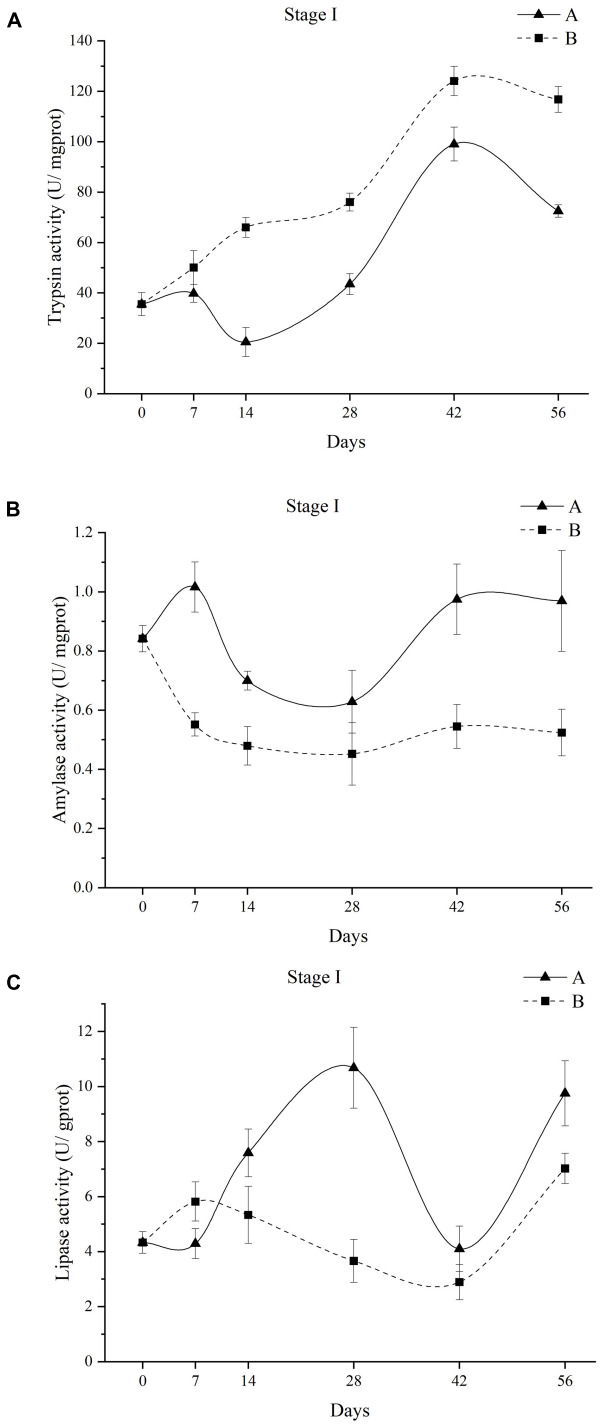

FIGURE 2 | Changes in trypsin activities of *A. japonicus* feeding different diets in stage I **(A)**; Changes in amylase activities of *A. japonicus* feeding different diets in stage I **(B)**. Changes in lipase activities of *A. japonicus* feeding different diets in stage I **(C)**. Data are presented as mean ± SD (n = 3).

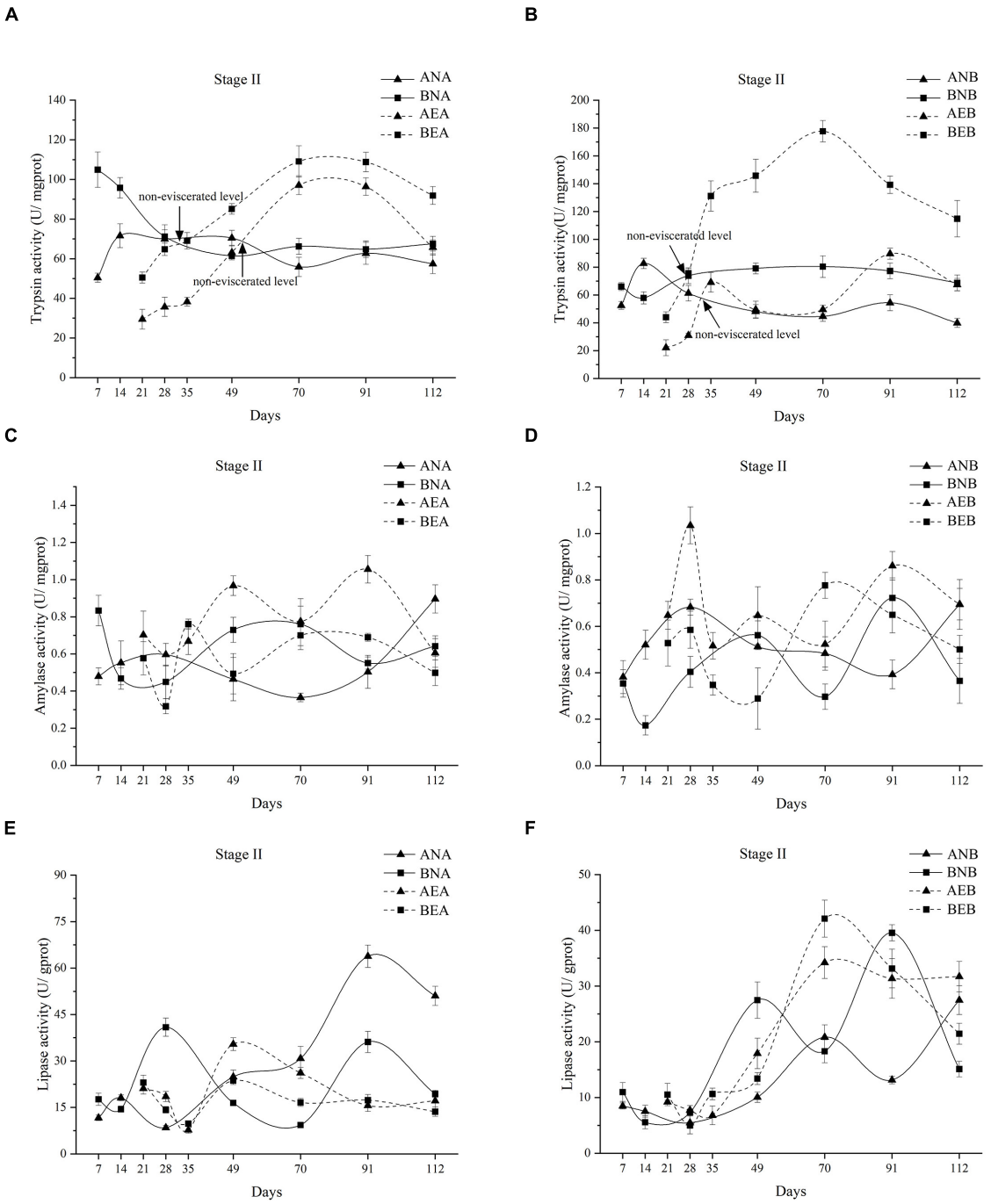

FIGURE 3 | Changes in trypsin activities of *A. japonicus* feeding different diets in stage II **(A,B)**. Non-eviscerated levels of trypsin activities indicate the intersection of the eviscerated group and its corresponding non-eviscerated group. Changes in amylase activities of *A. japonicus* feeding different diets in stage II **(C,D)**. Changes in lipase activities of *A. japonicus* feeding different diets in stage II **(E,F)**. Data are presented as mean ± SD (n = 3).

of *A. japonicus* in group A showed an abrupt increase in the first 7 days and then gradually decreased until day 28, followed by an increasing trend to the end of stage I (**Figure 2B**). The amylase activities of *A. japonicus* in group B decreased first and then showed a slight fluctuation until the end of stage I. Meanwhile, the amylase activities of *A. japonicus* in group A were higher than those of individuals in group B ($P < 0.05$). As shown in **Figure 2C**, the lipase activities of *A. japonicus* in group A quite fluctuated throughout stage I, with the highest value on day 28 and the lowest on day 42. For *A. japonicus* in group B, an increase in the lipase activity was observed before 7 days. After gradually decreasing between

TABLE 2 | The growth performance of *A. japonicus* in stage I.

Groups	Final body weight	Body weight gain
A	14.96 ± 0.44^{a}	70.00 ± 1.08^{a}
B	17.21 ± 0.72^{b}	95.52 ± 3.45^{b}

Data are presented as mean ± SD (n = 3). The letters a and b indicate significant differences between different groups at the significance level of 0.05 ($P < 0.05$).

day 7 and day 42, a further increase in lipase activities was observed until the end of stage I. *A. japonicus* fed diet A showed higher lipase activities than those fed diet B from day 14 to the end of stage I.

As shown in **Figure 3A**, the individuals in the non-eviscerated and eviscerated groups presented similar trends. *A. japonicus* in group BNA showed significantly higher trypsin activities than those in group ANA, except on days 28, 49, and 91 ($P < 0.05$, **Figure 3A**). The trypsin activities of *A. japonicus* in group BEA were significantly higher than those of individuals in group AEA ($P < 0.05$, **Figure 3A**), although this difference was non-significant on day 70. The trypsin activities of *A. japonicus* in group BEA increased rapidly and returned to non-eviscerated levels in less time than the activities of those in group AEA. As shown in **Figure 3B**, little changes were found in trypsin activities of *A. japonicus* in groups ANB and BNB. In contrast, the trypsin activities in eviscerated groups, particularly group BEB, greatly changed. *A. japonicus* in groups BNB and BEB exhibited significantly higher trypsin activities than those in groups ANB and AEB ($P < 0.05$). For eviscerated *A. japonicus*, the trypsin activities in group BEB increased rapidly and took 28 days to reach non-eviscerated levels, while the trypsin activities in group AEB returned to non-eviscerated levels after 35 days.

Dramatic fluctuations were observed in the amylase activities of *A. japonicus* in stage II (**Figures 3C,D**). Significant differences were found in amylase activities between *A. japonicus* in group ANA and BNA on days 7, 49, 70, and 112, but the amylase activities of *A. japonicus* in group ANA were higher than those of individuals in group BNA only on day 112 ($P < 0.05$, **Figure 3C**). For eviscerated *A. japonicus*, significantly higher amylase activities were observed in group AEA than in group BEA on days 28, 49, and 91 ($P < 0.05$, **Figure 3C**). As shown in **Figure 3D**, the amylase activities of *A. japonicus* in group ANB were higher than those of individuals in group BNB on days 14, 28, 70, and 112 ($P < 0.05$). *A. japonicus* in group AEB exhibited higher amylase activities than those in group BEB except on days 21 and 70 ($P < 0.05$).

The lipase activities of *A. japonicus* fed diet A in stage II fluctuated; only the lipase activities of *A. japonicus* in group ANA gradually increased during stage II (**Figure 3E**). *A. japonicus* in group ANA showed significantly higher lipase activities than those in group BNA from day 49 to the end of stage II ($P < 0.05$, **Figure 3E**). For eviscerated *A. japonicus*, similar values of lipase activities were observed in groups AEA and BEA during stage II except for days 28, 49, and 70, with higher lipase activities in group AEA than in group BEA ($P < 0.05$, **Figure 3E**). As shown in **Figure 3F**, the lipase activities of all *A. japonicus* fed diet B in stage II increased during the whole stage II. Notably, the lipase activities of *A. japonicus* in group ANB were significantly higher than those of individuals in group BNB only on day 112 ($P < 0.05$). The higher lipase activities were found in *A. japonicus* in group AEB than those in group BEB only on days 28 and 112 ($P < 0.05$).

Growth Performance

The FBW and BWG of *A. japonicus* in stage I are shown in **Table 2**. *A. japonicus* in group B showed significantly higher FBW and BWG than those in group A ($P < 0.05$). As shown in **Table 3**, diet in stage I, diet in stage II, and evisceration showed an influence on the FBW and BWG of *A. japonicus*, but their interaction did not affect the FBW and BWG. *A. japonicus* fed diet B, regardless of the stage, exhibited significantly higher FBW and BWG compared with those fed diet A in stage I or II ($P < 0.05$). The FBW of non-eviscerated *A. japonicus* was significantly higher rather than that of eviscerated *A. japonicus*, while the BWG of non-eviscerated *A. japonicus* was significantly lower than that of eviscerated *A. japonicus* ($P < 0.05$). The growth rates of *A. japonicus* in stage I are presented in **Table 4**. *A. japonicus* in group B showed a significantly higher growth rate than those in group A ($P < 0.05$). The growth performance of *A. japonicus* in stage II is shown in **Table 4**. The growth rates of *A. japonicus* in groups BNA, BNB, BEA, and BEB were higher than those of individuals in groups ANA, ANB, AEA, and AEA, respectively, despite non-significant differences in *A. japonicus* fed the same diet in stage II ($P > 0.05$).

Time-Dependent Isotopic Turnover and Metabolism

The carbon isotope ratios of diet A and B were measured as -17.97 ± 0.06 and -18.09 ± 0.05, respectively. The trends of carbon isotope ratios in the body walls of *A. japonicus* were determined and are visualized in **Figure 4**. In stage I, group A not only exhibited higher carbon isotope ratios but also responded more quickly than group B (**Figure 4A**). In stage II, different trends of carbon isotope ratios were observed in different groups, which were caused by the historic and new diets (**Figures 4B–E**). However, the carbon isotope ratios of *A. japonicus* fed diet A in stage I were always higher than those of individuals fed diet B in stage I. The changes in ^{13}C were modeled as functions of time (time models). The related parameters for different groups are presented in **Table 4**. In stage I, the higher MR was observed in *A. japonicus* in group A than in group B ($P < 0.05$). In stage II, *A. japonicus* in groups ANB, AEA, and AEB exhibited significantly higher MR than in groups BNB, BEA, and BEB, respectively ($P < 0.05$).

Relationships Between Enzymic Activities, SGRs, and MRs

Linear regression was applied to examine the relationship between enzymic activities, SGRs, and MRs (**Figure 5**). A positive relationship was identified between the trypsin activities and

SGRs ($P < 0.05$), but a non-significant relationship was found between trypsin activities and MRs (**Figures 5A,B**). The amylase activities of *A. japonicus* negatively correlated with the SGRs but positively correlated with the MRs of *A. japonicus* ($P < 0.05$, **Figures 5C,D**). No significant relationships between lipase activities, SGRs, and MRs were observed in *A. japonicus* (**Figures 5E,F**).

DISCUSSION

The present study explored the changes in the digestive enzyme activities, SGRs, and MRs of *A. japonicus* resulting from diet switch before and after evisceration. It revealed that the short-term plasticity of trypsin and amylase in response to historic diets would persist in non-regenerated and regenerated intestines, suggesting the long-term effect of historic diets on the digestion of *A. japonicus*. The correlation between the digestive enzyme activities, SGRs, and MRs suggested the effects of the changes in enzymatic activities induced by different diets on the SGR and MR of *A. japonicus*. This study provided a comprehensive view of the persistence of the plasticity of digestive enzymes in regenerated intestines and revealed the long-term effects of historic diets on digestion, growth, and metabolism of *A. japonicus*.

In stage I, enzymatic activities induced by distinct diets were different. High trypsin and amylase activities were observed in *A. japonicus* fed diet B and *A. japonicus* fed diet A, respectively. It was concluded that the activities of trypsin and amylase were positively affected by the dietary protein and carbohydrate contents, respectively, which were largely consistent with previous studies about white shrimp (*Litopenaeus vannamei*) (Muhlia-Almazan et al., 2003), sea bass (Peres et al., 1996), and large yellow croaker (*Pseudosciaena Crocea*) (Yu et al., 2012). And Xia et al. (2015a) also reported that the amylase activity of *A. japonicus* increased with the elevating dietary carbohydrate levels from 25.61 to 45.31%. However, the lipase activities of *A. japonicus* in group B fed a lipid-rich diet (diet B) were lower than those of group A fed a low-lipid diet (diet A), which was contrary to the previous study that the increasing lipase activities of *A. japonicus* were accompanied by the enhanced dietary lipid levels (Liao et al., 2015a). The positive relationship between lipase activities and dietary lipid content in that study was determined based on the changes in lipase activities of *A. japonicus* fed the isonitrogenous diet. Thus, in the present study, the changes in lipase activities of *A. japonicus* fed different diets might be not only affected by dietary lipids but also other dietary nutrients, which was furtherly supported by previous findings that the dietary proteins, carbohydrates, and lipids had interactive effects on the lipase activities (Xia et al., 2015a; Huang et al., 2019). According to the changes in digestive enzyme activities induced by different diets, great plasticity of the digestive enzymes under exposure to different diets was found in *A. japonicus* in stage I.

In stage II, although *A. japonicus* in groups BNA and ANA, groups BNB and ANB, groups BEA and AEA, as well as groups BEB and AEB were fed the same diet, higher trypsin activities were observed in groups BNA, BNB, BEA, and BEB than in groups ANA, ANB, AEA, and AEB, respectively. It suggested that the plasticity of trypsin induced by diet in stage I could persist in the non-regenerated and regenerated intestines. The persistence of plasticity was also detected in the amylase activities of eviscerated *A. japonicus*. In detail, *A. japonicus* in group AEA showed higher amylase activities than those in group BEA despite all individuals fed diet A in stage II. Also, *A. japonicus* in group AEB showed higher amylase activities than those in group BEB despite individuals fed diet B in stage II. The persistence of digestion plasticity has already been reported at molecular levels by Geurden et al. (2007), who found that the early hyperglucidic diet could rapidly increase the expression of α-amylase of larvae rainbow trout, and for juveniles subjected to the challenge test with a 25% dextrin diet, the higher expression of α-amylase was observed in rainbow trout that experienced the hyperglucidic stimulus compared with those fed a hypoglucidic diet at first feeding, suggesting that short-term digestive plasticity of fish resulting from dietary carbohydrates in the early feeding stage would persist in the later life. Therefore, in the present study, it was hypothesized that the genes related to trypsin and amylase activities could be affected by diet in stage I and subsequently regulated the trypsin activities and amylase activities in stage II. However, further studies need to be conducted to prove this hypothesis. Additionally, the lipase activities of *A. japonicus* exhibited no persistence of plasticity, which might be explained by the poor demand of dietary lipids and low lipase activities of *A. japonicus* (Liao et al., 2015a; Ye et al., 2019).

Changes in the digestive enzyme activities of *A. japonicus* before and after evisceration indicated the long-term effects of historic diets on digestion; the long-term effects of diets have been reported by numerous studies. For example, rainbow trout fry fed a plant-based diet for a short time enhanced the acceptance and use of a plant-based diet during their later life (Geurden et al., 2013; Balasubramanian et al., 2016). Besides, the early hyperglucidic diet stimuli showed long-term effects on carbohydrate digestion of rainbow trout in later life, which resulted in the persistence of amylase plasticity of the organism (Geurden et al., 2007). Similarly, in the present study, the long-term effects of diet in stage I on trypsin and amylase activities led to the persistence of these two enzymatic activities of *A. japonicus*. In addition, the trypsin activities of eviscerated *A. japonicus* fed diet B in stage I returned to the non-eviscerated levels in less time than those fed diet A in stage I, regardless of which diet was fed to *A. japonicus* in stage II. This finding suggested that the high-protein diet (diet B) in stage I could accelerate the recovery of trypsin activities of eviscerated *A. japonicus*, which further confirmed the positive long-term effects of historic diets on the digestion of *A. japonicus*. Moreover, the trypsin activities of non-eviscerated *A. japonicus* in stage II were affected by the historic diets in stage I, while for eviscerated *A. japonicus*, both trypsin and amylase activities were affected by diet in stage I, suggesting that the digestion of regenerated intestines was more susceptible to the long-term effect of historic diets compared with that of non-regenerated intestines.

For the growth performance of *A. japonicus* in stage I, *A. japonicus* in group B possessed higher FBW, BWG, and

TABLE 3 | The growth performance of *A. japonicus* in stage II.

Source	*df*	*P*	Multiple comparison					
			Diet in stage I		Diet in stage II		Non-eviscerated (N) or eviscerated (E)	
			A	B	A	B	N	E
Final body weight								
Diet in stage I	1	< 0.001	35.25 ± 6.09^{a}	47.22 ± 7.97^{b}	37.62 ± 7.56^{a}	44.85 ± 9.64^{b}	45.93 ± 8.46^{b}	36.54 ± 7.68^{a}
Diet in stage II	1	< 0.001						
Evisceration	1	< 0.001						
Diet in stage I*II	1	NS						
Diet in stage I* Evisceration	1	NS						
Diet in stage II* Evisceration	1	NS						
Diet in stage I*II*Evisceration	1	NS						
Body weight gain								
Diet in stage I	1	0.038	214.13 ± 56.52^{a}	240.07 ± 63.86^{b}	205.88 ± 50.27^{a}	248.33 ± 62.56^{b}	185.06 ± 41.98^{a}	269.14 ± 44.04^{b}
Diet in stage II	1	0.002						
Evisceration	1	< 0.001						
Diet in stage I*II	1	NS						
Diet in stage I* Evisceration	1	NS						
Diet in stage II* Evisceration	1	NS						
Diet in stage I*II*Evisceration	1	NS						

See the text for a detailed description of the model. df represents the degree of freedom. The letters a and b indicate significant differences between different groups at the significance level of 0.05 ($P < 0.05$).

TABLE 4 | Summary of time-based stable isotope turnover models and related growth and metabolism parameters for body walls of *A. japonicus*.

	Groups	Equation	R^2	$k + m$	k (d^{-1})	m (d^{-1})
Groups in stage I	A	$C_t = -18.33\text{-}0.94e^{-0.04704t}$	0.55*	0.04704	0.0095 ± 0.0005^{a}	0.0375 ± 0.0005^{b}
	B	$C_t = -18.67\text{-}0.60e^{-0.03970t}$	0.80*	0.03970	0.0120 ± 0.0007^{b}	0.0277 ± 0.0007^{a}
Non- eviscerated groups in stage II	ANA	$C_t = -18.10\text{-}0.26e^{-0.02696t}$	0.56*	0.02696	0.0082 ± 0.0007^{a}	0.0187 ± 0.0007^{a}
	BNA	$C_t = -18.15\text{-}0.36e^{-0.04052t}$	0.54*	0.04052	0.0088 ± 0.0007^{a}	0.0317 ± 0.0007^{b}
	ANB	$C_t = -18.18\text{-}0.22e^{-0.02933t}$	0.86*	0.02933	0.0090 ± 0.0008^{a}	0.0203 ± 0.0008^{b}
	BNB	$C_t = -18.27\text{-}0.36e^{-0.01299t}$	0.78*	0.01299	0.0109 ± 0.0009^{a}	0.0021 ± 0.0009^{a}
Eviscerated groups in stage II	AEA	$C_t = -18.18\text{-}0.37e^{-0.04645t}$	0.81*	0.04645	0.0130 ± 0.0009^{a}	0.0335 ± 0.0009^{b}
	BEA	$C_t = -18.19\text{-}0.41e^{-0.03838t}$	0.71*	0.03838	0.0140 ± 0.0012^{a}	0.0244 ± 0.0012^{a}
	AEB	$C_t = -18.20\text{-}0.35e^{-0.03773t}$	0.79*	0.03773	0.0149 ± 0.0010^{a}	0.0228 ± 0.0010^{b}
	BEB	$C_t = -18.38\text{-}0.22e^{-0.03007t}$	0.61*	0.03007	0.0160 ± 0.0009^{a}	0.0141 ± 0.0009^{a}

*t represents the experimental time in days. C_t represents the carbon stable isotope ratio on day t. R^2 represents the goodness of fitting for time-based stable isotope turnover model. * means significant correlation at the significance level of 0.05 ($P < 0.05$). $k + m$ represents the isotopic turnover rate. k represents growth rate constant and m metabolic rate constant. See the text for a detailed description of the model. The letters a and b indicate significant differences between different groups at the significance level of 0.05 ($P < 0.05$).*

SGR than those in group A. Previous studies documented that adequate dietary protein contributed to the growth of *A. japonicus* (Seo et al., 2011; Li et al., 2021). For MR in stage I, *A. japonicus* fed diet B exhibited lower MR than those fed diet A, which might be related to the low carbohydrate content of diet B. Increased MR in response to high-carbohydrate diets was observed in the present study, which was consistent with that in Southern catfish (*Silurus meridionalis*) and black carp (*Mylopharyngdon piceus Richardson*) (ShiJian and XieXiao, 2007; Cai et al., 2010). Moreover, the negative relationship between MR and SGR of *A. japonicus* was reported by Xia et al. (2015a), in which *A. japonicus* fed a diet consisting of 75% *S. thunbergii* and 25% soybean meal showed higher MR but lower SGR than those of *A. japonicus* fed a diet consisting of 100% *S. thunbergii*. It suggested that the growth rate of *A. japonicus* would be restricted when more energy was provided to meet metabolic requirements.

In stage II, eviscerated *A. japonicus* exhibited higher BWG but lower FBW compared with the non-eviscerated *A. japonicus*. It was consistent with the previous finding that the growth performance would be rapidly improved after the intestinal regeneration of *A. japonicus*, although the body weights of eviscerated *A. japonicus* could not exceed that of non-eviscerated individuals due to huge loss weights caused by evisceration (Zang et al., 2012). The growth performance of *A. japonicus* in stage II was affected by not only evisceration

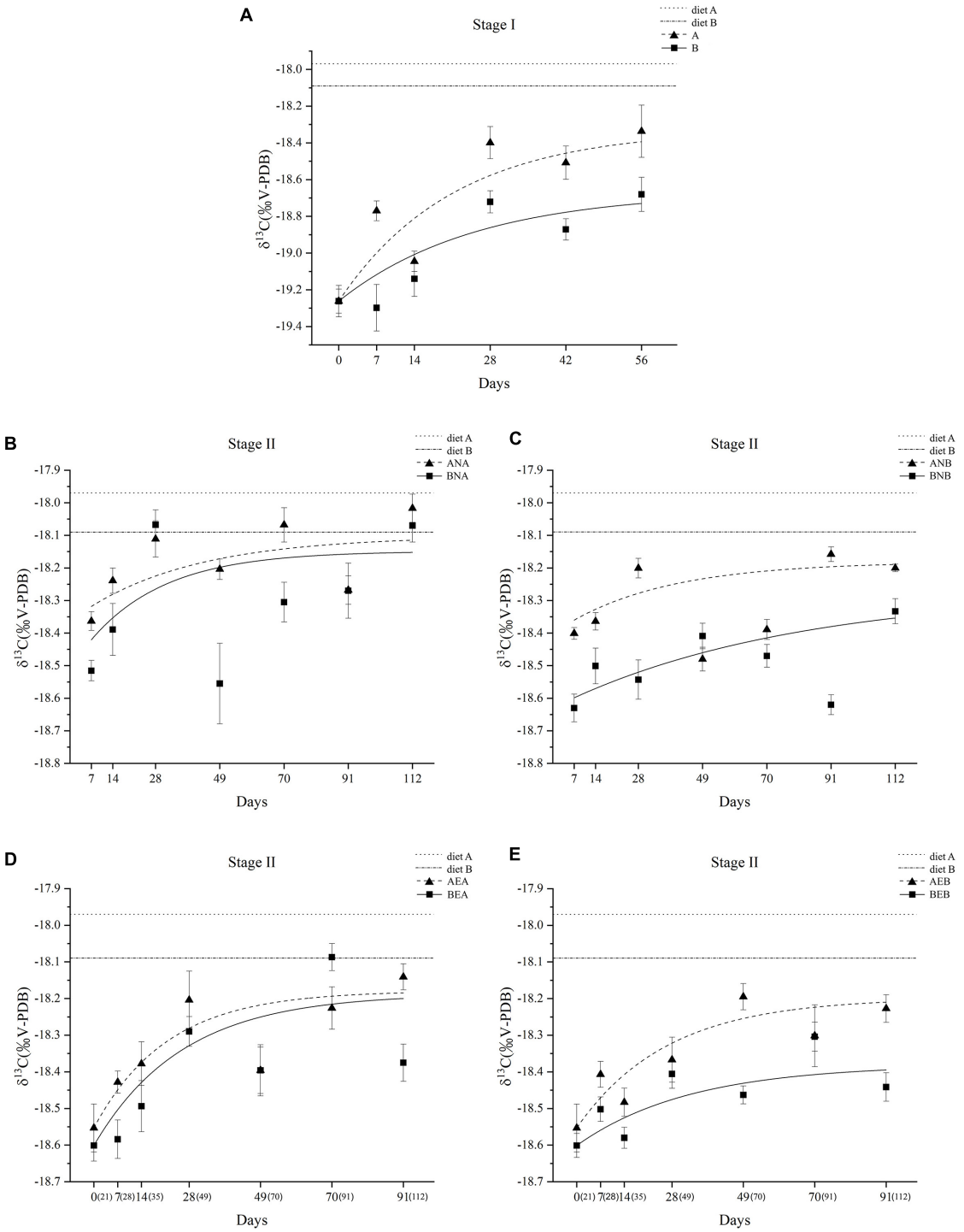

FIGURE 4 | Changes in observed $\delta^{13}C$ values of body walls of *A. japonicus* in stage I **(A)** Changes in observed $\delta^{13}C$ values of body walls of *A. japonicus* in stage II **(B-E)**. Data are presented as mean ± SD (n = 3).

but also the diet types in different stages. Diet B as diet in stages I or II would enhance FBW, BWG, and SGRs of all *A. japonicus* in stage II. Especially for *A. japonicus* fed diet B in stage II, it was more beneficial for their subsequent growth performance if diet B was included in stage I. However, for non-eviscerated or eviscerated groups, the differences in SGRs of *A. japonicus* fed the same diet in stage II were non-significant. It suggested that *A. japonicus* showed similar

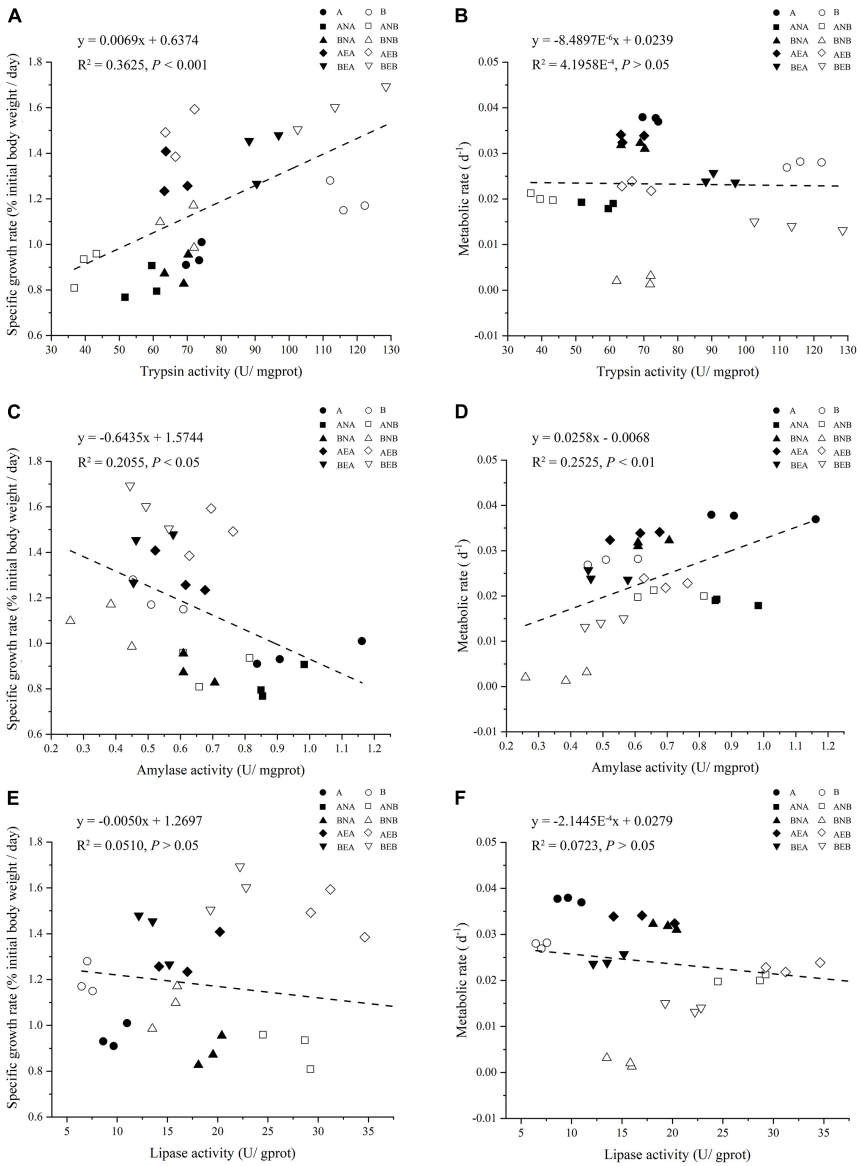

FIGURE 5 | Relationships between trypsin activities, specific growth rates, and metabolic rates of *A. japonicus* **(A,B)**; Relationships between amylase activities, specific growth rates, and metabolic rates of *A. japonicus* **(C,D)**; Relationships between lipase activities, specific growth rates, and metabolic rates of *A. japonicus* **(E,F)**.

SGRs when they consumed the same diet, and the SGRs of *A. japonicus* were slightly affected by the long-term effects of the historic diets.

The changes in MRs of *A. japonicus* fed the same diet in stage II indicated that the MRs of *A. japonicus* seemed to be affected by diet in stage I, which was in agreement with previous findings that the nutritional history had major impacts on metabolic processes to improve fish performance (Vera et al., 2017). Besides, Lage et al. (2020) also demonstrated that the early dietary restriction resulted in a long-term modification of the metabolism of white shrimp by regulating the mRNA levels. In addition, the enzymatic activities are important for digesting and assimilating nutrients and are closely associated with growth performance and metabolic status (Sunde et al., 2001; Rungruangsak-Torrissen et al., 2006). In the present study, the SGRs and MRs of *A. japonicus* were positively affected by trypsin and amylase activities, respectively, which were consistent with the positive relationship between trypsin activities and body weight observed in Atlantic salmon (Rungruangsak-Torrissen et al., 2006). Besides, Rungruangsak-Torrissen et al. (2006) and Murashita et al. (2013) reported that the changes in digestive enzyme activities could be applicable as the indicators for growth and metabolic studies of fish to reflect the growth and metabolism during the development of organisms. Therefore, in the present study, based on the relationship between digestive enzyme activities, growth, and

metabolism, the historic diets could produce long-term effects on the growth and metabolism of *A. japonicus* by affecting the enzyme activities in stage II.

In summary, the persistence of digestive enzyme activities induced by historic diets was found in non-eviscerated and eviscerated *A. japonicus*, indicating the long-term effects of historic diets on the digestion of *A. japonicus*. The digestion of regenerated intestines was more susceptible to the long-term effects of historic diets than those of non-regenerated intestines. The changes in SGRs and MRs, as well as their relationships with digestive enzyme activities, revealed that the historic diets showed long-term effects on the growth and metabolism of *A. japonicus* through long-term effects of historic diets on digestive enzyme activities. Further studies are required to investigate the molecular mechanism of the persistence of digestive enzyme activities and the long-term effects of historic diets on *A. japonicus*.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

XL was in charge of feeding experiments, data analysis, and manuscript writing. QG and YT were in charge of experimental guidance and manuscript revision. YM, YX, and YC were in charge of feeding experiments. All authors designated in this manuscript actively participated in this research.

FUNDING

This study presented in the manuscript was funded by the National Natural Science Foundation of China (Grant No. 31672657) and Taishan Industrial Leading Talent Program (Project No. LJNY201710).

ACKNOWLEDGMENTS

Thanks to Tianheng aquaculture company for the supply of sea cucumber. Meanwhile, thanks to the postgraduates of the Function Laboratory for Marine Fisheries Science and Food Production Processes for their assistance in sample collection.

REFERENCES

Antonio, E. S., and Richoux, N. B. (2016). Influence of diet on the metabolic turnover of stable isotope ratios and fatty acids in the omnivorous shrimp *Palaemon peringueyi*. *Mar. Biol.* 163, 1–12. doi: 10.1007/s00227-016-2930-y

Bae, J., Hamidoghli, A., Won, S., Choi, W., Lim, S., Kim, K., et al. (2020). Evaluation of seven different functional feed additives in a low fish meal diet for olive flounder, *Paralichthys olivaceus*. *Aquaculture* 525:e735333. doi: 10.1016/j.aquaculture.2020.735333

Bai, Y., Zhang, L., Liu, S., Ru, X., Xing, L., Cao, X., et al. (2015). The effect of salinity on the growth, energy budget and physiological performance of green, white and purple color morphs of sea cucumber, *Apostichopus japonicus*. *Aquaculture* 437, 297–303. doi: 10.1016/j.aquaculture.2014.12.020

Balasubramanian, M. N., Panserat, S., Dupont-Nivet, M., Quillet, E., Montfort, J., Le Cam, A., et al. (2016). Molecular pathways associated with the nutritional programming of plant-based diet acceptance in rainbow trout following an early feeding exposure. *BMC Genomics* 17:449. doi: 10.1186/s12864-016-2804-1

Bolasina, S., Pérez, A., and Yamashita, Y. (2006). Digestive enzymes activity during ontogenetic development and effect of starvation in Japanese flounder, *Paralichthys olivaceus*. *Aquaculture* 252, 503–515. doi: 10.1016/j.aquaculture.2005.07.015

Buchheister, A., and Latour, R. J. (2010). Turnover and fractionation of carbon and nitrogen stable isotopes in tissues of a migratory coastal predator, summer flounder (*Paralichthys dentatus*). *Can. J. Fish. Aquat. Sci.* 67, 445–461. doi: 10.1139/F09-196

Cai, C. F., Ye, Y. T., Chen, L. Q., Qin, J. G., and Wang, Y. L. (2010). Oxygen consumption and ammonia excretion of black carp (*Mylopharyngdon piceus* Richardson) and allogynogenetic crucian carp (*Carassius auratus gibelio* female x *Cyprinus carpio* male) fed different carbohydrate diets. *Fish Physiol. Biochem.* 36, 1191–1198. doi: 10.1007/s10695-010-9398-3

Ding, K., Zhang, L., Sun, L., Lin, C., Feng, Q., Zhang, S., et al. (2019). Transcriptome analysis provides insights into the molecular mechanisms responsible for evisceration behavior in the sea cucumber *Apostichopus japonicus*. *Comp. Biochem. Physiol. D-Genomics Proteomics* 30, 143–157. doi: 10.1016/j.cbd.2019.02.008

Fang, L., Liang, X., Zhou, Y., Guo, X., He, Y., Yi, T., et al. (2014). Programming effects of high-carbohydrate feeding of larvae on adult glucose metabolism in zebrafish, *Danio rerio*. *Br. J. Nutr.* 111, 808–818. doi: 10.1017/S0007114513003243

Fu, X., Xue, C., Miao, B., Li, Z., and Gao, X. (2006). Distribution and seasonal activity variation of proteases in digestive tract of sea cucumber *Stichopus japonicus*. *Fish. Sci.* 72, 1130–1132. doi: 10.1111/j.1444-2906.2006.01268.x

García-Arrarás, J. E., Estrada-Rodgers, L., Santiago, R., Torres, I. I., Díaz-Miranda, L., and Torres-Avillán, I. (1998). Cellular mechanisms of intestine regeneration in the sea cucumber, *Holothuria glaberrlma* Salenka (*Holothuroidea: Echinodermata*). *J. Exp. Zool.* 281, 288–304. doi: 10.1002/(SICI)1097-010X(19980701)281:43.0.CO;2-K

García-Arrarás, J. E., and Greenberg, M. J. (2001). Visceral regeneration in holothurians. *Microsc. Res. Tech.* 55, 438–451. doi: 10.1002/jemt.1189

Garland, T. (2011). The flexible phenotype: A Body-Centred integration of ecology, physiology, and behaviour. *Anim. Behav.* 82, 609–610. doi: 10.1016/j.anbehav.2011.06.012

Geurden, I., Aramendi, M., Zambonino-Infante, J., and Panserat, S. (2007). Early feeding of carnivorous rainbow trout (*Oncorhynchus mykiss*) with a hyperglucidic diet during a short period: Effect on dietary glucose utilization in juveniles. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 292, R2275–R2283. doi: 10.1152/ajpregu.00444.2006

Geurden, I., Borchert, P., Balasubramanian, M. N., Schrama, J. W., Dupont-Nivet, M., Quillet, E., et al. (2013). The positive impact of the Early-Feeding of a Plant-Based diet on its future acceptance and utilisation in rainbow trout. *PLoS One* 8:e83162. doi: 10.1371/journal.pone.0083162

Giri, S. S., Sukumaran, V., and Oviya, M. (2013). Potential probiotic *Lactobacillus plantarum* VSG3 improves the growth, immunity, and disease resistance of tropical freshwater fish, *Labeo rohita*. *Fish Shellfish Immunol.* 34, 660–666. doi: 10.1016/j.fsi.2012.12.008

Gisbert, E., Gimenez, G., Fernandez, I., Kotzamanis, Y., and Estevez, A. (2009). Development of digestive enzymes in common dentex *Dentex dentex* during early ontogeny. *Aquaculture* 287, 381–387. doi: 10.1016/j.aquaculture.2008.10.039

Gong, G., Xue, M., Wang, J., Wu, X., Zheng, Y., Han, F., et al. (2015). The regulation of gluconeogenesis in the Siberian sturgeon (*Acipenser during*)affected later in life by a short-term high-glucose programming during early life. *Aquaculture* 436, 127–136. doi: 10.1016/j.aquaculture.2014.10.044

Hassaan, M. S., Mahmoud, S. A., Jarmolowicz, S., El-Haroun, E. R., Mohammady, E. Y., and Davies, S. J. (2018). Effects of dietary baker's yeast extract on the

growth, blood indices and histology of Nile tilapia (*Oreochromis niloticus* L.) fingerlings. *Aquac. Nutr.* 24, 1709–1717. doi: 10.1111/anu.12805

Hesslein, R. H., Hallard, K. A., and Ramlal, P. (1993). Replacement of Sulfur, Carbon, and Nitrogen in Tissue of Growing Broad Whitefish (*Coregonus nasus*) in Response to a Change in Diet Traced by \u03b4 34 S, \u03b4 13 C, and \u03b4 15 N. *Can. J. Fish. Aquat. Sci.* 50, 2071–2076. doi: 10.1139/f93-230

Hidalgo, M. C., Urea, E., and Sanz, A. (1999). Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. *Aquaculture* 170, 267–283. doi: 10.1016/S0044-8486(98)00413-X

Huang, Q., Qin, D., Tan, B., Du, T., Yang, Y., Yang, Q., et al. (2019). The optimal dietary protein level of juvenile silver sillago *Sillago sihama* at three dietary lipid levels. *Aquac. Res.* 51, 816–827. doi: 10.1111/are.14431

Infante Zambonino, J. L., and Cahu, C. (1994). Development and response to a diet change of some digestive enzymes in sea bass (*Dicentrarchus labrax*) larvae. *Fish Physiol. Biochem.* 12, 399–408. doi: 10.1007/BF00004304

Kasiga, T., White, B. M., Bruce, T. J., and Brown, M. L. (2020). Effect of fish meal replacement with Carinata Brassica carinata in low animal protein diets of rainbow trout *Oncorhynchus mykiss* (Walbaum) on trypsin activity, protein and amino acid digestibility and bioavailability. *Aquac. Res.* 51, 2134–2149. doi: 10.1111/are.14564

Kürten, B., Painting, S. J., Struck, U., Polunin, N. V. C., and Middelburg, J. J. (2013). Tracking seasonal changes in North Sea zooplankton trophic dynamics using stable isotopes. *Biogeochemistry* 113, 167–187. doi: 10.1007/s10533-011-9630-y

Lage, L. P. A., Weissman, D., Serusier, M., Putrino, S. M., Baron, F., Guyonvarch, M., et al. (2020). Long-term impact of a 4-day feed restriction at the protozoea stage on metabolic gene expressions of whiteleg shrimp (*Litopenaeus vannamei*). *PeerJ* 8:e8715. doi: 10.7717/peerj.8715

Leibson, N. L. (1992). Regeneration of digestive tube in holothurians *Stichopus japonicus* and *Eupentacta fraudatrix*. *Monogr. Dev. Biol.* 23, 51–61.

Li, B. S., Han, X. J., Wang, J. Y., Song, Z. D., Sun, Y. Z., Wang, S. X., et al. (2021). Optimal dietary methionine requirement for juvenile sea cucumber *Apostichopus japonicus* selenka. *Aquac. Res.* 52, 1348–1358. doi: 10.1111/are.14989

Liao, M., Ren, T., He, L., Han, Y., and Jiang, Z. (2015b). Optimum dietary proportion of soybean meal with fish meal, and its effects on growth, digestibility, and digestive enzyme activity of juvenile sea cucumber *Apostichopus japonicus*. *Fish. Sci.* 81, 915–922. doi: 10.1007/s12562-015-0916-1

Liao, M., Ren, T., Chen, W., Han, Y., Liu, C., Jiang, Z., et al. (2015a). Effects of dietary lipid level on growth performance, body composition and digestive enzymes activity of juvenile sea cucumber, *Apostichopus japonicus*. *Aquac. Res.* 48, 92–101. doi: 10.1111/are.12864

Liu, Q. S., and Wang, D. H. (2007). Effects of diet quality on phenotypic flexibility of organ size and digestive function in Mongolian gerbils (*Meriones unguiculatus*). *J. Comp. Physiol. B* 177, 509–518. doi: 10.1007/s00360-007-0149-4

Luque De Castro, M. D., and Priego-Capote, F. (2010). Soxhlet extraction: Past and present panacea. *J. Chromatogr. A* 1217, 2383–2389. doi: 10.1016/j.chroma.2009.11.027

Macavoy, S. E., Arneson, L. S., and Bassett, E. (2006). Correlation of metabolism with tissue carbon and nitrogen turnover rate in small mammals. *Oecologia* 150, 190–201. doi: 10.1007/s00442-006-0522-0

Ministry of Agriculture and Rural Affairs of People's Republic of China (2011). *China Fisheries statistical Yearbook*. Beijing, China: China Agriculture Press.

Ministry of Agriculture and Rural Affairs of People's Republic of China (2021). *China Fisheries statistical Yearbook*. Beijing, China: China Agriculture Press.

Mir, I. N., Srivastava, P. P., Bhat, I. A., Muralidhar, A. P., Varghese, T., Gireesh-Babu, P., et al. (2018). Expression and activity of trypsin and pepsin during larval development of Indian walking catfish (*Clarias magur*). *Aquaculture* 491, 266–272. doi: 10.1016/j.aquaculture.2018.03.049

Moghadam, H., Mørkøre, T., and Robinson, N. (2015). Epigenetics—potential for programming fish for aquaculture? *J. Mar. Sci. Eng.* 3, 175–192. doi: 10.3390/jmse3020175

Muhlia-Almazan, A., Garcia-Carreno, F. L., Sanchez-Paz, J. A., Yepiz-Plascencia, G., and Peregrino-Uriarte, A. B. (2003). Effects of dietary protein on the activity and mRNA level of trypsin in the midgut gland of the white shrimp *Penaeus vannamei*. *Comp. Biochem. Physiol. B* 135, 373–383. doi: 10.1016/S1096-4959(03)00091-5

Murashita, K., Furuita, H., Matsunari, H., Yamamoto, T., Awaji, M., Nomura, K., et al. (2013). Partial characterization and ontogenetic development of pancreatic digestive enzymes in Japanese eel *Anguilla japonica* larvae. *Fish Physiol. Biochem.* 39, 895–905. doi: 10.1007/s10695-012-9749-3

Nazemroaya, S., Yazdanparast, R., Nematollahi, M. A., Farahmand, H., and Mirzadeh, Q. (2015). Ontogenetic development of digestive enzymes in Sobaity sea bream *Sparidentex hasta* larvae under culture condition. *Aquaculture* 448, 545–551. doi: 10.1016/j.aquaculture.2015.06.038

Norusis, M. (2008). *SPSS statistics 17.0: Guide to data analysis*. Upper Saddle River: Prentice-Hall.

Parma, L., Yufera, M., Navarro-Guillen, C., Moyano, F. J., Soverini, M., D'Amico, F., et al. (2019). Effects of calcium carbonate inclusion in low fishmeal diets on growth, gastrointestinal pH, digestive enzyme activity and gut bacterial community of European sea bass (*Dicentrarchus labrax* L.) juveniles. *Aquaculture* 510, 283–292. doi: 10.1016/j.aquaculture.2019.05.064

Patel, M. S., and Srinivasan, M. (2002). Metabolic programming: Causes and consequences. *J. Biol. Chem.* 277, 1629–1632. doi: 10.1074/jbc.R100017200

Peres, A., Cahu, C. L., Infante, J., LeGall, M. M., and Quazuguel, P. (1996). Amylase and trypsin responses to intake of dietary carbohydrate and protein depend on the developmental stage in sea bass (*Dicentrarchus labrax*) larvae. *Fish Physiol. Biochem.* 15, 237–242. doi: 10.1007/BF01875574

Peterson, B., and Fry, B. (1987). Stable isotopes in ecosystem studies. *Annu. Rev. Ecol. Syst.* 18, 293–320. doi: 10.1146/annurev.es.18.110187.001453

Ru, X., Zhang, L., Li, X., Liu, S., and Yang, H. (2019). Development strategies for the sea cucumber industry in China. *J. Oceanol. Limnol.* 37, 300–312. doi: 10.1007/s00343-019-7344-5

Rungruangsak-Torrissen, K., Moss, R., Andresen, L. H., Berg, A., and Waagbo, R. (2006). Different expressions of trypsin and chymotrypsin in relation to growth in Atlantic salmon (*Salmo salar* L). *Fish Physiol. Biochem.* 32, 7–23. doi: 10.1007/s10695-005-0630-5

Sabat, P., Lagos, J. A., and Bozinovic, F. (1999). Test of the adaptive modulation hypothesis in rodents: Dietary flexibility and enzyme plasticity. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 123, 83–87. doi: 10.1016/S1095-6433(99)00042-2

Seo, J. Y., Shin, I. S., and Lee, S. M. (2011). Effect of dietary inclusion of various plant ingredients as an alternative for *Sargassum thunbergii* on growth and body composition of juvenile sea cucumber *Apostichopus japonicus*. *Aquac. Nutr.* 17, 549–556. doi: 10.1111/j.1365-2095.2010.00849.x

Shi, C., Dong, S., Pei, S., Wang, F., Tian, X., and Gao, Q. (2015). Effects of diatom concentration in prepared feeds on growth and energy budget of the sea cucumber *Apostichopus japonicus* (Selenka). *Aquac. Res.* 46, 609–617. doi: 10.1111/are.12206

ShiJian, F., and XieXiao, J. (2007). Effect of Dietary Carbohydrate Levels on Routine Metabolic Rate in *Silurus meridionalis* Chen. *J. Anhui Agric. Sci.* 35, 7857–7859. doi: 10.13989/j.cnki.0517-6611.2007.25.094

Sudong, X., Hongsheng, Y., Yong, L., Shilin, L., Yi, Z., and Lili, Z. (2012). Effects of different seaweed diets on growth, digestibility, and ammonia-nitrogen production of the sea cucumber *Apostichopus japonicus* (Selenka). *Aquaculture* 338, 304–308. doi: 10.1016/j.aquaculture.2012.01.010

Sun, Z. L., Gao, Q. F., Dong, S. L., Shin, P., and Wang, F. (2012). Estimates of carbon turnover rates in the sea cucumber *Apostichopus japonicus* (Selenka) using stable isotope analysis: The role of metabolism and growth. *Mar. Ecol. Prog.* 457, 101–112. doi: 10.3354/meps09760

Sunde, J., Taranger, G. L., and Rungruangsak-Torrissen, K. (2001). Digestive protease activities and free amino acids in white muscle as indicators for feed conversion efficiency and growth rate in Atlantic salmon (*Salmo salar* L). *Fish Physiol. Biochem.* 25, 335–345. doi: 10.1023/A:1023233024001

Sveinsdóttir, H., Thorarensen, H., and Gudmundsdóttir, Á (2006). Involvement of trypsin and chymotrypsin activities in Atlantic cod (*Gadus morhua*) embryogenesis. *Aquaculture* 260, 307–314. doi: 10.1016/j.aquaculture.2006.06.009

Symonds, M. E., Sebert, S. P., Hyatt, M. A., and Budge, H. (2009). Nutritional programming of the metabolic syndrome. *Nat. Rev. Endocrinol.* 5, 604–610. doi: 10.1038/nrendo.2009.195

Tarboush, R. A., MacAvoy, S. E., Macko, S. A., and Connaughton, V. (2006). Contribution of catabolic tissue replacement to the turnover of stable isotopes in *Danio rerio*. *Can. J. Zool.* 84, 1453–1460. doi: 10.1139/z06-136

Turkmen, S., Luis Castro, P., Jose Caballero, M., Maria Hernandez-Cruz, C., Saleh, R., Jesus Zamorano, M., et al. (2015). Nutritional stimuli of gilthead seabream

(*Sparus aurata*) larvae by dietary fatty acids: Effects on larval performance, gene expression and neurogenesis. *Aquac. Res.* 48, 202–213. doi: 10.1111/are.12874

Vander Zanden, M. J., Clayton, M. K., Moody, E. K., Solomon, C. T., and Weidel, B. C. (2015). Stable isotope turnover and Half-Life in animal tissues: A literature synthesis. *PLoS One* 10:e0116182. doi: 10.1371/journal.pone.0116182

Vera, L. M., Metochis, C., Taylor, J. F., Clarkson, M., Skjaerven, K. H., Migaud, H., et al. (2017). Early nutritional programming affects liver transcriptome in diploid and triploid Atlantic salmon, *Salmo salar*. *BMC Genomics* 18:886. doi: 10.1186/s12864-017-4264-7

Wang, T., Sun, L., and Chen, M. (2015). Aestivation and regeneration. *Dev. Aquac. Fish. Sci.* 39, 177–209. doi: 10.1016/B978-0-12-799953-1.00011-8

Wen, B., Gao, Q., Dong, S., Hou, Y., Yu, H., and Li, W. (2016b). Effects of dietary inclusion of benthic matter on feed utilization, digestive and immune enzyme activities of sea cucumber *Apostichopus japonicus* (Selenka). *Aquaculture* 458, 1–7. doi: 10.1016/j.aquaculture.2016.01.028

Wen, B., Gao, Q., Dong, S., Hou, Y., and Yu, H. (2016a). Uptake of benthic matter by sea cucumber *Apostichopus japonicus* (Selenka): Insights from carbon stable isotopes and fatty acid profiles. *J. Exp. Mar. Biol. Ecol.* 474, 46–53. doi: 10.1016/j.jembe.2015.10.003

Winter, E. R., Nolan, E. T., Busst, G. M. A., and Britton, J. R. (2019). Estimating stable isotope turnover rates of epidermal mucus and dorsal muscle for an omnivorous fish using a diet-switch experiment. *Hydrobiologia* 828, 245–258. doi: 10.1007/s10750-018-3816-4

Xia, B., Gao, Q., Wang, J., Li, P., Zhang, L., and Zhang, Z. (2015a). Effects of dietary carbohydrate level on growth, biochemical composition and glucose metabolism of juvenile sea cucumber *Apostichopus japonicus* (Selenka). *Aquaculture* 448, 63–70. doi: 10.1016/j.aquaculture.2015.05.038

Xia, B., Wang, J., Gao, Q., Sun, Y., Zhang, L., Ma, J., et al. (2015b). The nutritional contributions of dietary protein sources to tissue growth and metabolism of sea cucumber *Apostichopus japonicus* (Selenka): Evidence from nitrogen stable isotope analysis. *Aquaculture* 435, 237–244. doi: 10.1016/j.aquaculture.2014.09.042

Xionggao, S., Xiaoqin, L., Xiangjun, L., Chonggui, T., Bo, L., Xianqi, C., et al. (2014). The improvement of growth, digestive enzyme activity and disease resistance of white shrimp by the dietary citric acid. *Aquac. Int.* 22, 1823–1835. doi: 10.1007/s10499-014-9785-3

Yang, H., Yuan, X., Zhou, Y., Mao, Y., Zhang, T., and Liu, Y. (2015). Effects of body size and water temperature on food consumption and growth in the sea cucumber *Apostichopus japonicus* (Selenka) with special reference to aestivation. *Aquac. Res.* 36, 1085–1092. doi: 10.1111/j.1365-2109.2005.01325.x

Ye, Z., Hui, L., Qing, W., and Bingjun, L. (2019). The influence of three antibiotics on the growth, intestinal enzyme activities, and immune response of the juvenile sea cucumber *Apostichopus japonicus* selenka. *Fish Shellfish Immunol.* 84, 434–440. doi: 10.1016/j.fsi.2018.10.022

Ying, L., Shuanglin, D., Xiangli, T., Fang, W., and Qinfeng, G. (2009). Effects of dietary sea mud and yellow soil on growth and energy budget of the sea cucumber *Apostichopus japonicus* (Selenka). *Aquaculture* 286, 266–270. doi: 10.1016/j.aquaculture.2008.09.029

Ying, L., Shuanglin, D., Xiangli, T., Fang, W., and Qinfeng, G. (2010). The effect of different macroalgae on the growth of sea cucumbers (*Apostichopus japonicus Selenka*). *Aquac. Res.* 41, e881–e885. doi: 10.1111/j.1365-2109.2010.02582.x

Yu, H., Ai, Q., Mai, K., Ma, H., Cahu, C. L., and Infante, J. L. Z. (2012). Effects of dietary protein levels on the growth, survival, amylase and trypsin activities in large yellow croaker, *Pseudosciaena Crocea* R., Larvae. *Aquac. Res.* 43, 178–186. doi: 10.1111/j.1365-2109.2011.02814.x

Zang, Y., Tian, X., Dong, S., and Dong, Y. (2012). Growth, metabolism and immune responses to evisceration and the regeneration of viscera in sea cucumber, *Apostichopus japonicus*. *Aquaculture* 358, 50–60. doi: 10.1016/j.aquaculture.2012.06.007

Zar, J. H. (1999). *Biostatistical Analysis (Fourth Edition). Fundamentals of air pollution*. Englewood Cliffs: Prentice Hall international.

Zhu, W., Mai, K., Zhang, B., Wang, F., and Xu, G. (2005). Study on dietary protein and lipid requirement for sea cucumber, *Stichopus japonicus*. *Mar. Sci.* 29, 54–58. doi: 10.1360/biodiv.050121

Conflict of Interest: YS and WJ were employed by Homey Group Co., Ltd.

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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frontiers in Marine Science

REVIEW
published: 08 March 2022
doi: 10.3389/fmars.2022.849870

Potential of Ascidians as Extractive Species and Their Added Value in Marine Integrated Multitrophic Aquaculture Systems–From Pests to Valuable Blue Bioresources

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OPEN ACCESS

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Reviewed by:
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Specialty section:
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

Received: *06 January 2022*
Accepted: *24 January 2022*
Published: *08 March 2022*

Citation:
Marques L, Calado R and Lillebø AI (2022) Potential of Ascidians as Extractive Species and Their Added Value in Marine Integrated Multitrophic Aquaculture Systems–From Pests to Valuable Blue Bioresources. Front. Mar. Sci. 9:849870. doi: 10.3389/fmars.2022.849870

Ascidians are considered as filter-feeder biofouling pests that negatively affect aquaculture facilities. However, they can also be recognized as a potential co-cultured/extractive species for integrated multi-trophic aquaculture (IMTA) with potential added value as bioresources. A systematic review aiming to understand the ecological importance of ascidians as efficient filter-feeders [What?]; their potential contribution as extractive species [How?]; and to set the benchmark for their nutritional value and potential added value to the aquaculture industry [For what?] is a timely contribution to advance the state of the art on these largely overlooked bioresources. In the last two decades, there has been an overall increase in publications addressing ascidians in aquaculture, namely, their negative impacts through biofouling, as well as their role in IMTA, environmental status, and microbiology. While *Ciona intestinalis*, a solitary ascidian, has been the most studied species, overall, most ascidians present high filtration and fast-growth rates. As ascidians perform well under IMTA, competition for resources and space with other filter-feeders might occur, which may require additional management actions to optimize production. Studies addressing their bioactive products show that ascidians hold great potential as premium ingredients for aquafeed formulations, as well as dietary supplements (e.g., amino acids, fatty acids). Further research on the potential use of ascidians in IMTA frameworks should focus on systems carrying capacity.

Keywords: tunicates, bioresource, IMTA, filtration rate, fatty acids, retention efficiency

INTRODUCTION

Aquaculture is an important source of food, nutrition, income, and livelihoods for hundreds of millions of people worldwide (FAO, 2020). With the continuous increase of the world's population, aquaculture production needs to increase by 21–44 million tons by the year 2050 (Costello et al., 2020). An idealistic scenario, and a major challenge for the aquaculture industry, is to be profitable,

product-diversified, socially beneficial, and yet ecologically efficient and environmentally friendly, i.e., to cope with the principles of sustainable development. Integrated multi-trophic aquaculture (IMTA) has the potential to achieve such a goal.

An IMTA framework is a nature-based solution in which the by-products, wastes, uneaten feed, and nutrients from one species are recycled and converted to become fertilizer, feed, and energy for the growth of another (Naylor and Burke, 2005). These systems can be land-based or open-water, use marine, brackish or freshwater, and may include several different combinations of co-cultured species (Neori et al., 2004). IMTA aims at mimicking a natural ecosystem by combining and incorporating complementary species from different trophic or nutritional levels in the same productive environment. In an operational IMTA system, extractive species uptake organic and inorganic matter contributing to reduce costs and comply with environmental regulations (Reid et al., 2020).

In addition, their potential market value (e.g., food, feed, pharma) might provide extra economic benefits to farmers (Barrington et al., 2009; Béné et al., 2015). Selected species should be cultured at densities that optimize nutrient uptake, promote a stable balance between biological and chemical processes improving the ecosystem's health, and should be economically important as aquaculture products (Alexander et al., 2016).

However, implementing a healthful and balanced concept can present multiple challenges to farmers. Nonetheless, an IMTA framework also presents numerous benefits, including the decrease in waste outputs from overall farming activities, the additional production of a marketable product for little or no additional input cost, and more importantly, environmentally sustainable farming operations (Barrington et al., 2009; Troell et al., 2009).

The open key question concerns the optimization of the uptake of particulate and dissolved organic matter from uneaten/undigested feed and feces. Organic nutrients can nitrify the benthic-pelagic community (Albert et al., 2021) and the excess of inorganic nutrients (ammonia, nitrate, nitrite) may ultimately create a bio-deposit and lead to eutrophicated waters (Chopin et al., 2001) and/or represent an economic burden to fish farmers (Fry et al., 2016).

Ascidians, commonly known as sea squirts or tunicates, are found in all marine habitats from shallow water to the deep sea (Shenkar and Swalla, 2011) and there are approximately 3000 described species (Shenkar and Swalla, 2011). Currently classed under Phylum Chordata, ascidians hold a unique evolutionary position as the sister group of vertebrates. These organisms are benthic suspension feeders that filter particulate organic matter from the water column via an oral siphon and expelled filtered water through the atrial siphon (Jørgensen and Goldberg, 1953; Jørgensen, 1954). They present a wide variety of forms (from small colonies to big solitary forms), colors, shapes (from cone-shaped, elongated, globular, or oval), and sizes (generally from 0.5 to 200 mm) (Petersen, 2007; Shenkar and Swalla, 2011).

The body is always covered with a tunic, a protective layer that may be translucid, brightly colored or dull, covered by various kinds of spines, and contain calcareous spicules (Lambert and Lambert, 1987), or even be covered by a dense layer of sand grains (Young, 1989). Most solitary ascidians are hermaphrodites and reproduce by external fertilization (Honegger, 1986). They develop a free-swimming tadpole-like larva that swims during a short period, settles on a wide variety of habitats, and finally matures into a sessile adult (Shenkar and Swalla, 2011). Colonial specimens can reproduce both sexually and asexually (Gasparini et al., 2015). Ascidians often present an invasive behavior, representing the most dominant fouling species worldwide, colonizing natural and artificial substrates (Ordóñez et al., 2013).

While several ascidian species present a preference to settle on natural substrates (Hirose and Sensui, 2021), others settle on artificial structures, such as ship hulls, floating docks (Zvyagintsev et al., 2007), and aquaculture infrastructures (Hodson et al., 2000; Khalaman, 2001; Bullard et al., 2013; Rosa et al., 2013), process known as biofouling. At times some species even grow on other organisms being farmed, such as on the shells of mollusks (Dijkstra and Nolan, 2017; Casso et al., 2018).

Hereupon, these organisms hold great potential as co-cultured/extractive species in IMTA frameworks, with potential to contribute to more efficient, profitable, and sustainable aquaculture systems. Benthic fish contribute to sediment resuspension while searching for food or shelter (Yahel et al., 2008; Carvajalino-Fernández et al., 2020). Although these resuspension events can be brief and localized (Yahel et al., 2002), in an IMTA scenario ascidians, as excellent filter-feeders, can rapidly uptake nutrient recycling and contribute to a positive outcome.

The main objective of this systematic review is to understand how ascidians may no longer be regarded as pest organisms, who's biofouling negatively impacts aquaculture ventures, but rather as important extractive species in IMTA frameworks that yield premium biomass for high-end uses. To this purpose, we surveyed the scientific literature to answer the following three questions: [What do we know?] To better understand the biological and ecological importance of ascidians as filter-feeders in an IMTA framework; [How do ascidians perform in IMTA?] to evaluate which combination of species will contribute the most to enhance the performance of ascidians in IMTA frameworks; and [For what kind of bioactive products?] to recognize ascidians as potential bioresources in different high-end fields, namely, blue biotechnology and human nutrition.

Here, special attention will be given to fatty acids, as both omega-3 (*n*-3) and omega-6 (*n*-6) fatty acids are essential components for food, feed, and pharma industries. The analysis of these three questions will enable us to discuss and conclude on the potential of ascidians as extractive species and their added value in marine IMTA frameworks.

LITERATURE REVIEW

In January 2020, a systematic literature review, with no year restriction, was performed using the databases Thomson Reuters Web of Science (Core Collection) (Topic) and Scopus (Article

title, Abstract, Keywords). The strategy used was to search within a combination of specific terms: Filtration AND (tunicate OR ascidian OR "sea squirt*"); Aquaculture AND (tunicate OR ascidian OR "sea squirt*"); Fatty acid* AND (tunicate OR ascidian OR "sea squirt*") to achieve the review's goal.

A schematic representation of the selection process is summarized in **Figure 1**. Overall, a total of 566 publications were retrieved (after excluding duplicates from the two databases), and a spreadsheet with the bibliographic information of each reference was created for further analysis to ascertain their relevance for this study. The review selection consists of two sections.

The first article selection aimed to retain publications with, at least, the abstract in the English language, peer-reviewed academic journals, book chapters, meetings abstracts, short communications, and reports on ascidians within Class Ascidiacea. When studies addressed ascidians in a general way, these were registered as "Ascidiacea." Furthermore, studies addressing the following topics were excluded from the present review: (i) first reports on the occurrence of a new species of ascidian in a given location and their geographic distributions, (ii) embryonic development, (iii) fertilization, reproduction, and associated topics, (iv) invasive behavior of ascidians and mitigation measures.

A total of 148 publications (**Supplementary Table 1**) were considered relevant and selected for further analysis. Ten research categories (aquaculture, biochemistry, biofouling, biology, biotechnology/methods, diseases, environmental, IMTA, microbiology, and review) were created and assigned to each of the 148 publications, with a maximum of four categories being attributed per publication.

The rationale for this procedure is detailed in **Table 1**. Additionally, each publication was also assigned to one of the three questions (occasionally two) initially established: (question 1 [What?]: 75 publications, question 2 [How?]: 31 publications, and question 3 [For what?]: 62 publications). Subsequently, each of the publications assigned to each of the three questions was further screened as detailed in **Figure 1**.

Briefly, concerning question 1, only publications addressing filtration, retention, ingestion, clearance, digestion, and water pumping rates were selected, for a total of 29 publications. Regarding question 2, only publications addressing topics such as aquaculture, IMTA, and growth rates were included, for a total of 19 publications. Finally, for question 3, publications referring to fatty acids and other potential co-products were considered, for a total of 28 publications. Blue biotechnology may focus on a plethora of potentially bioactive compounds (Vieira et al., 2020).

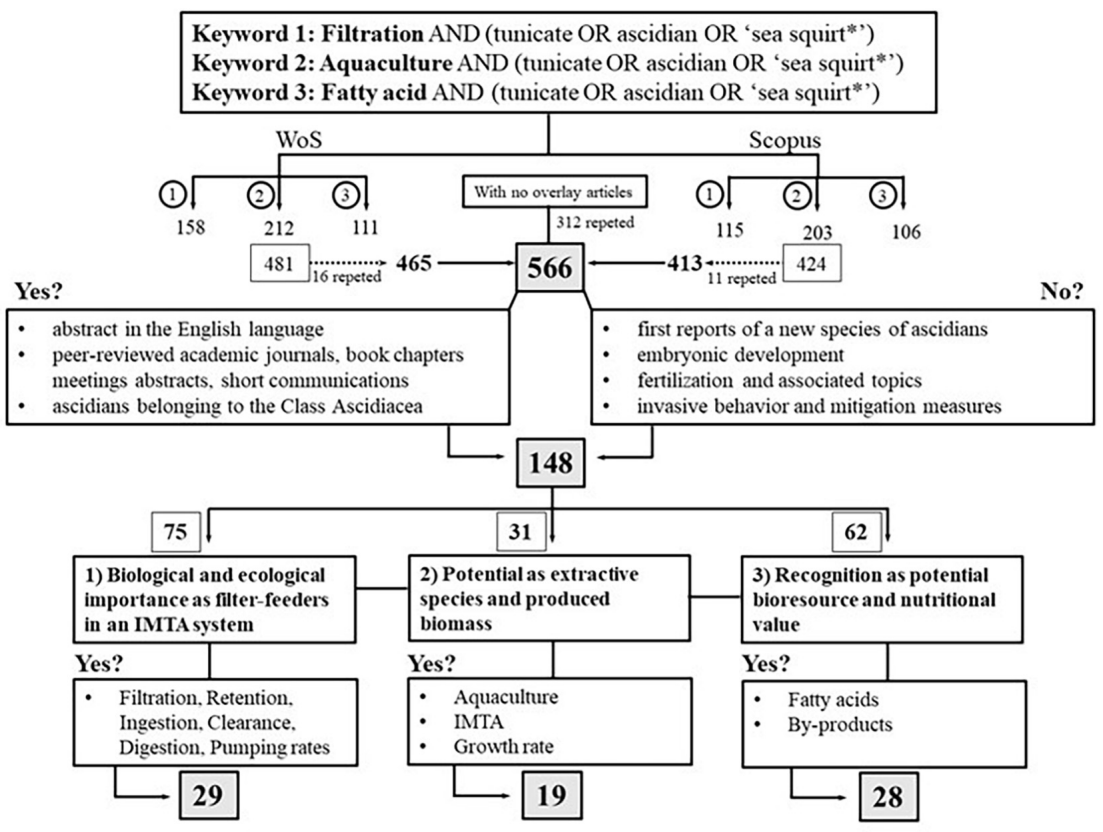

FIGURE 1 | Schematic representation of the process employed for the selection of relevant publications retrieved from each database (Web of Science, WoS; Scopus).

TABLE 1 | Research categories considered and their respective criteria.

Research category	
Aquaculture	Refers to farming of ascidians, impacts that other species may have and economic value
Biochemistry	Refers to proximate composition, lipid composition, fatty acid identification and nutrition information
Biofouling	Refers to biofouling ascidians in aquaculture sites and its impacts on produced species
Biology	Refers to biological and ecological traits such as growth, filtration, clearance, retention rates, natural diets, population interactions, and habitat preferences
Biotechnology/ Methods	Refers to models created and tested, development of technology toward the study of ascidians
Diseases	Refers to diseases associated with ascidians
Environmental	Refers to environmental parameters and their impact on ascidians, pollution, toxicity, and bioremediation
IMTA	Refers to farming ascidians with one or more different trophic groups, along with their interactions and impacts
Microbiology	Refers to the identification, characterization, and isolation of bacteria from ascidians
Review	Refers to any published review on ascidians

As this review targets marine species and they can be seen as sources of the essential omega-3 (*n*-3) and omega-6 (*n*-6) fatty acids, special attention will be given to these bioactive compounds as they can represent an added value for food, feed, and pharma industries.

RESULTS

Out of the 148 publications, 80 fell into the research category "Biology," hence demonstrating the importance of understanding morphology, biology, and anatomy of ascidians in a general manner (**Figure 2**). Since the 21st century, an overall increase in all research categories is noted, but it is worth mentioning a gradual and joint increase of publications in "Aquaculture," "Biofouling," and "IMTA" categories (44, 25, and 7%, respectively) as these are correlated with each other.

In addition, bacteria and associated diseases with ascidians are a growing concern, as seen with the increase in the number of publications within the category "Microbiology." A total of 45 species, belonging to 3 orders and 12 families (**Supplementary Table 2**) were present in this review, in which solitary ascidians represented 72% and merely 28% were colonial ascidians (**Figure 3**).

Despite the high number of ascidians from the marine realm, solely three species dominated the focus of scientists throughout the years. *Ciona intestinalis*, a translucent column-like tunicate, was by far the most studied species, followed by *Halocynthia roretzi* and *Styela clava* (**Figure 3**). A detailed analysis was performed regarding the three questions (**Table 2**). Ascidians *C. intestinalis* and *S. clava* were the two most studied species for their biological and ecological importance as filter-feeders

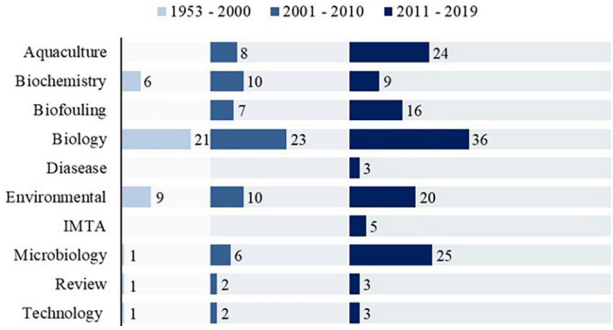

FIGURE 2 | Number of publications ($n = 148$), from 1953 to 2019, assigned to each research category.

[What?] and on the most effective combination of species for IMTA [How?]; while *H. roretzi* and *Halocynthia aurantium*, were mostly studied for their potential as bioresource [For what?] (26 and 11%, respectively).

[What Do We Know?] (Question 1)

To comprehend the role of ascidians as filter-feeders and their importance, a better understanding of basic biology is needed. Essentially, water filtrations rates were present in most of the 29 publications analyzed (**Figure 1**), with these referring to 31 different species (allocated to 17 different genera). Solitary ascidians, such as *C. intestinalis*, *Phallusia mammillata*, and *Styela plicata*, were the most investigated species accounting for 17.5, 9.5, and 7.9% of the publications, respectively. France is in the leadership both in the number of ascidians species being studied, as well as in the number of studies performed (**Table 3**).

Filtration rates presented a great variability between the different species of ascidians addressed, with intraspecific variability also being recorded, for example, for *C. intestinalis* with values ranging from 3.5 to 11.9 L h^{-1} (Fiala-Médioni, 1974; Petersen and Riisgärd, 1992) and for *P. mammillata* with values ranging from 4.4 to 11.9 L h^{-1} (Fiala-Médioni, 1973; Hily, 1991; **Table 3**). Nakai et al. (2018) demonstrated that water filtration rate increases with size, while Ribes et al. (1998) showed that filtration rates may vary seasonally, displaying an increase with rising water temperatures. Just 30% of publications registered retention rate values. A total of 17 species were investigated, with only two species being colonial ascidians. Particle retention varied from 1.7 to 4.71 μm (mean value).

[How Do Ascidians Perform in Integrated Multi-Trophic Aquaculture?] (Question 2)

Question 2 focused on understanding which combination of species with ascidians contributes the most to enhance the performance of IMTA frameworks, their extractive ability, and their impacts on other cultured species. Only five out of the 19 relevant publications (**Figure 1**) specifically addressed IMTA. These five publications presented similar aspects, as they were all performed in China and addressed *S. clava*.

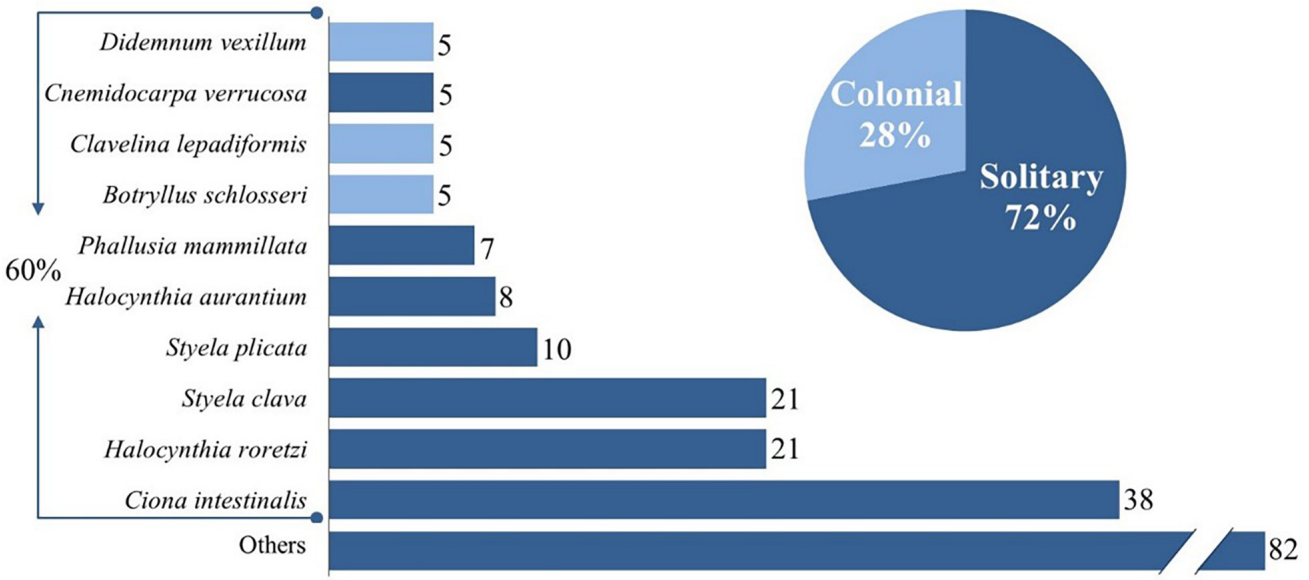

FIGURE 3 | (A) Top ten studied ascidian species and total number of publications addressing them. Light blue represents colonial species, dark blue represents solitary species; **(B)** Percentage of solitary and colonial ascidians addressed in the 148 publications surveyed. See **Supplementary Material** for complete list of studied species (**Supplementary Table 1**).

The main goal of these publications was to optimize the commercial production and growth of the sea cucumber *Apostichopus japonicus* (**Figure 4**). The remaining 14 publications addressed issues associated with the impact of biofouling promoted by ascidians on cultured species, namely, mussels, oysters, and scallops were addressed (**Table 4**). *C. intestinalis* was the most discussed ascidian regarding this topic.

[For What Kind of Bioactive Products?] (Question 3)

The recognition of the nutritional value *sensu lato* of ascidians and their potential as bioresources was considered in 28 publications (**Figure 1**), addressing 25 species belonging to 15 genera.

Ascidians being addressed under this scope mostly originated from Asian countries (China, Japan, South Korea, and North Korea), with a major focus on *C. intestinalis*, *Halocynthia* sp., and *Styela* sp. Amongst the various studies, 16 of them addressed specifically the fatty acid composition of ascidians, including 20 species belonging to 13 genera. From these, few analyzed the tunic and inner body separately (Zhao et al., 2015; Zhao and Li, 2016), with the remaining analyzing the whole body of ascidians.

A wide range of fatty acids was identified with percentages varying from 0.06 to 44% total fatty acid (Jeong et al., 1996; Zlatanos et al., 2009), nonetheless, palmitic acid (16:0), stearic acid (18:0), arachidonic acid [AA-20:4 (*n*-6)], eicosapentaenoic acid (EPA–20:5*n*-3) and docosahexaenoic acid (DHA–22:6*n*-3) were consistently recorded (**Figure 5**), see **Supplementary Table 3** for further detail. Fatty acids 16:0 and 18:0 were constantly higher in all studied ascidians, however, in several species, EPA and DHA presented high values as well (Carballeira et al., 1995; Jeong et al., 1996; Zhao and Li, 2016).

Out of the studies analyzed, biocompounds such as didemnilactones A and B and neodidemnilactone (Niwa et al., 1994), 2,3-dihydroxy fatty acid glycosphingolipids (Aiello et al., 2003), anticancer ecteinascidin 743 (Mendola, 2003), pentylphenols, cyclopropane fatty acid, and cyclopentenones (Rob et al., 2011) were proven to originate from ascidians (**Table 5**). Cytotoxicity against human solid tumor cell lines (Bao et al., 2009), against HCT116 cells (human colon cancer cells), and inhibition of the division of fertilized sea urchin eggs

TABLE 2 | Percentages of the top two research categories, countries, and ascidian species that most contributed to each of the questions [What?], [How?], [For what?], addressed in the 148 publications surveyed.

Questions	Category	Country	Species
(1) [What?]	Biology (52%)	France (18%)	*Ciona intestinalis* (21%)
	Environmental (21%)	Canada (12%)	*Styela clava* (8%)
(2) [How?]	Aquaculture (30%)	Canada (36%)	*Ciona intestinalis* (33%)
	Biology (27%)	China (21%)	*Styela clava* (28%)
(3) [For what?]	Biochemistry (44%)	South Korea (27%)	*Halocynthia roretzi* (26%)
	Microbiology (43%)	Japan (21%)	*Halocyhthia aurantium* (11%)

TABLE 3 | Summary of the main features of filtration, pumping, and retention rate of the studied ascidians addressed in the 29 publications selected regarding question 1 [What?].

Studied species	Country	Filtration rate/Pumping rate	Retention efficiency	References
Ascidiacea	NA	similar in different species suspension feeding is high efficient		Petersen, 2007
Ascidia challengeri	Antarctic	304 ml.h AFDW*	1.2–2 μm	Kowalke, 1999
Ascidia virginea	Sweden	5.2 $L.h^{-1}.g^{-1}$**		Petersen and Svane, 2002
Ascidiella aspersa	Denmark	5.4 $L.h^{-1}.g^{-1}$**	2–3 μm completely retained; RE decreased 70% for 1 μm	Randløv and Riisgärd, 1979
Ascidiella aspersa	France	6.28 h^{-1}		Hily, 1991
Ascidiella aspersa	United Kingdom	5.26 $L.h^{-1}.g^{-1}$ at about 10,000 cells ml^{-1}	decrease > 4.5 μm	Pascoe et al., 2007
Ascidiella scabra	United Kingdom	at LPS: 0.71 h^{-1}; decreased with increasing suspension load		Robbins, 1983
Ascidiella scabra	United Kingdom	FE: at HPS unchanged		Robbins, 1984
Boltenia echinata	Sweden	3.8 $L.h^{-1}.g^{-1}$**		Petersen and Svane, 2002
Ciona intestinalis	United States		1–2 μm	Jørgensen and Goldberg, 1953
Ciona intestinalis	France	3.5 $L.h^{-1}.g^{-1}$		Fiala-Médioni, 1974
Ciona intestinalis	France	4.3 $L.h^{-1}.g^{-1}$; FE(mean) = 74% 5.9 $L.h^{-1}.g^{-1}$*		Fiala-Médioni, 1978a
Ciona intestinalis	Sweden	7.7 $L.h^{-1}.g^{-1}$ **	2–3 μm completely retained; RE decreased 70% for 1 μm	Randløv and Riisgärd, 1979
Ciona intestinalis	United Kingdom	at LPS: 0.21 h^{-1} (mud); 0.11 h^{-1} (*Fucus*); decreased with increasing suspension load		Robbins, 1983
Ciona intestinalis	United Kingdom	FE: at HPS unchanged		Robbins, 1984
Ciona intestinalis	Denmark	11.9 $L.h^{-1}.g^{-1}$ ** 4–21°C increased, >21°C decrease		Petersen and Riisgärd, 1992
Ciona intestinalis	Sweden	8.4 $L.h^{-1}.g^{-1}$**		Petersen and Svane, 2002
Ciona intestinalis	United Kingdom	4.61 $L.h^{-1}.g^{-1}$ at about 5000 cells ml^{-1}	similar to 2–5.5 μm	Pascoe et al., 2007
Ciona intestinalis	United States	0.07–0.97 $L.h^{-1}$*		Clos et al., 2017
Ciona intestinalis	France	positively related to food concentration		Hoxha et al., 2018
Ciona robusta	France	positively related to food concentration CR higher than *C. intestinalis*		Hoxha et al., 2018
Ciona savignyi	Japan	0.125 $L.h^{-1}$ ind^{-1} (ind 3.5 cm) 0.359 $L.h^{-1}$ ind^{-1} (ind 5.3 cm) 1.05 $L.h^{-1}$ ind^{-1} (ind 6.4 cm) optimal at 24–25°C		Nakai et al., 2018
Clavelina lepadiformis	France	2.5 $L.h^{-1}.g^{-1}$		Fiala-Médioni, 1974
Clavelina lepadiformis	Denmark		2–3 μm completely retained; RE decreased 70% for 1 μm	Randløv and Riisgärd, 1979
Clavelina lepadiformis	Sweden	8.9 $L^{-1}.g^{-1}$**		Petersen and Svane, 2002
Cnemidocarpa verrucosa	Antarctic	348 ml.h AFDW*	1.4–4 μm	Kowalke, 1999
Cnemidocarpa verrucosa	Antarctic		0.2–2 μm	Lesser and Slattery, 2015
Corella eumyota	Antarctic	251 ml.h AFDW*	1.2–5 μm	Kowalke, 1999
Corella parallelogramma	Sweden	7.0 $L.h^{-1}.g^{-1}$**		Petersen and Svane, 2002
Didemnum sp.	Australia	reduced heterotrophic bacteria		Pile, 2005
Halocynthia papillosa	France	6.3 $L.h^{-1}.g^{-1}$		Fiala-Médioni, 1974
Halocynthia papillosa	Spain	3.0–3.6 $L.h^{-1}.g^{-1}$**	0.6–7 μm	Ribes et al., 1998
Halocynthia pyriformis	Canada	136 $ml.min^{-1}$ DW (1 g)	2–5 μm: increased 5–15 μm: decreased	Armsworthy et al., 2001
Halocynthia sp.	Australia	only reduced < 3 μm		Pile, 2005
Halocynthia spinosa	Israel		1 μm at 95% efficiency; 0.3 μm at 50% efficiency	Jacobi, 2018
Herdmania momus	Israel		1 μm at 95% efficiency; 0.3 μm at 50% efficiency	Jacobi, 2018

(Continued)

TABLE 3 | (Continued)

Studied species	Country	Filtration rate/Pumping rate	Retention efficiency	References
Microcosmus sabatieri	France	6.9 $L.h^{-1}.g^{-1}$		Fiala-Médioni, 1974
Microcosmus exasperatus	Israel		1 μm at 95% efficiency; 0.3 μm at 50% efficiency	Jacobi, 2018
Molgula manhattensis	Denmark	Higher than *C. intestinalis* and *A. aspersa*	2–3 μm completely retained; RE decreased 70% for 1 μm	Randløv and Riisgård, 1979
	Sweden	2.1 $L.h^{-1}.g^{-1}$**		Petersen and Svane, 2002
Molgula pedunculata	Antarctic	349 ml.h AFDW*	1.2–6.5 μm	Kowalke, 1999
Phallusia julinea	Australia	reduced heterotrophic bacteria		Pile, 2005
Phallusia mammillata	France	4.4 $L.h^{-1}.g^{-1}$ (ind 10–12 cm)		Fiala-Médioni, 1973
Phallusia mammillata	France	4.8 $L.h^{-1}.g^{-1}$: FE(mean) = 76% 6.3 $L.h^{-1}.g^{-1}$ *		Fiala-Médioni, 1978a
Phallusia mammillata	France	15°C: 4.3 $L.h^{-1}.g^{-1}$** 20°C: 1.6 $L.h^{-1}.g^{-1}$** (mean): 10°C: 3.56, 15°C: 5.79, 20°C: 2.63 $ml.h^{-1}.g^{-1}$ DW*		Fiala-Médioni, 1978b
Phallusia mammillata	France	pO_2 > 119 mg Hg: decrease pO_2 > 98 mg Hg: decrease faster FE: 77–79%		Fiala-Médioni, 1979
Phallusia mammillata	France	11.9 $L.h^{-1}$ g^{-1}		Hily et al., 1992
Phallusia mammillata	NA	825–5100 ml.h (ind 8–128 g WW)		Carlisle, 1966
Phallusia nigra	Israel		1 μm at 95% efficiency; 0.3 μm at 50% efficiency	Jacobi, 2018
Polyandrocarpa zorritensis	Italy	max: 1.745 $L.h^{-1}.g^{-1}$ DW	RE: 41%, removed bacterial biomass of 16.34 + 1.71 $\mu g.C.L^{-1}.g^{-1}$ DW)	Stabili et al., 2016
Polycarpa mytiligera	Israel		1 μm at 95% efficiency; 0.3 μm at 50% efficiency	Jacobi, 2018
Polycarpa pedunculata	Australia	only reduced < 3 μm		Pile, 2005
Polycarpa sp.	Australia	reduced heterotrophic bacteria		Pile, 2005
Pyura microcosmus	France	1.94 h^{-1}		Hily, 1991
Pyura sp.	Australia	only reduced < 3 μm		Pile, 2005
Pyura tessellata	Sweden	3.0 $L.h^{-1}.g^{-1}$**		Petersen and Svane, 2002
Styela clava	New Zealand	declined after 3 weeks (sedimentation)		Lohrer et al., 2006
Styela clava	South Korea	0.477 J d^{-1} mean DW (310 mg) at 5–15°C 0.687 J d^{-1} mean DW (310 mg) at 15–25°C		Kang et al., 2015
Styela plicata	France	8.8 $L.h^{-1}.g^{-1}$: FE(mean) = 80% (mean): 10.7 $L.h^{-1}.g^{-1}$ *		Fiala-Médioni, 1978a
Styela plicata	United States	*Nannochloropsis* sp.: $10^5 + 10^6$ cells: 3158 $ml.h^{-1}$; *Escherichia coli*: $10^5 + 10^6$ cells: 3475 $ml.h^{-1}$;		Draughon et al., 2010
Styela plicata	United States	<10 μm: decreased (fast and slow flow speeds); >10 μm: decreased (flow speed from 3 to 22 $cm.s^{-1}$) maximal at intermediate flow speeds 12 $cm.s^{-1}$		Sumerel and Finelli, 2014
Styela plicata	Italy	max: 1.4 $L.h^{-1}.g^{-1}$ DW	RE: 81% removed bacterial biomass of 32.28 + 2.15 μg $C.L^{-1}.g^{-1}$ DW	Stabili et al., 2016
Styela plicata	Israel		1 μm at 95% efficiency; 0.3 μm at 50% efficiency	Jacobi, 2018

LPS, Low particulate suspension; HPS, High particulate suspension; DR, Digestion rate; RE, Retention efficiency; CR, Clearance rate; FE, Filtration efficiency; IR, Ingestion rate.
**Pumping rate; **Adapted from Petersen (2007).*

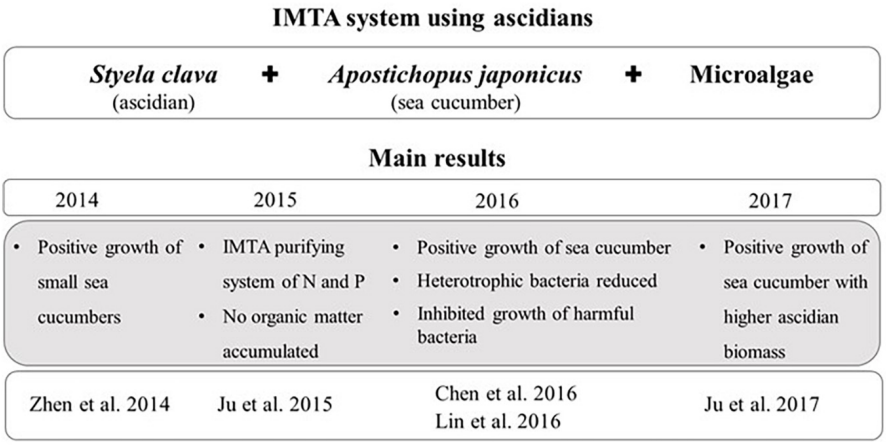

FIGURE 4 | Schematic representation of the main features of integrated multitrophic aquaculture (IMTA) frameworks using ascidians addressed in the 5 of the 19 publications selected regarding question 2 [How?].

