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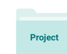
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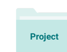
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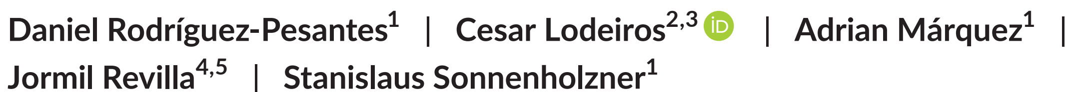

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Abstract

Monoalgal and bialgal diet were tried in 2 stages of larval development of *Striostrea prismatica*: phase I from mixotrofic to early umbonated veliger larva and phase II until eyespot larvae. The monoalgal diets in phase I were *Tisochrysis lutea*, *Pavlova lutheri* and *Nannochloropsis* sp. In phase II, *Tetraselmis suecica*, *T. chuii*, *Chaetoceros gracilis* and *Ch. muelleri* were incorporated. The bialgal diets (combination of *P. lutheri* + *Ch. gracilis* in proportions 1:3, 1:1 and 3:1) were chosen according to the results of the monoalgal trials and were evaluated against the classic diet = control (*T. lutea* + *Ch. gracilis*). The microalgae size and their fatty acid composition were determined. We recommend the combination *T. lutea* + *Ch. gracilis*, initially using *T. lutea* for mixotrophic larvae (6 days Post Fertilization-PF), then incorporating *Ch. gracilis* in a ratio of 1:1 until completing phase I (8–10 PF). In phase II, the ratio should change to 1:3 until larvae with eyespot are obtained (18–20 PF). Suggestions were based on the higher demand for DHA in initial larvae and the need for EPA from the early umbonated phase. Finally, as a third experiment, microsubstrates (pieces of shells, sandpaper, polycarbonate and ground rubber) were evaluated to promote the settlement of pediveliger larvae. A settlement of 20% was obtained in pieces of shells of the same species. We consider that the settlement technology has not been optimized; therefore, evaluations directed at the behaviour of the species are recommended.

KEYWORDS

bivalve molluscs, fatty acids, larval growth, substrates metamorphosis, tropical Pacific Ocean

1 | INTRODUCTION

The rock oyster, *Striostrea prismatica* (Gray, 1825), is distributed in the American Tropical and subtropical Pacific Ocean, from Baja California, Mexico, to Mancora, Peru, associated with rocky inter and subtidal substrates (Coan & Valentich-Scott, 2012). Although it represents an important natural resource for coastal communities, the species is currently overexploited due to the uncontrolled extraction (Argüello-Guevara, Loor, & Sonnenholzner, 2013; Hernández-Covarrubias, Patiño-Valencia, & Aguirre-Villaseñor, 2013; Ríos-González, López-Uriarte, Chong-Carrillo, Vega-Villasante, & Chávez-Villalba, 2018).

Given the need to restore their populations and to promote aquaculture production, Loor and Sonnenholzner (2016) studied this oyster's reproduction in wild populations, establishing that temperature and salinity are the principal factors in the reproductive modulation. Argüello-Guevara et al. (2013) provide a strategy to condition broodstock in the laboratory, achieving maturation and spawning in captivity. These techniques allowed characterizing the

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embryonic and larval development as well as the production of 5 mm spat, establishing initial cultivation methods by Lodeiros, Márquez, Revilla, Rodríguez-Pesantes, and Sonnenholzner (2017). However, more research is needed to optimize the massive spat production of *S. prismatica*, particularly to find appropriate microalgal diets and substrates for larval settlement, which are two of the main causes hindering the commercial activity of bivalve molluscs (Loosanoff & Davis, 1963; Helm & Bourne, 2004).

On a global scale, microalgae are already important commercial sources of high-value chemicals, whose uses vary from obtaining photosynthetic pigments (β-carotene, astaxanthin and phycocyanin), finding sources of renewable energy fuels, and are even being used as nutritional supplements in humans (Malcata, Pinto, & Guedes, 2018). Also, they are an essential part of aquaculture, being one of the most important food sources in the early stages of fish, molluscs, crustaceans and echinoderms, given their nutritional value and ability to synthesize and accumulate large amounts of essentials fatty acids (Patil et al., 2005). Malcata et al. (2018) consider their high levels of fatty acids as potential future sources of these nutrients with applications in human health, while Ohse et al. (2014) propose their use as vitamins, minerals, salts, pigments and lipids supplies.

There are several microalgae identified as suitable for feeding bivalve molluscs under cultivation (Brown & Blackburn, 2013; Coutteau & Sorgeloos, 1992), where the mix between a flagellate and a diatom, for example, is the most commonly used diet (Blanchard, Pechenik, Giudicelli, Connan, & Robert, 2008; Campa-Córdova, Luna-Gonzalez, Ascencio, Cortés-Jacinto, & Cáceres-Martínez, 2006; Helm & Laing, 1987), which provides a better 'balance' of nutrients (Lora-Vilchis & Doktor, 2001). However, for optimal intake and digestion, characteristics of the algae such as size, morphology and nutritional profile should be considered, since they are influential factors which require to be previously evaluated to formulate efficient diets (Aranda-Burgos, da Costa, Nóvoa, Ojea, & Martínez-Patiño, 2014) and to guarantee that these factors do not hinder the ingestion of the larvae both in early and late developmental stages (Marshall, McKinley, & Pearce, 2010). Previous studies have revealed that some bivalves can distinguish their food from various types of particles, preferably ingesting those of high quality, while rejecting undesirable ones (Ward & Shumway, 2004). In practice, this suggests supplementing the diets with mixtures of 2 or more microalgae to satisfy the nutritional needs of the larvae (Miao, Wu, & Yang, 2004).