(Rob et al., 2011) are just some examples of these compound functionalities.

DISCUSSION

Ascidians as Organic Matter Extractive Species

Over the years, ascidian's biology and functionality have been of growing interest and several studies have addressed water filtration, clearance, retention, pumping, ingestion, and digestion rates. According to Fiala-Médioni (1978a), the definition of filtration rate is the volume of water that has been cleared of particles in a given time frame. Authors have gradually replaced the term "filtration rate" for clearance rate and although this topic has been widely addressed, previous reports have shown considerable variation in the results being reported.

Petersen (2007) complied information on the suspension-feeding of ascidians and concluded that "filtration rates in different species at identical conditions will not vary more than within the same species of different sizes" and also suggests that ascidians are more efficient in non-turbid conditions. Moreover, this present review revealed that since Petersen's (2007) work, there is a generalized lack of studies on this topic. In the last decade, only six new publications have addressed filtration rates, mainly on genus *Styela* and *Ciona* (Draughon et al., 2010; Sumerel and Finelli, 2014; Kang et al., 2015; Stabili et al., 2016; Hoxha et al., 2018; Nakai et al., 2018), and therefore further research is urgently needed.

Testing filtration rates can be very complex in several ways and several variables must be taken into consideration. Robbins (1983) suggested that with an increase in food concentration, the filtration rate would decrease. Randløv and Riisgärd (1979) observed that the presence of a folded pharynx in *Molgula manhattensis* increased the area of the water transporting structure, thus allowing for higher filtration rates. The lag-phase phenomenon was not perceived by Randløv and Riisgärd (1979) leading to lower rates being reported and ultimately to an overall misinterpretation of their findings and not allowing comparison with other studies. Therefore, the need for a lag phase with an appropriate time (20–140 min) is highly recommended (Petersen and Riisgärd, 1992). Moreover, Petersen and Svane (2002) measured the filtration rate of seven ascidians and concluded that the area of the branchial basket and the length of the ciliary band lining the stigmata openings also contributes to higher filtration rates.

Ascidians are very sensitive organisms to any chemical or mechanical disturbance, which can cause them to close their siphons and thereby stop filtration, thus generating unrealistic filtration rates. Several studies in the 1970s (Fiala-Médioni, 1973, 1974, 1978a,b; Randløv and Riisgärd, 1979) concluded that undisturbed ascidians filter water very efficiently and at constant rates, a feature that will unquestionably optimize their performance if these are employed in the IMTA framework.

Most often, it is not easy to evaluate if filtration rates are at their optimal by merely recording the appearance of ascidians (unlike what occurs for some bivalves, such as mussels) (Petersen and Riisgärd, 1992). As environmental variables play an important role in the filtration process, several investigations aimed to elucidate the relationship between filtration rate, body size, temperature, and particle concentration (Fiala-Médioni, 1978b; Petersen and Riisgärd, 1992; Kang et al., 2015). In sum, the standardization of the methodology used to investigate filtration rate is at a high demand to better evaluate and compare data from different research.

Consistent results were observed allowing to affirm that as ascidians increase in size, their filtration rate will also increase, and filtration rate declined with temperatures above 20–21°C, this being true for ascidians from temperate waters. Moreover, the optimal temperature for ascidians' filtration rate may vary with the species being addressed and with the local conditions. Nakai et al. (2018) registered an optimal filtration at temperatures of 24–25°C for *Ciona savignyi*. Several reports focus the deleterious effects of biofouling by ascidians on mussel

TABLE 4 | Summary of the main impacts produced by ascidians in aquaculture scenarios addressed in the 19 publications selected regarding question 2 [How?].

Studied species	Country	Aquaculture species	Main results	References
Ciona intestinalis	Australia	*Mytilus galloprovincialis*	Small mussels: 4% shorter in shell length; 21% reduced flesh weight;	Sievers et al., 2013
			Large mussels: 3.9% shorter in shell length, flesh weights not reduced	
Styela clava			Large mussels: 4.4% shorter in shell length, flesh weights not reduced	
Botrylloides violaceus	Canada	Mussel	*C. intestinalis*: 80% more coverage on unfouled plates	Paetzold et al., 2012
Botryllus schlosseri			*C. intestinalis*: <10% coverage on pre-settled plates	
Ciona intestinalis			Higher individual growth on pre-settled plates than on unfouled plates	
Ciona intestinalis	Canada	*Mytilus edulis*	*C. intestinalis* has a negative impact,	Ramsay et al., 2008a
Styela clava			replacing *S. clava*	
Ciona intestinalis	Canada	*Mytilus edulis*	*C. intestinalis* was marginally higher in August;	Ramsay et al., 2008b
			Mussel loss 50–60% for all treatments	
Ciona intestinalis	Canada	*Mytilus edulis*	*C. intestinalis:* abundance: 98.4–828.6 ind/0.3 m mussel sock;	Lutz-Collins et al., 2009
			Negative effect on epifaunal species, primarily on sessile organisms	
Molgula sp.			*Molgula* sp.: colonized the mussel socks in lower numbers and an opposite spatial pattern of *C. intestinalis*	
Ciona intestinalis	Canada	*Mytilus edulis*	Size and condition decreased with increasing ascidian densities; 50% mussel mortality observed under heavy ascidian fouling	Daigle and Herbinger, 2009
Ciona intestinalis	Canada	*Mytilus edulis*	*C. intestinalis* can dominate mussel biomass and contribute to organic sedimentation	Guyondet et al., 2016
Didemnum sp.	France	*Pinctada margaritifera*	Competition between oysters and ascidians was not a limiting factor,	Lacoste et al., 2016
Herdmania momus			in spite of a diet overlap for nanophytoplankton	
Ascidiella aspersa	Japan	Scallop	*A. aspersa* settle as larvae in early summer, and grows well until winter, resulting in overgrowth on scallops in the harvest season	Kanamori et al., 2017
Ciona savignyi	Japan	*Mizuhopecten yessoensis*	Filtration increased with size increase; *C. savignyi* has the potential to negatively impact the growth of the Japanese scallop through competition for food.	Nakai et al., 2018
Didemnum vexillum	New Zealand	*Perna canaliculus*	Mussels may only be vulnerable to direct *D. vexillum* fouling impacts at early stages of production	Fletcher et al., 2013
Ciona intestinalis	Norway	*Mytilus edulis*	In forced upwelling conditions: positive effect on both species; ascidians would be more efficient at extracting resources due to their lower metabolic cost and higher filtration capacity.	Filgueira et al., 2019
Ciona intestinalis	South Africa	*Mytilus galloprovincialis*	Competitive exclusion of the mussel in dark, sheltered areas and physiological exclusion of the ascidian elsewhere	Rius et al., 2011
Ascidiacea	Spain	Oyster	15 spp. were identified	Casso et al., 2018

farming and their potential competition as filter feeders for trophic resources. One study compared ascidian and mussel filtration rates and highlighted that at 16 and 19°C these are similar (Daigle and Herbinger, 2009).

Conversely, allied with the filtration process is particle retention efficiency. Various approaches have shown that the diet of ascidians mainly comes from smaller particles [particulate organic matter (POM) < 20 μm] (Ju et al., 2015, 2016), picophytoplankton (<2 μm), and phytoplankton biomass (Riisgärd and Larsen, 2016). Moreover, Lacoste et al. (2016) verified an overall lack of food selectivity. The retention efficiency increased for particle sizes 2–5 μm (Armsworthy et al., 2001), in which particles from 2 to 3 μm were completely retained (Randløv and Riisgärd, 1979) and retention efficiency decreased for particles above 4.5 μm (Pascoe et al., 2007).

In general, a threshold of 2–4 μm is observed. In a more recent study, with an *in situ* experiment using 6 different ascidian species, a 95% retention efficiency was registered for 1 μm particles and 50% efficiency for submicron particles (0.3 μm), thus widening ascidians scope (Jacobi, 2018). The ability of *S. plicata* and *Polyandrocarpa zorritensis* to remove *Vibrio alginolyticus* from seawater has also been tested, with *S. plicata* showing a higher efficiency for bioremediation and restoring seawater quality (Stabili et al., 2016). The same authors

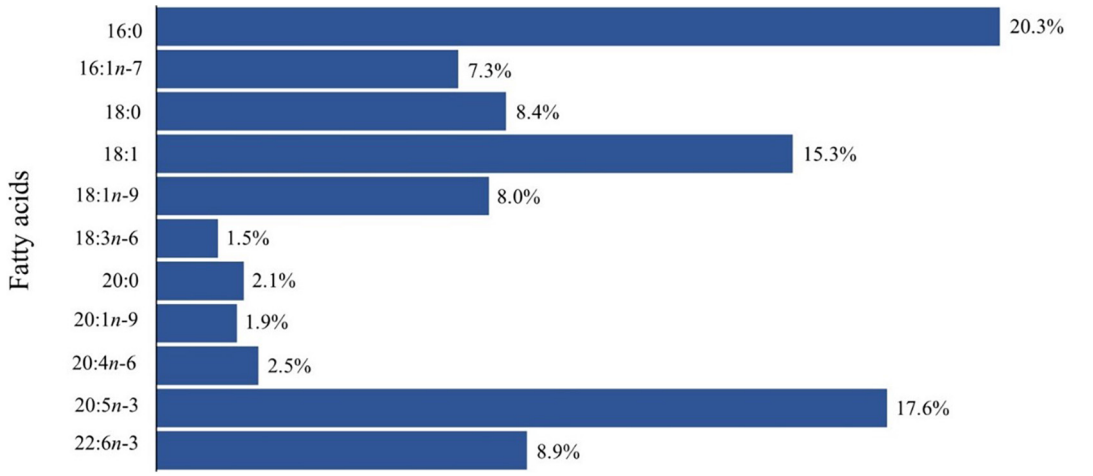

FIGURE 5 | Mean relative percentage values of total fatty acids of the ascidians addressed in this review. See **Supplementary Table 2** for further details.

also demonstrated that retention efficiency was higher in solitary ascidians (81%) than in colonial ones (41%). Lefebvre et al. (2000) used oysters in a land-based fish-farm effluent and confirmed that suspended feeders can improve water quality and add economic value. However, the retention efficiency of filter-feeders in an IMTA scenario must be dealt with caution as many parameters may influence the settling velocity of the suspended particles of organic matter (Reid et al., 2009).

Nonetheless, further research is much needed considering that our systematic review revealed the existence of few publications addressing this topic in colonial ascidians.

Ascidians Incorporated in Integrated Multi-Trophic Aquaculture Frameworks

Despite the increase in interest in IMTA frameworks over the last years, ascidians have rarely been addressed under this scope. Most publications on aquaculture mostly focus on -ascidians as pests due to biofouling features and negative impacts on aquaculture facilities, mainly on shellfish productions (Carver et al., 2003). Cultured shellfish can be negatively affected by ascidian fouling in many ways, with these causing a reduction in mussel growth, flesh weight, and reduced overall size and condition (Daigle and Herbinger, 2009; Sievers et al., 2013; Guyondet et al., 2016; Nakai et al., 2018).

In extreme conditions, this may even lead to mussel mortality (Daigle and Herbinger, 2009). However, this scenario cannot be generalized, as Cordell et al. (2013) did not record any negative effects on mussel growth at four different locations and Sievers et al. (2013) observed no reduction of flesh weight was seen in larger mussels. Moreover, Lacoste et al. (2016) found that food competition between oysters and ascidians was not a limiting factor, which advises caution on making generalized assumptions on the negative impacts of ascidians on the farming of bivalves.

Indeed, several factors such as location, species involved, environmental parameters, sampling, and experiment conditions, among others must also be considered (Fletcher et al., 2013). Furthermore, some ascidians present invasive traits, growing quickly and therefore must be supervised to not overwhelm and overgrow the other culture species.

The solitary ascidian, *C. intestinalis*, was investigated in 60% of publications in this field, given that this is one of the most studied ascidian species. As an example, they present high tolerance to a wide range of salinities and temperatures (Lutzen, 1999; Shenkar and Swalla, 2011), allowing them a worldwide spatial distribution. This biofouling ascidian, with a fast-growing rate (Ramsay et al., 2008b, Lutz-Collins et al., 2009), that contributes to organic sedimentation (Guyondet et al., 2016), and prefers unfouled sites, dark and sheltered areas (Paetzold et al., 2012) does not necessarily have negative impacts on all bivalves or other organisms, further research is needed. Recently, some studies investigated the impacts of the presence of ascidian *S. clava* in an IMTA framework to optimize the growth of the sea cucumber *A. japonicus* (Zhen et al., 2014; Ju et al., 2015, 2016). These studies have shown that an IMTA framework consisting of ascidian-sea cucumbers-microalgae, not only has the potential to reduce organic matter in the surrounding sediment (Ju et al., 2015), it can also reduce harmful bacteria (Lin et al., 2016) and purify the water body from dissolved nutrients such as nitrogen and phosphorus (Ju et al., 2015).

Moreover, this framework can also have a positive impact on the growth performance of these sea cucumber species (Zhen et al., 2014; Chen et al., 2015; Ju et al., 2016). Available literature shows that only one ascidian species (*S. clava*) was addressed in these studies, and yet with very positive results.

How to incorporate and manage ascidians in an IMTA framework is an important issue with many critical factors that must be considered. Growth rate, spawning season, number of generations, settlement locations, and life span are some of these factors. As an example of how contrasting can these factors be for different ascidians, *C. intestinalis* can produce from 12000 to 100000 eggs over different spawning periods, whereas the colonial ascidian *Botryllus schlosseri* can only produce

TABLE 5 | Summary of the main attributes of the bioactive compounds of ascidians and other features addressed in the 28 publications selected regarding question 3 [For what?].

Country	Studied species	Bioactive compounds and others	References
Greece	*Microcosmus sulcatus*	protein 0.8%, moisture: 81.1%, fat: 1.0%, ash: 7.5%;	Zlatanos et al., 2009
		glutamic acid: 1.05 g.100 g freeze-dried	
Italy	*Microcosmus sulcatus*	2,3-dihydroxy fatty acid glycosphingolipids	Aiello et al., 2003
India	*Didemnum psammathodes*	protein: 3.78 $\mu g.ml^{-1}$; total carbohydrate: 2.15 $\mu g.ml^{-1}$;	Sri Kumaran and Bragadeeswaran, 2014
		crude fiber: 9.2 $\mu g.ml^{-1}$; total free amino acid: 3.2 $\mu g.ml^{-1}$;	
		leucine: 540.9 mg.g, arginine: 401.2 mg.g, lysine: 385.4 mg.g	
	Eudistoma viride	protein: 3.62 $\mu g.ml^{-1}$; total carbohydrate: 12.2 $\mu g.ml^{-1}$;	
		crude fiber: 7.9 $\mu g.ml^{-1}$; total free amino acid: 3.9 $\mu g.ml^{-1}$;	
		leucine: 582.3 mg.g, arginine: 365.4 mg.g, lysine: 344.5 mg.g	
Japan	*Didemnum moseleyi*	Didemnilactone and Neodidemnilactone	Niwa et al., 1991
Japan	*Didemnum moseleyi*	Didemnilactones A and B and Neodidemnilactone	Niwa et al., 1994
Japan	*Diplosoma* sp.	Pentylphenols 1 (inhibited the division of fertilized sea urchin eggs) and 2, cyclopropane fatty acid 3, and cyclopentenones 4 (cytotoxity against HCT116 cells) and 5	Rob et al., 2011
Morocco	*Cynthia savignyi*	Cholesterol was the main sterol: 40.8%	Maoufoud et al., 2009
	Cynthia squamulata	Cholesterol was the main sterol: 59.5%	
NA	Ascidiacea	Man-made glue	Pennati and Rothbächer, 2015
NA	Ascidiacea	edible ascidians: raw, cooked, dried, or pickled	Lambert et al., 2016
Norway	*Ciona intestinalis*	Cellulose: 96%;	Hassanzadeh, 2014
		(g.100 g DW): glutamic acid: 5.27; leucine: 2.54; glycine: 2.31	
Norway	*Ciona intestinalis*	Cholestanol: (32.54% tunic,15.81% inner body);	Zhao et al., 2015
		Cholesterol (29.63% tunic, 33.11% inner body)	
South Korea	*Halocynthia roretzi*	Up to 80% of fishmeal could be replaced with tunic meal of sea squirt without retardation in growth. Optimal growth was fishmeal 20 diet	Choi et al., 2018
South Korea	Polyclinidae	1-Aplidic acid A; 2-Aplidic acid B; 3-4Z-Aplidic acid B; 4-Aplidic acid C; 5-4Z-Aplidic acid C; 6-Aplidamide A	Bao et al., 2009
South Korea	*Halocynthia roretzi*	Abalone fed the sea tangle (ST) 400 diet achieved the best growth	Jang et al., 2017
Turkey	*Phallusia* sp.	Cholesterol: 32%; Volatiles: Hydrocarbons: 48.4%	Slantchev et al., 2002
	Styela sp.	Cholesterol: 42.3%; Volatiles: Phenols: 46.2%	
United States	*Styela clava*	US retail price (frozen): ($3.63/kg)	Karney and Rhee, 2009
United States	*Ecteinascidia turbinate*	Anticancer ecteinascidin 743; Commercial-scale in-sea proved cost effective	Mendola, 2003

up to 50 eggs in 3 months (Paetzold et al., 2012). Solitary ascidians *Ascidiella aspersa*, *C. intestinalis* (Millar, 1952), and *Corella willmeriana* (Lambert, 1968) can develop into mature adults in just 3 months reach up to 50, 120, and 12 mm, respectively, with 1 or 2 generations and a life span of 12–18 months (*A. aspersa* and *C. intestinalis*) and 3 months (*C. willmeriana*).

The difficulty arises in the management of these biological and ecological characteristics due to the range of intra and interspecific variability and the potential environmental impacts that using ascidians may bring (e.g., biofouling). The existence of a specific area that may promote the settlement of ascidians, such as longlines or PVC plates, can be a simple solution to foster the production of biomass of these organisms and allow to easily remove their biomass for multiple applications.

Exploring the possibility of using multiple combinations of different ascidian species with other taxa, such as fish, shellfish, or echinoderms (namely, sea cucumbers) is paramount to test innovative IMTA frameworks with enhanced socio-economic and environmental performance.

Ascidians as Bioresources for High-End Uses

Considering the increase of wild-harvested or cultured ascidians for human consumption, mainly in Japan, South Korea, and Chile, knowledge on the proximate composition, biocompounds, food safety issues are of greater relevance.

Over the last decade, an increasing concern on food safety issues associated with ascidians has led prompt several studies on the identification of bacteria associated with edible

ascidians such as *H. aurantium* (Chen et al., 2018) and *H. roretzi* (Kumagai et al., 2011). Bacteria associated with ascidians can also be a source of bioactive secondary metabolites and biosurfactants with diverse biotechnology applications in the food-processing industry, among other high-end markets (Achieng et al., 2017).

Several natural products have been isolated from ascidians, for example, the cellulose that is present almost exclusively in the ascidian's tunic and it is rich in carbohydrate contents (Zhao and Li, 2016), whereas the inner body is protein-rich (Berrill and Ray Society, 2005; Hassanzadeh, 2014). Many other compounds, for example, alkaloids, cyclic peptides, and polyketides, collagens, sulfated polysaccharides, glycosaminoglycans, sterols, among others, can be exploited as by-products in the pharmaceutical and chemical industry (Hassanzadeh, 2014; Monmai et al., 2018) due to their antibacterial, antifungal, antitumor and anti-inflammatory activities (Chen et al., 2018). Numerous biocompounds have successfully been retrieved from ascidians, a recent review on this matter describes "about 160 molecules endowed with antimicrobial activity produced by ascidians and/or by their associated microorganisms" (Casertano et al., 2020).

In recent years, the search for new chemical constituents derived from marine invertebrates has increased intensity (Datta et al., 2015). For instance, Pennati and Rothbächer (2015) investigated ascidian's larval bioadhesion properties to develop man-made glues and fouling resistant surfaces from solitary and colonial ascidians. Nowadays, ascidians are used in multiple applications such as fishing bait, health supplement tablets (Lambert et al., 2016), and as ornamental species for marine aquaria, fetching high prices online.[1,2]

Looking at fatty acids in more detail, our review revealed that approximately 70% of publications regarding fatty acids focused on solitary ascidians and once again ascidian *C. intestinalis* was the main focus. Many studies have drawn their attention to establishing ascidians as a new bioresource for *n*-3 fatty acids-rich marine lipids (Hassanzadeh, 2014; Zhao et al., 2015; Zhao and Li, 2016). Nonetheless, the profiling of fatty acids in ascidians, in general, is still poorly explored. Our study retrieved information from 20 species, with 13 ascidian species being addressed only once.

The overall results suggest that ascidians can be a good source of *n*-3 polyunsaturated fatty acids, namely, essential fatty acids such as EPA and DHA, which were detected in most ascidians surveyed (Dagorn et al., 2010; Zhao et al., 2015). Therefore, ascidians present a high nutritional value, they are a healthy seafood choice due to their high protein levels and low calories (Lee et al., 1995; Kang et al., 2011). Hassanzadeh (2014) concluded that the composition profile of ascidian fatty acids seems to be similar to fish oil. Therefore, ascidians biomass may eventually be a good alternative to fish oil and fish meal in formulated aquafeeds.

Moreover, ascidians present amino acid composition similar to egg albumin, suggesting a great potential and capability to be weighed as marine organisms' feed (Hassanzadeh, 2014). Indeed, the replacement of fish meal with ascidian's biomass in aquafeeds has already started being addressed with Jang et al. (2017) and Choi et al. (2018) having partially or fully replaced the fish meal with the tunic of the ascidian *H. roretzi* in aquafeeds for the abalone *Haliotis discus* with compromising its growth performance.

CONCLUSION

In the past two decades, considerable insights have been achieved on ascidians' ecology and biology, including filtration and retention efficiencies. Their nutritional value and potential role in IMTA frameworks are also starting to be thoroughly investigated.

Despite the intra and interspecific variability recorded for ascidians filtration rates, there is a consensus that these organisms do display high filtration rates, that they can retain submicron and picoplankton particles, and they also present a fast-growing rate. As available scientific evidence suggests that these organisms are capable to perform well under an IMTA framework, however, it is important to investigate if competition with other filter-feeders for trophic resources and space can occur, namely, with mussels, scallops, and oysters.

Furthermore, available studies to date suggest that ascidians achieve higher growth performances in IMTA frameworks when in the presence of sea cucumbers and fish. The development of innovative IMTA frameworks is important to maximize the systems carrying capacity.

Finally, among other potentially bioactive compounds, ascidians represent a rich source of EPA and DHA, both being essential fatty acids paramount for human consumption, marine fish, and shrimp nutrition. Despite some cultural barriers in western countries, ascidians are increasingly regarded as a healthy seafood for human consumption, being an interesting source of essential amino and fatty acids. The use of ascidians as an alternative ingredient for the formulation of aquafeeds also looks promising and will certainly deserve further attention in coming years.

AUTHOR CONTRIBUTIONS

LM contributed to the main investigation, writing the original draft, reviewing, and editing the final version. RC provided supervision of the writing, reviewing, editing, and validation. AL contributed to supervision of the writing, reviewing and editing process, validation, and funding acquisition. All authors contributed to the article and approved the submitted version.

FUNDING

We are thankful to the Integrated Program of SR&TD "Smart Valorization of Endogenous Marine Biological Resources Under a Changing Climate" (reference Centro-01-0145-FEDER-000018)

[1] https://www.reefcleaners.org/

[2] https://www.mysaltwaterfishstore.com/

and to FCT (Portuguese Foundation for Science and Technology) for the financial support through a Ph.D. grant attributed to LM (PD/BD/127918/2016). We acknowledge financial support to CESAM by FCT/MEC (UIDP/50017/2020 + UIDB/50017/2020) through national funds.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.849870/full#supplementary-material

REFERENCES

Achieng, A. O., Rawat, M., Ogutu, B., Guyah, B., Ong'echa, J. M., Perkins, D. J., et al. (2017). Ascidians: an emerging marine model for drug discovery and screening. *Curr. Top. Med. Chem.* 17, 2056–2066. doi: 10.2174/1568026617666170130

Aiello, A., Fattorusso, E., Mangoni, A., and Menna, M. (2003). Three new 2, 3-dihydroxy fatty acid glycosphingolipids from the Mediterranean tunicate *Microcosmus sulcatus*. *Eur. J. Org. Chem.* 4, 734–739.

Albert, S., Bonaglia, S., Stjärnkvist, N., Winder, M., Thamdrup, B., and Nascimento, F. J. A. (2021). Influence of settling organic matter quantity and quality on benthic nitrogen cycling. *Limnol. Oceanogr.* 66, 1882–1895. doi: 10.1002/LNO.11730

Alexander, K. A., Angel, D., Freeman, S., Israel, D., Johansen, J., Kletou, D., et al. (2016). Improving sustainability of aquaculture in Europe: stakeholder dialogues on Integrated Multi-trophic Aquaculture (IMTA). *Environ. Sci. Policy* 55, 96–106. doi: 10.1016/j.envsci.2015.09.006

Armsworthy, S. L., Macdonald, B. A., and Ward, J. E. (2001). Feeding activity, absorption efficiency and suspension feeding processes in the ascidian, *Halocynthia pyriformis* (Stolidobranchia: Ascidiacea): responses to variations in diet quantity and quality. *J. Exp. Biol. Ecol.* 260, 41–69. doi: 10.1016/s0022-0981(01)00238-6

Bao, B., The, H., Zhang, P., Hong, J., Lee, C., Young, H., et al. (2009). Bicyclic α,ω-dicarboxylic acid derivatives from a colonial tunicate of the family Polyclinidae. *Bioorg. Med. Chem. Lett.* 19, 6205–6208. doi: 10.1016/j.bmcl.2009.08.094

Barrington, K., Chopin, T., and Robinson, S. (2009). "Integrated multi-trophic aquaculture (IMTA) in marine temperate waters," in *Integrated Mariculture: a Global Review*. (ed.) D. Soto, (Rome: FAO).

Béné, C., Barange, M., Subasinghe, R., Pinstrup-Andersen, P., Merino, G., Hemre, G. I., et al. (2015). Feeding 9 billion by 2050 - putting fish back on the menu. *Food Secur.* 7, 261–274. doi: 10.1007/s12571-015-0427-z

Berrill, N. J., and Ray Society (2005). *The Tunicata With an Account of the British Species*. Lymington: Pisces Conservation.

Bullard, S. G., Davis, C. V., and Shumway, S. E. (2013). Seasonal patterns of ascidian settlement at an aquaculture facility in the Damariscotte River, Maine. *J. Shellfish Res.* 32, 255–264. doi: 10.2983/035.032.0202

Carballeira, N. M., Shalabi, F., Stefanov, K., and Dimitrov, K. (1995). Comparison of the fatty acids of the tunicate Botryllus schlosseri from the Black Sea with two associated bacterial strains. *Lipids* 30, 677–679. doi: 10.1007/BF02537006

Carlisle, D. B. (1966). The ciliary current of Phallusia [Ascidiacea] and the squirting of sea squirts. *J. Mar. Biol. Assoc. United Kingdom* 46, 125–127.

Carvajalino-Fernández, M. A., Sævik, P. N., Johnsen, I. A., Albretsen, J., and Keeley, N. B. (2020). Simulating particle organic matter dispersal beneath Atlantic salmon fish farms using different resuspension approaches. *Mar. Pollut. Bull.* 161:111685. doi: 10.1016/J.MARPOLBUL.2020.111685

Carver, C. E., Chisholm, A., and Mallet, A. L. (2003). Strategies to mitigate the impact of *Ciona intestinalis* (L.) biofouling on shellfish production. *J. Shellfish Res.* 22, 621–631.

Casertano, M., Menna, M., and Imperatore, C. (2020). The ascidian-derived metabolites with antimicrobial properties. *Antibiotics* 9:510. doi: 10.3390/antibiotics9080510

Casso, M., Navarro, M., Ordóñez, V., and Fernández-tejedor, M. (2018). Seasonal patterns of settlement and growth of introduced and native ascidians in bivalve cultures in the Ebro Delta (NE Iberian Peninsula). *Reg. Stud. Mar. Sci.* 23, 12–22. doi: 10.1016/j.rsma.2017.11.002

Chen, L., Hu, J., Xu, J., Shao, C., and Wang, G. (2018). Biological and chemical diversity of ascidian-associated microorganisms. *Mar. Drugs* 743:362. doi: 10.3390/md16100362

Chen, L., Xing, R., Jiang, A., Teng, L., and Wang, C. (2015). A preliminary study on the potential value of a novel integrated aquaculture system on water purification. *Aquac. Int.* 24, 879–892. doi: 10.1007/s10499-015-9958-9958

Choi, D. G., Kim, J., Yun, A., and Cho, S. H. (2018). Dietary substitution effect of fishmeal with tunic meal of sea squirt, *Halocynthia roretzi*, Drasche, on growth and soft body composition of juvenile abalone, Haliotis discus, Reeve 1846. *J. World Aquac. Soc.* 49, 1095–1104. doi: 10.1111/jwas.12537

Chopin, T., Buschmann, A. H., Halling, C., Troell, M., Kautsky, N., Neori, A., et al. (2001). Integrating seaweeds into marine aquaculture systems: a key toward sustainability. *J. Phycol.* 37, 975–986.

Clos, K. T. D., Jones, I. T., Carrier, T. J., Damian, C., and Jumars, P. A. (2017). Model-assisted measurements of suspension-feeding flow velocities. *J. Exp. Biol.* 220, 2096–2107. doi: 10.1242/jeb.147934

Cordell, J. R., Levy, C., and Toft, J. D. (2013). Ecological implications of invasive tunicates associated with artificial structures in Puget Sound, Washington, USA. *Biol. Invasions* 15, 1303–1318. doi: 10.1007/s10530-012-0366-y

Costello, C., Cao, L., Gelcich, S., Cisneros-mata, M. Á., Free, C. M., Froehlich, H. E., et al. (2020). The future of food from the sea. *Nature* 588, 95–100. doi: 10.1038/s41586-020-2616-y

Dagorn, F., Dumay, J., Monniot, C., Rabesaotra, V., and Barnathan, G. (2010). Phospholipid distribution and phospholipid fatty acids of the tropical tunicates *Eudistoma sp*. and *Leptoclinides uniorbis*. *Lipids* 45, 253-261. doi: 10.1007/s11745-010-3389-3380

Daigle, R. M., and Herbinger, C. M. (2009). Ecological interactions between the vase tunicate (*Ciona intestinalis*) and the farmed blue mussel (*Mytilus edulis*) in Nova Scotia, Canada. *Aquat. Invasions* 4, 177–187. doi: 10.3391/ai.2009.4.1.18

Datta, D., Nath Talapatra, S., and Swarnakar, S. (2015). Bioactive compounds from marine invertebrates for potential medicines - an overview. *Int. Lett. Nat. Sci.* 34, 42–61.

Dijkstra, J. A., and Nolan, R. (2017). Potential of the invasive colonial ascidian, *Didemnum vexillum*, to limit escape response of the sea scallop, *Placopecten magellanicus*. *Aquactic Invasions* 6, 451–456. doi: 10.3391/ai.2011.6.4.10

Draughon, L. D., Scarpa, J., Hartmann, J. X., Draughon, L. D., Scarpa, J., and Hartmann, J. X. (2010). Are filtration rates for the rough tunicate Styela plicata independent of weight or size? *J. Environ. Sci. Heal. Part A* 45, 168–176. doi: 10.1080/10934520903429816

FAO (2020). *The State of World Fisheries and Aquaculture*. Rome: FAO.

Fiala-Médioni, A. (1973). Ethologie alimentaire d' invertébrés benthiques filtreurs (ascidies). I. Dispositif expérimental. Taux de filtration et de digestion chez *Phallusia mammillata*. *Mar. Biol.* 23, 137–145.

Fiala-Médioni, A. (1974). Ethologie alimentaire d' invertébrés benthiques filtreurs (ascidies). II . variations des taux de filtration et de digestion en fonction de l' espèce. *Mar. Biol.* 28, 199–206. doi: 10.1007/BF00387298

Fiala-Médioni, A. (1978a). Filter-feeding ethology of benthic invertebrates (Ascidians). IV. pumping rate, filtration rate, filtration efficiency. *Mar. Biol.* 48, 243–249.

Fiala-Médioni, A. (1978b). Filter-feeding ethology of benthic invertebrates (Ascidians). V. Influence of temperature on pumping, filtration and digestion rates and rhythms in *Phallusia mamillata*. *Mar. Biol.* 48, 251–259.

Fiala-Médioni, A. (1979). Effects of oxygen tension on pumping, filtration and oxygen uptake in the ascidian *Phallusia mammillata*. *Mar. Ecol. Prog. Ser.* 1, 49–53.

Filgueira, R., Strople, L. C., Strohmeier, T., Rastrick, S., and Strand, O. (2019). Mussels or tunicates: that is the question. evaluating efficient and sustainable resource use by low-trophic species in aquaculture settings. *J. Clean. Prod.* 231, 132–143. doi: 10.1016/j.jclepro.2019.05.173

Fletcher, L. M., Forrest, B. M., and Bell, J. J. (2013). Impacts of the invasive ascidian Didemnum vexillum on green-lipped mussel *Perna canaliculus* aquaculture in New Zealand. *Aquac. Environ. Interact.* 4, 17–30. doi: 10.3354/aei00069

Fry, J. P., Love, D. C., MacDonald, G. K., West, P. C., Engstrom, P. M., Nachman, K. E., et al. (2016). Environmental health impacts of feeding crops to farmed fish. *Environ. Int.* 91, 201–214. doi: 10.1016/j.envint.2016.02.022

Gasparini, F., Manni, L., Cima, F., Zaniolo, G., Burighel, P., Caicci, F., et al. (2015). Sexual and asexual reproduction in the colonial ascidian *Botryllus schlosseri*. *Genesis* 53, 105–120. doi: 10.1002/dvg.22802

Guyondet, T., Patanasatienkul, T., Comeau, L. A., Landry, T., and Davidson, J. (2016). Preliminary model of tunicate infestation impacts on seston availability and organic sedimentation in longline mussel farms. *Aquaculture* 465, 387–394. doi: 10.1016/j.aquaculture.2016.09.026

Hassanzadeh, M. (2014). Unique marine organism: identification of some methods for biomaterial production. *Chem. Eng. Trans.* 37, 385–390. doi: 10.3303/CET1437065

Hily, C. (1991). Is the activity of benthic suspension feeders a factor controlling water quality in the Bay of Brest? *Mar. Ecol. Prog. Ser.* 69, 179–188.

Hily, C., Potin, P., and Floc'h, J. Y. (1992). Structure of subtidal algal assemblages on soft-bottom sediments: fauna/flora interactions and role of disturbances in the Bay of Brest, France. *Mar. Ecol. Prog. Ser.* 85, 115–130. doi: 10.3354/meps085115.

Hirose, E., and Sensui, N. (2021). Substrate selection of ascidian larva: wettability and nano-structures. *J. Mar. Sci. Eng.* 9:634. doi: 10.3390/JMSE9060634

Hodson, S. L., Burke, C. M., and Bissett, A. P. (2000). Biofouling of fish-cage netting: the efficacy of a silicone coating and the effect of netting colour. *Aquaculture* 184, 277–290. doi: 10.1016/S0044-8486(99)00328-322

Honegger, T. G. (1986). Fertilization in ascidians: studies on the egg envelope, sperm and gamete interactions in Phallusia mammillata. *Dev. Biol.* 118, 118–128. doi: 10.1016/0012-1606(86)90079-5

Hoxha, T., Crookes, S., Lejeusne, C., Dick, J. T. A., Chang, X., Bouchemousse, S., et al. (2018). Comparative feeding rates of native and invasive ascidians. *Mar. Pollut. Bull.* 135, 1067–1071. doi: 10.1016/j.marpolbul.2018.08.039

Jacobi, Y. (2018). Efficient filtration of micron and submicron particles by ascidians from oligotrophic waters. *Limnol. Oceanogr.* 63, 267–279. doi: 10.1002/lno.10736

Jang, B., Kim, P. Y., Kim, H. S., Lee, K. W., Kim, H. J., Choi, D. G., et al. (2017). Substitution effect of sea tangle (ST) (*Laminaria japonica*) with tunic of sea squirt (SS) (*Halocynthia roretzi*) in diet on growth and carcass composition of juvenile abalone (*Haliotis discus*, Reeve 1846). *Aquac. Nutr.* 1–8. doi: 10.1111/anu.12593

Jeong, B. Y., Ohshima, T., and Koizumi, C. (1996). Hydrocarbon chain distribution of ether phospholipids of the ascidian Halocynthia roretzi and the sea urchin Strongylocentrotus intermedius. *Lipids* 31, 9–18. doi: 10.1007/BF02522404

Jørgensen, B. Y. C. B. (1954). Quantitative aspects of filter feeding in invertebrates. *Biol. Rev.* 30, 391–453. doi: 10.1128/AEM.02007-20

Jørgensen, B., and Goldberg, E. D. . (1953). Particle filtration in some ascidians and lamellibranchs. *Biol. Bull.* 105, 477–489.

Ju, B., Chen, L., Xing, R., and Jiang, A. (2015). A new integrated multi-trophic aquaculture system consisting of *Styela clava*, microalgae, and *Stichopus japonicus*. *Aquac. Int.* 23, 471–497. doi: 10.1007/s10499-014-9829-9828

Ju, B., Jiang, A., Xing, R., Chen, L., and Teng, L. (2016). Optimization of conditions for an integrated multi-trophic aquaculture system consisting of sea cucumber *Apostichopus japonicus* and ascidian *Styela clava*. *Aquac. Int.* 25, 265–286. doi: 10.1007/s10499-016-0027-28

Kanamori, M., Baba, K., Natsuike, M., and Goshima, S. (2017). Life history traits and population dynamics of the invasive ascidian, *Ascidiella aspersa*, on cultured scallops in Funka Bay, Hokkaido, northern Japan. *J. Mar. Biol. Assoc. United Kingdom* 97, 387–399. doi: 10.1017/S0025315416000497

Kang, C., Jung, E., Chan, W., Jung, N., Park, H., and Choi, K. (2011). Physiological energetics and gross biochemical composition of the ascidian *Styela clava* cultured in suspension in a temperate bay of Korea. *Aquaculture* 319, 168–177. doi: 10.1016/j.aquaculture.2011.06.016

Kang, C., Lee, Y., Han, E., Park, H., Yun, S., and Chan, W. (2015). Effects of temperature and body size on the physiological energetics of the stalked sea squirt *Styela clava*. *J. Exp. Mar. Bio. Ecol.* 462, 105–112. doi: 10.1016/j.jembe.2014.10.026

Karney, R. C., and Rhee, W. Y. (2009). Market potential for *Styela clava*, a non-indigenous pest invading New England coastal waters. *Aquactic Invasions* 4, 295–297. doi: 10.3391/ai.2009.4.1.31

Khalaman, V. V. (2001). Fouling communities of mussel aquaculture installations in the White Sea. *Russ. J. Mar. Biol.* 27, 227–237.

Kowalke, J. (1999). Filtration in antarctic ascidians - striking a balance. *J. Exp. Mar. Bio. Ecol.* 242, 233–244. doi: 10.1016/s0022-0981(99)00108-2

Kumagai, A., Suto, A., Ito, H., Tanabe, T., Song, J., Kitamura, S., et al. (2011). Soft tunic syndrome in the edible ascidian Halocynthia roretzi is caused by a kinetoplastid protist. *Dis. Aquat. Organ.* 95, 153–161. doi: 10.3354/dao02372

Lacoste, É., Raimbault, P., Harmelin-vivien, M., and Gaertner-mazouni, N. (2016). Trophic relationships between the farmed pearl oyster *Pinctada margaritifera* and its epibionts revealed by stable isotopes and feeding experiments. *Aquac. Environ. Interact.* 8, 55–66. doi: 10.3354/aei00157

Lambert, G. (1968). The general ecology and growth of a solitary ascidian, *Corella willmeriana*. *Biol. Bull.* 135, 296–307. doi: 10.2307/1539783

Lambert, G., and Lambert, C. C. (1987). Spicule formation in the solitary ascidian, *Herdmania momus*. *J. Morphol.* 192, 145–159. doi: 10.1002/jmor.1051920206

Lambert, G., Karney, R. C., Rhee, W. Y., Carman, M. R., Shellfish, V., Box, P. O., et al. (2016). Wild and cultured edible tunicates: a review. Manag. Biol. Invasions 7, 59–66.

Lee, K. H., Hong, B. I., Jung, B. C., and Cho, H. S. (1995). Seasonal variations of nutrients in warty sea squirt (*Styela clava*). *J. Korean Soc. Food Nutr. (Korea Republic)* 24, 268–273.

Lefebvre, S., Barillé, L., and Clerc, M. (2000). Pacific oyster (*Crassostrea gigas*) feeding responses to a fish-farm effluent. *Aquaculture* 187, 185–198. doi: 10.1016/S0044-8486(99)00390-397

Lesser, M. P., and Slattery, M. (2015). Picoplankton consumption supports the ascidian *Cnemidocarpa verrucosa* in McMurdo Sound, Antarctica. *Mar. Ecol. Prog. Ser.* 525, 117–126. doi: 10.3354/meps11215

Lin, J., Ju, B., Yao, Y., Lin, X., Xing, R., Teng, L., et al. (2016). Microbial community in a multi-trophic aquaculture system of *Apostichopus japonicus*, *Styela clava* and microalgae. *Aquac. Int.* 24, 1119–1140. doi: 10.1007/s10499-016-9975-9972

Lohrer, A. M., Hewitt, J. E., and Thrush, S. F. (2006). Assessing far-field effects of terrigenous sediment loading in the coastal marine environment. *Natl. Inst. Water Atmos. Res.* 315, 13–18. doi: 10.3354/meps315013

Lutz-Collins, V., Ramsay, A., Quijón, P. A., and Davidson, J. (2009). Invasive tunicates fouling mussel lines: evidence of their impact on native tunicates and other epifaunal invertebrates. *Aquat. Invasions* 4, 213–220. doi: 10.3391/ai.2009.4.1.22

Lutzen, J. (1999). *Styela clava* Herdman (Urochordata, Ascidiacea), a successful immigrant to North West Europe: ecology, propagation and chronology of spread. *Helgoländer Meeresuntersuchungen* 52, 383–391.

Maoufoud, S., Abdelmjid, A., Abboud, Y., and Tarik, A. (2009). Chemical composition of fatty acids and sterols from tunicates *Cynthia savignyi*, *Cynthia squamulata* and from the brown alga *Cystoseira tamariscifolia*. *Phys. Chem. News* 47, 115–119.

Mendola, D. (2003). Aquaculture of three phyla of marine invertebrates to yield bioactive metabolites: process developments and economics. *Biomol. Eng.* 20, 441–458. doi: 10.1016/S1389-0344(03)00075-73

Millar, R. H. (1952). The annual growth and reproductive cycle in four ascidians. *J. Mar. Biol. Assoc. United Kingdom* 31, 41–61. doi: 10.1017/S0025315400003672

Monmai, C., Go, S. H., Shin, I. -S., You, S. G., Lee, H., Kang, S. B., et al. (2018). Immune-enhancement and anti-inflammatory activities of fatty acids extracted from *Halocynthia aurantium* tunic in RAW264.7 cells. *Mar. Drugs* 16:309. doi: 10.3390/md16090309

Nakai, S., Shibata;, J., Umehara, A., Okuda, T., and Nishijima, W. (2018). Filtration rate of the ascidian *Ciona savignyi* and its possible impact. *Thalassas* 34, 271–277. doi: 10.1007/s41208-017-0061-y

Naylor, R., and Burke, M. (2005). Aquaculture and ocean resources: raising tigers of the sea. *Annu. Rev. Environ. Resour.* 30, 185–218. doi: 10.1146/annurev.energy.30.081804.121034

Neori, A., Chopin, T., Troell, M., Buschmann, A. H., Kraemer, G. P., Halling, C., et al. (2004). Integrated aquaculture: rationale, evolution and state of the art emphasizing seaweed biofiltration in modern mariculture. *Aquaculture* 231, 361–391. doi: 10.1016/j.aquaculture.2003.11.015

Niwa, H., Inagaki, H., and Yamada, K. (1991). Didemnilactone and neodidemnilactone, two new fatty acid metabolites possessing a 10-membered lactone from the tunicate *Didemnum moseleyi* (Herdman). *Tetrahedron Lett.* 32, 5127–5128.

Niwa, H., Watanabe, M., Inagaki, H., and Yamada, K. (1994). Didemnilactones A and B and Neodidemnilactone, three new fatty acid metabolites isolated from the tunicate *Didemnum moseleyi* (Herdman). *Tetrahedron* 50, 7385–7400.

Ordóñez, V., Pascual, M., Rius, M., and Turon, X. (2013). Mixed but not admixed: a spatial analysis of genetic variation of an invasive ascidian on natural and artificial substrates. *Mar. Biol.* 160, 1645–1660. doi: 10.1007/s00227-013-2217-2215

Paetzold, S. C., Giberson, D. J., Hill, J., Davidson, J. D. P., and Davidson, J. (2012). Effect of colonial tunicate presence on Ciona intestinalis recruitment within a mussel farming environment. *Manag. Biol. Invasions* 3, 15–23.

Pascoe, P. L., Parry, H. E., and Hawkins, A. J. S. (2007). Dynamic filter-feeding responses in fouling organisms. *Aquat. Biol.* 1, 177–185. doi: 10.3354/ab00022

Pennati, R., and Rothbächer, U. (2015). Bioadhesion in ascidians: a developmental and functional genomics perspective. *Interface Focus* 2, 1–10. doi: 10.1098/rsfs.2014.0061

Petersen, J. K. (2007). Ascidian suspension feeding. *Rome* 342, 127–137. doi: 10.1016/j.jembe.2006.10.023

Petersen, J. K., and Riisgärd, H. U. (1992). Filtration capacity of the ascidian *Ciona intestinalis* and its grazing impact in a s hallow fjord. *Mar. Ecol. Prog. Ser.* 88, 9–17. doi: 10.3354/meps088009

Petersen, J. K., and Svane, I. (2002). Filtration rate in seven Scandinavian ascidians: implications of the morphology of the gill sac. *Mar. Biol.* 140, 397–402. doi: 10.1007/s002270100706

Pile, A. J. (2005). Overlap in diet between co-occuring active suspension feeders on tropical and temperate reefs. *Bull. Mar. Sci.* 76, 743–749.

Ramsay, A., Davidson, J., Landry, T., and Arsenault, G. (2008a). Process of invasiveness among exotic tunicates in Prince Edward Island, Canada. *Biol. Invasions* 10, 1311–1316. doi: 10.1007/s10530-007-9205-y

Ramsay, A., Davidson, J., Landry, T., and Stryhn, H. (2008b). The effect of mussel seed density on tunicate settlement and growth for the cultured mussel, *Mytilus edulis*. *Aquaculture* 275, 194–200. doi: 10.1016/j.aquaculture.2008.01.024

Randløv, A., and Riisgärd, H. U. (1979). Efficiency of particle retention and filtration rate in four species of ascidians. *Mar. Ecol. Prog. Ser.* 1, 55–59.

Reid, G. K., Lefebvre, S., Filgueira, R., Robinson, S. M. C., Broch, O. J., Dumas, A., et al. (2020). Performance measures and models for open-water integrated multi-trophic aquaculture. *Rev. Aquac.* 12, 47–75. doi: 10.1111/RAQ.12304

Reid, G. K., Liutkus, M., Robinson, S. M. C., Chopin, T. R., Blair, T., Lander, T., et al. (2009). A review of the biophysical properties of salmonid faeces: implications for aquaculture waste dispersal models and integrated multi-trophic aquaculture. *Aquac. Res.* 40, 257–273. doi: 10.1111/J.1365-2109.2008.02065.X

Ribes, M., Coma, R., and Gili, J. (1998). Seasonal variation of in situ feeding rates by the temperate ascidian *Halocynthia papillosa*. *Mar. Ecol. Prog. Ser.* 175, 201–213.

Riisgärd, H. U., and Larsen, P. S. (2016). "Filter-feeding zoobenthos and hydrodynamics," in *Marine Animal Forests* (ed.) S. Rossi (Switcherland: Springer International Publishing)

Rius, M., Heasman, K. G., and Mcquaid, C. D. (2011). Long-term coexistence of non-indigenous species in aquaculture facilities. *Mar. Pollut. Bull.* 62, 2395–2403. doi: 10.1016/j.marpolbul.2011.08.030

Rob, T., Ogi, T., Maarisit, W., Taira, J., and Ueda, K. (2011). Isolation of C11 compounds and a cyclopropane fatty acid from an *Okinawan ascidian*, *Diplosoma sp*. *Molecules* 16, 9972–9982. doi: 10.3390/molecules16129972

Robbins, I. J. (1983). The effects of body size, temperature, and suspension density on the filtration and ingestion of inorganic particulate suspensions by ascidians. *J. Exp. Mar. Bio. Ecol.* 70, 65–78. doi: 10.1016/0022-0981(83)90149-1

Robbins, J. (1984). The regulation of ingestion rate, at high suspended particulate concentrations, by some phleobranchiate ascidians. *J. Exp. Mar. Bio. Ecol.* 82, 1–10.

Rosa, M., Holohan, B. A., Shumway, S. E., Bullard, S. G., Wikfors, G. H., Morton, S., et al. (2013). Biofouling ascidians on aquaculture gear as potential vectors of harmful algal introductions. *Harmful Algae* 23, 1–7. doi: 10.1016/j.hal.2012.11.008

Shenkar, N., and Swalla, B. J. (2011). Global diversity of *Ascidiacea*. *PLoS One* 6:e20657. doi: 10.1371/journal.pone.0020657

Sievers, M., Fitridge, I., Dempster, T., and Keough, M. J. (2013). Biofouling leads to reduced shell growth and flesh weight in the cultured mussel *Mytilus galloprovincialis*. *Biofouling* 29, 97–107. doi: 10.1080/08927014.2012.749869

Slantchev, K., Yalçın, F., Ersöz, T., Nechev, J., Çalis, I., Stefanov, K., et al. (2002). Composition of lipophylic extracts from two tunicates, *Styela sp*. and *Phallusia sp*. from the Eastern Mediterranean. *Z. Naturforsch* 57, 534–540. doi: 10.1515/znc-2002-5-623

Sri Kumaran, N., and Bragadeeswaran, S. (2014). Nutritional composition of the colonial ascidian Eudistoma viride and *Didemnum psammathodes*. *Biosci. Biotechnol. Res. Asia* 11, 331–338. doi: 10.13005/bbra/1427

Stabili, L., Licciano, M., Flavia, M., and Giangrande, A. (2016). Filtering activity on a pure culture of *Vibrio alginolyticus* by the solitary ascidian *Styela plicata* and the colonial ascidian *Polyandrocarpa zorritensis*: a potential service to improve microbiological seawater quality economically. *Sci. Total Environ.* 573, 11–18. doi: 10.1016/j.scitotenv.2016.07.216

Sumerel, A. N., and Finelli, C. M. (2014). Particle size, flow speed, and body size interactions determine feeding rates of a solitary ascidian *Styela plicata*: a flume experiment. *Mar. Ecol. Prog. Ser.* 495, 193–204. doi: 10.3354/meps10571

Troell, M., Joyce, A., Chopin, T., Neori, A., Buschmann, A. H., and Fang, J. G. (2009). Ecological engineering in aquaculture - potential for integrated multi-trophic aquaculture (IMTA) in marine offshore systems. *Aquaculture* 297, 1–9. doi: 10.1016/j.aquaculture.2009.09.010

Vieira, H., Leal, M. C., and Calado, R. (2020). Fifty shades of blue: how blue biotechnology is shaping the bioeconomy. *Trends Biotechnol.* 38, 940–943. doi: 10.1016/j.tibtech.2020.03.011

Yahel, G., Yahel, R., Katz, T., Lazar, B., Herut, B., and Tunnicliffe, V. (2008). Fish activity: a major mechanism for sediment resuspension and organic matter remineralization in coastal marine sediments. *Mar. Ecol. Prog. Ser.* 372, 195–209. doi: 10.3354/MEPS07688

Yahel, R., Yahel, G., and Genin, A. (2002). Daily cycles of suspended sand at coral reefs: a biological control. *Limnol. Oceanogr.* 47, 1071–1083. doi: 10.4319/LO.2002.47.4.1071

Young, C. M. (1989). Distribution and dynamics of an intertidal ascidian pseudopopulation. *Bull. Mar. Sci.* 45, 288–303.

Zhao, Y., and Li, J. (2016). Ascidian bioresources: common and variant chemical compositions and exploitation strategy - examples of *Halocynthia roretzi*, *Styela plicata*, *Ascidia sp*. and *Ciona intestinalis*. *Zeitschrift Naturforsch. - Sect. C J. Biosci.* 71, 165–180. doi: 10.1515/znc-2016-2012

Zhao, Y., Wang, M., Lindström, M. E., and Li, J. (2015). Fatty acid and lipid profiles with emphasis on n-3 fatty acids and phospholipids from *Ciona intestinalis*. *Lipids* 50, 1009–1027. doi: 10.1007/s11745-015-4049-4041

Zhen, Y., Aili, J., and Bao, J. (2014). Effect of the ascidian *Styela clava* on the growth of small holothurians of the species *Apostichopus japonicus*. *Mar. Freshw. Behav. Physiol.* 47, 211–226. doi: 10.1080/10236244.2014.929800

Zlatanos, S., Laskaridis, K., and Sagredos, A. (2009). Determination of proximate composition, fatty acid content and amino acid profile of five lesser-common sea organisms from the Mediterranean Sea. *Int. J. Food Sci. Technol.* 44, 1590–1594. doi: 10.1111/j.1365-2621.2008.01870.x

Zvyagintsev, A. Y., Sanamyan, K. E., and Kashenko, S. D. (2007). On the introduction of the ascidian *Ciona savignyi* Herdman, 1882 into Peter the Great Bay, Sea of Japan. *Russ. J. Mar. Biol.* 33, 133–136. doi: 10.1134/S1063074007020083

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.

frontiers | Frontiers in Marine Science

ORIGINAL RESEARCH
published: 13 May 2022
doi: 10.3389/fmars.2022.911383

Successful Culture of *Pelagia noctiluca* (Cnidaria: Scyphozoa) Over Time: A Continuous Supply of the Holoplanktonic Jellyfish for Research and Industrial Applications

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OPEN ACCESS

Edited by:
Sílvia Lourenço, Instituto Politécnico de Leiria, Portugal

Reviewed by:
Ricardo Calado, University of Aveiro, Santiago University Campus, Portugal
Mar Bosch Belmar, University of Palermo, Italy

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Specialty section:
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

Received: *02 April 2022*
Accepted: *20 April 2022*
Published: *13 May 2022*

Citation:
Ballesteros A, Páez D, Santín A, García A, Martín Y, Alonso E, Jourdan E and Gili J-M (2022) Successful Culture of Pelagia noctiluca (Cnidaria: Scyphozoa) Over Time: A Continuous Supply of the Holoplanktonic Jellyfish for Research and Industrial Applications. Front. Mar. Sci. 9:911383. doi: 10.3389/fmars.2022.911383

The scyphozoan *Pelagia noctiluca* has potential value for research and a wide variety of industrial applications. However, its holoplanktonic life cycle makes it difficult to maintain a permanent stock in aquaculture, unlike the continuous supply of newly-released ephyrae from jellyfish species with a benthic life stage. We investigated optimal feeding conditions for the captive breeding of *P. noctiluca*, monitoring total body diameter, morphological development, survival rate, and instantaneous growth rate. Diets of different items encapsulated in gelatin or non-motile prey were not suitable for successful growth of the early stages (ephyra and metaephyra), whereas rotifers were a sufficient food source for healthy growth. Fertilized eggs from sexually-mature jellyfish were obtained at 84.5 ± 2.1 and 87.0 ± 9.9 days post-fecundation in the presence and absence of gelatinous prey (*Aurelia* sp.), respectively. In contrast with the general consensus, we demonstrate that the incorporation of medusivorous prey was not essential for proper growth and closure of *P. noctiluca* life cycle. Instead, we recommend the inclusion of white shrimps (*Acetes* sp.) for the formation and maturation of gonads and production of eggs. Instantaneous growth rates (57.0% d^{-1}), the highest reported to date for this species in captive conditions, were higher during the early stages. A third generation of cultured *P. noctiluca* was obtained for the first time (169 – 179 experimental days), demonstrating the durability of the culture, providing permanent stock and halving the production time of mature individuals compared to previous studies. Finally, we validated the use of captive-bred specimens through quantification of the hemolytic area after the sting process using the tentacle skin blood agarose assay methodology.

Keywords: captivity, early stage, generation, growth rate, kreisel, life cycle, rotifer, venom

Abbreviations: DTW, dynamic time warping; TBD, total body diameter; TSBAA, tentacle skin blood agarose assay.

1 INTRODUCTION

Since marine bioprospecting began, the world's oceans and their ecosystems have been considered valuable sources to explore for new natural products with a variety of biotechnological applications (Rocha et al., 2011; Merquiol et al., 2019). Although sponges are the foremost providers of marine bioactive compounds, animals belonging to the phylum Cnidaria (such as jellyfish, sea anemones and corals) are also promising reservoirs of compounds with potential drug activity (Oliveira et al., 2018; Merquiol et al., 2019).

Most of the bioactive compounds are extracted from cnidarians derived from benthic species, while a limited number come from pelagic cnidarians such as scyphozoan jellyfish (Rocha et al., 2011). Although the full exploitation of jellyfish is still in its early stages, recent efforts have investigated the use of scyphozoan jellyfish as a natural source of collagen, fatty acids and bioactive compounds mainly extracted from the venom stored in their cnidocytes (Frazão et al., 2017; Merquiol et al., 2019; Duarte et al., 2021).

Jellyfish blooms may have been increasing in frequency and magnitude in some areas of the world (Brotz et al., 2012) but the scarcity of historical datasets covering large temporal and spatial scales makes it difficult to determine the biomass increase on a global scale (Marambio et al., 2021). Moreover, the variability of environmental conditions and the seasonality of most species do not guarantee their availability when needed (Purcell et al., 2013). Under this premise, wild jellyfish can be a useful resource, but their production by means of aquaculture techniques is key to guarantee a regular supply for research, aquarium exhibitions, stock enhancement for fisheries and successful commercial exploitation (Dong et al., 2009; Purcell et al., 2013; Duarte et al., 2021).

The mauve stinger *Pelagia noctiluca* is an oceanic jellyfish, widely distributed in the open waters of the Mediterranean Sea (Mariottini et al., 2008; Canepa et al., 2014; Marambio et al., 2021). The lack of a polyp phase, a peculiarity within its life cycle (Sandrini and Avian, 1983; Helm, 2018; Ballesteros et al., 2021b), allows it to inhabit oceanic as well as coastal ecosystems (Canepa et al., 2014). This scyphozoan is considered the most important jellyfish in the Mediterranean basin; its massive outbreaks and the high toxicity of its venom (Mariottini et al., 2008) lead to serious negative effects on tourism, fisheries, aquaculture, and the energy industry (Canepa et al., 2014; Bosch-Belmar et al., 2020).

Pelagia noctiluca is an ideal subject for research in biomedical, cosmetic, and pharmacological industries due to the potential value of the bioactive compounds from toxins identified in its venom (Ayed et al., 2012; Ayed et al., 2016; Frazão et al., 2017). The crude venom, stored in 4 nematocyst types (a-isorhiza, A-isorhiza, O-isorhiza and eurytele) (Ballesteros et al., 2021b), has hemolytic, cytotoxic, dermonecrotic and local tissue damage activity (D'Ambra and Lauritano, 2020). Its proteome, including not only the venom from its nematocysts, but also tissue proteins, revealed 68 different proteins (Frazão et al., 2017), with a wide range of biomedical implications in fields such as oncology, diabetes, HIV, immunology, as well as cardiovascular, musculoskeletal, neurological and hematological disorders (Ayed et al., 2012; Ayed et al., 2016; Frazão et al., 2017). Red fluorescent protein, a protein with high biotechnological importance in molecular biology and previously reported in anthozoans (Verkhusha and Lukyanov, 2004), was first discovered in scyphozoans in *P. noctiluca*, as was peroxiredoxin, a potential natural source of antioxidants and anti-UV radiation agents (Frazão et al., 2017).

The sting of *P. noctiluca* is painful and inconvenient for beach users and represents a high proportion of cases of those seeking help for jellyfish stings on the beaches of the Mediterranean Sea (Mariottini et al., 2008; De Donno et al., 2014). Although it is not considered a life-threatening species, its severe sting generates edema, burning sensation, vesicles and/or scabs immediately after inoculation of the venom (Montgomery et al., 2016; Hall et al., 2018). Hence, the cosmetic and pharmaceutical industries research and sell products to prevent and ameliorate its sting (Hall et al., 2018; Morabito et al., 2020).

Pelagia noctiluca is present all year in the Mediterranean Sea in diverse stages of its life cycle, and reproductive medusae appear in different periods in the basin (Rosa et al., 2013; Canepa et al., 2014; Milisenda et al., 2018a). Its oceanic distribution, vertical migrations, and spatiotemporal variability along the coast (Marambio et al., 2021; Pastor-Prieto et al., 2021) make sampling a difficult task. Moreover, many individuals stranded on the beaches are not in optimal conditions to be used for research or commercial purposes. Under these premises, and expanding its use to a larger and more sustainable scale, it is essential to establish *P. noctiluca* cultures. The holoplanktonic life cycle of *P. noctiluca*, which lacks a benthic stage (Sandrini and Avian, 1983; Ballesteros et al., 2021b), has limited scope for research in captive conditions. Maintaining its culture presents a host of challenges (Ramondenc et al., 2019) not only for academic and industrial research but also for aquarium exhibitions. Ephyrae released from polyp colonies through the strobilation process (Helm, 2018) represent a continuous source of individuals, an advantage that has allowed improvements in husbandry techniques, for example with the well-known moon jellyfish *Aurelia* spp. (Schaadt et al., 2017) and the upside-down jellyfish *Cassiopea* spp. (Pierce, 2005). To our knowledge, only two studies have shown long-term culture of *P. noctiluca*, but none of them managed to reproduce the second generation to ensure durability of the culture (Lilley et al., 2014; Ramondenc et al., 2019).

Considering this approach, and the great value of *P. noctiluca* in aquarium exhibitions, research and industrial applications, the objectives of this research were: (I) to determine the optimal food regimen for culture, (II) to obtain a third generation of *P. noctiluca* to guarantee durability of the culture, and (III) to validate the use of captive-bred *P. noctiluca* specimens by evaluating the hemolytic area after a sting event. In this study, we halved the production time of mature *P. noctiluca* individuals, acquiring a third generation in the presence and absence of gelatinous prey. The use of captive-bred *P. noctiluca* specimens for future venom experiments using the tentacle skin blood agarose assay was validated. The results represent an important contribution to the improvement of nutrition, production time and breeding techniques in aquaculture of the most important jellyfish in the Mediterranean Sea.

2 MATERIAL AND METHODS

2.1 Breeding of *Pelagia noctiluca* in Captivity

2.1.1 Culture Establishment

Mucus strips with fertilized eggs were collected at ∼ 11:00 AM from a mature jellyfish aquarium in Loro Parque Aquarium (Tenerife, Spain), 3 hours after the photoperiod was switched on (post-fecundation day 0). The fertilized eggs were transferred to a plastic container with 8 L of natural filtered seawater (34–36 salinity) and abundant aeration (**Figure 1A**) placed in a water bath (22°C) and using a wide plastic pipette. 22°C of temperature was chosen for being the water inlet temperature from the ocean to the Loro Parque Aquarium during the period of the experiments. Within 3 days, the eggs metamorphosed into the ephyra stage and the experiments could be started (post-fecundation day 3).

2.1.2 Maintenance and Feeding

Early stages. Four groups of 150 randomly-selected ephyrae, all from the same fecundation event, were transferred to four air-kreisel tanks (**Figure 1B**) with 12 L of natural filtered seawater (34–36 salinity), which were placed in a water bath to reach 22°C. The kreisel aquarium is a well-known tank designed to keep gelatinous zooplankton (Purcell et al., 2013). The air inlet, helped by its circular shape, creates a continuous circular water flow, allowing animals to remain in the water column. The light cycle was 12h light/12h dark. The early stages were cultivated in a closed water system. Every day, 15% of the seawater was renewed. Every three days, a complete deep cleaning was performed coinciding with the renewal of 100% of the seawater to ensure optimal culture conditions (e.g., pH and nitrogen and oxygen concentrations).

Dispensing with additional cultures such as *Brachionus* spp. (rotifers) or *Artemia* spp., helps optimize time in captive husbandry. Under this premise, we used two diets that did not involve additional cultures: Culture 1 (gelatin) was encapsulated food, made by us, inside a neutral gelatin (Promolac). It was composed of razor shell (*Ensis directus*), shrimp (*Neomysis* sp.), sardine (*Sardina pilchardus*), mussel (*Mytilus galloprovincialis*), hake (*Merluccius hubbsi*), pacific krill (*Euphausia pacifi*ca, Ocean Nutrition™) and red plankton (*Calanus finmarchicus*, Ocean Nutrition™). Culture 2 (non-motile prey) was formed of frozen *Artemia* sp. nauplii and codfish eggs (*Gadus morhua*). Amounts of food and feeding times used for each experimental week can be consulted in **Supplementary Material Tables 1, 2**.

Gelatinous zooplankton, such as the scyphozoan *Aurelia* spp. and *Salpa democratica*, seem to be important in the farming of medusivorous jellyfish such as *P. noctiluca* (Purcell et al., 2013). Two more types of food regimens were added. Culture 3 (medusivorous) was based on the presence of mucus and small pieces of *Aurelia* sp. as an addition to rotifers; both came from Loro Parque Aquarium cultures. Culture 4 (non-medusivorous) contained only rotifers. Amounts of food and feeding times used

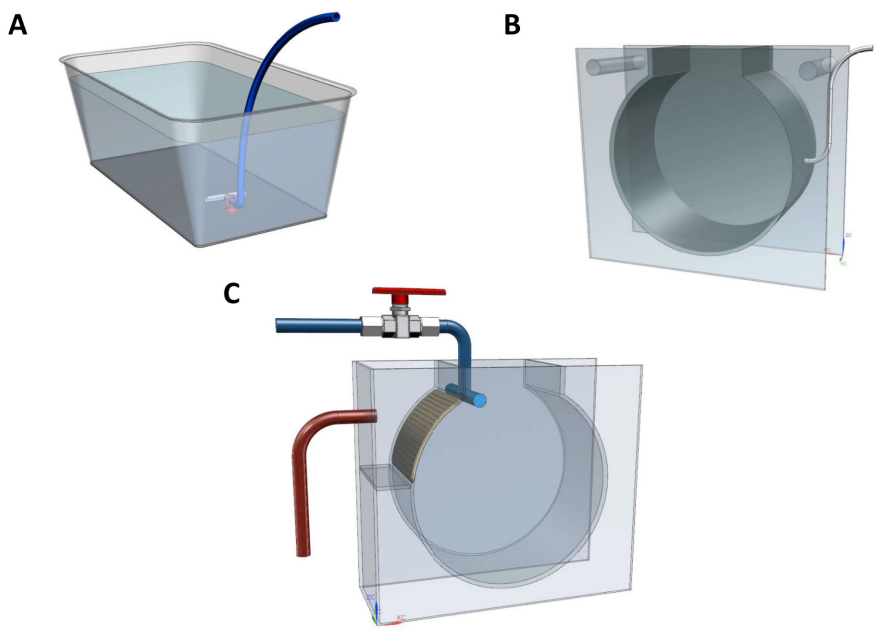

FIGURE 1 | Types of rearing vessels and tanks used for *Pelagia noctiluca* during the experiments. **(A)** Culture establishment from fertilized eggs to first ephyrae. **(B)** Air-kreisel tank for the farming of early stages. **(C)** Water-kreisel tank for the breeding of advanced stages. Illustrations by Carlos Mengod.

for each experimental week can be found in **Supplementary Material Tables 1, 2**.

Advanced stages. The optimal cultures, at day 31 of the experiments, were considered juvenile medusae and they were easily recognized for their typical brown coloration – detailed morphological descriptions can be consulted in Ballesteros et al. (2021b) –. Juvenile medusae were transferred to a water-kreisel tank (190 L) with a direct inlet of filtered natural seawater (34–36 salinity and 22°C temperature) (**Figure 1C**). The light cycle was 12h light/12h dark. The cultures were deep cleaned on a weekly basis.

Culture 3 (medusivorous) was separated into two cultures. The first, culture 3 (medusivorous), continued the research line in the presence of pieces of *Aurelia* sp. along with *Artemia* sp. nauplii, codfish eggs, encapsulated food in gelatin (described above) and frozen white shrimp (*Acetes* sp.). The other, culture 3.1 (gelatin), was fed only with encapsulated food in gelatin. Finally, culture 4 (non-medusivorous), with no *Aurelia* sp., was fed with *Artemia* sp. nauplii, codfish eggs, encapsulated food in gelatin and white shrimp. Amounts of food, feeding and times for each experimental week can be consulted in **Supplementary Material Tables 1, 2**.

2.1.3 Morphological Descriptions, Measurements, Statistical Analysis and Graphical Representation

To describe and monitor the growth of the jellyfish on each food regimen, each week, 10 living individuals were randomly collected from each culture. Ephyrae and metaephyrae were relaxed with a menthol solution (4% w/v), transferred into a ventral position on a glass slide and photographed using a camera (Imaging Source) attached to a binocular loupe (ZUZI 220). Afterwards, the individuals were carefully returned to their respective cultures. The morphology and the total body diameter (TBD) were described and measured (from lappet to lappet) as in Ballesteros et al. (2021b) by photo-analysis with the Fiji version of ImageJ software (Schindelin et al., 2012). For advanced stages, jellyfish were transferred to a plastic tray with a small amount of seawater. TBD was measured directly with a digital caliper with the animals in a ventral position. As in the early stages, individuals were carefully returned to each culture. A total of 602 individuals were measured and analyzed during the research.

The growth of *P. noctiluca* (TBD; mm) over time (days post-fecundation) was represented graphically by means of boxplots using the R software platform (R Core Team, 2017). Instantaneous growth rate (μ; % d^{-1}) was estimated as in Lilley et al. (2014). TBD measures were converted into carbon weight (CW) according to the formula (Lilley et al., 2014):

$$CW = 0.235 \cdot TBD^{3.115}$$

and μ was calculated as:

$$\mu = \frac{\frac{\ln(CW1}{CW2)}}{t2 - t1} \cdot 100$$

The differences between μ for successful food regimens were calculated using dynamic time warping (DTW). DTW provides a similarity score or distance between two sequences by means of the following formula (Giorgino, 2009):

$$M(i,j) = |X(i) - Y(j)| + \min(M(i-1,j-1),\ M(i,j-1), M(i-1,j))$$

where M is a matrix of X and Y series, *i* is the iterator for series X and *j* is the iterator for series Y.

A DTW distance of zero implies that the compared series are almost identical, whereas high distance values imply dissimilarities between the compared series (Giorgino, 2009). This was performed using the *dtw* package available in the R software platform (R Core Team, 2017).

Finally, during the deep cleaning of the systems, every three days for the early stages and once a week for the advanced stages, individuals were counted to calculate the survival rate (%):

$$SR = \frac{Nt}{N0} \cdot 100$$

where *Nt* is the individual number at that time point and *N0* is the number of individuals at the beginning of the culture.

2.2 Validation of the Hemolytic Activity of the Venom

In order to be able to use *P. noctiluca* bred in captivity in future toxicity tests, the hemolytic property of wild and captivity jellyfish venoms was compared using the tentacle skin blood agarose assay (TSBAA) (Yanagihara et al., 2016).

2.2.1 Origin of Jellyfish

Mature jellyfish from the open sea were collected in 2019 in Menorca (Spain) using hand nets and plastic jars. The individuals were then transferred to plastic bags full of seawater, avoiding air bubbles to ensure good conditions during transport. For the captive jellyfish, we used mature jellyfish from culture 3 (medusivorous), once the third generation was achieved. The culture 3 (medusivorous) was chosen because it represented a traditional diet in presence of gelatinous prey (*Aurelia* sp.).

2.2.2 Tentacle Skin Blood Agarose Assay

The venom load was evaluated by the hemolytic area in a modified protocol adapted from the TSBAA method (Yanagihara et al., 2016). Briefly, an agarose gel preparation incorporating sheep red blood cells (SRBCs) (Thermo Fisher Scientific) was used, covered by a thin tissue of pig small intestine to simulate the effect of the human skin barrier (Ballesteros et al., 2021a).

Cutting tentacles to the same size is a complex process due to their contraction and expansion after manipulation. With the aim of improving the methodology and standardizing the sting area, we designed a plastic mold (**Supplementary Material Figure 1**). These plastic molds were placed on top of the agarose rectangles with pig intestine. Consecutively, the sting process was carried out with tentacles from wild and captive jellyfish for 10 min. The sting diameter was 57.4 mm^2. A total of 8 replicates for wild jellyfish and 10 for captive jellyfish were

performed. After this natural-method sting event, the plastic molds and the intestine sections were removed and the SRBC agarose rectangles were stored in the humidification chamber at room temperature.

2.2.3 Hemolytic Areas, Statistical Analysis and Graphical Representation

After a period of 22 hours from the sting process, images of the hemolytic areas were obtained with a camera. The data was tested for normality and homogeneity using the *stats* package available for the R software platform (R Core Team, 2017). As data did not display homogenous variances, a Mann-Whitney test was applied by means of the *wilcox.test* function to test if significant differences existed between hemolytic areas. Finally, a graphical representation was performed using the *ggplot2* package (Wickham, 2016) from the R software platform (R Core Team, 2017).

3 RESULTS

3.1 Morphology, Total Body Diameter and Survival Rate

3.1.1 First Generation

Culture 1 (gelatin). Early stages. Feeding with gelatin was not satisfactory for early stages. The culture was maintained only up to 18 days post-fecundation with a 0% survival rate (**Figures 2**, **3A** and **Table 1**). No gelatin pieces were observed in the ephyrae gastric system. Throughout the culture there was an absence of gastric filaments, velar canals, and nematocyst batteries. Additional information is presented in **Supplementary Material Table 1**.

Culture 2 (non-motile prey). Early stages. A food regimen based on non-motile prey was insufficient for growth during the early stages. As with culture 1 (gelatin), this culture was stopped on the 18th day post-fecundation with a survival rate of 0% due to the absence of gastric filaments, velar canals, and nematocyst batteries (**Figures 2**, **3A** and **Table 1**). Additional information can be consulted in **Supplementary Material Table 1**.

Culture 3 (medusivorous). Early and advanced stages. First gastric filaments and velar canals were observed on the 10th day post-fecundation (mean TBD for this culture was 3.3 ± 0.3 mm). Tentacle buds or incipient tentacles and nematocyst batteries appeared on the 17th day after fecundation (mean TBD 6.3 ± 1.5 mm). Marginal tentacles fully developed in some individuals at the 24th day post-fecundation (mean TBD of 6.4 ± 1.5 mm) with a 64.7% survival rate (**Figures 2**, **3A**). Culture 3 was split at 31 days post-fecundation into two cultures, named culture 3 (medusivorous) and culture 3.1 (gelatin) (**Figure 2**), each with 45 individuals. Culture 3 (medusivorous), which included culture 3 early stages, was maintained for a period of 86 days post-fecundation. Gonad formation began 52 days after the fecundation event (mean TBD for this culture, 38.6 ± 3.9 mm), and incorporation of white shrimp as an additional protein was essential (**Supplementary Material Table 1**). Fertilized eggs from 25 mature jellyfish were observed at ~ 11:00 AM, closing the *P. noctiluca* life cycle in laboratory conditions at 86 days post-fecundation (survival rate of 55.6%) (**Figure 3B** and **Table 1**). Eggs were observed daily. Ephyrae of the second generation were obtained 3 days after egg production. Additional information is presented in **Supplementary Material Table 1**.

Culture 3.1 (gelatin). Advanced stages. 45 juvenile medusae (survival rate of 100%) (**Figure 3B**) from culture 3 (medusivorous) early stages were fed only with pieces of gelatin from the 31st day post-fecundation. Unfortunately, gelatin alone was also not a suitable food for the advanced stages. Young medusae did not grow. They were deformed and lost their typical juvenile brownish coloration. The culture was stopped on the 52nd day post-fecundation (survival rate of 4.4%) with a mean TBD of 11.4 ± 1.4 mm, compared with culture 3 (medusivorous) which had a mean TBD of 38.6 ± 3.9 (survival rate of 62.2%) and was formed of healthy juvenile medusae that produced gonads (**Figures 2**, **3B** and **Table 1**). Additional information can be consulted in **Supplementary Material Table 1**.

Culture 4 (non-medusivorous). Early and advanced stages. The culture was maintained for a period of 83 days post-fecundation. As in culture 3 (medusivorous), the first gastric filaments and velar canals were observed at the 10th day post-fecundation (mean TBD of 3.2 ± 0.7 mm). Tentacle buds or incipient marginal tentacles and nematocyst batteries appeared at 17 days post-fecundation (mean TBD of 5.7 ± 0.8 mm). Marginal tentacles fully developed in some metaephyrae at 24 days after fecundation with a survival rate of 28.7% (mean TBD of 6.3 ± 1.3 mm) (**Figures 2**, **3B**). Rotifers as a single food source were sufficient for normal development in each early stage. Gonad formation started 52 days after fecundation (mean TBD of 31.2 ± 4.5 mm). As in culture 3 (medusivorous), white shrimps were added to the diet as a rich source of protein. Fertilized eggs were observed from 21 mature jellyfish, on the 83rd day post-fecundation, at ~ 11:00 AM (**Table 1**). The life cycle was closed in laboratory conditions without the presence of medusae in the food regimen. Release of eggs and sperm occurred daily. Eggs metamorphosed into ephyrae (second generation) 3 days after egg production. Additional information is presented in **Supplementary Material Table 1**.

3.1.2 Second Generation

Culture 3 (medusivorous). Early and advanced stages. The culture was maintained for a period of 80 days (**Table 1**). First gastric filaments and velar canals were observed on the 10th day post-fecundation (mean TBD of 4.0 ± 0.6 mm). As in the first generation, tentacle buds or incipient marginal tentacles and nematocyst batteries were observed at 17 days after fecundation (mean TBD of 9.4 ± 1.6 mm). Marginal tentacles were fully developed at the 24th day post-fecundation (mean TBD of 12.3 ± 1.2 mm). Gonad formation occurred at 52 days after fecundation (mean TBD of 39.6 ± 6.3 mm). Fertilized eggs were collected on the 80th day post-fecundation with 28 jellyfish (survival rate of 28%) (**Figure 3C** and **Table 1**). Eggs metamorphosed into ephyrae (third generation) 3 days after fecundation, closing the second generation. Additional information is presented in **Supplementary Material Table 2**.

Culture 4 (non-medusivorous). Early and advanced stages. The culture was maintained until 94 days post-fecundation. First gastric filaments and velar canals appeared on the 10th day post-fecundation (mean TBD of 5.1 ± 0.4 mm). Tentacle buds or

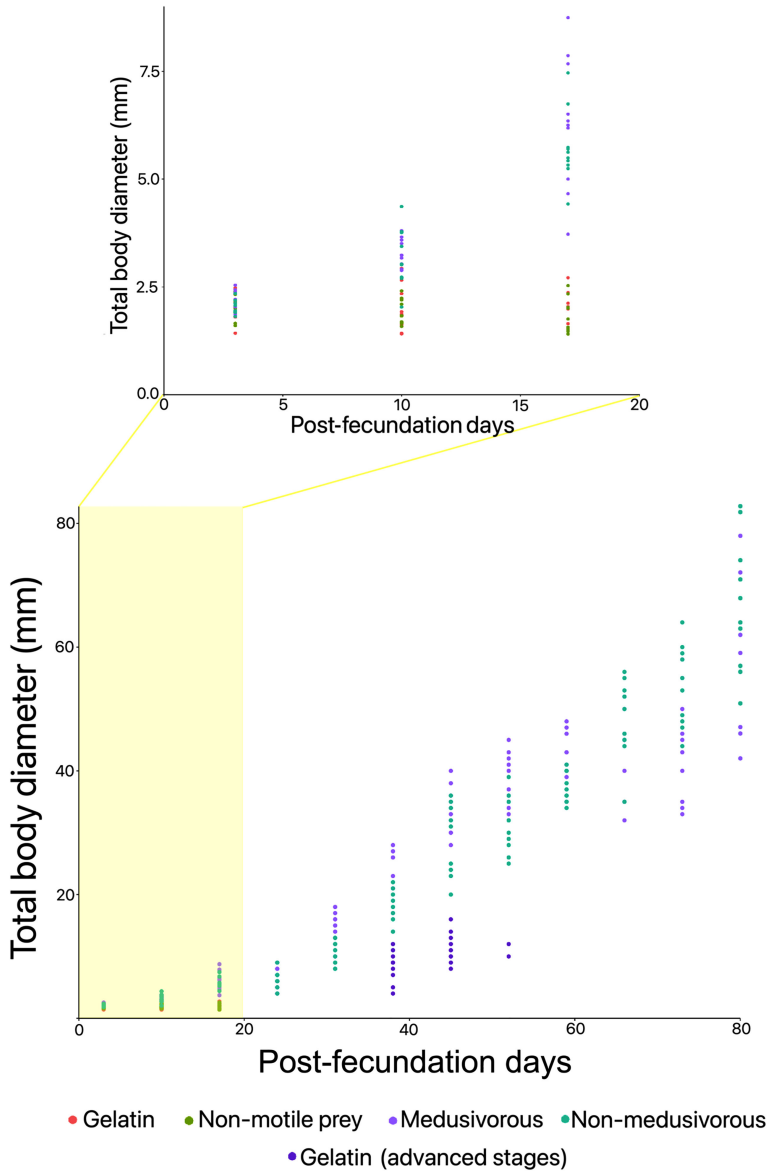

FIGURE 2 | Total body diameter over time in the presence of different food regimens for the first generation of *Pelagia noctiluca*. Food regimens: Culture 1, encapsulated food in gelatin; Culture 2, non-motile prey; Culture 3, medusivorous diet (presence of *Aurelia* sp.); Culture 3.1, medusivorous diet during early stages and gelatin from advanced stages; and Culture 4, non-medusivorous diet (absence of *Aurelia* sp.).

incipient marginal tentacles and nematocyst batteries were observed at 17 days after fecundation (mean TBD of 9.2 ± 0.8 mm). Marginal tentacles were fully developed at the 24th day post-fecundation (mean TBD of 10.1 ± 1.3 mm). Gonad formation occurred at 66 days after fecundation (mean TBD of 43.0 ± 8.9 mm), and again, white shrimps were incorporated in the diet. Fertilized eggs were collected on the 94th day post-fecundation from 23 jellyfish (survival rate of 23%) (**Figure 3C** and **Table 1**). Eggs metamorphosed into ephyrae (third generation) 3 days after fecundation, closing the second generation. Additional information can be consulted in **Supplementary Material Table 2**.

3.1.3 Third Generation

Culture 3 (medusivorous). The experiment was stopped after the observation of a healthy third generation of ephyrae (stage 1) with a mean TBD of 3.1 ± 0.2 mm, demonstrating the durability of the culture. The ephyrae were obtained after a total of 169 experimental days (**Figures 4**, **5**). Additional information can be consulted in **Supplementary Material Table 2**.

Culture 4 (non-medusivorous). The third generation of healthy ephyrae (stage 1), with a mean TBD of 2.7 ± 0.3 mm, was obtained after a total of 179 experimental days (**Figure 4**). As in culture 3 (medusivorous), the experiment was then stopped.

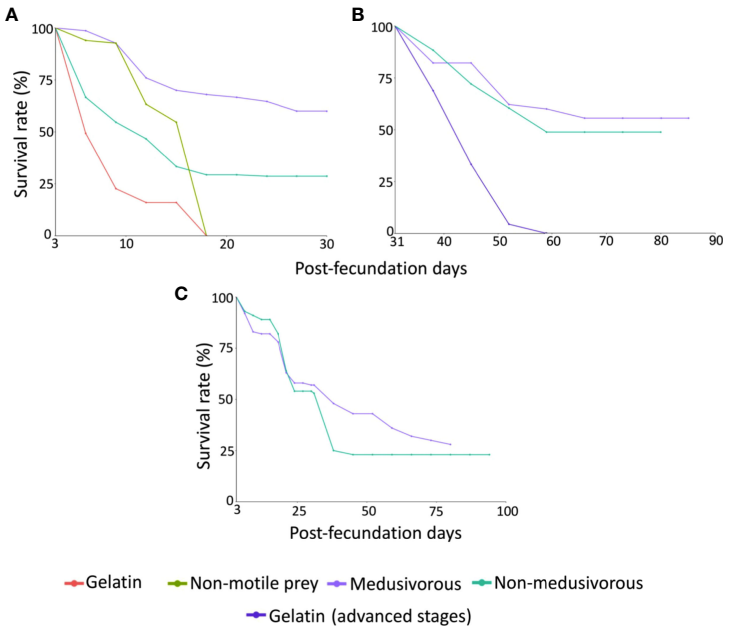

FIGURE 3 | Survival rates (%) of *Pelagia noctiluca* for: **(A)** Early stages of the first generation (ephyra and metaephyra stages), **(B)** Advanced stages of the first generation (juvenile and adult stages), **(C)** Early and advanced stages of the second generation. Food regimens: Culture 1, encapsulated food in gelatin; Culture 2, non-motile prey; Culture 3, medusivorous diet (presence of *Aurelia* sp.); Culture 3.1, medusivorous diet during early stages and gelatin from advanced stages; and Culture 4, non-medusivorous diet (absence of *Aurelia* sp.).

Additional information is presented in **Supplementary Material Table 2**.

3.2 Instantaneous Growth Rate

For the first generation, growth rates of *P. noctiluca* varied depending on the feeding regime (**Supplementary Material Table 1**). Changes in carbon weight in culture 1 (gelatin), culture 2 (non-motile prey) and advanced stages of culture 3.1 (gelatin) reflected the lack of growth and development of the gastric cavity (**Figure 2** and **Supplementary Material Table 1**). The growth rates with the successful feeding regimens in culture 3 (medusivorous) and culture 4 (non-medusivorous) were predominantly positive, with greater rates during the early stages of the life cycle (**Figure 6A**). Considering only positive changes in carbon weight, early stages grew up to 57.0% d^{-1} for culture 3 (medusivorous) and 48.9% d^{-1} for culture 4 (non-medusivorous). The instantaneous growth rates for advanced stages reached 33.0% d^{-1} for culture 3 (medusivorous) and 51.5% d^{-1} for culture 4 (**Figure 6A**). Additional information is presented in **Supplementary Material Table 1**.

The trends in instantaneous growth in the second generation were very similar to the first generation (**Figure 6**). Positive

TABLE 1 | Food regimens according to the life cycle stages, post-fecundation days and life cycle classification at the end of each experiment.

Culture code	Life cycle stage; food regimen*	Generation, fertilized eggs days obtained (mean ± S. D)	Life cycle stage classification+
Culture 1 (gelatin)	Early stages; gelatin: razor shell, shrimp, sardine, mussel, hake, pacific krill and red plankton	Stopped experiment at 18 days post-fecundation	Ephyra stage 1
Culture 2 (non-motile prey)	Early stage; frozen *Artemia* sp. nauplii and codfish eggs	Stopped experiment at 18 days post-fecundation	Ephyra stage 1
Culture 3 (medusivorous)	Early stages; *Aurelia* sp. and rotifer. Advanced stages; *Aurelia* sp., *Artemia* sp. nauplii, codfish eggs, gelatin and white shrimp	First generation, 86; Second generation, 83. (84.5 ± 2.1)	Adult
Culture 3.1 (gelatin)	Advanced stages; gelatin	Stopped experiment at 52 days post-fecundation	Metaephyra stage 4
Culture 4 (non-medusivorous 4)	Early stages; rotifer Advanced stages; *Artemia* sp. nauplii, codfish eggs, gelatin and white shrimp	First generation, 80; Second generation, 94. (87.0 ± 9.9)	Adult

Amounts and timing of food for each experimental week can be consulted in* ***Supplementary Material Tables 1 and 2.*
+Classification of the life cycle at the end of each experiment. Life cycle classification according to Ballesteros et al. (2021b).

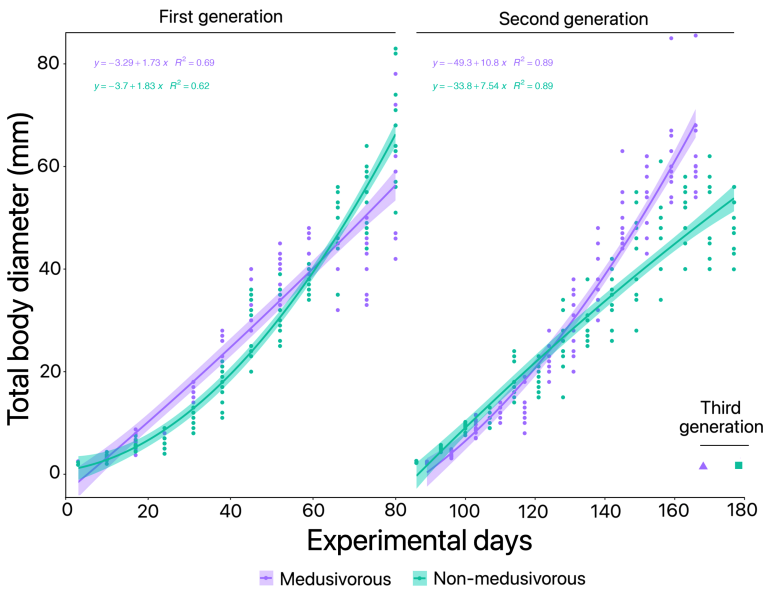

FIGURE 4 | Optimization of *Pelagia noctiluca* farming in the presence and absence of medusivorous prey. The three generations obtained from the beginning of the experiments are included. Culture 3, medusivorous diet (presence of *Aurelia* sp.) and Culture 4, non-medusivorous diet (absence of *Aurelia* sp.).

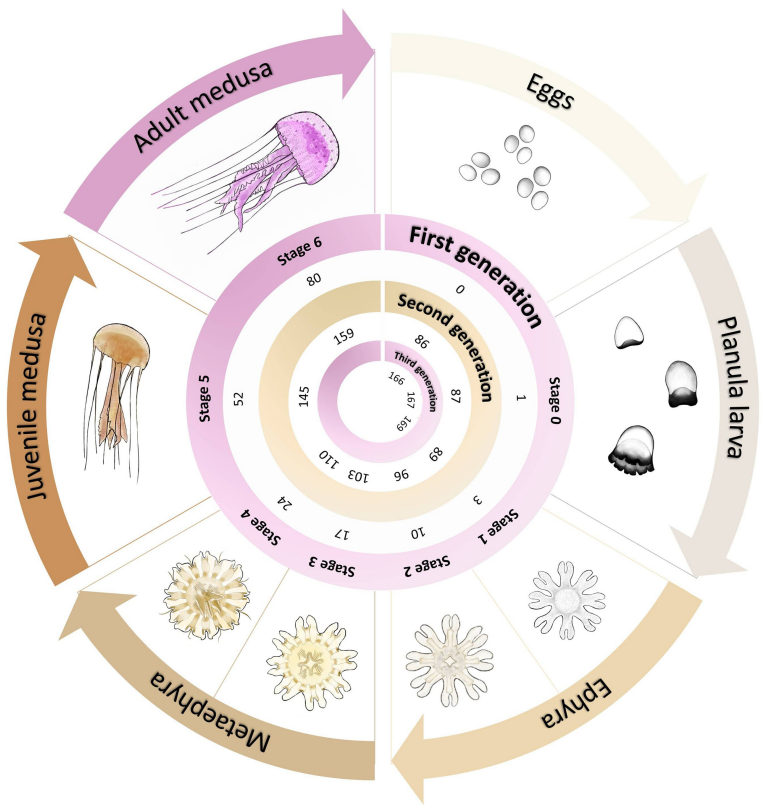

FIGURE 5 | Graphical representation of the *Pelagia noctiluca* life cycle optimization at 22°C. It includes the post-fecundation days until each stage of its life cycle is reached, when fed with the medusivorous diet (culture 3). Detailed morphological and cnidome descriptions for each life cycle stage can be consulted in Ballesteros et al. (2021b). Illustrations of the life cycle by Lau López.

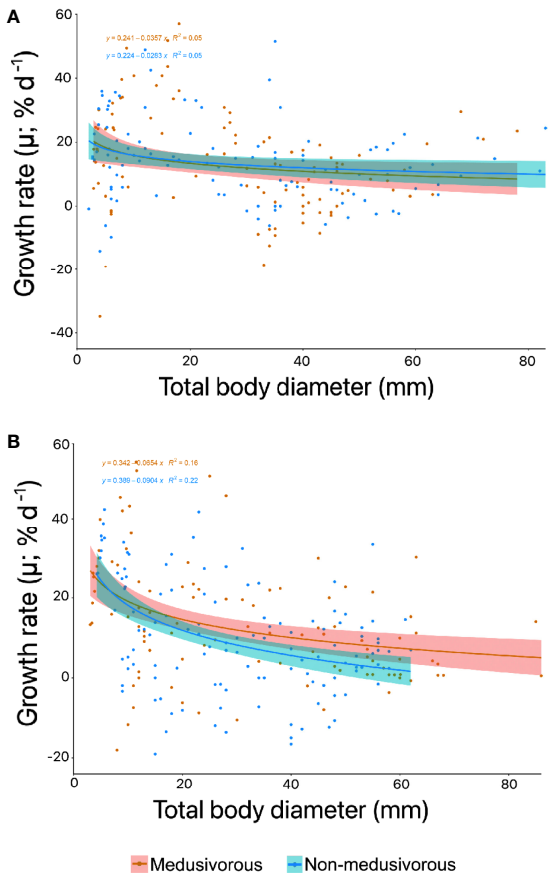

FIGURE 6 | Instantaneous growth rate (% d^{-1}) for the successful food regimens for *Pelagia noctiluca* in **(A)** first generation and **(B)** second generation. The decrease in growth rate with total body diameter (TBD) (solid line) represents a power relationship according to positive instantaneous growth rates. The shaded area indicates 95% confidence limits. Food regimens: Culture 3, medusivorous diet (presence of *Aurelia* sp.) and Culture 4, non-medusivorous diet (absence of *Aurelia* sp.).

instantaneous growth rates predominated, and greater rates corresponded to the early stages of the life cycle (**Figure 6B**). Early stages grew up to 54.2% d^{-1} for culture 3 (medusivorous) and 42.4% d^{-1} for culture 4 (non-medusivorous). Advanced stages reached a maximum growth rate of 50.7% d^{-1} for culture 3 (medusivorous) and 33.3% d^{-1} for culture 4 (non-medusivorous) (**Figure 6B**). Additional information can be consulted in **Supplementary Material Table 2**.

The comparison of growth rate data between non-medusivorous and medusivorous by means of DTW yielded a score of 0.2.

3.3 Validation of the Use of Captive Individuals in Venom Experiments

In order to validate the use of captive jellyfish in venom-related experiments, the hemolytic areas were evaluated after the sting process from captive-bred and wild jellyfish. The hemolytic area obtained a value of 44.3 ± 3.4% for wild jellyfish and 39.3 ± 10.2% for captive jellyfish (**Figure 7**). No significant difference (p value 0.2) was observed between wild and captive jellyfish.

4 DISCUSSION

The present study improves the culture of *P. noctiluca*. Here, we halved the production time of mature individuals and doubled the instantaneous growth rates, in the presence and absence of gelatinous prey (*Aurelia* sp.). This is the first time that a third generation of *P. noctiluca* has been obtained, guaranteeing its continuous availability, and the first time that the use of captive-bred individuals for venom-related experiments has been validated.

The culture of *P. noctiluca* is a challenge for most research institutions, aquariums and companies who wish to keep this species in captivity (Purcell et al., 2013; Ramondenc et al., 2019). To date, only two studies have focused on the culture of *P. noctiluca* over time (Lilley et al., 2014; Ramondenc et al., 2019). Lilley et al. (2014) cultured *P. noctiluca* eggs through to reproductive adults up to a maximum of ∼ 500 days post-fecundation. Although gamete release was observed, second generation was not achieved. Despite the use of a closed system with artificial seawater, a limitation for the cultivation of jellyfish, Ramondenc et al. (2019) improved on the results by Lilley et al. (2014), obtaining egg production in jellyfish with a mean TBD of ∼ 90 mm at 140 days post-fecundation. Comparing previous research (Lilley et al., 2014; Ramondenc et al., 2019) and optimizing the growth time of individuals, our study obtained fertilized eggs in 84.5 ± 2.1 and 87.0 ± 9.9 days post-fecundation in the presence and absence of gelatinous prey, respectively (**Figure 4** and **Table 1**). We halved the time of production of mature *P. noctiluca* individuals, growing two generations and obtaining a third generation in a period of 169 (medusivorous prey) and 179 (non-medusivorous prey) days (**Figures 4**, **5**). The TBDs we observed in mature individuals were comparable to wild *P. noctiluca* individuals (Avian, 1986; Milisenda et al., 2018a; Shtewi et al., 2022), and egg production was observed ∼ 3 hours after the light was switched on, as in previous studies (Lilley et al., 2014; Ramondenc et al., 2019).

Exponential somatic growth during the first weeks of development is a typical trend for jellyfish species (Widmer, 2005; Lilley et al., 2014; Ramondenc et al., 2019; Schäfer et al., 2021). Instantaneous growth rates were fastest during early stages and gradually slowed thereafter, when jellyfish invested more energy in the formation and maturation of gonads and egg production than in growth (**Figure 6**). Lilley et al. (2014) and Ramondenc et al. (2019) obtained growth rates up to 30% d^{-1} for the first weeks of development. In this study, we observed a maximum of 57.0% d^{-1} (**Figure 6A**), the highest values for captive-bred *P. noctiluca* early stages. Therefore, the satisfactory feeding regimens (**Figure 4** and **Table 1**) accompanied by the maintenance techniques used in the present research (see materials and methods section) represent an improvement in the aquaculture techniques of *P. noctiluca*.

The development from eggs to ephyrae of *P. noctiluca* is temperature-dependent, warmer waters favoring its growth

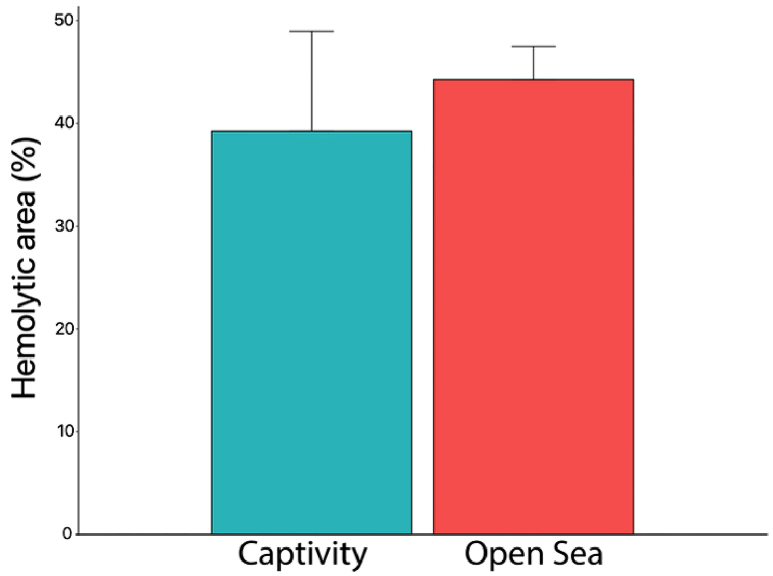

FIGURE 7 | Hemolytic area (%) produced by the venom from captive and open-sea jellyfish. No significant differences were observed between jellyfish from the open sea and those from captivity (p value 0.2) (Mann–Whitney test). Captivity replicates, n = 10; open sea replicates, n = 8.

(Avian, 1986; Purcell et al., 2013; Rosa et al., 2013). The temperature range for culture is from 9–24°C (Purcell et al., 2013; Duarte et al., 2021), although temperatures around 14°C slowed down the development of planulae (Avian, 1986; Rosa et al., 2013), and previous captivity studies considered 18°C the optimal temperature for experiments (Lilley et al., 2014; Ramondenc et al., 2019). In the present study we observed an improvement in the development time from planulae to ephyrae at 3 days post-fecundation (22°C) vs. the previously-reported 4 days post-fecundation at 18°C (Ramondenc et al., 2019; Ballesteros et al., 2021b). Regarding morphological descriptions, Ramondenc et al. (2019) identified first gastric filaments and marginal tentacles at 18 and 38 post-fecundation days, respectively, at 18°C. In our study, gastric filaments and marginal tentacles appeared at 10 and 24 post-fecundation days in the presence and absence of gelatinous prey, respectively. Similar morphological and cnidome descriptions were obtained in Ballesteros et al. (2021b) during the early stages at 18°C. Considering our results, we recommended a temperature of 22°C to encourage healthy growth (without any visible signs of stress) of *P. noctiluca* in captivity.

Unlike mixotrophic scyphozoa such as *Cotylorhiza tubercula*, *Phyllorhiza punctata* and *Cassiopea* spp. that obtain part of their nutrition from zooxanthellae (Raskoff et al., 2003; Pierce, 2005; Straehler-Pohl and Jarms, 2010; Crow et al., 2013), *P. noctiluca* is a heterotroph and opportunistic jellyfish that uses nematocysts to penetrate and entangle prey as an exclusive way of obtaining food (Larson, 1987; Milisenda et al., 2018b; Ballesteros et al., 2021b). The gut content analysis from wild *P. noctiluca* revealed a wide variety of zooplankton prey (e.g., Copepoda, Cladocera and Siphonophore) (Tilves et al., 2016), which has been provided in captive conditions through its daily collection to reproduce the natural diet (Lilley et al., 2014; Ramondenc et al., 2019). However, rotifers and *Artemia* spp. nauplii are the most widely used food for jellyfish farming (Raskoff et al., 2003; Crow et al., 2013; Purcell et al., 2013; Schaadt et al., 2017; Duarte et al., 2021). Previous studies identified good survival and ephyrae growth of *P. noctiluca* with *Artemia* spp. nauplii and small sea urchin eggs in the diet (Lilley et al., 2014; Ramondenc et al., 2019) while our results suggested rotifers or rotifers plus *Aurelia* sp. mucus was the best food regimen during early stages (**Figures 2**–**4**). The improvements observed in this study in terms of TBD, growth rates, development time of the gastric cavity and survival rates are linked with the ideal size and longevity of the rotifers offered during the early stages. *Pelagia noctiluca* does not have nematocyst batteries during ephyra stage 1 and 2 (Ballesteros et al., 2021b), thus it is preferable to feed them with smaller prey than *Artemia* spp. nauplii. The nematocyst batteries are a strategy to capture larger prey, as groups of nematocysts discharge simultaneously and operate more efficiently than a single nematocyst (Östman and Hydman, 1996; Ballesteros et al., 2021b). To avoid *Artemia* spp. nauplii escaping from young ephyrae of *P. noctiluca* as previously reported by Lilley et al. (2014), rotifers are fundamental for feeding in early stages, especially during the 3–17 days post-fecundation when nematocyst batteries are not yet formed and isolated a-isorhiza nematocysts dominate (Ballesteros et al., 2021b). Improved growth rate in the presence of rotifers or mix of rotifers and microalgae has been reported previously for some early stages of scyphozoans (Widmer et al., 2005; Miranda et al., 2016). *Artemia* spp. nauplii can be added as a nutritional complement from metaephyra stage 3, when the first nematocyst batteries appear, the gastric cavity is more developed and feeding structures such as the manubrium or marginal tentacles start to grow longitudinally (Ballesteros et al., 2021b).

Non-motile prey (**Table 1**) sunk rapidly to the bottom of the tank according to Crow et al. (2013). This short-lived food item explains the 0% survival rate at 18 days post-fecundation for gelatin and non-motile prey food regimens (**Figures 2**, **3A**). Contrary to Lilley et al. (2014), we do not recommend the use of immobile prey to feed early stages of *P. noctiluca*. According to the guidelines for jellyfish aquaculture techniques, a variety of frozen and immobile items (e.g., mysids, krill, bloodworms, mixed fish, fish eggs, and/or fish larvae) is suitable for advanced stages when feeding structures are fully developed, to provide additional nutritional value (Raskoff et al., 2003; Crow et al., 2013; Purcell et al., 2013; Duarte et al., 2021). Although the use of gelatin as the only food source was not satisfactory for *P. noctiluca* advanced stage (**Figures 2**, **3B**), encapsulating a variety of items in gelatin helped keep the water cleaner and offered a wide range of elements in a single feeding time; therefore, its use is suggested as a nutritional supplement in the food regimens for jellyfish husbandry.

Moon jellyfish *Aurelia* spp. and the upside-down jellyfish *Cassiopea* spp. are the most popular cultured gelatinous prey offered for the husbandry of medusivorous jellyfish such as *P. noctiluca, Cyanea* spp., *Chrysaora* spp., and *Phacellophora camtschatica* (Pierce, 2005; Purcell et al., 2013; Duarte et al., 2021). Until now, their use was necessary for proper growth of medusivorous jellyfish (Crow et al., 2013; Purcell et al., 2013; Duarte et al., 2021). In particular, previous studies highlighted high values of growth, gonadal maturity and release of eggs in the presence of gelatinous prey for captive-bred *P. noctiluca* (Lilley et al., 2014; Ramondenc et al., 2019). Here, we achieved a production of individuals that lasted over time without the presence of gelatinous prey (*Aurelia* sp.) (**Figure 4**). Besides, the comparison of growth rate between medusivorous and non-medusivorous cultures obtained a value of DTW = 0.2, so, it can be concluded that there were no differences in growth rate regardless of the presence of gelatinous prey (*Aurelia* sp.). This result is an important finding for jellyfish aquaculture techniques, since up until now all the growth manuals and guidelines for medusivorous jellyfish underlined the use of other jellyfish for good growth (Raskoff et al., 2003; Crow et al., 2013; Purcell et al., 2013; Duarte et al., 2021). *Aurelia* spp. pieces or mucus can be added as a nutritional supplement but are not an essential item to maintain a successful culture of *P. noctiluca*. The key is a varied diet in advanced stages (Raskoff et al., 2003; Crow et al., 2013; Purcell et al., 2013; Duarte et al., 2021), where the jellyfish invest energy in the production and maturation of gonads for reproduction. Here, the addition of white shrimp, when gonads began to be visible, was a sufficient energy source to ensure gonadal maturation and egg production and close the life cycle in captivity (**Figure 4** and **Supplementary Material Tables 1, 2**). Our research supports the use of white shrimp instead of gelatinous prey to avoid investing time in the culture of additional jellyfish destined solely for food (Pierce, 2005) or daily gelatinous plankton collection from the wild.

As with feeding, each stage of the life cycle requires different rearing vessels and aquarium tanks (Raskoff et al., 2003; Crow et al., 2013; Purcell et al., 2013; Duarte et al., 2021). Tank design, flow patterns and good regulation of suction velocity are critical factors for captive husbandry (Purcell et al., 2013). The ephyrae of most scyphozoans are successfully farmed in a closed system using glass dishes, beakers, plastic jugs and round glass tanks with or without aeration (Widmer et al., 2005; Purcell et al., 2013; Duarte et al., 2021). The early stages of *P. noctiluca* can be maintained in 5 L plastic jugs, round glass tanks or air-kreisel aquariums in a closed system with a flow of air (**Figure 1B**) or water that keeps them in suspension (Purcell et al., 2013; Ramondenc et al., 2019; Ballesteros et al., 2021b). Newly-developed ephyrae of *P. noctiluca* are transparent, in contrast to the colored newly-released ephyrae from polyps in some scyphozoan species (Straehler-Pohl and Jarms, 2010; Ballesteros et al., 2021b), a difference that causes even more time to be consumed during continuous water changes in a closed system due to the lack of visibility. Purcell et al. (2013) recommend the use of a kreisel aquarium with a gentle current for ephyrae of *P. noctiluca*; however, due to their small size, young ephyrae can get sucked in by the drain and trapped in the mesh screen (personal observation). From our experience, we do not recommend the use of conventional kreisel tanks for early stages of *P. noctiluca*. With the aim of reducing the time required for maintenance routines, novel system designs and safe techniques are required to grow the young ephyrae of *P. noctiluca* in an open system. Advanced stages of *P. noctiluca* can be maintained in conventional kreisel, pseudo-kreisel or rectangular tanks without any danger (Purcell et al., 2013; Duarte et al., 2021).

Jellyfish cultures allow availability of specimens when required and are a unique opportunity to study different areas of knowledge: from each stage of the life cycle and evolution of the cnidome (Holst, 2012; Heins et al., 2015; Ballesteros et al., 2021b) to the impact of climate change or ocean acidification on jellyfish populations (Olariaga et al., 2014). Without doubt, *P. noctiluca* stands out for its stinging capacity and the high number of incidents with beach-goers (Mariottini et al., 2008; De Donno et al., 2014), making it an ideal model for venom-toxicity experiments (Morabito et al., 2020; Ballesteros et al., 2021a). No significant differences were observed between the hemolytic areas caused by wild and captive specimens (p value 0.2) (**Figure 7**). Therefore, the optimization of its life cycle and the validation of the use of captive individuals in the TSBAA methodology may help clarify discrepancies among the scientific community about the best first-aid protocol to quickly and effectively treat its sting (Remigante et al., 2018; Ballesteros et al., 2021a), without depending on the presence of *P. noctiluca* in the marine environment. However, to definitively conclude the similarity in the properties of the venom, new lines of research are required, such as the study of the venom proteome of wild and captive jellyfish.

5 CONCLUSION

For the proper development of *P. noctiluca* in captive conditions, a diet that includes rotifers is recommended during the early life stages. Mucus from the jellyfish *Aurelia* spp. can be added but is

not essential. For advanced stages, *Artemia* spp., codfish eggs, a variety of different food items encapsulated inside a neutral gelatin (razor shell, shrimp, sardine, mussel, hake, pacific krill and red plankton) and white shrimp provide the food sources essential for proper development. The use of gelatin-encapsulated food is a suitable technique to supply additional nutritional value without compromising water quality. As with the early stages, the presence of gelatinous prey (pieces of *Aurelia* spp.) is not a necessary condition for the growth of *P. noctiluca* juvenile and adult stages, yet it can be used as a food supplement. When the gonads begin to be visible, inclusion of white shrimp is suggested to help ensure suitable development, maturation and egg production. Warmer waters reaching 22°C are beneficial for the growth of *P. noctiluca* without any sign of stress. Captive-bred individuals can be used in the TSBAA methodology, since the hemolytic areas caused by their sting do not show differences from the hemolytic areas caused by wild jellyfish.

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

AB and EA conceived the study and supervised the jellyfish cultures. DP, AG, YM, and EA cared for and fed the jellyfish cultures. AB and DP carried out the research and the data curation. AS performed the statistical analyses, and drew up the graphs. AB wrote and edited the original manuscript. DP, AS, AG, YM, EA, EJ, and J-MG revised the manuscript and contributed to its improvement. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by ISDIN (Barcelona, Spain).

ACKNOWLEDGMENTS

Many thanks to the late Verónica Fuentes for all the knowledge that she provided on jellyfish aquaculture techniques during her life. The authors would like to thank all the staff from Loro Parque who assisted with the experiments, as well as the Loro Parque Fundación. Authors AB, AS and J-MG acknowledge the institutional support of the 'Severo Ochoa Centre of Excellence' accreditation (CEX2019-000928-S). Finally, we want to thank to the reviewers for their comments and suggestions to improve the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.911383/full#supplementary-material

REFERENCES

Avian, M. (1986). Temperature Influence on *In Vitro* Reproduction and Development of *Pelagia Noctiluca* (Forskål). *Boll. Di. Zoolog.* 53, 385–391. doi: 10.1080/11250008609355528

Ayed, Y., Dellai, A., Mansour, H. B., Bacha, H., and Abid, S. (2012). Analgesic and Antibutyrylcholinestrasic Activities of the Venom Prepared From the Mediterranean Jellyfish *Pelagia Noctiluca* (Forsskal 1775). *Ann. Clin. Microbiol. Antimicrob.* 11, 1–8. doi: 10.1186/1476-0711-11-15

Ayed, Y., Sghaier, R. M., Laouini, D., and Bacha, H. (2016). Evaluation of Anti-Proliferative and Anti-Inflammatory Activities of *Pelagia Noctiluca* Venom in Lipopolysaccharide/Interferon-γ Stimulated RAW264.7 Macrophages. *Biomed. Pharmacoth.* 84, 1986–1991. doi: 10.1016/j.biopha.2016.11.010

Ballesteros, A., Marambio, M., Fuentes, V., Narda, M., Santín, A., and Gili, J. M. (2021a). Differing Effects of Vinegar on *Pelagia Noctiluca* (Cnidaria: Scyphozoa) and *Carybdea Marsupialis* (Cnidaria: Cubozoa) Stings—Implications for First Aid Protocols. *Toxins* 13(8), 509. doi: 10.3390/toxins13080509

Ballesteros, A., Östman, C., Santín, A., Marambio, M., Narda, M., and Gili, J.-M. (2021b). Cnidome and Morphological Features of *Pelagia Noctiluca* (Cnidaria: Scyphozoa) Throughout the Different Life Cycle Stages. *Front. Mar. Sci.* 8. doi: 10.3389/fmars.2021.714503

Bosch-Belmar, M., Milisenda, G., Basso, L., Doyle, T. K., Leone, A., and Piraino, S. (2020). Jellyfish Impacts on Marine Aquaculture and Fisheries. *Rev. Fish. Sci. Aquacult.* 29, 242–259. doi: 10.1080/23308249.2020.1806201

Brotz, L., Cheung, W. W. L., Kleisner, K., Pakhomov, E., and Pauly, D. (2012). Increasing Jellyfish Populations: Trends in Large Marine Ecosystems. *Hydrobiologia* 690, 3–20. doi: 10.1007/s10750-012-1039-7

Canepa, A., Fuentes, V., Sabateís, A., Piraino, S., Boero, F., and Gili, J. M. (2014). "Pelagia Noctiluca in the Mediterranean Sea" in *Jellyfish Blooms*. Eds. K. A. Pitt and C. H. Lucas (Dordrecht: Springer), 237–266. doi: 10.1007/978-94-007-7015-7_11

Crow, G., Howard, M., Levesque, V., Matsushige, L., Spina, S., Schaadt, M., et al. (2013). "Association of Zoos and Aquariums Aquatic Invertebrate TAG." In *Jellyfish (Cnidaria/ Ctenophora) Care Manual* Ed. M. Schaadt (Silver Spring), 5–79. doi: 10.13140/RG.2.1.4344.3045

D'Ambra, I., and Lauritano, C. (2020). A Review of Toxins From Cnidaria. *Mar. Drugs* 18, 507. doi: 10.3390/md18100507

De Donno, A., Idolo, A., Bagordo, F., Grassi, T., Leomanni, A., Serio, F., et al. (2014). Impact of Stinging Jellyfish Proliferations Along South Italian Coasts: Human Health Hazards, Treatment and Social Costs. *Int. J. Environ. Res. Public Health* 11, 2488–2503. doi: 10.3390/ijerph110302488

Dong, J., Jiang, L., Tan, K., Liu, H., Purcell, J. E., Li, P., et al. (2009). Stock Enhancement of the Edible Jellyfish (*Rhopilema Esculentum* Kishinouye) in Liaodong Bay, China: A Review. *Hydrobiologia* 616, 113–118. doi: 10.1007/s10750-008-9592-9

Duarte, I. M., Marques, S. C., Leandro, S. M., and Calado, R. (2021). An Overview of Jellyfish Aquaculture: For Food, Feed, Pharma and Fun. *Rev. Aquacult.* 14, 265–287. doi: 10.1111/raq.12597

Frazão, B., Campos, A., Osório, H., Thomas, B., Leandro, S., Teixeira, A., et al. (2017). Analysis of *Pelagia Noctiluca* Proteome Reveals a Red Fluorescent

Protein, a Zinc Metalloproteinase and a Peroxiredoxin. *Protein J.* 36, 77–97. doi: 10.1007/s10930-017-9695-0

Giorgino, T. (2009). Computing and Visualizing Dynamic Time Warping Alignments in R: The Dtw Package. *J. Stat. Softw.* 31, 1–24. doi: 10.18637/jss.v031.i07

Hall, A., Mathieu, L., and Blomet, J. (2018). *Pelagia Noctiluca* Jellyfish: Can Lesions and Symptoms be Prevented or Ameliorated? *J. Mar. Biol. Aquacult.* 4, 53–57. doi: 10.15436/2381-0750.18.1971

Heins, A., Glatzel, T., and Holst, S. (2015). Revised Descriptions of the Nematocysts and the Asexual Reproduction Modes of the Scyphozoan Jellyfish *Cassiopea Andromeda* (Forskål 1775). *Zoomorphology* 134, 351–366. doi: 10.1007/s00435-015-0263-x

Helm, R. R. (2018). Evolution and Development of Scyphozoan Jellyfish. *Biol. Rev.* 93, 1228–1250. doi: 10.1111/brv.12393

Holst, S. (2012). Morphology and Development of Benthic and Pelagic Life Stages of North Sea Jellyfish (Scyphozoa, Cnidaria) With Special Emphasis on the Identification of Ephyra Stages. *Mar. Biol.* 159, 2707–2722. doi: 10.1007/s00227-012-2028-0

Larson, R. J. (1987). A Note on the Feeding, Growth, and Reproduction of the Epipelagic Scyphomedusa *Pelagia Noctiluca* (Forskål). *Biol. Oceanogr.* 4, 447–454. doi: 10.1080/01965581.1987.10749501

Lilley, M. K. S., Ferraris, M., Elineau, A., Berline, L., Cuvilliers, P., Gilletta, L., et al. (2014). Culture and Growth of the Jellyfish *Pelagia Octiluca in* the Laboratory. *Mar. Ecol. Prog. Ser.* 510, 265–273. doi: 10.3354/meps10854

Marambio, M., Canepa, A., Lòpez, L., Gauci, A. A., Gueroun, S. K. M., Zampardi, S., et al. (2021). Unfolding Jellyfish Bloom Dynamics Along the Mediterranean Basin by Transnational Citizen Science Initiatives. *Diversity* 13, 274. doi: 10.3390/d13060274

Mariottini, G. L., Elisabetta, G., and Luigi, P. (2008). The Mauve Stinger *Pelagia Noctiluca* (Forsskål 1775). Distribution, Ecology, Toxicity and Epidemiology of Stings. A Review. *Mar. Drugs* 6, 496–513. doi: 10.3390/md20080025

Merquiol, L., Romano, G., Ianora, A., and D'Ambra, I. (2019). Biotechnological Applications of Scyphomedusae. *Mar. Drugs* 17, 604. doi: 10.3390/md17110604

Milisenda, G., Martinez-Quintana, A., Fuentes, V. L., Bosch-Belmar, M., Aglieri, G., Boero, F., et al. (2018a). Reproductive and Bloom Patterns of *Pelagia Noctiluca* in the Strait of Messina, Italy. *Estuar. Coast. Shelf. Sci.* 201, 29–39. doi: 10.1016/j.ecss.2016.01.002

Milisenda, G., Rossi, S., Vizzini, S., Fuentes, V. L., Purcell, J. E., Tilves, U., et al. (2018b). Seasonal Variability of Diet and Trophic Level of the Gelatinous Predator *Pelagia Noctiluca* (Scyphozoa). *Sci. Rep.* 8 (1), 1–13. doi: 10.1038/s41598-018-30474-x

Miranda, F. S., Chambel, J., Almeida, C., Pires, D., Duarte, I., Esteves, L., et al. (2016). Effect of Different Diets on Growth and Survival of the White-Spotted Jellyfish, *Phyllorhiza Punctata*. *Front. Mar. Sci*. doi: 10.3389/conf.FMARS.2016.04.00042

Montgomery, L., Seys, J., and Mees, J. (2016). To Pee, or Not to Pee: A Review on Envenomation and Treatment in European Jellyfish Species. *Mar. Drugs* 14, 127. doi: 10.3390/md14070127

Morabito, R., Cornara, L., la Spada, G., Marino, A., Mariottini, G. L., Remigante, A., et al. (2020). Inhibitory Effect of Plant Extracts on the Cytotoxicity of Eurytele Nematocysts From *Pelagia Noctiluca*. *J. Biol. Res. (Italy)* 93, 96–101. doi: 10.4081/jbr.2020.9136

Olariaga, A., Guallart, E. F., Fuentes, V., López-Sanz, À., Canepa, A., Movilla, J., et al. (2014). Polyp Flats, a New System for Experimenting With Jellyfish Polyps, With Insights Into the Effects of Ocean Acidification. *Limnol. Oceanogr.: Methods* 12, 212–222. doi: 10.4319/LOM.2014.12.212/FORMAT/PDF

Oliveira, C. S., Caldeira, C. A. S., Diniz-Sousa, R., Romero, D. L., Marcussi, S., Moura, L. A., et al. (2018). Pharmacological Characterization of Cnidarian Extracts From the Caribbean Sea: Evaluation of Anti-Snake Venom and Antitumor Properties. *J. Venom. Anim. Toxin. Includ. Trop. Dis.* 24 (1), 22. doi: 10.1186/s40409-018-0161-z

Östman, C., and Hydman, J. (1996). Nematocyst Analysis of *Cyanea Capillata* and *Cyanea Lamarckii* (Scyphozoa, Cnidaria)*. *Scient. Mar.* 61, 313–344.

Pastor-Prieto, M., Bahamon, N., Sabatés, A., Canepa, A., Gili, J. M., Carreton, M., et al. (2021). Spatial Heterogeneity of *Pelagia Noctiluca* Ephyrae Linked to Water Masses in the Western Mediterranean. *PloS One* 16, e0249756. doi: 10.1371/journal.pone.0249756

Pierce, J. (2005). A System for Mass Culture of Upside-Down Jellyfish *Cassiopea* Spp as a Potential Food Item for Medusivores in Captivity. *Int. Zoo Yearbook* 39, 62–69. doi: 10.1111/j.1748-1090.2005.tb00005.x

Purcell, J. E., Baxter, E. J., and Fuentes, V. L. (2013). "Jellyfish as Products and Problems of Aquaculture," in *Advances in Aquaculture Hatchery Technology*. Eds. G. Allan and G. Burnell (Cambridge, United Kingdom: Elsevier), 404–430. doi: 10.1533/9780857097460.2.404

Ramondenc, S., Ferrieux, M., Collet, S., Benedetti, F., Guidi, L., and Lombard, F. (2019). From Egg to Maturity: A Closed System for Complete Life Cycle Studies of the Holopelagic Jellyfish *Pelagia Noctiluca*. *J. Plankt. Res.* 41, 207–217. doi: 10.1093/plankt/fbz013

Raskoff, K. A., Sommer, F. A., Hamner, W. M., and Cross, K. M. (2003). Collection and Culture Techniques for Gelatinous Zooplankton. *Biol. Bull.* 204, 68–80. doi: 10.2307/1543497.z

R Core Team (2017). *A Language and Environment for Statistical Computing* (Vienna: R Foundation for Statistical Computing).

Remigante, A., Costa, R., Morabito, R., la Spada, G., Marino, A., and Dossena, S. (2018). Impact of Scyphozoan Venoms on Human Health and Current First Aid Options for Stings. *Toxins.* 10, 133. doi: 10.3390/toxins10040133

Rocha, J., Peixe, L., Gomes, N. C. M., and Calado, R. (2011). Cnidarians as a Source of New Marine Bioactive Compounds—An Overview of the Last Decade and Future Steps for Bioprospecting. *Mar. Drugs* 9, 1860–1886. doi: 10.3390/md9101860

Rosa, S., Pansera, M., Granata, A., and Guglielmo, L. (2013). Interannual Variability, Growth, Reproduction and Feeding of *Pelagia Noctiluca* (Cnidaria: Scyphozoa) in the Straits of Messina (Central Mediterranean Sea): Linkages With Temperature and Diet. *J. Mar. Syst.* 111–112, 97–107. doi: 10.1016/j.jmarsys.2012.10.001

Sandrini, L. R., and Avian, M. (1983). Biological Cycle of *Pelagia Noctiluca*: Morphological Aspects of the Development From Planula to Ephyra. *Mar. Biol.* 74, 169–174. doi: 10.1007/BF00413920

Schaadt, M., Widmer, C. L., and Sowinski, N. (2017). "Chapter Jellyfish" in *Marine Ornamental Species Aquaculture*. Eds. R. Calado, I. Olivotto, M. Oliver and J. Holt (New Jersey: John Wiley & Sons Ltd), 457–473. doi: 10.1002/9781119169147.ch2

Schäfer, S., Gueroun, S. K. M., Andrade, C., and Canning-Clode, J. (2021). Combined Effects of Temperature and Salinityon Polyps and Ephyrae of *Aurelia Solida* (Cnidaria: Scyphozoa). *Diversity* 13, 573. doi: 10.3390/d13110573

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: An Open-Source Platform for Biological-Image Analysis. *Nat. Methods* 9, 676–682. doi: 10.1038/nmeth.2019

Shtewi, H., Abushaala, N., Elfituri, A., and Abdullah, A. B. (2022). Morphological Feature of *Pelagia Noctiluca* (Forskål 1775) (Cnidaria: Scyphozoa) in Western Libyan Coast, Tripoli. *J. Pure. Appl. Sci.* 21, 24–27. doi: 10.51984/jopas

Straehler-Pohl, I., and Jarms, G. (2010). Identification Key for Young Ephyrae: A First Step for Early Detection of Jellyfish Blooms. *Hydrobiologia* 645, 3–21. doi: 10.1007/s10750-010-0226-7

Tilves, U., Purcell, J. E., Fuentes, V. L., Torrents, A., Pascual, M., Raya, V., et al. (2016). Natural Diet and Predation Impacts of *Pelagia Noctiluca* on Fish Eggs and Larvae in the NW Mediterranean. *J. Plankt. Res.* 38, 1243–1254. doi: 10.1093/plankt/fbw059

Verkhusha, V. V., and Lukyanov, K. A. (2004). The Molecular Properties and Applications of Anthozoa Fluorescent Proteins and Chromoproteins. *Nat. Biotechnol.* 22, 289–296. doi: 10.1038/nbt943

Wickham, H. (2016). *Ggplot2 Elegant Graphics for Data Analysis* (New York, NY: Springer). doi: 10.1007/978-3-319-24277-4

Widmer, C. L. (2005). Effects of Temperature on Growth of North-East Pacific Moon Jellyfish Ephyrae, *Aurelia Labiata* (Cnidaria: Scyphozoa). *J. Mar. Biol. Assoc. Unite. Kingd.* 85, 569–573. doi: 10.1017/S0025315405011495

Widmer, C. L., Voorhees, J. P., Badger, M. A., Lambert, J. W., and Block, N. M. (2005). The Effects of Rearing Vessels and Laboratory Diets on Growth of Northeast Pacific Jellyfish Ephyrae (Cnidaria: Scyphozoa). *Drum. Croaker.* 36, 29–36.

Yanagihara, A., Wilcox, C., King, R., Hurwitz, K., and Castelfranco, A. (2016). Experimental Assays to Assess the Efficacy of Vinegar and Other Topical First-Aid Approaches on Cubozoan (*Alatina Alata*) Tentacle Firing and Venom Toxicity. *Toxins* 8, 19. doi: 10.3390/toxins8010019

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frontiers | Frontiers in Marine Science

METHODS
published: 15 June 2022
doi: 10.3389/fmars.2022.884262

Methodologies for Patellid Limpets' Aquaculture: From Broodstock Management to Juveniles

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OPEN ACCESS

Edited by:
Sílvia Lourenço, Instituto Politécnico de Leiria, Portugal

Reviewed by:
Maria Paola Ferranti, University of Genoa, Italy
Maria Flavia Gravina, University of Rome "Tor Vergata", Italy

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Specialty section:
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

Received: *26 February 2022*
Accepted: *05 May 2022*
Published: *15 June 2022*

Citation:
Castejón D, García L, Cañizares JM, De Girolamo M, Nunes C, Isidro E, Courtois de Viçose G, Nogueira N and Andrade CAP (2022) Methodologies for Patellid Limpets' Aquaculture: From Broodstock Management to Juveniles. Front. Mar. Sci. 9:884262. doi: 10.3389/fmars.2022.884262

The production of cultured limpets is a recent research field contributing to aquaculture diversification, focusing on low trophic species while reducing the carbon footprint. Limpets are gastropods that colonize rocky substrates and are mostly present on tidal and subtidal shores. This animal group is in high commercial demand and is endangered in several regions. The aquaculture production of limpets has been traditionally challenging. The most successful reproduction method has been gonadal dissection, as artificial spawning induction has shown limited success to date. Moreover, methods for larval culture, settlement, and juvenile growth have been poorly developed and remain largely unknown. In recent years, advances in this field have led to the optimization of methods to enhance larval production, larval culture, settlement induction of competent larvae, and management of post-larvae and juveniles. The present manuscript reviews these advances, obtained within the framework of AQUAINVERT project, focusing on broodstock management, gametes release, larval production, larviculture, settlement, and grow-out of post-larvae, and providing an update on the actual state of the art in limpets' aquaculture.

Keywords: patellids, larval production, larviculture, settlement induction, juvenile production

INTRODUCTION

True limpets (hereafter referred to as limpets) are a monophyletic group of gastropods belonging to the subclass Patellogastropoda (Harasewych and McArthur, 2000; Nakano and Ozawa, 2007). Limpets play a key top-down influence on the structure of rocky shore communities (Branch, 1981; Branch, 1985; Menge, 2000; Burgos-Rubio et al., 2015) as powerful grazers that prevent the recruitment of macroalgae by consuming their sporelings and propagules (Branch, 1971; Branch,

1981; Branch, 1985; Jernakoff, 1985; Benedetti-Cecchi, 2000; Bulleri et al., 2000; Coleman et al., 2006; Lorenzen, 2007). Limpets also contribute to the stability of the red encrusting coralline algae assemblages, a worldwide group of red algae that dominate rocky shorelines (Steneck, 1986; Dethier, 1994) and play an important role in carbon sequestration, reef building, invertebrate recruitment, and fish nursery grounds (McCoy and Kamenos, 2015). These encrusting algae evolved to tolerate intense animal herbivory pressure (limpets, chitons, and sea urchins) that removes algal competitors (Underwood, 1980; Branch, 1981; Steneck, 1982; Branch, 1985; Steneck, 1990; Piazzi et al., 2016).

The exploitation of limpets as fishery resources has been reported to exist since 10,000 years into the paleontological records of distant parts of the world, such as South Africa (Klein, 1979; Klein and Steele, 2013), California (Erlandson et al., 2011), North Spain (Muñoz-Colmenero et al., 2012), and several Mediterranean sites (Stiner et al., 1999; Colonese et al., 2011). Currently, the exploitation of limpets continues for either ornamental, food, or fishing bait collection purposes (Espinosa and Rivera-Ingraham, 2017; Firth, 2021). The exploited taxa include the genera *Cellana* (Lasiak, 1993; Harada et al., 1997; McCoy, 2008), *Cymbula* (Branch and Odendaal, 2003), *Lottia* (Pombo and Escofet, 1996; Sagarin et al., 2007), *Patella* (Guerra-García et al., 2004; Guallart et al., 2013c; Henriques et al., 2017; Sousa et al., 2019a; Sousa et al., 2020a), and *Scutellastra* (Carballo et al., 2020; Valdez-Cibrián et al., 2021). Overfishing has led to a detrimental impact on several populations of limpets worldwide, resulting in smaller specimens and population sizes, a reduced female:male ratio, and an overall plummet of biomass and reproductive/recruitment output (Branch, 1975; Branch and Odendaal, 2003; Guerra-García et al., 2004; Espinosa et al., 2006; Espinosa et al., 2009a; Sousa et al., 2019b; Sousa et al., 2020b). Consequently, several limpet populations are currently declared vulnerable, endangered, or extinct (Espinosa et al., 2014; Espinosa and Rivera-Ingraham, 2017; Henriques et al., 2017; Luque et al., 2018; Carballo et al., 2020). Different governmental authorities have implemented mitigation measures to reduce the overfishing impact, mostly based on the application of Marine Protected Areas (MPAs) and capture restrictions (Branch and Odendaal, 2003; Espinosa and Rivera-Ingraham, 2017; Sousa et al., 2019a; Sousa et al., 2020a).

Aquaculture could offer an opportunity to reduce the impact of overfishing on limpet populations. Moreover, limpets represent a low trophic group with a reduced carbon footprint, which is especially interesting for the diversification of aquaculture. The limpets could also be incorporated as one of the trophic levels to integrated multi-trophic aquaculture (IMTA) systems, similar to the abalone that has revealed potential for IMTA production in land-based (Nobre et al., 2010) and offshore mariculture systems (Viera et al., 2016). The ability to culture limpets might also provide an opportunity for restoration projects of endangered species (Guallart et al., 2020a; Ferranti et al., 2022). Today, limpets aquaculture is still in its infancy and at the experimental level, as "there are many issues in limpet aquaculture because of their sensitive nature and complex environmental and biological requirements, most of which is still unknown in laboratory environments" (Mau and Jha, 2018). Moreover, scientific literature is especially scarce regarding larval culture methods, settlement requirements, and post-larval and juvenile grow-out experiences (Nhan, 2014; Ferranti et al., 2018; Mau and Jha, 2018; Guallart et al., 2020b; Ferranti et al., 2022).

The AQUAINVERT project (INTERREG MAC 2014-2020) aims to enhance and promote the aquaculture of marine invertebrates in the Macaronesia region. One major goal of the project is the development of aquaculture protocols for the congeneric patellid limpet species *Patella aspera* Röding, 1798 (**Figures 1A–C**), and *Patella candei* d'Orbigny, 1840 (**Figures 1D–F**). These native species from the European Macaronesia (including the Azores, Madeira, and Canary

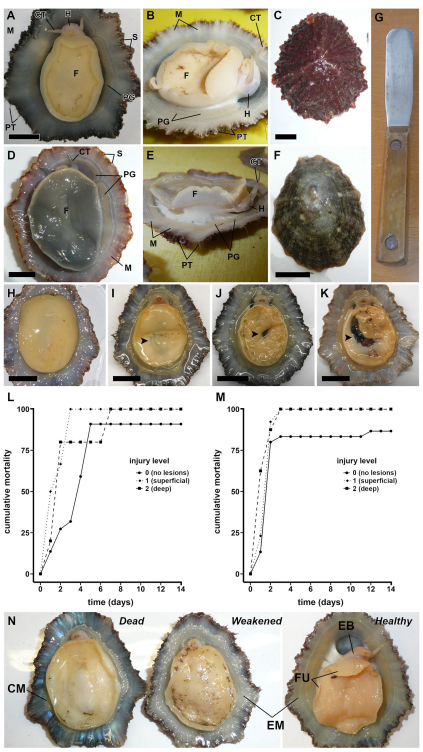

FIGURE 1 | Limpet species studied in the present study **(A–F)**. *Patella aspera* Röding, 1798 from Madeira **(A–C)**: ventral view **(A)**, lateral view **(B)**, and dorsal view of a shell covered by encrusting coralline algae **(C)**. *Patella candei* d'Orbigny, 1840 from Madeira **(D–F)**: ventral view **(D)**, lateral view **(E)**, and dorsal view of a shell with scarce epibionts **(F)**. Blunt knife known as "lapeira" used for the traditional fishing of limpets **(G)**. Impact of physical damage on broodstock survival **(H–M)**. Category level of the physical injuries using *P. aspera* as example **(H–K)**: level 0, no visible injuries **(H)**; level 1, superficial lesions **(I)**; level 2, deep lesions **(J)**; level 3, evisceration and/or decapitation **(K)**. Cumulative mortality during the first 2 weeks after the capture **(L, M)**: *P. aspera* **(L)** and *P. candei* **(M)**. Health condition of the specimens using *P. aspera* as example **(N)**. Scale bar = 10 mm. CM, contracted mantle; CT, cephalic tentacles; EB, extended body; EM, extended mantle; F, foot; FU, foot undulations; H, head; PG, pallial gills; PT, circumpallial tentacles; S, shell.

archipelagos) (Weber and Hawkins, 2002; Henriques et al., 2012; Sousa et al., 2017) represent important cultural, gastronomic, and economic resources. Nevertheless, both species endure relevant overfishing pressure, so legislative, restrictive, and protective measures have been promoted to reduce the anthropogenic impact on the native populations in Azores (Ferraz et al., 2001; Martins et al., 2011; Diogo et al., 2016; Martins et al., 2017), Madeira (Fernandes et al., 2019; Sousa et al., 2019a; Sousa et al., 2019b; Sousa et al., 2020a), and the Canary Islands (Navarro et al., 2005; López et al., 2012; González-Lorenzo et al., 2015; Parker et al., 2020).

The present manuscript thoroughly describes suitable methodologies developed for the culture of both limpet species, regarding broodstock management, larval production, and animal culture up to the grow-out phase. It reviews the methodologies already developed for other limpet species with an update and optimization in the framework of the AQUAINVERT project and introduces methodologies for settlement induction and post-larval management. Experimental data to validate the protocols and provide insight into the early life biology of the patellid limpets are included.

MATERIALS AND METHODS

Limpets' Collection and Research Facilities

The research work took place at two distinct facilities: the Mariculture Center of Calheta (CMC; Madeira) and the Experimental Aquaculture Laboratory (AquaLab; Azores). In both facilities, adult specimens of *P. aspera* and *P. candei* were captured during the reproductive period (October to April) (Góis et al., 2010; Henriques et al., 2012; Sousa et al., 2017). The specimens collected at CMC facilities were used for assays 1–3, 5–16, and 18, and those collected at AquaLab facilities were used for assays 4 and 17.

General Procedures

A total of 18 assays were realized with the following purposes: assays 1 to 2 deal with "Recollection and management of the broodstock"; assays 3 to 12 deal with "Gametes and larval production methods"; assays 13 to 15 deal with "Larval development and larviculture methods"; assays 16 to 17 deal with "Settlement and metamorphosis in limpets"; and assay 18 deals with "Management of the post-larvae and grow-out".

The adults dissected for larval production (assays 7–12), larviculture (assays 13–15), settlement (assay 16), and post-larval management (assay 18) were characterized using the average shell length, total mass, and gonadosomatic index (**Supplementary Table 1** for details). The gonadosomatic index was calculated following Sousa et al. (2017): $GSI = GM \times (TM - SM)^{-1} \times 100$, where GM is the gonadal mass, TM is the total mass, and SM is the shell mass.

For alkaline bath, incubation, and larval culture, we used glass beakers (600 ml) (**Figures 2A, B**) and commercial plastic cups (80–100 ml) (**Figures 2D, E**). For settlement, we used culture cell plates (**Figure 2G**), Petri dishes (**Figure 2H**), and tanks with transparent fiberglass plates (**Figure 2F**). The water employed was filtered (5–20 μm) seawater sterilized with ultraviolet (acronym: FSS).

Larval morphology was used as a criterion to distinguish between viable and deformed larvae, e.g., trochophores (assays 6–15; **Figures 9A–C, J–K**) and pediveligers (assays 14–15; **Figures 9G–I, L–M**). Larval descriptions are detailed in "Section 3. Larval development and larviculture".

Assays 1–2: Recollection and Management of the Broodstock

Assay 1. Impact of physical damage on broodstock survival. The influence of physical injuries on the survival of *P. aspera* ($n = 45$) and *P. candei* ($n = 45$) in captivity was evaluated. This assay was performed between November 2019 and February 2020. The specimens were tagged (PVC sheets glued with cyanoacrylate; **Figures 3D, G, J**). Injuries were categorized into four levels: 0 = no visible injuries, 1 = superficial lesions, 2 = deep lesions, and 3 = evisceration and/or decapitation (**Figures 1H–K**). The specimens were placed in the culture tanks, and mortality was monitored daily to calculate the relative cumulative mortality for each injury level. A bias in this study was that a majority of

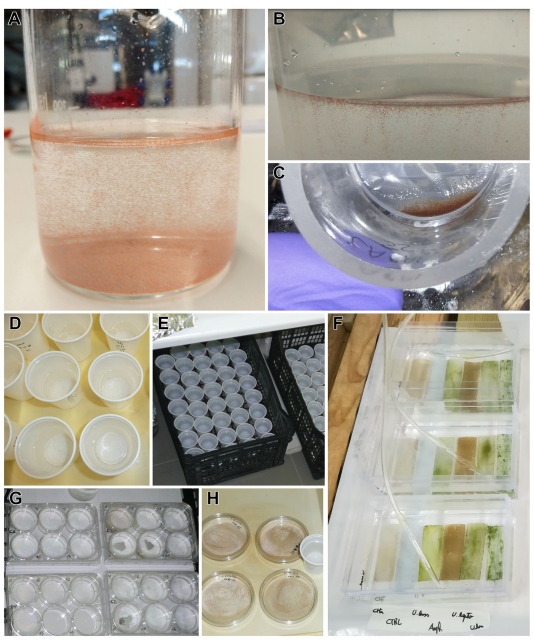

FIGURE 2 | Different containers used for the culture of limpets. Glass beaker (600 ml), used for alkaline treatment, fertilization, incubation, and larval culture **(A, B)**: glass beaker showing a pool of trochophores **(A)**, and trochophores swarming near the water surface **(B)**. Mesh (55 μm) used to gather the trochophores collected by siphoning **(C)**. Plastic cups (80–100 ml) used for alkaline treatment, fertilization, incubation, and larval culture (**D, E**). Tanks (1 L) with fiberglass plates covered by different biofilms used for settlement assays and grow-out of post-larvae **(F)**. Culture cell plates with different substrates used for settlement assays **(G)**. Petri dishes with *N. incerta* biofilms used for settlement assays and grow-out of post-larvae **(H)**.

FIGURE 3 | Culture tanks used for the maintenance of adult limpets and feeding trials. Culture tanks from the AquaLab facilities, Azores **(A, B)**. Culture tanks from the CMC facilities, Madeira **(C, D)**. Limpets attached to the culture tank walls above the water level **(E, F)**. Feeding trials for adult specimens **(G–L)**. Commercial fish flakes: flakes available for the adult limpets **(G)**, and resulting feces **(H, I)**. Artificial flour-based meal: prepared meal available for the adult limpets **(J)**, and resulting feces **(K, L)**. Scale bar = 1 mm. R, red coloration derived from a fish flake; P, *Porphyra* piece from the artificial meal.

healthy specimens (level 0; **Figure 1N**) were removed prematurely for experimental purposes.

Assay 2. Feeding trials on adult limpets. Acclimated adults of *P. aspera* and *P. candei* were placed in plastic baskets located inside the culture tanks. The food was kept for 2 days (**Figures 3G, J**) and tested: commercial fish flakes (ActivPet, Pingo Doce, Portugal) (**Figure 3G**) and artificial meals based on a flour mixture inspired by those developed for *Cellana sandwicensis* by Nhan (2014) (**Supplementary Table 2**; **Figure 3J**). The feces were examined using a dissecting microscope (**Figures 3H, I, K, L**).

Assays 3–12: Gametes and Larval Production Methods

Assay 3. Female fecundity. Female fecundity was calculated as total oocyte production per female in *P. aspera* (n = 73) and *P. candei* (n = 37) sampled from October 2020 to April 2021. The shell length was measured, and the gonadosomatic index was calculated. Each female gonad was placed inside a measuring cylinder, leveled up to 100 ml with FSS, and dropped in a beaker. The oocytes from the gonad were extracted (**Figures 5I–J**), and three samples (1 ml volume) were collected and fixed with 300 µl of formaldehyde 37%. The oocytes from the samples were counted to calculate oocyte production.

Assay 4. Spawning induction assays. This assay evaluates different treatments for spawning induction in *P. aspera* and *P. candei*. Specimens were collected from intertidal areas in Faial Island (Azores) and acclimatized for 2 weeks in 35-L tanks. Half an hour before the trial, specimens were placed on a tray, left upward, and covered with a wet towel. Trials were performed in an acclimatized room using 500 ml of transparent boxes as experimental containers. Water flow was kept running using filtered (1 µm) seawater (acronym: FSW) at ambient temperature (17.3 ± 0.4°C). All treatments had 8 independent replicates (individual limpets), except for one trial, which was conducted with 5 individuals for each treatment (**Table 1**). The treatments lasted between 02:00 and 04:30 h or until the first individuals started to spawn. At the end of each trial, the sex ratio was estimated by dissection of 25% of the treated specimens and calculated as: $N_{male} \times (N_{male} + N_{female})^{-1}$ (**Table 1**).

The protocol was mainly based on Ferranti et al. (2018) and included five treatments: intense bubbling, UV-light irradiation, hydrogen peroxide (H_2O_2), thermal shock, and a negative control group. The bubbling treatment used specimens placed in a cylindrical colander inside the boxes with FSW and was generated by aquarium air stones positioned under the colander. The UV-light irradiation treatment was tested as it induces spawning in different abalone species (Ebert and Houk, 1984; Moss et al., 1995). The specimens were placed in a flow-through system with UV-treated FSW with a calculated irradiation of 600 mW h L^{-1}. In the thermal shock treatment, the specimens were placed in boxes with FSW whose temperature was increased by 4–5°C. The temperature was maintained at a constant level using a thermostat that was switched off at the end of the treatment. Hydrogen peroxide treatment employed an experimental box filled with FSW in which the pH was increased to 9.1 using 2 M

TABLE 1 | Results of induction tests performed with different treatments: bubbling (BUB), thermic shock (T), hydrogen peroxide (H_2O_2), ultraviolet radiation (UV) and control (CNT). .

date	species	n specimens	SR	n treatments	sex/treatment	Exp. time
06/02/2021	*P. candei*	32	0.50	4	0	2h
25/03/2021	*P. candei*	40	0.67	5	1f/ H_2O_2	2h
05/04/2021	*P. aspera*	40	0.75	5	0	
26/04/2021	*P. candei*	40	0.71	5	1m/ H_2O_2 3m/BUB 1f/T	4h30min
02/12/2021	*P. candei*	40	0.37	5	0	4h30min
15/12/2021	*P. candei*	25	0.66	5	1f/BUB	4h30min

Sex Ratio (SR) is indicated. Sex/treatment indicate the number of individuals per sex (m = male, f = female) responding to the stimulus, zero value indicates none specimen responded to the stimulus. Exp. time, indicates the time that limpets were exposed to the treatments in each experimental event.

Tris. The specimens were placed in those boxes for 15 min before the addition of 3.2 ml of freshly prepared 10% H_2O_2 solution. The specimens of the control treatment were placed in experimental boxes filled with FSW. At the end of all treatments, seawater was changed in all boxes with FSW and maintained at room temperature until the end of the trial, which lasted 7 ± 1 h.

Assay 5. Measurement of the diameter of oocytes. The oocytes from ten specimens per limpet species (*P. aspera* and *P. candei*) were photographed immediately after their extraction using a dissecting microscope (Leica M165, Leica Microsystems, Wetzlar, Germany) connected to a camera and image analysis software (LAS V4.12; Leica Microsystems, Wetzlar, Germany). The oocytes from the same specimens were also measured after receiving an alkaline treatment (NaOH, pH 8.9, 3 h). The minimum and maximum Feret's diameters were measured for 40 oocytes per specimen using the image analysis software ImageJ 1.53k to calculate the average Feret's diameter, following Dopchiz et al. (2018).

Assay 6. Variability in female fertility. Larval production (as a ratio of viable trochophores) was analyzed individually in *P. aspera* (n = 40) and *P. candei* (n = 16) females sampled from November 2020 to April 2021. Two treatments were applied to the oocytes: control (FSS) and alkaline treatment (NaOH, pH 8.9, 3 h). The specimens were processed in batches of eight females and four males. The males were used to prepare four different sperm pools: ABC, ABD, ACD, and BCD; each letter represents a different male. Each sperm pool was used to fertilize two females. Fertilization was performed at a density of 10^5 sperm cells ml^{-1}, and incubation lasted 24 h (16 ± 2°C). Three replicates per treatment and per female were realized. Samples were fixed in formaldehyde 6%–8% and identified as viable trochophores (VT; **Figures 9A–C**), deformed specimens (**Figures 9J, K**), and unfertilized oocytes (**Figure 5O–S**). The ratio of viable trochophores was calculated as VT × total $specimens^{-1}$. Pairwise comparisons of the ratio of viable trochophores between control and alkaline treatments were performed in each female to calculate the percentage of specimens showing significant differences. The influence of the sperm pool was analyzed by pairwise comparisons between females fertilized with the same sperm pool.

Assays 7–8. Alkaline agent (NaOH vs. NH_4OH). This experiment was repeated twice to study the influence of different alkaline treatments on the larval production of *P. aspera*. The following factors were combined: two alkaline bases (NaOH and NH_4OH), two pH values (8.4 and 8.9), and six times (10, 20, 30, 60, 120 and 180 min), resulting in 24 combined treatments. In addition, a control treatment (FSS, pH 8.0) was applied at the same times specified before (six control treatments). Three replicates per combination were used. The oocytes were rinsed at the end of each corresponding time and kept in FSS. Fertilization was simultaneous for all oocytes (10^5 sperm cells ml^{-1}). Incubation lasted 24 h (17 ± 1°C). The specimens were fixed and examined to calculate the ratio of viable trochophores, ratio of normal development, and ratio of fertilization (Cañizares et al., 2021).

Assays 9–12. Sperm density. For consistency of the results, evaluation of the optimal sperm density in *P. aspera* was done in four separate assays, applying six treatments using logarithmic increments of sperm concentration: 10^N sperm cells ml^{-1}; being n = 2, 3, 4, 5, 6, and 7. Four replicates per treatment were used. The oocytes were alkaline treated (NaOH, pH 8.4, 3 h). Incubation lasted 24 h (16 ± 1°C). The specimens were fixed and examined to calculate the ratio of viable trochophores, ratio of normal development, and ratio of fertilization (Cañizares et al., 2021).

Assays 13–15: Larval Development and Larviculture Methods

Assays 13–15. Validation of the larviculture protocol. Assay 13 compared the viability of the trochophores (24 h post-fertilization) obtained from non-alkalinized oocytes (control using FSS) and from alkalinized oocytes (NaOH, pH 8.9, 3 h). The trochophores were distributed in eight plastic cups per treatment; four cups per treatment were sampled immediately and fixed to calculate (1) the initial density of trochophores and (2) the ratio of viable trochophores. The remaining cups were cultured for 48 h and fixed to calculate (1) the final density of pediveligers and (2) the ratio of viable pediveligers as viable pediveligers × $total\ specimens^{-1}$. Student's t-test ($p < 0.05$) was used to analyze pairwise comparisons of the ratios of viable larvae and the densities of larvae.

Assays 14 and 15 were performed with oocytes from two different treatments: control (FSS) and alkaline bath (NH_4OH, pH 9, 10 min) and were divided into two sequential parts. First, for each treatment, the oocytes were distributed in four plastic cups (40 ml FSS) and three glass beakers (500 ml FSS) using 100 oocytes ml^{-1}. The beakers were sampled to calculate the initial quantity of oocytes in each beaker. Fertilization used 10^5 sperm cells $ml^{-1,}$ and incubation lasted 24 h (16 ± 1°C). The plastic cups were sampled and fixed to calculate the ratio of viable trochophores. In each glass beaker, the upper 400 ml was siphoned, and specimens were gathered using a 55-μm mesh, resuspended in 100 ml of FSS and sampled to calculate the ratio of collected trochophores relative to the initial quantity of oocytes.

At continuation, the collected trochophores from each treatment were pooled and distributed in eight plastic cups (40 ml FSS) and four glass beakers (500 ml FSS) using 4 ± 1 trochophores ml^{-1}. Four cups per treatment were sampled and fixed as described in assay 13. The beakers were sampled to calculate the initial quantity of trochophores per beaker. The remaining cups and the beakers were cultured for 48 h. Then, the plastic cups were sampled and fixed following assay 13. In each glass beaker, the upper 400 ml was siphoned, and specimens were gathered using a 55-μm mesh, resuspended in 50 ml FSS, and sampled to calculate the ratio of collected pediveligers relative to the initial quantity of trochophores.

Assays 16–17: Settlement and Metamorphosis in Limpets

Assay 16 studied the settlement in *P. candei* following the methodology described by Castejón et al. (2022). The pediveligers were obtained from alkalinized oocytes (NaOH, pH 8.9, 3 h) and trochophores cultured in glass beakers (12 ± 4 larvae ml^{-1}). The assay was performed by placing pediveligers (72 h post-fertilization) on cell culture plates (**Figure 2G**) using 8 ml of FSS and 4 ± 2 larvae

ml^{-1}. The assay lasted 13 days. Six treatments with six replicates each were tested: (1) negative control; (2) shell pieces covered by light pink coralline crusts (143 ± 27 mm^2); (3) diatom *Halamphora coffeaeformis* biofilm; (4) diatom *Navicula incerta* biofilm; (5) free swimming haptophycean *Pavlova* sp.; and (6) combined *H. coffeaeformis* and *Pavlova* sp. The assay was monitored daily to calculate the ratio of swimming larvae, ratio of crawling larvae, ratio of settled specimens, and ratio of dead specimens. Settlement success was analyzed at Day 13 as the ratio of juveniles (post-larvae with teleoconch).

Assay 17 used gametes of *P. candei* obtained from a spawning induction in April 2020 (one female and three males; **Table 1**). The oocytes were suitable for fertilization, so alkaline treatment was not required (Nunes et al., 2021). Fertilization was performed using 29 ± 6 oocytes ml^{-1} and 4.7 × 10^5 sperm cells ml^{-1} and lasted 3 h. Pediveligers 72 h post-fertilization were placed in three rectangular tanks (1 L) with FSS at room temperature (17 ± 1°C). The treatments were performed on transparent fiberglass plates (2 × 7 cm; **Figure 2F**) in which different biofilms were grown: *Amphora* sp. (BEA 1588B), *Chaetoceros* sp. (BEA 0419B), *Ulvella lens* (CS-801-19), *Ulvella leptochaete* (BEA 0702B), and *Ulva lactuca*, in addition to a plate without biofilm as a negative control. The tanks were filled with 400 ml of FSS, placing ca. 200 pediveligers per tank. For 1 week, 50 ml of FSW was added daily to maintain healthy experimental conditions. From Day 8 to Day 64 post-fertilization, the tanks were topped up to 1 L and switched to a flow-through system at constant temperature (17 ± 1°C).

Assay 18: Petri Dishes as Culture Containers for Limpet Post-Larvae

Assay 18. Petri dishes with grown *N. incerta* biofilms were tested as culture containers for limpet post-larvae. The same algal culture was used as a settlement inducer in both *P. aspera* and *P. candei*. The pediveligers (72 h post-fertilization) were obtained from alkalinized oocytes (NH_4OH, pH 9, 10 min). Larvae were cultured in glass beakers (4 ± 1 larvae ml^{-1}). The culture media used for *N. incerta* was removed before the start of the assay and replaced with 25 ml of FSS. The initial density was 3 ± 1 pediveligers ml^{-1}. The assay was monitored weekly.

Statistical Analyses

All statistical analyses were performed using the statistical software R version 4.1.0. Yuen's test for trimmed means (package "WRS2 1.1–3") was used in assays 5–6 as a nonparametric approach for pairwise comparisons (null hypothesis rejected when $p < 0.01$). The homogeneity of the variances was analyzed using Levene's test (package "car 3.0-12"), and the normality of the residuals was analyzed using the Shapiro–Wilk normality test (null hypothesis rejected when $p < 0.05$). The Tukey HSD was used in assays 7–8 to establish the significant differences relative to the treatment with the highest value following Pérez et al. (2016); in assay 6, two combined treatments (NaOH × pH 8.9 × 3 h; NH_4OH × pH 8.4 × 3 h) were removed from the statistical analysis because two replicates from each treatment were accidentally lost. The Tukey HSD was used in assays 9–12 as a parametric method for pairwise comparisons among treatments (null hypothesis rejected when $p < 0.05$). The Games–Howell test (package "rstatix 0.7.0") was used in assays 9–10 as an alternative for the Tukey HSD test when the data were normal but not the homogeneity of variances (null hypothesis rejected when $p < 0.01$). Student's *t*-test was used in assays 13–15 as a parametric method to analyze pairwise comparisons (null hypothesis rejected when $p < 0.05$). Assay 16 analyzed the ratio of juveniles performing the post-hoc Tukey HSD test (null hypothesis rejected when $p < 0.05$) on arcsine square root transformed data to fit the normality and homoscedasticity assumptions. The HAL +PAV treatment was removed since zero values were obtained in all replicates.

RESULTS AND DISCUSSION

Recollection and Management of the Broodstock

Recollection and Transport

To date, adult limpets can only be obtained from wild populations because technologies to produce captive bred animals have yet to be developed (Mau and Jha, 2018). Wild limpets form an isolation barrier consisting of the limpets' shell, the substrate, and the mucus attachment (Davies, 1969; Branch, 1971; Wolcott, 1973), with the purpose of surviving different levels of environmental stressors such as insolation, temperature, and dehydration in the littoral zone (Orton, 1929; Davies, 1969; Branch, 1971; Wolcott, 1973; Harley et al., 2009). Breeders, either for production or for experimental purposes, might consider that simple detachment exposes the limpets to external stressors, increasing the risk of mortality (Mau and Jha, 2018). Wild specimens arrived at the culture facilities within a few hours after capture (usually less than 2 h) and were directly delivered by the fishermen in bags or nets. In contrast, for long transport, it has been suggested to cover the specimens with pieces of cloth soaked with seawater and place them inside portable refrigerators with coolers (Guallart et al., 2020b; Ferranti et al., 2022).

The physical damage that occurred during the capture influenced the survival in culture conditions (assay 1): specimens eviscerated or decapitated did not survive longer than 24 h, while injured specimens showed 100% mortality in approximately 1 week (**Figures 1L, M**). Non-injured specimens were the only ones that survived longer than 2 weeks, showing the necessity to avoid physical injuries to minimize mortality (Guallart et al., 2013a; Guallart et al., 2013b; Mau and Jha, 2018). Distinct levels of health status were identified (**Figure 1N**): dead specimens show a contracted mantle, absence of mantle reflexes to physical contact, and no activity; weakened specimens have a partially extended mantle and mantle reflexes but little or no activity; healthy specimens show an extended mantle, mantle reflexes, and notorious activity (**Video 1**).

Tank Acclimation

Broodstocks of *P. aspera* and *P. candei* were maintained using the same conditions for both species. At the AquaLab facilities (Azores), the adults were kept at a density ranging from 15 to 30

specimens in 55-L cylindrical tanks covered by plastic liners naturally colonized by algae (**Figures 3A, B**). At CMC facilities (Madeira), the adults were kept at a density of 30 to 35 specimens in 200-L cylindrical tanks; in this case, neither plastic liners nor algal growths were employed (**Figures 3C, D**). The environmental conditions were the same at both installations and for both limpet species: flow-through system (1–4 L min^{-1}), at ambient temperature (19 ± 2°C) and salinity (37 ± 1 g L^{-1}), and under a photoperiod of 12 h light:12 h dark. Aeration was provided to maintain oxygen saturation above 90%. Shallow tanks with flowing water at ambient temperature have also been used to keep different limpets' species (Hodgson et al., 2007; Ribeiro, 2008; Mau et al., 2018). Other authors preferred recirculation systems (Ferranti et al., 2018; Nakano et al., 2020; Ferranti et al., 2022).

Cleaning and Maintenance

Tank cleaning was performed routinely (every 1–2 days during the first week and every 2–3 days during the second week) to remove feces, mucus, and dead specimens.

The limpets are prone to injuries; consequently, it is suggested to minimize the manipulation as much as possible. If animal manipulations are needed, we suggest to use a blunt knife (**Figure 1G**) to exert a smooth pressure below the shell to detach the specimens, supporting previous studies (Guallart et al., 2013a). The use of plastic liners is another measure that facilitates the detachment of the broodstock (**Figures 3A, B, E**), as suggested by previous studies (Nhan, 2014; Mau et al., 2018; Mau and Jha, 2018). *P. aspera* and *P. candei* can move above the upper water level (**Figures 3E, F**). This behavior did not cause associated problems, yet other authors preferred to place artificial turfs at the water level to limit animal movement (Nhan, 2014; Mau et al., 2018; Mau and Jha, 2018) or to generate artificial tides to reduce air exposure (Guallart et al., 2020b; Ferranti et al., 2022).

Broodstock of *P. aspera* and *P. candei* were not fed due to the unavailability of diets for these species. However, short feeding trials showed that limpets can accept certain food items (assay 2; **Figures 3G, J**). The examination of the feces revealed content related to the commercial fish flakes (**Figures 3H, I**) and the flour-based meals (**Figures 3K, L**). These observations support the limpets as generally opportunistic feeders with an unrestricted diet (Branch, 1971; Della Santina et al., 1993; Burgos-Rubio et al., 2015), which would facilitate the development of diets for aquaculture purposes.

Gametes and Larval Production Methods

The first limiting factor to start the production of limpets is the availability of mature specimens. Limpets are generally protandrous hermaphrodites in which the smaller and younger specimens tend to mature as males, while the larger and older specimens tend to mature as females (Orton et al., 1956; Wright and Lindberg, 1982; Creese et al., 1990; Espinosa et al., 2006; Lindberg, 2007; Espinosa et al., 2009b; Sousa et al., 2019b). Overfishing affects the sex ratio toward a higher proportion of males and smaller, less productive females (Martins et al., 2017; Sousa et al., 2019b), a factor to be considered when working with specimens from exploited areas. However, the most important factor is the reproductive season. In Azores, the populations of *P. candei* reproduce during the whole year, peaking in summer (Curdia et al., 2005). In Madeira, the populations *P. aspera* and *P. candei* are winter breeders (Góis et al., 2010; Henriques et al., 2012; Sousa et al., 2017), seriously limiting the acquisition of reproductive stocks due to the frequent adverse sea conditions (Torres and Andrade, 2010).

Sex Determination

Limpets have been traditionally considered animals with no secondary sex characteristics, so sex determination has relied on invasive techniques such as biopsies or dissection (Dodd, 1956; Blackmore, 1969; Rao, 1973; Wright and Lindberg, 1979; Wright and Lindberg, 1982; Guallart et al., 2013b; Ferranti et al., 2022). Nevertheless, we found some visual cues that enable sex identification. In *P. aspera*, the foot should be highly pressed toward the animal body to identify the hue of the ventral mid-left side of the foot (**Figures 4A, D**): dark hue in females (**Figures 4A, B**) and pale hue in males (**Figures 4C, D**). From the selected batch ($n = 15$), both males ($n = 5$) and females ($n = 9$) were successfully identified, with only a single unidentified specimen due to unclear features. In *P. candei*, the foot should be highly extended upward while clearly showing the abductor musculature, with the purpose of identifying the hue of the lateral left side of the foot (**Figures 4E–H**): females were harder to identify, showing no clear coloration changes (**Figures 4E, F**), while males showed a cream-color hue (**Figures 4G, H**). From the randomly selected batch ($n = 21$), 100% of males ($n = 5$) were successfully identified, while 87.5% of females ($n = 8$) were identified. The remaining specimens ($n = 8$) did not show clear visual cues. This method is based on the observation of the gonad through the animal body and could be useful for the management of specimens from endangered populations.

The visual determination was shown to be neither infallible nor applicable for all the specimens. The gonadal biopsy designed by Wright and Lindberg (1979) is a non-lethal and minimally invasive alternative. It is performed using a syringe with a hypodermic needle (0.5 × 16 mm) and pinching through the foot or the mantle (**Figures 4I–L**). Guallart et al. (2013b) reported that a quick puncture through the mantle allows a survival rate higher than 90%.

Female Fecundity

The fecundity (assay 3) in females of *P. aspera* was on average 59,000 oocytes, ranging from less than 1,000 to ca. 280,000 oocytes for specimens ranging from 34 to 53 mm shell length (**Figures 4M, N**), while in females of *P. candei*, it was on average 186,000 oocytes, ranging from ca. 12,000 to ca. 1.2×10^6 oocytes for specimens ranging from 37- to 57-mm shell length (**Figures 4O, P**). The greater fecundity values increased with the animal size, yet the correlation was not clear (**Figures 4M, O**), probably masked by differential gonadal development (**Figures 4N, P**). In this sense, fecundity increased with animal size in the limpet *Patella ferruginea* (Espinosa et al., 2006; Guallart et al., 2020b).

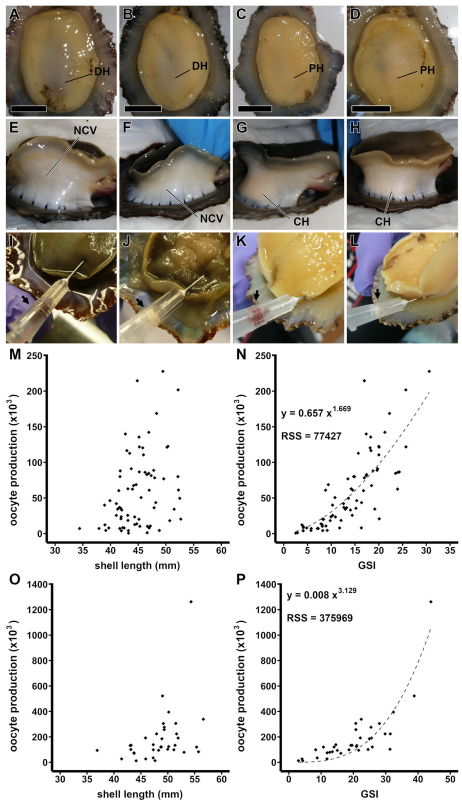

FIGURE 4 | Characters identified for the sex determination of limpets by visual examination. *P. aspera*, coloration of the ventral side of the foot when highly contracted **(A–D)**: females, dark hue **(A, B)**, and males, pale hue **(C, D)**. *P. candei*, coloration of the lateral-left side of the foot when highly extended **(E–H)**: females, no color variation **(E, F)**, and males, cream-color hue **(G, H)**. Biopsy as method to determine the sex in limpets. Biopsy realized through the ventral-left side of the foot in *P. candei* **(I, J)**: female **(I)** and male **(J)**. Biopsy realized through the left side of the foot basis in *P. aspera* **(K, L)**: female **(K)** and male **(L)**. Female fecundity as oocyte production ($\times 10^3$) per specimen **(M–P)**. *P. aspera* **(M, N)**: oocyte production per female ordered by shell length **(M)** and by gonadosomatic index **(N)**. *P. candei* **(O, P)**: oocyte production per female ordered by shell length **(O)** and by gonadosomatic index **(P)**. Scale bar = 10 mm. CH, cream-color hue; DH, dark hue; NCV, no color variation; PH, pale hue.

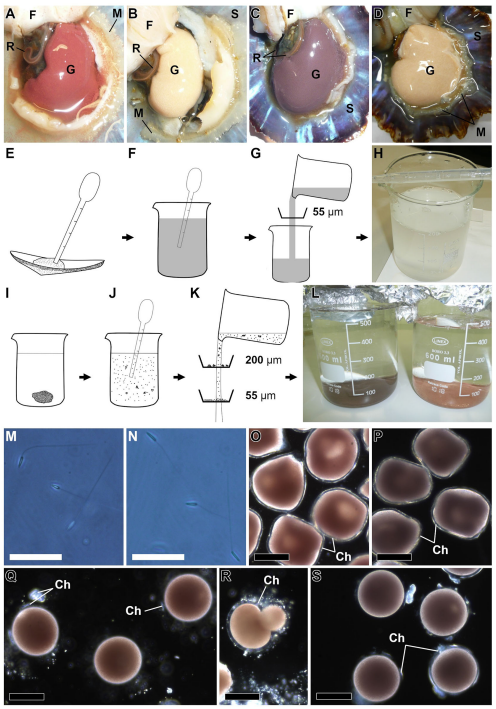

FIGURE 5 | Method for obtaining the gametes of the limpet species *P. aspera* and *P. candei*. Gonad of *P. aspera* exposed by dissection **(A, B)**: female **(A)** and male **(B)**. Gonad of *P. candei* exposed by dissection **(C, D)**: female **(C)** and male **(D)**. Method for obtaining the sperm **(E–H)**: sperm collected using a Pasteur's pipette **(E)**, dilution of the sperm in seawater **(F)**, filtration (55 µm) of the sperm solution **(G)**, and example for sperm solution **(H)**. Method for obtaining the oocytes **(I–L)**: female gonad inside a glass beaker **(I)**, oocytes released using a Pasteur's pipette **(J)**, filtration using a 200- and a 55-µm mesh **(K)**, and example of pool of oocytes of *P. aspera* (right) and *P. candei* (left) **(L)**. Sperm cells **(M, N)**: *P. aspera* **(M)** and *P. candei* **(N)**. Oocytes just after the extraction showing polyhedral shape and the chorion membrane **(O, P)**: *P. aspera* **(O)** and *P. candei* **(P)**. Oocytes just after the alkaline treatment showing spherical shape and the degradation of the chorion membrane **(Q, S)**: *P. aspera* **(Q)** and *P. candei* **(S)**. Oocyte of *P. aspera* deformed due to the alkaline treatment **(R)**. Scale bar = 25 µm **(M, N)**, 100 µm **(O–S)**. Ch, chorion; F, foot; G, gonad; M, mantle; R, radula; S, shell.

Regarding the gamete release for the limpets, two methodologies are available: (1) spawning induction and (2) dissection of the adult specimens to extract the gonads.

Spawning Induction Methods

Spawning induction techniques are based on the exposure of mature specimens to physical or chemical factors able to induce gamete release and are used successfully in marine gastropods with aquaculture interest, such as abalone (Morse et al., 1977; Uki and Kikuchi, 1984; Moss et al., 1995; Courtois de Vicose et al., 2007). The factors tested in limpets include air desiccation, chemical exposition (H_2O_2, KCl), osmolarity shock, thermal shock, vigorous aeration, and different combinations of these; however, only a small fraction of stimulated adults actually released gametes (Kay and Emlet, 2002; Ferranti et al., 2018; Ferranti et al., 2022). Spawning induction has been tested in *P. aspera* and *P. candei* (assay 4), with the higher number of gametes released reported in the bubbling treatments (**Table 1**), a physical factor that could simulate the environmental conditions favorable for limpets' reproduction (Kay and Emlet, 2002; Ferranti et al., 2018). Recently, Ferranti et al. (2022) developed a potentially successful protocol for *P. ferruginea* consisting of maintaining specimens upturned in cold (5°C) and dry conditions, performing a gonad biopsy followed by cold bubbling for at least 1 h (temperature 5°C lower than ambient seawater temperature), air drying upturned specimens at room temperature, and performing a final immersion in seawater at environmental temperature and without aeration, waiting for spawning. Spawning success is probably influenced by gonadal maturation stages, as mature specimens are the most prone to release gametes (Uki and Kikuchi, 1984). Magnetic resonance is a promising non-lethal technique to determine

gonadal maturation in endangered limpet populations (Guallart et al., 2020a).

As an alternative approach, Mau et al. (2018) tested the viability of intramuscular injections of salmon-gonadotropin-releasing hormone analog (250 ng g^{-1} for priming and 500 ng g^{-1} for resolving) in the limpet *C. sandwicensis*, reporting a spawning success between 10% and 13%. Ferranti et al. (2022) used injections of human chorionic gonadotropin (5 µl g^{-1}) and luteinizing hormone-releasing hormone (1 µg g^{-1}) in *P. ferruginea*, but spawning was unsuccessful except for a single male individual. The use of analogous hormones is an interesting approach that requires more studies in different limpet species using different hormones and concentrations.

Dissection and Fertilization *In Vitro*

The most traditional method to produce limpets' larvae is based on the dissection of mature specimens to extract the gonad and obtain gametes (Smith, 1935; Dodd, 1957). The dissection proceeded with specimens upturned over a flat surface. A scalpel was used to cut the perimeter of the foot musculature from the left to the right side of the animal. The foot was removed, exposing the gonads (**Figures 5A–D**). Sex is determined by gonad coloration. The females of *P. aspera* and *P. candei* showed a red–orange and brown–purple colored gonad, respectively (**Figures 5A, C**), with the oocytes visible to the naked eye. The males of both species showed a cream-white gonad and seminal fluid (**Figures 5B, D**).

The protocol to obtain the gametes described hereby is an improved version of the one used in previous studies (Cañizares et al., 2021; Castejón et al., 2021). The sperm were collected directly from the male gonad with a plastic Pasteur pipette (**Figure 5E**) and immediately diluted in FSS (**Figure 5F**). This step should be carefully performed to avoid damaging the underlying organs and collecting debris. It is recommended to pool the sperm of several males to enhance the chances for successful fertilization and genetic variability. The sperm solution can be filtered through a 55- to 100-µm mesh to remove large debris (**Figures 5G**). The sperm started to activate immediately after dilution in seawater. This method enabled sperm solutions of 50–200 ml with a density ranging from 10^7 to more than 10^8 sperm cells ml^{-1} (**Figures 5H, M, N**). Following Cañizares et al. (2021), the sperm can be placed in the fridge (5 ± 2°C) for more than 6 h without adverse consequences for fertilization. Active sperm cells have been reported up to 24 h after being diluted in seawater at 16 ± 1°C.

The oocytes were extracted by applying repeated suction-release pressure efforts using a plastic Pasteur pipette to break the female gonad inside a glass beaker with FSS (**Figures 5I, J**). The resulting solution was a mixture of oocytes, hemolymph, and tissue debris (**Figure 5J**). The diameter of the oocytes was 156 ± 11 µm in *P. aspera* and 161 ± 10 µm in *P. candei* (**Figures 5O, P**; assay 5). Then, the oocytes were washed using a 200-µm mesh to retain large debris and a 55-µm mesh to retain the oocytes (**Figure 5K**). It is recommended to pool the oocytes of several females (**Figure 5L**), as fertility is highly variable (**Figure 6**). Limpets' oocytes are denser than seawater and immediately sink, so the supernatant was removed by siphoning and replaced with FSS, which increases the water quality for the incubation (Ferranti et al., 2022).

The recently extracted limpet oocytes show a polyhedral shape and a membrane covering called the chorion (**Figures 5O, P**), exhibiting low fertility. Since the study by Corpuz (1981), it has been known that fertilization success can be enhanced using an artificial maturation treatment consisting of an alkaline seawater bath, generally consisting of NaOH solutions at pH 9.0–9.5 for several hours (Aquino De Souza et al., 2009; Pérez et al., 2016; Guallart et al., 2020b; Cañizares et al., 2021; Castejón et al., 2021), or based on NH_4OH solutions at pH 8.5–9.0 lasting no more than 10 min (Wanninger et al., 1999; Gould et al., 2001; Hodgson et al., 2007; Ribeiro, 2008; Pérez et al., 2016; Seabra et al., 2019; Nakano et al., 2020). The alkaline treatment promoted chorion removal and the acquisition of a spherical shape and significantly reduced the oocyte diameter to 141 ± 6 µm in *P. aspera* (Yuen's test, *p*-value = 0) and 146 ± 6 µm in *P. candei* (Yuen's test, *p*-value = 0) (**Figures 5Q, S**; assay 5) when using NaOH at pH 8.4–9.0 for 3 h on these species (Cañizares et al., 2021; Castejón et al., 2021; Castejón et al., 2022). The duration of the alkaline bath should be controlled to avoid the degradation of the oocytes (**Figure 5R**) and to limit the ratio of abnormal development (**Figures 9J, K**) (Cañizares et al., 2021; Castejón et al., 2021). For this reason, the oocytes should be washed to remove the alkaline agent after the finalization of the bath; we recommend using a 55-µm mesh and abundant FSS. The density of oocytes used during the alkaline bath ranged from 100 to 500 oocytes ml^{-1}, representing from 4 to 50 oocytes mm^2 at the bottom of the containers.

The fertilization of the oocytes of *P. aspera* and *P. candei* was realized by adding the sperm directly to the oocytes at a density of 10^5–10^6 sperm cells ml^{-1} (Cañizares et al., 2021; Castejón et al., 2021; Castejón et al., 2022), which was within the range used for other patellid limpet species (Hodgson et al., 2007; Ferranti et al., 2018; Guallart et al., 2020b; Ferranti et al., 2022). The fertilization period is highly variable in the literature, from 30 min for *Patella ulyssiponensis* (Hodgson et al., 2007) to 3 h for *Patella vulgata* (Pérez et al., 2016), and intermediate values for other patellids (González-Novoa, 2014; Seabra et al., 2019; Guallart et al., 2020b). However, the sperm can be left with the oocytes during the entire incubation period (24 ± 2 h) without adverse effects on the culture, such as increased polyspermy (Cañizares et al., 2021; Castejón et al., 2021; Castejón et al., 2022), saving additional rinses and losses of oocytes. The incubation was realized in static conditions, i.e., neither aeration nor stirring was provided. The density of oocytes used during the incubation ranged from 30 to 150 oocytes ml^{-1}, representing from 1 to 15 oocytes mm^2 at the bottom of the containers.