In this sense, the search for microalgal diets with high nutritional value for larval development of bivalve molluscs is one of the major steps to establish a protocol for spat production (Rico-Villa, Le Coz, Mingant, & Robert, 2006; Uriarte & Farías, 1999), particularly due to the lipid-rich diets are beneficial for larval growth. The importance of polyunsaturated fatty acids like 20:5(n − 3) and 22:6(n − 3) on the larval cultivation of molluscs has been shown in some bivalves, that is *Crassostrea gigas* (Langdon & Waldock, 1981), *Ostrea edulis* (Labarta, Fernández-Reiriz, & Pérez-Camacho, 1999), *Mytilus galloprovincialis* (Sánchez-Lazo & Martínez-Pita, 2014), *Perna perna* (Aarab et al., 2013) and *Nodipecten nodosus* (Sühnel, Lagreze, Zanette, Magalhães, & Ferreira, 2012). Another important fatty acid on molluscs larval development is the 20:4(n − 6) which has also been associated with higher growth rates, survival and less stress (Pernet, Bricelj, & Parrish, 2005).

This study evaluated the effect of monoalgal and bialgal diets on the survival and larval development, as well as different substrates for the settlement of competent larvae, in the search of optimal procedures for mass production of *S. prismatica* spat for aquaculture or ecological restoration purposes.

2 | MATERIALS AND METHODS

2.1 | Microalgae

The microalgae used for evaluation of diets were *Tetraselmis chuii* (Butcher, 1959)-Tch, *Tetraselmis suecica* (Kylin) (Butcher, 1959)-Ts, *Tisochrysis lutea* (Bendif & Probert, 2013)-Tl, *Pavlova lutheri* (Droop) (J.C. Green, 1975)-Pl, *Chaetoceros gracilis* (Parocek, 1892)-Cg, *Chaetoceros muelleri* (Lemmermann, 1898)-Cm and *Nannochloropsis* sp.-N, from the microalgae bank of Centro Nacional de Acuicultura e Investigaciones Marinas of Escuela Superior Politécnica del Litoral (CENAIM-ESPOL). The cultures were made using filtered seawater (0.45 μm) and irradiated with UV light (FSW), under conditions of permanent fluorescent light (47–67 μmol/photons m^2 s) and constant aeration. We use the medium f/2 (Guillard, 1975), enriched with sodium metasilicate 1% only for diatoms cultivation. Temperature and salinity in the cultures were 20 ± 0.5°C and 34 ± 0.1 PSU (±*SD*).

To determine the diet ration and prevent differences in the amount of food provided, the microalgae dry mass was estimated. For this, a sample of 200 ml of each monoalgal culture in exponential phase of growth was filtered through Whatman GF/F glass fibre filters using a Millipore vacuum equipment. Retained algae were washed with 4 ml of ammonium formate (0.5 M) to remove salts and then oven-dried at 60°C until constant weight. The weight was determined in an analytical balance (0.00001 g accuracy). A subsample of each culture was preserved with formalin (3.7%) for measuring cell characteristics with a compound microscope at 100 X, using a digital camera and Nahwoo iWorks 2.0 software. The measured characteristics were dry mass, cell size, appendages-flagella and setae (Table 1).

2.2 | Production of veliger larvae stock

Broodstock of *Striostrea prismatica* was collected from natural banks in the Bay of Ayangue, Santa Elena, Ecuador (1°59.0'S; 80°45.58'W), and transported in a container with FSW to the Mollusks Laboratory of CENAIM-ESPOL. Then, the oysters were conditioned to spawning and larval development following Argüello-Guevara et al. (2013) and Lodeiros et al. (2017). All

TABLE 1 Characteristics of the microalgae species used in the experiments, with their identification code, cell size, size of appendages-flagella and dry mass

Class	Species	Id. Code	Mean cell size[a] (μm cell)	Mean appendages and flagella size[a] (μm)	Cell dry mass (pg)
Chlorophyceae	*Tetraselmis chuii*	Tch	8.8 ± 1.43	10.8 ± 1.01	178
	Tetraselmis suecica	Ts	8.9 ± 1.30	12.1 ± 0.93	166
Prymnesiophyceae	*Tisochrysis lutea*	Tl	5.0 ± 0.55	ND	40
	Pavlova lutheri	Pl	5.0 ± 0.65	ND	53
Bacillariophyceae	*Chaetoceros gracilis*	Cg	6.6 ± 0.76	15.1 ± 1.58	62
	Chaetoceros muelleri	Cm	7.0 ± 1.22	29.6 ± 6.11	82
Eustigmatophyceae	*Nannochloropsis* sp.	N	2.8 ± 0.25	ND	13