Variability in Female Fertility

Individual fertility of the females was studied in *P. aspera* (n = 40) and *P. candei* (n = 16) (assay 6). Current results are preliminary as a study is still ongoing when publishing this manuscript, yet this information could be valuable in the current context. Both species showed a high variability in the ratio of viable trochophores obtained in the control and alkaline treatments (**Figure 6**). The alkaline treatment significantly

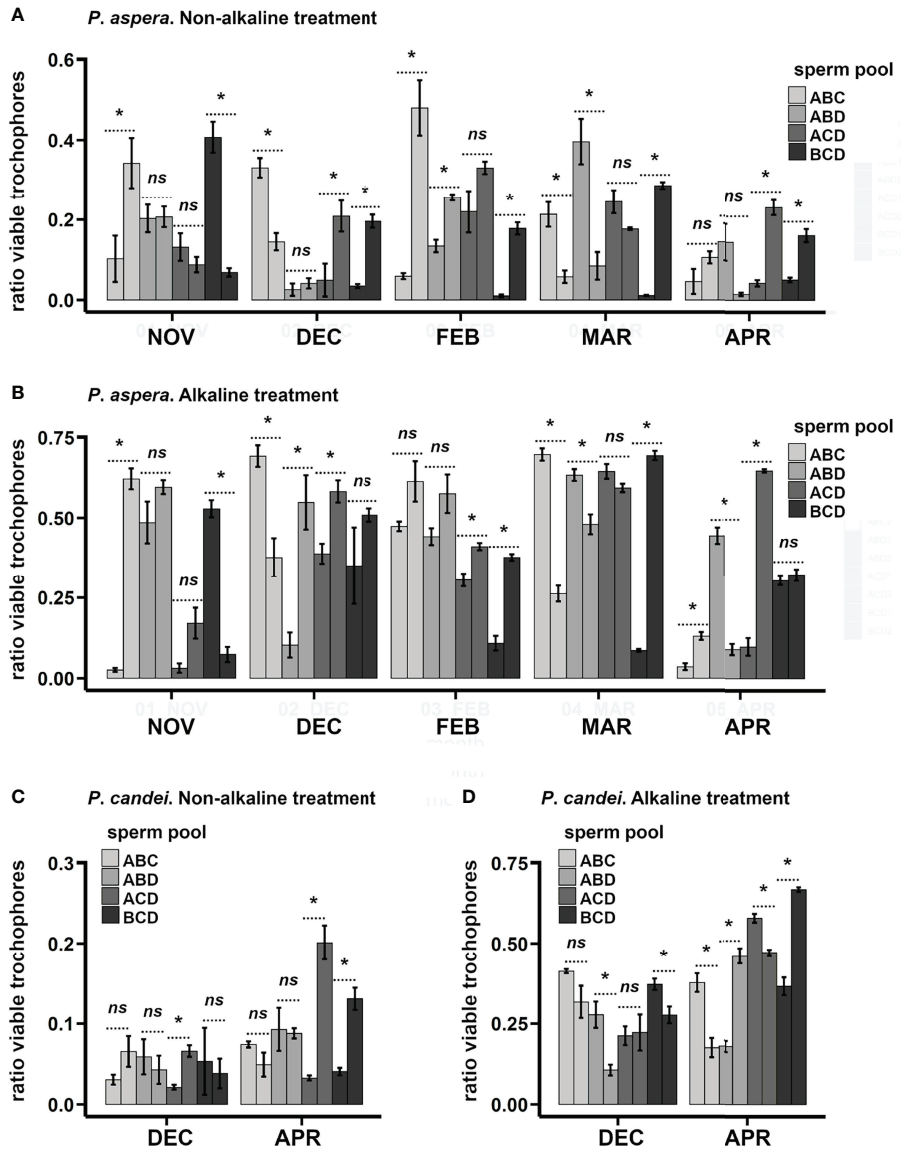

FIGURE 6 | Individual variation of the fertility among female limpets, studied as a ratio of viable trochophores, and comparison between non-alkaline (control) and alkaline treatments. Eight females were analyzed each month. Each bar represents the average ± SD ratio of viable trochophores obtained in each female. The females of each species follow the same order in non-alkaline and alkaline treatments. The differences in ratio of viable trochophores within pairs of females fertilized using the same sperm pool were analyzed statistically (n.s., not significant differences; *$p < 0.01$). Ratio of viable trochophores in different females of *P. aspera* **(A, B)**: non-alkaline treatment **(A)** and alkaline treatment **(B)**. Ratio of viable trochophores in different females of *P. candei* **(C, D)**: non-alkaline treatment **(C)** and alkaline treatment **(D)**.

increased the ratio of viable trochophores in 55% of the specimens in *P. aspera* (**Figures 6A, B**) and 81% of the specimens in *P. candei* (**Figures 6C, D**). The sperm pool cannot explain such variability because significant differences occurred in the majority of the pairwise comparisons between females fertilized with the same sperm pool: 65% in *P. aspera* (**Figures 6A, B**) and 75% in *P. candei* when using alkaline treatment (**Figure 6D**). Altogether, these results support that female quality is a key factor to be considered for obtaining good larval production yields.

Alkaline Agent (NaOH vs. NH_4OH)

Ammonium hydroxide (NH_4OH) has been used as an alkaline agent in several limpet species, showing greater larval production yields while requiring shorter application times than sodium hydroxide (NaOH) (Wanninger et al., 1999; Gould et al., 2001; Hodgson et al., 2007; Pérez et al., 2016; Seabra et al., 2019; Nakano et al., 2020). The effectiveness of NH_4OH to produce viable trochophores in *P. aspera* was studied (assays 7–8). The optimal treatment was NH_4OH at pH 8.9 for 10 min, which was not significantly different from the highest value (**Figure 7**;

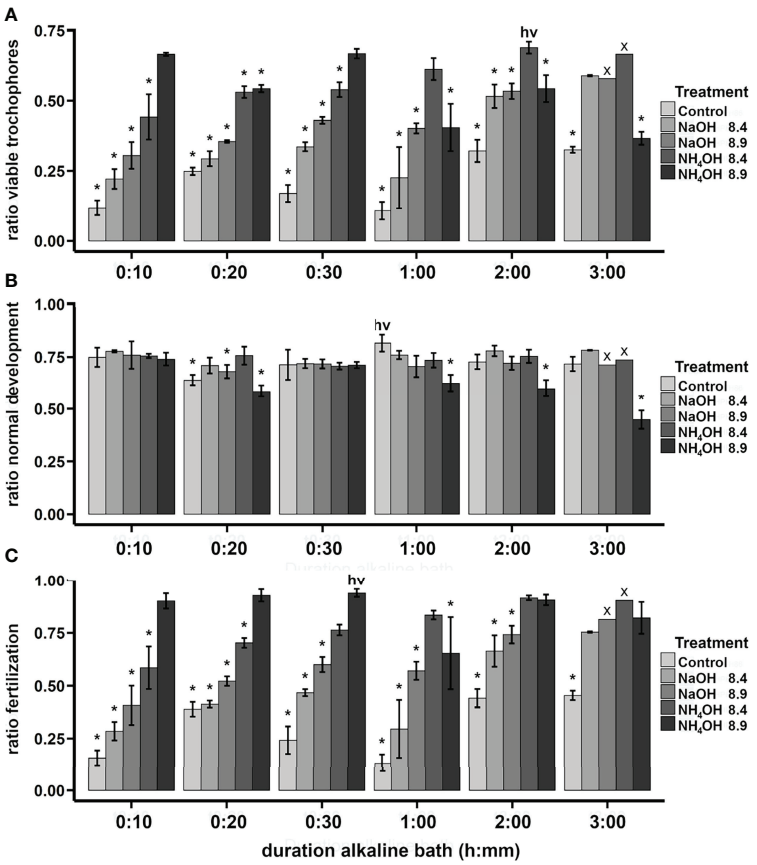

FIGURE 7 | *P. aspera*. First test to study the influence of different alkaline baths on the larval production. Treatments combined different alkaline agents (NaOH and NH_4OH), pH (8.4 and 8.9), and bath durations (10, 20, 30, 60, 120, and 180 min), plus negative control treatments. Bars indicate average ± SD. Asterisks indicate significant differences ($p < 0.05$) with the treatment with highest value (hv). Marked treatments (x) were excluded from the analyses due to the lack of replicates. Ratio of viable trochophores **(A)**, ratio of normal development **(B)**, and ratio of fertilization **(C)**.

Supplementary Figure 1). NH_4OH at pH 8.9 should not be used longer than 30 min because it showed adverse effects on larval production (**Figures 7A, B**; **Supplementary Figures 1A, B**).

Sperm Concentration

The optimal sperm concentration for larval production (as a ratio of viable trochophores) was tested on *P. aspera* (assays 9–12). The optimal sperm concentration was 10^5 sperm cells ml^{-1} (**Figure 8A**). The ratio of normal development usually decreased with higher sperm concentrations (**Figure 8B**), while the ratio of fertilization decreased with lower sperm concentrations (**Figure 8C**). Similar results were described in *P. ferruginea* (Guallart et al., 2020b).

Larval Development and Larviculture

The larval development of *P. aspera* and *P. candei* is similar, in terms of both larval morphology and developmental time (**Figure 9**), except for the body coloration, which resembles that of the oocytes (**Figures 5O–S**). Incubation and larval culture were performed at 17 ± 1°C, and trochophores were observed 17 h post-fertilization. Trochophores showed an oval shape, an apical tuft of cilia, and prototroch cilia (**Figures 9A–C**). The veliger stage was observed 48 h post-fertilization, showing an oval velum and globular protoconch (**Figures 9D–F**). The pediveliger (last larval stage) was observed 72 h post-fertilization, showing eyespots, cephalic tentacles, foot, and operculum (**Figures 9G–I**). The larval morphology of *P. aspera* and *P. candei* and the timing of development resemble those described in other limpet species, e.g., *Lottia asmi* (Kay and Emlet, 2002), *L. digitalis* (Kay and Emlet, 2002), *L. persona* (Kolbin and Kulikova, 2011), *L. tenuisculpta* (Nakano et al., 2020), *Patella depressa* (Ribeiro, 2008), *P. ferruginea* (Guallart et al., 2020b; Ferranti et al., 2022), *P. ulyssiponensis* (Ribeiro, 2008), and *P. vulgata* (Aquino De Souza et al., 2009).

Larviculture Methodology

Larval culture of both *P. aspera* and *P. candei* started with trochophores within 24 ± 2 h post-fertilization. The trochophores were found to generally swarm near the water surface (**Figures 2A, B**; **Video 2**), allowing for siphoning using a plastic tube (4 mm diameter). The siphon output was released over a 55-µm mesh to capture the trochophores, and it is

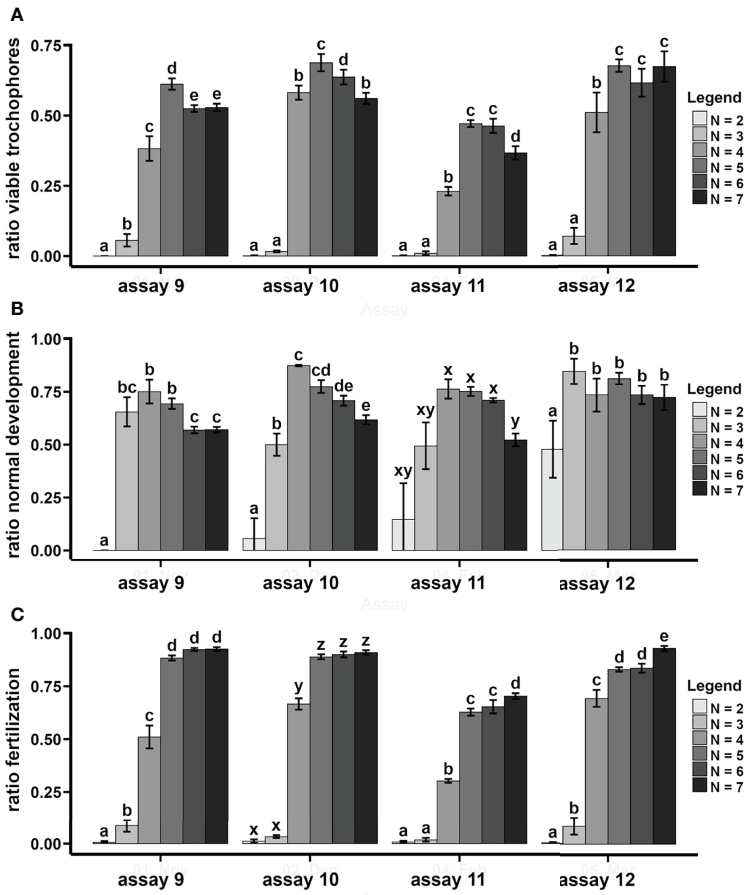

FIGURE 8 | *P. aspera*. Influence of the sperm concentration on the larval production. Treatments were based on logarithmic increments on the sperm concentration: 10^N sperm cells ml^{-1}; *N* = 2, 3, 4, 5, 6 and 7. Bars indicate average ± SD. Different letters indicate significant differences among treatments ($p < 0.05$). Ratio of viable trochophores **(A)**, ratio of normal development **(B)**, and ratio of fertilization **(C)**.

important to keep the mesh partially immersed to minimize the air exposure of the larvae (**Figure 2C**; **Video 3**). Siphoning the bottom of the culture containers should be avoided, as it is composed of debris, abnormal larvae, and unfertilized oocytes. In this sense, good results were obtained by siphoning the first 400 ml of seawater from beakers filled with 500 ml of seawater.

The same type of containers used for the alkaline treatment and the incubation were used successfully for larviculture during several trials. Previous studies used larval densities ranging from 1 larvae ml^{-1} (Ribeiro, 2008), 3–7 larvae ml^{-1} (Seabra et al., 2019), to 10–15 larvae ml^{-1} (Mau et al., 2018; Castejón et al., 2022; Ferranti et al., 2022). Nevertheless, particularly good results were obtained when using an initial density of approximately 5 larvae ml^{-1}. The conditions for the larval culture were as follows: filtered seawater sterilized by ultraviolet or autoclaved (acronym: FSS), no aeration or water circulation (static), temperature ranging from 15 to 18°C, and natural salinity (36 ± 1 g L^{-1}). Some authors have tried feeding limpet larvae to increase their survival and to test whether it was essential to feed them (Dodd, 1957; Ribeiro, 2008; Ferranti et al., 2018; Nhan and Ako, 2019). However, *P. aspera* and *P. candei* larvae were not fed, as they are probably lecitotrophic animals (Castejón et al., 2022), as in other limpet species (Seabra et al., 2019; Guallart et al., 2020b). The trochophores placed in the larviculture conditions previously described reached the pediveliger stage at 48 h (equivalent to 72 h post-fertilization). The pediveligers swim in the water column, so the same procedure used to collect the trochophores (siphoning and gathering using a 55-μm mesh) can be employed. It is recommended to collect the pediveligers at Day 3 post-fertilization because the crawling behavior of older larvae would interfere with the efficiency of the present methodology.

The present larviculture protocol shares certain similarities with previous protocols. In *P. ferruginea*, the trochophores were transferred (by siphoning or decantation) to different vessels, providing clean seawater for culture (Kay and Emlet, 2002; Ferranti et al., 2018; Guallart et al., 2020b; Ferranti et al., 2022). Some authors used a mesh to transfer the larvae to the culture containers (Ribeiro, 2008; Ferranti et al., 2018), while other authors preferred to remove the debris of the bottom instead of the larvae (Nakano et al., 2020). The water for larviculture can be exchanged routinely for maintenance

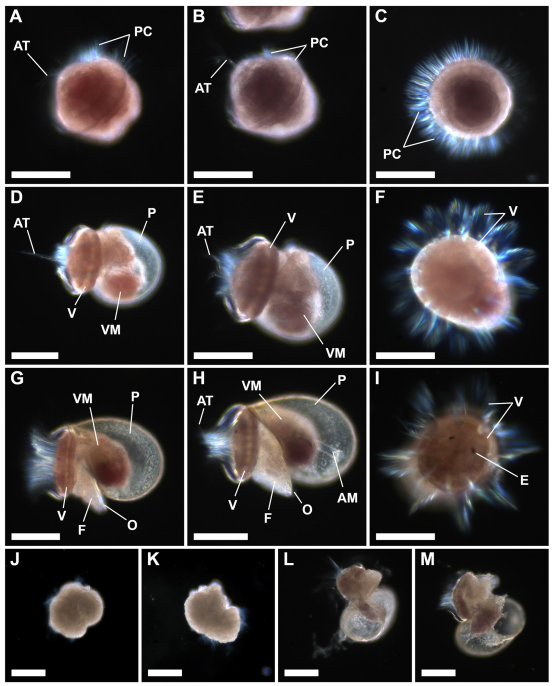

FIGURE 9 | Larval development of the limpet species *P. aspera* and *P. candei*. Trochophore stage ca. 17–24 h post-fertilization **(A–C)**: *P. aspera* in lateral view **(A)**, *P. candei* in lateral view **(B)**, and *P. candei* in frontal view **(C)**. Veliger stage ca. 40–48 h post-fertilization **(D–F)**: *P. aspera* in lateral view **(D)**, *P. candei* in lateral view **(E)**, and *P. aspera* in frontal view **(F)**. Pediveliger stage ca. 72 h post-fertilization **(G–I)**: *P. aspera* in lateral view **(G)**, *P. candei* in lateral view **(H)**, and *P. aspera* in frontal view **(I)**. Deformed trochophores **(J, K)**. Deformed pediveligers **(L, M)**. Scale bar = 100 µm. AM, abductor muscles; AT, apical tuft; E, eyespot; P, protoconch; PC, prototroch cilia; V, velum; VM, visceral mass.

(Ribeiro, 2008; Ferranti et al., 2018; Seabra et al., 2019); this step was not required for our research. The use of antibiotics such as streptomycin and penicillin to prevent fungal and microbial infections has been used and proposed (Wanninger et al., 1999), but larval development was completed successfully without using them.

Validation of the Larviculture Protocol

The present larviculture protocol has been used successfully for obtaining limpets larvae ready to settle and metamorphose (Castejón et al., 2022). Regardless of this good result, *P. aspera* was used as a model to test its reliability in terms of production yields.

Assay 13 showed that the final ratio of viable pediveligers was similar to the initial ratio of viable trochophores, with little or no variation between the initial and final larval densities (**Table 2**). Moreover, the final ratio of viable pediveligers did not vary with the original treatment of the oocytes (control: 0.71 ± 0.03; alkaline: 0.76 ± 0.03; *t*-test $p = 0.06$).

Assays 14–15 showed a significantly increased ratio of viable trochophores when alkaline treatment was applied (assay 14: control = 0.28 ± 0.05, alkaline = 0.72 ± 0.02, *t*-test $p < 0.001$; assay 15: control = 0.11 ± 0.01, alkaline = 0.35 ± 0.08, *U*-test $p = 0.03$), as reported in previous studies (Cañizares et al., 2021; Castejón et al., 2021). Regarding the efficiency of the siphoning method, the ratio of collected trochophores relative to the initial quantity of oocytes was greater when alkaline treatment was applied (assay 14: control = 0.31 ± 0.03, alkaline = 0.50 ± 0.08, $p = 0.04$; assay 15: control = 0.11 ± 0.03, alkaline = 0.36 ± 0.05, *t*-test $p < 0.001$).

In assays 14–15, the larviculture in plastic cups obtained similar results to that described in assay 13. The ratio of viable larvae was similar between the starting trochophores and the final pediveligers, without negative influence by the treatment of

TABLE 2 | Validation of the larviculture protocol.

	Initial ratio viable trochophores	Final ratio viable pediveligers	Statistics
pH N	0.77 ± 0.03	0.71 ± 0.03	$p = 0.06$
pH A	0.74 ± 0.05	0.76 ± 0.03	$p = 0.50$
	Initial density of trochophores	Final density of pediveligers	Statistics
pH N	4.1 ± 1.0	4.2 ± 0.5	$p = 0.85$
pH A	3.5 ± 0.4	4.4 ± 0.3	$p = 0.01$*
Assay 14			
	Initial ratio viable trochophores	Final ratio viable pediveligers	Statistics
pH N	0.86 ± 0.04	0.82 ± 0.03	$p = 0.14$
pH A	0.92 ± 0.02	0.86 ± 0.03	$p = 0.01$*
	Initial density of trochophores	Final density of pediveligers	Statistics
pH N	5.6 ± 0.4	5.4 ± 0.2	$p = 0.32$
pH A	4.3 ± 0.7	4.2 ± 0.5	$p = 0.69$
Assay 15			
	Initial ratio viable trochophores	Final ratio viable pediveligers	Statistics
pH N	0.89 ± 0.01	0.92 ± 0.02	$p = 0.04$*
pH A	0.89 ± 0.02	0.92 ± 0.02	$p = 0.11$
	Initial density of trochophores	Final density of pediveligers	Statistics
pH N	5.1 ± 0.5	4.7 ± 0.5	$p = 0.32$
pH A	4.4 ± 0.5	4.9 ± 1.2	$p = 0.49$

Analysis of the larval development in plastic cups (80 ml). Results are shown as average ± SD. Treatments were control treatment (pH N) and alkaline treatment (pH A; assay 13: NaOH, pH 8.9, 3 h; assays 14–15: NH_4OH, pH 9, 10 min). Statistical analyses realized using t*-test. Asterisks indicate significant differences ($p < 0.05$).*

the oocytes (assay 14: *t*-test $p = 0.07$; assay 15: *t*-test $p = 0.77$; **Table 2**). Regarding the reliability of the glass beakers for the larval culture, the ratio of collected pediveligers relative to the initial quantity of trochophores was similar between treatments (assay 14: control = 0.64 ± 0.14, alkaline = 0.67 ± 0.15, *t*-test $p = 0.82$; assay 15: control = 0.41 ± 0.11, alkaline = 0.32 ± 0.08, *t*-test $p = 0.22$). Moreover, in assay 15, the quality of the pediveligers collected from the water column was greater than those from the bottom (control: column = 1.00 ± 0.01, bottom = 0.84 ± 0.03, *t*-test $p < 0.001$; alkaline: column = 0.99 ± 0.01, bottom = 0.77 ± 0.12, *t*-test $p = 0.03$).

Several conclusions can be extrapolated from these assays. The morphology is a valid criterion to recognize the viability of the trochophores. The reduced variation in larval density during culture suggests minimal mortality, as reported in *Cellana exarata* (Corpuz, 1981). The alkaline bath has no "carry over" effects on the resulting trochophores, supporting the safety of this technique to increase larval production. The limpets' larvae might also be highly resilient, being able to tolerate several management techniques, including siphoning, filtering, pipetting, stirring, and resuspension. The first factor affecting larval production was female fertility, as it influences the production of viable trochophores. The second factor was the pediveliger behavior, as the quantity of swimming pediveligers influenced the efficiency of the siphoning. For laboratory studies, the stimulation of swimming in pediveligers would be useful for better production yields, i.e., the pediveligers from the water column showed a better quality.

Settlement and Metamorphosis in Limpets

Settlement and recruitment are key processes in which the planktonic larvae of benthic species must find a suitable site on the benthos to settle and metamorphose to the first post-larval stage (Rodríguez et al., 1993; Jenkins et al., 2009). The loss of the velum, the pediveliger swimming organ, has been used to define the onset of metamorphosis in several marine gastropods (McGee and Targett, 1989; Inestrosa et al., 1992; Searcy-Bernal et al., 1992; Davis, 1994; Roberts and Nicholson, 1997; Gallardo and Sánchez, 2001; Zhao and Qian, 2002; Salas-Garza et al., 2009; Courtois de Vicose et al., 2010), including different limpet species, e.g., *P. aspera* (Castejón et al., 2022), *P. caerulea* (Dodd, 1957; Wanninger et al., 1999), *P. ferruginea* (Guallart et al., 2020b; Ferranti et al., 2022), and *P. vulgata* (Dodd, 1957; Wanninger et al., 1999). In limpets, teleoconches are another key characteristic whose presence is restricted to post-larvae (Kay and Emlet, 2002; Ferranti et al., 2018; Nakano et al., 2020; Castejón et al., 2022; Ferranti et al., 2022).

P. aspera and *P. candei* showed morphological and behavioral similarities during the settlement and metamorphic processes (**Figure 10**). The pediveligers showed several behaviors: swimming propelled by the velum cilia (**Figures 10 A, E**; **Video 4**), crawling using the foot (**Figures 10B, F**; **Video 5**), withdrawal inside the shell and enclosed by the operculum (**Figures 10C, G**), and resting inside the shell while supported by the foot (**Figures 10D, H**). The early post-larvae is the first post-larval and post-metamorphic stage (Castejón et al., 2022), identified by the loss of the velum and the operculum, and commonly found resting over different surfaces (**Figures 10I, J, L, M**; **Video 6**). The sooner settlement-competency might occur approximately 6 days post-fertilization, since early post-larvae were rarely observed sooner (Castejón et al., 2022). The morphology and behavior of the early post-larvae suggest a distinctive stage involving transformative changes for benthic life, including the development of the digestive structures required to ingest and digest particulate food (Castejón et al., 2022). The next post-larval stage is the juvenile stage (post-larvae with teleoconches), which is reported to be ca. 72 h post-settlement. The juveniles have a wider head, teleoconch, and marked digestive system (**Figures 10K, N**) (Castejón et al., 2022). The juveniles, as active grazers (**Videos 7–8**), represent the starting point for the grow-out phase.

Traditionally, the settlement process in limpets has been considered a bottleneck associated with high mortality (Corpuz, 1981), with little or no knowledge regarding their requirements and associated settlement cues (Mau and Jha, 2018). Considering the fundamental role of settlement and metamorphosis in the successful development of limpets

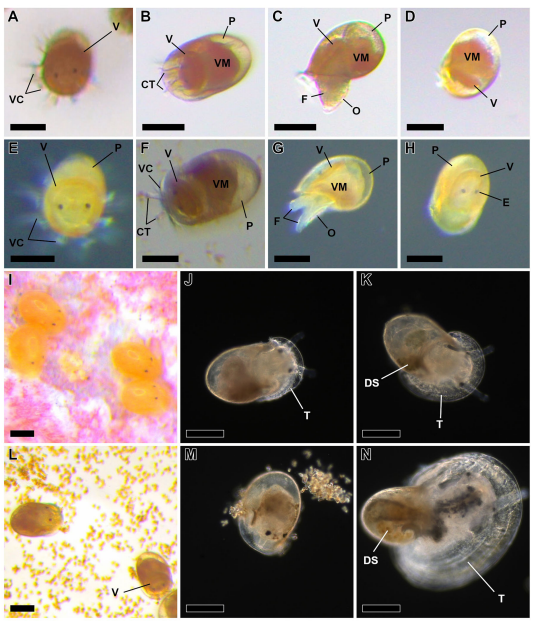

FIGURE 10 | Settlement and metamorphosis in the limpet species *P. aspera* and *P. candei*. Key characters for the identification of the pediveligers and typical behaviors of this stage **(A–H)**. *P. aspera* 4 days post-fertilization **(A–D)**: swimming **(A)**, crawling **(B)**, withdraw **(C)**, and resting **(D)**. *P. candei* 6 days post-fertilization **(E–H)**: swimming **(E)**, crawling **(F)**, withdraw **(G)**, and resting **(H)**. Comparison between the pediveliger and the post-larval stages **(I–N)**. *P. aspera* **(I–K)**: group of early post-larvae settled on encrusting coralline algae, 7 days post-fertilization **(I)**; early post-larvae, 8 days post-fertilization **(J)**; juvenile, 15 days post-fertilization **(K)**. *P. candei* **(L–N)**: early post-larvae (left) and crawling pediveliger (right) on *Navicula incerta* biofilm, 7 days post-fertilization **(L)**; early post-larvae, 39 days post-fertilization **(M)**; juvenile, 39 days post-fertilization **(N)**. Scale bar = 100 μm. CT, cephalic tentacle; DS, digestive system; E, eyespot; F, foot; O, operculum; P, protoconch; T, teleoconch; V, velum; VC, velum cilia; VM, visceral mass.

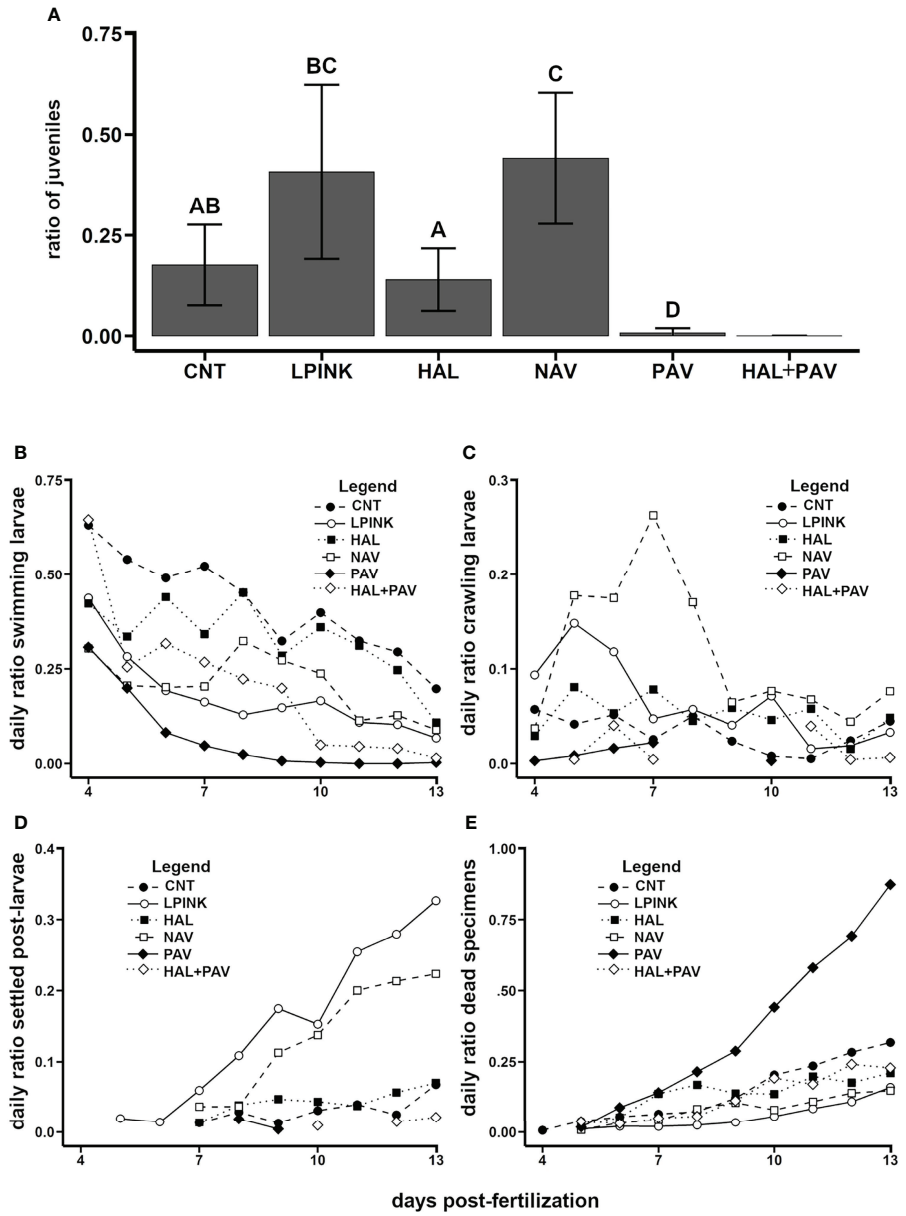

FIGURE 11 | *P. candei*. Influence of different cultured algal strains and encrusting coralline algae on the settlement (study realized in Madeira). Ratio of juveniles obtained at the end of the assay, bars indicate average ± SD and different letters indicate significant differences ($p < 0.01$) **(A)**. Daily average ratio: swimming **(B)** and crawling pediveligers **(C)**, settled specimens (early post-larvae + juveniles) **(D)** and dead specimens (empty shells) **(E)**. CNT, negative control treatment; HAL, diatom *Halamphora coffeaeformis* biofilm; HAL+PAV, combination of the treatments HAL and PAV; LPINK, coralline algae formed by light pink crusts; NAV, diatom *Navicula incerta* biofilm; PAV, free swimming algae *Pavlova* sp.

aquaculture, the search for adequate settlement inducers was a major objective of the present research.

Settlement and Metamorphosis in *P. aspera*

Castejón et al. (2022) studied the suitability of several substrates as settlement inducers for *P. aspera* larvae. The results showed that encrusting coralline algae (CCA) of the order Corallinales shortened the timing for the earliest settlers, increased the daily number of settled specimens, and increased the final ratio of juveniles. In contrast, the same algal strains used for assay 16 did not show any influence on the settlement.

Settlement and Metamorphosis in *P. candei*

The results obtained in CMC (Madeira; assay 16) showed that *N. incerta* biofilms significantly promoted a greater ratio of juveniles (**Figure 11A**), reduced swimming activity (**Figure 11B**), peaked

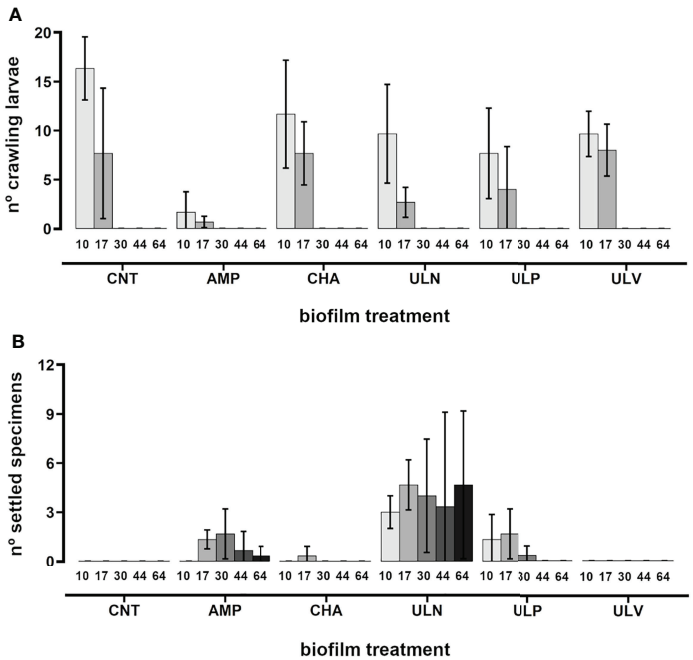

FIGURE 12 | *P. candei*. Influence of different cultured algal strains on the settlement (study realized in Azores). Pediveliger larvae (no. of crawling pediveligers **(A)** and metamorphosed post-larvae and juveniles (no. of settled specimens **(B)** recorded during different days post-fertilization (10, 17, 30, 44, and 64 days) on different cultured algal biofilms. Bars indicate average ± SD. AMP, diatom *Amphora* sp.; CHA, diatom *Chaetoceros* sp.; CNT, negative control treatment; ULN, chlorophyte *Ulvella lens*; ULP, chlorophyte *Ulvella leptochaete*; ULV, chlorophyte *Ulva* sp.

crawling activity (**Figure 11C**), and increased the ratio of settlers over time (**Figure 11D**). The CCA showed the second highest value for juveniles, but it was not significantly different from the control (**Figure 11A**). The CCA also reduced swimming activity (**Figure 11B**) and increased the ratio of settlers over time (**Figure 11D**). Moreover, the treatments of N. incerta biofilms and CCA showed a low mortality (**Figure 11E**). Overall, these results showed that biofilms of *N. incerta* can be useful as settlement substrates for the limpet *P. candei*, while the encrusting coralline algae showed promising potential to be elucidated in future studies.

The results obtained in AquaLab (Azores; assay 17) reported a crawling phase extended up to Day 17 post-fertilization (**Figure 12A**), in which the pediveligers were recorded on all biofilms showing an exploratory behavior with no evident preference. Settlement and metamorphosis occurred between 17 and 30 days post-fertilization (**Figure 12B**), similar to the results reported in *P. ferruginea* when using cultured algae as a settlement substrate (Ferranti et al., 2022). The juveniles were recorded only on plates covered with *Amphora* sp. (1.7 ± 1.5 specimens), *Ulvella lens* (4.0 ± 3.5 specimens), and *Ulvella leptochaete* (0.3 ± 0.6 specimens) (**Figure 12B**; Video 8). After 2 months (64 days post-fertilization), the juveniles ($n = 14$) increased greatly in size (shell size length = 1.76 ± 0.46 mm), being observed exclusively on plates with *U. lens* (**Figures 12B**, **13C**).

Altogether, these results reveal important information for future studies focused on the settlement of different limpet species. Limpets' larvae originally obtained from spawning induction (Nunes et al., 2021) or alkalinized oocytes (Castejón et al., 2022) develop successfully, reaching post-larval stages. The settlement response was triggered in *P. aspera* exclusively by encrusting coralline algae, whose effectiveness is influenced by the following factors: species, area coverage, and health status (Castejón et al., 2022). The lack of one or more of these factors could explain the unclear effectiveness of the CCA as a settlement inducer in *P. candei*. The importance of the Corallinales cannot be neglected, since this algal group can trigger mass settlement events (Video 6) and are recognized settlement inducers for several limpet species (Ribeiro, 2008) and other marine gastropods (Roberts and Nicholson, 1997; Daume et al., 1999; Moss, 1999; Roberts et al., 2004). In *P. candei*, the settlement response was triggered by biofilms formed by the diatom *N. incerta* and the chlorophyte *U. lens*. Such results are promising, as they are fast-growing algae that are easy to culture. Moreover, the post-larvae cultured with biofilms of *U. lens* biofilms reported fast growth, as they are tenfold the shell length in 2 months, implying the possibility of using this alga as an effective feeding source for the development of grow-out culture systems for limpets.

Management of the Post-Larvae and Grow-Out

The scientific literature provides scarce information about the management of the early post-larvae and juveniles of limpets, partly due to the limited success reaching these stages (Ferranti et al., 2018; Mau and Jha, 2018; Guallart et al., 2020b; Ferranti

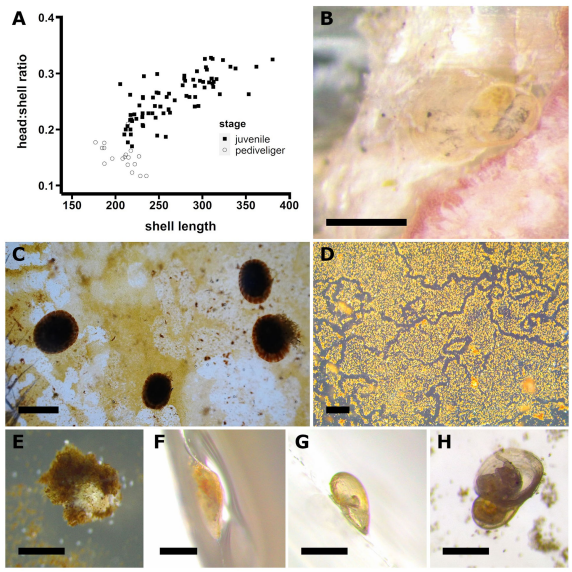

FIGURE 13 | Post-metamorphic morphologic and behavioral changes observed in limpets. Variation of the head:shell ratio in relationship with the animal growth in *P. aspera*; head size was measured as eye distance relative to the shell length and shell length was measured as: protoconch + teleoconch **(A)**. *P. aspera* juvenile 10 days post-settlement showing body pigmentation **(B)**. *P. candei* juveniles around 2 months post-settlement showing shell pigmentation and grazing behavior on *Ulvella lens* biofilm **(C)**. Grazing marks left by the juveniles of *P. candei* on *Navicula incerta* biofilm, 7 days post-settlement **(D)**. *P. aspera* juvenile with the shell totally covered by surrounding environmental elements, ca. 7 weeks post-settlement **(E)**. *P. candei* juvenile placed vertically and reaching the water level **(F)**. *P. candei* juvenile desiccated due to stay above the water level **(G)**. *P. aspera* juvenile accidentally upturned, 1 month post-settlement **(H)**. Scale bar = 200 µm **(B, F–H)**, 500 µm **(E)**, and 1 mm **(C, D)**.

et al., 2022). This section resumes the major findings obtained during the present research in relation to the management of the post-larvae using *P. aspera* and *P. candei* as model species, which could be helpful for the development of improved protocols.

Post-metamorphic development includes several morphological changes. The head widening is proportionally greater than the animal growth in length (**Figures 10I–N** and **13A**). The teleoconch starts as a thin line (**Figure 10J**), which gradually encloses posteriorly as a plane shield surrounding the animal body (**Figures 10K, N**). Body pigmentation was reported approximately 10 days post-settlement (**Figure 13B**), and shell pigmentation was reported 2 months post-settlement (**Figure 13C**). The timing for those developmental changes is more affected by the time after the settlement rather than animal age, i.e., specimens with the same post-fertilization age can show extreme differences if metamorphosis is delayed over time (**Figures 10M, N**).

The juveniles of *P. aspera* and *P. candei* showed features and behaviors to be considered for either production or experimental purposes. The widening of the head mentioned before (**Figure 13A**) probably implies the development of the radula and other mouth structures required to actively graze the surroundings, which include shedding of encrusting coralline algae (Castejón et al., 2022) and cultured biofilms (**Figures 13C, D**; **Videos 7–8**), suggesting non-selective grazing, as observed in adults (Branch, 1971; Della Santina et al., 1993; Burgos-Rubio et al., 2015). Several specimens were found to be partially or totally covered by different environmental elements resembling a cryptic strategy to avoid predation (**Figure 13E**; **Video 7**). The juvenile activity was restricted to a 2D environment where the specimens attach tightly and firmly to the surface below. The juveniles showed special affinity for the vertical surfaces, which increases the risk of mortality by desiccation (**Figures 13F, G**).

Direct manipulation of juveniles is not recommended since no reliable methods for animal detachment are available. Moreover, juveniles lying on the back of their shell are unable to recover their normal position (**Figure 13H**; **Video 9**). If individual management is needed, selective pipetting (using a plastic pipette with the tip cut) showed limited success on already detached specimens (**Video 10**). The juveniles attach firmly to the surface when threatened, so the optimal solution would be to move the surfaces instead of the specimens themselves.

Petri Dishes as Culture Containers for Limpet Post-Larvae (Assay 18)

In *P. candei*, approximately 1 month after the start of the assay (Day 30 post-fertilization), the ratio of juveniles was 0.37 ± 0.04, corresponding to 111 juveniles distributed on four Petri dishes. Swimming pediveligers and early post-larvae were reported as well. This observation suggests that limpets can maintain the pediveliger stage for a long time without disabling metamorphosis, as reported by Ferranti et al. (2022), which would allow long dispersal in wild conditions. More than 80% of the juveniles were observed over the vertical wall of the Petri dishes, limiting the possibilities to measure the animal growth (**Figure 13F**) and increasing the mortality by desiccation (**Figure 13G**). The assay was terminated because the biofilm destabilized without recovery. In conclusion, we do not recommend the Petri dishes as culture containers for limpet post-larvae. Alternatively, the Petri dishes have been used as additional surfaces placed inside larger culture containers (Guallart et al., 2017; Guallart et al., 2020b; Ferranti et al., 2022). The use of vertical plates in culture tanks is an alternative to be tested.

Although the same algal culture and management conditions were used for *P. aspera* and *P. candei*, *P. aspera* did not show any settlement response in *N. incerta* biofilms, coinciding with a previous study (Castejón et al., 2022).

Challenges and Future Goals

Traditionally, several bottlenecks were present in limpets when considering adult management, gamete release, larviculture and settlement, and metamorphosis induction. The present manuscript represents a significant advance in all these fields, marking a starting point for the aquaculture of different limpet species. Several methodologies were tested, adapted, and optimized for the native species of the Macaronesia region, *P. aspera* and *P. candei*, i.e., a methodology for experimental larval production is presented, establishes a larviculture protocol, presents successful settlement inducers, and shows the management and feeding requirements of the post-metamorphic stages. In a few years, it might be possible to close the life cycle of different limpet species and establish large-scale production.

However, researchers have a profuse field to be explored. We acknowledge those authors who previously worked hard on the subject and highlighted many of the specializations and limpets' requirements. The spawning methods should be polished and optimized; in this sense, the studies realized in *P. ferruginea* show potential, and the use of hormonal injections should be deeply studied. The determination of female fertility would be important to determine oocyte quality and expected larval productivity. Larviculture methods need to be expanded to allow reliable large-scale production of larvae. The settlement and the grow-out of juveniles are fully experimental, requiring more research before reaching large-scale production levels. In this sense, some of the techniques used for abalone could be adapted for limpets.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon reasonable request.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

AUTHOR CONTRIBUTIONS

DC: animal management, experimental design, experiment realization, sampling, analysis of samples, statistical analyses, pictures design, and drafted paper. MG: animal management, experiment realization, sampling, analysis of samples, and drafted paper review. JC: animal management, experimental design, sampling, analysis of samples, and drafted paper review. CN: animal management, experiment realization, sampling, analysis of samples, and drafted paper review. MG: animal management, experimental design, experiment realization, sampling, analysis of samples, statistical analyses, pictures design, drafted paper review, and project elaboration. EI: drafted paper review, project elaboration, coordination, and direction. GV: drafted paper review, project elaboration, coordination, and direction. NN: drafted paper review and project elaboration. CA: drafted paper review, project elaboration, coordination, and direction. All authors contributed to the article and approved the submitted version.

FUNDING

Financial support was provided by the projects: 1) "AQUAINVERT - Development of sustainable, integrated and innovative aquaculture in Macaronesia. Research and Development to promote the production of marine invertebrates of commercial interest (MAC/1. 1ª/282)" founded by FEDER under the INTERREG MAC 2014-2020 program; and 2) "ISLANDAP ADVANCED - R+D+I towards aquaponic development in the up islands and the circular economy. Interregional Forward Challenges (MAC2/1.1a/299)". DC was supported by a grant under the project AQUAINVERT.

ACKNOWLEDGMENTS

The sampling of limpets for scientific purposes during closed season was authorized by the Fisheries Directorate, Secretary for the Sea and Fisheries, Regional Government of Madeira. The authors would like to thank the technicians at the Mariculture Center of Calheta (CMC) and at the Experimental Laboratory of Aquaculture (AquaLab) for their assistance. The authors would like to thank the local fishermen of Calheta for helping provide the specimens. The authors would also like to thank Alfonso A. Ramos, Francisca Giménez, Carmen Barberá, and Andrés Izquierdo-Muñoz, for the opportunity to use the installations of the Research Marine Centre in Santa Pola (Spain).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.884262/full#supplementary-material

Supplementary Figure 1 | P. *aspera*. Second test to study the influence of different alkaline baths on the larval production. Treatments combined different alkaline agents (NaOH and NH_4OH), pH (8.4 and 8.9), and bath durations (10, 20, 30, 60, 120, and 180 min), plus negative control treatments. Bars indicate average ± SD. Asterisks indicate significant differences ($p < 0.05$) with the treatment with highest value (hv). Ratio of viable trochophores **(A)**, ratio of normal development **(B)**, and ratio of fertilization **(C)**.

REFERENCES

Aquino De Souza, R., Tyler, P., and Hawkins, S. J. (2009). Artificial Oocyte Maturation in *Patella Depressa* and *Patella Vulgata* Using NaOH-Alkalinized Seawater. *Mar. Biol. Res.* 5 (5), 503–510. doi: 10.1080/17451000802603645

Benedetti-Cecchi, L. (2000). Predicting Direct and Indirect Interactions During Succession in a Mid-Littoral Rocky Shore Assemblage. *Ecol. Monogr.* 70 (1), 45–72. doi: 10.1890/0012-9615(2000)070[0045:PDAIID]2.0.CO;2

Blackmore, D. (1969). Studies of *Patella Vulgata* L. I. Growth, Reproduction and Zonal Distribution. *J. Exp. Mar. Biol.* 3 (2), 200–213. doi: 10.1016/0022-0981(69)90018-5

Branch, G. M. (1971). The Ecology of *Patella* Linnaeus From the Cape Peninsula, South Africa I. Zonation, Movements and Feeding. *Afr. Zoology* 6 (1), 1–38. doi: 10.1080/00445096.1971.11447402

Branch, G. M. (1975). Notes on the Ecology of *Patella Concolor* and *Cellana Capensis*, and the Effects of Human Consumption on Limpet Populations. *Afr. Zoology* 10 (1), 75–85. doi: 10.1080/00445096.1975.11447494

Branch, G. M. (1981). The Biology of Limpets: Physical Factors, Energy Flow, and Ecological Interactions. *Oceanog. Mar. Biol.: Annu. Rev.* 192, 235–280.

Branch, G. M. (1985). "Limpets: Evolution and Adaptation," in *The Mollusca*. Eds. E. Trueman and M. Clarke (New York: Academic Press, Inc), 187–220.

Branch, G., and Odendaal, F. (2003). The Effects of Marine Protected Areas on the Population Dynamics of a South African Limpet, *Cymbula Oculus*, Relative to

the Influence of Wave Action. *Biol. Conserv.* 114 (2), 255–269. doi: 10.1016/S0006-3207(03)00045-4

Bulleri, F., Menconi, M., Cinelli, F., and Benedetti-Cecchi, L. (2000). Grazing by Two Species of Limpets on Artificial Reefs in the Northwest Mediterranean. *J. Exp. Mar. Biol. Ecol.* 255 (1), 1–19. doi: 10.1016/S0022-0981(00)00275-6

Burgos-Rubio, V., de la Rosa, J., Altamirano, M., and Espinosa, F. (2015). The Role of Patellid Limpets as Omnivorous Grazers: A New Insight Into Intertidal Ecology. *Mar. Biol.* 162 (10), 2093–2106. doi: 10.1007/s00227-015-2739-0

Cañizares, J. M., Castejón, D., Haroun, R., Nogueira, N., and Andrade, C. A. P. (2021). Enhancing Oocyte Maturation and Fertilisation in the Black-Foot Limpet *Patella Candei* D'Orbigny 1840 (Patellidae, Mollusca). *Aquacul. Rep.* 21, 100856. doi: 10.1016/j.aqrep.2021.100856

Carballo, J. L., Yáñez, B., Bautista-Guerrero, E., García-Gómez, J. C., Espinosa, F., Tortolero-Langarica, J. J., et al. (2020). Decimation of a Population of the Endangered Species *Scutellastra Mexicana* (Broderip and Sowerby 1829) (Mollusca, Gastropoda) in the Marías Island (Eastern Ocean Pacific) Biosphere Reserve. *Aquat. Conservation: Mar. Freshw. Ecosyst.* 30 (1), 20–30. doi: 10.1002/aqc.3239

Castejón, D., Cañizares, J. M., Nogueira, N., and Andrade, C. A. P. (2021). Artificial Maturation and Larval Production of the Limpet *Patella Aspera* Röding 1798(Patellogastropoda, Mollusca): Enhancing Fertilization Success of Oocytes Using NaOH-Alkalinized Seawater. *Aquacul. Res.* 52 (5), 1904–1914. doi: 10.1111/are.15039

Castejón, D., Nogueira, N., and Andrade, C. A. P. (2022). Limpet Larvae (*Patella Aspera* Röding 1798), Obtained by Gonad Dissection and Fecundation *In Vitro*, Settled and Metamorphosed on Crustose Coralline Algae. *J. Mar. Biol. Assoc. UK* 101(7), 991–1002. doi: 10.1017/S0025315421000916

Coleman, R. A., Underwood, A. J., Benedetti-Cecchi, L., Åberg, P., Arenas, F., Arrontes, J., et al. (2006). A Continental Scale Evaluation of the Role of Limpet Grazing on Rocky Shores. *Oecologia* 147 (3), 556–564. doi: 10.1007/s00442-005-0296-9

Colonese, A. C., Mannino, M. A., Mayer, D. B.-Y., Fa, D., Finlayson, J., Lubell, D., et al. (2011). Marine Mollusc Exploitation in Mediterranean Prehistory: An Overview. *Quat. Int.* 239 (1-2), 86–103. doi: 10.1016/j.quaint.2010.09.001

Corpuz, G. C. (1981). Laboratory Culture of *Cellana Exarata* Reeve (Gastropoda: Prosobranchia, Patellidae). *Aquaculture* 24, 219–231. doi: 10.1016/0044-8486(81)90058-2

Courtois de Vicose, G., Viera, M., Bilbao, A., and Izquierdo, M. (2007). Embryonic and Larval Development of *Haliotis Tuberculata Coccinea* Reeve: An Indexed Micro-Photographic Sequence. *J. Shellfish Res.* 26 (3), 847–854. doi: 10.2983/0730-8000(2007)26[847:EALDOH]2.0.CO;2

Courtois de Vicose, G., Viera, M., Bilbao, A., and Izquierdo, M. (2010). Larval Settlement of *Haliotis Tuberculata Coccinea* in Response to Different Inductive Cues and the Effect of Larval Density on Settlement, Early Growth, and Survival. *J. Shellfish Res.* 29 (3), 587–591. doi: 10.2983/035.029.0306

Creese, R., Schiel, D., and Kingsford, M. (1990). Sex Change in a Giant Endemic Limpet, *Patella Kermadecensis*, From the Kermadec Islands. *Mar. Biol.* 104 (3), 419–426. doi: 10.1007/BF01314345

Curdia, J., Rodrigues, A. S., Martins, A., and Costa, M. (2005). The Reproductive Cycle of *Patella Candei Gomesii* Drouët 1858 (Mollusca: Patellogastropoda), an Azorean Endemic Subspecies. *Invertebr. Reprod. Dev.* 48 (1-3), 137–145. doi: 10.1080/07924259.2005.9652180

Daume, S., Brand-Gardner, S., and Woelkerling, W. J. (1999). Preferential Settlement of Abalone Larvae: Diatom Films vs. non-Geniculate Coralline Red Algae. *Aquaculture* 174 (3), 243–254. doi: 10.1016/S0044-8486(99)00003-4

Davies, P. S. (1969). Physiological Ecology of *Patella*. III. Desiccation Effects. *J. Mar. Biol. Assoc. United Kingdom* 49 (2), 291–304. doi: 10.1017/S0025315400035918

Davis, M. (1994). Short-Term Competence in Larvae of Queen Conch *Strombus Gigas*: Shifts in Behavior, Morphology and Metamorphic Response. *Mar. Ecol. Prog. Ser.* 104, 101–101. doi: 10.3354/meps104101

Della Santina, P., Sonni, C., Sartoni, G., and Chelazzi, G. (1993). Food Availability and Diet Composition of Three Coexisting Mediterranean Limpets (*Patella* Spp.). *Mar. Biol.* 116 (1), 87–95. doi: 10.1007/BF00350735

Dethier, M. N. (1994). The Ecology of Intertidal Algal Crusts: Variation Within a Functional Group. *J. Exp. Mar. Biol. Ecol.* 177 (1), 37–71. doi: 10.1016/0022-0981(94)90143-0

Diogo, H., Pereira, J. G., and Schmiing, M. (2016). Catch Me If You can: Non-Compliance of Limpet Protection in the Azores. *Mar. Policy* 63, 92–99. doi: 10.1016/j.marpol.2015.10.007

Dodd, J. M. (1956). Studies on the Biology of Limpets: III. Hermaphroditism in the Three British Species of *Patella*. *J. Mar. Biol. Assoc. United Kingdom* 35 (2), 327–340. doi: 10.1017/S002531540001016X

Dodd, J. M. (1957). Artificial Fertilisation, Larval Development and Metamorphosis in *Patella Vulgata* L. And *Patella Caerulea* L. *Pubblicazioni della Stazione Zoologica di Napoli* 29, 172–185.

Dopchiz, L. P., Ansaldo, M., and Genovese, G. (2018). Gonadal Histology and Gametogenesis of the Antarctic Limpet *Nacella Concinna* (Patellogastropoda, Nacellidae) Collected at Potter Cove, 25 De Mayo (King George) Island, During Austral Summer. *Polar Biol.* 41 (9), 1751–1762. doi: 10.1007/s00300-018-2314-5

Ebert, E. E., and Houk, J. L. (1984). Elements and Innovations in the Cultivation of Red Abalone *Haliotis Rufescens*. *Aquaculture* 39 (1), 375–392. doi: 10.1016/0044-8486(84)90279-5

Erlandson, J. M., Braje, T. J., Rick, T. C., Jew, N. P., Kennett, D. J., Dwyer, N., et al. (2011). 10,000 Years of Human Predation and Size Changes in the Owl Limpet (*Lottia Gigantea*) on San Miguel Island, California. *J. Archaeol. Sci.* 38 (5), 1127–1134. doi: 10.1016/j.jas.2010.12.009

Espinosa, F., Guerra-Garcia, J. M., Fa, D., and Garcia-Gomez, J. C. (2006). Aspects of Reproduction and Their Implications for the Conservation of the Endangered Limpet, *Patella Ferruginea*. *Invertebr. Reprod. Dev.* 49 (1-2), 85–92. doi: 10.1080/07924259.2006.9652197

Espinosa, F., and Rivera-Ingraham, G. A. (2017). "Chapter Three - Biological Conservation of Giant Limpets: The Implications of Large Size," in *Advances in Marine Biology*. Ed. B. E. Curry (United Kingdom & United States: Academic Press), 105–155.

Espinosa, F., Rivera-Ingraham, G., Fa, D., and García-Gómez, J. (2009a). Effect of Human Pressure on Population Size Structures of the Endangered Ferruginean Limpet: Toward Future Management Measures. *J. Coastal Res.* 25(4 (4 (254), 857–863. doi: 10.2112/08-1005.1

Espinosa, F., Rivera-Ingraham, G., and García-Gómez, J. C. (2009b). Gonochorism or Protandrous Hermaphroditism? Evidence of Sex Change in the Endangered Limpet *Patella Ferruginea*. *Mar. Biodivers. Rec.* 2, e153. doi: 10.1017/S1755267209990790

Espinosa, F., Rivera-Ingraham, G. A., Maestre, M., González, A. R., Bazairi, H., and García-Gómez, J. C. (2014). Updated Global Distribution of the Threatened Marine Limpet *Patella Ferruginea* (Gastropoda: Patellidae): An Example of Biodiversity Loss in the Mediterranean. *Oryx* 48 (2), 266–275. doi: 10.1017/S0030605312000580

Fernandes, I., Fernandes, T., and Cordeiro, N. (2019). Nutritional Value and Fatty Acid Profile of Two Wild Edible Limpets From the Madeira Archipelago. *Eur. Food Res. Technol.* 245 (4), 895–905. doi: 10.1007/s00217-019-03234-y

Ferranti, M. P., Guallart, J., Fanciulli, G., Panzalis, P. A., and Chiantore, M. (2022). Advancements Towards Restoration of the Endangered Limpet *Patella Ferruginea* Gmelin 1791 Through Controlled Reproduction. *Aquacul. Res.* 53 (3), 782–798. doi: 10.1111/are.15614

Ferranti, M. P., Monteggia, D., Asnaghi, V., and Chiantore, M. (2018). Artificial Reproduction Protocol, From Spawning to Metamorphosis, Through Noninvasive Methods in *Patella Caerulea* Linnaeus 1758. *Aquacul. Res.* 49 (10), 3386–3391. doi: 10.1111/are.13802

Ferraz, R. R., Menezes, G. M., and Santos, R. S. (2001). Limpet (*Patella* Spp.) (Mollusca: Gastropoda) Exploitation in the Azores, During the Period 1993-1998. *Arquipélago. Life Mar. Sci.* Supplement 2 (Part B), 59–65.

Firth, L. B. (2021). What Have Limpets Ever Done for Us? On the Past and Present Provisioning and Cultural Services of Limpets. *Int. Rev. Environ. Hist.* 7 (2), 5–45. doi: 10.22459/IREH.07.02.2021.01

Gallardo, C. S., and Sánchez, K. A. (2001). Induction of Metamorphosis and its Effect on the Growth and Survival of Postmetamorphic Juveniles of *Chorus Giganteus* (Gastropoda: Muricidae). *Aquaculture* 201 (3-4), 241–250. doi: 10.1016/S0044-8486(01)00611-1

González-Lorenzo, G., Hernández, E. M., Pérez-Dionis, G., Hernández, A. B., Santos, B. G., and Diez, J. B. (2015). Ineffective Conservation Threatens *Patella Candei*, an Endangered Limpet Endemic to the Macaronesian Islands. *Biol. Conserv.* 192, 428–435. doi: 10.1016/j.biocon.2015.11.005

González-Novoa, P. R. (2014). Fecundación in vitro de gametos de lapa. Final Undergraduate Project, Universidade da Coruña.

Gould, M. C., Stephano, J. L., Ortíz-Barrón, B., and Pérez-Quezada, I. (2001). Maturation and Fertilization in Lottia Gigantea Oocytes: Intracellular Ph, Ca2+, and Electrophysiology. *J. Exp. Zoology* 290 (4), 411–420. doi: 10.1002/jez.1082

Guallart, J., Acevedo, I., Calvo Revuelta, M., and Machordom, A. (2013a). Protocolo No Letal Para La Obtención De Muestras De Tejido (Para Estudios Genéticos) En La Lapa Amenazada *Patella Ferruginea* (Mollusca, Patellidae). *Iberus* 31 (2), 171–174.

Guallart, J., Calvo Revuelta, M., Acevedo, I., and Templado, J. (2013b). Two-Way Sex Change in the Endangered Limpet *Patella Ferruginea* (Mollusca, Gastropoda). *Invertebr. Reprod. Dev.* 57, 247–253. doi: 10.1080/07924259.2012.754794

Guallart, J., Ferranti, M. P., Bacigalupo, L., and Chiantore, M. (2020a). *In Vivo* Magnetic Resonance Imaging to Assess the Sexual Maturity of the Endangered Limpet *Patella Ferruginea*. *J. Molluscan Stud.* 86 (4), 422–426. doi: 10.1093/mollus/eyaa021

Guallart, J., Luque, Á.A., Acevedo, I., and Calvo, M. (2013c). Distribución Y Censo Actualizado De La Lapa Ferrugínea (*Patella Ferruginea* Gmelin 1791) En El Litoral De Melilla (Mediterráneo Suroccidental). *Iberus* 31 (1), 21–51.

Guallart, J., Pena, J. B., Luque, A. A., and Templado, J. (2017). Where Have All the Youngest Gone? The Postlarval and Young Stages of the Mediterranean Endangered Limpet *Patella Ferruginea* Gmelin 1791. *Mediterr. Mar. Sci.* 18 (3), 385–392. doi: 10.12681/mms.2076

Guallart, J., Peña, J., Pérez-Larruscaín, J., Luque, Á., and Templado, J. (2020b). Filling Gaps: Closing the Life Cycle of the Endangered Mediterranean Limpet *Patella Ferruginea* Gmelin 1791 (Gastropoda, Patellidae). *Mediterr. Mar. Sci.* 21 (2), 400–419. doi: 10.12681/mms.22508

Guerra-García, J. M., Corzo, J., Espinosa, F., and García-Gómez, J. C. (2004). Assessing Habitat Use of the Endangered Marine Mollusc *Patella Ferruginea* (Gastropoda, Patellidae) in Northern Africa: Preliminary Results and Implications for Conservation. *Biol. Conserv.* 116 (3), 319–326. doi: 10.1016/S0006-3207(03)00201-5

Harada, K., Miyasaki, T., Maeda, H., and Satoh, K. (1997). Statistical Estimation of Probable Feeding Attractive Components in Fishing Baits for Abalone. *Aquacul. Sci.* 45 (4), 533–537. doi: 10.11233/aquaculturesci1953.47.455

Harasewych, M. G., and McArthur, A. (2000). A Molecular Phylogeny of the Patellogastropoda (Mollusca: Gastropoda). *Mar. Biol.* 137 (2), 183–194. doi: 10.1007/s002270000332

Harley, C. D. G., Denny, M. W., Mach, K. J., and Miller, L. P. (2009). Thermal Stress and Morphological Adaptations in Limpets. *Funct. Ecol.* 23 (2), 292–301. doi: 10.1111/j.1365-2435.2008.01496.x

Henriques, P., Delgado, J., Sousa, R., and Ray, S. (2017). "Patellid Limpets: An Overview of the Biology and Conservation of Keystone Species of the Rocky Shores," in *Organismal and Molecular Malacology* (London: Intech Croatia), 71–95.

Henriques, P., Sousa, R., Pinto, A., Delgado, J., Faria, G., Alves, A., et al. (2012). Life History Traits of the Exploited Limpet *Patella Candei* (Mollusca: Patellogastropoda) of the North-Eastern Atlantic. *J. Mar. Biol. Assoc. United Kingdom* 92 (6), 1379–1387. doi: 10.1017/S0025315411001068

Hodgson, A. N., Le Quesne, W. J., Hawkins, S. J., and Bishop, J. D. (2007). Factors Affecting Fertilization Success in Two Species of Patellid Limpet (Mollusca: Gastropoda) and Development of Fertilization Kinetics Models. *Mar. Biol.* 150 (3), 415–426. doi: 10.1007/s00227-006-0354-9

Inestrosa, N. C., Gonzalez, M., and Campos, E. O. (1992). Metamorfosis De Larvas Planctonicas De *Concholepas Concholepas* ("Loco") (Mollusca; Gastropoda; Muricidae). *Rev. Biología Marina y Oceanografía* 27 (2), 249–263.

Jenkins, S. R., Marshall, D., and Fraschetti, S. (2009). "Settlement and Recruitment," in *Marine Hard Bottom Communities: Patterns, Dynamics, Diversity, and Change*. Ed. M. Wahl (Berlin, Heidelberg: Springer Berlin Heidelberg), 177–190.

Jernakoff, P. (1985). Interactions Between the Limpet *Patelloida Latistrigata* and Algae on an Intertidal Rock Platform. *Mar. Ecol. Prog. series. Oldendorf* 23 (1), 71–78.

Kay, M. C., and Emlet, R. B. (2002). Laboratory Spawning, Larval Development, and Metamorphosis of the Limpets *Lottia Digitalis* and *Lottia Asmi* (Patellogastropoda, Lottiidae). *Invertebr. Biol.* 121 (1), 11–24. doi: 10.1111/j.1744-7410.2002.tb00125.x

Klein, R. G. (1979). Stone Age Exploitation of Animals in Southern Africa: Middle Stone Age People Living in Southern Africa More Than 30,000 Years Ago Exploited Local Animals Less Effectively Than the Later Stone Age People Who Succeeded Them. *Am. Sci.* 67 (2), 151–160.

Klein, R. G., and Steele, T. E. (2013). Archaeological Shellfish Size and Later Human Evolution in Africa. *Proc. Natl. Acad. Sci.* 110 (27), 10910–10915. doi: 10.1073/pnas.1304750110%

Kolbin, K. G., and Kulikova, V. A. (2011). Reproduction and Larval Development of the Limpet *Lottia Persona* (Rathke 1833) (Gastropoda: Lottiidae). *Russian J. Mar. Biol.* 37 (3), 239–242. doi: 10.1134/s1063074011030072

Lasiak, T. (1993). The Shellfish-Gathering Practices of Indigenous Coastal People in Transkei: Patterns, Preferences and Perceptions. *South Afr. J. Ethnol.* 16 (4), 115–120. doi: 10520/AJA02580144_669

Lindberg, D. R. (2007). Reproduction, Ecology, and Evolution of the Indo-Pacific Limpet *Scutellastra Flexuosa*. *Bull. Mar. Sci.* 81 (2), 219–234.

López, C., Poladura, A., Hernández, J. C., Martín, L., Concepción, L., Sangil, C., et al. (2012). Contrasting Effects of Protection From Harvesting in Populations of Two Limpet Species in a Recently Established Marine Protected Area. *Scientia. Mar.* 76 (4), 799–807. doi: 10.3989/scimar.03601.15C

Lorenzen, S. (2007). The Limpet *Patella Vulgata* L. At Night in Air: Effective Feeding on *Ascophyllum Nodosum* Monocultures and Stranded Seaweeds. *J. Molluscan Stud.* 73 (3), 267–274. doi: 10.1093/mollus/eym022

Luque, Á.A., Guallart, J., González, J. T., and Pola, M. (2018). *Recopilación Y Análisis De La Información Científica Disponible Sobre Patella Ferruginea* (Spain: Sociedad Española de Malacología).

Martins, G. M., Borges, C. D., Vale, M., Ribeiro, P. A., Ferraz, R. R., Martins, H. R., et al. (2017). Exploitation Promotes Earlier Sex Change in a Protandrous Patellid Limpet, *Patella Aspera* Röding 1798. *Ecol. Evol.* 7 (10), 3616–3622. doi: 10.1002/ece3.2925

Martins, G. M., Jenkins, S. R., Hawkins, S. J., Neto, A. I., Medeiros, A. R., and Thompson, R. C. (2011). Illegal Harvesting Affects the Success of Fishing Closure Areas. *J. Mar. Biol. Assoc. United Kingdom* 91 (4), 929–937. doi: 10.1017/S0025315410001189

Mau, A., Bingham, J.-P., Soller, F., and Jha, R. (2018). Maturation, Spawning, and Larval Development in Captive Yellowfoot Limpets (*Cellana Sandwicensis*). *Invertebrate Reprod. Dev.* 62 (4), 239–247. doi: 10.1080/07924259.2018.1505670

Mau, A., and Jha, R. (2018). Aquaculture of Two Commercially Important Molluscs (Abalone and Limpet): Existing Knowledge and Future Prospects. *Rev. Aquacul.* 10 (3), 611–625. doi: 10.1111/raq.12190

McCoy, M. D. (2008). Hawaiian Limpet Harvesting in Historical Perspective: A Review of Modern and Archaeological Data on Genus Cellana Spp. From the Kalaupapa Peninsula, Moloka'i Island. *Pac. Sci.* 62 (1), 21–38. doi: 10.2984/1534-6188(2008)62[21:HLHIHP]2.0.CO;2

McCoy, S. J., and Kamenos, N. A. (2015). Coralline Algae (Rhodophyta) in a Changing World: Integrating Ecological, Physiological, and Geochemical Responses to Global Change. *J. phycol.* 51 (1), 6–24. doi: 10.1111/jpy.12262

McGee, B. L., and Targett, N. M. (1989). Larval Habitat Selection in *Crepidula* (L.) and its Effect on Adult Distribution Patterns. *J. Exp. Mar. Biol. Ecol.* 131 (3), 195–214. doi: 10.1016/0022-0981(89)90112-3

Menge, B. A. (2000). Top-Down and Bottom-Up Community Regulation in Marine Rocky Intertidal Habitats. *J. Exp. Mar. Biol. Ecol.* 250 (1), 257–289. doi: 10.1016/S0022-0981(00)00200-8

Morse, D. E., Duncan, H., Hooker, N., and Morse, A. (1977). Hydrogen Peroxide Induces Spawning in Mollusks, With Activation of Prostaglandin Endoperoxide Synthetase. *Science* 196 (4287), 298–300. doi: 10.1126/science.403609

Moss, G. A. (1999). Factors Affecting Settlement and Early Post-Settlement Survival of the New Zealand Abalone *Haliotis Australis*. *New Z. J. Mar. Freshw. Res.* 33 (2), 271–278. doi: 10.1080/00288330.1999.9516876

Moss, G. A., Illingworth, J., and Tong, L. J. (1995). Comparing Two Simple Methods to Induce Spawning in the New Zealand Abalone (Paua), *Haliotis Iris*. *New Z. J. Mar. Freshw. Res.* 29 (3), 329–333. doi: 10.1080/00288330.1995.9516667

Muñoz-Colmenero, M., Turrero, P., Horreo, J. L., and Garcia-Vazquez, E. (2012). Evolution of Limpet Assemblages Driven by Environmental Changes and Harvesting in North Iberia. *Mar. Ecol. Prog. Ser.* 466, 121–131. doi: 10.3354/meps09906

Nakano, T., Nakayama, R., and Takahashi, Y. (2020). Artificial Fertilisation and Early Development of a Limpet *Lottia Tenuisculpta* (Patellogastropoda: Lottiidae). *Molluscan Res.* 40 (1), 52–59. doi: 10.1080/13235818.2019.1688752

Nakano, T., and Ozawa, T. (2007). Worldwide Phylogeography of Limpets of the Order Patellogastropoda: Molecular, Morphological and Palaeontological Evidence. *J. Molluscan Stud.* 73 (1), 79–99. doi: 10.1093/mollus/eym001

Navarro, P. G., Ramírez, R., Tuya, F., Fernandez-gil, C., Sanchez-jerez, P., and Haroun, R. J. (2005). Hierarchical Analysis of Spatial Distribution Patterns of Patellid Limpets in the Canary Islands. *J. Molluscan Stud.* 71 (1), 67–73. doi: 10.1093/mollus/eyi009%J

Nhan, H. T. (2014). *Development of Aquaculture Technology for the Hawaiian Opihi Cellana Spp* (Honolulu: University of Hawaii).

Nhan, H. T., and Ako, H. (2019). Reproductive Biology, Seed Production, and Culture of the Hawaiian Limpet *Cellana Sandwicensis* (Pease 1861). *Invertebr. Ecophysiol. Manage.* 1–19. doi: 10.5772/intechopen.87128

Nobre, A., Robertson-Andersson, D., Neori, A., and Sankar, K. (2010). Ecological–economic Assessment of Aquaculture Options: Comparison Between Abalone Monoculture and Integrated Multi-Trophic Aquaculture of Abalone and Seaweeds. *Aquaculture* 306 (1-4), 116–126. doi: 10.1016/j.aquaculture.2010.06.002

Nunes, C., Ramirez, A., Rodeia, J., De Girolamo, M., and Isidro, E. (2021). "Invasive vs. non-Invasive Methods of Reproduction in Patella Candei (D'orbigny 1840): From Fertilization to Settlement," in *Aquaculture Europe 2021 – Oceans of Opportunity* (Funchal, Madeira, Portugal: European Aquaculture Society).

Orton, J. H. (1929). Observations on *Patella Vulgata*. Part III. Habitat and Habits. *J. Mar. Biol. Assoc. United Kingdom* 16 (1), 277–288. doi: 10.1017/S0025315400029805

Orton, J. H., Southward, A. J., and Dodd, J. M. (1956). Studies on the Biology of Limpets: II. The Breeding of *Patella Vulgata* L. @ in Britain. *J. Mar. Biol. Assoc. United Kingdom* 35 (1), 149–176. doi: 10.1017/S0025315400009036

Parker, W., Yanes, Y., Mesa Hernández, E., Hernández Marrero, J. C., Pais, J., Soto Contreras, N., et al. (2020). Shellfish Exploitation in the Western Canary Islands Over the Last Two Millennia. *Environ. Archaeol.* 25 (1), 14–36. doi: 10.1080/14614103.2018.1497821

Pérez, S., Fernández, N., and Ribeiro, P. A. (2016). Standardization of a *Patella* Spp.(Mollusca, Gastropoda) Embryo–Larval Bioassay and Advantages of its Use in Marine Ecotoxicology. *Ecotoxicol. Environ. Saf.* 127, 175–186. doi: 10.1016/j.ecoenv.2016.01.025

Piazzi, L., Bulleri, F., and Ceccherelli, G. (2016). Limpets Compensate Sea Urchin Decline and Enhance the Stability of Rocky Subtidal Barrens. *Mar. Environ. Res.* 115, 49–55. doi: 10.1016/j.marenvres.2016.01.009

Pinto, A. R., Alves, A., Faria, G., Reis, R., and Delgado, J. (2010). "Reproductive Biology of Patella Candei D'orbigny 1840 in Madeira Archipelago," in *XVI Simposio Iberico De Estudios De Biologia Marina* (Alicante: Departamento de Ciencias del Mar y Biología Aplicada de la Universidad de Alicante).

Pombo, O. A., and Escofet, A. (1996). Effect of Exploitation on the Limpet *Lottia Gigantea*: A Field Study in Baja California (Mexico) and California U.S.A. *Pac. Sci.* 50 (4), 393–403. doi: 10125/2914

Rao, M. B. (1973). Sex Phenomenon and Reproductive Cycle in the Limpet *Cellana Radiata* (Born) (Gastropoda: Prosobranchia). *J. Exp. Mar. Biol. Ecol.* 12 (3), 263–278. doi: 10.1016/0022-0981(73)90057-9

Ribeiro, P. (2008). *Dispersal and Connectivity of Northeastern Atlantic Patellid Limpets: A Multidisciplinary Approach. Thesis* (Southampton: University of Southampton).

Roberts, R. D., Kaspar, H. F., and Barker, R. J. (2004). Settlement of Abalone (*Haliotis Iris*) Larvae in Response to Five Species of Coralline Algae. *J. Shellfish Res.* 23 (4), 975–988.

Roberts, R. D., and Nicholson, C. M. (1997). Variable Response From Abalone Larvae *(Haliotis Iris*, *H. Virginea*) to a Range of Settlement Cues. *Molluscan Res.* 18 (2), 131–141. doi: 10.1080/13235818.1997.10673687

Rodríguez, S., Ojeda, F. P., and Inestrosa, N. C. (1993). Settlement of Benthic Marine Invertebrates. *Mar. Ecol. Prog. Ser.* 97, 197–207. doi: 10.3354/meps097193

Sagarin, R. D., Ambrose, R. F., Becker, B. J., Engle, J. M., Kido, J., Lee, S. F., et al. (2007). Ecological Impacts on the Limpet *Lottia Gigantea* Populations: Human Pressure Over a Broad Scale on Island and Mainland Intertidal Zones. *Mar. Biol.* 150 (3), 399–413. doi: 10.1007/s00227-006-0341-1

Salas-Garza, A., Parés-Sierra, G., Gómez-Rigalt, R., and Carpizo-Ituarte, E. (2009). The Larval Development, Metamorphosis and Juvenile Growth of the Turban Snail *Lithopoma (Astraea) Undosa* (Wood 1828) (Gastropoda: Turbinidae). *J. World Aquacul. Soc.* 40 (4), 460–471. doi: 10.1111/j.1749-7345.2009.00268.x

Seabra, M. I., Cruz, T., Fernandes, J. N., Silva, T., and Hawkins, S. (2019). Recruitment of the Limpet *Patella Ulyssiponensis* and its Relationship With Crustose Coralline Algae: Patterns of Juvenile Distribution and Larval Settlement. *J. Mar. Biol. Assoc. United Kingdom* 99 (8), 1787–1796. doi: 10.1017/S0025315419000869

Searcy-Bernal, R., Salas-Garza, A. E., Flores-Aguilar, R. A., and Hinojosa-Rivera, P. R. (1992). Simultaneous Comparison of Methods for Settlement and Metamorphosis Induction in the Red Abalone (*Haliotis Rufescens*). *Aquaculture* 105 (3-4), 241–250. doi: 10.1016/0044-8486(92)90090-8

Smith, F. G. W. (1935). III-The Development of *Patella Vulgata*. Philosophical Transactions of the Royal Society of London. *Ser. B Biol. Sci.* 225 (520), 95–125. doi: 10.1098/rstb.1935.0008

Sousa, R., Delgado, J., Pinto, A. R., and Henriques, P. (2017). Growth and Reproduction of the North-Eastern Atlantic Keystone Species *Patella Aspera* (Mollusca: Patellogastropoda). *Helgoland Mar. Res.* 71 (1), 8. doi: 10.1186/s10152-017-0488-9

Sousa, R., Henriques, P., Vasconcelos, J., Pinto, A. R., Delgado, J., and Riera, R. (2020a). The Protection Effects of Marine Protected Areas on Exploited Molluscs From an Oceanic Archipelago. *Aquat. Conservation: Mar. Freshw. Ecosystems* 30 (4), 717–729. doi: 10.1002/aqc.3285

Sousa, R., Pinto, A., Vasconcelos, J., and Riera, R. (2020b). Does Harvesting Affect the Relative Growth in *Patella Aspera* Röding 1798? *Eur. Zoological J.* 87 (1), 395–401. doi: 10.1080/24750263.2020.1791266

Sousa, R., Vasconcelos, J., Henriques, P., Pinto, A. R., Delgado, J., and Riera, R. (2019a). Long-Term Population Status of Two Harvested Intertidal Grazers (*Patella Aspera* and *Patella Candei*), Before, (1996–2006) and After, (2007–2017) the Implementation of Management Measures. *J. sea Res.* 144, 33–38. doi: 10.1016/j.seares.2018.11.002

Sousa, R., Vasconcelos, J., Riera, R., Pinto, A. R., Delgado, J., and Henriques, P. (2019b). Potential Impact of Harvesting Management Measures on the Reproductive Parameters of the Limpets *Patella Aspera* and *Patella Candei* From Madeira Island. *Estuarine Coastal Shelf Sci.* 226, 106264. doi: 10.1016/j.ecss.2019.106264

Steneck, R. S. (1982). A Limpet-Coralline Alga Association: Adaptations and Defenses Between a Selective Herbivore and its Prey. *Ecology* 63 (2), 507–522. doi: 10.2307/1938967

Steneck, R. S. (1986). The Ecology of Coralline Algal Crusts: Convergent Patterns and Adaptative Strategies. *Annu. Rev. Ecol. systematics* 17 (1), 273–303. doi: 10.1146/annurev.es.17.110186.001421

Steneck, R. S. (1990). "Herbivory and the Evolution of Nongeniculate Coralline Algae (Rhodophyta, Corallinales) in the North Atlantic and North Pacific," in *Evolutionary Biogeography of the Marine Algae of the North Atlantic*. Eds. D. J. Garbary and G. R. South (Berlin Heidelberg: Springer), 107–129.

Stiner, M. C., Munro, N. D., Surovell, T. A., Tchernov, E., and Bar-Yosef, O. (1999). Paleolithic Population Growth Pulses Evidenced by Small Animal Exploitation. *Science* 283 (5399), 190–194. doi: 10.1126/science.283.5399.190

Torres, C., and Andrade, C. (2010). Processo De Decisão De Análise Espacial Na Selecção De Áreas Óptimas Para a Aquacultura Marinha: O Exemplo Da Ilha Da Madeira. *Rev. Gestão Costeira Integrada-Journal Integ. Coastal Zone Manage.* 10 (3), 321–330. doi: 10.5894/rgci180

Uki, N., and Kikuchi, S. (1984). Regulation of Maturation and Spawning of an Abalone, *Haliotis* (Gastropoda) by External Environmental Factors. *Aquaculture* 39 (1), 247–261. doi: 10.1016/0044-8486(84)90270-9

Underwood, A. J. (1980). The Effects of Grazing by Gastropods and Physical Factors on the Upper Limits of Distribution of Intertidal Macroalgae. *Oecologia* 46 (2), 201–213. doi: 10.1007/BF00540127

Valdez-Cibrián, A., Michel-Morfín, J. E., Gómez-Torres, M., Landa-Jaime, V., Gómez-Gastelum, L., and Carballo, J. L. (2021). Population Status of the Endangered Giant Mexican Limpet *Scutellastra Mexicana* in the Central Mexican Pacific. *Aquat. Conservation: Mar. Freshw. Ecosyst.* 31 (3), 531–542. doi: 10.1002/aqc.3480

Viera, M., Courtois de Viçose, G., Fernández-Palacios, H., and Izquierdo, M. (2016). Grow-Out Culture of Abalone Haliotis Tuberculata Coccinea Reeve, Fed Land-Based IMTA Produced Macroalgae, in a Combined Fish/Abalone Offshore Mariculture System: Effect of Stocking Density. *Aquacul. Res.* 47 (1), 71–81. doi: 10.1111/are.12467

Wanninger, A., Ruthensteiner, B., Lobenwein, S., Salvenmoser, W., Dictus, W. J., and Haszprunar, G. (1999). Development of the Musculature in the Limpet *Patella* (Mollusca, Patellogastropoda). *Dev. Genes Evol.* 209 (4), 226–238. doi: 10.1007/s004270050247

Weber, L. I., and Hawkins, S. J. (2002). Evolution of the Limpet *Patella Candei* d'Orbigny (Mollusca, Patellidae) in Atlantic Archipelagos: Human Intervention and Natural Processes. *Biol. J. Linn. Soc.* 77 (3), 341–353. doi: 10.1046/j.1095-8312.2002.00102.x

Wolcott, T. G. (1973). Physiological Ecology and Intertidal Zonation in Limpets (Acmaea): A Critical Look at "Limiting Factors". *Biol. Bull.* 145 (2), 389–422. doi: 10.2307/1540048

Wright, W. G., and Lindberg, D. R. (1979). A non-Fatal Method of Sex Determination for Patellacean Gastropods. *J. Mar. Biol. Assoc. United Kingdom* 59 (3), 803–803. doi: 10.1017/S0025315400045793

Wright, W. G., and Lindberg, D. R. (1982). Direct Observation of Sex Change in the Patellacean Limpet *Lottia Gigantea*. *J. Mar. Biol. Assoc. United Kingdom* 62 (3), 737–738. doi: 10.1017/S0025315400019895

Zhao, B., and Qian, P.-Y. (2002). Larval Settlement and Metamorphosis in the Slipper Limpet *Crepidula Onyx* (Sowerby) in Response to Conspecific Cues and the Cues From Biofilm. *J. Exp. Mar. Biol. Ecol.* 269 (1), 39–51. doi: 10.1016/S0022-0981(01)00391-4

Conflict of Interest: The handling editor SL declared a past co-authorship with the author CA.

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.

frontiers | Frontiers in Marine Science

ORIGINAL RESEARCH
published: 24 June 2022
doi: 10.3389/fmars.2022.865450

Trophic Requirements of the Sea Urchin *Paracentrotus lividus* Varies at Different Life Stages: Comprehension of Species Ecology and Implications for Effective Feeding Formulations

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Investigations on trophic requirements of different life cycle stages of *Paracentrotus lividus* are crucial for the comprehension of species ecology and for its artificial rearing. The future success of echinoculture depends heavily on the development of suitable and cost-effective diets that are specifically designed to maximize somatic growth during the early life stages and gonadal production in the later stages. In this context, a considerable number of studies have recommended animal sources as supplements in sea urchin diets. However, with the exception of Fernandez and Boudouresque (2000), no studies have investigated the dietary requirements over the different life stages of the sea urchin. In the present study, the growth and nutrition of three life stages of *P. lividus* (juveniles: 15-25 mm; subadults: 25-35 mm; adults: 45-55 mm) were analyzed over a 4-month rearing experiment. Three experimental diets, with 0%, 20% and 40% of animal sourced enrichments, were tested in parallel in sea urchin three size classes. The food conversion ratio, somatic and gonadal growth were assessed in each condition in order to evaluate the optimal level of animal-sourced supplements for each life stage. A general growth model covering the full post-metamorphic *P. lividus* life cycle was defined for each condition. During the juvenile stage *P. lividus* requires higher animal supply (40%), while a feeding requirement shift takes place toward lower animal supply (20%) in sub-adult and adult stages. Our results evidenced that the progressive increase in size after the metamorphosis led to a consequent variation of trophic requirements and food energy allocation in the sea urchin *P. lividus*. Macronutrient requirements varied widely during the

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

Received: *29 January 2022*
Accepted: *09 May 2022*
Published: *24 June 2022*

Citation:
Grosso L, Rakaj A, Fianchini A, Tancioni L, Vizzini S, Boudouresque C-F and Scardi M (2022) Trophic Requirements of the Sea Urchin Paracentrotus lividus Varies at Different Life Stages: Comprehension of Species Ecology and Implications for Effective Feeding Formulations. Front. Mar. Sci. 9:865450. doi: 10.3389/fmars.2022.865450

different life stages, in response to changes in the energy allocation from somatic growth to reproductive investment. This study sheds light on *P. lividus* trophic ecology, broadening our basic knowledge of the dietary requirements of juveniles, sub-adults and adults as a function of their behavior also in the natural environment.

Keywords: *Paracentrotus lividus*, sea urchin, echinoculture, life stages, ecology and behavior, aquaculture, feeding requirements

INTRODUCTION

The sea urchin *Paracentrotus lividus* (Lamarck, 1816) is an edible echinoid, which is widespread throughout the Mediterranean coasts and in the northeastern Atlantic, from Scotland to southern Morocco (Tortonese, 1965). Since the time of ancient Greece, its gonads (sea urchin roe), which are the edible part of this organism, have been considered a seafood delicacy in the Mediterranean Sea. Indeed, in some Italian localities, the reddish-orange gonads of *P. lividus* are still called "red gold" to indicate a luxury sea food of an intense color and unique flavor (Sartori and Gaion, 2016). Nowadays, *P. lividus* is the most commercially exploited and high market value echinoid in Europe and its gonads can be sold for up to 150 € kg^{-1} (Carboni et al., 2012). The increasing market value of this species, however, has caused the species to be targeted in the huge spread of illegal, unreported and unregulated fishing that is leading to the fast decline or in some cases even the collapse of local stocks (Guidetti et al., 2004; Ceccherelli et al., 2009; Rakaj et al., 2021). This phenomenon is very harmful also for the biodiversity of the benthic communities where this species inhabits since this sea urchin has an important ecological role, strictly connected to its trophic strategy (Tortonese, 1965; Yeruham et al., 2015). *P. lividus* is, in fact, a voracious and generalist herbivore, as well as a prey for a number of fish, starfish, and mollusks, and hence it is a key species in the dynamics of sublittoral ecosystems (Boudouresque and Verlaque, 2020).

In this context, aquaculture is a promising solution, offering a sustainable alternative to wild exploitation for meeting the current market demand (McBride, 2005; Bartley and Bell, 2008). However, the economic effectiveness of this solution, which is a crucial factor for the feasibility of industrial plants, is still hindered by the lack of excellent feeds, able to guarantee sea urchin fast growth and gonadal maturation (Spirlet et al., 1998; Cook and Kelly, 2009; Shpigel et al., 2018; Zupo et al., 2019). In fact, food quality and quantity to administrate at each developmental stages are still among the main challenges in echinoculture research to overcome the bottlenecks in commercial scale productions (Kelly, 2004). The development of successful sea urchin aquaculture therefore requires a basic understanding of the dietary requirements during both planktonic and benthonic life stages. More specifically, after the metamorphosis, the progressive variation in sea urchin size leads to a consequent variation in dietary requirements and food energy allocation (McCarron et al., 2009; Boudouresque and Verlaque, 2020). Sea urchins may require different levels of dietary protein and/or carbohydrate at different life stages (Heflin et al., 2012) and the level of energy allocated for reproduction increases with the increase in size, with a concurrent decrease in the allocation to the test and lantern (Fernandez and Boudouresque, 2000). Several studies have already demonstrated that formulated feeds can better satisfy specific nutrient requirements when compared with the food sources traditionally used in echinoculture (e.g. macroalgae) (Pearce et al., 2002; Daggett et al., 2005; Schlosser et al., 2005; Sartori and Gaion, 2016; Prato et al., 2018; Ruocco et al., 2018; Santos et al., 2020a). For this reason, modern aquaculture is turning to formulated diets with more sustainable, low-cost and constantly available feedstuffs than natural sources. Vegetable and animal ingredients mixed in a formulated diet can, in fact, maximize sea urchin growth performance, reducing time for the production of high-quality marketable gonads (Vizzini et al., 2019). To date, several attempts have been made to obtain a good market product using a balanced mix of vegetable and animal sources, as alternative diets to traditional ones (Vizzini et al., 2015; Sartori and Gaion, 2016; Cirino et al., 2017; Fabbrocini et al., 2019; Raposo et al., 2019; Zupo et al., 2019; Ciriminna et al., 2020; Santos et al., 2020b; Ciriminna et al., 2021; Lourenço et al., 2021). However, as far as we know, only Fernandez and Boudouresque (2000) focused on a comparative investigation of the dietary requirements of the sea urchin during different life stages. In addition, they analyzed the effects of a wide range of fishmeal enrichment, testing vegetable, mixed and animal feed (respectively with 0.0%, 44.7% and 89.4% of fish meal). Although levels of animal-sourced supplements between 0 and 50% are recommended, no investigations have so far defined more specifically the requirements of these supplements in the different life stages of *P. lividus*. Hence, this issue is still crucial in order to reduce food waste in echinoculture activities, whilst maximizing growth performance and reducing the environmental footprint.

To address this gap, the present study aimed to evaluate the effects of a narrow range of animal supply (0%; 20%; 40%) in a vegetable based diet, on three size-classes of *P. lividus* (15-25 mm; 25-35 mm; 45-55 mm). Maize, carrots, and soy, were selected as vegetable ingredients for the diet base due to their complementary profiles in terms of macronutrients (Hammer et al., 2012; Sartori and Gaion, 2016). Fish-meal was chosen as animal supply to be tested among the different life stages because of its large literature background on *P. lividus* rearing (Fernandez and Boudouresque, 2000; Spirlet et al., 2001; Fabbrocini et al., 2012; Sartori and Gaion, 2016; Ruocco et al., 2018; Volpe et al., 2018; Fabbrocini et al., 2019; Zupo et al., 2019; Ciriminna et al., 2021; Lourenço et al., 2021).

The division of sea urchins into discrete life stages is a controversial topic in literature. Several studies, in fact, have

adopted heterogeneous criteria such as test diameter, age, reproductive maturity or trophic behavior to classify juveniles, sub-adults and adults in *P. lividus* (Sala, 1997; Grosjean et al., 1998; Cook and Kelly, 2007; McCarron et al., 2010; Boudouresque and Verlaque, 2020). In this study sea urchins were allocated into three size groups (test diameter) according to Fernandez and Boudouresque (2000). Based on their growth phase and gonads maturity, small specimens with Test Diameter TD < 25 mm were termed "juveniles" as they belong to the exponential growth phase and are not reproductively active; intermediate specimens with TD between 25 and 35 mm were termed "sub-adults" since they belong to the end of the exponential growth phase, but their gonads may be able to produce small amount of gametes; large specimens with TD > 35 mm were termed "adults", since their gonads are fully reproductively active producing large quantities of healthy gametes. On the basis of this division, the effect of each experimental diet was evaluated in terms of somatic growth for the three sea urchin size classes and in terms of gonad yield for sub-adults and adults. In addition, these investigations were associated with the study of the Feed Conversion Ratio (FCR), in order to identify the best performing animal-sourced supplement for each sea urchin size-class, able to guarantee the highest growth with lowest food input.

MATERIALS AND METHODS

Specimen Collection and Maintenance

Three size classes of *P. lividus*: 15 – 25 mm (juveniles); 25 – 35 mm (sub-adults) and 45 – 55 mm (adults) were reared in a four-month experiment (15 May - 20 September 2020). Sea urchins of the smallest size-class (15 – 25 mm, juveniles) were obtained by artificial reproduction, carried out in 2019 at the Laboratory of Experimental Ecology and Aquaculture (LESA, University of Rome "Tor Vergata") in the frame of an echinoderm hatchery production project (PO FEAMP 2.1 2014/2020; See Supplementary Material). The other two size classes were collected by snorkeling (1–8 m depth) at Santa Marinella in the central Tyrrhenian Sea, Italy (42°03′00″ North, 11°49′09″ East) in May 2020. Specimens were then transported to the LESA inside 30 L tanks equipped with aerators and dry ice (Rakaj et al., 2018; Rakaj et al., 2019). Once in the lab, the specimens were acclimatized in floating baskets inside 600 L indoor tanks (Morroni et al., 2020). Water temperature was kept at 20 ± 0.5°C (Spirlet et al., 1998), which was the rearing temperature also used during the experiment. Sea urchins of each size class were starved for two weeks in the maintaining system before undergoing the experimental trials (Prato et al., 2018)

Experimental Design

The experiment was run in the same recirculating aquaculture system (RAS) made up of 40 L aquaria each connected in tandem (Grosso et al., 2021). This RAS was specifically designed for feeding investigations, as it was equipped with an efficient waste removal system. The water leaving the aquaria was firstly subjected to a mechanical filtration phase in a drum filter, in order to remove waste particles greater than 20 μm. In the second step, organic compounds, such as proteins and amino acids, were removed from a strong protein skimming. In the next step, the residual particles (such as nitrogen compounds) were retained and oxidized in fine sand filters (first) and biofilters with moving bed media (after). In the final filtration phase, 5 μm cartridge filter allowed fine particle removal and a UV sterilizer eliminated bacteria load (See Supplementary material). This rearing system guaranteed a flow rate of 120 L h^{-1} (in each aquarium) and ensured similar physico-chemical parameters: temperature (20 ± 1°C); salinity (36 ± 1‰); pH (7.95-8.25), and natural photoperiod. Water exchange of 50% of the whole volume was undertaken at least twice a week using 5 μm filtered and UV-sterilized seawater. Ammonia, nitrate and nitrite concentration were monitored every two days by means of spectrophotometer (Hach Lange D3900), to maintain the rearing condition within a healthy range (Huguenin and Colt, 2002; Mortensen et al., 2012). Additionally, pH, temperature and salinity were monitored daily through a probe (EUTECH PCD 650).

18 sub-adults and 18 adults were sacrificed prior to the experiment in order to assess the baseline of the Gonad Index (GI %) at time T0. After starvation, 108 specimens of *P. lividus* were sorted into the three size classes - 36 in each size class - by measuring their test diameters with a digital calliper (± 0.01 mm accuracy). Then each size class was reared separately, with juveniles, sub-adults and adults allocated in different 40 L aquaria, inside perforated single floating baskets (**Figure 1**). The mean test diameter and wet weight of the three size classes at the beginning of the experiment are shown in **Table 1**.

Diet Preparation and Feeding Parameters

Three experimental diets were tested in this study:

- D-0 (0% fish meal, 100% vegetables)
- D-20 (20% fish meal, 80% vegetables)
- D-40 (40% fish meal, 60% vegetables)

Carrots, maize and soy were employed to make up the vegetable base of all the experimental diets. In each diet, 119.80 g (carrots), 33.73 g (maize) and 46.46 g (soy) respectively, were used to obtain the same proportion (1: 1: 1) of vegetables in dry weight (**Table 2**) (Grosso et al., 2021). The vegetables were steamed, blended and extruded with different levels of fish-meal enrichment (0%, 20% and 40%) using agar-agar (Nature et Plantes - St Paul Les Dax, France) as a binder to minimize food dispersion (Fabbrocini et al., 2012; Volpe et al., 2018). All diet ingredients were certified for human consumption complying the HACCP principles. The mixtures were rolled out into moist blocks (1 cm x side) and administered fresh to the sea urchins. Each week, the diets were freshly prepared and stored at 4°C to preserve the nutritional profile and avoid bacteria growth. Diets were administered once a day ad libitum (around 1% of sea urchins weight in food dry weight) to sea urchins of each

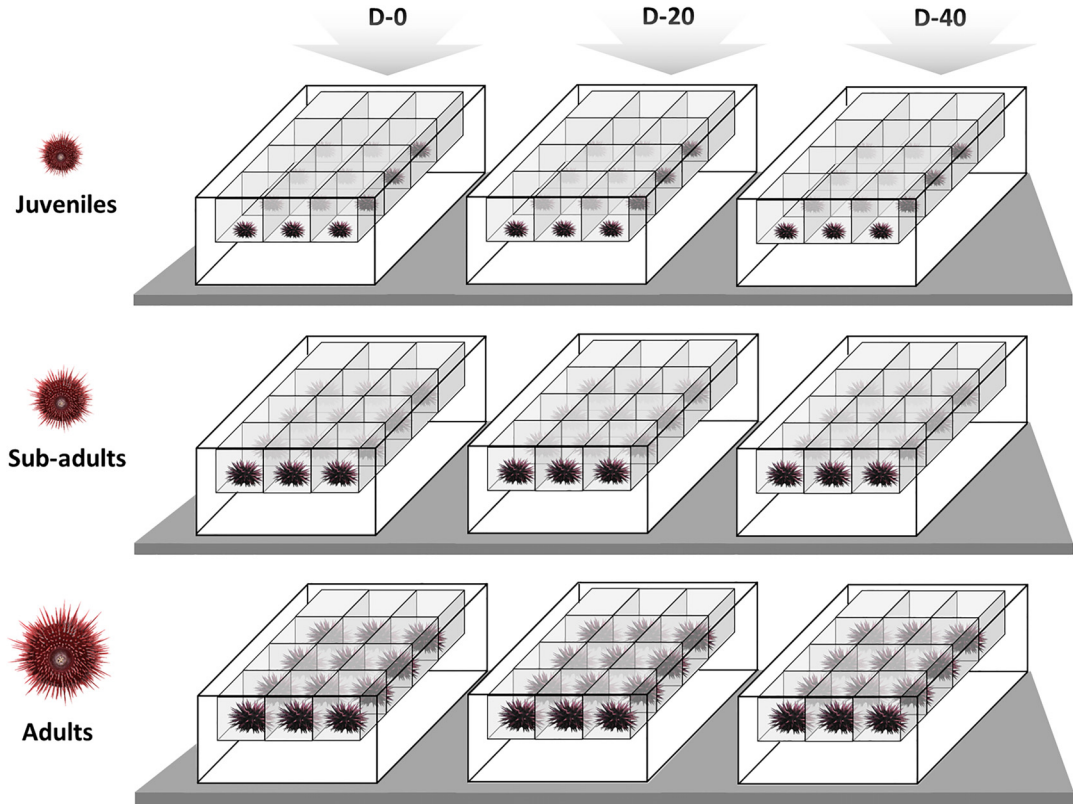

FIGURE 1 | Representation of the experimental design: above the three experimental diets administrated, D-0 (100% vegetables, 0% fish meal); D-20 (80% vegetables, 20% fish meal); D-40 (60% vegetables, 40% fish meal), and at each level the respective *P. lividus* size classes, juveniles (15-25 mm); sub-adults (25-35 mm); adults (45-55 mm). Twelve sea urchins were employed in each condition, individually placed in feeding boxes within same RAS.

size-class. Over a period of four weeks (one week for each month), the ingestion was calculated for each specimen by weighing the administered food (wet weight) and leftover food produced after 24 h (in dry weight). The conversion of administrated food from wet to dry weight was obtained weekly by drying 5 replicates for each diet. Finally, the Feed Conversion Ratio (FCR) was calculated by combining sea urchin ingestion and growth data.

TABLE 1 | Biometric measures (mean ± SE) and growth performances of *P. lividus* size classes fed with the three experimental diets (D-0, D-20 and D-40).

Biometric variables	**Sea urchin size-classes**								
	Juveniles			**Sub-adults**			**Adults**		
	D-0	**D-20**	**D-40**	**D-0**	**D-20**	**D-40**	**D-0**	**D-20**	**D-40**
WW_{T0} (g)	6.58 ± 0.45	7.36 ± 0.66	6.61 ± 0.68	14.19 ± 0.82	12.74 ± 0.55	15.03 ± 0.72	56.70 ± 2.82	57.68 ± 2.83	63.05 ± 3.12
WW_{T1} (g)	10.67 ± 0.77	13.67 ± 0.95	12.09 ± 1.25	21.21 ± 1.19	21.38 ± 0.58	24.66 ± 0.89	62.85 ± 3.07	66.80 ± 3.45	71.03 ± 3.41
TD_{T0} (mm)	20.23 ± 0.78	21.62 ± 0.84	20.89 ± 0.70	29.79 ± 0.80	29.94 ± 0.29	31.05 ± 0.47	50.04 ± 0.85	50.18 ± 0.76	50.22 ± 0.82
TD_{T1} (mm)	27.72 ± 0.87	30.78 ± 0.67	29.73 ± 0.92	34.44 ± 0.70	34.66 ± 0.35	36.46 ± 0.59	52.23 ± 0.83	53.70 ± 0.85	53.66 ± 0.93
GI_{T0}				4.72 ± 0.92	4.72 ± 0.92	4.72 ± 0.92	3.52 ± 0.97	3.52 ± 0.97	3.52 ± 0.97
GI_{T1}				10.59 ± 1.31^{a}	18.90 ± 0.80^{b}	18.25 ± 0.83^{b}	7.21 ± 0.63^{a}	13.58 ± 0.93^{b}	8.66 ± 0.73^{a}
SGR	0.40 ± 0.01^{a}	0.53 ± 0.02^{b}	0.59 ± 0.05^{b}	0.34 ± 0.01^{a}	0.44 ± 0.01^{b}	0.42 ± 0.01^{b}	0.09 ± 0.00^{a}	0.12 ± 0.01^{b}	0.10 ± 0.01^{a}
FCRs	2.23 ± 0.33^{a}	1.22 ± 0.06^{b}	1.42 ± 0.15^{b}	1.40 ± 0.08^{a}	1.03 ± 0.01^{b}	1.23 ± 0.03^{a}	2.18 ± 0.13^{a}	1.62 ± 0.15^{b}	1.75 ± 0.10^{ab}
FCRg				3.66 ± 0.42^{a}	1.70 ± 0.08^{b}	2.07 ± 0.11^{b}	7.37 ± 1.40^{a}	2.26 ± 0.31^{b}	4.13 ± 0.51^{b}

The different letters indicate significant differences ($p < 0.05$) among the experimental diets (one-way ANOVA followed by Turkey's test).
WW_{T0}: initial somatic wet weight; WW_{T1}: final somatic wet weight; TD_{T0}: initial test diameter; TD_{T1}: Final test diameter; GI_{T0}: initial Gonad Index (%); GI_{T1}: final Gonad Index (%); SGR: Somatic Growth Rate (%); FCRs: Feed Conversion Ratio in wet weight; FCRg: Feed Conversion Rate in gonads.

TABLE 2 | Formulation and proximate composition of the three experimental diets.

	Experimental diets		
	D-0	**D-20**	**D-40**
Ingredients (%)			
Fish meal	0,00%	20,00%	40,00%
Carrots	31,67%	25,00%	18,33%
Maize	31,67%	25,00%	18,33%
Soy	31,67%	25,00%	18,33%
Agar-agar	5,00%	5,00%	5,00%
Proximate Composition (%)			
% carbohydrates	56.19	42.68	34.42
% proteins	18.32	29.92	37.01
% lipids	7.08	10.97	13.34
% fiber	18.41	16.44	15.23

Proximate composition source: BDA nutritional database 2021

Growth Rates

During the experiment, diets performances were investigated through different measurements for each life stage: in juveniles, since gonads were immature, we focused on somatic growth performances (Somatic Growth Rates SGR and Feed Conversion Ration in wet weight FCRs); in sub-adults and adults, in terms of both somatic growth (SGR and FCRs) and gonad yield (Gonad Index GI and Feed Conversion Ratio in gonads FCRg). The somatic growth of juvenile, sub-adult and adult sea urchins was followed, measuring the wet weight of specimens, as described above. At the end of the experiment, the sea urchins were starved for 48 h in order to void their digestive tract before being weighed to calculate the Somatic Growth Rate SGR % (Grosso et al., 2021) as follows:

$$\bullet \qquad \mathrm{SGR}:((\ln W_f - \ln W_i)/t) * 100 \qquad Z$$

Where W_f and W_i are the final and initial wet weights (g) of juvenile or subadult sea urchins and t represents time in days of the experiment.

The Gonad Index GI % (Vizzini et al., 2015) was calculated for sub-adult and adult sea urchins, as follows:

$$\bullet \qquad \mathrm{GI}:\left(W_g/W_t\right) * 100 \qquad Z$$

Where W_g is the wet weight (g) of the gonad and W_t is the total wet weight (g) of the sea urchin.

Finally, combining the data collected regarding the ingestion and growth of the three sea urchin size-classes, Feed Conversion Ratio in gonads (FCRg) and Feed Conversion Ratio in somatic weight (FCRs) were calculated:

$$\bullet \qquad \mathrm{FCRg}:(DW_i - DW_f)/\left(WW_{fg} - WW_{ig}\right) \qquad Z$$

Where DW_i is the total dry weight of feed added to the aquaria and DWf is the total dry weight of the uneaten feed; WW_{fg} and WW_{ig} are the final and initial wet weights of sub-adult and adult *P. lividus* gonads.

$$\bullet \qquad \mathrm{FCRs}:(DW_i - DW_f)/(WW_{fs} - WW_{is}) \qquad Z$$

Where DW_i is the total dry weight of feed added to the aquaria and DW_f is the total dry weight of the uneaten feed; WW_{fs} and WW_{is} are the final and initial wet weights of juvenile and sub-adult *P. lividus*.

Statistical Analysis

Prior to analysis, raw data were diagnosed for normality of distribution and homogeneity of variance by means of a Levene's test and a Kolmogorov-Smirnov test respectively (Whitlock and Schluter, 2010). Samples were close to a normal distribution (Kolmogorov-Smirnov $p > 0.05$) with similar variances (Levene's $p > 0.05$). One-way analysis of variance ANOVA was carried out to analyze the effects of experimental diets on somatic and gonadic growth performances and the feeding parameters of each sea urchin size-class. Where p values were generated in ANOVA, Turkey multiple comparison tests were used to evaluate differences among pair-wise means ($p < 0.05$). Local regression analysis (LOESS) was then performed in order to estimate the Somatic Growth Rate (%) for a continuous distribution of sea urchin test diameters in relation to each experimental diet (**Figure 3**). Statistical analyses were performed with PAST 3.0 (Hammer et al., 2013).

RESULTS

All the experimental diets were found to be suitable to sustain the breeding of juvenile, sub-adult and adult *P. lividus*. In fact, during the experiment specimens from all the sea urchin size-classes showed a 100% survival rate and positive somatic or gonadal growth in all the feeding conditions. However, the cross-comparison analysis highlighted significant differences in growth performances among the sea urchin size classes related to the three diets administrated.

Somatic Growth

The results of this experiment showed an overall reduction in the somatic growth rate with the increase in the size class. In fact, in all feeding trials (D-0, D-20, D-40), juveniles obtained the highest values of Somatic Growth Rate (SGR%) among the sea urchin size classes, followed by sub-adults and adults. Maximal growth was observed for juveniles fed with the 40% fishmeal-enriched diet (D-40; SGR = 0.59%) and the 20% fishmeal-enriched diet (D-20, SGR = 0.53%), which did not differ each other but were higher than SGR value obtained with the entirely vegetable diet (D-0, SGR = 0.40%) (**Figure 2**). Similarly, in sub-adults the maximal growth was obtained for D-20 (SGR = 0.44%) and D-40 (SGR = 0.42%), while D-0 yielded a lower growth rate (SGR = 0.34%) (**Figure 2**). Finally, for adult sea urchins the highest SGR value was obtained for D-20 (0.12%), which was higher than that obtained for D-40 and D-0 (0.10% and 0.09% respectively) (**Figure 2**).

In addition, the estimation of the maximum growth of *P. lividus* within a continuous distribution of test diameter for each of the three experimental diets was carried out through local regression analysis (LOESS) (**Figure 3**). The highest growth level in the sea urchins with smaller test diameters was obtained for the D-40 diet. However, the increase in test diameter was related to a decrease in the requirement of animal-source in the diet, as shown by the progressive slope inversion between the equation-lines of D-20 and D-40 (**Figure 3**). In fact, the sub-adult sea urchins obtained similar SGR % when fed with D-20 and D-40.

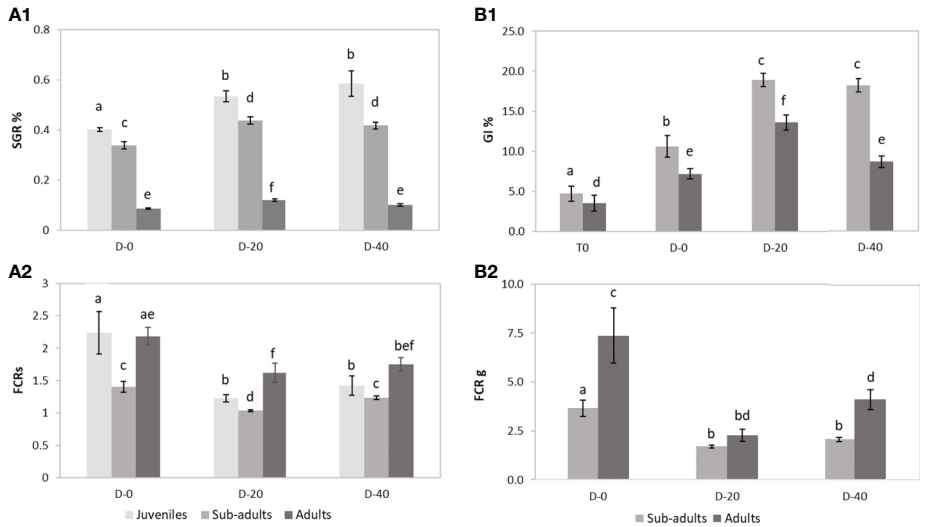

FIGURE 2 | Somatic and gonadal growth performances in a four-month experiment across three P. lividus life stages (juveniles, sub-adults and adults) fed with three different diets (D-0, D-20 and D-40) (n=12 sea urchins for each condition). **(A1)** Somatic growth rate (SGR); **(A2)** Feed conversion ratio in somatic weight (FCRs), based in dry weight; **(B1)** Gonad index (GI%); **(B2)** Feed conversion ratio in gonads (FCRg), based in dry weight. Error bars indicate standard errors of the means. Columns with the same letter are not significantly different (p> 0.05).

Instead, the adults showed higher SGR values when fed with D-20. Finally, the sea urchin of all test diameters fed with D-0 showed the lowest growth.

These results were also confirmed by the FCRs (**Figure 2**). In fact, for all the three sea urchin size classes, the lowest FCRs values were obtained for D-20 (1.23, 1.04, 1.62, respectively for juveniles, sub-adults and adults) and D-40 (1.42, 1.23, 1.75 respectively), which did not differ each other, while values for D-0 were significantly higher (2.24, 1.40, 2.18 respectively).

Comparison analysis across size classes evidenced that sub-adults yielded lower FCRs in all feeding conditions (**Figure 2B**). Juveniles, on the other hand, obtained lower FCRs than adults in enriched animal diets (D-20 and D-40), although for the entirely vegetable diet (D-0), FCRs were similar between the two groups (**Figure 2B**).

Gonadal Growth

At the end of the experiment, only sub-adult and adult specimens showed gonadal production. In fact, for these two sea urchin size classes the final Gonad Index was significantly higher than the initial values (**Figure 2**). However, ANOVA highlighted significant differences in gonadal growth performances among the diets administrated for each size class. Sub-adults showed higher GI values when fed with D-20 (18.90) and D-40 (18.25) than with D-0 (10.60) (**Figure 2**). Instead, the maximal value for the adults was recorded for D-20 (13.58), which was higher than those for D-40 (8.66) and D-0 (7.21) (**Figure 2**).

These results were also confirmed by the FCRg (**Figure 2**), which was lower for sub-adults fed with D-20 (1.70) and D-40 (2.08) than with D-0 (3.66) and for adults fed with D-20 (2.26) than both those fed with D-40 (4.12) and D-0 (7.36).

Finally, cross-comparison analysis evidenced that in all feeding condition (D-0, D-20 and D-40) the GI and FCRg detected for sub-adults were respectively higher and lower than values obtained in adults (**Figure 3**).

DISCUSSION

Experimental Setting

Many animal species change their feeding habits during their various life cycle stages, in relation to variations in their dietary requirements. High dietary variations may occur in the transition from the growth to the reproductive phases. In this context, animal protein sources are very important macronutrients for omnivorous species such as *P. lividus* since they provide essential amino acids for growth and reproduction (Scheibling and Hatcher, 2013). Several investigations have suggested the pivotal role of animal proteins, underlining how the somatic and gonadal growth of sea urchins fed with animal-source enriched diets were higher than those fed with entirely vegetable diets (Fernandez and Pergent, 1998; Fernandez and Boudouresque, 2000). However, in these studies the highest growth performances, in terms of somatic and gonadal growth, were obtained with the mix of animal and vegetal sources, whereas the sole animal food had no beneficial effects. These results, although highlighting the importance of animal supply in formulated diets, have also suggested the existence of a specific threshold which must be still investigated in *P. lividus*. Moreover, excessive amounts of animal protein may substantially increase the costs of diet formulation and of the treatment of waste in farming. These protein sources are, in fact, the most expensive nutrients in aquafeeds and their use produces a high level of

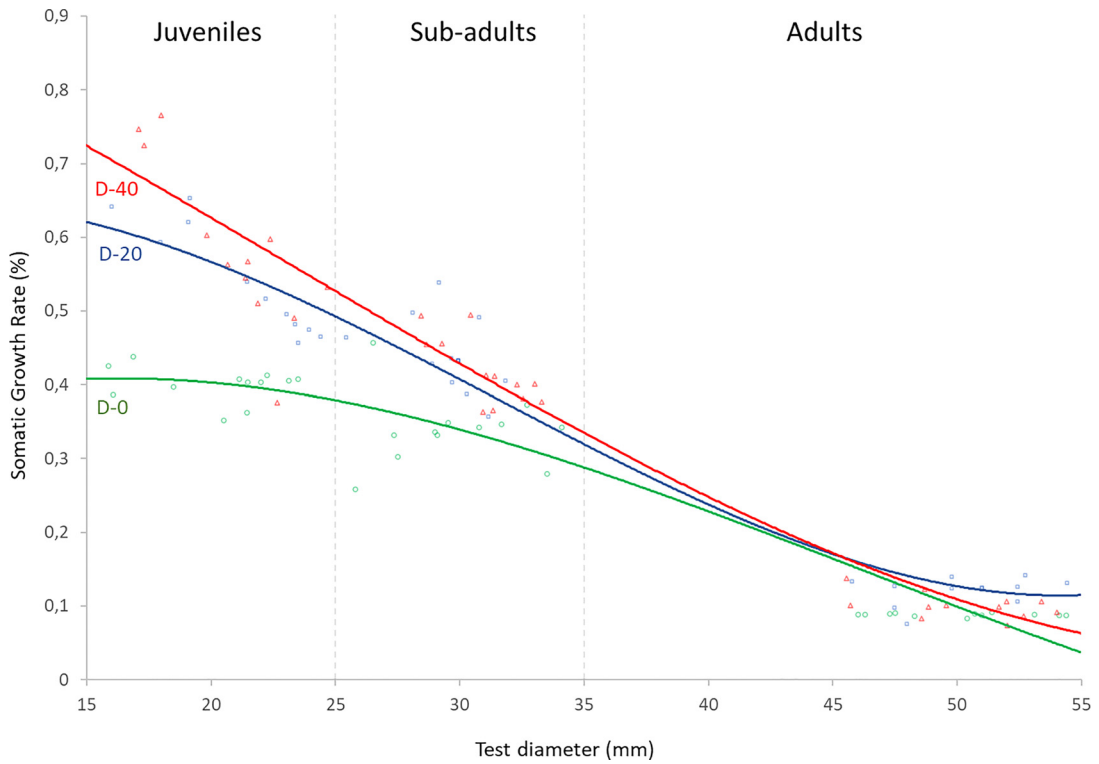

FIGURE 3 | LOESS curves describe the relationship between weight gain and diameter of *P. lividus* fed with three experimental diets. Y axis: Somatic Growth Rate of sea urchins during the four months of the experiment; X axis: diameter of sea urchins at the beginning of the experiment.

nitrogenous pollution, which has a heavy ecological footprint and decreases the quality of the rearing seawater. For this reason, finding an optimal integration level of animal supply in formulated diets is a key issue for the future development of echinoculture.

This study evaluated the adequate provision level of animal supply in three sea urchin life stages, broadening basic knowledge on the dietary requirements of *P. lividus* post-metamorphic rearing. The experimental diets were made up of the same vegetable base in order to investigate exclusively the effects of animal supply variation. Vegetable ingredients employed in this study (carrots, maize and soy) were selected for their market availability, low price and their nutritional profiles which can cover a wide range of dietary requirements. Carrots were chosen for their notable β-carotene content, which plays an important role in promoting egg production and gonad development (Sartori and Gaion, 2016). Soy, instead, is one of the most valuable vegetable protein sources in animal farming (Hammer et al., 2012). Finally, maize is a rich source of carbohydrates, which are the preferred energy source for sea urchins, as it is for most herbivores and omnivores (Hammer et al., 2012).

The present study was set up within a single RAS in order to standardize the environmental conditions and allow the evaluation of the experimental hypothesis without random variations among the tested conditions. This investigation approach is largely shared in current aquaculture literature and also in echinoculture (Spirlet et al., 2001; Fabbrocini and D'Adamo, 2010; Cirino et al., 2017; Baião et al., 2019; Ciriminna et al., 2021; Grosso et al., 2021). Conversely, in separate RASs a random variation in environmental factors (such as a different microbial proliferation, anomalies in the filtering systems, different exposure, etc.) is much more likely than within the same recirculating system.

Furthermore, since the waste residual signature usually represents the main bias in this type of investigation, we adopted strict solutions to avoid their influence on experimental conditions: i) lower biomass (< 5%) than the actual carrying capacity of the RAS was employed; ii) gelatin agar-agar was employed as the binder for the experimental diets to reduce food dissolving in seawater; iii) the leftover food and feces were removed daily from the system; iiii) a water change was carried out at least twice a week, although the water parameters never exceeded the healthy range during the experimental period.

Growth Performances

During the experiment, overall somatic growth (SGR) was higher in sea urchin juveniles than in sub-adults and adults (**Figure 2**).

These results are in agreement with the asymptotic model elaborated by Grosjean et al. (2003), which gives a good description of the growth of *P. lividus*. Positive results in survivorship and growth were obtained with all the experimental diets. However, statistical analysis highlighted that variations in the level of animal-sourced food in the experimental diets differentially affect sea urchin growth in the three life stages. In fact, the cross-comparison analysis showed significant differences in growth performance among juveniles, sub-adults and adults, confirming that each sea urchin size-class have specific dietary requirements.

In juveniles, animal enriched diets (D-40 and D-20) performed better than the entirely vegetal diet (D-0), as highlighted by both growth rate and FCRs values, lower and higher respectively in D-0. These results suggest that *P. lividus* requires a rich animal diet to promote somatic growth during the juvenile stage, suggesting that animal supply provides essential amino acids and fatty acids. Accordingly, Fernandez and Boudouresque (2000) observed the highest weight gain of the smallest size classes of *P. lividus* (TD: 20-25 mm) fed with an animal-diet; and Cook and Kelly (2007) observed that *P. lividus* in an IMTA experiment with *Salmo salar* may benefit from a high animal-enriched diet, up to a test diameter of 25 mm.

SGR value in D-0 resulted to be similar to that achieved by Vizzini et al. (2018), when testing *L. sativa* based diet. In D-20, we obtained SGR values comparable to Lourenço et al. (2021), who tested similar dietary animal supply (12,5%). Both these SGR values were, however, lower than that observed in D-40 in the present study, suggesting the better suitability of a high amount of animal supply (around 40%) to meet the dietary requirements of sea urchin juveniles. According to previous investigations on different sea urchin species, juvenile growth may be maximized with total protein levels around 20% or greater in the diets (Akiyama et al., 2001; Pearce et al., 2002; Kennedy et al., 2005; Heflin et al., 2016). This dietary requirement was highlighted in our study also for *P. lividus* juveniles, since the highest dietary protein levels tested (around 37% in D-40) promoted the maximum weight gain in this life stage. Furthermore, a protein/carbohydrate ratio of around 1:1 in D-40 yielded the best results, indicating that in this condition juveniles may have sufficient energy (from carbohydrates) to utilize protein mainly for growth with a consequent protein sparing.

With regards to sub-adults, SGR, although lower than in juveniles, was higher than in adults in each diet. This is in accordance with general growth theory, for which energy allocation (EA) changes with the variation in animal size. Therefore, the proportion of food energy allocated to somatic growth decreases with the increase in sea urchin size, whereas a higher allocation for reproduction is observed with size growth (Giese et al., 1966; Fernandez and Boudouresque, 2000; McCarron et al., 2009).

Our results, moreover, showed that the best somatic growth values in sub-adults were achieved in both animal enriched diets (D-20 and D-40). No difference was highlighted between the two enriched diets but both cases were significantly higher than the value obtained with the entirely vegetable diet (D-0). These results are comparable with those of Fernandez and Boudouresque (2000) which suggested that the limited amount of proteins versus the insoluble carbohydrates in vegetable meals negatively affects the nutrient uptake of *P. lividus*, causing growth to be lower with a purely vegetable diet. However, from our investigation, it emerged that sub-adult sea urchins require less animal supply than juveniles. This was also confirmed by the lower FCRs data for D-20, highlighting the high efficiency of sub-adult sea urchins consuming the intermediate enriched animal diet (20% of animal supply).

For this size class, the diet yields were investigated also in terms of gonadal production. Indeed, although most of the energy is allocated to somatic growth in this life stage, the sub-adult sea urchins show a trade-off in resource allocation between reproduction and growth (Grosjean et al., 1998). The GI values obtained with fishmeal enriched diets resulted higher than those observed in D-0, highlighting the benefits of animal supply on sub-adult growth. These results were also confirmed by comparison with the current literature. In fact, previous studies that administrated entirely vegetable diets to similar sea urchin size class (Vizzini et al., 2015; Santos et al., 2020b), obtained comparable GI values to those obtained in this study with D-0, but lower GI compared to D-20 and D-40. Furthermore, among the animal enriched diets tested in the present study, D-20 yielded the highest value of gonad index, as well as the lowest value of FCRg. The gonad growth in this feeding condition was also higher than the values obtained with 44.7% and 88.9% of fishmeal supply by Fernandez and Boudouresque (2000). These results, therefore, suggested the better suitability of a low amount of animal supply (around 20%) to meet the dietary requirements for somatic and gonadic growth in this sea urchin size class.

Considering the macronutrient proximate composition of the experimental diets, the best growth performances in sub-adults were achieved with dietary proteins ranging between 29.92 - 37.01%, in D-20 and D-40 respectively. This is in line with the optimal protein range found by Fernandez and Boudouresque (2000) and Fabbrocini et al. (2019) for this sea urchin size class. Finally, conversely to juveniles, sub-adults benefitted from high levels of dietary carbohydrates (42.68% in D-20). This could be related to the different food allocation in these life stages. In fact, sub-adults begin to be reproductively active, and most dietary carbohydrates are generally stored in the gonads (Marsh et al., 2013).

In adult sea urchins, the best measure of well-being is the ability to produce healthy and large reproductive organs (Vadas, 1977). Since the adult gonads are of high market value and can be traded, the most important goal for aquaculture production is to guarantee high gonad quality and yields. During this life stage, sea urchins allocate the highest level of food energy to reproduction, and only a small part to somatic growth. In fact, during our experiment, adult sea urchins showed the lowest somatic growth rates of all the three size classes, although still in line with the SGR values already found in other studies (Baião et al., 2019; Lourenço et al., 2020). Hence, the diet which promoted the highest gonadal production (in terms of GI%) in

our study was assumed to be the best to satisfy the dietary requirements of adult sea urchins, providing key information on its feeding behaviour and nutritional needs. D-20 (20% of animal supply) gave the best result in terms of *P. lividus* GI% (13,58%) and FCRg, suggesting a better capacity of the sea urchins to transform food input into gonad production with this diet. Hence, 20% animal supply emerged as a threshold level for adult sea urchins at which gonad growth is maximized, and over which only disadvantages in terms of cost and water pollution were observed. This finding is also corroborated by the results we obtained with the entirely vegetal diet. Indeed, in adults, contrary to what is observed in sub-adults, D-0 showed similar GI values to those achieved with D-40, evidencing that adult sea urchins need a lower animal supply than in the previous life stages. Raposo et al. (2019) obtained comparable GI to ours in D-0, by feeding sea urchins with maize and spinach. However, adopting the same diet, Sartori and Gaion (2016) achieved a higher value after a four-month experiment in RAS. The different gonad growth detected among these studies may be related to specific experimental conditions, such as the rearing temperature. In our study, the GI value in D-20 resulted higher than in previous investigations with predominantly vegetable diets (Prato et al., 2018; Ruocco et al., 2018), but comparable to those achieved with similar fishmeal supply (Fernandez and Boudouresque, 2020; Ciriminna et al., 2021). Conversely, Cirino et al. (2017) evidenced higher gonad growth than we found in D-20, applying a similar animal supply (25%). This difference is probably due to the smaller size of the sea urchin specimens employed by the authors and/or to the macronutrient levels and ratios in the different experimental feeding conditions. The lipid/protein ratio in the diet tested by Cirino et al. (2017), in fact, was significantly lower than in ours and Hammer et al. (2010) have shown that high dietary levels of lipids (> 9%) may negatively influence growth. Hence, further investigations are still needed to clarify the optimal lipid/protein ratio to adopt in adult sea urchin diets.

Summarizing our results, as far as we know, Fernandez and Boudouresque (2000) were the only authors who compared in parallel the effects of formulated diets on different *P. lividus* life stages. Our study therefore expands on their previous investigation, identifying more specifically the possible optimal level of animal-sourced supplements for the three *P. lividus* life stages. The diet containing 40% animal supply showed high benefits for the juveniles, while levels around 20% performed very well for sub-adults and adults. In addition, by apply a Local regression analysis (LOESS) we were able to determine the most effective diets in terms of the highest growth performances for the full post-metamorphic *P. lividus* life cycle. These findings provide a practical tool for developing efficient and sustainable commercial feeds for *P. lividus* rearing, representing a guideline on how and when to change the level of animal source enrichment in diets during the sea urchin production phases.

General Considerations on *P. lividus* Ecology and Trophic Behavior

This controlled-environment experiment, evidencing the animal protein requirement of *P. lividus*, shed light also on its feeding ecology and local-scale migratory movements. Crook et al. (2000) suggested that daily migration between the sides, interstices, and the upper surface of rocks is size dependent. Previous investigations have claimed that sheltering from predators is the driver of the migratory behavior in the different life stages (Boudouresque and Verlaque, 2020). However, Rico (1989) showed that the percentage of time *P. lividus* spends in motion, depends on the type of food available, although the authors didn't find evidence of a clear relationship with food choice. Our results corroborated this theory, revealing that sea urchin spatial behavior may also reflect the variations in the dietary requirements. Indeed, the juvenile specimens live under rocks and refuges, where they can rely on protein-rich animal food sources (i.e., sponges, bryozoans and polychaetes). The sub-adults, instead, start performing foraging excursions on the photic zone where both algae and animal food sources become available. Adult sea urchins become dominant in algal beds where they graze on predominantly vegetal sources. This micro-habitat migration in *P. lividus* closely matches the feeding requirement shift that emerged in our study, leading us to hypothesize that this may be the root cause or a contributing cause of the sheltering behavior. Then, stable isotope analysis may be a promising tool in order to further investigate this topic as a function of resource availability and habitat features in the natural environment (Boncagni et al., 2019; Camps-Castellà et al., 2020; Rumolo et al., 2020)

CONCLUSION

A balanced mix of vegetal and animal sources in the diets is highly recommended to maximize the growth performance of the sea urchin species, reducing time to market and the production of high quality gonads (Vizzini et al., 2019). However, sea urchins change their feeding habits during their life cycle in relation to variations in dietary requirement. Therefore, finding the optimum level of animal-source supplements in each stage of the sea urchin life cycle is crucial in echinoculture research. Indeed, animal proteins, despite being a very important nitrogen and amino acid source, are also one of the most expensive nutrients in aquafeeds and their metabolism as an energy source is highly inefficient, producing a huge ecological footprint as a result of nitrogenous pollution.

The results of this study provide another step towards the development of a full life-cycle aquaculture of the sea urchin *P. lividus*. More specifically, this 4-month rearing experiment identified the optimal level of animal-sourced supplements in the diets for three *P. lividus* life stages, expanding on the investigation of Fernandez and Boudouresque (2000). Moreover, a general growth model covering the full post-metamorphic *P. lividus* life cycle was defined for each experimental diet, providing a practical tool for the

identification of a better level of animal-sourced supplement in each of the sea urchin rearing phases.

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

LG and AR conceived the experimental design and carried out the investigation, performed the statistical analysis, wrote the first draft of the manuscript. AF, SV, and MS wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

The research and publication materials was supported by FEAMP project: "E84I19000640002" and PON project: "E86C18001440008".

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.865450/full#supplementary-material

REFERENCES

Akiyama, T., Unuma, T., and Yamamoto, T. (2001). Optimum Protein Level in a Purified Diet for Young Red Sea Urchin Pseudocentrotus Depressus. *Fish. Sci.* 67 (2), 361–363. doi: 10.1046/j.1444-2906.2001.00252.x

Baião, L. F., Rocha, F., Costa, M., Sá, T., Oliveira, A., Maia, M. R. G., et al. (2019). Effect of Protein and Lipid Levels in Diets for Adult Sea Urchin Paracentrotus Lividus (Lamarck 1816). *Aquaculture* 506, 127–138. doi: 10.1016/j.aquaculture.2019.03.005

Bartley, D. M., and Bell, J. D. (2008). Restocking, Stock Enhancement, and Sea Ranching: Arenas of Progress. *Rev. Fish. Sci.* 16, 357–365. doi: 10.1080/10641260701678058

Boncagni, P., Rakaj, A., Fianchini, A., and Vizzini, S. (2019). Preferential Assimilation of Seagrass Detritus by Two Coexisting Mediterranean Sea Cucumbers: *Holothuria Polii* and *Holothuria Tubulosa*. *Estua. Coast. Shelf. Sci.* 231, 106464. doi: 10.1016/j.ecss.2019.106464

Boudouresque, C. F., and Verlaque, M. (2020). "Paracentrotus Lividus," in *Developments in Aquaculture and Fisheries Science* (London, United Kingdom: Elsevier B.V), pp. 447–485. doi: 10.1016/B978-0-12-819570-3.00026-3

Camps-Castellà, J., Romero, J., and Prado, P. (2020). Trophic Plasticity in the Sea Urchin Paracentrotus Lividus, as a Function of Resource Availability and Habitat Features. *Mar. Ecol. Prog. Ser.* 637, 71–85. doi: 10.3354/meps13235

Carboni, S., Vignier, J., Chiantore, M., Tocher, D. R., and Migaud, H. (2012). Effects of Dietary Microalgae on Growth, Survival and Fatty Acid Composition of Sea Urchin Paracentrotus Lividus Throughout Larval Development. *Aquaculture* 324–325, 250–258. doi: 10.1016/j.aquaculture.2011.10.037

Ceccherelli, G., Pinna, S., and Sechi, N. (2009). Evaluating the Effects of Protection on Paracentrotus Lividus Distribution in Two Contrasting Habitats. *Estua. Coast. Shelf. Sci.* 81, 59–64. doi: 10.1016/j.ecss.2008.10.007

Ciriminna, L., Signa, G., Vaccaro, A. M., Messina, C. M., Mazzola, A., and Vizzini, S. (2020). Formulation of a New Sustainable Feed From Food Industry Discards for Rearing the Purple Sea Urchin Paracentrotus Lividus. *Aquacult. Nutr.* 26, 1046–1057. doi: 10.1111/anu.13063

Ciriminna, L., Signa, G., Vaccaro, A. M., Visconti, G., Mazzola, A., and Vizzini, S. (2021). Turning Waste Into Gold: Sustainable Feed Made of Discards From the Food Industries Promotes Gonad Development and Colouration in the Commercial Sea Urchin Paracentrotus Lividus (Lamarck 1816). *Aquacult. Rep.* 21, 100881. doi: 10.1016/J.AQREP.2021.100881

Cirino, P., Ciaravolo, M., Paglialonga, A., and Toscano, A. (2017). Long-Term Maintenance of the Sea Urchin Paracentrotus Lividus in Culture. *Aquacult. Rep.* 7, 27–33. doi: 10.1016/j.aqrep.2017.04.003

Cook, E. J., and Kelly, M. S. (2007). Effect of Variation in the Protein Value of the Red Macroalga Palmaria Palmata on the Feeding, Growth and Gonad Composition of the Sea Urchins Psammechinus Miliaris and Paracentrotus Lividus (Echinodermata). *Aquaculture* 270, 207–217. doi: 10.1016/j.aquaculture.2007.01.026

Cook, E. J., and Kelly, M. S. (2009). Co-Culture of the Sea Urchin Paracentrotus Lividus and the Edible Mussel Mytilus Edulis L. On the West Coast of Scotland, United Kingdom. *J. Shellf. Res.* 28 (3), 553–559. doi: 10.2983/035.028.0318

Crook, A. C., Long, M., and Barnes, D. K. (2000). Quantifying Daily Migration in the Sea Urchin Paracentrotus Lividus. *J. Mar. Biol. Assoc. Unit. Kingd.* 80 (1), 177–178. doi: 10.1017/S0025315499001721

Daggett, T. L., Pearce, C. M., Tingley, M., Robinson, S. M. C., and Chopin, T. (2005). Effect of Prepared and Macroalgal Diets and Seed Stock Source on Somatic Growth of Juvenile Green Sea Urchins (Strongylocentrotus Droebachiensis). *Aquaculture* 244, 263–281. doi: 10.1016/j.aquaculture.2004.11.030

Fabbrocini, A., and D'Adamo, R.. (2010). Gamete Maturation and Gonad Growth in Fed and Starved Sea Urchin Paracentrotus lividus (Lamarck, 1816). *J. Shellfish Res.* 29 (4), 1051–1059. doi: 10.2983/035.029.0407

Fabbrocini, A., and D'Adamo, R.. (2012). Gamete Maturation and Gonad Growth in Fed and Starved Sea Urchin Paracentrotus Lividus (Lamarck, 1816). *J. Shellfish Res.* 29 (4), 1051–59. doi: 10.2983/035.029.0407

Fabbrocini, A., Volpe, M. G., di Stasio, M., D'Adamo, R., Maurizio, D., Coccia, E., et al. (2012). Agar-Based Pellets as Feed for Sea Urchins (Paracentrotus Lividus): Rheological Behaviour, Digestive Enzymes and Gonad Growth. *Aquacult. Res.* 43, 321–331. doi: 10.1111/j.1365-2109.2011.02831.x

Fabbrocini, A., Volpe, M. G., Hoseinifar, S. H., Siano, F., Coccia, E., Scordella, G., et al. (2019). Paracentrotus Lividus Roe Enhancement by a Short-Time Rearing in Offshore Cages Using Two Agar-Based Experimental Feed. *Int. J. Aquat. Biol.* 7, 155–165. doi: 10.22034/ijab.v7i3.626

Fernandez, C., and Boudouresque, C. (2000). Nutrition P. Lividus Artificial Food. *Mar. Ecol. Prog. Ser.* 204, 131–141. doi: 10.3354/meps204131

Fernandez, C., and Pergent, G. (1998). Effect of Different Formulated Diets and Rearing Conditions on Growth Parameters in the Sea Urchin Paracentrotus Lividus. *J. Shellf. Res.* 17, 1571–1581.

Giese, A. C., Farmanfarmaian, A., Hilden, S., and Doezema, P. (1966). Respiration During the Reproductive Cycle in the Sea Urchin, Strongylocentrotus Purpuratus. *Biol. Bull.* 130, 192–201. doi: 10.2307/1539696

Grosjean, P., Spirlet, C., Gosselin, P., Vaïtilingon, V., and Jangoux, M. (1998). Land-Based, Closed-Cycle Echiniculture of Paracentrotus Lividus (Lamarck) (Echinoidea: Echinodermata): A Long-Term Experiment at a Pilot Scale. *J. Shellf. Res.* 17 (5), 1523–1531.

Grosjean, P., Spirlet, C., and Jangoux, M. (2003). A Functional Growth Model With Intraspecific Competition Applied to a Sea Urchin, Paracentrotus Lividus. *Can. J. Fish. Aquat. Sci.* 60, 237–246. doi: 10.1139/F03-017

Grosso, L., Rakaj, A., Fianchini, A., Morroni, L., Cataudella, S., and Scardi, M. (2021). Integrated Multi-Trophic Aquaculture (IMTA) System Combining the Sea Urchin Paracentrotus Lividus, as Primary Species, and the Sea Cucumber Holothuria Tubulosa as Extractive Species. *Aquaculture* 534, 736268. doi: 10.1016/J.AQUACULTURE.2020.736268

Guidetti, P., Terlizzi, A., and Boero, F. (2004). Effects of the Edible Sea Urchin, Paracentrotus Lividus, Fishery Along the Apulian Rocky Coast (SE Italy, Mediterranean Sea). *Fish. Res.* 66, 287–297. doi: 10.1016/S0165-7836(03)00206-6

Hammer, Ø., Harper, D. A. T., and Ryan, P. D. (2013). "PAST: Paleontological Statistics," in *Version 3.0. National History Museum* (Oslo, Norway: University of Oslo).

Hammer, H., Powell, M., Gibbs, V., Jones, W., Watts, S., Lawrence, A., et al. (2010). Effect of Dietary Menhaden Oil and Soy Oil on Consumption,Somatic Growth and Gonad Production of the Sea Urchin, Lytechinus Variegatus. *Echinoderms*, 377–383.

Hammer, H. S., Powell, M. L., Jones, W. T., Gibbs, V. K., Lawrence, A. L., Lawrence, J. M., et al. (2012). Effect of Feed Protein and Carbohydrate Levels on Feed Intake, Growth, and Gonad Production of the Sea Urchin, Lytechinus Variegatus. *J. World Aquacult. Soc.* 43, 145–158. doi: 10.1111/j.1749-7345.2012.00562.x

Heflin, L. E., Gibbs, V. K., Powell, M. L., Makowsky, R., Lawrence, J. M., Lawrence, A. L., et al. (2012). Effect of Dietary Protein and Carbohydrate Levels on Weight Gain and Gonad Production in the Sea Urchin Lytechinus Variegatus. *Aquaculture* 358–359, 253–261. doi: 10.1016/j.aquaculture.2012.06.009

Heflin, L. E., Makowsky, R., Taylor, J. C., Williams, M. B., Lawrence, A. L., and Watts, S. A. (2016). Production and Economic Optimization of Dietary Protein and Carbohydrate in the Culture of Juvenile Sea Urchin Lytechinus Variegatus. *Aquaculture* 463, 51–60. doi: 10.1016/j.aquaculture.2016.05.023

Huguenin, J. E., and Colt, J. (2002). *Design and Operating Guide for Aquaculture Seawater Systems* (Amsterdam, Netherlands: Elsevier).

Kelly, M. S. (2004). *Sea Urchin Aquaculture: A Review and Outlook. Echinoderms* (London: Taylor & Francis Group), 283–289.

Kennedy, E. J., Robinson, S. M., Parsons, G. J., and Castell, J. D. (2005). Effect of Protein Source and Concentration on Somatic Growth of Juvenile Green Sea Urchins Strongylocentrotus Droebachiensis. *J. World Aquacult. Soc.* 36 (3), 320–336. doi: 10.1111/j.1749-7345.2005.tb00336.x

Lourenço, S., Cunha, B., Raposo, A., Neves, M., Santos, P. M., Gomes, A. S., et al. (2021). Somatic Growth and Gonadal Development of Paracentrotus Lividus (Lamarck 1816) Fed With Diets of Different Ingredient Sources. *Aquaculture* 539, 736589. doi: 10.1016/J.AQUACULTURE.2021.736589

Lourenço, S., José, R., Andrade, C., and Valente, L. M. P. (2020). Growth Performance and Gonad Yield of Sea Urchin Paracentrotus Lividus (Lamarck 1816) Fed With Diets of Increasing Protein: Energy Ratios. *Anim. Feed. Sci. Technol.* 270, 114690. doi: 10.1016/j.anifeedsci.2020.114690

Marsh, A. G., Powell, M., and Watts, S. (2013). "Energy Metabolism and Gonad Development," in *Sea Urchins: Biology and Ecology, Third Ed.* Ed. J. Lawrence (Amsterdam, The Netherlands: Elsevier BV), 35–50.

McBride, S. C. (2005). Sea Urchin Aquaculture. *Am. Fish. Soc.* 46, 179–208. doi: 10.1201/9780203970881.ch48

McCarron, E., Burnell, G., Kerry, J., and Mouzakitis, G. (2010). An Experimental Assessment on the Effects of Photoperiod Treatments on the Somatic and Gonadal Growth of the Juvenile European Purple Sea Urchin Paracentrotus Lividus. *Aquacult. Res.* 41 (7), 1072–1081. doi: 10.1111/j.1365-2109.2009.02392.x

McCarron, E., Burnell, G., and Mouzakitis, G. (2009). Growth Assessment on Three Size Classes of the Purple Sea Urchin Paracentrotus Lividus Using Continuous and Intermittent Feeding Regimes. *Aquaculture* 288, 83–91. doi: 10.1016/j.aquaculture.2008.11.025

Morroni, L., Rakaj, A., Grosso, L., Fianchini, A., Pellegrini, D., and Regoli, F. (2020). Sea Cucumber Holothuria Polii (Delle Chiaje 1823) as New Model for Embryo Bioassays in Ecotoxicological Studies. *Chemosphere* 240, 124819. doi: 10.1016/j.chemosphere.2019.124819

Mortensen, A., Siikavuopio, I., and James, P. (2012). Water Quality Requirements for Culture of the Green Sea Urchin, Strongylocentrotus Droebachiensis. *World Aquacult.* 46–50.

Pearce, C. M., Daggett, T. L., and Robinson, S. M. C. (2002). Effect of Protein Source Ratio and Protein Concentration in Prepared Diets on Gonad Yield and Quality of the Green Sea Urchin, Strongylocentrotus Droebachiensis. *Aquaculture* 214, 307–332. doi: 10.1016/S0044-8486(02)00041-8

Prato, E., Fanelli, G., Biandolino, F., Chiantore, M., Secci, M., Angioni, A., et al. (2018). Influence of a Prepared Diet and a Macroalga (Ulva Sp.) on the Growth, Nutritional and Sensory Qualities of Gonads of the Sea Urchin Paracentrotus Lividus. *Aquaculture* 493, 240–250. doi: 10.1016/j.aquaculture.2018.05.010

Rakaj, A., Fianchini, A., Boncagni, P., Lovatelli, A., Scardi, M., and Cataudella, S. (2018). Spawning and Rearing of Holothuria Tubulosa: A New Candidate for Aquaculture in the Mediterranean Region. *Aquacult. Res.* 49 (1), 557–568. doi: 10.1111/are.13487

Rakaj, A., Fianchini, A., Boncagni, P., Scardi, M., and Cataudella, S. (2019). Artificial Reproduction of Holothuria Polii: A New Candidate for Aquaculture. *Aquaculture* 498, 444–453. doi: 10.1016/j.aquaculture.2018.08.060

Rakaj, A., Morroni, L., Grosso, L., Fianchini, A., Pensa, D., Pellegrini, D., et al. (2021). Towards Sea Cucumbers as a New Model in Embryo-Larval Bioassays: Holothuria Tubulosa as Test Species for the Assessment of Marine Pollution. *Sci. Total. Environ.* 787, 147593. doi: 10.1016/j.scitotenv.2021.147593

Raposo, A. I. G., Ferreira, S. M. F., Ramos, R., Santos, P. M., Anjos, C., Baptista, T., et al. (2019). Effect of Three Diets on the Gametogenic Development and Fatty Acid Profile of Paracentrotus Lividus (Lamarck 1816) Gonads. *Aquacult. Res.* 50, 2023–2038. doi: 10.1111/are.14051

Rico, V. (1989). "Contribution a`L'E´Tude Des Preferenda Alimentaires Et Du Comportement Moteur De L'Oursin Re´Gulierparacentrotus Lividus," in *Advenced Degree Dissertation Oceanography* (Marseille: Universite´Aix-Marseille 2).

Rumolo, P., Zappes, I. A., Fabiani, A., Barra, M., Rakaj, A., Palozzi, R., et al. (2020). The Diet of Weddell Seals (Leptonychotes Weddellii) in Terra Nova Bay Using Stable Isotope Analysis. *Eur. Zool. J.* 87 (1), 94–104. doi: 10.1080/24750263.2020.1720832

Ruocco, N., Zupo, V., Caramiello, D., Glaviano, F., Polese, G., Albarano, L., et al. (2018). Experimental Evaluation of the Feeding Rate, Growth and Fertility of the Sea Urchins Paracentrotus Lividus. *Invertebr. Reprod. Dev.* 62, 209–220. doi: 10.1080/07924259.2018.1504125

Sala, E. (1997). Fish Predators and Scavengers of the Sea Urchin Paracentrotus Lividus in Protected Areas of the North-West Mediterranean Sea. *Mar. Biol.* 129 (3), 531–539. doi: 10.1007/s002270050194

Santos, P. M., Albano, P., Raposo, A., Ferreira, S. M. F., Costa, J. L., and Pombo, A. (2020a). The Effect of Temperature on Somatic and Gonadal Development of the Sea Urchin Paracentrotus Lividus (Lamarck 1816). *Aquaculture* 528, 735487. doi: 10.1016/J.AQUACULTURE.2020.735487

Santos, P. M., Ferreira, S. M. F., Albano, P., Raposo, A., Costa, J. L., and Pombo, A. (2020b). Can Artificial Diets be a Feasible Alternative for the Gonadal Growth and Maturation of the Sea Urchin Paracentrotus Lividus (Lamarck 1816)? *J. World Aquacult. Soc.* 51, 463–487. doi: 10.1111/jwas.12656

Sartori, D., and Gaion, A. (2016). Can Sea Urchins Benefit From an Artificial Diet? Physiological and Histological Assessment for Echinoculture Feasibility Evaluation. *Aquacult. Nutr.* 22, 1214–1221. doi: 10.1111/anu.12326

Scheibling, R. E., and Hatcher, B. G. (2013). "Strongylocentrotus Droebachiensis," in *Developments in Aquaculture and Fisheries Science*, vol. Vol. 38. (London, United Kingdom: Elsevier), pp. 381–412.

Schlosser, S. C., Lupatsch, I., Lawrence, J. M., Lawrence, A. L., and Shpigel, M. (2005). Protein and Energy Digestibility and Gonad Development of the European Sea Urchin Paracentrotus Lividus (Lamarck) Fed Algal and Prepared Diets During Spring and Fall. *Aquacult. Res.* 36, 972–982. doi: 10.1111/j.1365-2109.2005.01306.x

Shpigel, M., Shauli, L., Odintsov, V., Ben-Ezra, D., Neori, A., and Guttman, L. (2018). The Sea Urchin, Paracentrotus Lividus, in an Integrated Multi-Trophic Aquaculture (IMTA) System With Fish (Sparus Aurata) and Seaweed (Ulva Lactuca): Nitrogen Partitioning and Proportional Configurations. *Aquaculture* 490, 260–269. doi: 10.1016/j.aquaculture.2018.02.051

Spirlet, C., Grosjean, P., and Jangoux, M. (1998). Reproductive Cycle of the Echinoid Paracentrotus Lividus: Analysis by Means of the Maturity Index. *Invertebr. Reprod. Dev.* 34, 69–81. doi: 10.1080/07924259.1998.9652355

Spirlet, C., Grosjean, P., and Jangoux, M. (2001). Cultivation of Paracentrotus Lividus (Echinodermata: Echinoidea) on Extruded Feeds: Digestive Efficiency, Somatic and Gonadal Growth. *Aquacult. Nutr.* 7 (2), 91–100.

Tortonese, E. (1965). "Echinodermata," in *Fauna D'italia Iv.* (Bologna, Italy: Edizioni Calderini).

Vadas, R. L. (1977). Preferential Feeding : An Optimization Strategy in Sea Urchins. *Ecol. Soc. America Stable.* 47, 337–371. doi: 10.2307/1942173

Vizzini, S., Miccichè, L., Vaccaro, A., and Mazzola, A. (2015). Use of Fresh Vegetable Discards as Sea Urchin Diet: Effect on Gonad Index and Quality. *Aquacult. Int.* 23, 127–139. doi: 10.1007/s10499-014-9803-5

Vizzini, S., Visconti, G., Signa, G., Romano, S., and Mazzola, A. (2019). A New Sustainable Formulated Feed Based on Discards From Food Industries for

Rearing the Sea Urchin Paracentrotus Lividus (Lmk). *Aquacult. Nutr.* 25, 691–701. doi: 10.1111/anu.12890

Vizzini, S., Visconti, G., Vaccaro, A., and Mazzola, A. (2018). Experimental Rearing of the Sea Urchin Paracentrotus Lividus Fed With Discards of the Lettuce Lactuca Sativa in a Sea-Based System. *Aquacult. Res.* 49 (2), 631–636. doi: 10.1111/are.13492

Volpe, M. G., Fabbrocini, A., Siano, F., Coccia, E., Scordella, G., Licchelli, C., et al (2018). Gonad Quality of Sea Urchin Paracentrotus Lividus Cultured in an Offshore Pilot-Scale Trial on the South-East Italian Coast. *Aquacult. Nutr* 24 (5), 1444–1455. doi: 10.1111/anu.12681

Whitlock, M. C., and Schluter, D. (2010). *Analisi Statistica Dei Dati Biologici.* (Zanichelli).

Yeruham, E., Rilov, G., Shpigel, M., and Abelson, A. (2015). Collapse of the Echinoid Paracentrotus Lividus Populations in the Eastern Mediterranean - Result of Climate Change? *Sci. Rep.* 5 (1), 1–6. doi: 10.1038/srep13479

Zupo, V., Glaviano, F., Paolucci, M., Ruocco, N., Polese, G., di Cosmo, A., et al. (2019). Roe Enhancement of Paracentrotus Lividus: Nutritional Effects of Fresh and Formulated Diets. *Aquacult. Nutr.* 25, 26–38. doi: 10.1111/anu.12826

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.

The reviewer CS declared a past co-authorship with one of the authors C-FB to the handling Editor.

frontiers | Frontiers in Marine Science

ORIGINAL RESEARCH
published: 22 July 2022
doi: 10.3389/fmars.2022.932310

The Impact of Climate Change on Bivalve Farming: Combined Effect of Temperature and Salinity on Survival and Feeding Behavior of Clams *Ruditapes decussatus*

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European clam (*Ruditapes decussatus*) is one of the most relevant emergent bivalve species from the aquaculture sector in Europe with high economic value. Climate changes represent a potential limiting factor to this activity, directly interfering with the survival and behavior of bivalves. Severe fluctuations in temperature along with periods of heavy rainfall or periods of drought that significantly change the salinity can promote physiological stress in bivalves, resulting in changes in physiological and behavioral responses and, in extreme cases, leading to high mortalities. This study aimed to evaluate the combined effect of temperature and salinity on mortality and feeding behavior of *R. decussatus*. Juveniles and adults were exposed to combined ranges of temperature (5°C–35°C) and salinity (0–40). Mortality and feeding behavior were registered every 24 h of each 120-h trial. A control temperature range was set between 15°C and 23°C, where mortality and feeding behavior were considered as the normal scenario. Our data suggested salinity 15 as a "turning point," a point from which occurred distinct patterns in mortality and feeding behavior. The results evidently indicate that abrupt reductions in salinity and sharp increases in temperature will lead to high mortality of *R. decussatus.* Juveniles were revealed to be more sensitive to the increase of temperature in a less saline environment, to suffer greater and faster mortalities, and to be more resistant to extremely high temperatures under more saline conditions. The high temperatures and sporadic heavy rainfall that are predicted to occur in the south of Europe due to climate changes will contribute to compromise the recruitment of European clam, thus threatening the production of this species and consequently impacting the economic sector.

Keywords: European clam, heavy rainfalls, heatwaves, mortality, feeding behavior

OPEN ACCESS

Edited by:
Sílvia Lourenço, Instituto Politécnico de Leiria, Portugal

Reviewed by:
Bin Xia, Qingdao Agricultural University, China;
Rula Dominguez, Centro Tecnológico del Mar (CETMAR), Spain

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Specialty section:
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

Received: *29 April 2022*
Accepted: *21 June 2022*
Published: *22 July 2022*

Citation:
Rato A, Joaquim S, Matias AM, Roque C, Marques A and Matias D (2022) The Impact of Climate Change on Bivalve Farming: Combined Effect of Temperature and Salinity on Survival and Feeding Behavior of Clams Ruditapes decussatus. Front. Mar. Sci. 9:932310. doi: 10.3389/fmars.2022.932310

INTRODUCTION

Climate changes are of great concern, being the focus of scientists and policymakers (Verdelhos et al., 2015a). In the next 100 years, changes in climate are predicted to occur, at a global scale, through increases in sea and air temperature, water acidification, and changes in seawater salinity, sea level, and precipitation (Poulin and Mouritsen, 2006; Matozzo and Marin, 2011; IPCC, 2021).

According to the Intergovernmental Panel on Climate Change (IPCC) (2021), until the end of the century, it is hypothesized that, in the lower emissions scenario, the global temperature will rise by 1.5°C with increasing intensity and frequency of extreme rainfalls and heatwaves. In the worst possible scenario, global temperature may reach an increment of 4.4°C, with the potential to achieve 5.7°C, causing large-scale coastal inundation and extremely destructive weather with parts of the planet being uninhabitable during heat waves (IPCC, 2021).

Global climate changes also interfere with hydrological cycles, namely, through the occurrence and intensity of extreme events such as precipitation and/or drought (Fenoglio et al., 2010). In turn, these events may have a direct impact on salinity, mainly in coastal and estuarine areas. For instance, in coastal areas, warmer temperatures can cause an increase in seawater evaporation and a reduction in rainfall, thus increasing salinity or causing heavy tropical rainfall that may be formed by warmer temperatures, consequently decreasing salinity (Matozzo and Marin, 2011).

Even though species have dealt with climate changes throughout their evolutionary history, the fast rate at which changes in climate are occurring (Philippart et al., 2003) may comprise the capacity of organisms to adapt to these variations, therefore affecting their physiology, growth, behavior, and survival (Otero et al., 2013; Velez et al., 2016).

Temperature and salinity are two of the most important physical factors affecting intertidal organisms, such as bivalves, playing an important role on their abundance and distribution (Gosling, 2004; Booij, 2005; Gharbi et al., 2016). While temperature is considered to affect energy balance and level of activity (Sobral and Widdows, 1997), salinity has an influence on physiological processes (Navarro and Gonzalez, 1998; Carregosa et al., 2014).

Besides the spatial distribution of bivalves, several aspects of their biology, such as growth, reproduction, feeding, behavior, and respiration (among others), are also affected by changes in climate (Gosling, 2004). During emersion periods, bivalves also have to deal with hypoxia and the interruption of feeding, since they are filter feeders (Sobral and Widdows, 1997; Gharbi et al., 2016). When exposed to stressful conditions, such as severe variations in temperature and salinity, bivalves usually close the valves—as a mechanism of protection against osmotic stress (Carregosa et al., 2014)—reduce feeding activity, and show slower growth and respiration rates and, consequently, alterations in oxygen consumption (Navarro and Gonzalez, 1998; Sarà et al., 2008; Carregosa et al., 2014).

European clam (*Ruditapes decussatus*) is a commercially and ecologically important species, broadly distributed along the European, Mediterranean, and North African coastal and estuarine waters (de Sousa et al., 2011; Matias et al., 2011; Cravo et al., 2012). In Portugal, this species is crucial to the aquaculture's revenue, being extensively produced and harvested in Ria Formosa, representing 90% of the national production (Cravo et al., 2012; Matias et al., 2013; De Marchi et al., 2020). Ria Formosa is a highly productive mesotidal lagoon system located in the south coast of Portugal and, as an intertidal environment, is severely affected by temperature and salinity. The Ria Formosa lagoon is vital for local communities not only because of tourism and salt extraction but also due to bivalve harvesting and aquaculture (Matias et al., 2013).

Up to now, majority of the studies focused mainly on the variation of only one factor of climate change, even though, in the natural environment, organisms are affected by the variation of several factors combined. This way, hypothesizing the impact of changes in climate on lagoon areas, such as Ria Formosa, and considering the economic and ecological importance of *R. decussatus*, the current study was designed to evaluate the combined effects of different salinities (0–40) and temperatures (5°C–35°C) on the mortality and feeding behavior of *R. decussatus*. To better understand the effects of changes in climate in the life cycle of organisms and in their production, we chose to expose juveniles and adults to these combinations.

MATERIALS AND METHODS

Experimental Design

Juveniles and adults of clams *R. decussatus* (3 ± 0.9 mm and 31 ± 3.1 mm shell length, respectively) were captured in the Ria Formosa lagoon (36°59′31.46″N, 7°55′21.90″W) and exposed to different combinations of water temperatures and salinities.

The Ria Formosa lagoon is characterized by subtidal channels, tidal flats, and salt marshes that are protected by a system of barrier islands and interact with oceanic waters *via* several inlets (Ribeiro et al., 2008; Cravo et al., 2012). This lagoon does not have a significant freshwater input, except for the contribution of the small river Gilão (Cravo et al., 2012).

The annual water temperature variation in this coastal area over the last years (2016–2020) was between 14.14 ± 0.86°C and 22.20 ± 1.14°C [data obtained from Tunipex, S.A., Olhão, Portugal (Tunipex, 2022)], and salinity was usually around 35–37 (Cravo et al., 2012). Considering these factors, a control temperature range between 15°C and 23°C, inclusive, and salinity 35 were established. The temperatures tested were 5°C, 15°C, 20°C, 23°C, 26°C, 29°C, 32°C, and 35°C, while salinity ranged from 0 to 40 at intervals of 5. Extreme temperatures and salinities regarding control were chosen in order to simulate the predicted scenarios by the IPCC.

A total of 360 individuals of each size class were equally distributed into the experimental treatments: five individuals per treatment, in a total of 72 combinations of temperature and salinity. Each treatment tank (50 l), corresponding to a different temperature, was filled with water that was cooled down to 5°C and 15°C by a refrigerator or heated up to 35°C by a resistance provided with a thermostat. In each tank, nine beakers (V = 0.5 l) were placed and each one was filled with 1 μm of filtered seawater and adjusted to each desired salinity (0–40) (**Figure 1**). Each salinity was prepared by dissolving salt from the Ria Formosa lagoon in dechlorinated tap water. Tanks and beakers were continuously aerated to ensure oxygenation and to maintain constant temperature and salinity. Temperatures were verified twice a day throughout the experiment and adjusted if needed. The water of beakers was changed daily, and each one was refilled with water with the respective treatment of temperature and salinity.

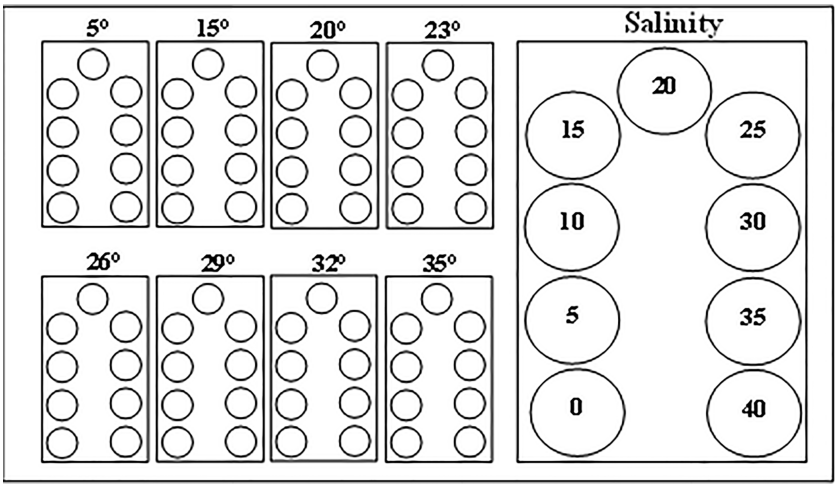

FIGURE 1 | Schematic representation of the experimental design.

Individuals were fed daily with 10 ml of the microalgae *Tisochrysis lutea* (approximately 60 cell/µl). The amount of food was calculated in such a way that it would not become a stress factor and to avoid salinity variations. Three and two trials of 120 h (Verdelhos et al., 2015a; Verdelhos et al., 2015b) were performed for adults and juveniles, respectively.

Mortality and Feeding Behavior

Mortality and feeding behavior were evaluated and registered every 24 h. Individuals were considered dead every time they were unable to close their valves after mechanical stimulus (Gosling, 2004), being then removed from the beakers.

Feeding behavior was evaluated through absence or presence of feces, scored as 0 or 1, respectively (Gosling, 2004).

Data Analysis

One-way ANOVA was applied to test for differences between trials. Since no statistical differences were detected between trials (ANOVA, $p > 0.05$), the results were analyzed considering the average of the different trials.

A preliminary analysis of mortality results suggested the existence of a "turning point" at salinity 15. Thus, to determine the median lethal temperature required for 50% of mortality (LC50) to occur after 120 h of exposure and the 95% confidence intervals (95% CI), mortality results were divided into two groups: salinities equal or below 15 ($S \leq 15$) and salinities above 15 ($S > 15$). Then, these grouped results were fitted to a logistic equation:

$$y = min + \frac{(max - min)}{1 + \left(\frac{x}{LC50}\right)^{-Hillslope}}$$

(where y = mortality; x= temperature; min, max, and Hillslope are estimated parameters).

Median lethal time (Lt50), which refers to the exposure time necessary for a given combination of temperature and salinity to cause 50% of mortality, was calculated through linear regressions.

The mean percentage of mortality after 120 h of exposure as well as Lt50 results of both size classes were plotted into contour graphs.

Feeding behavior was analyzed as: Feeding behavior= feces (24h + 48h + 72h + 96h + 120h)/5 ranging from 0 to 1.

Principal component analysis (PCA) was performed to determine the degree of association between factors (temperature, salinity, mortality, and feeding behavior).

All statistical analyses were performed using SigmaPlot 12.5 statistical package and XLSTAT 2022.1.2 statistical software for PCA analysis.

RESULTS

Mortality

Low salinities and high temperatures caused higher mortality rates, in both juveniles and adults, after 120 h of exposure (**Figure 2**).

In the control temperature range (15°C–23°C), juveniles were revealed to be more sensitive to the salinity variations, reaching 100% of mortality, mainly at the upper limit of the range (23°C) and at salinities lower than 15. As salinity increased, juveniles' mortality decreased, varying between 0% and 20%. In adults, the mortality rate reached 60% of the individuals at salinities lower than 15. Above this salinity and at this temperature range, there was no mortality.

Below 15°C, the observed mortality was not significant, being very close to zero, in nearly all of the salinities tested, for both juveniles and adults.

Above the upper limit of the control temperature range (23°C) and salinities inferior to 15, the observed mortality was almost always extremely high, for both size classes. In more saline waters, juveniles seemed to be more resistant than adults,

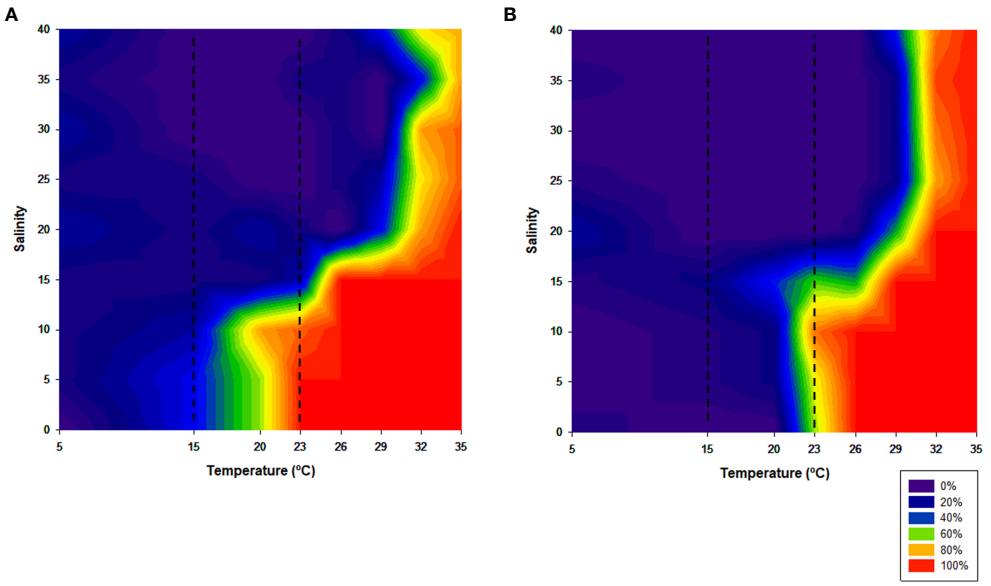

FIGURE 2 | Percentage of mortality after 120 h of exposure along the temperature and salinity ranges. **(A)** juveniles; **(B)** adults. Dotted lines represent the established control temperature range (15°C–23°C).

mainly at extreme temperatures (32°C and 35°C), where 100% of mortality of the adult population occurred.

Since our results showed that salinity 15 seemed to be a "turning point" for individuals' survival, thus LC50 at 120 h of exposure was calculated considering the group of salinities equal and inferior to 15 (S ≤15: 0, 5, 10, 15) and the group of salinities above (S >15: 20, 25, 30, 35, 40).

For both juveniles and adults, mortality results fitted into a logistic distribution within the tested temperature. LC50 at 120 h was estimated according to following equations:

$$Juveniles_{S\leq 15} : y = 10.88 + \frac{(103.67 - 10.88)}{1 + \left(\frac{x}{20.28}\right)^{-8.37}} \quad r^2 = 0.84;$$

$$Juveniles_{S>15} : y = 7.12 + \frac{(90.15 - 7.12)}{1 + \left(\frac{x}{31.08}\right)^{-30.56}} \quad r^2 = 0.90;$$

$$Adults_{S\leq 15} : y = 4.20 + \frac{(99.12 - 4.20)}{1 + \left(\frac{x}{22.29}\right)^{-16.79}} \quad r^2 = 0.94;$$

$$Adults_{S>15} : y = 1.46 + \frac{(100.56 - 1.46)}{1 + \left(\frac{x}{30.08}\right)^{-33.46}} \quad r^2 = 0.97;$$

For S ≤15, LC50 after 120 h of exposure was lower than that for S >15, for both juveniles (20.28°C; 95% CI: 18.36–22.19°C and 31.08°C; 95% CI: 30.36–31.80°C, respectively) and adults (22.29°C; 95% CI: 20.54–23.04°C and 30.08°C; 95% CI: 29.68–30.49°C, respectively).

In general, the time needed for 50% mortality (Lt50) of both juveniles and adults to occur is higher than 120 h, mainly until 23°C, with exception of juveniles that, for this temperature and salinities below or equal to 10, it would only take between 65 and 70 h (**Figure 3**).

In temperatures higher than 23°C and salinities less than or equal to 15, Lt50 substantially decreased as temperature increased, being that juveniles would only survive less than 20 h at extreme temperatures (32°C and 35°C). Adults appeared to be more resistant, surviving longer at this temperature and salinity combination. On the other hand, at higher salinities, the opposite occurred, i.e., juveniles seemed to be more tolerant than adults; however, the mortality of 50% of the adult population would never be expected to occur below 30 h of exposure.

Feeding Behavior

Feeding behavior seemed to be highly influenced by salinity, regardless of the temperature, for both juveniles and adults. In both size classes, at salinities lower than 15, it appeared that individuals did not feed, since the presence of feces was not observed. From this "turning point" and at the control temperature range, the feeding behavior of juveniles and adults was maximum, with juveniles exhibiting an index of 1 while that for adults was 0.93 (**Figure 4**).

Out of this range, juveniles were less active at lower temperatures and more active at extreme temperatures. On the other hand, adults showed the opposite pattern. For instance, at

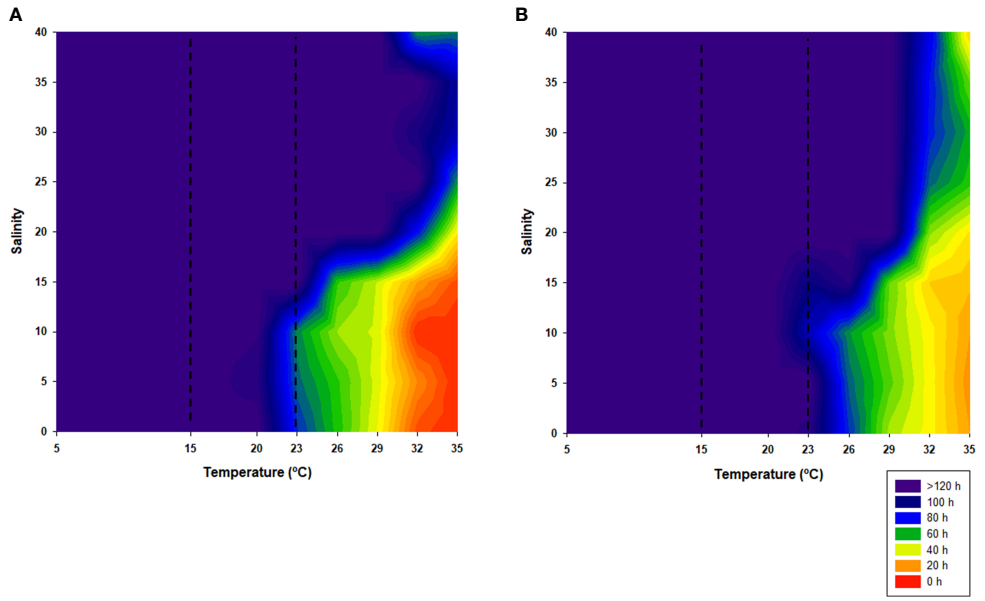

FIGURE 3 | Median lethal time (Lt50) for juveniles **(A)** and adults **(B)**. Dotted lines represent the established control temperature range (15°C–23°C).

lower temperatures, the feeding behavior index of juveniles was 0.1, while for adults this index was about 0.7, namely, at salinities higher than 25. As temperature increased to extreme values, the juveniles' feeding behavior index remained high, except for the higher salinity (40). Comparatively, at extreme temperatures (32°C and 35°C), adults apparently stop feeding.

Principal Component Analysis

The PCA analysis resulted in two principal components (PC1 and PC2) that together described 90.61% and 93.05% of the overall variability of the data (**Figure 5**), for juveniles and adults, respectively. For both juveniles and adults, the PCA biplot displays that mortality was positively correlated with temperature ($r_{juveniles}$ = 0.597; r_{adults} = 0.745; $p< 0.05$) while negatively with salinity ($r_{juveniles}$ = -0.552; r_{adults} = -0.383; $p< 0.05$) and feeding behavior ($r_{juveniles}$ = -0.667; r_{adults} = -0.603; $p< 0.05$). Salinity had a strong positive influence on feeding behavior ($r_{juveniles}$ = 0.753; r_{adults} =0.835; $p< 0.05$).

DISCUSSION

Besides global warming and the consequent increase in seawater temperature, the IPCC (2021) has predicted higher intensity and frequency of extreme climatic events, such as heavy rainfalls, heatwaves, and droughts. Variations in temperature and salinity caused by these events will have a great impact on the survival and behavior of organisms, mainly those inhabiting intertidal areas, like bivalves. The stress induced by temperature and salinity fluctuations are distinct from each other (Woodin et al., 2020), and each species responds differently. Indeed, this work revealed distinct effects on the mortality and feeding behavior of *R. decussatus* juveniles and adults for varied combinations of temperature and salinity. As expected for euryhaline species, clams revealed a higher tolerance to salinity fluctuations. However, the temperature had a great impact on clams' survival and behavior and, when combined with low salinities, revealed to be lethal.

In the current study, we have assumed a control range of temperatures (15°–23°C) and salinities (35–37), considering the annual variation in the Ria Formosa lagoon. It is important to underline that in this "normal" scenario, the mortality was residual, and the feeding behavior of both juveniles and adults was maximum.

Salinity 15 represents a "turning point," a point from which occurred a change in mortality and feeding behavior. Woodin et al. (2020) also proposed the existence of a "breaking point" between salinities 15 and 20, below which there was a significant reduction in the behavioral activity of three venerid species (*Venerupis corrugata*, *R. decussatus*, and *R. philippinarum*). Likewise, Domínguez et al. (2020) suggested salinity 15 as a threshold below which was observed a sharp reduction in activity (burrowing and pumping) and scope for growth, and a high frequency of valve closure of *Cerastoderma edule*, *R. decussatus*, *R. philippinarum*, and *V. corrugata*. Besides the effect on activity behavior, our data suggested that salinities below 15 also had an impact on mortality when combined with high temperatures.

Below the salinity "turning point," neither juvenile nor adult populations exhibited feeding behavior, therefore suggesting that salinity has a major impact on overall activity. Juveniles' feeding behavior differed from that of adults at extreme temperatures: juveniles were more active at high temperatures while adults were at lower ones. Sarà et al. (2008) also found that low temperature and salinity caused a reduction in the clearance rate

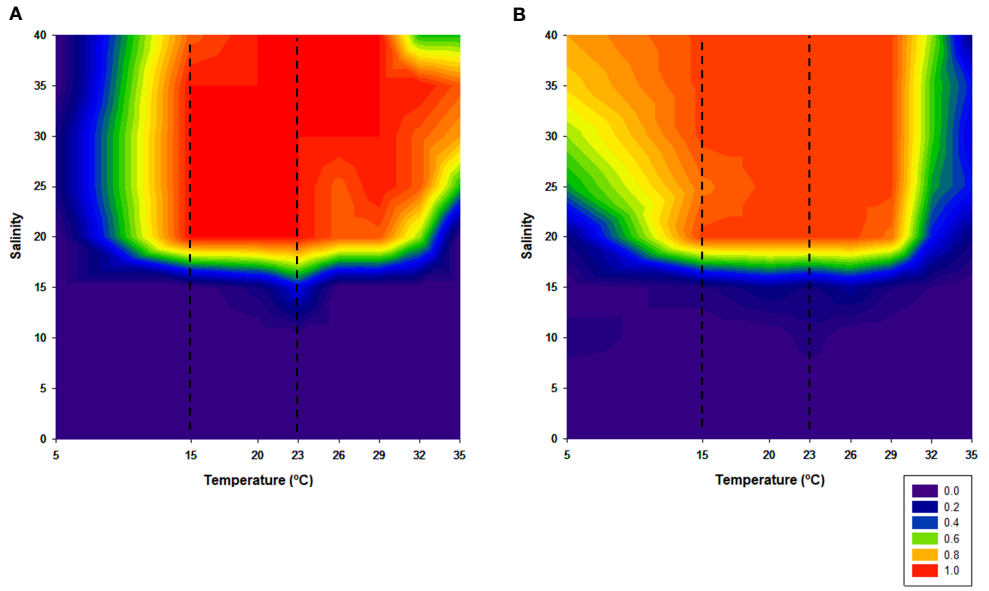

FIGURE 4 | Feeding behavior of juveniles **(A)** and adults **(B)**. Dotted lines represent the established control temperature range (15°C–23°C)

of the bivalve species *Brachidontes pharaonis*. The reduction in feeding behavior has severe consequences for growth, survival, and reproduction (Woodin et al., 2020).

The temperature had a great impact on clams' survival, exhibiting a strong positive correlation with mortality. Regardless of the salinity, low temperatures (<15°C) did not cause any mortality. As the temperature increased and below the salinity "turning point," there was a significant increase in clams' mortality, with juveniles more susceptible even within the control temperature range.

High temperatures and low salinities proved to be quite lethal to clams. Above the salinity "turning point," juveniles appeared to be more resistant than adults, mainly at extremely high temperatures (32° and 35°C).

These data are also supported by the median lethal temperature (LC50). The temperature needed to cause death to 50% of juveniles, at salinities ≤15, was inferior (20.28°C) than that of adults (22.29°C). At salinities higher than 15, both adults and juveniles can endure higher temperatures, with juveniles being more resilient, exhibiting 50% of mortality at 31.08°C while 50% of adults died at 30.08°C.

The effect of temperature and salinity on clams' mortality not only depends on the variations of the parameters themself but also on the time of individuals' exposure to it (Parada et al., 2012). The higher the exposure time to these variations, namely, to high temperatures, the greater the mortality. At low temperatures (≤23°C) and low salinities (≤15), it would take more than 120 h to cause 50% of mortality, maybe due to the clams' capacity to remain with the valves closed under these stressful conditions. The situation became more critical in warmer (>23°C) and less saline waters (≤15), especially for juveniles, where only 20 h of exposure to these conditions was needed for 50% of mortality in the juvenile population to occur. In more saline waters (>15), only extremely high temperatures (32°C–35°C) were lethal, with adults more sensitive than juveniles, taking about 30 h to cause 50% mortality. Under these thermal and saline stresses, valve closure seems to be the most important bivalve response and defined the median lethal time (Lt50) of *R. decussatus*. This mechanism protects the soft tissues by isolating the bivalve from the surrounding environment and, therefore, limiting exposure to osmotic stress (Woodin et al., 2020). Bivalves are able to tolerate valve closure for short and cyclic periods, like between tides (Nossier, 1986; Domínguez et al., 2020), tolerating hypoxia and reducing the necessity for energy adjustments due to osmotic stress (Shumway, 1977). However, prolonged periods of valve closure have severe implications on bivalves' performance since filtration activity ceases, reducing energy uptake and accumulating excretion products, which results in an increase in oxidative stress and therefore causing significant changes in their biochemical mechanisms (Domínguez et al., 2020).