Abbreviation: ND, Not determined.
[a]Mean ± *SD*, *n* = 30.

developing embryos were transferred to two 1000-L incubation tanks, with observations made regularly to ensure that embryonic progressed normally. Initially, mixotrofic D larvae produced 24 h post fertilization (PF) were collected through a 30-μm sieve, and transferred to 5 hatchery tanks of 1,000 L at a density of 6 larvae/mL, where they were cultivated in order to have a stock of larvae required for monoalgal diet assessments and settlement substrate evaluation. A second spawning and larval development was carried out under the same conditions for assessment of bialgal diets (Table 2). D larvae were fed with Tl during the first two days PF and afterwards were fed with classic mix named as control in this experiment, a combination Tl + Cg, as shown in Lodeiros et al. (2017). We started our evaluations on the third day PF looking for a selective classification of larvae, ensuring that only those of more than 70 μm (shell length) were kept for culture purposes.

2.3 | Experiments to evaluate mono and bialgal diets

Experiments were performed in two phases, according to the general description of the larval development in Lodeiros

TABLE 2 Culture days, growth rate per day (μm/day^{-1} ± SE) and % oculated larvae (±SE) of *Striostrea prismatica* in larval development phase I (veliger to early umbonated larval stage) to and II (umbonated to eyespot larval stage), exposed to different diets treatments

Experiment	Phase experimental	Diets	Culture Days	Growth rate (μm/day^{-1})	% larvae oculated
Monoalgal	I	Control (Cg + Tl)	6	4.64 ± 0.64[ab]	–
	I	*Pavlova lutheri*	6	4.29 ± 0.41[b]	–
	I	*Tisochrysis lutea*	6	5.63 ± 0.57[a]	–
	I	*Nannochloropsis* sp.	6	0.95 ± 0.07[c]	–
	II	Control (Cg + Tl)	10	12.17 ± 3.30[a]	44.82 ± 3.77[a]
	II	*Pavlova lutheri*	10	11.50 ± 2.46[a]	15.78 ± 2.20[b]
	II	*Tisochrysis lutea*	10	11.16 ± 0.49[a]	7.69 ± 0.68[bc]
	II	*Chaetoceros gracilis*	10	10.44 ± 3.52[a]	10.00 ± 4.36[b]
	II	*Tetraselmis chuii*	4	1.41 ± 3.85[a]	0
	II	*Nannochloropsis* sp.	4	0	0
	II	*Tetraselmis suecica*	10	3.46 ± 0.21[b]	0
	II	*Chaetoceros muelleri*	10	3.67 ± 0.36[b]	0
Bialgal	I	Control (Cg + Tl)	8	3.27 ± 0.6	–
	I	Tl + Pl 1:1	8	2.94 ± 0.18	–
	I	Tl + Pl 1:3	8	2.87 ± 0.10	–
	I	Tl + Pl 3:1	8	2.39 ± 0.26	–
	II	Control (Cg + Tl)	11	9.29 ± 1.83	44.69 ± 1.11[a]
	II	Pl + Cg	11	8.41 ± 1.64	40.97 ± 1.59[b]

Note: Different letters indicate statistical differences.
Abbreviations: Tl, *Tisochrysis lutea*, Pl, *Pavlova lutheri*, Cg, *Chaetoceros gracilis*.

et al. (2017). Phase I involved veliger larvae (3 days PF; 75–77 μm dorsoventral-DV length) to early umbonated larval stage (8–10 days PF); and phase II involved from umbonated stage (11 days PF; 120–150 μm DV length, ±*SD*) to eyespot larvae (18–20 days PF). In the monoalgal diet experiment (experiment 1) during phase I, the veliger larvae came from cultures in 1000-L tanks previously fed with TI as noted above and were first evaluated with TI, PI, N and the control diet (TI + Cg). For phase II, larvae came from same culture tanks but were fed with the control diet. In this phase, the same microalgae were evaluated, incorporating the larger species: Tch, Ts, Cg and Cm. All experiments (including bialgal) were performed in conical 50-L containers filled with FSW at an initial density of 2 larvae/mL using 3 replications per treatment and kept with moderate aeration. Also, water from all experimental units was replaced daily. Temperature and salinity during monoalgal experiments were 26 ± 1.5°C and 34 ± 0.1 PSU, and during bialgal experiments 24.5 ± 0.5°C and 34 ± 0.1 PSU (±*SD*). All feeding procedures for larvae were established based on previous calculation of microalgae dry mass, with values ranging from 1.0 to 2.2 mg/L.

The microalgae that showed better performance after the evaluation of monoalgal diets were used to test bialgal diets (experiment 2). In view of this, treatments were established under the same conditions, using the combination TI + PI in proportions 1:1, 1:3 and 3:1 (expressed in mg/L), and the control diet used during phase I. Since only the PI treatment showed adequate larval growth (ignoring Cg that belongs to the control diet) and survival during phase I, this microalga was used for phase II in combination with Cg (PI + Cg, 1:1) versus the control diet (TI + Cg, 1:1).