In summary, clams closed their valves when exposed to low temperatures (<15°C) and salinities (≤15), resulting in a high survival rate and absence of feeding behavior in both juveniles and adults. At salinities below the "turning point," as temperature increased to high values, clams were forced to open the valves, thus exposing the soft tissues to low salinities, and therefore to osmotic stress. The exposure to low salinities, which caused an osmotic shock, associated with high temperatures and consequent reduction in dissolved oxygen (Deutsch et al., 2015), led to severe mortalities in both size classes, with juveniles exhibiting a narrower thermal limit. Above the salinity "turning point," mortality decreased and feeding behavior increased, except for extremely high temperatures (32° and 35°C), in which feeding behavior was reduced and mortality occurred probably due to low dissolved oxygen. In fact, the oxygen consumption of intertidal species exhibits circatidal rhythms, which may be

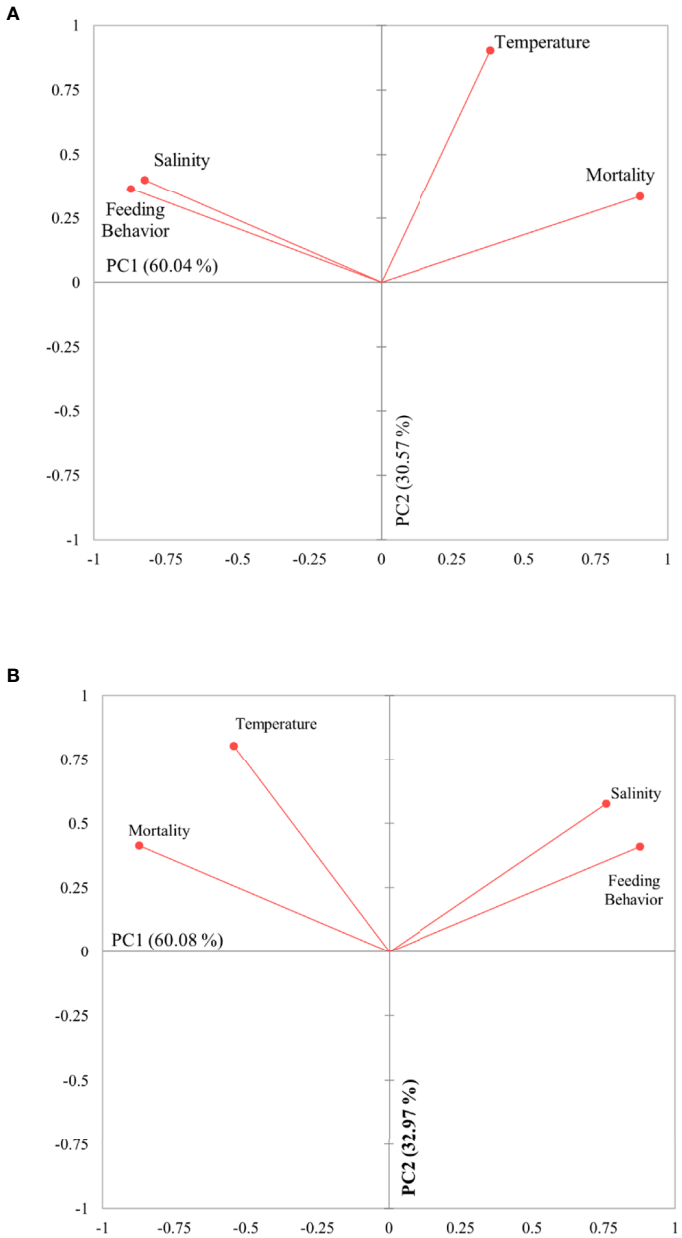

FIGURE 5 | Principal component analysis (PCA) on the parameters used to evaluate the effect of different combinations of temperature and salinity on mortality and feeding behavior. **(A)** juveniles; **(B)** adults.

modified when individuals are exposed to abrupt changes in salinity (Reyes-Martínez et al., 2020).

Although the shell acts as a boundary between the bivalve and the environment, valve closure does not protect the soft tissues of the bivalves from thermal stress caused by elevated temperatures. This is due to the high thermal diffusion of aragonite shells (Gómez-Martinez et al., 2002; Woodin et al., 2020), the main form of calcium carbonate ($CaCO_3$) of *Ruditapes* species (Mu et al., 2018).

In this context, it is important to highlight the importance of the substrate. The substrate acts as a thermal buffer (Macho et al., 2016) since the temperature at deeper sediment layers is cooler than at the surface (Befus et al., 2013). When temperatures are high, *R. decussatus* can be buried in the sediment, therefore escaping the impacts of elevated temperatures. In our study, to avoid the introduction of another variable, we excluded substrate. According to Macho et al. (2016), at a surface temperature of 40°C, *R. decussatus* buried at 6 or 8 cm will experience a temperature of 30°C–31°C. The authors demonstrated that temperatures of 32°C, in the presence of substrate, did not cause any mortality in the venerid species *R. decussatus* and *R. philippinarum* (Macho et al., 2016). In the current study, we observed mortalities at

32°C. Moreover, according to Matias et al. (2013), in the Ria Formosa lagoon the severe clam mortalities occurred during summer, probably due to the junction of high temperatures with a weak physiological condition of individuals after spawning. Vázquez et al. (2021) demonstrated that short-term episodes of low salinities in winter and spring, followed by heatwave in summer, could greatly compromise reproduction in bivalves. Low salinities (<15) during sexual resting and the beginning of gametogenesis caused a delay in the gametogenic cycle and changes in oocytes' morphology, while higher sediment temperatures (>32°C) caused an acute increase in hemocytes and gonadal reabsorption (Vázquez et al., 2021).

Moreover, warming and hyposalinity caused by changes in climate will promote the emergence, growth, and distribution of pathogenic microorganisms (e.g., viruses, bacteria, protozoans) and toxic microalgae (Zgouridou et al., 2022), therefore negatively impacting the immune and metabolic systems of bivalves (Matozzo and Marin, 2011; Turner et al., 2016), leading to severe mortalities. In fact, Reverter et al. (2020) reported that most of the infected marine organisms showed higher mortalities at warmer temperatures. It has been suggested that temperature and salinity play a critical role on disease outbreaks in marine organisms, by increasing the incidence and infection intensity by pathogens (Villalba et al., 2005; Macho et al., 2016), therefore leading to high clam mortality. In the case of *Perkinsus olseni*, a parasite of marine mollusks (Villalba et al., 2004), *in vitro* studies suggested that sporulation optimal temperature ranges between 24°C and 28°C and salinity between 25 and 35 (Auzoux-Bordenave et al., 1995), while it is inhibited below 15°C and slows at salinities between 10 and 15 (Kyoung and Ki, 2001). In conclusion, salinity fluctuations have a direct impact on feeding behavior, while temperature has a major impact on the survival of clams. Low salinities and high temperatures are lethal to *R. decussatus*. Juveniles are less resistant to the increase in temperature under less saline environments but are more resistant than adults to extremely high temperatures under more saline conditions. It is noteworthy that, in nature, these fluctuations in temperature and salinity may occur gradually, allowing clams to adapt. Likewise, the daily variation of temperature will also oscillate according to the tidal cycles and, therefore, clams will not be continuously exposed to extreme conditions (Sobral and Widdows, 1997). Nevertheless, considering the predicted climate change scenarios, juveniles will die faster when exposed to low salinities and high temperatures, consequences of heavy rainfall and heatwaves, never reaching the adult stage, thus compromising reproduction and consequently bivalve recruitment. On the other hand, these climate events may be coincident with the spawning season, between April and June (Matias et al., 2013), which may cause larval mortality and once more compromising the recruitment. The failure in bivalve recruitment will compromise the exploitation of the species, negatively impacting the economic sector.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

AM acquired the funding. SJ and DM designed the experimental work. AR, AMM, and CR performed the experimental work. AR, SJ, and AMM analyzed the data and wrote the manuscript draft. All the authors revised and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the projects CERES – Climate change and European aquatic RESources, funded by European Union's Horizon 2020 research and innovation program (grant agreement no. 678193), AIM (MAR – 02.01.01 – FEAMP – 0060), and SNMB-Sul-IV (MAR – 02.01.02 – FEAMP – 0221), funded by PO MAR2020. Ph.D. grant (SFRH/BD/147215/2019) was granted to AR by the Portuguese Foundation for Science and Technology (FCT).

ACKNOWLEDGMENTS

The authors are grateful to Ana Bela Dias and João Maurício Teixeira for all the support during the experimental trials. The authors are also very thankful to the reviewers for their constructive comments, which helped to improve the manuscript.

REFERENCES

Auzoux-Bordenave, S., Vigario, A. M., Ruano, F., Domart-Coulon, I. and Doumenc, D. (1995). *In Vitro* Sporulation of the Clam Pathogen *Perkinsus atlanticus* (Apicomplexa, Perkinsea) Under Various Environmental Condition. *J. Shellfish Res.* 14, 469–475.

Befus, K. M., Cardenas, M. B., Erler, D. V., Santos, I. R. and Eyre, B. D. (2013). Heat Transport Dynamics at a Sandy Intertidal Zone. *Water Resour. Res.* 49, 3770–3786. doi: 10.1002/wrcr.20325

Booij, M. J. (2005). Impact of Climate Change on River Flooding Assessed With Different Spatial Model Resolutions. *J. Hydrol.* 303, 176–198. doi: 10.1016/j.jhydrol.2004.07.013

Carregosa, V., Velez, C., Soares, A. M. V. M., Figueira, E. and Freitas, R. (2014). Physiological and Biochemical Responses of Three Veneridae Clams Exposed to Salinity Changes. *Comp. Biochem. Physiol. Part - B Biochem. Mol. Biol.* 177–178, 1–9. doi: 10.1016/j.cbpb.2014.08.001

Cravo, A., Pereira, C., Gomes, T., Cardoso, C., Serafim, A., Almeida, C., et al. (2012). A Multibiomarker Approach in the Clam *Ruditapes decussatus* to Assess the Impact of Pollution in the Ria Formosa Lagoon, South Coast of Portugal. *Mar. Environ. Res.* 75, 23–34. doi: 10.1016/j.marenvres.2011.09.012

De Marchi, L., Rocha, R. J. M., Rodrigues, A. C. M., Soares, A. M. V. M., Pretti, C., Chiellini, F., et al. (2020). Environmental Fate of Multistressors on Carpet Shell Clam *Ruditapes decussatus*: Carbon Nanoparticles and Temperature Variation. *Sustain.* 12, 1–15. doi: 10.3390/SU12124939

de Sousa, J. T., Matias, D., Joaquim, S., Ben-Hamadou, R. and Leitão, A. (2011). Growth Variation in Bivalves: New Insights Into Growth, Physiology and Somatic Aneuploidy in the Carpet Shell *Ruditapes decussatus*. *J. Exp. Mar. Bio. Ecol.* 406, 46–53. doi: 10.1016/j.jembe.2011.06.001

Deutsch, C., Ferrel, A., Seibel, B., Pörtner, H. O. and Huey, R. B. (2015). Climate Change Tightens a Metabolic Constraint on Marine Habitats. *Science* 348:1132–1135. doi: 10.1126/science.aaa1605

Domínguez, R., Vázquez, E., Woodin, S. A., Wethey, D. S., Peteiro, L. G., Macho, G., et al. (2020). Sublethal Responses of Four Commercially Important Bivalves to Low Salinity. *Ecol. Indic.* 111, 106031. doi: 10.1016/j.ecolind.2019.106031

Fenoglio, S., Bo, T., Cucco, M., Mercalli, L. and Malacarne, G. (2010). Effects of Global Climate Change on Freshwater Biota: A Review With Special Emphasis on the Italian Situation. *Ital. J. Zool.* 77, 374–383. doi: 10.1080/11250000903176497

Gharbi, A., Farcy, E., Van Wormhoudt, A. and Denis, F. (2016). Response of the Carpet Shell Clam (*Ruditapes decussatus*) and the Manila Clam (*Ruditapes philippinarum*) to Salinity Stress. *Biol. (Bratisl).* 71, 551–562. doi: 10.1515/biolog-2016-0072

Gómez-Martinez, O., Quintana, P., Pichardo, J. L. and Alvarado-Gil, J. J. (2002). Photoacoustic Determination of the Thermal Properties of Bivalve Mollusk Shells. *Mar. Biol.* 141, 911–914. doi: 10.1007/s00227-002-0880-z

Gosling, E. (2004). *Bivalve Molluscs : Biology, Ecology and Culture* (Oxford, UK: Blackwell).

IPCC (2021). "Summary for Policymakers," in *Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change*. Eds. R. Y, , Masson-Delmotte, B. Z., Zhai, V. ,. P., Pirani, A., Connors, S. L., Péan, C., Berger, S., Caud, N., Chen, Y., Goldfarb, L., Gomis, M. I., Huang, M., Leitzell, K., Lonnoy, E., Matthews, J. B. R., Maycock, T. K., Waterfield, T. and Yelekçi, O. Yu, R. and Zhou, B. (eds.) (Cambridge University Press).

Kyoung, J. A. and Ki, H. K. (2001). Effect of Temperature and Salinity on *In Vitro* Zoosporulation of Perkinsus Sp. In Manila Clams *Ruditapes philippinarum*. *Dis. Aquat. Organ.* 48, 43–46. doi: 10.3354/dao048043

Macho, G., Woodin, S. A., Wethey, D. S. and Vázquez, E. (2016). Impacts of Sublethal and Lethal High Temperatures on Clams Exploited in European. *Fisheries.* 35, 405–419. doi: 10.2983/035.035.0215

Matias, D., Joaquim, S., Matias, A. M., Moura, P., de Sousa, J. T., Sobral, P., et al. (2013). The Reproductive Cycle of the European Clam *Ruditapes decussatus* (L. 1758) in Two Portuguese Populations: Implications for Management and Aquaculture Programs. *Aquaculture* 406–407, 52–61. doi: 10.1016/j.aquaculture.2013.04.030

Matias, D., Joaquim, S., Ramos, M., Sobral, P. and Leitão, A. (2011). Biochemical Compounds' Dynamics During Larval Development of the Carpet-Shell Clam *Ruditapes decussatus* (Linnaeus 1758): Effects of Mono-Specific Diets and Starvation. *Helgol. Mar. Res.* 65, 369–379. doi: 10.1007/s10152-010-0230-3

Matozzo, V. and Marin, M. G. (2011). Bivalve Immune Responses and Climate Changes : Is There a Relationship ;? *ISJ* 8, 70–77.

Mu, G., Duan, F., Zhang, G., Li, X., Ding, X. and Zhang, L. (2018). Microstructure and Mechanical Property of *Ruditapes philippinarum* Shell. *J. Mech. Behav. Biomed. Mater.* 85, 209–217. doi: 10.1016/j.jmbbm.2018.06.012

Navarro, J. M. and Gonzalez, C. M. (1998). Physiological Responses of the Chilean Scallop Argopecten Purpuratus to Decreasing Salinities. *Aquaculture* 167, 315–327. doi: 10.1016/S0044-8486(98)00310-X

Nossier, M. A. (1986). Ecophysiological Responses of Cerastoderma Edule (L.) and C. Glaucum (Bruguière) to Different Salinity Regimes and Exposure to Air. *J. Molluscan Stud.* 52, 110–119. doi: 10.1093/mollus/52.2.110

Otero, M., Cebrian, E., Francour, P., Galil, B. and Savini, D. (2013). *Monitoring Marine Invasive Species in Mediterranean Marine Portected Areas (MPAs): A Strategy and Practical Guide for Managers* (Malaga, Spain: IUCN).

Parada, J. M., Molares, J. and Otero, X. (2012). Multispecies Mortality Patterns of Commercial Bivalves in Relation to Estuarine Salinity Fluctuation. *Estuaries and Coasts,* 35: 132–142. doi: 10.1007/s12237-011-9426-2

Philippart, C. J. M., Van Aken, H. M., Beukema, J. J., Bos, O. G., Cadée, G. C. and Dekker, R. (2003). Climate-Related Changes in Recruitment of the Bivalve Macoma Balthica. *Limnol. Oceanogr.* 48, 2171–2185. doi: 10.4319/lo.2003.48.6.2171

Poulin, R. and Mouritsen, K. N. (2006). Climate Change, Parasitism and the Structure of Intertidal Ecosystems. *J. Helminthol.* 80, 183–191. doi: 10.1079/joh2006341

Reverter, M., Sarter, S., Caruso, D., Avarre, J. C., Combe, M., Pepey, E., et al. (2020). Aquaculture at the Crossroads of Global Warming and Antimicrobial Resistance. *Nat. Commun.* 11, 1–8. doi: 10.1038/s41467-020-15735-6

Reyes-Martínez, M. J., Martínez-Pita, I., Soler-Navarro, D. and García-García, F. J. (2020). The Impact of Salinity Changes Associated with Size on the Wedge Clam *Donax trunculus* Linnaeus, 1758 (Mollusca: Bivalvia): A laboratory assay. *Estuar. Coast. Shelf Sci.* 241, 106838. doi: 10.1016/j.ecss.2020.106838. 8:42 PM.

Ribeiro, J., Monteiro, C. C., Monteiro, P., Bentes, L., Coelho, R., Gonçalves, J. M. S., et al. (2008). Long-Term Changes in Fish Communities of the Ria Formosa Coastal Lagoon (Southern Portugal) Based on Two Studies Made 20 Years Apart. *Estuar. Coast. Shelf Sci.* 76, 57–68. doi: 10.1016/j.ecss.2007.06.001

Sarà, G., Romano, C., Widdows, J. and Staff, F. J. (2008). Effect of Salinity and Temperature on Feeding Physiology and Scope for Growth of an Invasive Species (Brachidontes Pharaonis - MOLLUSCA: BIVALVIA) Within the Mediterranean Sea. *J. Exp. Mar. Bio. Ecol.* 363, 130–136. doi: 10.1016/j.jembe.2008.06.030

Shumway, S. E. (1977). Effect of Salinity Fluctuation on the Osmotic Pressure and Na+, Ca2+ and Mg2+ Ion Concentrations in the Hemolymph of Bivalve Molluscs. *Mar. Biol.* 41, 153–177. doi: 10.1007/BF00394023

Sobral, P. and Widdows, J. (1997). Effects of Copper Exposure on the Scope for Growth of the Clam *Ruditapes decussatus* From Southern Portugal. *Mar. Pollut. Bull.* 34, 992–1000. doi: 10.1016/S0025-326X(97)00116-1

Tunipex, S. A. (2022). Available at: http://www.tunipex.eu/pt/information_oceanic.php (Accessed January 28, 2022). Olhão, Portugal.

Turner, L. M., Alsterberg, C., Turner, A. D., Girisha, S. K., Rai, A., Havenhand, J. N., et al. (2016). Pathogenic Marine Microbes Influence the Effects of Climate Change on a Commercially Important Tropical Bivalve. *Sci. Rep.* 6, 1–10. doi: 10.1038/srep32413

Vázquez, E., Woodin, S. A., Wethey, D. S., Peteiro, L. G. and Olabarria, C. (2021). Reproduction Under Stress: Acute Effect of Low Salinities and Heat Waves on Reproductive Cycle of Four Ecologically and Commercially Important Bivalves. *Front. Mar. Sci.* 8. doi: 10.3389/fmars.2021.685282

Velez, C., Figueira, E., Soares, A. M. V. M. and Freitas, R. (2016). Native and Introduced Clams Biochemical Responses to Salinity and pH Changes. *Sci. Total Environ.* 566–567, 260–268. doi: 10.1016/j.scitotenv.2016.05.019

Verdelhos, T., Marques, J. C. and Anastácio, P. (2015a). Behavioral and Mortality Responses of the Bivalves Scrobicularia Plana and Cerastoderma Edule to Temperature, as Indicator of Climate Change's Potential Impacts. *Ecol. Indic.* 58, 95–103. doi: 10.1016/j.ecolind.2015.05.042

Verdelhos, T., Marques, J. C. and Anastácio, P. (2015b). The Impact of Estuarine Salinity Changes on the Bivalves Scrobicularia Plana and Cerastoderma Edule, Illustrated by Behavioral and Mortality Responses on a Laboratory Assay. *Ecol. Indic.* 52, 96–104. doi: 10.1016/j.ecolind.2014.11.022

Villalba, A., Casas, S. M., López, C. and Carballal, M. J. (2005). Study of Perkinsosis in the Carpet Shell Clam Tapes Decussatus in Galicia (NW Spain). II. Temporal Pattern of Disease Dynamics and Association With Clam Mortality. *Dis. Aquat. Organ.* 65, 257–267. doi: 10.3354/dao065257

Villalba, A., Reece, K. S., Ordás, M. C., Casas, S. M. and Figueras, A. (2004). Perkinsosis in Molluscs: A Review. *Aquat. Living Resour.* 17, 411–432. doi: 10.1051/alr:2004050

Woodin, S. A., Wethey, D. S., Olabarria, C., Vázquez, E., Domínguez, R., Macho, G., et al. (2020). Behavioral Responses of Three Venerid Bivalves to Fluctuating Salinity Stress. *J. Exp. Mar. Bio. Ecol.* 522, 151256. doi: 10.1016/j.jembe.2019.151256

Zgouridou, A., Tripidaki, E., Giantsis, I. A., Theodorou, J. A., Kalaitzidou, M., Raitsos, D. E., et al. (2022). The Current Situation and Potential Effects of Climate Change on the Microbial Load of Marine Bivalves of the Greek Coastlines: An Integrative Review. *Environ. Microbiol.* 24, 1012–1034. doi: 10.1111/1462-2920.15765

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frontiers | Frontiers in Marine Science

ORIGINAL RESEARCH
published: 22 July 2022
doi: 10.3389/fmars.2022.927116

Effects of Dietary Lipid Sources on the Growth, Gonad Development, Fatty Acid Composition and Spawning Performance of Broodstock, and Early Larvae Quality of Sea Urchin (*Strongylocentrotus intermedius*)

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

Received: *23 April 2022*
Accepted: *14 June 2022*
Published: *22 July 2022*

Citation:
Zuo R, Ning Y, Di W, Heqiu Y, Song J, Ding J and Chang Y (2022) Effects of Dietary Lipid Sources on the Growth, Gonad Development, Fatty Acid Composition and Spawning Performance of Broodstock, and Early Larvae Quality of Sea Urchin (Strongylocentrotus intermedius). Front. Mar. Sci. 9:927116. doi: 10.3389/fmars.2022.927116

This study was carried out to investigate the effects of five formulated feeds with different lipid sources (colza oil (CO), fish oil (FO), linseed oil (LO), soybean oil (SO), or palm oil (PO)) and kelp (*Laminaria japonica*) on the growth, reproductive performance of broodstock, and early larvae quality of sea urchin (*Strongylocentrotus intermedius*). The experimental diets were randomly allocated to a total of 48 (eight urchins per experimental group) individually cultured *S. intermedius* (initial weight 90.51 ± 0.82g) and the feeding period lasted for 12 weeks. The results showed that the weight gain rate of female sea urchins fed kelp was significantly higher than those fed formulated feeds while the gonadosomatic index of spawned female sea urchins fed kelp was significantly lower than those fed formulated feeds. *S. intermedius* broodstock fed FO showed the largest egg diameter and fecundity, which could be due to the abundant n-3 LC-PUFA deposited in the gonads of this group. Sea urchins fed SO showed the lowest fecundity and hatching rate, and the highest inflammation level. Sea urchins fed CO showed the highest content of oleic acid in the gonads and eggs, but the lowest fertilization rate. The highest hatching rate was observed in the kelp treatment, which was comparable to that in the LO and PO but was significantly higher than that in CO, FO, and SO. Before mouth opening, all prismatic larvae showed no significant differences in survival during the first 2 days post hatchery (DPH). At the 3 DPH, the survival of *S. intermedius* larvae was highest in the FO group, followed by those in the PO group, with the lowest survival observed in the kelp group. Thus, FO was accepted as the most ideal lipid source based on growth, reproductive performance, and early larval quality. These results could contribute to adopting an efficient feeding strategy to promote the reproductive performance and offspring quality by choosing the optimal lipid source for *S. intermedius* broodstock.

Keywords: lipid sources, *Strongylocentrotus intermedius*, growth, reproductive performance, offspring quality

INTRODUCTION

The gonads of sea urchins are highly accepted as luxury seafood products due to their delicious taste and high nutritional value (Xin et al., 2018; Loureno et al., 2019; Wang et al., 2019). In the last decades, the demand for sea urchins has seen a sharp increase worldwide, which directly causes overfishing and destruction of their wild stocks (Prato et al., 2018; Li et al., 2021; Nhan et al., 2020). To mitigate this issue, aquaculture of sea urchins has been attempted globally and has proven to be an effective alternative strategyforf producing gonads with high yield and market acceptance (Phillips et al., 2010; Onomu et al., 2020). *Strongylocentrotus intermedius*, which is naturally distributed in the coastal areas of North Japan and Far Eastern Russia, was first introduced to China from Japan by Dalian Ocean University in 1989 (Chang et al., 2012). Up to now, it has become one of the most important sea urchin species for aquaculture in the coastal areas of China (Zuo et al., 2018; Wang et al., 2019). At present, fresh kelp *Laminaria japonica* are used as the main food category for *S. intermedius* (Zuo et al., 2018; Li et al., 2020). However, the gonad development of *S. intermedius* was shown to be retarded when they were fed solely macroalgae and could be markedly accelerated after the addition of some mussel *Mytilus edulis* (Zhou et al., 2013). Thus, it is imperative to quantify the nutritional requirement and formulate specialized formulated feeds for the broodstock of this species.

It has been shown that the quality of broodstock diets and their nutrient levels have profound influences on the gonad maturation, reproductive performance, and subsequent offspring quality of numerous aquatic animals (Izquierdo et al., 2001; Mazorra et al., 2003; Sui et al., 2009; Tercero et al., 2015; Yldz et al., 2020). Lipids are not only one of the main energy sources, but also act as the providers of essential fatty acids, carriers of lipid-soluble vitamins, and the major structural components of cellular membranes (Zhou et al., 2007; Turchini et al., 2010; Wang et al., 2012; Gibbs et al., 2015). The level and fatty acid composition of dietary lipids are considered one of the key factors that affect the spawning performance and larvae quality of aquatic animals (Izquierdo et al., 2001). In particular, the absolute level and specific composition of long-chain polyunsaturated fatty acids (LC-PUFAs) and the ratio of members, such as arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3), and docosahexaenoic acid (DHA; 22:6n-3), have been proven to be critical factors in regulating the reproductive performance, egg, and larval viability of rainbow trout *Oncorhynchus mykiss* (Agh et al., 2019; Yldz et al., 2020; Yldz et al., 2021), Nile tilapia *Oreochromis niloticus* (Ng and Wang, 2011), Yucatan octopus *Octopus Maya* (Tercero et al., 2015), Chinese mitten crab *Eriocheir sinensis* (Xiao et al., 2002; Sui et al., 2009), tongue sole *Cynoglossus semilaevis* (Liang et al., 2014), Pearl gourami *Trichogarster leeri* (Mobaraki et al., 2020), European eel *Anguilla anguilla* (Kottmann et al., 2020), and Oriental river prawn *Macrobrachium nipponense* (Li et al., 2020). Fish oil (FO), as the major source of LC-PUFAs, will not be sufficient and continuous for the expanding aquaculture industry (Turchini and Francis, 2009; Ng and Wang, 2011; Tocher, 2015; Yuan et al., 2019). Vegetable oils (VOs), such as colza oil (CO), soybean oil (SO), linseed oil (LO), and palm oil (PO), are viable alternatives for FO due to their relatively stable supply, abundant PUFA, and low cost (Turchini and Francis, 2009; Gibbs et al., 2015; Cuesta-Gomez et al., 2020). However, little information is available about the effects of VO on the reproductive performance and larvae viability of sea urchins, especially when the requirement for LC-PUFA is guaranteed.

Gonads are the reproductive organ and sole edible part of sea urchins. Thus, different feed formulation should be designed for adult sea urchins to acquire market acceptance or reproductive success (Heflin et al., 2012; Brink-Hull et al., 2022). In a recent study, it was found that PO and CO showed comparable effects to FO for *S. intermedius* from the perspective of growth performance and gonad market acceptance (Ning et al., 2022). However, to the best of our knowledge, no information is available about the optimal lipid sources for achieving the best reproductive performance of *S. intermedius*. Thus, this study was conducted to investigate the effects of dietary lipid sources on the gonad development, fatty acid composition, spawning performance of broodstock, and early larvae quality of *S. intermedius*.

MATERIALS AND METHODS

Experimental Broodstock Diets

Five isoproteic (25%) and isolipidic (6.0%) formulated feeds and kelp (*L. japonica*) were used as the experimental diets for sea urchin broodstock in this study. All feed ingredients were ground and passed through a 150 μm mesh. After all the solid ingredients of each formulated feed were thoroughly mixed, one of the following lipid sources, colza oil (CO), fish oil (FO), linseed oil (LO), soybean oil (SO), or palm oil (PO), was added and blended well. At last, about 30% water was mixed with the ingredients above before they were pressed through a die with 2 mm pores by a pellet-making machine (DES-TS1280, Jinan, China). The pellets were then dried at 50°C, tightly packed in sealed bags, and stored at -20° until use. The formulation and nutritional profile of formulated feeds can be found in **Tables 1**, **2**.

Feeding Experiment

Adult *S. intermedius* with the age of 2.5 years were purchased from a local farm (Changhai County, Dalian, China). Then, sea urchins were reared in a rectangular tank (length: width: depth=180cm: 99cm: 70cm), where they were fed fresh kelp to acclimate the experimental conditions for 10 days. After that, sea urchins of similar size (initial weight 90.51 ± 0.82g) were individually assigned to 48 floating cages (22 cm × 28 cm × 48 cm) with each cage holding one individual. All cages were placed in fiber-reinforced rectangular plastic tanks (length: width: depth=95cm: 65cm: 55cm), with each tank holding eight cages. Each experimental diet was randomly allocated to eight urchins. To avoid feed waste, a Petri dish was placed at the bottom of each cage to prevent the feeds from falling through the gaps.

The experimental animals were handfed every day to a state of apparent satiation. To avoid the detrimental effects on

TABLE 1 | Formulation and proximate composition of the experimental diets (% dry weight).

Ingredients (%)	Formulated feeds with different lipid sources				
	CO	**FO**	**LO**	**SO**	**PO**
Fish meal[1]	4	4	4	4	4
Kelp meal[2]	10	10	10	10	10
Seaweed meal[3]	29.7	29.7	29.7	29.7	29.7
Soybean meal[4]	13	13	13	13	13
Wheat meal[5]	24	24	24	24	24
Wheat gluten[6]	10	10	10	10	10
Vitamin premix[7]	2	2	2	2	2
Mineral premix[8]	2	2	2	2	2
Calcium propionate	0.18	0.18	0.18	0.18	0.18
Choline chloride	0.1	0.1	0.1	0.1	0.1
Ethoxyquin	0.02	0.02	0.02	0.02	0.02
Soybean lecithin	1	1	1	1	1
Colza oil	4	–	–	–	–
Fish oil	–	4	–	–	–
Linseed oil	–	–	4	–	–
Soybean oil	–	–	–	4	–
Palm oil	–	–	–	–	4
Proximate composition					
Crude protein	25.12	25.20	25.05	25.23	25.01
Crude lipid	6.03	6.10	6.12	6.01	6.05

[1]Fish meal, crude protein 68.7% dry matter, crude lipid 9.7% dry matter.
[2]kelp meal, crude protein 19.31% dry matter.
[3]Seaweed meal, crude protein 8.15% dry matter.
[4]Soybean meal, crude protein 51.56% dry matter, crude lipid 0.9% dry matter.
[5]Wheat meal, crude protein 13.88% dry matter, crude lipid 1.0% dry matter.
[6]Wheat gluten, crude protein 80% dry matter, crude lipid 2.8% dry matter.
[7]Vitamin premix (mg or g kg^{-1} diet), vitamin D, 5 mg; vitamin K, 10 mg; vitamin B_{12}, 10 mg; vitamin B_6, 20 mg; folic acid, 20 mg; vitamin B_1, 25 mg; vitamin A, 32 mg; vitamin B_2, 45 mg; pantothenic acid, 60 mg; biotin, 60 mg; niacin acid, 200 mg; α-tocopherol, 240 mg; inositol, 800 mg; ascorbic acid, 2000 mg; microcrystalline cellulose, 16.47 g.
[8]Mineral premix (mg or g kg^{-1} diet), $CuSO_4 \cdot 5H_2O$, 10 mg; Na_2SeO_3 (1%), 25 mg; $ZnSO_4 \cdot H_2O$, 50 mg; $CoCl_2 \cdot 6H_2O$ (1%), 50 mg; $MnSO_4 \cdot H_2O$, 60 mg; $FeSO_4 \cdot H_2O$, 80 mg; $Ca(IO_3)_2$, 180 mg; $MgSO_4 \cdot 7H_2O$, 1200 mg; zeolite, 18.35 g.

water quality, residual feeds and feces were timely removed out of the tanks by siphoning about 6 hours after each feeding operation. Seawater was sand filtered, aerated, and preheated before they were filled into experimental tanks. Water in each tank was exchanged completely every 3 days. During the whole feeding experiment, the water quality parameters were monitored and recorded daily, with temperature maintained at 15 ± 1°C, salinity 33 ± 1‰, pH 8.0 ± 0.1, and dissolved oxygen >8.0 mg L^{-1}. The feeding period lasted for 12 weeks.

Spawning, Egg, and Gonad Collection

At the end of the feeding experiment, the sea urchins were individually weighed after fasting for 24 h, and then artificially induced for spawning as described by Liu et al. (2005). First, 1 ml KCl (0.5 M) was injected into the coelomic cavity *via* the peristomial membrane of each sea urchin. Eggs or sperms began to flood out of the genital pores in about 30 min. Then, three female sea urchins and three male sea urchins were chosen out from each dietary group. The eggs of each sea urchin fed the same diet were collected, counted, and divided into three parts. The first part (about 100 eggs) was put into a 1.5 mL tube with 10% formalin for measuring the egg diameter. The second part was placed in 1.5 ml sterile tubes and then stored at -80°C for later analysis of fatty acids. The third part was placed in a sterile beaker with filtered seawater for the larval culture experiment. The collected sperm of broodstock fed the same diet were mixed and diluted with sterile seawater. Then, the diluted sperm were mixed with the eggs of the same dietary group (sperms/eggs ≈ 1000:1).

Fertilized eggs were hatched and incubated according to the method reported by Chang et al. (2012). Briefly, fertilized eggs collected from each female sea urchin were incubated in a 5L plastic tank (15 eggs/mL), where the water temperature was kept at 15°C ± 0.5°C. After hatching, healthy and strong prismatic larvae were carefully gathered by dragging a net across the one-third upper layer of each tank. Among them, some were transferred to a new 5L container for subsequent larval culture with a density of 1.0 individual/mL. The remaining healthy prismatic larvae in each tank were collected, pooled into a 1.5 tube, and stored at -80°C for later analysis of their fatty acids in response to different maternal nutrition. During the larval culture, the water temperature was maintained at 15 ± 1°C, salinity was 33 ± 1‰, pH was 8.0 ± 0.1, and oxygen was above 7 mg/L. The larval container was illuminated by less than 300 lx of incandescent light, with a natural photoperiod. During the larval culture, no feeding was carried out, and the quality of the prismatic larvae was evaluated by a starvation tolerance test (Quintana et al., 2015). Larvae in each tank were daily sampled to monitor the quantity, developmental stages, body length, and width. If four-armed larvae occurred in any tank, the starvation tolerance test was terminated. At this time, larvae within three tanks of each dietary treatment were

TABLE 2 | Fatty acid composition (g/kg dry matter) of formulated feeds[1].

Fatty acids	Formulated feeds with different lipid sources				
	CO	FO	LO	SO	PO
C14:0	0.57	2.35	0.56	0.78	0.90
C15:0	0.07	0.30	0.06	0.09	0.07
C16:0	7.92	12.09	8.68	10.9	17.6
C17:0	0.08	0.18	0.08	0.12	0.09
C18:0	1.79	2.15	2.53	2.94	2.30
C20:0	0.21	0.18	0.18	0.24	0.20
C21:0	–	–	–	0.16	–
C22:0	0.11	0.09	0.08	0.24	0.09
C23:0	0.04	–	–	0.06	0.04
C24:0	0.17	0.12	0.12	0.13	0.13
ΣSFA[2]	10.94	17.46	12.29	15.66	21.41
C14:1	–	0.06	–	–	–
C16:1	0.41	1.84	0.35	0.53	0.43
C17:1	0.05	0.19	0.03	0.06	0.05
C18:1	23.08	7.4	10.14	11.52	22.26
C20:1	0.60	1.74	0.13	0.36	0.20
C22:1	0.41	0.98	0.13	0.30	0.13
C24:1	0.11	0.24	0.04	0.06	–
ΣMUFA[3]	24.65	12.45	10.82	12.82	23.07
C18:3n-3	–	–	8.03	2.20	0.78
C20:5n-3	0.26	1.82	0.17	0.44	0.25
C22:6n-3	0.37	3.23	0.25	0.68	0.36
∑n-3PUFA[4]	0.63	5.05	8.44	3.32	1.39
C18:2n-6	15.24	8.39	10.63	23.76	13.17
C18:3n-6	–	0.08	–	–	–
C20:3n-6	–	0.05	–	–	0.04
C20:4n-6	0.22	3.06	0.24	0.63	0.27
C22:2n-6	0.04	0.08	–	0.04	–
∑n-6PUFA	15.49	11.65	10.86	24.43	13.48
n-3/n-6PUFA	0.04	0.43	0.78	0.14	0.10
ARA/EPA	0.83	1.68	1.44	1.42	1.08
DHA/EPA	1.39	1.77	1.48	1.53	1.45

[1]Some fatty acids, of which the contents are minor, trace amount or not detected, such as C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C15:1, were not listed in the table.
[2]SFA saturated fatty acids.
[3]MUFA mono-unsaturated fatty acids.
[4]PUFA poly-unsaturated fatty acids.

pooled into one tube, flash frozen, and stored at -80 °C for later fatty acid analysis.

After spawning, three female sea urchins were weighed and then dissected for gonads and digestive tract. First, the digestive tract of each sea urchin was weighed to calculate the digestive tract index and was placed in a 1.5 ml tube and stored at -80°C until they were used for the analysis of related gene transcription. Then, gonads from three urchins were individually weighed to calculate the gonadosomatic index. After that, a small part of the gonad was carefully put in a 5 ml tube with Bouin's reagent for subsequent histological analysis; the second part was placed in a 1.5 ml tube and stored at -80°C for analysis of gene transcription; the remaining gonads were pooled and then stored at -80°C for later determining the nutritional composition.

Gonadal Histology

The gonad slices were made by following the method of Ning et al. (2022). Briefly, the Bouin's reagent fixed gonad was dehydrated by sinking them into a graded ethanol series (75%–100%) before they were embedded in paraffin. Tissue sections (4 μm in thickness) were prepared by using a microtome (Leica, RM2016). After that, slices were stained with hematoxylin and eosin, which were observed under an optical microscope (40 ×, Leica, Germany).

Nutritional Composition Analysis

The proximate composition of experimental feeds and gonads was analyzed according to AOAC (1995). Briefly, the contents of crude protein and crude lipids were detected by following the methods of Soxhlet and Kjeldahl, respectively. The content of moisture was determined by calculating the mass loss of the samples which were dried at 105°C.

Fatty acid composition of samples was assayed by following the method of Li et al. (2020). Here, the procedures were introduced briefly. First, the fatty acids in the samples were esterified into methyl esters, which were separated with hexane and transferred to a new tube. Before injection, certain amounts of C19:0 were added to each sample obtained above. Finally, the fatty acids were quantified by using gas chromatography (Thermo Fisher Trace 1310 ISQ). The temperature of the injector and detector was

maintained at 290°C. The oven temperature rising procedures were set as follows: 80°C to 200°C at a speed of 10°C/min, 200°C to 250°C at a speed of 5°C/min, and 250°C to 270°C at a speed of 2°C/min. The fatty acid composition (g/kg) was calculated based on their relative peak area.

RNA Extraction and Real-Time Quantitative PCR

The sequences of the specific primers are presented in **Table 3**. The procedures of RNA extraction and real-time quantitative PCR have been previously described (Zuo et al., 2017). Briefly, total RNA was extracted and was reverse transcribed to cDNA by using a commercial kit (TaKaRa, Beijing, China). Then, the reaction regime (20 μL) was prepared as follows: 2 μL of cDNA, 0.8 μL of forward primer and reverse primer (10 mM), 10 μL of FastStart Essential DNA Green Master, and 6.4 μL of sterile distilled water. The quantitative PCR was performed by using the LightCycler® 96 real-time PCR system with the following reaction conditions: 95°C for 10 min; 95°C for 15 s; and 60°C for 60 s (45 cycles); 95°C for 10 s; 65°C for 60 s; and 97°C for 1 s. The formula of $2^{-\Delta\Delta CT}$ was used to calculate the relative gene expression of target genes (Kenneth and Thomas, 2002).

Calculations and Statistical Analysis

Survival rate (SR, %) = $N_f/N_i \times 100$

Weight growth rate (WGR, %) = $(W_b - W_i)/W_i \times 100$

Gonadosomatic index (GSI, %) = $W_g/W_f \times 100$

Digestive tract index (DTI, %) = $W_d/W_f \times 100$

Relative fecundity (RF, 10^3 eggs/g) = E_t/W_b

Fertilization rate (FR, %) = $E_2/E_t \times 100$

Hatching rate (HR, %) = $E_p/E_2 \times 100$

Larvae survival rate (LSR, %) = $N_d/N_t \times 100$

where N_i and N_f were the initial and final number of sea urchins in each dietary group. W_i was the initial body weight of each female sea urchin; W_b was the body weight of each female sea urchin before spawning; W_f, W_g and W_d were the body weight, gonad weight, and digestive tract weight of each female sea urchin after spawning. E_t was the total number of eggs produced by each female sea urchin; E_2 was the number of two-cell stage embryos in the same batch of eggs after fertilization; E_p was the number of healthy prismatic larvae after hatchery. N_t and N_d were the initial number of healthy prismatic larvae and number of live larvae in each tank during the first 3 days post hatchery.

The data were checked for normal distribution and homogeneity of variance before they were statistically analyzed by ANOVA with SPSS 22.0 software. When a significance ($P<0.05$) was detected, Duncan's multiple comparison test was used to compare differences in the means between different treatments. The data were presented as means ± S.E.M (standard error of means).

RESULTS

Survival and Growth Performance

No mortality was observed in any dietary group. Before spawning, the WGR of female sea urchins fed kelp was significantly higher than those fed formulated feeds ($P<0.05$). Among the formulated feed groups, sea urchins fed PO showed a marginally higher WGR than other lipid source groups ($P>0.05$). After spawning, the body weight of female sea urchins fed PO was comparable to those fed LO ($P>0.05$) but was significantly higher than those fed CO, FO, SO, and kelp ($P<0.05$). The gonad weight and GSI of spawned female sea urchins fed kelp were the lowest, which were significantly lower than those fed formulated feeds ($P<0.05$). Among the formulated feed groups, the highest gonad weight and GSI were observed in the PO group, which were significantly higher than those in the other groups ($P<0.05$). The DTI of female sea urchins fed FO was comparable to that of sea urchins fed LO and PO ($P>0.05$) but was significantly higher than those fed CO, SO, and kelp ($P<0.05$) (**Table 4**).

Ovary Histological Observation

A small number of ova were loosely distributed in the space vacated by spawned ova. In the ovary of sea urchins fed kelp, there were still many vitellogenic oocytes that were distributed along the ovarian wall. By contrast, there were only a small number of vitellogenic oocytes observed in the ovaries of sea

TABLE 3 | List of primers used for real time PCR in this study.

Name	Sequence (5'-3')	AT[1] (°C)	Reference
18s rRNA-F	GTTCGAAGGCGATCAGATAC		Li et al. (2020)
18s rRNA-R	CTGTCAATCCTCACTGTGTC	52	
COX-2-F	GAGGTGGATAACCGATTGA		MH516324
COX-2-R	AGCATTGCCCATAGAACAG	60	
AIF-1-F	TCGAACGTGCAAGGTGGCAAG		MH516330
AIF-1-R	CGTCATTGTCATCGAGGTCTCCAC	60	
TNF-α-F	GCTGTAACGGCGTTCGTCTCC		MH516331
TNF-α-R	TGGTGTACTTGTGCTGGTTGTTGG	61.5	
MYP-F	ACCATATGGACTGACGT		LC170478.1
MYP-R	GGGTTCTACCTCGGAGTTGAC	51	

[1]*AT, annealing temperature; COX-2, cyclooxygenase-2; AIF-1, allograft inflammatory factor-1; TNF-α, tumor necrosis factor-α; MYP, major yolk protein.*

TABLE 4 | Effects of different lipid sources on survival and growth performance of female adult sea urchin (*Strongylocentrotus intermedius*) (mean ± SEM, n=3)[1].

	Formulated feeds with different lipid sources					Kelp
	CO	FO	LO	SO	PO	
SR (%)	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00
IBW (g)	89.71 ± 0.70	90.05 ± 0.18	90.21 ± 0.75	90.80 ± 0.83	92.53 ± 1.37	89.77 ± 1.07
BFW (g)	96.53 ± 0.69^{c}	97.47 ± 0.78^{bc}	96.99 ± 1.99^{bc}	97.40 ± 1.02^{bc}	101.68 ± 0.54^{a}	100.74 ± 0.77^{ab}
WGR (%)	7.63 ± 0.46^{b}	8.38 ± 0.05^{b}	8.03 ± 0.69^{b}	7.57 ± 0.70^{b}	10.06 ± 1.02^{b}	12.83 ± 0.79^{a}
AFW (g)	91.62 ± 1.11^{b}	93.06 ± 0.24^{b}	93.56 ± 0.71^{ab}	93.24 ± 0.27^{b}	96.11 ± 0.50^{a}	91.33 ± 0.92^{b}
GW (g)	17.71 ± 0.78^{b}	16.77 ± 0.46^{b}	18.05 ± 1.58^{b}	15.99 ± 0.67^{b}	23.21 ± 1.19^{a}	8.95 ± 0.50^{c}
GSI (%)	18.91 ± 0.50^{b}	17.98 ± 0.94^{b}	18.96 ± 0.86^{b}	19.41 ± 0.53^{b}	24.22 ± 0.71^{a}	9.88 ± 0.94^{c}
DTW (g)	2.87 ± 0.37^{b}	4.38 ± 0.60^{a}	3.42 ± 0.42^{ab}	3.20 ± 0.24^{ab}	3.49 ± 0.05^{ab}	3.12 ± 0.40^{b}
DTI (%)	3.13 ± 0.38^{b}	4.70 ± 0.65^{a}	3.67 ± 0.14^{ab}	3.29 ± 0.34^{b}	3.64 ± 0.06^{ab}	3.42 ± 0.43^{b}

[1]*Mean values with the different superscript letters within the same row are significantly different at P<0.05.*
SR, survival rate; IBW, initial body weight; BFW, before fecundity weight; WGR, weight gain rate; AFW, after fecundity weight; GW, gonad weight; GSI, gonadosomatic index; DTW, digestive tract weight; DTI, digestive tract index.

urchins fed formulated feeds. Generally, sea urchins fed FO showed less vitellogenic oocytes in their ovaries than those fed other lipid sources. The nutritive phagocytes (NP) in the ovary of sea urchins fed the formulated feeds showed a thin and pale meshwork structure, while the nutritive phagocytes in the kelp group showed a dense network structure (**Figure 1**).

Reproductive Performance

The relative fecundity was not significantly affected by diet categories or lipid sources, with a relatively higher value observed in the FO and kelp groups (P>0.05) (**Figure 2A**).

Before fertilization, the egg diameter in the FO group was significantly larger than that in the PO group (P<0.05). After fertilization, the egg diameter in the SO group was significantly larger than that in the other dietary groups (P<0.05). The lowest egg diameter was observed in the PO group, which was comparable to that in FO and CO groups

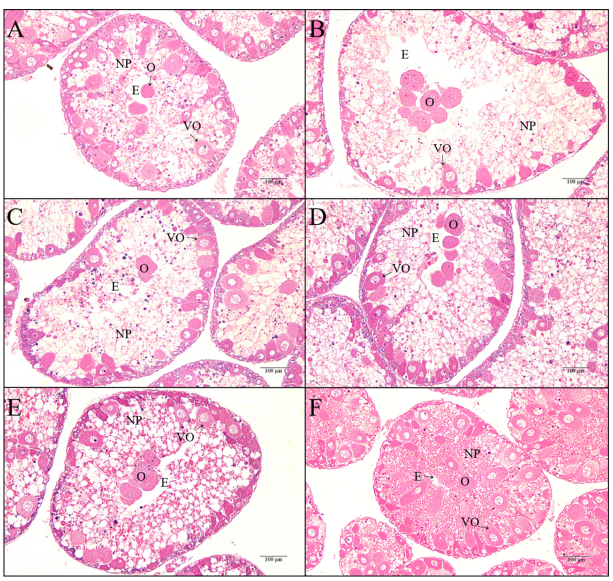

FIGURE 1 | Histology observations in the ovaries of female adult sea urchin (*Strongylocentrotus intermedius*) fed diets with different lipid sources (colza oil (CO), fish oil (FO), linseed oil (LO), soybean oil (SO), or palm oil (PO)). **(A)** Colza oil, stage V; **(B)** Fish oil, stage V; **(C)** Linseed oil, stage V; **(D)** Soybean oil, stage V; **(E)** Palm oil, stage V; **(F)** Feed kelp, stage V. NP, nutritive phagocyte. VO, vitellogenic oocyte. O, ova. E, empty spaces.

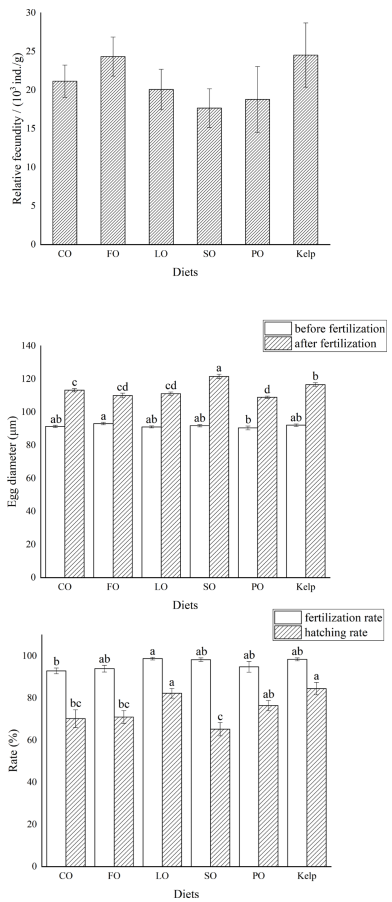

FIGURE 2 | Effects of different lipid sources (colza oil (CO), fish oil (FO), linseed oil (LO), soybean oil (SO), or palm oil (PO)) on the relative fecundity, egg diameter, as well as fertilization rate and hatching rate of sea urchin (*Strongylocentrotus intermedius*) (mean ± SEM, n=3). Mean value bars within the same parameter of each chart bearing with different lowercase letters are significantly different at P < 0.05.

(P>0.05) but was significantly lower than that in the other groups (P<0.05) (**Figure 2**).

The fertilization rate was highest in the LO group, which was only significantly higher than that in the CO group (P<0.05). The hatching rate was highest in the kelp group, which was comparable to that in LO and PO (P>0.05) but was significantly higher than that in CO, FO, and SO groups (P<0.05) (**Figure 2**).

Prismatic Larvae Quality

The body length of prismatic larvae was highest in the treatment of LO, which was only significantly higher than that in CO and SO (P<0.05). The body width of prismatic larvae was highest in the kelp group, which was comparable to that in CO (P>0.05) but was significantly higher than that in FO, LO, SO, and PO groups (P<0.05) (**Table 5**).

During the first 2 days post hatching (DPH), no significant difference was observed in the prismatic larvae survival rate (LSR) among dietary groups (P>0.05). On the 3rd DPH, the LSR was significantly decreased in all dietary groups (P<0.05). The LSR in the kelp group was significantly lower than that in the FO group (P<0.05). Among formulated feed groups, LSR was highest in the FO group, followed by PO, CO, SO, and LO groups. However, no significant difference was observed in SR among different formulated feed groups (P>0.05) (**Table 6**).

Transcription of Proinflammatory Genes

The transcription of *COX-2* was highest in the spawned ovary of sea urchins fed SO, which was significantly higher than that in the other groups (P<0.05). The transcription of *AIF-1* was highest in the ovary of sea urchins fed SO, but there was no significant difference among all dietary treatments (P>0.05). The transcription of *TNF-α* was highest in the ovary of sea urchins fed SO, which was comparable to that in CO (P>0.05) but was significantly higher than that in kelp, FO, LO, or PO groups (P<0.05) (**Figure 3**).

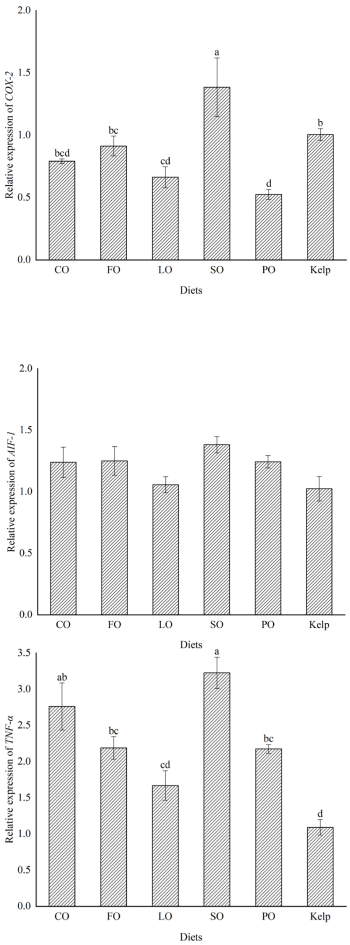

FIGURE 3 | Effects of dietary lipid sources (colza oil (CO), fish oil (FO), linseed oil (LO), soybean oil (SO), or palm oil (PO)) on the inflammatory related gene expression in the ovary of sea urchin (*Strongylocentrotus intermedius*) (mean ± SEM, n=3). COX-2, cyclooxygenase-2. AIF-1, allograft inflammatory factor-1. TNF-α, tumor necrosis factor-α. Mean value bars bearing with different lowercase letters are significantly different at P < 0.05.

Transcription of Major Yolk Protein (MYP)

The transcription of *MYP* was highest in the digestive tract of female adult sea urchins fed FO, which was comparable to that in LO (P>0.05) but was significantly higher than that in kelp, CO, SO, or PO groups (P<0.05). The mRNA level of *MYP* was the highest in the ovary of the FO group, which was approximately 2-fold higher than that in the other dietary groups (P<0.05) (**Figure 4**).

Proximate Composition of Ovary

The ovary of sea urchins fed kelp showed significantly lower moisture, but higher protein, than that of sea urchins fed the formulated feeds (P<0.05). Crude lipid in the ovary of sea urchins

TABLE 5 | Effects of different lipid sources on prismatic larvae size of sea urchin (*Strongylocentrotus intermedius*) (mean ± SEM, n=3)[1].

	Formulated feeds with different lipid sources					Kelp
	CO	FO	LO	SO	PO	
Length (μm)	221.1±9.7^{c}	257.7±10.9^{ab}	269.1±9.1^{a}	232.0±6.3^{bc}	239.7±9.9^{abc}	246.5±3.4^{abc}
Width (μm)	146.5±3.8^{ab}	134.5±3.8^{c}	143.4±2.8^{bc}	135.7±1.9^{bc}	141.64±1.9^{bc}	156.2±7.3^{a}

[1]*Mean values with the different superscript letters within the same row are significantly different at P<0.05.*

TABLE 6 | Effects of different lipid sources on the prismatic larvae survival of sea urchin (*Strongylocentrotus intermedius)* at different day post hatching (DPH) (mean ± SEM, n=3)[1].

DPH	Formulated feeds with different lipid sources					Kelp
	CO	FO	LO	SO	PO	
1	100 ± 0^{A}	100 ± 0^{A}	100 ± 0^{A}	100 ± 0^{A}	100 ± 0^{A}	100 ± 0^{A}
2	96.0 ± 4.0^{A}	100 ± 0.0^{A}	100 ± 0.0^{A}	93.3 ± 6.7^{A}	96. 7 ± 3.3^{A}	100 ± 0.0^{A}
3	56.0 ± 11.6Bab	72.6 ± 11.3Ba	47.0 ± 6.0Bab	55.0 ± 5.0Bab	65.4 ± 7.0Bab	40.4 ± 1.4Bb

[1]*Mean values with the different superscript capital letters within the same column are significantly different at P<0.05. Mean values with the different superscript lowercase letters within the same row are significantly different at P<0.05.*

TABLE 7 | Effects of different lipid sources on the proximate composition (% wet weight) in the ovary of adult sea urchin (*Strongylocentrotus intermedius*) (mean ± SEM, n=3)[1].

	Formulated feeds with different lipid sources					Kelp
	CO	FO	LO	SO	PO	
Moisture	74.68 ± 0.33^{a}	74.86 ± 0.46^{a}	73.04 ± 0.63^{b}	74.3 ± 0.80^{ab}	73.64 ± 0.34^{ab}	69.19 ± 0.15^{c}
Crude lipid	5.47 ± 0.42^{a}	5.79 ± 0.37^{a}	5.76 ± 0.16^{a}	5.49 ± 0.15^{a}	5.35 ± 0.01^{ab}	4.38 ± 0.05^{b}
Crude protein	12.26 ± 0.27^{b}	11.67 ± 0.04^{b}	12.10 ± 0.27^{b}	12.00 ± 0.25^{b}	11.83 ± 0.10^{b}	13.70 ± 0.58^{a}

[1]*Mean values with the different superscript letters within the same row are significantly different at P<0.05.*

fed kelp was significantly lower than that of formulated feed groups except for the PO group (P<0.05). Among the formulated feed groups, the moisture was highest in the ovary of sea urchins fed FO and CO, which was only significantly higher than that of sea urchins fed LO (P<0.05). However, there were no significant differences in the protein and lipid among the formulated feed groups (P>0.05) (**Table 7**).

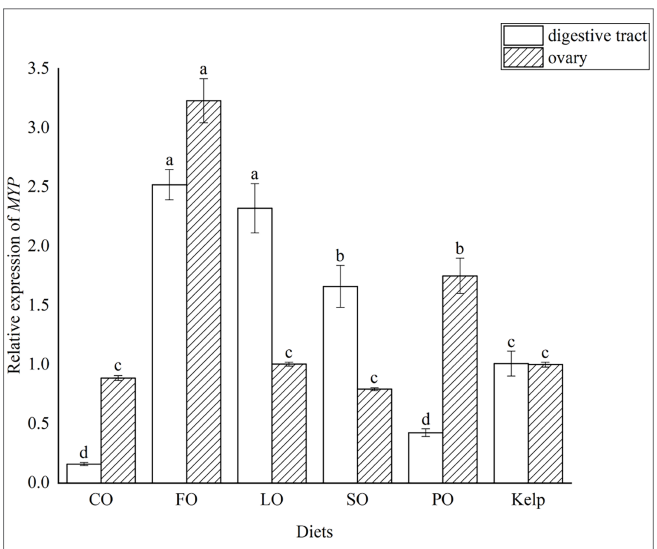

FIGURE 4 | Effects of dietary lipid sources (colza oil (CO), fish oil (FO), linseed oil (LO), soybean oil (SO), or palm oil (PO)) on the major yolk protein (MYP) expression in sea urchin (*Strongylocentrotus intermedius*) (mean ± SEM, n=3). Mean value bars bearing with different lowercase letters are significantly different at P < 0.05

Fatty Acid Composition of the Spawned Ovary, Eggs, and Prismatic Larvae

Generally, the spawned ovary of sea urchins showed characteristic fatty acid categories of the ingested lipid sources, with the highest amounts of oleic acid (OA, C18:1n-9), EPA (C20:5n-3) and DHA (C22:6n-3), linolenic acid (LNA, C18:3n-3), linoleic acid (LA, C18:2n-6) and palmitic acid (PA, C16:0) detected in the CO, FO, LO, SO, and PO groups, respectively. Sea urchins fed kelp showed significantly higher EPA and n-3/n-6 PUFA but lower LA in the spawned ovary than those fed formulated feeds (P<0.05). The ARA in the spawned ovary of sea urchins fed CO, FO, and kelp was significantly higher than that in the other groups (P<0.05). ARA/EPA of the spawned ovary was highest in the PO group, which was comparable to that in the CO group (P>0.05) but was significantly higher than the other groups (P<0.05). The lowest ARA/EPA was observed in the spawned ovary of sea urchins fed kelp, which was comparable to that in the FO and LO groups (P>0.05) but was significantly lower than that in the other groups (P<0.05). DHA/EPA was highest in the FO group, followed by the CO group, which were significantly higher than that in the other groups (P<0.05). Sea urchins fed kelp showed the lowest DHA/EPA in the spawned ovary, which was significantly lower than that in the other groups (**Table 8**).

The EPA content in the eggs of sea urchins fed kelp was significantly higher than that in the formulated feed groups (P<0.05). The DHA and ARA contents were highest in the eggs of sea urchins fed FO, which were significantly higher than that in the other dietary groups (P<0.05). However, sea urchins fed LO and CO produced eggs with significantly (P<0.05) higher n-3 PUFA and n-6 PUFA, respectively. The n-3/n-6 PUFA in the eggs of sea urchins fed kelp was significantly higher than that in the formulated feed groups (P<0.05). The ARA/EPA and DHA/EPA showed a similar changing

TABLE 8 | Effects of different lipid sources on the fatty acid composition (g/kg dry matter) in the ovary of adult sea urchin (*Strongylocentrotus intermedius*) (mean ± SEM, n=3)[1].

FA	Formulated feeds with different lipid sources					Kelp
	CO	FO	LO	SO	PO	
C14:0	15.52 ± 0.72b	18.76 ± 0.44a	18.98 ± 0.74a	14.14 ± 0.60b	15.07 ± 0.56b	14.84 ± 0.94b
C15:0	0.76 ± 0.18	0.93 ± 0.10	0.57 ± 0.09	0.76 ± 0.26	0.45 ± 0.03	0.64 ± 0.05
C16:0	39.1 ± 1.31ab	37.9 ± 0.77b	37.48 ± 1.74b	31.17 ± 1.08c	37.11 ± 1.27b	42.07 ± 0.85a
C17:0	0.31 ± 0.04	0.30 ± 0.04	0.24 ± 0.03	0.26 ± 0.03	0.23 ± 0.02	0.24 ± 0.02
C18:0	5.47 ± 0.37b	4.82 ± 0.47b	5.52 ± 0.69b	5.16 ± 0.36b	4.33 ± 0.41b	7.45 ± 0.23a
C20:0	0.63 ± 0.06b	0.56 ± 0.03b	0.65 ± 0.07b	0.64 ± 0.10b	0.42 ± 0.05b	2.92 ± 0.17a
C21:0	35.51 ± 0.21a	21.42 ± 1.24c	19.91 ± 0.80cd	18.34 ± 0.70d	27.04 ± 1.05b	12.49 ± 0.51e
C22:0	0.34 ± 0.03a	0.27 ± 0.04abc	0.22 ± 0.03bc	0.28 ± 0.03ab	0.18 ± 0.01c	0.29 ± 0.02ab
C23:0	0.77 ± 0.13a	0.54 ± 0.03ab	0.43 ± 0.09b	0.43 ± 0.05b	0.58 ± 0.06ab	0.34 ± 0.03b
C24:0	0.19 ± 0.03ab	0.12 ± 0.01b	0.17 ± 0.04ab	0.22 ± 0.00a	0.15 ± 0.01ab	0.14 ± 0.02b
ΣSFA[2]	98.59 ± 2.44a	85.63 ± 0.96b	84.16 ± 2.80b	71.4 ± 2.87c	85.57 ± 1.86b	81.43 ± 0.88b
C14:1	0.91 ± 0.20ab	1.27 ± 0.11a	1.35 ± 0.15a	0.87 ± 0.21ab	1.33 ± 0.19a	0.68 ± 0.16b
C16:1	8.87 ± 0.60ab	11.02 ± 0.61a	7.74 ± 0.96b	6.54 ± 1.16b	8.99 ± 0.55ab	8.28 ± 0.56b
C18:1	29.95 ± 0.82a	8.65 ± 0.81d	11.43 ± 0.65c	12.99 ± 1.03c	26.69 ± 0.67b	5.22 ± 0.43e
C20:1	12.61 ± 0.78a	7.74 ± 0.89b	6.37 ± 0.81b	6.70 ± 0.99b	8.82 ± 0.60b	8.35 ± 0.39b
C22:1	18.66 ± 0.57b	17.97 ± 1.69b	14.28 ± 0.95c	21.37 ± 0.92b	12.98 ± 1.07c	28.89 ± 1.08a
C24:1	0.72 ± 0.12a	0.55 ± 0.05ab	0.32 ± 0.06b	0.38 ± 0.09b	0.47 ± 0.02b	0.40 ± 0.03b
ΣMUFA[3]	71.73 ± 1.43a	47.20 ± 0.66c	41.48 ± 1.55d	48.84 ± 1.19c	59.29 ± 2.8b	51.81 ± 1.41c
C18:3n-3	8.46 ± 0.47b	2.13 ± 0.25d	19.72 ± 0.77a	4.28 ± 0.65c	1.64 ± 0.12d	4.84 ± 0.33c
C20:3n-3	1.47 ± 0.13c	0.93 ± 0.06cd	4.05 ± 0.43b	1.33 ± 0.36c	0.33 ± 0.04d	7.72 ± 0.44a
C20:5n-3	5.75 ± 0.65cd	11.61 ± 1.21b	8.26 ± 0.64c	5.45 ± 1.51cd	2.86 ± 0.37d	26.49 ± 0.39a
C22:6n-3	1.31 ± 0.14b	10.34 ± 1.74a	1.01 ± 0.09b	1.21 ± 0.18b	1.41 ± 0.27b	0.48 ± 0.08b
∑n-3PUFA	16.99 ± 0.48d	25.01 ± 3.24c	33.04 ± 1.86b	12.28 ± 1.91d	6.24 ± 0.77e	39.53 ± 0.32a
C18:2n-6	31.32 ± 0.60b	13.25 ± 1.96e	19.13 ± 0.71d	36.89 ± 1.37a	24.22 ± 0.73c	3.12 ± 0.24f
C18:3n-6	0.13 ± 0.02b	0.22 ± 0.00b	0.11 ± 0.02b	0.20 ± 0.06b	0.11 ± 0.03b	0.63 ± 0.13a
C20:2n-6	6.85 ± 0.58b	5.58 ± 1.02bc	6.23 ± 0.87bc	9.90 ± 0.80a	4.19 ± 0.31c	5.75 ± 0.34bc
C20:3n-6	4.05 ± 0.36a	2.26 ± 0.32c	2.80 ± 0.28bc	3.74 ± 0.70ab	2.75 ± 0.07bc	1.58 ± 0.15c
C20:4n-6	12.2 ± 0.24a	10.27 ± 1.20a	7.20 ± 0.62b	7.79 ± 0.59b	7.71 ± 0.47b	10.96 ± 0.44a
C22:2n-6	0.26 ± 0.04a	0.24 ± 0.05ab	0.27 ± 0.06a	0.33 ± 0.02a	0.12 ± 0.01b	0.26 ± 0.01a
∑n-6PUFA	54.82 ± 0.84^{a}	31.82 ± 1.97^{c}	35.72 ± 0.92^{bc}	58.84 ± 3.03^{a}	39.1 ± 0.62^{b}	22.29 ± 0.84^{d}
ΣPUFA[4]	71.81 ± 1.29a	56.83 ± 2.16c	68.76 ± 2.47ab	71.11 ± 4.78a	45.34 ± 0.64d	61.82 ± 0.53bc
n-3/n-6PUFA	0.31 ± 0.00^{c}	0.80 ± 0.15^{b}	0.92 ± 0.05^{b}	0.21 ± 0.03^{c}	0.16 ± 0.02^{c}	1.78 ± 0.08^{a}
ARA/EPA	2.17 ± 0.20ab	0.88 ± 0.06c	0.87 ± 0.03c	1.63 ± 0.37b	2.78 ± 0.35a	0.41 ± 0.02c
DHA/EPA	0.23 ± 0.00c	0.88 ± 0.07a	0.12 ± 0.01cd	0.26 ± 0.08c	0.49 ± 0.04b	0.02 ± 0.00d

[1]Mean values with the different superscript letters within the same row are significantly different at P<0.05. Some fatty acids, of which the contents are minor, trace amount or not detected, such as C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C15:1, C17:1, were not listed in the table.

[2]SFA, saturated fatty acids.

[3]MUFA, mono-unsaturated fatty acids.

[4]PUFA, poly-unsaturated fatty acids.

tendency to that of spawned ovary in all dietary groups, with significantly higher ratios observed in the PO and FO, respectively (P<0.05) (**Table 9**).

The EPA content of the prismatic larvae was highest in the kelp group, followed by that of FO, LO, and CO groups, with the lowest EPA observed in SO and PO groups. Although no statistical analysis was made, DHA content in the prismatic larvae of the FO group was extremely higher than that in the other dietary groups. ARA of prismatic larvae showed comparable levels among dietary groups, with marginally higher value observed in PO and kelp groups. The prismatic larvae in the kelp group showed the lowest n-6 PUFA and the highest n-3 PUFA and n-3/n-6 PUFA among all dietary groups. Among the prismatic larvae of formulated feed groups, the lowest n-6 PUFA and the highest n-3 PUFA and n-3/n-6 PUFA were observed in the FO group. The highest ARA/EPA of prismatic larvae was observed in the PO group, followed by the SO and CO groups. While DHA/EPA of prismatic larvae was highest in the FO group, followed by the PO group, with the lowest ratio observed in the kelp group (**Table 10**).

DISCUSSION

In the present study, the WGR of female adult *S. intermedius* fed kelp was significantly higher than those fed formulated feeds. Similar results have been obtained in the juvenile *S. intermedius* (Li et al., 2020). This could be due to the presence of unknown immune and growth stimulant substances in the fresh kelp. A low molecular weight fucoidan, obtained from the kelp *Laminaria japonica*, played an important role in antioxidant and anticoagulant activity (Wang et al., 2010). In the present study, female sea urchins fed SO showed the worst growth performance. Similar results have been reported by Gibbs et al. (2015), who found that the WGR of juvenile *Lytechinus variegatus* fed SO was lower than that of sea urchins fed FO. Li et al. (2020) reported

TABLE 9 | Effects of different lipid sources on the fatty acid composition (g/kg dry matter) of eggs of adult sea urchin (*Strongylocentrotus intermedius*) (mean ± SEM, n=3)[1].

FA	Formulated feeds with different lipid sources					Kelp
	CO	FO	LO	SO	PO	
C14:0	10.88 ± 0.21^{c}	19.21 ± 0.31^{a}	12.21 ± 0.45^{b}	9.06 ± 0.26^{d}	10.18 ± 0.17^{c}	10.94 ± 0.21^{c}
C15:0	0.63 ± 0.03^{b}	1.01 ± 0.01^{a}	0.53 ± 0.03^{c}	0.38 ± 0.04^{d}	0.36 ± 0.00^{d}	0.41 ± 0.02^{d}
C16:0	32.64 ± 0.62^{b}	42.71 ± 0.71^{a}	30.25 ± 1.12^{cd}	22.8 ± 0.64^{e}	31.33 ± 0.54^{bc}	28.5 ± 0.60^{d}
C17:0	0.28 ± 0.01^{b}	0.39 ± 0.02^{a}	0.26 ± 0.01^{b}	0.20 ± 0.00^{cd}	0.21 ± 0.01^{c}	0.17 ± 0.01^{d}
C18:0	5.65 ± 0.09^{b}	7.45 ± 0.12^{a}	7.03 ± 0.26^{a}	4.85 ± 0.12^{c}	4.56 ± 0.10^{c}	5.98 ± 0.12^{b}
C20:0	0.48 ± 0.01^{c}	0.69 ± 0.01^{b}	0.63 ± 0.02^{b}	0.45 ± 0.01^{cd}	0.39 ± 0.02^{d}	1.95 ± 0.05^{a}
C21:0	33.89 ± 0.60^{a}	27.51 ± 0.57^{b}	19.76 ± 0.72^{d}	14.46 ± 0.42^{e}	24.32 ± 0.47^{c}	8.71 ± 0.20^{f}
C22:0	0.27 ± 0.01^{b}	0.33 ± 0.03^{a}	0.27 ± 0.01^{b}	0.22 ± 0.01^{c}	0.18 ± 0.00^{c}	0.21 ± 0.01^{c}
C23:0	1.06 ± 0.02^{b}	0.97 ± 0.04^{c}	0.67 ± 0.03^{d}	0.37 ± 0.01^{e}	1.46 ± 0.01^{a}	0.22 ± 0.01^{f}
C24:0	0.16 ± 0.00^{b}	0.18 ± 0.00^{b}	0.22 ± 0.00^{a}	0.21 ± 0.00^{a}	0.17 ± 0.00^{b}	0.12 ± 0.02^{c}
ΣSFA[2]	85.94 ± 1.60^{b}	100.45 ± 1.76^{a}	71.83 ± 2.63^{c}	53.00 ± 1.50^{d}	73.15 ± 1.28^{c}	57.21 ± 1.20^{d}
C14:1	0.68 ± 0.05^{c}	1.34 ± 0.04^{a}	0.69 ± 0.07^{c}	0.58 ± 0.06^{c}	0.89 ± 0.03^{b}	0.43 ± 0.02^{d}
C16:1	7.65 ± 0.14^{b}	12.43 ± 0.15^{a}	5.26 ± 0.20^{d}	4.65 ± 0.15^{e}	7.05 ± 0.10^{c}	5.42 ± 0.13^{d}
C18:1	28.02 ± 0.52^{a}	9.94 ± 0.13^{cd}	10.62 ± 0.39^{c}	9.22 ± 0.27^{d}	24.21 ± 0.46^{b}	3.03 ± 0.07^{e}
C20:1	9.47 ± 0.17^{a}	8.55 ± 0.16^{b}	5.13 ± 0.18^{d}	3.86 ± 0.11^{e}	6.84 ± 0.13^{c}	4.89 ± 0.10^{d}
C22:1	18.76 ± 0.30^{c}	25.83 ± 0.63^{a}	19.59 ± 0.70^{c}	19.68 ± 0.54^{c}	14.75 ± 0.31^{d}	22.59 ± 0.52^{b}
C24:1	0.93 ± 0.02^{b}	1.01 ± 0.03^{a}	0.54 ± 0.02^{d}	0.39 ± 0.01^{e}	0.85 ± 0.02^{c}	0.44 ± 0.02^{e}
ΣMUFA[3]	65.51 ± 1.19^{a}	59.10 ± 1.05^{b}	41.83 ± 1.54^{d}	38.38 ± 1.13^{de}	54.6 ± 0.97^{c}	36.79 ± 0.81^{e}
C18:3n-3	7.94 ± 0.15^{b}	1.97 ± 0.05^{e}	19.79 ± 0.76^{a}	6.44 ± 0.18^{c}	1.53 ± 0.02^{e}	3.92 ± 0.09^{d}
C20:3n-3	1.46 ± 0.02^{c}	0.97 ± 0.04^{d}	5.19 ± 0.19^{b}	1.44 ± 0.04^{c}	0.26 ± 0.00^{e}	6.01 ± 0.14^{a}
C20:5n-3	5.41 ± 0.09^{d}	15.94 ± 0.27^{b}	10.15 ± 0.35^{c}	4.47 ± 0.13^{e}	2.57 ± 0.05^{f}	22.35 ± 0.49^{a}
C22:6n-3	1.44 ± 0.02^{b}	12.69 ± 0.22^{a}	1.18 ± 0.03^{bc}	0.93 ± 0.03^{c}	1.14 ± 0.03^{c}	0.28 ± 0.01^{d}
∑n-3 PUFA	16.26 ± 0.28^{c}	31.57 ± 0.49^{b}	36.32 ± 1.33^{a}	13.28 ± 0.37^{d}	5.49 ± 0.11^{e}	32.55 ± 0.72^{b}
C18:2n-6	29.8 ± 0.55^{a}	15.74 ± 0.22^{e}	19.16 ± 0.70^{d}	26.00 ± 0.75^{b}	22.52 ± 0.42^{c}	1.92 ± 0.05^{f}
C18:3n-6	0.12 ± 0.00^{c}	0.22 ± 0.01^{b}	0.09 ± 0.01^{d}	0.10 ± 0.01^{cd}	0.09 ± 0.01^{d}	0.37 ± 0.01^{a}
C20:2n-6	7.72 ± 0.13^{ab}	8.24 ± 0.13^{a}	7.97 ± 0.28^{a}	7.37 ± 0.24^{b}	4.67 ± 0.10^{c}	3.83 ± 0.09^{d}
C20:3n-6	2.61 ± 0.05^{a}	2.32 ± 0.09^{b}	1.91 ± 0.07^{d}	2.15 ± 0.06^{bc}	1.99 ± 0.04^{cd}	0.94 ± 0.03^{e}
C20:4n-6	10.63 ± 0.21^{b}	11.51 ± 0.21^{a}	7.23 ± 0.26^{c}	4.99 ± 0.14^{d}	7.11 ± 0.13^{c}	7.00 ± 0.12^{c}
C22:2n-6	0.26 ± 0.01^{b}	0.27 ± 0.01^{b}	0.44 ± 0.01^{a}	0.27 ± 0.00^{b}	0.16 ± 0.01^{d}	0.21 ± 0.01^{c}
∑n-6 PUFA	51.13 ± 0.95^{a}	38.30 ± 0.65^{bc}	36.80 ± 1.31^{c}	40.88 ± 1.18^{b}	36.53 ± 0.70^{c}	14.27 ± 0.30^{d}
ΣPUFA[4]	67.39 ± 1.23^{b}	69.86 ± 1.14^{ab}	73.12 ± 2.64^{a}	54.16 ± 1.55^{c}	42.01 ± 0.80^{e}	46.83 ± 1.02^{d}
n-3/n-6 PUFA	0.32 ± 0.00^{e}	0.82 ± 0.00^{c}	0.99 ± 0.00^{b}	0.32 ± 0.00^{d}	0.15 ± 0.00^{f}	2.28 ± 0.00^{a}
ARA/EPA	1.96 ± 0.01^{b}	0.72 ± 0.00^{d}	0.71 ± 0.00^{d}	1.12 ± 0.00^{c}	2.77 ± 0.01^{a}	0.31 ± 0.00^{e}
DHA/EPA	0.27 ± 0.00^{c}	0.80 ± 0.00^{a}	0.12 ± 0.00^{e}	0.21 ± 0.00^{d}	0.44 ± 0.00^{b}	0.01 ± 0.00^{f}

[1]Mean values with the different superscript letters within the same row are significantly different at P<0.05. Some fatty acids, of which the contents are minor, trace amount or not detected, such as C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C15:1, C17:1, were not listed in the table.

[2] SFA, saturated fatty acids.

[3] MUFA, mono-unsaturated fatty acids.

[4] PUFA, poly-unsaturated fatty acids.

that the WGR of juvenile *S. intermedius* fed SO was lower than those fed LO and FO. In a recent study, we also found that the WGR of adult *S. intermedius* fed SO was lower than those fed PO, CO, and FO (Ning et al., 2022). This indicated that a high amount of linoleic acid (LA, 18:2n-6) had detrimental effects on the growth performance of sea urchins, just as observed in most marine carnivorous fish species (Zuo et al., 2015). The GSI of female *S. intermedius* fed kelp was significantly lower than those fed formulated feeds, which was consistent with the findings of a variety of previous studies (Ning et al., 2022). Although n-3 LC-PUFA is essential for normal gonad development, results of this study and some previous studies showed that sea urchins seem to have a low n-3 LC-PUFA requirement for sustaining normal gonad development (Lv et al., 2020). In this study, sea urchins fed PO showed higher GSI than those fed other lipid sources. This could be due to the abundant PA in PO, which has also been found in high quantities in the gonads of wild sea urchins (Wang et al., 2019).

Although sea urchins belong to inveterate, they are similar to fish in some physiological processes, including fatty acid metabolism, gonad development, and reproduction (Roöttinger et al., 2008; Silvia et al., 2015; Wang et al., 2021). Fecundity generally depends on the species, age, size, and nutritional conditions of spawners (Izquierdo et al., 2001; Kamler, 2005; Wanke et al., 2017). In the present study, the relative fecundity of sea urchins was not significantly affected by the experimental diets. This was consistent with the findings on rainbow trout *Oncorhynchus mykiss*, which showed that the relative fecundity of female individuals was not significantly affected by dietary lipid sources (Yldz et al., 2020). However, some studies have shown that dietary lipids can significantly improve the fecundity of broodstock, such as zebrafish *Danio rerio* (Jaya-Ram et al., 2008), yellowfin sea bream *Acanthopagrus latus* (Zakeri et al., 2009), Atlantic cod *Gadus morhua* (Rjbek et al., 2014), and three-spot gourami *Trichopodus trichopterus* (Berenjestanaki et al., 2014). Egg size (may be expressed as egg diameter) has been

TABLE 10 | Effects of different lipid sources on the fatty acid composition (g/kg dry matter) in the prismatic larvae of sea urchin (*Strongylocentrotus intermedius*) (n=1)[1].

FA	Formulated feeds with different lipid sources					Kelp
	CO	FO	LO	SO	PO	
C14:0	5.60	7.96	5.36	3.79	5.25	9.53
C15:0	0.31	0.45	0.30	0.34	0.27	0.48
C16:0	16.44	17.38	14.12	9.95	17.89	29.95
C17:0	0.13	0.18	0.16	0.10	0.12	0.21
C18:0	3.35	3.65	4.09	3.00	3.80	5.73
C20:0	0.35	0.37	0.35	0.22	0.22	1.63
C21:0	15.94	10.87	8.96	5.86	12.33	7.27
C22:0	0.20	0.19	0.20	–	0.28	0.17
C23:0	0.51	0.39	0.33	0.27	0.41	0.20
C24:0	0.18	0.14	0.17	0.40	0.47	0.25
ΣSFA[2]	43.02	41.58	34.02	23.91	41.02	55.41
C14:1	0.31	0.51	0.28	0.17	0.34	0.22
C16:1	3.57	5.05	2.43	1.67	3.31	4.73
C18:1	13.32	4.40	5.53	4.26	14.97	2.94
C20:1	4.73	3.42	2.35	1.81	3.51	4.98
C22:1	11.96	13.29	13.89	10.02	9.33	19.30
C24:1	0.62	0.52	0.35	0.32	0.61	0.41
ΣMUFA[3]	34.51	27.20	24.82	18.23	32.05	32.58
C18:3n-3	3.54	0.90	5.74	1.06	0.76	3.15
C20:3n-3	0.72	0.43	1.69	0.57	0.21	5.53
C20:5n-3	3.22	7.82	4.47	2.20	1.72	19.25
C22:6n-3	0.71	5.38	0.58	0.37	0.71	0.20
∑n-3PUFA	8.18	14.54	12.47	4.20	3.40	28.13
C18:2n-6	13.73	6.49	12.97	10.40	12.36	1.63
C20:2n-6	3.81	3.25	4.17	3.72	2.37	3.49
C20:3n-6	1.42	0.92	1.16	0.67	0.93	0.77
C20:4n-6	6.90	6.08	4.54	5.63	7.96	7.31
C22:2n-6	0.15	0.15	0.24	0.28	0.19	0.23
∑n-6PUFA	26.01	16.88	23.07	20.70	23.81	13.43
ΣPUFA[4]	34.19	31.42	35.54	24.89	27.21	41.56
n-3/n-6PUFA	0.31	0.86	0.54	0.20	0.14	2.10
ARA/EPA	2.14	0.78	1.02	2.56	4.62	0.38
DHA/EPA	0.22	0.69	0.13	0.17	0.41	0.01

[1]*Some fatty acids, of which the contents are minor, trace amount or not detected, such as C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C15:1, C17:1, were not listed in the table.*

[2]*SFA, saturated fatty acids.*

[3]*MUFA, mono-unsaturated fatty acids.* [4]*PUFA, poly-unsaturated fatty acids.*

shown to be an indicator of evaluating reproductive performance in most aquatic animals (Lund et al., 2008; Wanke et al., 2017; Stuart et al., 2020), with the assumption that larger eggs represent a higher reserve of metabolic energy substrates (Brooks et al., 1997; Kamler, 2005; Nazari et al., 2009; Stuart et al., 2020). In the present study, sea urchins fed FO produced eggs with the largest size. This was consistent with the findings of Xu et al. (2017) who found that the egg diameter of tongue sole *Cynoglossus semilaevis* was significantly increased by feeding diets enriched with n-3 LC-PUFA. On one hand, the n-3 LC-PUFA could be used for the formation of cell membranes and substances reserved inside the eggs. On the other hand, n-3 LC-PUFA could promote the synthesis, transportation, and deposition of yolk protein in the eggs. Indeed, results of this study showed that the mRNA level of *MYP* in the ovaries of sea urchins fed FO was approximately 2-fold higher than that in the other groups. However, the egg size of Nile tilapia (Ng and Wang, 2011), flame angelfish *Centropyge loriculus* (Callan et al., 2014), and three-spot gourami (Berenjestanaki et al., 2014) was not significantly affected by dietary LC-PUFA levels. The discrepancies could be due to differences in oil droplet presence and egg size between species.

In the present study, the fertilization rate and hatching rate of sea urchins fed LO were relatively high, which could be related to the relatively higher ratio of n-3/n-6 PUFA in the eggs of sea urchins. Jaya-Ram et al. (2008) reported that egg quality and hatching rate of zebrafish were positively correlated with dietary n-3/n-6 PUFA. On the other hand, this could be due to the relatively lower inflammation in the ovary of sea urchins fed LO. Crespo et al. (2010) reported that the pro-inflammatory cytokines (e.g., *TNF-α*) in the ovaries of brown trout *Salmo trutta* broodstock negatively affected the ovarian function and egg quality. In this study, sea urchin broodstock fed SO had a poor reproductive performance. This could be due to the increased linolic acid derived inflammation, which has been found to be responsible for the infertility. It was previously found that auraptene, as a coumarin derivative with anti-inflammatory properties, was effective in improvement of oocyte maturation and fertilization rate in polycystic ovary syndrome patients

(Abizadeh et al., 2020). Furthermore, the sperm quality is important for the success of fertilization. Vassallo-Agius et al. (2001) found that semen obtained from rainbow trout fish fed n-3 PUFA-sufficient showed higher sperm motility rates. Butts et al. (2015) reported that European eel fed diets with deficient n-3 fatty acids showed lower sperm motility. Therefore, the higher fertilization in the LO group of this study could be due to the better sperm quality of sea urchins. However, sperm quality parameters were not analyzed in this study and following studies are needed to clarify this point. It is well known that the steps of fertilization include the acrosome reaction, cell membrane fusion of both gametes, and genetic materials interchange (Pomin, 2015). Previous studies have shown that membrane fluidity plays a critical role in the fusion process of gametes, which is positively correlated with the content of n-3 PUFAs (Asturiano et al., 2001). Despite high n-3 LC-PUFA promoting fertilization, fertilization was lower in the FO group, which was possibly due to oxidation of seminal plasma (Jedrzejczak et al., 2005). This indicated that oxidation levels should be assayed when sea urchin broodstock are conditioned with fish oil to acquire satisfactory fertilization rates.

Larvae survival during a short period of starvation has been widely used as an index of evaluating their viability (Quintana et al., 2015). In the present study, at the 3rd day post hatching (DPH), the larvae survival was the highest in the FO treatment, which paralleled with egg size, n-3 LC-PUFA content, and MYP expression level. Tamada and Iwata (2005) reported that the survival rate of *Rhinogobius* larvae was positively correlated with the egg size during a period of 72 h starvation. Stuart et al. (2020) reported that the starvation-resistant capacity of California yellowtail *Seriola dorsalis* larvae was positively correlated with their egg size. It is commonly accepted that larger larvae can live longer under the starvation test (Miller et al., 1998; Kamler, 2005). However, body length and body width of prismatic larvae were almost the highest in the kelp group, which was inconsistent with the poorest survival performance of this group. Thus, in addition to body size, body nutritional composition was also important for the survival of larvae before mouth opening. Carboni et al. (2012) showed that the increasing levels of DHA of sea urchin *Paracentrotus lividus* larvae fed with *Pleurochrysis carterae* and *Cricosphaera elongata* promoted better larval performance. It was previously found that *P. lividus* larvae has specific dietary requirements for high level of n-3 LC-PUFA, including DHA and EPA, low DHA/EPA and high EPA/ARA (Liu et al., 2007; Carboni et al., 2012). In this study, prismatic larvae with high DHA/EPA showed a higher survival rate. Thus, DHA/EPA could be used as a sensitive indicator of evaluating the survival of early larvae which still relies on endogenous nutrition. Furthermore, survival of larvae in the CO group was the lowest among all dietary groups. This could be due to the high amount of oleic acid (OA, 18:1n-9) in the CO. Samaee (2010) reported that the increasing level of OA in eggs reduced the survival rate of early larvae of common dentex *Dentex dentex*. Callan et al. (2012) reported that the increasing level of oleic acid in eggs has a negative impact on the egg vitality of flame angelfish *Centropyge loriculus*.

In conclusion, sea urchins fed PO showed higher GSI than those fed other lipid sources. Egg diameter and fecundity of *S. intermedius* were largest in the FO group, which could be due to the abundant n-3 LC-PUFA deposited in their gonads. The higher inflammation level could account for lower fecundity and hatching rate of sea urchins fed SO. Sea urchins fed CO showed the highest content of oleic acid in the gonads and eggs, and the lowest fertilization rate. The highest hatching rate was observed in the kelp treatment, which was comparable to that in the LO and PO but was significantly higher than that in CO, FO, and SO. At the 3rd DPH, the survival of *S. intermedius* larvae was highest in the FO treatment, which was only significantly higher than those in Kelp. Thus, FO was accepted as the most ideal lipid source based on growth, reproductive performance, and early larval quality. These results could contribute to adopting an efficient feeding strategy to promoting the reproductive performance and offspring quality by choosing the optimal lipid source for *S. intermedius* broodstock.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

AUTHOR CONTRIBUTIONS

RZ and YN designed the experiment under the help of JD. YN and RZ performed the feeding experiment. YN, WD, and YH analyzed the data. YN drafted the manuscript. RZ, JD, JS, and YC revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by the National Key Research and Development Program of China (2018YFD0900400, 2018YFD0901603), National Natural Foundation of China (Grant no.: 41606180), and Young Elite Scientists Sponsorship of China (YESS20150157).

REFERENCES

Abizadeh, M., Novin, M. G., Amidi, F., Ziaei, S. A., Abdollahifar, M. A. and Nazarian, H. (2020). Potential of Auraptene in Improvement of Oocyte Maturation, Fertilization Rate, and Inflammation in Polycystic Ovary Syndrome Mouse Model. *Reprod. Sci.* 27 (1), 1–10. doi: 10.1007/s43032-020-00168-9

Agh, N., Jafari, F., Jalili, R., Noori, F. and Mozanzadeh, M. T. (2019). Replacing Dietary Fish Oil With Vegetable Oil Blends in Female Rainbow Trout Broodstock Does Not Affect Breeding Quality. *Lipids* 54 (2-3), 149–161. doi: 10.1002/lipd.12138

AOAC (Association of Official Analytical Chemists) (1995). *Official Methods of Analysis of Official Analytical Chemists International.16th Edition* (Arlington, VA: Assoc. Off. Anal. Chem), 16–26.