To evaluate the effect of diets, interdaily samples were taken from each experimental unit to register dorsoventral length according to Bayne (2017), and survival. For this, larvae from 50-L tanks were concentrated with a 30-μm sieve and released in a 1-L glass beaker filled with FSW, homogenized and 3 aliquots of 1 ml were taken with a micropipette. The number of larvae from each experimental unit was calculated volumetrically. Samples obtained were analysed under a microscope to estimate survival, dorsoventral length and the presence of larvae with eyespot. Images were captured with a LANOPTIK camera model MDX503 connected to an Olympus tri-ocular microscope model CX31RTSF and processed with Nahwoo iWorks 2.0 software. In order to confirm the ingestion of diets, periodic direct microscopic observations (100 X) were made on the digestive gland.

2.4 | Fatty acids

To evaluate the quality of the diets on larval development, the fatty acid profiles of the seven microalgae species were determined. Microalgae were collected in their exponential growth phase prior to their use as feed. For this, 1.5 L of each microalgae culture was retained in Whatman GF/F filters using a Millipore vacuum pump, washed with ammonium formiate, and then oven-dried at 60°C for 48 h until constant weight. The final dry mass was quantified using an analytical balance (0.00001 g accuracy). The fatty acids profiles were analysed using gas chromatography with detection of ionization flame according to Folch, Lees, and Stanley (1957) and Ackman (1969). The identification and quantification of fatty acids and methyl esters were carried out by comparison with vegetable origin standards.

2.5 | Experiment to evaluate the substrates for settlement

On day 22nd PF, when the cultivated larvae (raised in 1,000-L tanks and fed with the control mixture TI + Cg diet) were competent for metamorphosis presenting eyespot, foot and a dorsoventral length of 298.5 ± 14.92 μm, according to Lodeiros et al. (2017), they were transferred to the settling systems (2-L plastic containers, with a bottom of 150-μm mesh, and operational volume of 1.5 L) at a density of 1 larvae/mL by triplicate for evaluation of 5 substrates. Each substrate was previously autoclaved and distributed in the containers occupying 25% of the bottom area. The tested substrates were shells of *S. prismatica* crushed into 10 mm^2 pieces, shells of *S. prismatica* ground to 0.5 mm^2, pieces of sandpaper (pieces of fabric with particles attached that gave a rough surface) with 70 mm^2 in area and pieces of polycarbonate plastic with 70 and 3 mm^2 ground rubber. Complete daily refills with FSW were performed in this experiment, including a minimum dose of daily food of 15,000 cells/mL TI and Cg in proportion 1:3 (0.6 mg/L of TI and 1 mg/L of Cg). The experimental units were kept with moderate aeration for 10 days, to verify the preference of the larvae on the evaluated substrates, settlement percentage (relationship between the number of spat obtained and the number of larvae initially planted) and size of the settled postlarvae were analysed through observation of the substrates under a stereoscopic microscope Olympus model SZ2-ILST, determining the dorsoventral and anterior–posterior lengths as described previously. The daily growth rate (μm/day) was determined following the recommendations outlined in Abasolo-Pacheco, Mazón-Suástegui, and Saucedo (2009).

2.6 | Statistical analyses

The effects of treatments on the maximum length reached, survival and the proportion of larvae reaching early umbonated stage in the phase I and with eyespot stage in the phase II at the end of the diet experiments (mono and bialgal), as well as the effect of substrates on the percentage of settlement and the dorsoventral length of larvae, were analysed using one-way ANOVA. Previously, normality of data and homogeneity of variances were verified with the Shapiro–Wilk and Bartlett tests respectively. To detect differences among treatments by means of multiple comparisons, the Tukey–Cramer a posteriori test was used (Zar, 2010). For all tests, a $p < .05$ was used for significance.

2.7 | Ethics statement

All the procedures followed the guidelines for ethical and responsible research using in vivo animals for experiments (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010).

3 | RESULTS

3.1 | Diets

3.1.1 | Monoalgal diets Phase I

The larval growth with Tl diet showed significant highest sizes (109 ± 1.9 μm), followed by control and Pl diets with no significant difference between them (103 ± 2.1 and 101 ± 2.2 μm respectively; Figure 1a). The daily growth rate was greater for Tl (5.6 μm/day), followed by control and Pl (4.6 and 4.3 μm/day respectively) and minimum for N (1 μm/day) (Table 2). Growth of larvae fed with N was practically null during the experiment (81 ± 1.9 μm).

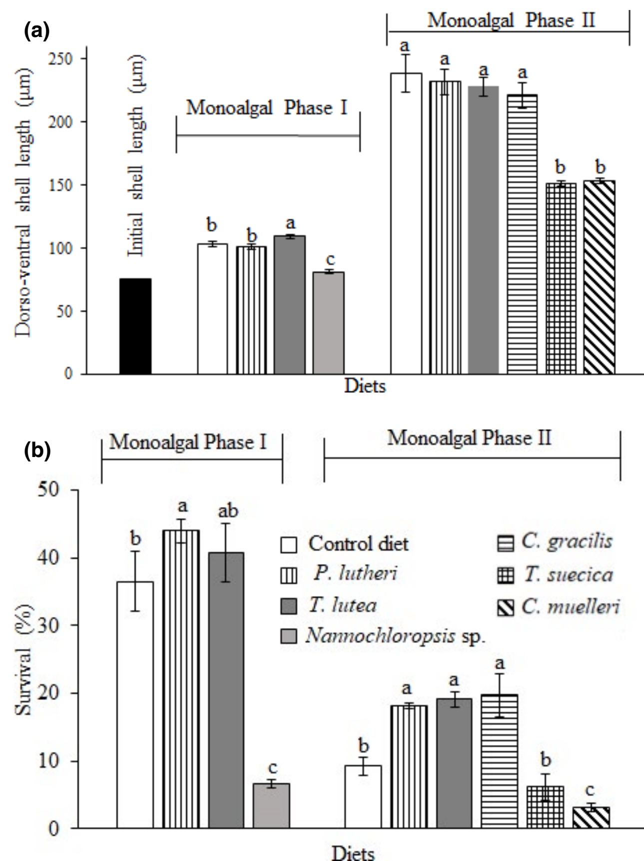

FIGURE 1 Dorsoventral shell growth (a) and survival (b) of *Stiostrea prismatica* larvae at the end of phases I (8th day PF) and II (21th day PF), fed with monoalgal diets. Bars indicate standard errors. Tl + Cg = control diet (*Tisochrysis lutea* + *Chaetoceros gracilis*). In phase II, the microalgae *Tetraselmis chuii* and *Nannochloropsis* sp. did not survive until the end, so they are not shown. The letters on the bars indicate statistical differences between the treatments

Survival of the larvae showed a decrease in all treatments (Figure 1b). At the end of phase I, treatments Pl, Tl and control (Tl + Cg) had a significant higher survival (44.0 ± 1.8; 40.8 ± 4.3 and 36.6 ± 4.4% respectively) than N (6.7 ± 0.6%).

3.1.2 | Monoalgal diets Phase II

Non-significant differences among daily growth rates were observed in treatment control, Pl, Tl and Cg (224–274 μm equivalent to 10–13 μm/day), which were significantly higher than Ts and Cm (~155 μm; equivalent to ~4 μm/day) (Figure 1a and Table 2). The experiment was ended on day 20th PF after observing that 44.8% of larvae had eyespot from control diet and was significantly higher than the treatments with diets Pl, Cg and Tl (15.8, 10.0 and 7.7% respectively). No larvae with eyespot were observed in the treatments fed with Ts and Cm (Table 2).

Survival decreased abruptly to values < 20% at the 5th day of phase II (16th PF of larval development), reaching total mortality in the treatment with N microalga on the 3rd day of this phase (14th PF of larval development; Figure 1b). The survival was 18%–19% with Cg, Tl and Pl, which were significantly higher than survival rates achieved by treatments control, Ts and Cm (<10%).

3.2 | Bialgal diets

3.2.1 | Bialgal diets Phase I

The lengths reached by the larvae at the end of this phase (~125 μm) showed no statistical differences among the evaluated treatments (Figure 2a). Also, no statistical differences were observed in the daily growth rates (2.5–3.3 μm/day).

At the end of the phase I (day 8th), the survival in the control diet (Tl + Cg) reached 50.1 ± 1.81%, which was significantly higher than the other diets (14%–25%; Figure 2b).

3.2.2 | Bialgal diets Phase II

The bialgal combinations used (control diet and Pl + Cg) showed the same behaviour for both growth and survival (Figure 2a,b), with no significant differences in dorsoventral length (257 ± 8.1 and 247 ± 6.9 μm respectively), daily growth rate (9.3 ± 1.8 and 8.4 ± 1.6 μm/day) and survival (12 ± 6.5 and 13 ± 4.5% respectively). However, the control diet showed a significant higher percentage of larvae with eyespot than the Pl + Cg diet (44 ± 1.1 and 41 ± 1.6% respectively) (Table 2).

3.3 | Microalgal fatty acids profiles

The highest levels of the fatty acids found were 14:0, 14:1, 16:0, 16:1(n − 9), 18:0, 18:1(n − 9), 18:2(n − 6), 18:3(n − 3), 20:5(n − 3) and