Asturiano, J. F., Sorbera, L. A., Carrillo, M., Zanuy, S., Ramos, J., Navarro, J. C., et al. (2001). Reproductive Performance in Male European Sea Bass (Dicentrarchus Labrax, L.) Fed Two Pufa-Enriched Experimental Diets: A Comparison With Males Fed a Wet Diet. *Aquaculture* 194 (1-2), 173–190. doi: 10.1016/S0044-8486(00)00515-9

Berenjestanaki, S. S., Fereidouni, A. E., Ouraji, H. and Khalili, K. J. (2014). Influence of Dietary Lipid Sources on Growth, Reproductive Performance and Fatty Acid Compositions of Muscle and Egg in Three-Spot Gourami (Trichopodus Trichopterus) (Pallas 1770). *Aquac. Nutr.* 20 (5), 494–504. doi: 10.1111/anu.12102

Brink-Hull, M., Cyrus, M. D., Macey, B. M., Rhode, C., Hull, K. L. and Roodt-Wilding, R. (2022). Dietary Effects on the Reproductive Performance of the Sea Urchin Tripneustes Gratilla L: Implications for Broodstock Conditioning. *Aquaculture* 552, 738035. doi: 10.1016/j.aquaculture.2022.738035

Brooks, S., Tyler, C. R. and Sumpter, J. P. (1997). Egg Quality Infish: What Makes a Good Egg? *Rev. Fish Biol. Fisher.* 7 (4), 387–416. doi: 10.1023/A:1018400130692

Butts, I., Baeza, R., Støtrup, J. G., Krüger-Johnsen, M., Jacobsen, C., Pérez, L., et al. (2015). Impact of Dietary Fatty Acids on Muscle Composition, Liver Lipids, Milt Composition and Sperm Performance in European Eel. *Comp. Biochem. Phys. A* 183, 87–96. doi: 10.1016/j.cbpa.2015.01.015

Callan, C. K., Laidley, C. W., Forster, I. P., Liu, K. M., Kling, L. J. and Place, A. R. (2012). Examination of Broodstock Diet Effects on Egg Production and Egg Quality in Flame Angelfish (Centropyge Loriculus). *Aquac. Res.* 43 (5), 696–705. doi: 10.1111/j.1365-2109.2011.02877.x

Callan, C. K., Laidley, C. W., Kling, L. J., Breen, N. E. and Rhyne, A. L. (2014). The Effects of Dietary Hufa Level on Flame Angelfish (Centropyge Loriculus) Spawning, Egg Quality and Early Larval Characteristics. *Aquac. Res.* 45 (7), 1176–1186. doi: 10.1111/are.12063

Carboni, S., Vignier, J., Chiantore, M., Tocher, D. R. and Migaud, H. (2012). Effects of Dietary Microalgae on Growth, Survival and Fatty Acid Composition of Sea Urchin Paracentrotus Lividus Throughout Larval Development. *Aquaculture* 324-325 (12), 250–258. doi: 10.1016/j.aquaculture.2011.10.037

Chang, Y. Q., Zhang, W. J., Zhao, C. and Song, J. (2012). Estimates of Heritabilities and Genetic Correlations for Growth and Gonad Traits in the Sea Urchin Strongylocentrotus Intermedius. *Aquac. Res.* 43 (2), 271–280. doi: 10.1111/j.1365-2109.2011.02825.x

Crespo, D., Bonnet, E., Roher, N., Mackenzie, S. A., Krasnov, A., Goetz, F. W., et al. (2010). Cellular and Molecular Evidence for a Role of Tumor Necrosis Factor Alpha in the Ovulatory Mechanism of Trout. *Reprod. Biol. Endocrin.* 8 (1), 34–34. doi: 10.1186/1477-7827-8-34

Cuesta-Gomez, D., Lazo, J. P. and Sánchez-Saavedra, M. (2020). Effects of Dietary Fish Oil and Soya Bean Lecithin on Gonad Index, Colour and Biochemical Composition of the Purple Sea Urchin, Strongylocentrotus Purpuratus (Stimpson 1857). *Aquac. Res.* 51 (6), 3384–3402. doi: 10.1111/are.14674

Gibbs, V. K., Heflin, L. E., Jones, W. T., Powell, M. L., Lawrence, A. L., Makowsky, R., et al. (2015). Optimizing Dietary Levels of Menhaden and Soybean Oils and Soybean Lecithin for Pre-Gonadal Somatic Growth in Juveniles of the Sea Urchin Lytechinus Variegatus. *Aquaculture* 446 (1), 198–205. doi: 10.1016/j.aquaculture.2015.05.013

Heflin, L. E., Gibbs, V. K., Powell, M. L., Makowsky, R., Lawrence, J. M., Lawrence, A. L., et al. (2012). Effect of Dietary Protein and Carbohydrate Levels on Weight Gain and Gonad Production in the Sea Urchin (Lytechinus Variegatus). *Aquaculture* 358-359 (2), 253–261. doi: 10.1016/j.aquaculture.2012.06.009

Izquierdo, M. S., Fernández-Palacios, H. and Tacon, A. (2001). Effect of Broodstock Nutrition on Reproductive Performance of Fish. *Aquaculture* 197 (1-4), 25–42. doi: 10.1016/S0044-8486(01)00581-6

Jaya-Ram, A., Kuah, M. K., Lim, P. S., Kolkovski, S. and Shu-Chien, A. C. (2008). Influence of Dietary HUFA Levels on Reproductive Performance, Tissue Fatty Acid Profile and Desaturase and Elongase mRNAs Expression in Female Zebrafish (Danio Rerio). *Aquaculture* 277 (3-4), 275–281. doi: 10.1016/j.aquaculture.2008.02.027

Jedrzejczak, P., Fraczek, M., Szumaa-kakol, A., Taszarek-hauke, G., Pawelczyk, L. and Kurpisz, M. (2005). Consequences of Semen Inflammation and Lipid Peroxidation on Fertilization Capacity of Spermatozoa in *In Vitro* Conditions. *Int. J. Androl.* 28 (5), 275–283. doi: 10.1111/j.1365-2605.2005.00547.x

Kamler, E. (2005). Parent-Egg-Progeny Relationships in Teleost Fishes: An Energetics Perspective. *Rev. Fish Biol. Fisher.* 15 (14), 399–421. doi: 10.1007/s11160-006-0002-y

Kenneth, J. L. and Thomas, D. S. (2002). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-ΔΔCT Method. *Methods* 25, 402–408. doi:10.1006/meth.2001

Kottmann, J. S., Tomkiewicz, J., Butts, I. A. E., Lund, I., Jacobsen, C., Josianne, G., et al. (2020). Effects of Essential Fatty Acids and Feeding Regimes on Egg and Offspring Quality of European Eel: Comparing Reproductive Success of Farm-Raised and Wild-Caught Broodstock. *Aquaculture* 529, 1–16. doi: 10.1016/j.aquaculture.2020.735581

Liang, M. Q., Lu, Q. C., Qian, C., Zheng, K. K. and Wang, X. X. (2014). Effects of Dietary N-3 to N-6 Fatty Acid Ratios on Spawning Performance and Larval Quality in Tongue Sole Cynoglossus Semilaevis. *Aquac. Nutr.* 20 (1), 79–89. doi: 10.1111/anu.12054

Liu, X. L., Chang, Y. Q., Xiang, J. H. and Cao, X. B. (2005). Estimates of Genetic Parameters for Growth Traits of the Sea Urchin, Strongylocentrotus Intermedius. *Aquaculture* 243 (1-4), 27–32. doi: 10.1016/j.aquaculture.2004.10.014

Liu, H., Kelly, M. S., Cook, E. J., Black, K., Orr, H., Zhu, J. X., et al. (2007). The Effect of Diet Type on Growth and Fatty-Acid Composition of Sea Urchin Larvae, I. Paracentrotus Lividus (Lamarck 1816) (Echinodermata). *Aquaculture* 264 (1), 247–262. doi: 10.1016/j.aquaculture.2006.12.021

Li, L., Wang, W., Yusuf, A., Zhu, Y., Zhou, Y., Ji, P., et al. (2020). Effects of Dietary Lipid Levels on the Growth, Fatty Acid Profile and Fecundity in the Oriental River Prawn, Macrobrachium Nipponense. *Aquac Res.* 51 (2), 1–10. doi: 10.1111/are.14539

Li, M., Zhang, F., Ding, J., Zuo, R. and Chang, Y. (2021). Effects of Lipid Sources on the Growth Performance, Gonad Development, Fatty Acid Profile and Transcription of Related Genes in Juvenile Sea Urchin (Strongylocentrotus Intermedius). *Aquac. Nutr.* 27 (1), 28–38. doi: 10.1111/anu.13162

Loureno, S., Valente, L. and Andrade, C. (2019). Meta-Analysis on Nutrition Studies Modulating Sea Urchin Roe Growth, Colour and Taste. *Rev. Aquacult.* 11 (3), 776–781. doi: 10.1111/raq.12256

Lund, I., Steenfeldt, S. J., Suhr, K. I. and Hansen, B. W. (2008). A Comparison of Fatty Acid Composition and Quality Aspects of Eggs and Larvae From Cultured and Wild Broodstock of Common Sole (Solea soleaL.). *Aquac. Nutr.* 14 (6), 544–555. doi: 10.1111/j.1365-2095.2007.00560.x

Lv, D., Zhang, F., Ding, J., Chang, Y. and Zuo, R. (2020). Effects of Dietary N-3 LC-PUFA on the Growth Performance, Gonad Development, Fatty Acid Profile, Transcription of Related Genes and Intestinal Microflora in Adult Sea Urchin (Strongylocentrotus Intermedius). *Aquac. Res.* 52 (5), 1431–1441. doi: 10.1111/are.14997

Mazorra, C., Bruce, M., Bell, J. G., Davie, A., Alorend, E., Jordan, N., et al. (2003). Dietary Lipid Enhancement of Broodstock Reproductive Performance and Egg and Larval Quality in Atlantic Halibut (Hippoglossus Hippoglossus). *Aquaculture* 227 (1), 21–33. doi: 10.1016/S0044-8486(03)00493-9

Mobaraki, M., Kenari, A. A., Gorji, S. B. and Esmaeili, M. (2020). Effect of Dietary Fish and Vegetable Oil on the Growth Performance, Body Composition, Fatty Acids Profile, Reproductive Performance and Larval Resistance in Pearl Gourami (Trichogaster Leeri). *Aquac. Nutr.* 26 (3), 1–14. doi: 10.1111/anu.13048

Nazari, R. M., Sohrabnejad, M., Ghomi, M. R., Modanloo, M., Ovissipour, M. and Kalantarian, H. (2009). Correlations Between Egg Size and Dependent Variables Related to Larval Stage in Persian Sturgeon Acipenser Persicus. *Mar. Freshw. Behav. Phy.* 42 (2), 147–155. doi: 10.1080/10236240902846796

Ng, W. K. and Wang, Y. (2011). Inclusion of Crude Palm Oil in the Broodstock Diets of Female Nile Tilapia, Oreochromis Niloticus, Resulted in Enhanced Reproductive Performance Compared to Broodfish Fed Diets With Added Fish Oil or Linseed Oil. *Aquaculture* 314 (1-4), 122–131. doi: 10.1016/j.aquaculture.2011.01.034

Nhan, H. T., Nhu, T. Q., Duc, P. M., Liew, H. J., Ako, H. and Jha, R. (2020). Effects of Dietary Arachidonic Acid on Final Maturation, Spawning and Composition of Gonad of Black Sea Urchin (Diadema Setosum) (Leske 1778). *Aquac. Nutr.* 26 (2), 1771–1779. doi: 10.1111/anu.13127

Ning, Y. C., Zhang, F., Tang, L., Song, J., Ding, J., Chang, Y. Q., et al. (2022). Effects of Dietary Lipid Sources on the Growth, Gonad Development, Nutritional and Organoleptic Quality, Transcription of Fatty Acid Synthesis Related Genes and Antioxidant Capacity During Cold Storage in Adult Sea Urchin (Strongylocentrotus Intermedius). *Aquaculture* 548, 1–14. doi: 10.1016/j.aquaculture.2021.737688

Onomu, A. J., Vine, N. G., Cyrus, M., Macey, B. M. and Bolton, J. J. (2020). The Effect of Fresh Seaweed and a Formulated Diet Supplemented With Seaweed on the Growth and Gonad Quality of the Collector Sea Urchin, Tripneustes Gratilla, Under Farm Conditions. *Aquac. Res.* 51 (2), 1–16. doi: 10.1111/are.14752

Phillips, K., Hamida, N., Silcocka, P., Sewellb, M. A., Barkerc, M., Weaverc, A., et al. (2010). Effect of Manufactured Diets on the Yield, Biochemical Composition and Sensory Quality of Evechinus Chloroticus Sea Urchin Gonads. *Aquaculture* 308 (1-2), 49–59. doi: 10.1016/j.aquaculture.2010.07.030

Pomin, V. H. (2015). Sulfated Glycans in Sea Urchin Fertilization. *Glycoconjugate J.* 32 (1-2), 9–15. doi: 10.1007/s10719-015-9573-y

Prato, E., Fanelli, G., Alberto, A., Biandolino, F. and Piero, A. (2018). Influence of a Prepared Diet and a Macroalga (Ulva Sp.) on the Growth, Nutritional and Sensory Qualities of Gonads of the Sea Urchin Paracentrotus Lividus. *Aquaculture* 493, 240–250. doi: 10.1016/j.aquaculture.2018.05.010

Quintana, D., Márquez, L., Arévalo, J., Lorenzo, A. and Almansa, E. (2015). Relationships Between Spawn Quality and Biochemical Composition of Eggs and Hatchlings of Octopus Vulgaris Under Different Parental Diets. *Aquaculture* 446 (1), 206–216. doi: 10.1016/j.aquaculture.2015.04.023

Rjbek, M. C., Stttrup, J. G., Jacobsen, C., Tomkiewicz, J., Nielsen, A. and Trippel, E. A. (2014). Effects of Dietary Fatty Acids on the Production and Quality of Eggs and Larvae of Atlantic Cod (Gadus Morhua L.). *Aquac. Nutr.* 20 (6), 654–666. doi: 10.1111/anu.12124

Röttinger, E., Saudemont, A., Duboc, V., Besnardeau, L., McClay, D. and Lepage, T. (2008). FGF Signals Guide Migration of Mesenchymal Cells, Control Skeletal Morphogenesis and Regulate Gastrulation During Sea Urchin Development. *Development.* 135 (2), 353–365. doi: 10.1242/dev.014282

Samaee, S. M. (2010). "Quantitative Composition of Egg Protein, Lipid,Fatty Acid, and Free Amino Acid in Common Dentex (Dentex Dentex L.) and Their Relations to Viability and Larval Development," in *Ph.D Dissertation* (Austria: Department of Organismic Biology, University of Salzburg), 205pp.

Silvia, M., Paolo, T., Nobile, M., Denise, F., Cinta, P. and Michela, S. (2015). Unraveling Estradiol Metabolism and Involvement in the Reproductive Cycle of non-Vertebrate Animals: The Sea Urchin Model. *Steroids.* 104, 25–36. doi: 10.1016/j.steroids.2015.08.008

Stuart, K. R., Armbruster, L., Johnson, R. and Drawbridge, M. A. (2020). Egg Diameter as a Predictor for Egg Quality of California Yellowtail (Seriola Dorsalis). *Aquaculture* 522 (8), 1–6. doi: 10.1016/j.aquaculture.2020.735154

Sui, L. Y., Wu, X. G., Wille, M., Cheng, Y. X. and Sorgeloos, P. (2009). Effect of Dietary Soybean Lecithin on Reproductive Performance of Chinese Mitten Crab Eriocheir Sinensis (H. Milne-Edwards) Broodstock. *Aquacult. Int.* 17 (1), 45–56. doi: 10.1007/s10499-008-9178-6

Tamada, K. and Iwata, K. (2005). Intra-Specific Variations of Egg Size, Clutch Size and Larval Survival Related to Maternal Size in Amphidromous Rhinogobius Goby. *Environ. Biol. Fish.* 73 (4), 379–389. doi: 10.1007/s10641-005-2230-8

Tercero, J. F., Rosas, C., Mascaro, M., Poot, G. and Gallardo, P. (2015). Effects of Parental Diets Supplemented With Different Lipid Sources on Octopus Maya Embryo and Hatching Quality. *Aquaculture* 448, 234–242. doi: 10.1016/j.aquaculture.2015.05.023

Tocher, D. R. (2015). Omega-3 Long-Chain Polyunsaturated Fatty Acids and Aquaculture in Perspective. *Aquaculture* 449, 94–107. doi: 10.1016/j.aquaculture.2015.01.010

Turchini, G. M. and Francis, D. S. (2009). Fatty Acid Metabolism (Desaturation, Elongation and β-Oxidation) in Rainbow Trout Fed Fish Oil- or Linseed Oil-Based Diets. *Br. J. Nutr.* 102 (1), 69–81. doi: 10.1017/S0007114508137874

Turchini, G. M., Mentasti, T., Caprino, F., Panseri, S., Moretti, V. M. and Valfr, F. (2010). Effects of Dietary Lipid Sources on Flavour Volatile Compounds of Brown Trout (Salmo Trutta L.) Fillet. *J. Appl. Ichthyol.* 20 (1), 71–75. doi: 10.1046/j.0175-8659.2003.00522.x

Vassallo-Agius, R., Watanabe, T., Yoshizaki, G., Satoh, S. and Takeuchi, Y. (2001). Quality of Eggs and Spermatozoa of Rainbow Trout Fed an N-3 Essential Fatty Acid-Deficient Diet and its Effects on the Lipid and Fatty Acid Components of Eggs, Semen and Livers. *Fisheries Sci.* 67 (5), 818–827. doi: 10.1046/j.1444-2906.2001.00328.x

Wang, H., Ding, J., Ding, S. and Chang, Y. (2019). Metabolomic Changes and Polyunsaturated Fatty Acid Biosynthesis During Gonadal Growth and Development in the Sea Urchin Strongylocentrotus Intermedius. *Comp. Biochem. Phys. D* 32, 1–15. doi: 10.1016/j.cbd.2019.100611

Wang, X. X., Li, Y. J., Hou, C. L., Gao, Y. and Wang, Y. Z. (2012). Influence of Different Dietary Lipid Sources on the Growth, Tissue Fatty Acid Composition, Histological Changes and Peroxisome Proliferator-Activated Receptor γ Gene Expression in Large Yellow Croaker (Pseudosciaena Crocea R.). *Aquac. Res.* 43 (2), 281–291. doi: 10.1111/j.1365-2109.2011.02826.x

Wang, H., Zhao, W., Ding, B., Zhang, Y., Huang, X., Liu, X., et al. (2021). Comparative Lipidomics Profiling of the Sea Urchin, Strongylocentrotus Intermedius. *Comp. Biochem. Phys. D.* 40, 100900. doi: 10.1016/j.cbd.2021.100900

Wanke, T., Brämick, U. and Mehner, T. (2017). High Stock Density Impairs Growth, Female Condition and Fecundity, But Not Quality of Early Reproductive Stages in Vendace (Coregonus Albula). *Fish. Res.* 186 (1), 159–167. doi: 10.1016/j.fishres.2016.08.028

Xiao, B. W., Li, Q. C., Zhong, L. Z., Ai, C. X. and Deng, G. Y. (2002). Reproduction Response of Chinese Mitten-Handed Crab (Eriocheir Sinensis) Fed Different Sources of Dietary Lipid. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 131 (3), 675–681. doi: 10.1016/S1095-6433(01)00515-3

Xin, Z., Zhou, D. Y., Lu, T., Liu, Z. Y., Zhao, Q., Liu, Y. X., et al. (2018). Characterization of Lipids in Three Species of Sea Urchin. *Food Chem.* 241 (2), 97–103. doi: 10.1016/j.foodchem.2017.08.076

Xu, H. G., Zhao, M., Zheng, K. K., Wei, Y. L., Yan, L. and Liang, M. Q. (2017). Antarctic Krill (Euphausia Superba) Meal in the Diets Improved the Reproductive Performance of Tongue Sole (Cynoglossus Semilaevis) Broodstock. *Aquac. Nutr.* 23 (6), 1287–1295. doi: 10.1111/anu.12503

Yldz, M., Oforic-Mensah, S., Arslan, M., Yamaner, G., Ekici, A., Baltaci, M. A., et al. (2021). Effects of Different Dietary Lipid Resources on Sperm Quality and Reproductive Success in Rainbow Trout (Oncorhynchus Mykiss). *Aquac. Res.* 52 (8), 1–11. doi: 10.1111/are.15226

Yldz, M., Ofori-Mensah, S., Arslan, M., Ekici, A. and Korkmaz, F. (2020). Effects of Different Dietary Oils on Egg Quality and Reproductive Performance in Rainbow Trout Oncorhynchus Mykiss. *Anim. Reprod. Sci.* 221 (2), 1–11. doi: 10.1016/j.anireprosci.2020.106545

Yuan, Y., Wang, X., Jin, M., Jiao, L. and Zhou, Q. (2019). Modification of Nutritional Values and. Flavor Qualities of Muscle of Swimming Crab (Portunus Trituberculatus): Application of a Dietary Lipid Nutrition Strategy. *Food Chem.* 308, 125607. doi: 10.1016/j.foodchem.2019.125607

Zakeri, M., Marammazi, J. G., Kochanian, P., Savari, A., Yavari, V. and Haghi, M. (2009). Effects of Protein and Lipid Concentrations in Broodstock Diets on Growth, Spawning Performance and Egg Quality of Yellowfin Sea Bream (Acanthopagrus Latus). *Aquaculture* 295 (1-2), 99–105. doi: 10.1016/j.aquaculture.2009.06.026

Zhou, H. S., Chang, Y. Q., Luo, S. B., Tan, X. F., Zhang, W. J. and Zhao, C. (2013). Effects of Three Feeding Models on Growth, Gonad Yield and Gonad Quality of the Sea Urchin (Strongylocentrotus Intermedius). *J. Fish. China* 37 (7), 1050–1057. doi: 10.3724/SP.J.1231.2013.38485

Zhou, Q. C., Liu, C. W., Chi, S. Y. and Yang, Q. H. (2007). Effects of Dietary Lipid Sources on Growth and Fatty Acid Composition of Juvenile Shrimp, Litopenaeus Vannamei. *Aquac. Nutr.* 13 (3), 222–229. doi: 10.1111/j.1365-2095.2007.00470.x

Zuo, R., Hou, S., Wu, F., Song, J., Zhang, W., Zhao, C., et al. (2017). Higher Dietary Protein Increases Growth Performance, Anti-Oxidative Enzymes Activity and Expression of Heat Shock Protein 70 in the Juvenile Sea Urchin (Strongylocentrotus Intermedius) Under a Heat Stress. *Aquac. Fish.* 2 (1), 18–23. doi: 10.1016/j.aaf.2017.01.005

Zuo, R., Li, M., Ding, J. and Chang, Y. (2018). Higher Dietary Arachidonic Acid Levels Improved the Growth Performance, Gonad Development, Nutritional Value, and Antioxidant Enzyme Activities of Adult Sea Urchin (Strongylocentrotus Intermedius). *J. Ocean Univ. China* 17 (4), 932–940. doi: 10.1007/s11802-018-3606-7

Zuo, R. T., Mai, K. S., Xu, W., Turchini, G. M. and Ai, Q. H. (2015). Dietary ALA, But Not LNA, Increase Growth, Reduce Inflammatory Processes,

and Increase Anti-Oxidant Capacity in the Marine Finfish Larimichthys Crocea. *Lipids* 50 (2), 149–163. doi: 10.1007/s11745-014-3970-z

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frontiers | Frontiers in Marine Science

TYPE Original Research
PUBLISHED 01 August 2022
DOI 10.3389/fmars.2022.895061

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

RECEIVED 12 March 2022
ACCEPTED 29 June 2022
PUBLISHED 01 August 2022

CITATION
Suckling CC, Zavell MD, Byczynski AL and Takeda BT (2022) Assessing the potential of the unexploited Atlantic purple sea urchin, *Arbacia punctulata*, for the edible market.
Front. Mar. Sci. 9:895061.
doi: 10.3389/fmars.2022.895061

Assessing the potential of the unexploited Atlantic purple sea urchin, *Arbacia punctulata*, for the edible market

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The global demand for sea urchin as seafood is currently unmet. Despite exploitation of > 40 species across the world, there is a need to identify other candidate species, especially in regions where diversification in production is sought where species are considered native. The Eastern US presents an opportunity to determine the marketability of the currently unexploited *Arbacia punctulata* which is naturally distributed from Massachusetts and southwards into the Gulf of Mexico. To determine whether *A. punctulata* had market potential, it was fed one of the following diets to determine whether the gonad tissue (uni) could be manipulated to increase gonad mass and improve gonad color for the market: dried *Ulva lactuca*, Salmon pellets (Skretting), Tilapia pellets (Ziegler) or an Urchinomics diet designed for sea urchins either fed for 8 weeks or 12 weeks. All of the pelleted feeds (Salmon, Tilapia and Urchinomics) increased gonad mass and altered the color. The colors of the uni were generally darker than the colors that the market would typically prefer but some individuals did exhibit colors which have been classed as acceptable to the European market. This work highlights that further research is worthwhile to assess the market potential of *A. punctulata*.

KEYWORDS

aquaculture, echinoderm, economic, emerging species, low-trophic, novel species, sustainability

Introduction

There is a globally unmet demand for the luxury seafood product, sea urchin gonads (termed roe by the industry or uni in Japan). This is largely driven by the Asian market, but urchins are grown, sold, and consumed across the world, including regions such as North America (Eddy et al., 2015; Sun and Chiang, 2015; Stefánsson et al., 2017). Regional demand can range from local restaurants serving uni raw as sushi or

incorporating it into a sauce served with cooked pasta, to regional processors who can remove and prepare uni into aesthetically appealing packaged trays in preparation for shipping. Marketable uni should ideally have a firm and non-gamete-shedding texture and bright orange or yellow in color with a pleasant sweet-salty flavor (Sun and Chiang, 2015). Sea urchins have been wild harvested for at least six decades, with highest yields produced during the times when sushi became popular during the 1960s, however, most catches dropped dramatically following this period due to overexploitation (Stefánsson et al., 2017). Harvesting pressure to meet market demand led to over 40 species populations to overexploitation (Andrew et al., 2002; McBride, 2005) and there is pressure to identify other candidate species and to also rely more heavily on aquaculture production to meet this demand. In recent years new or emerging species have included the European Sea urchin species, *Psammechinus miliaris* (Suckling et al., 2011; Suckling et al., 2018; Suckling et al., 2020a; Suckling, 2021) and *Sphaerechinus granularis* (José et al., 2019), but there remain many species which have not yet been investigated for market potential.

The Atlantic purple sea urchin, *Arbacia punctulata* (Lamarck, 1816) is a common model species for toxicology studies (Ward et al., 2006; Nelson et al., 2010; Barron et al., 2020) but is not fished commercially at all and little is known about its market potential. It is a regular echinoid with a similar morphology to other echinoids which are currently commercially exploited, and has a wide distribution in the western Atlantic Ocean, from Massachusetts through the Gulf of Mexico and along the coast of Central and South America towards Belize, from the low tide line down to approximately 230 m depth (Kier, 1975; Serafy, 1979; Hendler & Pawson, 2000). A combination of literature reviewing and pilot sampling (Suckling, unpublished data) of sea urchins collected in the Cape Cod and Narragansett Bay regions (Rhode Island, USA) have highlighted that *A. punctulata* are gonochoristic and generally spawn in the Summer (Harvey, 1956) with firm non-gamete-shedding gonads during the austral winter indicating a lack of distinct gametes (Suckling et al., 2011). While the broad reproductive stages for *A. punctulata* need further investigation this indicates a similar reproductive stage pattern to many other temperate/sub-tropical sea urchin species (e.g. *Psammechinus miliaris* and *Paracentrotus lividus*; Byrne, 1990; Kelly et al, 2000) where firm roe are marketable within the late fall through to early spring months (**Figure 1**). Pilot sampling of sea urchins sampled Vineyard Sound (Massachusetts) and Narragansett Bay (Rhode Island, U.S.A) also identified that *A. punctulata* uni were small and undesirable in market color indicating that fishing alone would not meet market demand (Suckling, unpublished data) and is typical for animals found within habitats with low food supplies (Hughes et al., 2006; Symonds et al., 2009; Suckling et al., 2011). Their omnivorous feeding habits on a range of

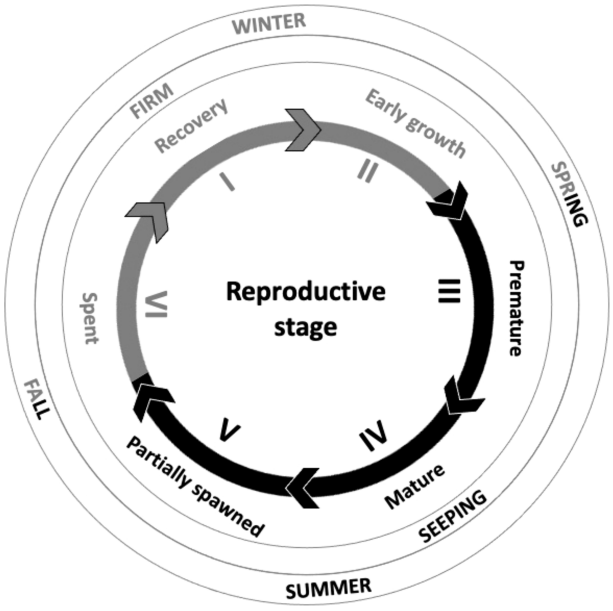

FIGURE 1
Schematic of the reproductive stages of a mature sea urchin which are best suited for the seafood market (light grey lines and text) when uni are firm and not seeping gametes, and those that are not suited for the market (black lines and text) when near their reproductive peak and uni readily seep gametes. Based on Northern hemisphere species such as *Arbacia punctulata, Psammechinus miliaris* and *Paracentrotus lividus* (adapted from Fuji, 1960; Byrne, 1990; Kelly et al., 2000; Suckling et al., 2011; Suckling, 2012). This schematic presents a general overview only, to illustrate how the reproductive cycle can be linked to the market and season, and does not align exactly with seasonal timings for *A. punctulata*.

animal and algal food sources (Lawrence, 1975; Wahl & Hay, 1995) indicate a strong ability to digest various compounds. This therefore strongly suggests that intervention using formulated feeds could be feasible, an approach used to influence and yield uni with marketable attributes for some existing commercially exploited sea urchin species (e.g. large size, firm texture and appealing bright colors; Robinson et al., 2002; Pearce et al., 2002; McBride et al., 2004; Shpigel et al., 2005; Symonds et al. 2007; Suckling et al., 2011).

A proprietary diet specifically designed for sea urchins has been emerging in recent years. This was initially developed by and referred to as the 'Nofima' diet [Norway (e.g. Siikavuopio and Mortensen, 2015)], but has since been globally licensed to Urchinomics (www.urchinomics.com) and undergone further formulation developments and is now a different diet. This diet has shown extremely promising results, yielding marketable uni in wild collected sea urchins collected to protect diminishing kelp forests within as little as 8-12 weeks. At present this feed is not available as an off the shelf diet with selective commercial agreements with the company required. Determining the suitability of alternative off the shelf options would therefore be valuable for growers. A range of available high protein (e.g. fishmeal and or soya bean protein sources) formulated feeds designed for other aquaculture species such as salmon have shown to be palatable by many sea urchin species and to yield large uni (Brown and Eddy, 2015; Fernandez and Boudouresque, 2000; Suckling et al., 2011). These feeds often incorporate natural carotenoids such as astaxanthin to promote the red/pink salmon flesh color prized by consumers, but these do not always translate well to promote marketable uni colors in sea urchins (Suckling et al., 2020b), often instead needing β-carotene supplementation through the provision of macroalgae (e.g. Shpigel et al., 2005; Carrier et al., 2017) and microalgae (McLaughlin and Kelly, 2001; Shpigel et al., 2006). Commercially available proprietary formulated feeds designed for Tilapia (e.g. Zeigler) incorporate both high protein inclusions as well as algae which would likely include desirable carotenoids which could positively influence uni color (Shpigel et al., 2005; Suckling et al., 2020b), but these remain untested in sea urchins to date.

With species such as *A. punctulata* where the effect of formulated feeds on uni development is currently unknown, some of the initial steps are to determine palatability and whether uni size and color can be influenced. Pilot trials indicate that the above listed formulated feeds are palatable and ingested and processed by *A. punctulata* (Suckling, unpublished data) and therefore the next step is to determine whether prolonged feeding can enhance uni marketable attributes. The aim of this study was to therefore assess the potential commercial prospects of *A. punctulata* by providing a range of food treatments for a period of up to 3 months, a period known to be sensitive enough to measure nutritional influences on the development of the uni (e.g. Suckling et al., 2011).

Materials and methods

Animal collection and maintenance

Adult *Arbacia punctulata* were collected in early September 2019 off the coast of Falmouth, Massachusetts, U.S.A in Vineyard Sound (41°31'38.1"N 70°38'08.1"W). Specimens were collected by bottom dredge using a 5-foot-wide scallop dredge at a depth of 9 meters and stored in coolers with aeration until their return to land within approximately 1-2 hours. Following collection, specimens were housed at the Marine Biological Laboratory (MBL), Marine Resource Center in Woods Hole (Massachusetts, USA) for 48 hours within a flow through system and fed *Ulva* sp. and *Saccharina latissima*. Specimens were then transported in insulated 95 L coolers containing ~ 56 L of seawater (~ 23°C) with aeration to the University of Rhode Island's (URI) Bay Campus aquarium facilities, Narragansett Rhode Island, U.S.A. Upon arrival at the URI Bay Campus (within approximately 2 hours), inspection of the coolers showed no sign of spawning had occurred during transportation (e.g. no milky appearance from sperm release). Seawater from the URI Bay Campus seawater supply was gradually added to the coolers across a 30-minute period to acclimate the animals to the new seawater supply. Seawater provided to the URI Bay Campus aquarium facilities was ambient with Southeastern Narragansett Bay (ambient temperature ~ 23.1°C, salinity 34 psu, pH ~ 7.9). Specimens were then held across eight 60 L (60.5 x 30.5 x 40 cm) holding tanks (25 specimens per aquaria) for two weeks supplied with ambient sand filtered flow through seawater (flow rate ~ 560 ml/min; 23.1°C; 34 psu from southern Narragansett Bay) and with aeration and under an ambient photoperiod with fluorescent lighting. During this period sea urchins were fed a combination of *Ulva* sp., *Palmaria palmata* and *Grateloupia turuturu ad libitum*.

Feed trial setup

The sea urchins were randomly allocated across eighteen 60 L glass experimental tanks (60.5 x 30.5 x 40 cm) flow through aquaria (seawater flow rate ~ 560 ml/min) until 10 specimens per aquarium was achieved. These animals were then starved for a period of two weeks to assure empty alimentary canals and to standardize their nutritional state (Vadas, 1977). Animal test diameters and whole animal wet mass were measured at the experimental start and were found to be homogenous across all experimental tanks thus showing strong initial experimental control (test diameter (mean ± SD) = 31.92 ± 3.53 mm, $F_{17, 179} = 0.26$, $p = 0.999$; wet mass = 17.24 ± 5.67g, $F_{17, 179} = 0.454$, $p = 0.501$).

The tanks (n = 10 sea urchins per replicate) were randomly allocated to baseline sampling or one of five diet treatments (3 replicate tanks per treatment and baseline group). One diet

comprised of *Ulva lactuca* ("*Ulva*") was used due to various *Ulva* species having shown to act as a feeding stimulant and enhance gonad growth and quality in sea urchins when added as an additive to pelleted feeds (Cyrus et al., 2015a; Cyrus et al., 2015b; Shpigel et al., 2018; Cyrus et al., 2019). Due to its abundance in coastal areas around the globe *U. lactuca* may be a cheap and easily available alternative to other diets for raising sea urchins and is easily dried as a supplement for pelleted feeds (Cyrus et al., 2014). *U. lactuca* was collected from the east passage of Narragansett Bay at Beavertail State Park (41.4535°N, 71.3976°W) at low tide once a week throughout September and October of 2019. All epiphytes were removed from the surface and *U. lactuca* was then oven dried at 60° C for one week and then frozen at -20°C. *U. lactuca* was dried to enable medium term storage. High protein diets were also assessed because, like other sea urchin species, *A. punctulata* is omnivorous with which include carnivory habits (Lawrence, 1975; Wahl & Hay, 1995; Gianguzza, 2020). Furthermore, previous studies have highlighted that gonad index can be substantially enhanced with high protein diets (e.g. Pearce et al., 2002; Robinson et al., 2002; Suckling et al., 2011). Sea urchins were fed a commercially available "Salmon" pelleted diet treatment (Skretting Salmon Sink 1.6 mm, Tooele, UT, USA; **Table 1**) with the primary sources of protein comprising of fish meal/oil and poultry meal/oil. A "Tilapia" diet treatment (Zeigler Finfish Broodstock 38-10, Gardners, PA, USA; **Table 1**) was also used with proteins comprising primarily of fish and poultry meal as well as wheat, corn, and soybeans. This diet also contains a proprietary mix of the carotenoids (Zeigler Bros., Inc, personal communication) due to the inclusion of algae in this pelleted feed, known to be important in immunity defense (Ito et al., 1992). Furthermore, carotenoids (e.g. β-carotene) have been widely shown to enhance the color of the gonads for the market (Robinson et al., 2002; Shpigel et al., 2005; Symonds et al. 2007; Symonds et al., 2009; Suckling et al., 2011; Suckling et al., 2020b).

The final diet treatments comprised of an "Urchinomics" diet. This is a proprietary sea urchin diet currently globally licensed by Urchinomics (https://www.urchinomics.com/). It has previously been identified as the "NOFIMA" diet (Siikavuopio and Mortensen, 2015) but has since undergone further development. During its identity as the Nofima diet, it was shown to successfully enhance somatic and gonad growth within commercial sea urchin species such as *Strongylocentrotus droebachiensis* and *Paracentrotus lividus* and enhanced gonad color due to its high inclusion rate of macroalgae containing β-carotene (e.g. Siikavuopio and Mortensen, 2015; Prato et al., 2018). Since these studies and licensing to Urchinomics, this diet has undergone further changes through research and development, thus meaning it now has a different composition to its previous identity as the NOFIMA diet and its current status will now be referred to as the "Urchinomics" diet. Wild caught sea urchins can be fed the Urchinomics diet for a period of at least eight weeks to show gonad enhancement for the commercial market, but most food trials have been conducted for 12 weeks (e.g. Pearce et al., 2007; Suckling et al., 2011; Shpigel and Erez, 2020). This study incorporated both time frames for the Urchinomics diet (8 and 12 weeks; 'Urchinomics-8' and "Urchomics-12" respectively) and started feeding sea urchins from the start of the experimental period, with the 'Urchinomics-8' treatment group ending 4 weeks earlier than all other diet treatments. The start of these feed trials conformed to the start of the reproductive cycle and both time frames (8 and 12 weeks) were within what would be considered as the harvesting period, when gonad is firm and not leaching gametes.

Sea urchins were fed diet treatments at 3% wet body mass, three times a week for three months following the protocols outlined in Suckling et al. (2011). Aquaria were cleaned and siphoned three times a week and allowed to refill before the sea urchins were fed. Frozen *U. lactuca* was defrosted before being fed. Proximate analyses of the diets were provided by the manufacturer except for *Ulva* (**Table 1**). Ulva for proximate analysis was homogenized into a fine powder and three 50 mg samples were sent to New Jersey Feed Lab Inc (Trenton, NJ, USA) for a proximate and caloric analysis. The feed trials were conducted at ambient temperature and a 12L:12D photoperiod with fluorescent lighting was used. Tank seawater parameters (salinity and temperature) were monitored and measured twice a week (Mettler Toledo Portable SG3 pH Meter and TMC Aquarium V2 Handheld Refractometer). The seawater salinity and temperature remained similar throughout the experiment (**Table 2**).

Sea urchin data collection

Thirty sea urchins were dissected at the start of the experiment for baseline samples (3 replicates of 10 urchins,

TABLE 1 Percent proximate analysis and caloric value, of the diets provided throughout the feeding trial.

Diet	Protein(%)	Fibre(%)	Fat(%)	Phosphorus(%)	Calories(Kcal/100g)	48-h Stability
Ulva	12.52	5.29	0.93	0.17	192.43	Fully Intact
Salmon	45	3	19	1.4	4.16e-7	Partially Intact
Tilapia	38	4.5	10	1.0	448	Partially Intact
Urchinomics*	12.5	–	1.6	–	–	Fully Intact

* Calculated values provided by Urchinomics.
A qualitative assessment of diet stability when exposed to seawater for 48 hours is also provided.

TABLE 2 Mean (± SE) seawater parameters measured throughout the feed trial within aquaria.

Diet	Temperature (°C)	Salinity (psu)
Ulva	8.24 ± 0.2	34 ± 0
Salmon	7.93 ± 0.2	34 ± 0
Tilapia	7.82 ± 0.2	34 ± 0
Urchinomics-8	8.55 ± 0.4	34 ± 0
Urchinomics-12	7.85 ± 0.2	34 ± 0
p-value	0.080	0.830

total = 30) and an additional 30 sea urchins (3 replicates of 10 urchins, total = 30) were sampled at the end of the experiment for each food treatment group following a ten-day starvation period to empty the digestive tract from remaining food and fecal materials to allow for tissue comparisons (Vadas, 1977). Prior to dissection, excess seawater was removed from the sea urchin body by briefly drip drying on clean paper towels. Test diameter (mm ± 0.01) was measured three times using vernier calipers and the mean test diameter was used for analysis. Whole animal wet mass was then measured (g ± 0.01) after which they were dissected in half using dissection scissors and tweezers to remove the gonad segments and alimentary canal. Immediately following dissection, gonads were qualitatively assessed by compared against a gonad color chart comprising of Pantone color chips for assessing the market value developed by Cook (1999); Symonds et al. (2009) and Suckling et al. (2011). In summary, colors ranked as acceptable (bright orange to yellow and pale colors) and unacceptable (dark brown). The gonad tissue was removed and following recording the wet gonad mass (g ± 0.01), one segment was selected at random and removed, and CIE L*a*b color values were measured using a chromometer (Minolta Chroma Meter CR-300; Suckling et al., 2011). The L*a*b color space is a way to quantifiably define colors (C.I.E., 1931) and is commonly used in the food industry (Hutchings, 1994) and used to define sea urchin gonads (Agatsuma, 1998; Robinson et al., 2002; McBride et al., 2004). L* represents the intensity or lightness of a samples (L* = 60 is white), while a* represents the hue or redness in a sample (+0 – 60), and b is the chroma or yellowness in a sample (+0 -60; McBride et al., 2004; Suckling et al., 2020b). Gonad index (GI) was calculated by dividing the wet gonad mass by the whole animal wet mass and expressed as a percentage.

One of the gonad segments was fixed in a 4% formalin solution until it was dehydrated, stained with a hematoxylin and eosin dye (H/E) which stained the gametogenic cells and nutritive phagocytes differently (Byrne, 1990), sectioned, and placed on positively charged slides (MAS Histology Services, Worcester, Massachusetts, USA). Upon return the samples were photographed using a compound trinocular microscope with mounted Omax (A35180U3) 18 mp digital camera. Each sample was photographed at 4x magnification. The images were then used to determine the sex and reproductive stage following the descriptions outlined by Fuji (1960) and Byrne (1990). "Stage I" is the recovering period and the follicle is contracted with "rumples", "Stage II" is the growing period with oocytes between 40-60 µm and many spermatocytes in testes, "Stage III" is the pre-mature stage and in females the follicle is occupied by the primary oocyte and in males there are "sperm patches", "Stage IV" are mature and there is no empty space in the follicle with numerous secondary oocytes while male follicles are filled with spermatozoa, finally "Stage V" is spent and in both sexes there is a large empty space in the follicle.

The alimentary canal was removed and placed onto tissue paper briefly to remove excess moisture, and weighed (g ± 0.01). The alimentary index was then calculated by dividing the alimentary mass by the whole animal wet mass and expressed as a percentage.

Statistical analysis

Data was stored in Microsoft Excel (V.16.0.12730.20188), analyzed in Minitab (v17). Proportional data were arcsine transformed before analysis (Kelly et al., 2000). The data were tested for homogeneity of variance (Levene's) and if these assumptions were met then a general linear model was used to assess the factor of treatment and nesting replicate tanks for test diameter, whole animal wet mass, alimentary index, and color measurements (CIE L*a*b values). A one-way ANOVA was conducted for survival and marketable gonad color data. After significant results, Tukey's pairwise comparisons were conducted to determine treatment differences. If data did not fit the assumptions of ANOVA following either a log or square root transformation, then a nonparametric Kruskal Wallis test was conducted (gonad index, reproductive stage). Where significant differences occurred, a Mann Whitney *post-hoc* comparison test was conducted to identify the treatment differences. Due to multiple testing, a Bonferroni correction was applied to reduce the occurrence of type I errors.

Results

Survival and somatic growth

No significant differences in survival were found between the food treatments ($F_{4,14}$ = 2.00, p = 0.171; **Figure 2A**). With

respect to somatic growth, test diameters of *A. punctulata* fed the Urchinomics diet for 8 weeks (Urchinomics-8) was significantly lower than the *Ulva*, Tilapia and Urchinomics diet for 12 weeks (Urchnomics-12) treatments, likely due to the shorter experimental time utilized for this treatment group (i.e. 8 vs 12 weeks) (Treatment: $F_{4,\ 147} = 7.62$, $p < 0.001$; Treatment (Tank): $F_{10,147} = 3.15$, $p = 0.001$; **Figure 2B**). The provision of different diet treatments did not significantly impact whole animal wet mass (Treatment: $F_{4,\ 147} = 0.62$, $p = 0.647$; Treatment (Tank): $F_{4,147} = 3.05$, $p = 0.002$; **Figure 2C**). Specimens fed the pelleted diets had significantly higher alimentary indices (A.I.) compared to the baselines and those fed *Ulva* ($F_{5,177} = 12.23$, $p < 0.001$; Treatment (Tank): $F_{12,177} = 2.90$, $p = 0.179$; **Figure 2D**).

Gonad growth and quality

The pelleted diet treatments (Salmon, Tilapia, Urchinomics-8 and Urchinomics-12) gave rise to the largest GI and were significantly greater compared to the *Ulva* diet and baseline samples (Treatment: $F_{5,177} = 18.71$, $p < 0.001$; Treatment (Tank): $F_{12,177} = 1.48$, $p = 0.135$; **Figure 3A**). A significant lightening (CIE L*) of gonad color was seen from the baseline samples for sea urchins fed the salmon diet diets ($F_{5,\ 177} = 5.55$, $p < 0.001$; Treatment (Tank): $F_{12,177} = 2.90$, $p = 0.001$; **Figure 3B**). A decrease in redness (CIE a*) was observed for sea urchins fed *Ulva* compared to the baseline samples ($F_{5,\ 177} = 7.25$, $p < 0.001$; Treatment (Tank): $F_{12,177} = 1.08$, $p = 0.382$; **Figure 3C**). Compared to baseline samples, a decrease in yellowness (CIE b) of gonad color was observed in sea urchins fed the Ulva diet, and an increase in yellowness was observed in sea urchins fed the Tilapia diet ($F_{5,\ 177} = 6.62$, $p < 0.001$; Treatment (Tank): $F_{12,177} = 2.12$, $p = 0.019$; **Figure 3D**). There were no significant differences in the percentage of sea urchins with gonad colors acceptable for the market across the experiment ($F_{5,17} = 2.83$, $p = 0.065$; **Figure 3E**). The reproductive stage of the sea urchins remained statistically similar throughout the experimental period, regardless of the diet provided ($H_5 = 9.69$, $p = 0.140$; **Figure 3F**).

Discussion

This study highlights that the gonad index of *A. punctulata* can be positively enhanced using pelleted feed which is in agreement with a wide number of studies (e.g. de Jong-Westman et al., 1995; Fernandez et al., 1997; Lawrence et al.,

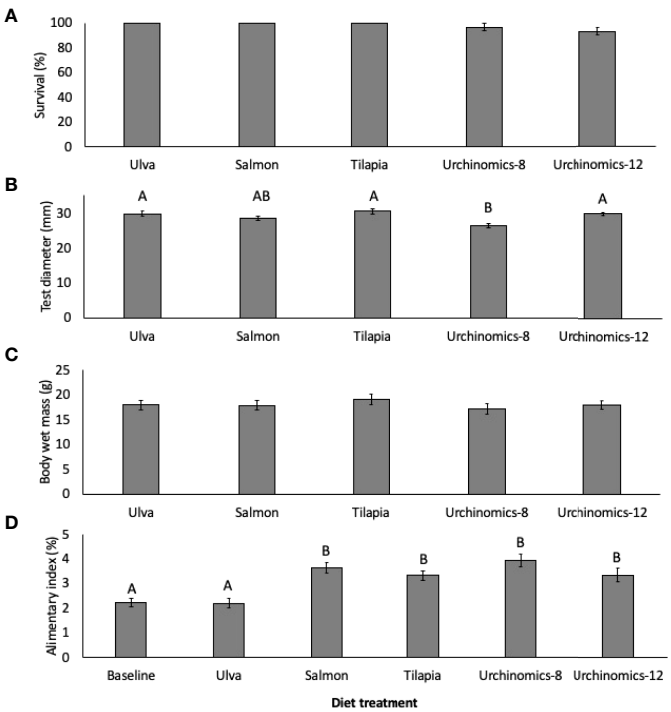

FIGURE 2
Mean (± SE) survival (%; **A**), test diameter (mm; **B**), whole animal wet mass (g; **C**), and alimentary index (%; **D**) of *Arbacia punctulata* fed different diet treatments. Letters above bars represent groups that are significantly different from each other, and where there are no letters indicate no significant treatment effect.

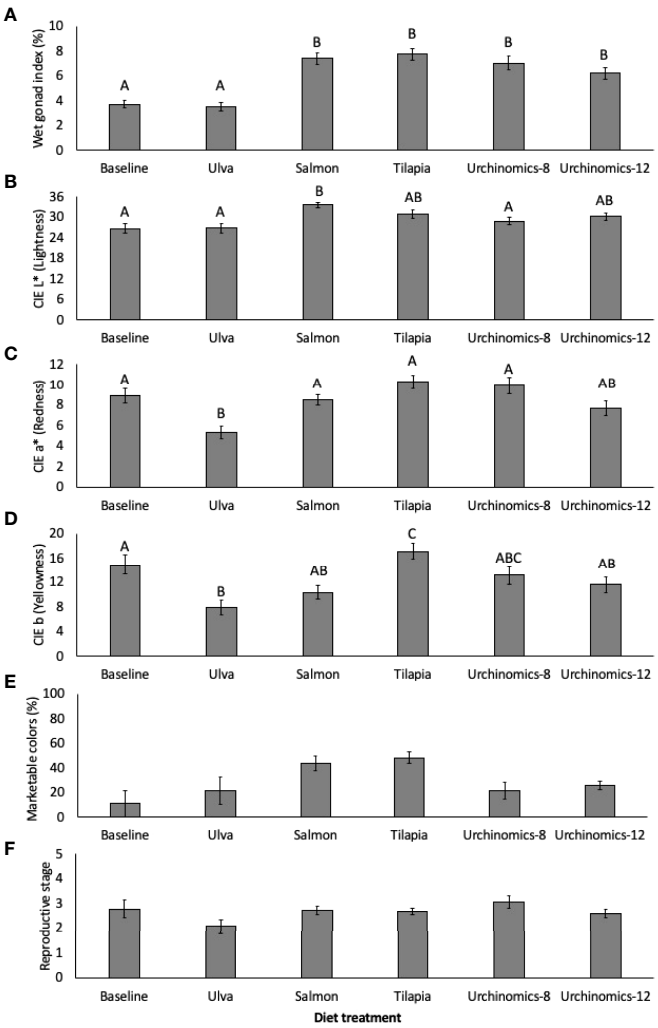

FIGURE 3
Mean (± SE) wet gonad index (%; **A**), CIE L* (lightness; **B**), CIE a* (redness; **C**), CIE b (yellowness; **D**), acceptable market gonad colors (%; **E**) and median (± IQR) reproductive stage **(F)** of *Arbacia punctulata* fed different diet treatments. Letters above bars represent groups that are significantly different from each other, and where there are no letters indicate no significant treatment effect.

1997; Akiyama et al., 2001; Robinson et al, 2002; Suckling et al., 2011; Zupo et al., 2018). While there was no significant improvement on marketable gonad colors, there was a subtle increase which highlights potential to manipulate gonad color through diet in this species and would need further trials to elucidate this. The colors of the uni were a darker range of colors (i.e. dark shades of purple, brown, red, orange and yellow) than the market would typically prefer from established marketable species (Suckling and Zavell, personal observation). At present we do not know for certain whether this species can achieve the familiar and preferred market uni colors typically being bright orange or yellow. But some individuals did exhibit darker pink/cream/red colors which have been classed as acceptable to the European market for *P. miliaris* and may provide an interesting new color aesthetic experience for the sushi market (Suckling et al., 2011). It is possible that the carotenoids included within these diets might not be compatible for biochemical conversion to color in the uni, and this has been shown to differ across species (Tsushima et al., 1997; Suckling et al., 2020b). Further work assessing the influence of differing carotenoids included into a base diet such those described by Robinson et al. (2002) and Suckling et al. (2011) would be required to determine this. Despite the darker uni colors, this study highlights that there was a clear influence of diet on gonad coloration. Additionally, the uni were observed to be at reproductive stages which would be conducive to typical late harvesting periods (**Figure 1**), thus showing that *A. punctulata* has potential for the market.

While it was clear that diet could influence and enhance gonadal growth in *A. punctulata*, in comparison to commercially exploited or emerging species, this growth was markedly slow

(e.g. de Jong-Westman et al., 1995; Fernandez et al., 1997; Lawrence et al., 1997; Akiyama et al., 2001; Robinson et al, 2002; Suckling et al., 2011; Zupo et al., 2018). This could in part be explained by the collection method, scallop dredging. During this collection method, the harvested animals are caught within a mesh collection bag and dragged some distance with other dredged materials which can incur injury and high stress. Scallop dredging has been used in the past to collect *Strongylocentrotus droebachiensis* for the US market with some success (Scattergood, 1961), but the animals were sold and consumed within a short period of time after harvesting, thus omitting the longer-term impacts of this method. Dredging is used in other sea urchin fisheries across the world (e.g. Greenland and Iceland) but can be modified to reduce the stress and damage on the harvested sea urchins (James and Hannon, 2017). It is therefore possible that the harvested *A. punctulata* could have been highly stressed and therefore physiologically compromised and/or needing to allocate energy towards repair, despite efforts to use only healthy intact and undamaged looking animals in this study. Although survival data was not significantly different, this could also explain the mortalities recorded for both Urchinomics diet treatments (**Figure 2**), however, only a single animal died in a replicate tank thus representing 10% of that population and therefore was overall a low number. The Urchinomics diet has shown strong unanimous successes in its use (www.urchinomics.com) and would unlikely have been the cause of mortality in this trial.

Slow gonadal growth could also be explained by the geographical distribution and sourcing of this species. These animals were collected from and reared within the coldest northernmost range of their natural distribution (Serafy, 1979). This study was also carried out during the seasonally low winter temperatures (**Table 2**) meaning that this species was likely to be functioning within its lowest metabolic scope (Clarke and Johnston, 1999; Addo-Bediako et al., 2000; Suckling et al., 2020a). In turn the collection method or cooler temperatures could have limited the carotenoid utilization from the diets thus leading to the darker uni colors. Therefore, it is recommended that more trials be conducted using less impactful harvesting methods and within warmer temperatures than those used in the current study and/or across longer periods of time and using different carotenoids (e.g., lutein and zeaxanthin; Suckling et al., 2011; Suckling et al., 2020b) to determine whether gonad growth and color in *A. punctulata* can be further enhanced. While these remain the most likely factors of influence, there is also the possibility the diets provided within this study were not optimized for this species, thus warranting further investigations on differing food supplies.

Numerous sea urchin species (e.g. *Arbacia lixula, Strongylocentrotus droebachiensis, Psammechinus miliaris*), have been shown to be resilient to projected variability in ocean conditions expected within the next few decades (e.g. alteration to CO_2 and temperature; Wangensteen et al., 2013; Suckling et al., 2014; Ross et al., 2015). The cellular processes of acid base buffering, that counteract increased CO_2, can be achieved within 7-10 days (Stumpp et al., 2012) and they have shown to be reproductive and marketable under medium to long term exposures to laboratory simulated climate change scenarios (Dupont et al., 2013; Suckling et al., 2014; Suckling et al., 2020a) across several generations (Suckling, unpublished data). Therefore, the production of sea urchins presents a potential sustainable option for growers, and to support this industry it is important that new (e.g. *A. punctulata*) and emerging species (e.g. *P. miliaris*, and *S. granularis*;Suckling et al., 2011; Suckling et al., 2018; José et al., 2019; Suckling et al., 2020a), are identified and investigated. This mitigation strategy of diversifying production (i.e. with tolerant sea urchin species) is increasing in interest and uptake by growers in the US (Reid et al., 2019) allowing for contingency against losses already being observed from climate change (e.g. shellfish production; Barton et al., 2012; Clements & Chopin, 2016).

At present on the Eastern US coast, the green sea urchin (*Strongylocentrotus droebachiensis*) is one of the most valuable sea urchin species. It is a cold-water species and is currently produced through wild fisheries capture and is emerging through aquaculture in the State of Maine (ME). But recent work funded by the Northeastern Regional Aquaculture Center led by lead author Suckling shows that there is interest to expand this production into other New England states (New Hampshire, Massachusetts, and Rhode Island). At present the Rhode Island governing bodies are uncertain of the native status of *S. droebachiensis* with unconfirmed reports provided from regional recreational scuba divers and one confirmed report in 1998 in a coastal pond (Rhode Island Department of Environmental Management). Therefore, there is a potential niche and interest for *A. punctulata* production in areas where sea urchin cultivation is not yet practiced expanding southwards comfortably within this species' natural distribution range. However, further work is first needed to fully determine the economic potential for *A. punctulata.*

Data availability statement

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

Author contributions

CS, MZ, and BT contributed to conception and design of the study. CS and MZ performed the statistical analysis, and wrote the first draft of the manuscript. AB conducted and contributed sex and reproductive stage determination data. AB and BT wrote sections of the manuscript. BT contributed the Urchinomics diet for the trial. All authors contributed to manuscript revision, read, and approved the submitted version.

Funding

This work was partly funded by the United States Department of Agriculture, National Institute of Food and Agriculture (Project #RI0019-H020), Northeastern Regional Aquaculture Center and the University of Rhode Island Department of Undergraduate Research and Innovation (URI[2]).

Acknowledgments

Thank you to Urchinomics and Yuma Yamamoto for supplying the diet for use in this study. Thank you to Alli McKenna and Tyler Pelletier for animal husbandry assistance and to Sara Lacourciere, Ellie Mulligan, and Kelsey Mudry for assistance in the laboratory. This work was generated through an undergraduate research skills course (AFS491) within the University of Rhode Island.

Conflict of interest

Author BT was employed by Urchinomics BV.

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.

Publisher's note

References

Addo-Bediako, A., Chown, S. L., and Gaston, K. J. (2000). Thermal tolerance, climatic variability and latitude. *Proc. Biol. Sci.* 267, 739–745. doi: 10.1098/rspb.2000.1065

Agatsuma, Y. (1998). Aquaculture of the sea urchin (Strongylocentrotus nudus) transplanted from coralline flats in hoikkaido Sea. *J. Shellfish. Res.* 17, 1541–1547.

Akiyama, T., Uhuma, T., and Yamamota, T. (2001). Optimum protein level in a purified diet for young red sea urchin pseudocentrotus depressus. *Fish. Sci.* 67, 361–363. doi: 10.1046/j.1444-2906.2001.00252.x

Andrew, N. L., Agastusma, Y., Ballesteros, E., Bazhin, A. G., Creaser, E. P., Barnes, D. K. A., et al. (2002). Status and management of world sea urchin fisheries. *Oceanogr. Mar. Biol.* 40, 343–425. doi: 10.1201/9780203180594-26

Barron, M. G., Bejarano, A. C., Conmy, R. N., Sundaravadivelu, D., and Meyer, P. (2020). Toxicity of oil spill response agents and crude oils to five aquatic test species. *Mar. pollut. Bull.* 153, 110954. doi: 10.1016/j.marpolbul.2020.110954

Barton, A., Hales, B., Waldbusser, G. G., Langdon, C., and Feely, R. A. (2012). The pacific oysters, crassostrea gigas, shows negative correlation to naturally elevated carbon dioxide levels: implications for near-term ocean acidification effects. *Limnol. Ocean.* 57, 698–710. doi: 10.4319/lo.2012.57.3.0698

Brown, N., and Eddy, S. *Echinoderm aquaculture* (Hoboken, New Jersey, USA: Wiley Blackwell).

Byrne, M. (1990). Annual reproductive cycles of the commercial sea urchin paracentrotus lividus from an exposed intertidal and a sheltered subtidal habitat on the west coast of Ireland. *Mar. Biol.* 104, 275–289. doi: 10.1007/BF01313269

Carrier, T. J., Eddy, S. D., and Redmond, S. (2017). Solar-dried kelp as potential feed in sea urchin aquaculture. *Aquac. Intl.* 25, 355–366. doi: 10.1007/s10499-016-0033-x

C.I.E (1931). *Comission internationale de l'Eclairage: Proceedings 8th session. Cambridge, England, 19 to 29 September 1931* (Paris, France: Bureau Central de la C.I.E.).

Clarke, A., and Johnston, N. M. (1999). Scaling of metabolic rate with body mass and temperature in teleost fish. *J. Anim. Ecol.* 68, 893–905. doi: 10.1046/j.1365-2656.1999.00337.x

Clements, J. C., and Chopin, T. (2016). Ocean acidification and marine aquaculture in north America: potential impacts and mitigation strategies. *Rev. Aqua.* 9, 326–341. doi: 10.1111/raq.12140

Cook, E. J. (1999). *Psammechinus miliaris (Gmelin) (Echinodermata: Echinoidea): factors affecting its somatic growth and gonadal growth and development* (Edinburgh, UK: Napier University).

Cyrus, M. D., Bolton, J. J., De Wat, L., and Macey, B. M. (2014). The development of a formulated feed containing ulva (Chlorophyta) to promote rapid growth and enhanced production of high quality roe in the sea urchin tripneustes gratilla (Linnaeus). *Aquac. Res.* 45, 159–176. doi: 10.1111/j.1365-2109.2012.03219.x

Cyrus, M. D., Bolton, J. J., and Macey, B. M. (2015a). The role of green seaweed ulva as a dietary supplement for full life cycle grow-out of tripneustes gratilla. *Aquaculture* 446, 187–197. doi: 10.1016/j.aquaculture.2015.05.002

Cyrus, M. D., Bolton, J. J., and Macey, B. M. (2019). The use of stable isotope ratios δ13C and δ15N to track the incorporation of ulva and other important dietary ingredients into the gonads of the sea urchin tripneustes gratilla. *Aquac. Nutr.* 26, 174–1185. doi: 10.1111/anu.12979

Cyrus, M. D., Bolton, J. J., Scholtz, R., and Macey, B. M. (2015b). The advantages of ulva (Chlorophyta) as an additive in sea urchin formulated feeds: effects on palatability, consumption and digestibility. *Aquac. Nutr.* 21, 578–591. doi: 10.1111/anu.12182

de Jong-Westman, M., March, B. E., and Carefoot, T. H. (1995). The effect of different nutrient formulations in artificial diets on gonad growth in the sea urchin strongylocentrotus droebachiensis. *Can. J. Zool.* 73, 1495–1502. doi: 10.1139/z95-177

Dupont, S., Dorey, N., Stumpp, M., Melzner, F., and Thorndyke, M. (2013). Long-term & trans-life-cycle effects of exposure to ocean acidification in the green sea urchin strongylocentrotus droebachiensis. *Mar. Biol.* 160, 1835–1843. doi: 10.1007/s00227-012-1921-x

Eddy, S. D., Brown, N. P., and Harris, L. G. (2015). "Aquaculture of the green Sea urchin strongylocentrotus droebachiensis in north America," in *Echinoderm aquaculture*. Eds. N. P. Brown and S. D. Eddy (Hoboken, NJ: Wiley-Blackwell), 175–209. doi: 10.1002/9781119005810.ch8

Fernandez, C., and Boudouresque, C.-F. (2000). Nutrition of the sea urchin paracentrotus lividus (Echinodermata: Echinoidea) fed different artificial food. *Mar. Ecol. Prog. Ser.* 204, 131–141. doi: 10.3354/meps204131

Fernandez, C., Dombrowski, E., and Caltagirone, A. (1997). "Gonadic growth of adult sea urchins, paracentrotus lividus (Echinodermata: Echinoidea) in rearing: the effect of different diet types," in *Echinoderm research*. Eds. R. Emson, A. Smith and A. Campbell (Rotterdam: Balkema), 269–275.

Fuji, A. (1960). *Studies on the biology of the sea urchin: I. superficial and histological gonadal changes in gametogenic process of two sea urchins, strongylocentrotus nudus and s. intermedius* Vol. 11 (Bulletin of Faculty of Fishes, Hokkaido University), 1–14. Available at: https://eprints.lib.hokudai.ac.jp/dspace/bitstream/2115/23091/1/11(1)_P1-14.pdf.

Gianguzza, P. (2020). "Chapter 24: Arbacia," in *Sea Urchins: Biology and ecology, 4th ed.* Ed. J. M. Lawrence (Amsterdam: Elsevier B.V.), 419–429. doi: 10.1016/B978-0-12-819570-3.00024-X

Harvey, E. B. (1956). *The American arbacia and other sea urchins* (Princeton, New Jersey: Princeton University Press). doi: 10.5962/bhl.title.7234

Hendler, G., and Pawson, D. L. (2000). Echinoderms of the rhomboidal cays, Belize: Biodiversity, distribution, and ecology. *Atoll. Res. Bull.* 479, 275–299.

Hughes, A. D., Kelly, M., Barnes, D. K. A., Catarino, A. I., and Black, K. D. (2006). The dual function of sea urchin gonads are reflected in the temporal variations of their biochemistry. *Mar. Biol.* 148, 789–798. doi: 10.1007/s00227-005-0124-0

Hutchings, J. B. (1994). *Food colour and appearance* (New York: Springer US). doi: 10.1007/978-1-4615-2123-5

Ito, T., Matsutani, T., Mori, K., and Nomura, T. (1992). Phagocytosis and hydrogen peroxide production by phagocytes the sea urchin strongylocentrotus nudus. *Dev. Comp. Immunol.* 16, 287–294. doi: 10.1016/0145-305x(92)90003-u

James, P., and Hannon, C. (2017). "Cost/benefit analysis of sea urchin fishing techniques," in *Technical report activity A3.2.2 of the NPA URCHIN project* (Tromsø: Nofima). doi: 10.13140/RG.2.2.10724.78728

José, R., Ribeiro, C., Neves, P., and Lourenço, S. (2019). "First assessment of the population structure and reproductive cycle of the sea urchin sphaerechinus granularis (Lamarck 1816) in Madeira island: a potential new candidate to echinoculture," in *IMMR'18 | International Meeting on Marine Research*, Peniche, 2018. doi: 10.3389/conf.FMARS.2018.06.00133

Kelly, S. M., Hunter, A. J., Scholfield, C. L., and McKenzie, J. D. (2000). Morphology and survivorship of larval psammechinus miliaris (Gmelin) (Echinodermata: Echinoidea) in response to varying food quantity and quality. *Aquaculture* 183, 223–240. doi: 10.1016/S0044-8486(99)00296-3

Kier, P. M. (1975). *The echinoids of Carrie bow cay, belize. smithson. contrib. zool.* NO. 206, 1–45. Smithsonian Institute.

Lawrence, J. M. (1975). On the relation between marine plants and sea urchins. *Oceanogr. Mar. Biol.* 13, 213–286.

Lawrence, J. M., Olave, S., Otaiza, R., Lawrence, A. L., and Bustos, E. (1997). Enhancement of gonad production in the sea urchin loxechinus albus in Chile fed extruded feeds. *J. World Aquac. Soc* 28, 91–96. doi: 10.1111/j.1749-7345.1997.tb00966.x

Mcbride, S. C. (2005). Sea Urchin aquaculture. *Am. Fish. Soc Symp.* 46, 179–208.

McBride, S. C., Price, R. J., Tom, P. D., Lawrence, J. M., and Lawrence, A. L. (2004). Comparison of gonad quality factors: color, hardness, and resilience, of strongylocentrotus franciscanus between sea urchins fed prepared feed or algal diets and sea urchins harvested from the northern California fishery. *Aquaculture* 231, 405–422. doi: 10.1016/j.aquaculture.2003.10.014

McLaughlin, G., and Kelly, S. M. (2001). Effect of artificial diets containing carotenoid-rich microalgae on gonad growth and colour in the sea urchin psammechinus miliaris (Gmelin). *J. Shellfish. Res.* 20, 377–382.

Nelson, R., Nipper, M., Lawrence, A., and Watts, S. (2010). Parental dietary effect on embryological development response to toxicants with the sea urchin arbacia punctulata. *Bull. Environ. Contam. Toxicol.* 84, 71–75. doi: 10.1007/s00128-009-9909-z

Pearce, C. M., Daggett, T. L., and Robinson, S. M. C. (2002). Effect of binder type and concentration on prepared feed stability and gonad yield and quality of the green sea urchin, strongylocentrotus droebachiensis. *Aquaculture* 205, 301–323. doi: 10.1016/s0044-8486(01)00685-8

Pearce, C. M., Daggett, T., and Robinson, S. M. C. (2007). Optimizing prepared feed ration for gonad production of the green sea urchin strongylocentrotus droebachiensis. *J. World Aquac. Soc* 33, 268–277. doi: 10.1111/j.1749-7345.2002.tb00503.x

Prato, E., Chiantore, M., Kelly, M. S., Hughes, A. D., James, P., Ferranti, M. P., et al. (2018). Effect of formulated diets on the proximate composition and fatty acid profiles of sea urchin paracentrotus lividus gonad. *Aquac. Int.* 26, 185–202. doi: 10.1007/s10499-017-0203-5

Reid, G. K., Gurney-Smith, H. J., Flaherty, M., Garber, A. F., Forster, I., Brewer-Dalton, K., et al (2019). Climate change and aquaculture: considering adaptation potential. *Aquac. Environ. Interact.* 11, 603–624. doi: 10.3354/aei00333

Robinson, S. M. C., Castell, J. D., and Kennedy, E. J. (2002). Developing suitable colour in the gonads of cultured green sea urchins (Strongylocentrotus droebachiensis). *Aquaculture* 206, 289–303. doi: 10.1016/S0044-8486(01)00723-2

Ross, P., Parker, L., and Byrne, M. (2015). Transgenerational responses of molluscs and echinoderms to changing ocean conditions. *ICES J. Mar. Sci.* 73, 537–549. doi: 10.1093/icesjms/fsv254

Scattergood, L. W. (1961). *The sea urchin fishery* (Washington D.C: The United States Department of the Interior, Fish and Wildlife Service Bureau of Commercial Fisheries), 20240.

Serafy, D. K. (1979). *Memoirs of the hourglass cruises: Echinoids (Echinodermata: Echinoidea), volume 5* (St. Petersberg, Florida: Florida Department of Natural Resources, Marine Research Laboratory). Available at: http://hdl.handle.net/1834/18639.

Shpigel, M., and Erez, J. (2020). Effect of diets and light regimes on calcification and somatic growth of the sea urchin tripneustes gratilla elantensis. *Aquaculture* 529, 735547. doi: 10.1016/j.aquaculture.2020.735547

Shpigel, M., McBride, S. C., Marciano, S., Ron, S., and Ben-Amotz, A. (2005). Improving gonad color and somatic index in the European sea urchin paracentrotus lividus. *Aquaculture* 318, 335–342. doi: 10.1016/j.aquaculture.2004.11.043

Shpigel, M., Schlosser, S. C., Ben-Amotz, A., Lawrence, A. L., and Lawrence, J. M. (2006). Effects of dietary carotenoid on the gut and the gonad of the sea urchin paracentrotus lividus. *Aquaculture* 261, 1269–1280. doi: 10.1016/j.aquaculture.2006.08.029

Shpigel, M., Shauli, L., Odintsov, V., Ashkenazi, N., and Ben-Ezra, D. (2018). Ulva lactuca biofilter from a land-based integrated multi trophic aquaculture (IMTA) system as a sole food source for the tropical sea urchin tripneustes gratilla elatensis. *Aquaculture* 496, 221–231. doi: 10.1016/j.aquaculture.2018.06.038

Siikavuopio, S. I., and Mortensen, A. (2015). "Sea Urchin aquaculture in Norway," in *Echinoderm aquaculture*. Eds. N. Brown and S. Eddy (Hoboken, New Jersey: Wiley Blackwell), 147–174.

Stefánsson, G., Krisinsson, H., Ziemer, N., Hannon, C., and James, P. (2017). "Markets for sea urchins: a review of lobal supply and markets," in *Matís report* (Reykjavik: Skýrsluágrip Matís ohf - Icelandic Food and Biotech R&D). doi: 10.13140/RG.2.2.12657.99683

Stumpp, M., Trubenbach, K., Brennecke, D., Hu, M. Y., and Melzner, F. (2012). Resource allocation and extracellular acid-base status in the sea urchin strongylocentrotus droebachiensis in response to CO2 induced seawater acidification. *Aquat. Toxicol.* 110-111, 194–207. doi: 10.1016/j.aquatox.2011.12.020

Suckling, C. C. (2012). *Calcified marine invertebrates: the effects of ocean acidification* (Cambridge: University of Cambridge, UK). doi: 10.1016/j.aquaculture.2011.05.042

Suckling, C. C. (2021). Responses to environmentally relevant microplastics are species-specific with dietary habit as a potential sensitivity indicator. *Sci. Total Environ.* 751, 142341. doi: 10.1016/j.scitotenv.2020.142341

Suckling, C. C., Clark, M. S., Beveridge, C., Brunner, L., Hughes, A., Cook, E., et al. (2014). Experimental influence of pH on the early life-stages of sea urchins II: increasing parental exposure times gives rise to different responses. *Invertebr. Reprod. Dev.* 58, 161–175. doi: 10.1080/07924259.2013.875951

Suckling, C. C., Czachur, M. V., Ellis, J. E., and Davies, A. J. (2020a). European Sea urchin somatic and gonadal growth responses to differing stocking densities and seawater flow rates: A case study for experimental design consideration. *J. Shellfish. Res.* 39, 159–171. doi: 10.2983/035.039.0115

Suckling, C. C., Kelly, M. S., and Symonds, R. C. (2020b). "Chapter 11: Carotenoids in sea urchins," in *Sea Urchins: Biology and ecology, 4th ed.* Ed. J. M. Lawrence (Amsterdam: Elsevier B.V.), 209–217. doi: 10.1016/B978-0-12-819570-3.00011-1

Suckling, C. C., Symonds, R. C., Kelly, M. S., and Young, A. J. (2011). The effect of artificial diets on the gonad colour and biomass in the edible sea urchin psammechinus miliaris. *Aquaculture* 318, 355 – 342.

Suckling, C. C., Terrey, D., and Davies, A. J. (2018). Optimising stocking density for the commercial cultivation of sea urchin larvae. *Aquaculture* 488, 96–104. doi: 10.1016/j.aquaculture.2018.01.022

Sun, J., and Chiang, F.-S. (2015). "Use and exploitation of sea urchin," in *Echinoderm aquaculture*. Eds. N. P. Brown and S. D. Eddy (Hoboken, New Jersey: Wiley-Blackwell), 25–45.

Symonds, R. C., Kelly, M., Caris-Veryat, C., and Young, A. (2007). Carotenoids in the sea urchin paracentrotus lividus: occurrence of 9'-cis-echinenone as the dominant carotenoid in gonad colour determination. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 148, 432–444. doi: 10.1016/j.cbpb.2007.07.012

Symonds, R. C., Kelly, M. S., Suckling, C. C., and Young, A. J. (2009). Carotenoids in the gonad and gut of the edible sea urchin psmamechinus miliaris. *Aquaculture* 288, 120–125. doi: 10.1016/j.aquaculture.2008.11.018

Tsushima, M., Kawakami, T., Mine, M., and Matsuno, T. (1997). The role of carotenoids in the development of the sea urchin pseudocentrotus depressus. *Invertebr. Reprod. Dev.* 32, 149–153. doi: 10.1080/07924259.1997.9672616

Vadas, R. L. (1977). Preferential feeding: an optimization strategy in sea urchins. *Ecol. Monogr.* 47, 337–371. doi: 10.2307/1942173

Wahl, M., and Hay, M. E. (1995). Associational resistance and shared doom: effects of epibosis on herbivory. *Oecologia.* 102, 329–340. doi: 10.1007/bf00329800

Wangensteen, O. S., Turon, X., Casso, M., and Palacín, C. (2013). The reproductive cycle of the sea urchin arbacia lixula in Northwest Mediterranean: potential influence of temperature and photoperiod. *Mar. Biol.* 160, 3157–3168. doi: 10.1007/s00227-013-2303-8

Ward, T. J., Kramer, J. R., Boeri, R. L., and Gorsuch, J. W. (2006). Chronic toxicity of silver to the sea urchin (Arbacia punctulata). *Environ. Toxicol. Chem.* 25, 1568–1573. doi: 10.1897/05-299r.1

Zupo, V., Glaviano, F., Paolucci, M., Ruocco, N., Polese, G., Di Cosmo, A., et al. (2018). Roe enhancement of paracentrotus lividus: Nutritional effects of fresh and formulated diets. *Aquac. Nutr.* 25, 26–38. doi: 10.1111/anu.12826

frontiers | Frontiers in Marine Science

TYPE Original Research
PUBLISHED 04 August 2022
DOI 10.3389/fmars.2022.931991

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

RECEIVED 29 April 2022
ACCEPTED 13 July 2022
PUBLISHED 04 August 2022

CITATION
Ribes-Navarro A, Alberts-Hubatsch H, Monroig Ó, Hontoria F and Navarro JC (2022) Effects of diet and temperature on the fatty acid composition of the gammarid *Gammarus locusta* fed alternative terrestrial feeds. *Front. Mar. Sci.* 9:931991. doi: 10.3389/fmars.2022.931991

Effects of diet and temperature on the fatty acid composition of the gammarid *Gammarus locusta* fed alternative terrestrial feeds

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The fast and remarkable growth of global aquaculture in recent years has created new challenges, such as guaranteeing a sustainable supply of raw materials used for aquafeed formulation. Gammarids are low-trophic crustaceans with an increasing interest in aquaculture due to their high nutritional profiles and their capacity to grow under high-density conditions. Moreover, gammarids have the ability to thrive on a wide range of sidestreams while accumulating relatively high levels of long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA). In the present study, juveniles of the marine gammarid *Gammarus locusta* were cultured at four different temperatures (5°C, 10°C, 15°C, and 20°C) for 21 days and fed three diets, including the seaweed *Fucus* sp. as control, and carrot leaves and coconut flesh representing two agri-food industry sidestreams. Our results indicate that both the survival and biomass of *G. locusta* were highly affected by diet, with coconut showing the lowest growth performance. The temperature had no effect on biomass, although high temperature (20°C) resulted in a decrease in survival. The effects of temperature on the gammarid fatty acids were not evident, with diet being the main modulator of the profiles. Furthermore, the results also reveal that the *Fucus* sp. diet was associated with relatively high percentages of n-3 and n-6 LC-PUFA. Interestingly, essential LC-PUFA such as eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic (22:6n-3, DHA) acids were detected in gammarids fed on either *Fucus* sp. or any of the sidestreams irrespectively of their presence in the diets. These results suggest an ability of *G. locusta* for LC-PUFA biosynthesis (trophic upgrading) and/or retention, making this species a promising candidate for the production of high-value ingredients for aquafeeds.

KEYWORDS

agri-food sidestreams, circular economy, *gammarus locusta*, long-chain polyunsaturated fatty acids, novel marine ingredients

Introduction

Rapidly expanding aquaculture worldwide has created new economic and ecological challenges (FAO, 2020). With regard to fish farming, such challenges have been mostly linked to modifying the supply of raw materials used for feed formulation in order to reduce the current usage of finite resources such as the so-called marine ingredients fishmeal (FM) and fish oil (FO). FM and FO are regarded as major sources of essential nutrients for aquafeeds, including long-chain (C_{20-24}) polyunsaturated fatty acids (LC-PUFA) (Tocher, 2015; Shepherd et al., 2017). The current production of FM and FO largely relies on feed-grade species fisheries and, with the expansion of aquaculture worldwide, pressure on these fisheries has grown to an extent that they may not sustainably fulfill the increasing demand (Naylor et al., 2009; FAO, 2020).

Several efforts have been made to find alternative feeding sources to alleviate the abovementioned pressure on fisheries and reduce the dependence upon FM and FO in finfish aquaculture (Naylor et al., 2009; Turchini et al., 2011b; Jannathulla et al., 2019). Up to the present, the use of raw materials derived from animals or plants has become a widely extended practice (Turchini et al., 2009; Jannathulla et al., 2019; Galkanda-Arachchige et al., 2020). However, the replacement of FM and FO with nonmarine ingredients has often been associated with decreased nutritional value of fish farming products, including reduced levels of the health-promoting n-3 LC-PUFA eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), as well as suboptimal growth (Bell et al., 2001; Mourente and Bell, 2006; Turchini et al., 2011a; Yıldız et al., 2018; Romano et al., 2020). Therefore, high-quality alternative ingredients are needed in order to successfully replace traditional sources of FM and FO in aquafeed while maintaining growth performance and nutritional value (i.e., levels of n-3 LC-PUFA) of farmed fish (Alberts-Hubatsch et al., 2019). Indeed, the search for novel aquatic ingredients for aquafeed is a priority within the EU, as is their production through Integrative Multi-Trophic Aquaculture (IMTA) strategies (Guerra-García et al., 2016).

Ingredients derived from biomasses of low-trophic marine crustaceans such as gammarids, krill, and copepods have been regarded as promising candidates for aquafeed formulations due to their balanced profiles of essential nutrients, including n-3 LC-PUFA (McKinnon et al., 2003; Suontama et al., 2007; Naylor et al., 2009; Dhont et al., 2013; Harlıoğlu and Farhadi, 2018). While the exploitation of wild populations of marine crustaceans poses negative ecological impacts similar to those of wild-capture fisheries alluded to above, biomass production using intensive aquaculture systems arises as an interesting strategy. Gammarids are abundant in benthic communities and inhabit practically all aquatic environments, which is reflected in their high diversity of feeding habits (Costa and Costa, 2000; Harlıoğlu and Farhadi, 2018). Moreover, gammarids are fish's natural prey, and their use as an alternative ingredient for fish feeding has been explored (Ashour et al., 2021). Gammarids can be grown and maintained in laboratory cultures and adapt well to different culture conditions and diets (Sexton, 1928; Costa and Costa, 2000; Neuparth et al., 2002; Ahyong and Hughes, 2016; Beermann et al., 2018; Alberts-Hubatsch et al., 2019). Interestingly, recent investigations have also demonstrated that gammarids can be fed on a wide range of sidestreams from bioindustries such as agriculture and aquaculture itself (Alberts-Hubatsch et al., 2019; Jiménez-Prada et al., 2020). Such sidestreams are characterized by being deprived or having low contents of LC-PUFA, but, intriguingly, gammarids fed on these sidestreams have shown relatively high levels of LC-PUFA, suggesting that gammarids have some capacity for trophic upgrading *via* endogenous lipid metabolism (Alberts-Hubatsch et al., 2019; Jiménez-Prada et al., 2020). Thus, applying circular bioeconomy strategies by which sidestreams derived from bioindustries are used for the production of high nutritional value gammarid biomasses as potential fish feed ingredients has been proposed (Jiménez-Prada et al., 2020). However, up to date, little is known about the optimal culture conditions for reliable large-scale cultures of marine gammarids (Alberts-Hubatsch et al., 2019; Jiménez-Prada et al., 2020). Several factors, including diet, temperature, and salinity can modulate LC-PUFA metabolism, growth, and survival of aquatic invertebrates (Neuparth et al., 2002; Monroig and Kabeya, 2018; Alberts-Hubatsch et al., 2019). Hence, it is necessary to fine tune the culture conditions for the production of LC-PUFA–rich gammarid biomass.

A previous study comparing the effects of several diets on two gammarid species, namely *Gammarus locusta* and *Echinogammarus marinus*, suggested that *G. locusta* is the best candidate regarding LC-PUFA composition, growth, and survival when fed sidestreams (Alberts-Hubatsch et al., 2019). However, to the best of our knowledge, information on the combined effects of diet and environmental factors, on survival, growth, and LC-PUFA content of marine gammarids is lacking. The main goal of this study was to elucidate the effects of three diets and four temperatures on the FA profile and growth performance of the marine gammarid *G. locusta*. For this purpose, cultures were carried out at 5°C, 10°C, 15°C, and 20°C, representing ambient temperatures in the wild, with *G. locusta* occurring in coastal areas of the North Atlantic (Costa and Costa, 2000). *Fucus* spp. was used as a natural marine diet, and carrot leaves and coconut flesh as two different nonmarine diets, mimicking agriculture sidestreams of different nutritional values.

Materials and methods

Animal collection and culture

All *G. locusta* specimens used in the experiments were obtained from a laboratory culture at the Alfred Wegener Institute, with stock cultures originating from the German Bight, North Sea. Specimens were reared in the laboratory prior to the experiment at temperatures of 18°C, a salinity of 33–34 ppt, and pH 8. Broodstock cultures and juvenile *G. locusta* were raised on a mixture of dried *Fucus* spp. and vegetable greens (mainly carrot greens and kale leaves). Juvenile *G. locusta* (28 days posthatch) were collected from the same batch culture, maintained at 10°C for 5 days, and starved for 24 h prior to the experiment.

Experimental setup

The juvenile gammarids (5.327 ± 1.54 mm SD, n = 960) were randomly transferred into white 1-L buckets, filled with 500 ml of freshly filtered seawater (5µm tube filter) at 33–34 ppt and equipped with a mesh (70 × 70 mm, 5 mm mesh size) and an oyster shell as substrate. Each container was stocked with 20 specimens at 10°C and randomly allocated to the respective temperatures (5°C, 10°C, 15°C, and 20°C) and diet in quadruplicates. The temperature was slowly adjusted by transferring the containers into water baths at the desired temperature. Three different diets were prepared: one natural marine food source, thalli of *Fucus* spp. containing LC-PUFA (hereafter referred to as Fucus), and two nonmarine diets: carrot leaves (hereafter referred to as "Carrot"), an agricultural sidestream, (high shorter-chain (<C20) PUFA content), and a diet that mimics a potential sidestream rich in saturated fatty acids (SFA) consisting on coconut flesh (hereafter referred to as "Coco"). All diets were rinsed in fresh water and dried at 55°C for 24 h. The temperature in the experimental containers was recorded twice a day, and water exchange with fresh seawater adjusted to the respective temperature was done every second day. Feeding was done *ad libitum* with remaining food items removed and replaced during water exchange. Dead individuals were removed on a daily basis. Gammarids were cultured at the corresponding diet vs. temperature combination for 21 days, until sexual maturity was reached in the higher temperature treatments.

Growth and survival

Initially, a subsample of 100 juvenile gammarids (average 5 mm) was analyzed. Total body lengths of gammarids were measured at the beginning and end of the experiment by analyzing pictures of each replicate taken on scale paper using Fiji ImageJ (vers. 1.53q, Schneider et al., 2012). Total lengths were measured from the basal point of the antennae to the third urosome segment. Specific growth rate (SGR, in % day^{-1}) was calculated as length gain per experimental duration for each pool as follows:

SGR = 100 × [ln (final length) − ln (initial length)/time interval]. Initial individual weights could not be taken at the beginning of the experiment due to the vulnerability of the early life stages. Therefore, at the end of the experiment, all remaining specimens were counted and wet biomass per replicate was recorded using an analytical scale (Sartorius Practum 213-S1, d = 0.001 g). The gammarids were rinsed in Milli-Q water twice, placed into Eppendorf tubes, and immediately frozen at −80°C. The frozen samples were then freeze-dried at −52°C within 2 weeks after the experiment and thereafter stored at −80°C until further analyses.

Fatty acid analysis

Total lipids and FA were analyzed from gammarid samples as well as from experimental diets. Briefly, total lipids were extracted from the homogenized samples using the Folch method (Folch et al., 1957). Subsequently, total lipids were used to prepare fatty acid methyl esters (FAME), which were analyzed using a Thermo Trace GC Ultra Gas Chromatograph (Thermo Electron Corporation, Waltham, MA, USA), equipped with a fused silica 30 m × 0.25 mm open tubular column (Tracer, TR-WAX, film thickness: 0.25 µm, Teknokroma, Sant-Cugat del Vallés, Spain), fitted with an on-column injection system, using helium as a carrier gas, and a flame ionization detector (FID). The analytical temperature was programmed from 50°C to 220° C. Chromatograms were integrated and analyzed with Azur Datlys (St Martin d'Heres, France) software. FAs were identified by comparison of retention times of each peak with those of well-characterized standards.

Statistical analysis

All data were subjected to statistical analyses using PAST (vers. 4.09) (Hammer et al., 2001). Prior to the analyses, data were tested for normality (Shapiro–Wilk) and homogeneity (Levene's test). After assuring that normality and homogeneity criteria were met, data were analyzed using analysis of variance (fixed effects two-way ANOVA) with Tukey's *post-hoc* test for multiple comparisons. Principal component analysis (PCA) was used to analyze and visualize the relationship between diet and FA profiles of gammarids. Differences in FA levels among treatments were tested by using a two-way permutational multivariate analysis of variance (PERMANOVA) with factors: "Temperature," four different conditions (5°C, 10°C, 15°C, and 20°C), and "Diet," three different treatments (Fucus, Carrot, Coco). One-way PERMANOVA was further used to compare the scores of the dietary groups. Ellipses were fitted to the scores

at 95% confidence. Additionally, the percentage of similarity analysis (SIMPER) was used to determine the FA responsible for dissimilarities between conditions within the dietary groups. Unless otherwise stated, statistical significance was tested at 95% confidence level ($p \leq 0.05$). The Unsaturation Index (UI) was used to establish a relationship between the FA levels of diets used and the FA levels of gammarids fed on the corresponding diets in order to determine how diet can affect the gammarids' FA profiles and to ascertain previous retention. The UI was calculated according to the following formula: Σ [area of fatty acid * number of unsaturations].

Results

Growth and survival

The two-way ANOVA revealed that both temperature (F (3, 36) = 12.43, $p < 0.0001$) and diet (F (2, 36) = 18.08, $p < 0.0001$) had a significant effect on the survival of *G. locusta*, although no interacting effects of temperature and diet on survival were observed (F (6, 36) = 0.38, $p = 0.89$). Pairwise comparisons of the survival of gammarids at different temperatures revealed that the highest temperature (20°C) resulted in significantly lower survival rates, whereas no differences were observed between the temperatures (Table 1). Regarding the diets, Coco resulted in significantly lower survival of gammarids, whereas no differences between the effects of Carrot and Fucus were found. The highest mortality was obtained with the Coco diet at 20°C (Table 1).