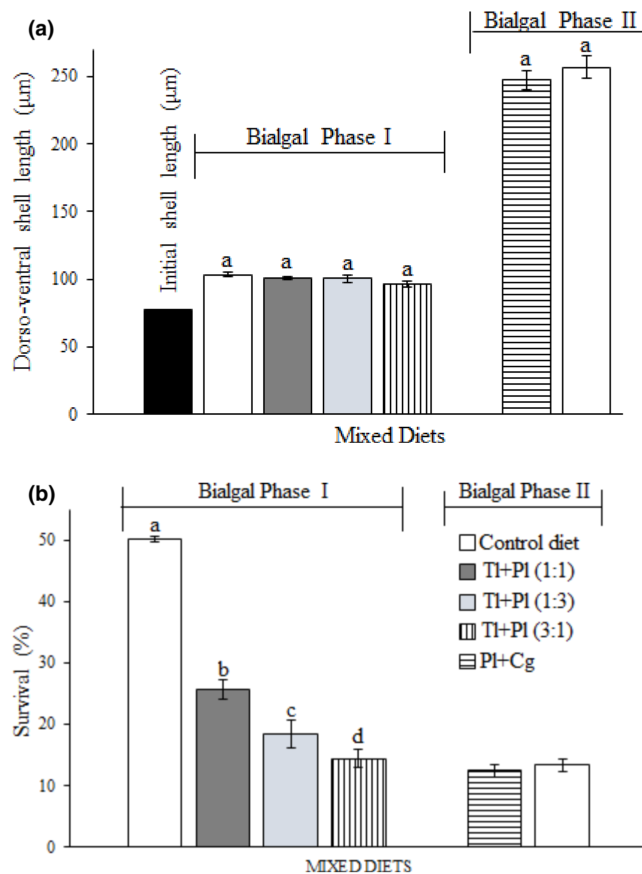

FIGURE 2 Dorsoventral shell growth (a) and survival (b) of *Striostrea prismatica* larvae at the end of phases I (8th day PF) and II (21th day PF), fed with bialgal diets. Bars indicate standard errors. Tl + Cg = control diet (*Tisochrysis lutea* + *Chaetoceros gracilis*), Tl + Pl 1:1; 1:3; 3:1 = *Tisochrysis lutea* + *Pavlova lutheri* in different proportions, Pl + Cg =*Pavlova lutheri* + *Chaetoceros gracilis* in equal proportions to those suggested for control by Lodeiros et al. (2017). The letters on the bars indicate statistical differences between the treatments

22:6(n − 3) (Table 3). Chlorophyceae microalgae (*T. chuii* and *T. suecica*) lack or had very low concentrations of 14:0, 14:1, 18:0, 20:5(n − 3) and 22:6(n − 3), but instead were very rich in other fatty acids such as 16:0, 16:1(n − 9), 18:1(n − 9), 18:2(n − 6), 18:3(n − 3), 16:2(n − 4) and 20:0. In contrast, the smaller microalgae, *Nannochloropsis* sp., showed high values of 12:0, 14:0, 16:0, 16:1(n − 9), 18:1(n − 9) and 20:5(n − 3) but no presence of 22:6(n − 3).

The highest content of saturated fatty acids was observed in diatoms (Cg = 46.3 and Cm = 58.2%), while the monounsaturated fatty acids were found in high percentages in all the tested microalgae. Likewise, polyunsaturated fatty acids were found in important percentages in all microalgae except *Ch. muelleri*. No arachidonic fatty acid (AA) was detected in the tested microalgae, except Cm, although in very low proportion (1.1%), while docosahexaenoic (DHA) was found in Pl and Tl, in concentrations of 5.0 and 6.3% respectively; eicosapentaenoic (EPA) and myristic fatty acid (14:0) were found in important quantities in the tested microalgae except Ts.

3.4 | Substrates evaluation for settling

The dorsoventral length of postlarvae and percentage of metamorphosis are summarized in Table 4. The substrate made of *S. prismatica* shell pieces with the greater surface area (10 mm^2) showed the highest larval settlement (19 ± 0.19%), significantly higher than the rest of the substrates (1.0 ± 0.09; 4.0 ± 0.23 and 4.0 ± 0.13% for sand paper, shell pieces of 0.5 mm^2 and ground rubber respectively). As for the length of the postlarvae, those that settled on sandpaper had significantly higher size (2.4 ± 0.18 mm), followed by the postlarvae on shell pieces of greater surface area and ground rubber, whose sizes did not differ significantly (1.6 ± 0.16 and 1.3 ± 0.07 mm respectively). The smallest postlarvae were found on shell pieces of 0.5 mm^2 surface (0.9 ± 0.08 mm). No settlement was observed on polycarbonate pieces.

4 | DISCUSSION

The larval development of *Striostrea prismatica* was influenced by the type of diet, both mono and bialgal, which we also associated with the nutritional composition and morphology of the microalgae. The microalgae used in the experiments have been widely tested for cultivation of bivalve molluscs (Brown & Blackburn, 2013; Helm & Bourne, 2004) and their selection was carried out considering the microalgae size, to ensure that this factor would not hinder ingestion by larvae (Marshall et al., 2010). Among the tested microalgae, *Nannochloropsis* sp. (with the highest concentration of EPA in this experiment) showed a low or undetectable larval performance. Similar results were observed in the larval feeding of *Pteria sterna* and *Argopecten ventricosus*, since although they ingested the microalgal cells did not show evidence of digestion, raising the hypothesis that the rigid-fibrose glycogen cell wall of this microalga is a limiting factor for larval digestion (Lora-Vilchis & Maeda-Martinez, 1997; Martínez-Fernández, Acosta-Salmón, & Rangel-Dávalos, 2004).

It is possible that *S. prismatica* larvae do not possess efficient specific digestive enzymes for the digestion of *Nannochloropsis* sp., justifying dismissing it as feed for larvae of this bivalve species. Furthermore, its nutritional quality general seems to be limited considering the lack of essential fatty acids like DHA, AA and other important fatty acids such as 16:2 (n − 4), 20:0, 18.3 (n − 3), 18:4 (n − 3) and 22:4 (n − 6) as observed in this study and also reported by Volkman, Brown, Dunstan, and Jeffrey (1993). Despite this, there are reports of better performance when used in combination with *Tetraselmis* spp. in the cultivation of other marine organisms such as rotifers (Hemaiswarya, Raja, Kumar, Ganesan, & Anbazhagan, 2011).

Among the microalgae used as monoalgal diets in phase I, the best growth was recorded with *T. lutea* (rich in DHA), also allowing a 41% larvae survival. This was statistically similar to the maximum achieved survival (44% for *P. lutheri* rich in DHA too) and was even statistically higher than the survival observed with the bialgal diet

TABLE 3 Fatty acid composition of the microalgae used in the experiments

	Pavlova lutheri	*Tisochrysis lutea*	*Nannochloropsis* sp.	*Chaetoceros gracilis*	*Tetraselmis suecica*	*Tetraselmis chuii*	*Chaetoceros muelleri*	
12:0	N.D.	N.D.	7.77	N.D.	N.D.	N.D.	N.D.	12:0
14:0	15.12	11.78	7.38	20.26	N.D.	3.30	10.36	14:0
14:1	0.64	1.06	N.D.	7.38	N.D.	2.52	N.D.	14:1
16:0	23.35	17.59	27.70	21.73	21.08	22.67	43.29	16:0
16:1(*n*−9)	3.71	4.02	27.32	22.36	7.24	8.19	31.19	16:1(*n*−9)
16:2(*n*−4)	0.35	N.D.	N.D.	6.54	11.54	11.29	2.21	16:2(*n*−4)
18:0	4.73	5.92	1.54	4.35	2.96	1.84	1.68	18:0
18:1(*n*−9)	19.46	23.28	6.26	4.86	6.55	4.73	1.04	18:1(*n*−9)
18:2(*n*−6)	8.887	10.58	2.66	6.16	8.69	5.13	1.57	18:2(*n*−6)
20:0	1.69	4.33	N.D.	N.D.	3.68	3.12	N.D.	20:0
18:3(*n*−3)	9.06	11.36	N.D.	8.44	33.03	32.04	0.82	18:3(*n*−3)
18:4(*n*−3)	4.41	1.07	N.D.	N.D.	N.D.	N.D.	N.D.	18:4(*n*−3)
20:4(*n*−6) AA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.10	20:4(*n*−6) AA
20:5(*n*−3) EPA	0.84	1.01	12.40	5.30	N.D.	1.69	3.89	20:5(*n*−3) EPA
22:4(*n*−6)	1.21	0.95	N.D.	N.D.	N.D.	N.D.	N.D.	22:4(*n*−6)
22:6(*n*−3) DHA	5.01	6.29	N.D.	N.D.	N.D.	N.D.	N.D.	22:6(*n*−3) DHA
Σ *n*−3	19.63	18.66	12.40	13.74	33.03	33.73	4.71	Σ *n*−3
Σ *n*−6	10.07	11.54	5.63	6.16	8.69	5.13	2.67	Σ *n*−6
Σ *n*−9	24.14	29.54	33.58	27.22	13.79	15.45	32.23	Σ *n*−9
n−3/*n*−6	1.95	1.62	2.20	2.23	3.80	6.58	1.76	*n*−3/*n*−6
Σ MUFA	24.14	29.54	33.58	27.22	13.79	15.45	32.23	Σ MUFA
Σ SAFA	45.81	40.26	45.81	46.34	32.96	34.40	58.18	Σ SAFA
Σ PUFA	30.06	30.20	18.03	26.44	53.26	50.15	9.59	Σ PUFA
Σ INSAT	54.19	59.74	51.60	53.66	67.04	65.60	41.82	Σ INSAT
Σ HUFA	7.36	8.25	12.40	5.30	N.D.	1.69	3.89	Σ HUFA

Note: Microalgae for the analyses were taken in the exponential phase of their production. The values are expressed as a percentage of methyl esters of fatty acids in relation to total lipids.
Abbreviation: N.D., Not detected.

TABLE 4 Settlement rate (% ± SE) and shell size (mm ± SE) of pediveliger larvae of *Striostrea prismatica* exposed to different types of substrate

Treatments	Settlement (%)	Dorsoventral length (μm)
S. prismatica shells 10 mm^2	19 ± 0.19[a]	1.6 ± 0.16[b]
S. prismatica shells 0.5 mm^2	4 ± 0.23[b]	0.9 ± 0.08[c]
Sand paper 70 mm^2	1 ± 0.09[c]	2.4 ± 0.18[a]
Polycarbonate 70 mm^2	0	NS
Ground rubber 3 mm^2	4 ± 0.13[b]	1.3 ± 0.07[b]

Note: Different letters indicate significant differences among treatments ($p < .05$).
Abbreviation: NS, no settling.

used as a control that includes 75% of this microalgae in combination with *Ch. gracilis* (rich in EPA). However, it had low EPA amounts (but higher compared with *P. lutheri*). Our finding demonstrates a greater importance of DHA with respect to EPA in the first 6–8 days of larval culture and coincides with what was reported by Farías and Uriarte (2002).