Regarding total biomass, there was a significant interacting effect of temperature and diet as revealed by the two-way ANOVA (F (6, 36) = 3.17, $p = 0.01$). Here, our analysis shows no significance of temperature (F (3, 36) = 1.63, $p = 0.20$) but a highly significant effect of diet (F (2, 36) = 29.07, $p < 0.001$). Pairwise comparisons of the biomass output revealed highly significant differences ($p < 0.005$) between all diet groups, with the Fucus diet resulting in the highest (Table 1). No significant differences were found in the gammarid biomasses when comparing temperature groups.

The final length was not affected by the interaction of temperature and diet (F (6,35) = 1.47, $p = 0.22$). In this case, the diet had no effect on final length (F (2, 35) = 3.03, $p = 0.06$), in contrast to temperature, which strongly affected final length (F (3, 35) = 20, 73, $p < 0.001$). When looking at pairwise comparisons, it becomes evident that this effect was caused by the 20°C treatment, which resulted in significantly higher final lengths compared to all other temperature groups ($p < 0.001$), whereas no differences were found between the other groups (Table 1).

The SGR followed a similar pattern as the final length data, with temperature having a highly significant effect on SGR (F (3, 35) = 31.83, $p < 0.001$). This was also explained by the higher SGR in the 20°C treatment, which was significantly different from all other temperature treatments ($p < 0.001$) while the other temperatures were not different (Table 1). Diet had a significant effect on SGR (F (2, 35) = 5.26, $p = 0.01$), which was caused by the low SGR when regarding pairwise comparisons, in which Coco was significantly different from Fucus but not from Carrot (Table 1). This was biased by the very high mortality in this treatment group (see above). Similarly, a significant interaction effect of diet and temperature on SGR (F (6, 35) = 3.44, $p = 0.008$) was observed.

TABLE 1 Survival, total biomass, and total length of *G. locusta* in response to different diets and temperatures after the 21-day feeding trial.

	5°C	10°C	15°C	20°C
% Survival (± SD)				
Fucus	73.75 ± 10.31 a, A	65.00 ± 10.80 a, A	71.25 ± 14.93 a, A	45.00 ± 28.28 b, A
Carrot	71.25 ± 6.29 a, A	60.00 ± 10.80 a, A	66.25 ± 27.80 a, A	32.50 ± 10.41 b, A
Coco	47.50 ± 8.66 a, B	23.75 ± 23.94 a, B	47.50 ± 8.66 a, B	8.75 ± 4.79 b, B
Biomass (mg ± SD)				
Fucus	76.00 ± 11.86 a, A	122.50 ± 31.03 ab, A	121.25 ± 38.35 b, A	159.00 ± 36.39 ab, A
Carrot	67.50 ± 20.04 a, B	78.00 ± 26.89 ab, B	95.75 ± 41.10 b, B	86.50 ± 27.74 ab, B
Coco	64.00 ± 43.14 a, C	27.25 ± 9.00 ab, C	51.25 ± 16.66 b, C	27.25 ± 12.47 ab, C
Length (mm ± SD)				
Fucus	6.92 ± 0.85 a	8.28 ± 1.44 a	8.24 ± 0.1 a	11.59 ± 2.45 b
Carrot	6.85 ± 0.73 a	7.10 ± 0.32 a	7.62 ± 1.05 a	9.81 ± 1.73 b
Coco	7.26 ± 2.63 a	5.31 ± 1.57 a	6.75 ± 0.3 a	10.89 ± 8.0 b
Specific growth rate (SGR ± SD)				
Fucus	0.37 ± 0.12 a, A	0.89 ± 0.11 a, A	0.89 ± 0.13 a, A	1.68 ± 0.32 b, A
Carrot	0.53 ± 0.08 a, AB	0.71 ± 0.04 a, AB	0.74 ± 0.04 a, AB	1.29 ± 0.44 b, AB
Coco	0.43 ± 0.36 a, B	−0.10 ± 0.58 a, B	0.66 ± 0.22 a, B	1.48 ± 0.39 b, B

Fucus, Fucus spp.; Carrot, carrot leaves; Coco, coconut flesh. Data are expressed as mean of the different replicates per condition ± standard deviation (SD). Statistical differences (Tukey's pairwise comparisons, $p \leq 0.05$) are indicated by different letters: lowercase letters (a–d) indicate differences in temperature and uppercase letters (A–D) indicate differences in diet.

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Fatty acid profiles of gammarids

The FA analysis of the diets revealed that Fucus was rich in oleic acid (18:1n-9) (29.8%), whereas α-linolenic acid (ALA, 18:3n-3) was the most abundant FA in carrot leaves (26.5%). On the other hand, the Coco diet showed high levels of SFA (90.4%), such as lauric acid (12:0) (48.7%) and myristic acid (14:0) (19.9%) (Table 2). Low levels of DHA (22:6n-3) were detected only in the Fucus diet (1.1%), whereas EPA (20:5n-3) was also present in Fucus (4.8%) and only in trace amounts (0.2%) in Carrot. Moreover, the Coco diet had the lowest UI (6.2) whereas the Fucus and Carrot diets showed similar levels (150.4 and 138.3, respectively), reflecting that the former is very poor in PUFA and MUFA and rich in SFA, as compared to the other treatments.

FA analysis of cultured *G. locusta* showed similar profiles when different temperature treatments were compared (Table 3). However, the two-way ANOVA of the FA analyses only showed significant differences when comparing the different dietary treatments (Table 4). One-way ANOVA of gammarids fed on the different diets (Table 5) did not show significant differences in EPA and DHA levels. The two-way PERMANOVA results did not show differences in FA among different temperatures ($F(3, 28) = 1.20$, $p = 0.29$), or the interaction temperature and diet ($F(6, 28) = 0.73$, $p = 0.74$). Moreover, the two-way PERMANOVA revealed diet as the main modulator of FA profiles ($F(2, 28) = 8.97$, $p < 0.001$). PCA revealed that the first component (PC1) accounted for the 60.81% of variance of this dataset, whereas the PC2 accounted for the 19.19% of variance (Figure 1). The PCA loading plot showed saturated fatty acids such as lauric acid (12:0) and myristic acid (16:0) are separated on the negative side of PC1 from unsaturated and polyunsaturated fatty acids including LC-PUFA such as arachidonic acid (20:4n-6, ARA), EPA (20:5n-3), docosapentaenoic acid (22:5n-3, DPA), and DHA (22:6n-3), which load on the positive side (Figure 1). On the other hand, 16:1n-7, LA (18:2n-6), and ALA (18:3n-3) load on the negative side of PC2. This variable distribution drives the scores segregation (Figure 1). The FA profiles of *G. locusta* fed on Coco are correlated with SFA (Figure 1). Those fed carrots are associated with LA and ALA, whereas those fed the Fucus diet are associated with LC-PUFA, among others. It is also interesting to note that the scores of the FA profiles of gammarids fed on the Fucus diet showed less dispersion than those fed the carrot leaves and coconut diets (95% ellipses, Figure 1). One-way PERMANOVA ($F = 9.38$, $p < 0.0001$), however, showed that the three dietary groups were significantly different (Supplementary Table S1). No significant differences were found for PC2. Additional SIMPER analysis (Supplementary Tables S2-S4) revealed that oleic acid (18:1n-9, OA) was the FA with more dissimilarity between the Fucus diet and the other diet groups (Carrot and Coco). On the other hand, the SIMPER analysis comparing Carrot vs. Coco showed that the SFA were the FA characteristic of the Coco diet.

Generally, *G. locusta* fed on Fucus showed FA profiles richer in LC-PUFA (C_{20-24}) than those fed on Carrot or Coco, whereas

TABLE 2 Relative FA composition (% of total FA) of diets used in the feeding trial.

	Fucus	Carrot	Coco
12:0	1.1	1.1	48.7
14:0	11.4	1.4	19.9
16:0	13.6	17.5	9.3
16:1n-7	1.5	0.2	0.1
18:0	0.7	1.3	3.5
18:1n-9 (OA)	29.8	2.1	4.7
18:2n-6 (LA)	8.8	14.3	0.7
18:3n-3 (ALA)	3.3	26.5	0.1
20:4n-6 (ARA)	12.0	0.4	nd
20:5n-3 (EPA)	4.8	0.2	nd
22:5n-3 (DPA)	nd	nd	nd
22:6n-3 (DHA)	1.1	nd	nd
SFA	27.7	23.2	90.4
MUFA	32.4	2.8	4.7
n-3 PUFA	11.6	26.7	0.1
n-6 PUFA	21.6	14.9	0.7
n-3 LC-PUFA	6.0	0.3	nd
n-6 LC-PUFA	12.8	0.6	nd
UI	150.4	138.3	6.2

Fucus, Fucus spp.; Carrot, carrot leaves; Coco, coconut flesh; OA, oleic acid; LA, linolenic acid; ALA, α-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids; UI, unsaturation index; nd, not detected.

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TABLE 3 Relative fatty acid (FA) composition (% of total FA) of samples of *G. locusta* fed different diets at four temperatures.

	5°C Fucus	5°C Carrot	5°C Coco	10°C Fucus	10°C Carrot	10°C Coco	15°C Fucus	15°C Carrot	15°C Coco	20°C Fucus	20°C Carrot	20°C Coco
12:0	0.2 ± 0.1	0.1 ± 0.08	6.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	11.6	0.7 ± 0.2	0.2 ± 0.1	8.2	0.2 ± 0.2	0.3 ± 0.1	8.7 ± 1.7
14:0	3.0 ± 0.4	0.7 ± 0.1	4.9 ± 0.1	3.4 ± 0.7	1.0 ± 0.2	12.9	2.8 ± 0.2	1.0 ± 0.3	9.1	2.4 ± 1.0	1.1 ± 0.5	7.3 ± 2.0
16:0	15.0 ± 0.3	17.2 ± 1.3	15.7 ± 0.8	16.2 ± 0.6	15.3 ± 1.5	31.8	16.6 ± 0.2	16.4 ± 0.6	37.0	18.9 ± 1.4	18.3 ± 2.6	19.7 ± 2.9
16:1n-7	2 ± 0.5	1.5 ± 0.5	1.7 ± 0.9	2.2 ± 0.5	2.5 ± 1.4	0.8	2.0 ± 0.6	3.4 ± 0.4	0.9	1.5 ± 0.2	2.4 ± 0.6	1.8 ± 0.7
18:0	2.5 ± 1.4	3.4 ± 0.6	3.9 ± 0.69	2.1 ± 0.2	2.8 ± 0.4	9.1	3.6 ± 0.9	3.3 ± 0.5	12.5	3.3 ± 1.0	5.6 ± 1.6	5.2 ± 0.7
18:1n-9 (OA)	29.7 ± 4.0	17.5 ± 2.5	26.2 ± 2.4	27.2 ± 3.4	17.6 ± 6.1	18.3	27.7 ± 3.0	14.0 ± 2.7	8.7	24.9 ± 2.6	16.1 ± 2.7	14.2 ± 4.9
18:2n-6 (LA)	7.3 ± 0.7	8.6 ± 1.7	4.5 ± 0.2	6.7 ± 1.1	10.0 ± 0.0	2.2	6.0 ± 0.6	13.9 ± 1.4	0.7	5.5 ± 0.8	7.8 ± 3.3	2.4 ± 0.1
18:3n-3 (ALA)	2.3 ± 0.7	5.4 ± 1.9	0.4 ± 0.0	2.6 ± 0.3	8.5 ± 2.4	nd	2.1 ± 0.6	12.0 ± 1.5	1.1	1.8 ± 0.4	5.9 ± 0.8	0.3
20:4n-6 (ARA)	10.1 ± 3.6	10.9 ± 2.6	13.2 ± 0.3	12.1 ± 0.8	8.3 ± 1.3	3.5	12.3 ± 1.3	4.8 ± 1.0	0.8	13.5 ± 1.9	4.1 ± 1.6	4.6 ± 0.1
20:5n-3 (EPA)	7.1 ± 3.5	7.5 ± 2.3	8.9 ± 0.3	9.9 ± 0.8	6.3 ± 1.1	1.7	10.8 ± 1.4	4.1 ± 0.7	1.4	12.6 ± 3.7	5.9 ± 0.8	9.7 ± 2.1
22:5n-3 (DPA)	0.4 ± 0.0	0.2 ± 0.0	0.1 ± 0.2	0.5 ± 0.1	0.1 ± 0.2	nd	0.6 ± 0.1	0.2 ± 0.1	nd	0.7 ± 0.3	0.2 ± 0.1	0.3 ± 0.1
22:6n-3 (DHA)	2.3 ± 0.6	3.3 ± 0.9	2.3 ± 1.7	2.4 ± 0.7	3.0 ± 0.4	0.6	2.6 ± 0.9	1.7 ± 0.4	0.4	3.0 ± 0.8	3.0 ± 1.1	3.0 ± 0.6
SFA	20.9 ± 0.7	22.1 ± 1.6	32.0 ± 1.6	22.2 ± 0.9	22.3 ± 0.9	66.8	24.1 ± 0.2	22.3 ± 0.7	69.2	25.8 ± 3.7	24.6 ± 4.9	37.1 ± 11.4
MUFA	36.0 ± 4.0	23.7 ± 3.0	31.9 ± 0.2	35.7 ± 1.8	25.3 ± 8.8	22.4	34.2 ± 2.0	24.0 ± 3.8	12.5	30.9 ± 2.3	23.9 ± 1.9	23.7 ± 2.7
n-3 PUFA	16.6 ± 1.3	20.0 ± 1.2	12.8 ± 0.3	18.3 ± 1.6	20.2 ± 1.1	2.3	17.6 ± 1.5	21.8 ± 3.1	2.9	19.7 ± 4.1	21.1 ± 6.5	15.2 ± 1.9
n-6 PUFA	21.2 ± 0.3	23.0 ± 2.3	19.0 ± 0.1	20.8 ± 0.9	20.1 ± 1.0	6.0	19.6 ± 1.3	20.4 ± 2.0	1.6	20.5 ± 1.4	19.0 ± 1.9	10.8 ± 5.5
n-3 LC-PUFA	12.2 ± 0.4	13.8 ± 0.6	12.4 ± 0.3	13.6 ± 1.4	11.6 ± 1.1	2.3	14.3 ± 2.3	9.7 ± 4.0	1.8	16.5 ± 4.8	12.7 ± 6.2	13.2 ± 2.4
n-6 LC-PUFA	13.5 ± 0.3	12.6 ± 2.6	14.5 ± 0.1	13.6 ± 0.8	10.1 ± 1.0	3.8	13.8 ± 1.3	6.5 ± 1.4	0.8	15.0 ± 2.1	9.4 ± 3.3	5.3 ± 0.2
UI	184.0 ± 2.2	193.7 ± 9.1	165.0 ± 2.1	189.5 ± 9.9	165.3 ± 2.2	53.8	184.5 ± 9.6	162.1 ± 11.8	30.0	196.7 ± 26.6	184.4 ± 40.6	136.9 ± 19.3

Fucus, Fucus spp.; Carrot, carrot leaves; Coco, coconut flesh.; OA, oleic acid; LA, linolenic acid; ALA, α-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids; UI, unsaturation index; nd, not detected.

G. locusta fed on Carrot showed the highest levels of PUFA ($<C_{20}$) such as LA and ALA (Table 3). Notably, levels of EPA and DHA were detected in *G. locusta* fed on either Carrot or Coco regardless of these FA not being present in those diets. Regarding SFA, *G. locusta* fed on Coco showed higher percentages as compared to *G. locusta* fed on either Fucus or Carrot (Table 3). It is important to note as well that lower percentages of SFA were detected in cultured *G. locusta* fed on Coco in comparison with the Coco diet itself, while maintaining moderate LC-PUFA levels.

Discussion

The nutritional profiles of gammarids have been studied as alternative sustainable ingredients for aquafeeds (Kolanowski et al., 2007; Baeza-Rojano et al., 2010; Baeza-Rojano et al., 2013; Baeza-Rojano et al., 2014; Jiménez-Prada et al., 2018) due to the potential of these organisms to feed on a wide range of sidestreams (Harlıoğlu and Farhadi, 2018). However, only a few investigations have focused on culture strategies based on the use of aquaculture and/or agriculture waste as primary food (Alberts-Hubatsch et al., 2019; Jiménez-Prada et al., 2020), and up to date, there are no investigations reporting on the effect of food and different environmental conditions on the fatty acid profile, growth, and survival of these organisms. In the present study, juveniles of *G. locusta* survived and reached sexual maturity in all the conditions tested for both temperature and diet groups. Interestingly, the survival of *G. locusta* was significantly lower at 20°C than at the rest of the temperatures. This result supports the hypothesis that at 20°C there is an acceleration of growth and reduction of the life cycle of *G. locusta*, resulting in a faster length growth and therefore metabolism, which results in an increased mortality (Neuparth et al., 2002). Regarding the effect of different diets, survival was significantly lower in *G. locusta* fed on Coco,

TABLE 4 Two-way ANOVA results of the fatty acid composition of samples of *G. locusta* fed different diets at four temperatures.

	Temperature		Diet		Interaction	
	F-test (2, 38)	*p*-value	*F*-test (3, 37)	*p*-value	*F*-test (6, 40)	*p*-value
12:0	0.56	0.65	19.78	< 0.001	0.32	0.92
14:0	0.27	0.84	14.09	< 0.001	0.25	0.96
16:0	0.32	0.81	1.15	0.329	0.34	0.91
16:1n-7	0.63	0.60	1.34	0.275	1.38	0.26
18:0	0.75	0.53	9.24	< 0.001	1.02	0.43
18:1n-9 (OA)	1.23	0.31	32.13	< 0.001	0.89	0.51
18:2n-6 (LA)	1.59	0.21	8.79	< 0.001	1.84	0.12
18:3n-3 (ALA)	1.87	0.15	11.71	< 0.001	1.60	0.18
20:4n-6 (ARA)	1.19	0.33	8.96	< 0.001	1.79	0.14
20:5n-3 (EPA)	1.56	0.22	4.28	0.021	0.74	0.62
22:5n-3 (DPA)	1.07	0.37	8.82	< 0.001	0.45	0.84
22:6n-3 (DHA)	2.06	0.12	0.85	0.437	0.75	0.61
SFA	0.19	0.90	7.09	0.002	0.30	0.93
MUFA	0.80	0.50	25.03	< 0.001	0.54	0.78
n-3 PUFA	0.13	0.94	4.36	0.019	0.49	0.81
n-6 PUFA	0.84	0.48	7.38	0.002	0.76	0.60
n-3 LC-PUFA	1.19	0.33	2.93	0.065	0.61	0.72
n-6 LC-PUFA	1.14	0.35	8.72	< 0.001	0.99	0.44

OA, oleic acid; LA, linolenic acid; ALA, α-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids.

whereas no differences were found between those of gammarids fed on the Carrot and Fucus diets. The absence of physiologically essential compounds such as LC-PUFA in the coconut diet may explain this difference (Jump, 2002; Monroig et al., 2022). Indeed, it is tempting to hypothesize that the high content of SFA in the diet may be related to an

TABLE 5 Tukey's *post-hoc* test after ANOVA of the fatty acid composition of *G. locusta* fed different diets.

	p-value		
	Fucus vs. Carrot	Fucus vs. Coco	Carrot vs. Coco
12:0	0.95	<0.001	<0.001
14:0	0.25	<0.001	<0.001
16:0	0.52	0.34	0.90
16:1n-7	0.63	0.69	0.25
18:0	0.04	<0.001	0.13
18:1n-9 (OA)	<0.001	<0.001	0.45
18:2n-6 (LA)	0.08	0.08	<0.001
18:3n-3 (ALA)	<0.001	0.77	<0.001
20:4n-6 (ARA)	0.004	0.002	0.002
20:5n-3 (EPA)	0.033	0.07	1.00
22:5n-3 (DPA)	0.010	<0.001	<0.001
22:6n-3 (DHA)	0.94	0.41	0.61
SFA	0.58	0.002	0.023
MUFA	<0.001	<0.001	<0.001
n-3 PUFA	0.99	0.03	0.04
n-6 PUFA	0.49	<0.001	0.03
n-3 LC-PUFA	0.21	0.07	0.76
n-6 LC-PUFA	0.01	<0.001	0.62

OA, oleic acid; LA, linolenic acid; ALA, α-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids.

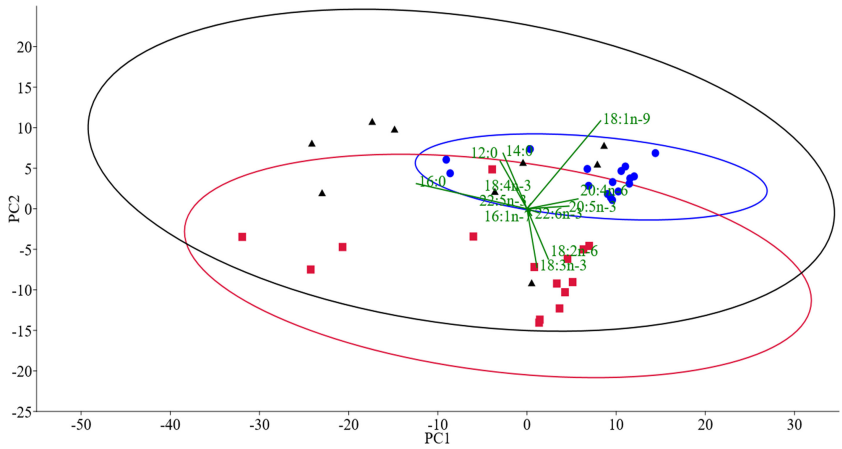

FIGURE 1
PCA of the fatty acid composition of *G. locusta* fed different diets and reared at different temperatures. Dots (blue), squares (red), and triangles (black) are the scores of *G. locusta* fed on *Fucus* spp., carrot leaves, and coconut, respectively. Fatty acids responsible for the grouping pattern are displayed in the biplot (green vectors); 95% confidence ellipses are shown. For the sake of clarity, and due to the lack of statistical significance, temperature labels are not shown.

impairment of the membrane composition and properties, ultimately resulting in an increase in mortality under the most stressful temperature conditions (Hazel and Eugene Williams, 1990; Hazel, 1995; Ernst et al., 2016; Laila, 2021; Zinoöcker et al., 2021). Noticeably, cannibalistic behavior was observed in gammarids fed on a coconut diet, suggesting a potential corrective mechanism towards the nutritional impairment. Although LC-PUFA was low in carrot leaves, the survival of gammarids fed on this diet was similar to that obtained with Fucus. As previously reported by Alberts-Hubatsch et al. (2019), this higher survival may be partially explained by the presence of other health-promoting nutrients like carotenoids that can thus compensate for suboptimal levels of LC-PUFA (Saini et al., 2015; González-Peña et al., 2021). Indeed, carotenoids are regarded as biomolecules that protect against unspecific and oxidant stress conditions such as suboptimal temperatures, which can ultimately cause increased mortality in gammarids (Agnew and Taylor, 1986; Neuparth et al., 2002; Wijnhoven et al., 2003). Significant differences in total gammarid biomass outputs were found when comparing different diets, and, regarding temperature, only significant differences were found between 5°C and 15°C, the latter having been reported as the optimal growth temperature for *G. locusta* (Neuparth et al., 2002). Interestingly, culturing gammarids at 20°C and fed on Fucus resulted in the highest output of biomass as well as final length and SGR. This may be due to the combined effects of the high nutritional value of the Fucus diet (Alberts-Hubatsch et al., 2019) as well as the increased growth performance of *G. locusta* when grown at 20°C (Neuparth et al., 2002) that compensates for the biomass loss associated with the abovementioned high mortality rates detected at 20°C.

Under the present experimental conditions, *G. locusta* showed similar FA profiles regardless of the rearing temperature. Only slight differences among temperatures within the same diet were evident, indicating either an apparent lack of temperature effect or a much more predominant dietary phenotypic impact. The FA dietary fingerprint was noticeable in the Fucus treatment in the LC-PUFA of the gammarid's profiles, whereas Carrot treatment resulted in higher levels of LA and ALA, and Coco induced higher amounts of SFA such as lauric and myristic acids in the final biomass. Interestingly, *G. locusta* fed on either Carrot or Coco still showed EPA and DHA percentages similar to those of *G. locusta* fed on Fucus, regardless of the absence of these LC-PUFA in the diets. This finding is in agreement with the potential capacity of gammarids for trophic upgrading, as has been suggested by several authors (Baeza-Rojano et al., 2013; Baeza-Rojano et al., 2014; Jiménez-Prada et al., 2018; Alberts-Hubatsch et al., 2019; Jiménez-Prada et al., 2020), indicating that these animals could bioconvert the short-chain FA present in sidestreams into high nutritional value LC-PUFA *via* their biosynthetic pathway.

LC-PUFA biosynthesis relies especially upon the gene repertoire and in the coordinated action of two types of enzymes (Monroig and Kabeya, 2018; Monroig et al., 2022). These two enzymes, elongation of very long-chain-fatty acid proteins (commonly known as "elongases"), and front-end desaturases (also named "desaturases"), need to be present and active in the organism in order to efficiently biosynthesize LC-

PUFA such as EPA and DHA from dietary FA (Castro et al., 2016; Monroig and Kabeya, 2018; Monroig et al., 2022). A recent report on the three different elongases in the marine gammarid *E. marinus* (Ribes-Navarro et al., 2021) indicates the potential capacity for endogenous production of LC-PUFA in marine gammarids, but the presence and activity of desaturases remain to be elucidated. The presence of EPA and DHA can be due to reasons other than endogenous biosynthesis, such as the accumulation and retention of these FA from sources other than the diet provided, like the occasional and practically unavoidable presence of prey organisms such as copepods and rotifers in the culture tanks during the experiment. These organisms, particularly copepods, are characterized by having high LC-PUFA contents (McKinnon et al., 2003; Miller et al., 2008; Naylor et al., 2009; Nielsen et al., 2019; Boyen et al., 2020; Kabeya et al., 2021). It should be noted, however, that during the experiment, it was evident that gammarids fed actively on the vegetal substrates, and although impossible to rule out, the possible contribution of the above-mentioned accompanying fauna entering the system as well as that of the rapid development of biofilm seems at most testimonial. Other factors, like the fungal contribution described in the digestive process for the freshwater *G. pulex* (Chamier and Willoughby, 1986), can also be invoked to explain the presence of unexpected components in the final profiles, but this can only be clarified in experiments run in controlled conditions, far beyond the scope of the present experimental design.

In summary, the present study demonstrates that gammarids can be fed on agricultural sidestreams (carrot leaves) without drastically affecting their growth performance and survival when reared at temperatures ranging from 5°C to 15°C. A culture at 20°C is not recommended due to the impairment between growth and survival. This study is the first study of this kind to show interactions between rearing temperatures and novel feed ingredients for this species, further steps (e.g., other under-used sidestreams from agriculture) need to be taken to optimize biomass gain in addition to survival. In particular, this study shows that *G. locusta* can be a potential candidate for applying circular economy principles by which sidestreams such as carrot leaves can be used as the main feed for gammarid culture and potential biomass generation. The aim of this study was also to get independent from marine resources, such as macroalgae, that can be costly or not sustainable to use for feeding gammarids. Thus, showing that at least carrot greens are providing sufficient nutrients for a successful culture of *G. locusta* gives important directions in terms of sustainability. Moreover, under the present experimental conditions tested herein, diet has been established as the main modulator of FA profiling in *G. locusta*. However, a combination of agricultural and aquaculture sidestreams, along with other modulating conditions such as salinity, should be considered in order to set the best environment for large-scale gammarid production. Aside from the strong phenotypic effect of diet on the final FA profile, the presence of LC-PUFA like EPA and DHA, detected on *G. locusta* fed on diets devoid of these compounds, points towards unknown mechanisms of trophic upgrading beyond the theoretical endogenous biosynthetic capacity of the species.

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.

Ethics statement

All experiments were carried out under the European Directive 2010/63/EU (European Parliament, Council of the European Union, 2010) for the protection of animals used for experimentation and other scientific purposes, from which invertebrates other than cephalopods are excluded. The experiments were run bearing in mind the 3R guidelines.

Author contributions

Conceptualization: HA-H and JN. Methodology: AR-N, HA-H, JN, and ÓM. Formal analysis: AR-N, HA-H, JN, and ÓM. Investigation: AR-N, HA-H, JN, FH, and ÓM. Resources: HA-H. Writing—original draft preparation: AR-N, HA-H, and JN. Writing—review and editing: AR-N, FH, HA-H, JN, and ÓM. Project administration: FH, HA-H, JN, and ÓM. Funding acquisition: FH, HA-H, JN, and ÓM. All authors contributed to the article and approved the submitted version.

Funding

This research was supported by the ERA-NET BlueBio COFUND Project SIDESTREAM [Grant ID 68], cofunded through national funds provided by the Agencia Española de Investigación, Spain, grant no. PCI2020-111960/MCIN/AEI/10.13039/501100011033 and the EU NextGenerationEU/PRTR, and the Funding by the German Federal Ministry of Education and Research (BMBF), FKZ161B0950B. Additional funding was received through the project IMPROMEGA Agencia Española de Investigación, Spain, grant no. RTI2018-095119-B-100, MCIU/AEI/FEDER/UE/MCIN/AEI/10.13039/501100011033/and FEDER "A way to make Europe."

Acknowledgments

This is a short text to acknowledge the contributions of specific colleagues, institutions, or agencies that aided the efforts of the authors.

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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.931991/full#supplementary-material

SUPPLEMENTARY TABLE 1
Calculated *p* values for the one-way PERMANOVA (Bonferroni corrected *p* values).

SUPPLEMENTARY TABLE 2
SIMPER analysis of the dietary groups Fucus *vs* Carrot. All FA detected were included in the analysis and those above the cumulative cutoff of 70% were not shown in the table, except for the first one.

SUPPLEMENTARY TABLE 3
SIMPER analysis of the dietary groups Fucus *vs* Coco. All FA detected were included in the analysis and those above the cumulative cut-off of 70% were not shown in the table, except for the first one.

SUPPLEMENTARY TABLE 4
SIMPER analysis of the dietary groups Carrot *vs* Coco. All FA detected were included in the analysis and those above the cumulative cut-off of 70% were not shown in the table, except for the first one.

References

Agnew, D. J., and Taylor, A. C. (1986). Effects of oxygen tension, temperature, salinity, and humidity on the survival of two intertidal gammarid amphipods. *Mar. Ecol. Prog. Ser.* 32, 27–33.

Ahyong, S. T., and Hughes, L. E. (2016). Collecting and processing amphipods. *J. Crustac. Biol.* 36, 584–588. doi: 10.1163/1937240X-00002450

Alberts-Hubatsch, H., Slater, M. J., and Beermann, J. (2019). Effect of diet on growth, survival and fatty acid profile of marine amphipods: Implications for utilisation as a feed ingredient for sustainable aquaculture. *Aquac. Environ. Interact.* 11, 481–491. doi: 10.3354/aei00329

Ashour, M., Abo-Taleb, H. A., Hassan, A. K. M., Abdelzaher, O. F., Mabrouk, M. M., Elokaby, M. A., et al. (2021). Valorization use of amphipod meal, gammarus pulex, as a fishmeal substitute on growth performance, feed utilization, histological and histometric indices of the gut, and economic revenue of grey mullet. *J. Mar. Sci. Eng.* 9(12), 1336. doi: 10.3390/jmse9121336

Baeza-Rojano, E., Domingues, P., Guerra-García, J. M., Capella, S., Noreña-Barroso, E., Caamal-Monsreal, C., et al. (2013). Marine gammarids (Crustacea: Amphipoda): a new live prey to culture octopus maya hatchlings. *Aquac. Res.* 44, 1602–1612. doi: 10.1111/j.1365-2109.2012.03169.x

Baeza-Rojano, E., García, S., Garrido, D., Guerra-García, J. M., and Domingues, P. (2010). Use of amphipods as alternative prey to culture cuttlefish (Sepia officinalis) hatchlings. *Aquaculture* 300, 243–246. doi: 10.1016/j.aquaculture.2009.12.029

Baeza-Rojano, E., Hachero-Cruzado, I., and Guerra-García, J. M. (2014). Nutritional analysis of freshwater and marine amphipods from the strait of Gibraltar and potential aquaculture applications. *J. Sea Res.* 85, 29–36. doi: 10.1016/j.seares.2013.09.007

Beermann, J., Boos, K., Gutow, L., Boersma, M., and Peralta, A. C. (2018). Combined effects of predator cues and competition define habitat choice and food consumption of amphipod mesograzers. *Oecologia* 186, 645–654. doi: 10.1007/s00442-017-4056-4

Bell, J. G., McEvoy, J., Tocher, D. R., McGhee, F., Campbell, P. J., and Sargent, J. R. (2001). Replacement of fish oil with rapeseed oil in diets of Atlantic salmon (Salmo salar) affects tissue lipid compositions and hepatocyte fatty acid metabolism. *J. Nutr.* 131, 1535–1543. doi: 10.1093/jn/131.5.1535

Boyen, J., Fink, P., Mensens, C., Hablützel, P. I., and De Troch, M. (2020). Fatty acid bioconversion in harpacticoid copepods in a changing environment: A transcriptomic approach. *Philos. Trans. R. Soc B Biol. Sci.* 375. doi: 10.1098/rstb.2019.0645

Castro, L. F. C., Tocher, D. R., and Monroig, O. (2016). Long-chain polyunsaturated fatty acid biosynthesis in chordates: Insights into the evolution of fads and elovl gene repertoire. *Prog. Lipid Res.* 62, 25–40. doi: 10.1016/j.plipres.2016.01.001

Chamier, A., and Willoughby, L. G. (1986). The role of fungi in the diet of the amphipod gammarus pulex (L.): an enzymatic study. *Freshw. Biol.* 16, 197–208. doi: 10.1111/j.1365-2427.1986.tb00964.x

Costa, F. O., and Costa, M. H. (2000). Review of the ecology of gammarus locusta (L.). *Pol. Arch. Hydrobiol.* 47, 541–559.

Dhont, J., Dierckens, K., Støttrup, J., Van Stappen, G., Wille, M., and Sorgeloos, P. (2013). ""Rotifers, artemia and copepods as live feeds for fish larvae in aquaculture,"," in *Advances in aquaculture hatchery technology* (Cambridge, UK: Woodhead Publishing Limited), 157–202. doi: 10.1533/9780857097460.1.157

Ernst, R., Ejsing, C. S., and Antonny, B. (2016). Homeoviscous adaptation and the regulation of membrane lipids. *J. Mol. Biol.* 428, 4776–4791. doi: 10.1016/j.jmb.2016.08.013

FAO (2020). *The state of world fisheries and aquaculture 2020. sustainability in action*. (Rome, Italy: FAO). doi: 10.4060/ca9229en

Folch, J., Lees, M., and Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* 226, 497–509. doi: 10.1016/S0021-9258(18)64849-5

Galkanda-Arachchige, H. S. C., Wilson, A. E., and Davis, D. A. (2020). Success of fishmeal replacement through poultry by-product meal in aquaculture feed formulations: a meta-analysis. *Rev. Aquac.* 12, 1624–1636. doi: 10.1111/raq.12401

González-Peña, M. A., Lozada-Ramírez, J. D., and Ortega-Regules, A. E. (2021). Carotenoids from mamey (Pouteria sapota) and carrot (Daucus carota) increase the oxidative stress resistance of caenorhabditis elegans. *Biochem. Biophys. Rep.* 26. doi: 10.1016/j.bbrep.2021.100989

Guerra-García, J. M., Hachero-Cruzado, I., González-Romero, P., Jiménez-Prada, P., Cassell, C., and Ros, M. (2016). Towards integrated multi-trophic aquaculture: Lessons from caprellids (Crustacea: Amphipoda). *PloS One* 11, e0154776. doi: 10.1371/journal.pone.0154776

Hammer, Ø., Harper, D. A. T., and Ryan, P. D. (2001). *PAST : Paleontological statistics software package for education and data analysis*, Vol. 4. (California: Palaentologia Electronica, Coquina Press) 1–9.

Harlıoğlu, M. M., and Farhadi, A. (2018). Importance of gammarus in aquaculture. *Aquac. Int.* 26, 1327–1338. doi: 10.1007/s10499-018-0287-6

Hazel, J. R. (1995). Thermal adaptation in biological membranes: Is homeoviscous adaptation the explanation? *Annu. Rev. Physiol.* 57, 19–42. doi: 10.1146/annurev.ph.57.030195.000315

Hazel, J. R., and Eugene Williams, E. (1990). The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. *Prog. Lipid Res.* 29, 167–227. doi: 10.1016/0163-7827(90)90002-3

Jannathulla, R., Rajaram, V., Kalanjiam, R., Ambasankar, K., Muralidhar, M., and Dayal, J. S. (2019). Fishmeal availability in the scenarios of climate change: Inevitability of fishmeal replacement in aquafeeds and approaches for the utilization of plant protein sources. *Aquac. Res.* 50, 3493–3506. doi: 10.1111/are.14324

Jiménez-Prada, P., Hachero-Cruzado, I., Giráldez, I., Fernández-Diaz, C., Vilas, C., Cañavate, J. P., et al. (2018). Crustacean amphipods from marsh ponds: A nutritious feed resource with potential for application in integrated multi-trophic aquaculture. *PeerJ* 2018. doi: 10.7717/peerj.4194

Jiménez-Prada, P., Hachero-Cruzado, I., and Guerra-García, J. M. (2020). Aquaculture waste as food for amphipods: the case of gammarus insensibilis in marsh ponds from southern Spain. *Aquac. Int.* 139–153. doi: 10.1007/s10499-020-00615-z

Jump, D. B. (2002). The biochemistry of n-3 polyunsaturated fatty acids. *J. Biol. Chem.* 277, 8755–8758. doi: 10.1074/jbc.R100062200

Kabeya, N., Ogino, M., Ushio, H., Haga, Y., Satoh, S., Navarro, J. C., et al. (2021). A complete enzymatic capacity for biosynthesis of docosahexaenoic acid (DHA, 22:6n-3) exists in the marine harpacticoida copepod tigriopus californicus. *Open Biol.* 11. doi: 10.1098/rsob.200402

Kolanowski, W., Stolyhwo, A., and Grabowski, M. (2007). Fatty acid composition of selected freshwater gammarids (Amphipoda, crustacea): A potentially innovative source of omega-3 LC PUFA. *JAOCS J. Am. Oil Chem. Soc* 84, 827–833. doi: 10.1007/s11746-007-1116-7

Laila, A. (2021). Limitations in the "homeoviscous adaptation to dietary lipids" model. *Am. J. Clin. Nutr.* 114, 822–823. doi: 10.1093/ajcn/nqab230

McKinnon, A. D., Duggan, S., Nichols, P. D., Rimmer, M. A., Semmens, G., and Robino, B. (2003). The potential of tropical paracalanid copepods as live feeds in aquaculture. *Aquaculture* 223, 89–106. doi: 10.1016/S0044-8486(03)00161-3

Miller, M. R., Nichols, P. D., and Carter, C. G. (2008). n-3 oil sources for use in aquaculture alternatives to the unsustainable harvest of wild fish. *Nutr. Res. Rev.* 21, 85–96. doi: 10.1017/S0954422408102414

Monroig, Ó., and Kabeya, N. (2018). Desaturases and elongases involved in polyunsaturated fatty acid biosynthesis in aquatic invertebrates: a comprehensive review. *Fish. Sci.* 84, 911–928. doi: 10.1007/s12562-018-1254-x

Monroig, Ó, Shu-Chien, A. C., Kabeya, N., Tocher, D. R., and Castro, L. F. C. (2022). Desaturases and elongases involved in long-chain polyunsaturated fatty acid biosynthesis in aquatic animals: From genes to functions. *Prog. Lipid Res.* 86, 101157. doi: 10.1016/j.plipres.2022.101157

Mourente, G., and Bell, J. G. (2006). Partial replacement of dietary fish oil with blends of vegetable oils (rapeseed, linseed and palm oils) in diets for European sea bass (Dicentrarchus labrax l.) over a long term growth study: Effects on muscle and liver fatty acid composition and e. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 145, 389–399. doi: 10.1016/j.cbpb.2006.08.012

Naylor, R. L., Hardy, R. W., Bureau, D. P., Chiu, A., Elliott, M., Farrell, A. P., et al. (2009). Feeding aquaculture in an era of finite resources. *Proc. Natl. Acad. Sci. U. S. A.* 106, 15103–15110. doi: 10.1073/pnas.0905235106

Neuparth, T., Costa, F. O., and Costa, M. H. (2002).). effects of temperature and salinity on life history of the marine amphipod gammarus locusta. implications for ecotoxicological testing. *Ecotoxicology* 11, 61–73. doi: 10.1023/A:1013797130740

Nielsen, B. L. H., Gøtterup, L., Jørgensen, T. S., Hansen, B. W., Hansen, L. H., Mortensen, J., et al. (2019). N-3 PUFA biosynthesis by the copepod apocyclops royi documented using fatty acid profile analysis and gene expression analysis. *Biol. Open* 8, 1–12. doi: 10.1242/bio.038331

Ribes-Navarro, A., Navarro, J. C., Hontoria, F., Kabeya, N., Standal, I. B., Evjemo, J. O., et al. (2021).). biosynthesis of long-chain polyunsaturated fatty acids in marine gammarids: Molecular cloning and functional characterisation of three fatty acyl elongases. *Mar. Drugs* 19. doi: 10.3390/MD19040226

Romano, N., Kumar, V., Yang, G., Kajbaf, K., Rubio, M. B., Overturf, K., et al. (2020). Bile acid metabolism in fish: disturbances caused by fishmeal alternatives and some mitigating effects from dietary bile inclusions. *Rev. Aquac.* 12, 1792–1817. doi: 10.1111/raq.12410

Saini, R. K., Nile, S. H., and Park, S. W. (2015). Carotenoids from fruits and vegetables: Chemistry, analysis, occurrence, bioavailability and biological activities. *Food Res. Int.* 76, 735–750. doi: 10.1016/j.foodres.2015.07.047

Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* 9, 671–675. doi: 10.1038/nmeth.2089

Sexton, E. W. (1928). On the rearing and breeding of gammarus in laboratory conditions. *J. Mar. Biol. Assoc. United Kingdom* 15, 33–55. doi: 10.1017/S0025315400055521

Shepherd, C. J., Monroig, O., and Tocher, D. R. (2017). Future availability of raw materials for salmon feeds and supply chain implications: The case of Scottish farmed salmon. *Aquaculture* 467, 49–62. doi: 10.1016/j.aquaculture.2016.08.021

Suontama, J., Kiessling, A., Melle, W., Waagbø, R., and Olsen, R. E. (2007). Protein from northern krill (Thysanoessa inermis), Antarctic krill (Euphausia superba) and the Arctic amphipod (Themisto libellula) can partially replace fish meal in diets to Atlantic salmon (Salmo salar) without affecting pro. *Aquac. Nutr.* 13, 50–58. doi: 10.1111/j.1365-2095.2007.00453.x

Tocher, D. R. (2015). Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. *Aquaculture* 449, 94–107. doi: 10.1016/j.aquaculture.2015.01.010

Turchini, G. M., Francis, D. S., Senadheera, S. P. S. D., Thanuthong, T., and De Silva, S. S. (2011a). Fish oil replacement with different vegetable oils in Murray cod: Evidence of an "omega-3 sparing effect" by other dietary fatty acids. *Aquaculture* 315, 250–259. doi: 10.1016/j.aquaculture.2011.02.016

Turchini, G. M., Ng, W., and Tocher, D. (2011b). *Fish oil replacement and alternative lipid sources in aquaculture feeds* (Boca Rat: CRC Press).

Turchini, G. M., Torstensen, B. E., and Ng, W. K. (2009). Fish oil replacement in finfish nutrition. *Rev. Aquac.* 1, 10–57. doi: 10.1111/j.1753-5131.2008.01001.x

Wijnhoven, S., Van Riel, M. C., and van der Velde, G. (2003). Exotic and indigenous freshwater gammarid species: Physiological tolerance to water temperature in relation to ionic content of the water. *Aquat. Ecol.* 37, 151–158. doi: 10.1023/A:1023982200529

Yıldız, M., Eroldoğan, T. O., Ofori-Mensah, S., Engin, K., and Baltacı, M. A. (2018). The effects of fish oil replacement by vegetable oils on growth performance and fatty acid profile of rainbow trout: Re-feeding with fish oil finishing diet improved the fatty acid composition. *Aquaculture* 488, 123–133. doi: 10.1016/j.aquaculture.2017.12.030

Zinoöcker, M. K., Svendsen, K., and Dankel, S. N. (2021). The homeoviscous adaptation to dietary lipids (HADL) model explains controversies over saturated fat, cholesterol, and cardiovascular disease risk. *Am. J. Clin. Nutr.* 113, 277–289. doi: 10.1093/ajcn/nqaa322

frontiers | Frontiers in Marine Science

TYPE Technology and Code
PUBLISHED 18 August 2022
DOI 10.3389/fmars.2022.942094

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

RECEIVED 12 May 2022
ACCEPTED 02 August 2022
PUBLISHED 18 August 2022

CITATION
Ballesteros A, Siles P, Jourdan E and Gili J-M (2022) Versatile aquarium for jellyfish: A rearing system for the biomass production of early life stages in flow-through or closed systems. *Front. Mar. Sci.* 9:942094. doi: 10.3389/fmars.2022.942094

Versatile aquarium for jellyfish: A rearing system for the biomass production of early life stages in flow-through or closed systems

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Interest in the rearing of jellyfish has grown exponentially over recent years due to their indisputable potential in a wide variety of uses and research. Here, we describe the design and operation of an effective modified kreisel aquarium that allows to grow of the early planktonic life stages of jellyfish in both flow-through or closed systems. Thanks to its versatile and innovative approach, the aquarium operates for species with a metagenetic life cycle, and allows the metamorphosis of ephyrae from fertilized eggs for species with a holoplanktonic life cycle, such as *Pelagia noctiluca*. In addition, its configuration allows the mesh screen to be changed *in situ*, adjusting the size of the mesh according to the growth of the jellyfish and the size of the prey offered. An accessory, named the polyp plate, suspends the polyps in a downward or sideways position, facilitating prey capture, strobilae formation and release of ephyrae. The improvements of this modified kreisel reduce the time-involvement for staff in maintenance routines, and it is an important contribution to jellyfish husbandry techniques and biomass production. These improvements especially target to the culture of the blooming and stinging *P. noctiluca*, which has been noted to present a host of challenges to the scientific and aquarist communities.

KEYWORDS

aquaculture, ephyra, kreisel, metaephyra, *Pelagia noctiluca*, polyp, strobilation, tank

Introduction

Jellyfish are considered an important fishery commodity for the food industry (Omori and Nakano, 2001; Dong et al., 2009) and have a high value as raw material in a wide variety of biotechnological applications (Merquiol et al., 2019; D'Ambra and Merquiol, 2022). Although wild jellyfish are exploited as a marine resource for different proposals (Omori and Nakano, 2001; Dong et al., 2009; Elliott et al., 2017), they are considered unpredictable due to the seasonality and spatiotemporal variability of their life cycles (Purcell et al., 2013; Gueroun et al., 2021; Marambio et al., 2021). From this point of view, biomass production through aquaculture activity can offer a permanent stock to supplement the fished biomass, as well as, to assures individuals not contaminated with unknown pollutants and safeguards traceability; a plus when aiming to target premium sectors associated with pharmaceutical, cosmeceutical and biomedical industries.

To raise jellyfish successfully, knowledge on the biology of target species is required including their feeding and reproductive behavior (Fu et al., 2014; Lilley et al., 2014b; Gueroun et al., 2021; Camacho-Pacheco et al., 2022). The majority of the scyphozoan species (Cnidaria: Scyphozoa) have a well-known metagenetic life cycle. Sexually mature jellyfish produce eggs or sperm which fuse to develop a ciliated planulae. Planulae settle to the marine substrate and metamorphose into the scyphistoma or polyp (benthic phase) (Helm, 2018). Through the process of strobilation, the polyps fission perpendicularly (strobilae) to release small ephyrae into the water column (planktonic phase). Ephyrae grow into metaephyrae, juvenile medusae and finally, sexually mature adults that produce eggs and sperm, completing the life cycle (Fuentes et al., 2011; Helm, 2018). Despite being the most common life cycle in scyphozoans, some species, such as the holoplanktonic *Pelagia noctiluca*, lack a benthic phase, and the fertilized eggs metamorphose directly into ephyrae in the water column (Sandrini and Avian, 1983; Canepa et al., 2014).

Aquaculture techniques, employed to produce jellyfish, vary according to the species and life cycle stages (Raskoff et al., 2003; Pierce, 2005; Purcell et al., 2013; Schaadt et al., 2017; Duarte et al., 2021). Polyp colonies are a continuous source of jellyfish through the strobilation process (Helm, 2018; Duarte et al., 2021), hence keeping colonies healthy is strategic for continuous production over time. Their maintenance is easier and much less time-consuming than early planktonic life stages (ephyrae and metaephyrae), which are the most complicated stages in jellyfish aquaculture and consume the majority of staff time (Duarte et al., 2021). However, although polyps are resistant to some anthropogenic stressors in marine habitats (e.g., pesticides, Olguín-Jacobson et al., 2020), to ensure successful breeding in aquaculture, it is important to maintain adequate water quality and suitable nutrition (Raskoff et al., 2003; Crow et al., 2013; Duarte et al., 2021).

Regarding life support system in jellyfish aquaculture, polyps and jellyfish can be maintained in flow-through or closed systems (Crow et al., 2013). While in a flow-through is the water is constantly replaced with new water from a source such as the ocean, in a closed system, the water needs to be recirculated (Duarte et al., 2021). In the case of a close system, the outflow drain should lead to a sump with proper mechanical and biological filtration, and UV sterilisation (Duarte et al., 2021, e.g., Figure 6). Both systems have advantages and disadvantages, but one of the drawbacks of using a closed system is the time spent on maintenance routines (Crow et al., 2013).

Problem description

Depending on the life cycle stage, individuals are grown in a variety of culture vessels or aquariums in a flow-through or closed systems (Widmer et al., 2005; Purcell et al., 2013; Duarte et al., 2021, e.g., Figure 5). The best-known type of aquarium in jellyfish rearing is the kreisel tank (Greve, 1968). The kreisel is a circular aquarium with a flat back and front that contains an inlet and an outlet water chamber separated by a screen. The water inlet is generally provided by a spray bar with holes creating a circular flow that moves jellyfish away from the screen (Purcell et al., 2013, e.g., Figure 13.3). The separation of the chambers along with the water inlet allows jellyfish to swim freely without danger of being sucked down the drain (Purcell et al., 2013; Duarte et al., 2021). Despite their wide use in jellyfish aquaculture, usually, kreisel aquariums are not used as early stage grow-out tanks (Duarte et al., 2021, e.g., Figure 4). Ephyrae and metaephyrae, which are small and thin (Straehler-Pohl and Jarms, 2010; Straehler-Pohl et al., 2011), can easily be retained in the mesh screen due to the suction produced by the drain. Under this premise and to ensure their safety, they are often cultured successfully in vessels such as flasks, dishes or balls glass and plastic jars (Widmer et al., 2005; Crow et al., 2013), as well as, in larger tanks without water outlet (Duarte et al., 2021). All of these rearing systems require daily water changes to ensure its quality (Duarte et al., 2021). However, from the perspective of biomass production and scalability of jellyfish cultures, the time spent on recurrent water changes, involving the pipetting of each ephyra one by one at discrete time intervals, is unfeasible.

With the aim of reducing the time required for maintenance routines, and exploring new technologies to scale jellyfish cultures and biomass production, we present a versatile modified kreisel aquarium that allows safe cultivation early jellyfish life stages in both flow-through and closed systems. Our prototype aquarium is an important contribution to jellyfish husbandry techniques, especially for *P. noctiluca*, a species whose culture represents a challenge for the scientific and

aquarist community due to the lack of polyp stage in its life cycle (Ramondenc et al., 2019; Ballesteros et al., 2022). Additionally, due to its versatility and accessories, this system can be used for polyps, strobilae, eggs, planulae and grow-out ephyrae and metaephyrae tanks.

Aquarium design

Tank configuration

The aquarium (350 mm x 350 mm x 150 mm) comprises an 8 L main PVC and methacrylate circular tank (main chamber) with a rectangular upper frame on top, through which the water inlet and jellyfish are introduced (Figures 1A–C, 2A, B). A second chamber (drainage chamber) adjoins the main chamber, separated by a long, narrow interchangeable mesh screen (Figures 1B, D, 2C). The mesh screen allows the water to enter from the main chamber to the drainage chamber (Figure 2D). An air duct (Q_{air} = 11.5 l/h), placed in the bottom-center of the mesh screen (Figures 1A, B), introduces air into the first tank, creating a flow of bubbles which covers almost the entire mesh screen to prevent small ephyrae and metaephyrae from approaching (Figures 2E, F). The air bubbles, push the animals into the main chamber.

The interchangeable mesh screen (150, 250, 350 and 500 µm and 1 mm) (Figures 1B, D, 2D) allows the changing of different sizes of mesh screen throughout the growth of the early jellyfish stages *in situ* without removing individuals from the tank.

Polyp plate

An accessory, named the polyp plate (Figures 1A, E, 3A, B, E), can be easily placed to keep polyps suspended in the water column in a downward or sideways position, facilitating prey capture, strobilae formation and release of ephyrae (Figures 3C, D). The strobilation process can be induced in the tank. In jellyfish aquaculture, different methods are used to trigger the strobilation process (e.g., addition of chemicals or the up- or down-regulation of water temperature or salinity, Kuniyoshi et al., 2012; Treible and Condon, 2019). In this case, and in order to keep the desired chemical concentrations in a

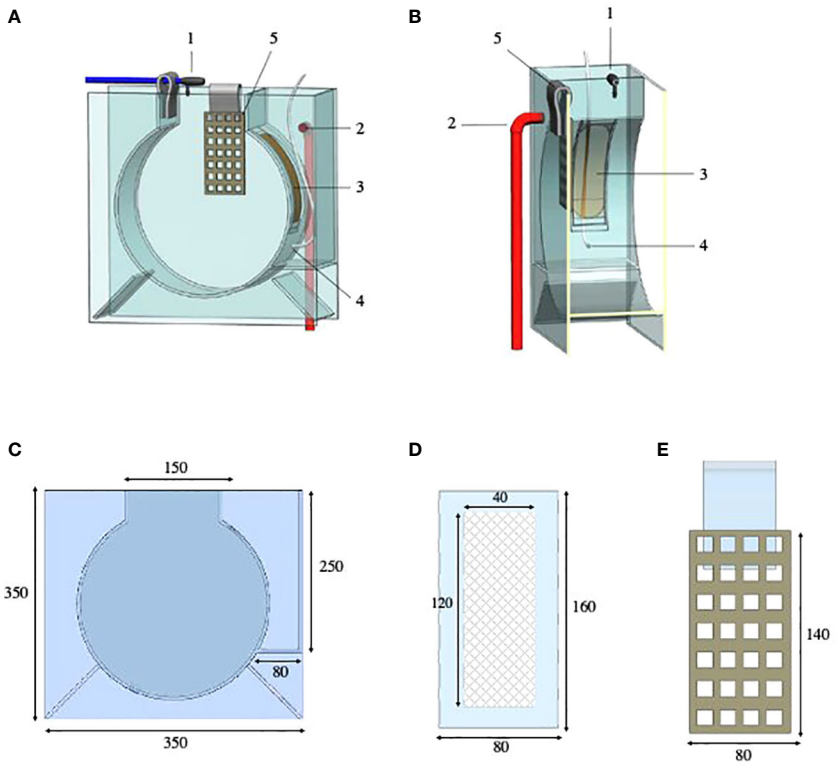

FIGURE 1
Schematic representation of the aquarium and accessories for the growth of the early stages of jellyfish: **(A)** Frontal view. **(B)** Transverse modified section. **(C)** Aquarium measures (mm). **(D)** Interchangeable mesh screen measures (mm). **(E)** Polyp plate measures (mm). Aquarium configuration: 1, water inlet in the main chamber (blue pipe, Ø ext.=8 mm); 2, water outlet in the drainage chamber (red pipe, Ø ext.=20 mm); 3, interchangeable mesh screen (150, 250, 350 and 500 µm and 1 mm); 4, air inlet in the main chamber (white tube, Ø ext.=5 mm; 5, polyp plate (square grid, 20x20 mm). Polyp plate dimensions can be varied as desired. Schematic representation by Carlos Mengod.

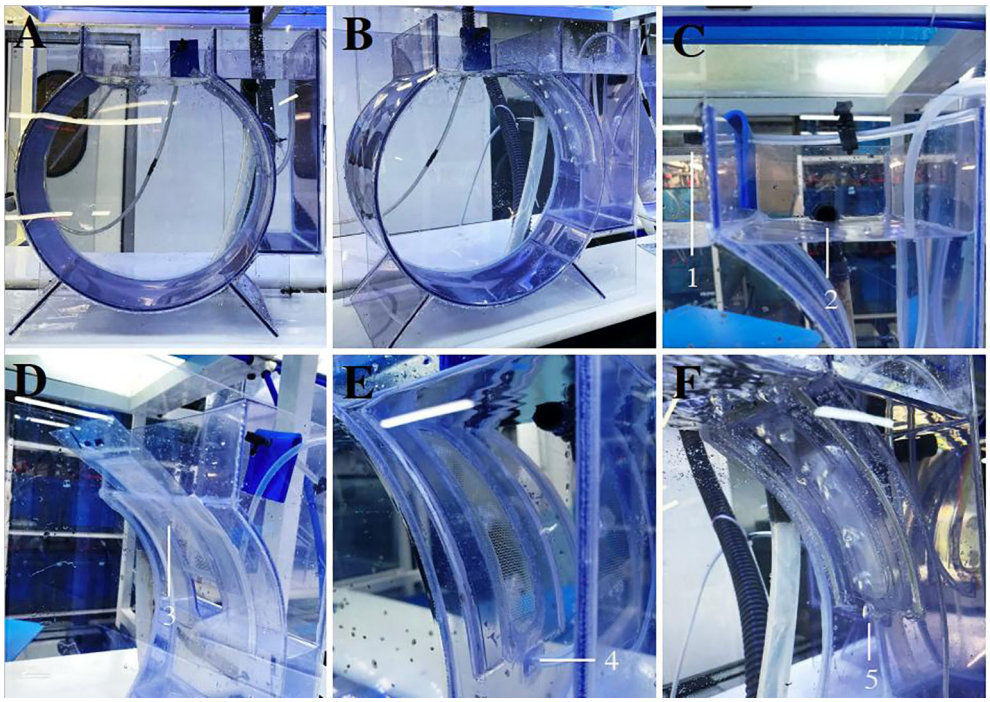

FIGURE 2
Aquarium for the growth out of the early jellyfish life stages: **(A, B)** Frontal view. **(C)** Drainage chamber. **(D)** Interchangeable mesh screen (150, 250, 350 and 500 µm and 1 mm). **(E)** Air inlet in the main chamber. **(F)** Air bubbles covering the interchangeable mesh screen. Aquarium configuration: 1, water inlet; 2, water outlet; 3, interchangeable mesh screen; 4, air inlet; 5, air bubbles.

closed system, water entry to the system can be stopped with stopcocks.

Biomass production in a flow-through system

In order to increase biomass, the aquariums are placed in series in metal galvanized shelves in a temperature-controlled chamber (Figure 4). The water inlet is supplied with seawater that has been pretreated in a distribution system, obtained from a facility that provides access to high-quality seawater (e.g., Aquaria and Experimental Chambers of the Institute of Marine Science (ICM-CSIC)). The water is pretreated by mechanical (decantation and filtration with artificial polymers) and chemical (activated carbon) processes, as well as UV sterilized to remove unwanted organisms. For greater safety in case of any specific climatic event (e.g., storm), a filter system comprising filter cartridges of 20, 10 and 1 µm – the 1 µm filter cartridge can be replaced by a 5 µm cartridge – is placed before the water enters the sump (Figure 4A). For ease of emptying, there is a screw cap at the bottom (Figure 4B).

From the sump, water is pumped through the pipes to each aquarium individually, and the inlet flow can be regulated by individual stopcocks (Figure 4A). Like the water inlet, the air inlet comes from the general system for each aquarium (Figure 4A). The air bubble flow can also be controlled with stopcocks. Lastly, the excess water stored in the drainage chamber flows into laboratory sewers through the system's drain pipes (Figure 4B). Before being returned to the sea, the water is once again pretreated at the facility through mechanical and biological filtrations, pH control and UV sterilization.

Finally, and proving its versatility, this system has been successfully used for metagenetic species such as *Aurelia* sp. or *Rhizostoma pulmo* (Table 1), as well as for the breeding of the holoplanktonic jellyfish *P. noctiluca* in a flow-through system (Table 2). For *P. noctiluca* culture, fertilized eggs were transferred from sexually mature jellyfish to the aquariums. After three (22°C) or four days (18°C) the first ephyrae were observed in the tank, and they were grown until they reach the juvenile stage in flow-through aquariums (Figure 4). The conditions for its culture from fertilized eggs to metaephyrae in a flow-through system are given in Table 2.

Discussion

Jellyfish early life stages are commonly cultured in receptacles (e.g., balls glass and plastic jars) which involve recurring water

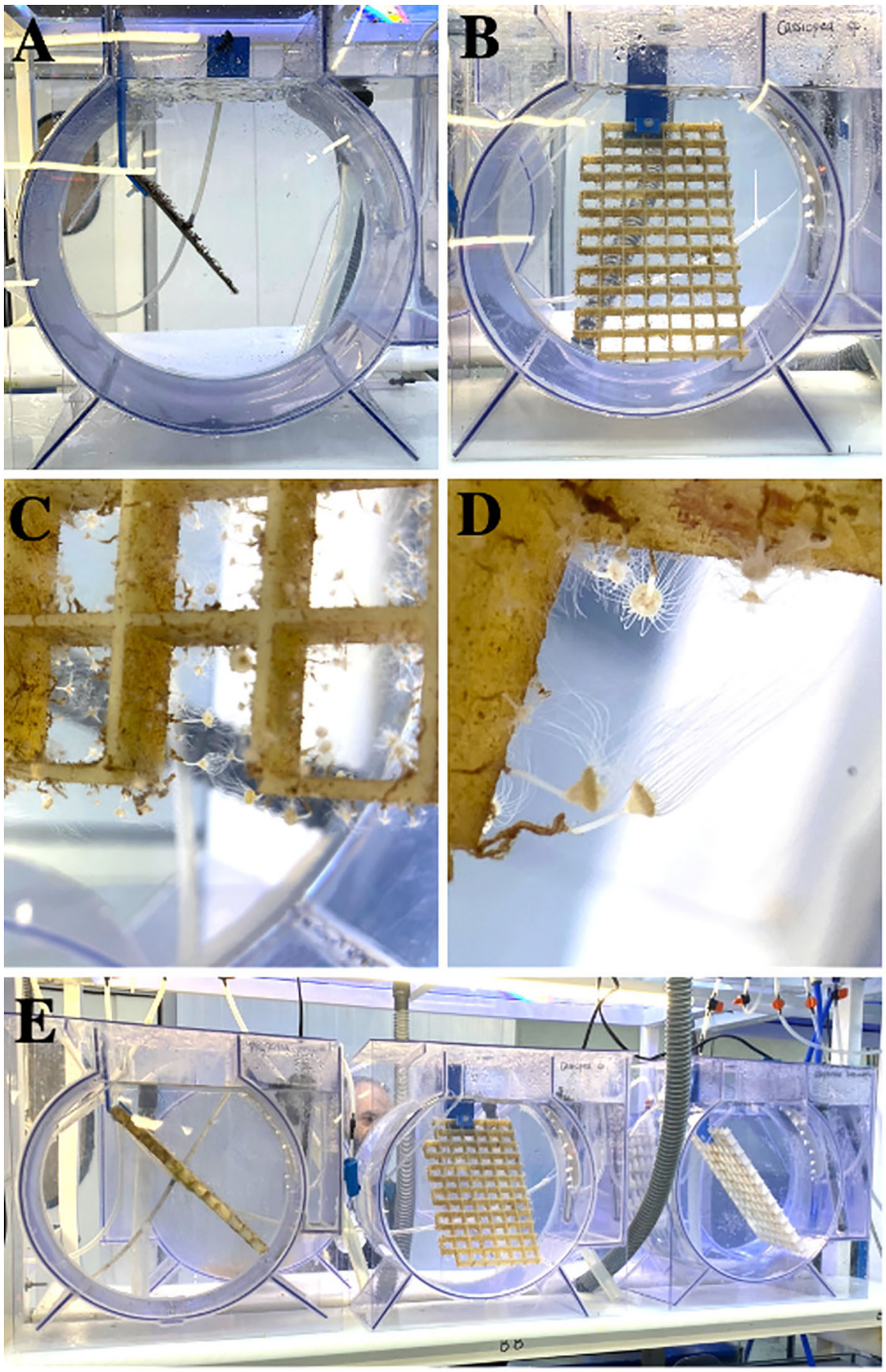

FIGURE 3
Polyp plates in flow-through aquarium: **(A)** Polyp plate of *Aurelia* spp. **(B)** Grid polyp plate of *Cassiopea* spp. **(C)** Suspended polyps in a downward or sideways position, enhancing prey capture, strobilae formation and release of ephyrae. **(D)** Tentacles of Cassiopea spp. polyps fully extended. **(E)** Polyp plates with *Phyllorhiza punctata* (left), *Cassiopea* spp. (middle) and *Cotylorhiza tuberculata* (right) species. Notes: The *Cassiopea* spp. polyp plates can be placed in rectangular tanks; The use of grid plates prevents the massive growth of algae; Multiple polyp plates can be placed in the same aquarium.

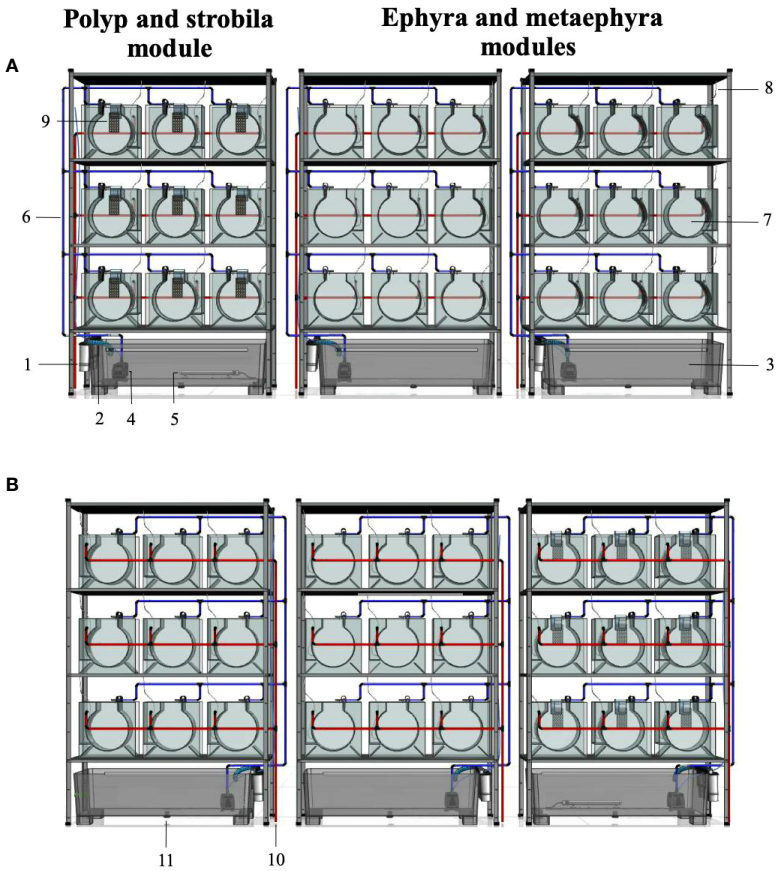

FIGURE 4
Schematic representation of a flow-through system of jellyfish biomass production for metagenetic and holoplanktonic species. **(A)** Frontal view. **(B)** Posterior view. System configuration: 1, filter system composed of filter cartridges; 2, water inlet towards the sump; 3, sump; 4, water pump; 5, heater; 6, individual water inlet to each kreisel (blue pipe); 7, kreisel; 8, individual air inlet to each kreisel; 9, polyp plate; 10, water drainage (red pipe); 11, screw cap for emptying the sump. Notes: Polyp plates with strobilae can be easily moved to the ephyra and metaephyra modules during the strobilation process; Fertilized eggs of *Pelagia noctiluca* can be metamorphosed into the ephyra and metaephyra modules.

changes as well as more staff time on maintenance routines (Duarte et al., 2021). In the recent review of jellyfish husbandry by Duarte et al. (2021), different types of ephyrae grow-out tanks have been proposed. However, of all of them, only the rectangular and V-shaped bottom aquariums allow growth in flow-through (Duarte et al., 2021, Figure 4 and Table 1). Here, we present a modified kreisel for rearing of the early planktonic stages of jellyfish in both flow-through and closed systems. The aquariums have been manufactured for an 8 L water capacity, but larger volumes can be mass-produced keeping the same configuration (Figure 1).

Collecting newly released ephyrae from polyp tanks represents a large investment of time for research staff and aquarists, who must chase ephyrae around the polyp tank to transfer them to new aquariums (Schaadt et al., 2017). To optimize jellyfish aquaculture techniques, polyp and ephyrae aquariums have been connected to each other in order to sweep ephyrae after release from the polyp towards ephyrae catch aquariums in a flow-through. An example of an ephyrae catch tank was proposed by Schaadt et al. (2017) for *Aurelia* spp. The system was composed of a small plastic vessel with a large parabolic arc-shaped mesh screen to one side of the tank followed by a hole allowing water drainage. Similar designs can be consulted in Raskoff et al. (2003) and Duarte et al. (2021) for any scyphozoan with a metagenetic life cycle. Here, the easily removable polyp plates allow movement between tanks (Figures 1A, 3A, B), and they can be returned to the polyp module once the strobilation process is finished (Figure 4). The suspension of the polyp plate in the kreisel allows the scyphistome to reside in a position where the tentacles are suspended downward or sideways as in the wild, improving prey capture, strobilae formation and release of ephyrae (Figures 3C, D) (Brewer, 1976). In addition, it also prevents the polyp from being covered by debris. However, for the rearing of *Cassiopea* spp., a benthic jellyfish, the use of the current kreisel design is not recommended, but to benefit from the

TABLE 1 Culture conditions for polyps, ephyrae and metaephyrae of some metagenetic species in flow-through system.

Scyphozoan species	T (°C)+	Salinity+	Bubble flow	Mesh size			Number of feedings times, amounts and food offered+		
				P	E	ME	P	E	ME
Aurelia spp.	13–25	36–37	2 bubbles/second	350 µm	350 µm	1 mm	(x1) 50000 rotifers/100 ml seawater	(x2) 50000 rotifers/100 ml seawater	(x1) 50000 rotifers/100 ml seawater, (x2) 14000 *Artemia* sp./100 ml seawater
*Cotylorhiza tuberculata**	13–25	36–37	2 bubbles/second	350 µm	350 µm	1 mm	(x1) 50000 rotifers/100 ml seawater	(x2) 50000 rotifers/100 ml seawater	(x1) 50000 rotifers/100 ml seawater, (x2) 14000 *Artemia* sp./100 ml seawater
*Phyllorhiza punctata**	13–25	36–37	2 bubbles/second	350 µm	350 µm	1 mm	(x1) 50000 rotifers/100 ml seawater	(x2) 50000 rotifers/100 ml seawater	(x1) 50000 rotifers/100 ml seawater, (x2) 14000 *Artemia* sp./100 ml seawater
Rhizostoma pulmo	13–25	36–37	2 bubbles/second	350 µm	350 µm	1 mm	(x1) 50000 rotifers/100 ml seawater	(x2) 50000 rotifers/100 ml seawater	(x1) 50000 rotifers/100 ml seawater, (x2) 14000 *Artemia* sp./100 ml seawater
Sanderia malayensis	13–25	36–37	2 bubbles/second	350 µm	350 µm	1 mm	(x1) 50000 rotifers/100 ml seawater	(x2) 50000 rotifers/100 ml seawater	(x1) 50000 rotifers/100 ml seawater, (x2) 14000 *Artemia* sp./100 ml seawater

P, polyp stage; E, ephyra stage; ME, metaephyra stage.
*Species with zooxanthellae. IED strip with photosynthetic spectrum (4500K and 80W). All life cycle stages require light.
+Additional temperature and salinity ranges and food offered can be consulted in Purcell et al. (2013) and Duarte et al. (2021).
An interval of at least 4 hours between feeding times is suggested. Amounts of food for a number of 150 individuals. Jellyfish can also be feed ad libitum.

improvements in polyp suspension (Figures 3C–E), the polyp plates can be placed in flow-through rectangular tanks. Those specifically interested in the culture of *Cassiopea* spp. should consult the system proposed by Pierce (2005).

The rearing of *P. noctiluca* is a challenge for the scientific and aquarist community (Ramondenc et al., 2019; Ballesteros et al., 2022). Lilley et al. (2014a) proposed a system composed of 5 to 15 L receptacles with motorized PVC paddles rotating to keep animals in suspension with recurrent water changes (Ramondenc et al., 2019, Figure 1A). According to Ramondenc et al. (2019), this system caused stress to the individuals and was not suitable for the farming of any jellyfish. Years later, Ramondenc et al. (2019) improved the rearing techniques for *P. noctiluca* and cultivated the individuals in a closed system kreisel aquarium, avoiding collision of the individuals with the vessel walls (Ramondenc et al., 2019, Figure 1B). The authors highlighted the difficulty of adjusting prey concentration to feed individuals *ad libitum* and avoiding the accumulation of debris from unassimilated food. The technical improvement of this aquarium (Figure 1) has allowed to provide large amounts of food or *ad libitum* thanks to the continuous renewal of new seawater (Tables 1, 2). In addition, for the first time to our knowledge, *P. noctiluca* to be safely maintained during their early life stages in a flow-through system (Table 2). The design of the screen mesh (Figures 1B, D), has allowed fertilized eggs metamorphose into ephyrae in the aquarium directly (Figure 4) without staff having to move the transparent ephyrae between receptacles as in Purcell et al. (2013), Lilley et al. (2014a), Ramondenc et al. (2019); Ballesteros et al. (2021) and Ballesteros et al. (2022). In disagreement with Purcell et al. (2013) and Duarte et al. (2021), we do not recommend the ephyrae grow-out of *P. noctiluca* in a flow-through system using conventional kreisels or rectangular tanks due to their risk of being retained on the mesh screen. Although our aquarium design has been used for different scyphomedusae (Table 1), specifically, we highlight the successful rearing of *P. noctiluca* from fertilized eggs to metaephyrae in flow-through, an important improvement and contribution to the rearing techniques of this scyphozoan (Table 2).

Jellyfish grow faster during the early stages of their planktonic life cycle (Widmer et al., 2005; Lilley et al., 2014a; Ramondenc et al., 2019), when the gastric system, prey capture structures and cnidome develop rapidly (Straehler-Pohl et al., 2011; Holst, 2012), increasing the possibility of eating larger amounts of food and capturing larger prey (Ballesteros et al., 2021). Like rearing vessels or aquariums, food regimens also influence the proper development of the gastric system and the growth and survival rates in jellyfish aquaculture (Widmer et al., 2005; Lilley et al., 2014a; Miranda et al., 2016; Ramondenc et al., 2019). Overall, enriched *Artemia* spp. nauplii and *Brachiounus* spp. (rotifers) are frequently used for husbandry (Raskoff et al., 2003; Purcell et al., 2013; Crow et al., 2013; Duarte et al., 2021) but some researchers have highlighted the importance of prey size for proper rearing, particularly during the polyp and early planktonic life cycle stages (Widmer et al., 2005; Purcell et al., 2013; Miranda et al., 2016; Ballesteros et al., 2022). In our system, the interchangeable mesh screen (Figures 1B, D, 2D) allows adaptation of the size of the mesh to the size of the prey in the aquarium. For example, if the prey offered is *Brachionus plicatilis* (≈ 100 – 200 µm) – an ideal size range for capture and ingestion by polyps (Raskoff et al., 2003) and ephyrae such as *Phyllorhiza punctata* (Miranda et al., 2016) and *P. noctiluca* (Ballesteros et al., 2022) – a mesh screen of 350 µm can be selected (Tables 1, 2). When *Artemia* sp. (≈ 400 µm) (Raskoff et al., 2003) is included for feeding larger individuals during the metaephyrae stage, the previous mesh screen (350 µm) can be changed for a 1 mm screen (Tables 1, 2). In this way, not only is the permanence of the feed in the tank in a flow-through

TABLE 2 Culture conditions for *Pelagia noctiluca* early life stages in flow-through system.

Life cycle stage*		T (°C)+	Salinity+	Bubble flow	Mesh size	Number of feedings times, amounts and food offered+
Fertilized eggs to planulae		13–24	36–37	2 bubbles/ second	250 µm	No food required
Ephyra, stage 1		13–24	36–37	2 bubbles/ second	350 µm	(x3) 50000 rotifers/100 ml seawater
Ephyra, stage 2		13–24	36–37	2 bubbles/ second	350 µm	(x3) 50000 rotifers/100 ml seawater
Metaephyra, stage 3		13–24	36–37	2 bubbles/ second	1 mm	(x2) 50000 rotifers/100 ml seawater, (x1) 14000 *Artemia* spp./100 ml seawater
Metaephyra, stage 4		13–24	36–37	2 bubbles/ second	1 mm	(x2) 50000 rotifers/100 ml seawater, (x1) 14000 *Artemia* spp./100 ml seawater

* Morphological and cnidome descriptions to identify each life stage can be consulted in Ballesteros et al. (2021).
+ Additional temperature and salinity ranges and food offered can be consulted in Purcell et al. (2013) and Duarte et al. (2021).
An interval of at least 4 hours between feeding times is suggested. Amounts of food for a number of 150 individuals. Jellyfish can also be feed ad libitum.

system improved, but it also allows the passage and drainage of debris, avoiding the mesh screen to become clogged. The air inlet moves the individuals inside the tank without any damage (Figures 5E, F), since the air bubbles are safe for individuals with a total body diameter less than 30 mm as reported by Raskoff et al. (2003). It is important to transfer juvenile medusae to their appropriate aquariums (Purcell et al., 2013; Duarte et al., 2021) to avoid any possible damage.

Despite the advantages of the mesh screen change, screens still collect debris quickly in any system and they should be scrubbed and cleaned regularly (Raskoff et al., 2003). When screens become clogged, organisms are more likely to stick to them, and inadequate drainage can even lead to overflow involving the total loss of jellyfish. Here, the mesh screen can be replaced *in situ* without stressing the jellyfish on a regular basis, allowing better cleaning and disinfection of the removable mesh screen and improving water quality, a critical requirement during the husbandry of early jellyfish stages (Raskoff et al., 2003; Duarte et al., 2021).

In summary, the improvements in this modified kreisel have made it possible to optimize the time invested by staff in rearing the early life-cycle stages of jellyfish with a metagenetic life cycle, such as *R. pulmo*, and species with a holoplanktonic life cycle such as *P. noctiluca*. Its configuration and accessories offer the aquarium a versatile and innovative approach in the field of jellyfish rearing techniques, allowing the use of the same aquarium for the maintenance of fertilized eggs, planulae, polyps, strobilae, ephyrae and metaephyrae in both flow-through and closed systems to safeguard the water quality.

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

AB and J-MG conceived and designed the aquarium prototype. PS manufactured the aquariums. AB wrote and edited the original manuscript. PS, EJ, and J-MG revised the manuscript and contributed to its improvement. All authors agreed to the published version of the manuscript.

Funding

The authors declare that this study received funding from ISDIN (Barcelona, Spain). The funder had the following involvement in the study: the writing of this article and the decision to submit it for publication. The funder was not involved in the study design, collection, analysis or interpretation of data.

Acknowledgments

Many thanks to the staff of the Experimental Aquarium Zone (ZAE) of the Institute of Marine Science (ICM-CSIC), especially to Elvira Martínez. The authors also thank Carlos Mengod for the schematic representation of the aquariums in

this manuscript. Authors AB, PS, and J-MG acknowledge the institutional support of the 'Severo Ochoa Centre of Excellence' accreditation (CEX2019-000928-S).

Conflict of interest

AB and J-MG are intellectual authors of the utility model (Industrial Property number: U202132533) solicited by the Spanish National Research Council (CSIC).

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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References

Ballesteros, A., Östman, C., Santín, A., Marambio, M., Narda, M., and Gili, J.-M. (2021). Cnidome and morphological features of *Pelagia noctiluca* (Cnidaria: Scyphozoa) throughout the different life cycle stages. *Front. Mar. Sci.* 8. doi: 10.3389/fmars.2021.714503

Ballesteros, A., Páez, D., Santin, A., García, A., Martín, Y., Alonso, E., et al. (2022). Successful culture of *Pelagia noctiluca* (Cnidaria: Scyphozoa) over time: a continuous supply of the holoplanktonic jellyfish for research and industrial applications. *Front. Mar. Sci.* 9. doi: 10.3389/fmars.2022.911383

Brewer, R. H. (1976). Larval settling behavior in *Cyanea capillata* (Cnidaria: Scyphozoa). *Biol. Bull.* 150, 183–199. doi: 10.2307/1540467

Camacho-Pacheco, A. V., Gómez-Salinas, L. C., Cisneros-Mata, M. Á., Rodríguez-Félix, D., Díaz-Tenorio, L. M., and Unzueta-Bustamante, M. L. (2022). Feeding behavior, shrinking, and the role of mucus in the cannonball jellyfish stomolophus sp. 2 in captivity. *Diversity* 14, 103. doi: 10.3390/d14020103

Canepa, A., Fuentes, V., Sabatés, A., Piraino, S., Boero, F., and Gili, J. M. (2014). "Pelagia noctiluca in the Mediterranean sea," in *Jellyfish blooms*. Eds. K. A. Pitt and C. H. Lucas (Dordrecht: Springer), 237–266. doi: 10.1007/978-94-007-7015-7_11

Crow, G., Howard, M., Levesque, V., Matsushige, L., Spina, S., Schaadt, M., et al. (2013). "Association of zoos and aquariums aquatic invertebrate TAG.," in *Jellyfish (Cnidaria/ ctenophora) care manual*. Ed. M. Schaadt (Silver Spring), 5–79. doi: 10.13140/RG.2.1.4344.3045

D'Ambra, I., and Merquiol, L. (2022). Jellyfish from fisheries by-catches as a sustainable source of high-value compounds with biotechnological applications. *Mar. Drugs* 20, 266. doi: 10.3390/MD20040266

Dong, J., Jiang, L. X., Tan, K. F., Liu, H. Y., Purcell, J. E., Li, P. J., et al. (2009). "Stock enhancement of the edible jellyfish (Rhopilema esculentum kishinouye) in liaodong bay, China: a review," in *Jellyfish blooms: Causes, consequences, and recent advances*. Eds. K. A. Pitt and J. E. Purcell (Dordrecht: Springer), 113–118. doi: 10.1007/978-1-4020-9749-2_8

Duarte, I. M., Marques, S. C., Leandro, S. M., and Calado, R. (2021). An overview of jellyfish aquaculture: for food, feed, pharma and fun. *Rev. Aquac.* 14, 265–287. doi: 10.1111/raq.12597

Elliott, A., Hobson, V., and Tang, K. W. (2017). Balancing fishery and conservation: a case study of the barrel jellyfish *Rhizostoma octopus* in south Wales. *ICES J. Mar. Sci.* 74, 234–241. doi: 10.1093/icesjms/fsw157

Fuentes, V., Straehler-Pohl, I., Atienza, D., Franco, I., Tilves, U., Gentile, M., et al. (2011). Life cycle of the jellyfish *Rhizostoma pulmo* (Scyphozoa: Rhizostomeae) and its distribution, seasonality and inter-annual variability along the Catalan coast and the mar menor (Spain, NW Mediterranean). *Mar. Biol.* 158, 2247–2266. doi: 10.1007/s00227-011-1730-7

Fu, Z., Shibata, M., Makabe, R., Ikeda, H., and Uye, S. I. (2014). Body size reduction under starvation, and the point of no return, in ephyrae of the moon jellyfish *Aurelia aurita*. *Mar. Ecol. Prog. Ser.* 510, 255–263. doi: 10.3354/meps10799

Gueroun, S. K., Torres, T. M., Dos Santos, A., Vasco-Rodrigues, N., Canning-Clode, J., and Andrade, C. (2021). *Catostylus tagi* (Class: Scyphozoa, order: Discomedusae, suborder: Rhizostomida, family: Catostylidae) life cycle and first insight into its ecology. *PeerJ* 9, e12056. doi: 10.7717/peerj.12056

Helm, R. R. (2018). Evolution and development of scyphozoan jellyfish. *Biol. Rev.* 93, 1228–1250. doi: 10.1111/brv.12393

Holst, S. (2012). Morphology and development of benthic and pelagic life stages of north Sea jellyfish (Scyphozoa, cnidaria) with special emphasis on the identification of ephyra stages. *Mar. Biol.* 159, 2707–2722. doi: 10.1007/s00227-012-2028-0

Kuniyoshi, H., Okumura, I., Kuroda, R., Tsujita, N., Arakawa, K., Shoji, J., et al. (2012). Indomethacin induction of metamorphosis from the asexual stage to sexual stage in the moon jellyfish, *Aurelia aurita*. *Biosci. Biotechnol. Biochem.* 76, 1397–1400. doi: 10.1271/bbb.120076

Lilley, M. K., Elineau, A., Ferraris, M., Thiéry, A., Stemmann, L., Gorsky, G., et al. (2014b). Individual shrinking to enhance population survival: quantifying the reproductive and metabolic expenditures of a starving jellyfish, *Pelagia noctiluca*. *J. Plankton Res.* 36, 1585–1597. doi: 10.1093/plankt/fbu079

Lilley, M. K. S., Ferraris, M., Elineau, A., Berline, L., Cuvilliers, P., Gilletta, L., et al. (2014a). Culture and growth of the jellyfish *Pelagia noctiluca* in the laboratory. *Mar. Ecol. Prog. Ser.* 510, 265–273. doi: 10.3354/meps10854

Marambio, M., Canepa, A., Lòpez, L., Gauci, A. A., Gueroun, S. K. M., Zampardi, S., et al. (2021). Unfolding jellyfish bloom dynamics along the Mediterranean basin by transnational citizen science initiatives. *Diversity* 13, 274. doi: 10.3390/d13060274

Merquiol, L., Romano, G., Ianora, A., and D'Ambra, I. (2019). Biotechnological applications of scyphomedusae. *Mar. Drugs* 17, 604. doi: 10.3390/md17110604

Miranda, F. S., Chambel, J., Almeida, C., Pires, D., Duarte, I., Esteves, L., et al. (2016). Effect of different diets on growth and survival of the white-spotted jellyfish, *Phyllorhiza punctata*. *Front. Mar. Sci.* doi: 10.3389/conf.FMARS.2016.04.00042

Olguín-Jacobson, C., Pitt, K. A., Carroll, A. R., and Melvin, S. D. (2020). Polyps of the jellyfish *Aurelia aurita* are unaffected by chronic exposure to a combination of pesticides. *Environ. Toxicol. Chem.* 39, 1685–1692. doi: 10.1002/etc.4750

Omori, M., and Nakano, E. (2001). Jellyfish fisheries in southeast Asia. *Hydrobiologia* 451, 19–26. doi: 10.1023/A:1011879821323

Pierce, J. (2005). A system for mass culture of upside-down jellyfish *Cassiopea* spp as a potential food item for medusivores in captivity. *Int. Zoo Yearb* 39, 62–69. doi: 10.1111/j.1748-1090.2005.tb00005.x

Purcell, J. E., Baxter, E. J., and Fuentes, V. L. (2013). "Jellyfish as products and problems of aquaculture," in *Advances in aquaculture hatchery technology*. Eds. G. Allan and G. Burnell (Cambridge, United Kingdom: Elsevier), 404–430. doi: 10.1533/9780857097460.2.404

Ramondenc, S., Ferrieux, M., Collet, S., Benedetti, F., Guidi, L., and Lombard, F. (2019). From egg to maturity: A closed system for complete life cycle studies of the holopelagic jellyfish *Pelagia noctiluca*. *J. Plankton Res.* 41, 207–217. doi: 10.1093/plankt/fbz013

Raskoff, K. A., Sommer, F. A., Hamner, W. M., and Cross, K. M. (2003). Collection and culture techniques for gelatinous zooplankton. *Biol. Bull.* 204, 68–80. doi: 10.2307/1543497

Sandrini, L. R., and Avian, M. (1983). Biological cycle of *Pelagia noctiluca*: morphological aspects of the development from planula to ephyra. *Mar. Biol.* 74, 169–174. doi: 10.1007/BF00413920

Schaadt, M., Widmer, C. L., and Sowinski, N. (2017). "Jellyfish," in *Marine ornamental species aquaculture*. Eds. R. Calado, I. Olivotto, M. Oliver and J. Holt (New Jersey: John Wiley & Sons Ltd), 457–473. doi: 10.1002/9781119169147

Straehler-Pohl, I., and Jarms, G. (2010). Identification key for young ephyrae: a first step for early detection of jellyfish blooms. *Hydrobiologia* 645, 3–21. doi: 10.1007/978-90-481-9541-1_2

Straehler-Pohl, I., Widmer, C. L., and Morandini, A. C. (2011). Characterizations of juvenile stages of some semaeostome scyphozoa (Cnidaria), with recognition of a new family (Phacellophoridae). *Zootaxa* 2741, 1–37. doi: 10.11646/zootaxa.2741.1.1

Treible, L. M., and Condon, R. H. (2019). Temperature-driven asexual reproduction and strobilation in three scyphozoan jellyfish polyps. *J. Exp. Mar. Biol. Ecol.* 520, 151204. doi: 10.1016/J.JEMBE.2019.151204

Widmer, C. L., Voorhees, J. P., Badger, M. A., Lambert, J. W., and Block, N. M. (2005). The effects of rearing vessels and laboratory diets on growth of northeast pacific jellyfish ephyrae (Cnidaria: Scyphozoa). *Drum Croaker* 36, 29–36.

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