In addition, although sizes of *T. lutea* and *Ch. gracilis* are similar, the setae of the diatom have an average length of 15 ± 1.6 μm and can disrupt filtration, particularly in the initial phase of mixotrofic stage, where the larvae are of small size and may not have the velum fully developed. This has also been referred to *Venerupis corrugata* when it was fed with *Ch. neogracile* (Fernández-Pardo et al., 2016). This decreases the quality of the control mixture in the phase I, since its contribution of EPA from *Ch. gracilis* was decreased justifying the lower survival percentage obtained.

The microalgae *T. lutea* together with *P. lutheri* and *Ch. gracilis* were the monoalgal diets with the best performance (daily rate growth and survival) in phase II. However, the percentage of larvae with eyespot, which indicates the beginning of the competent stage for metamorphosis, was significantly lower (7%–15%) than the one observed with the control diet (45%), which suggests that

the mixture of microalgae generates further development (Lora-Vilchis & Doktor, 2001). It indicates that the contribution of the essential fatty acids is directly correlated with the best performance to the settlement, same as reported by Pettersen, Turchini, Jahangard, Ingram, and Sherman (2010) in larvae of *M. galloprovincialis*. In this phase, the larvae fed diatom *Ch. gracilis* had better performance than *Ch. muelleri*. It is possible that ingestion of *Ch. gracilis* by larvae that reached a size of 120–130 μm in phase II (day 14th PF) was not hindered in spite of the long setae, in comparison with *Ch. muelleri* which has setae more than twice as long as *Ch. gracilis* (30 ± 6.1 μm). As a result, there was ingestion of *Ch. muelleri*, but the development of the larvae was limited, since they did not develop eyespot, which could be more associated with ingestion problems more than its nutritional quality, since *Ch. muelleri* was the third microalgae with the best proportion of EPA, and the only with AA. Similar contents were recorded by Ohse et al. (2014), and a similar non-acceptance showed when *Ruditapes decussatus* larvae were fed with that microalga (Aranda-Burgos et al., 2014).

In general, except *Nannochloropsis* sp. (low digestibility) and *Ch. muelleri* (long setae), *T. lutea* and *P. lutheri* provided the best results registered in monoalgal experiment of this study (at least in growth and survival). Similar acceptance reports are found when those flagellates are included in the diet in experiments with *Crassostrea gigas* (Rico-Villa et al., 2006; Wang, Li, Zhang, & Yu, 2018), *C. virginica* (Babinchak & Ukeles, 1979), *Pecten maximus* (Le Pennec & Rangel-Dávalos, 1985), *Argopecten ventricosus* (Lora-Vilchis & Maeda-Martínez, 1997), *Pteria sterna* (Martínez-Fernández et al., 2004), *Ruditapes decussatus* (Aranda-Burgos et al., 2014), *Placopecten magellanicus* (Gouda, Kenchington, Hatcher, & Vercaemer, 2006), *Pinctada margaritifera* (Martínez-Fernández, Acosta-Salmón, & Southgate, 2006) and *Ostrea edulis* (González-Araya & Robert, 2018).

In the second bioassay, a combination of *T. lutea* + *P. lutheri* was compared in three ratios (1:3; 1:1 and 3:1; first used by cell size) contrasting it with the control (*T. lutea* + *Ch. gracilis*) on phase I. The result showed that the control diet had a better performance for generating a greater survival at the end of phase I, due to a lower mortality, probably due to a better acceptance of the microalga Cg, at the end of the phase (inclusion of EPA in larvae). During phase II, classic mixtures used in shellfish farming were compared (Blanchard et al., 2008), finding no significant differences in survival, daily growth rate or growth between the *P. lutheri* + *Ch. gracilis* and *T. lutea* + *Ch. gracilis*. However, the larvae fed with the control diet showed a significantly larger proportion of larvae with eyespot. From a nutritional point of view, this is explained only by the lower ratio of 20:0; 18:3 (n − 3); 18:0; 18:1 (n − 9); 18:2 (n − 6); DHA and EPA in Pl + Cg mix.

Additionally, the control mix used provided most of fatty acids except 20:4 (n-6) (AA), which is reported as important factor in the larval phase of bivalves (Pernet et al., 2005), and suggests that the larvae of *S. prismatica* have the metabolic capacity to synthesize de novo these fatty acids from molecular sources. In this sense, Farías and Uriarte (2002) and Sánchez-Lazo and Martínez-Pita (2014) suggest that bivalve larvae may have some ability to obtain AA using 18:2(n − 6) (linoleic acid) or 18:3(n − 3) (Alpha-linolenic acid) as a precursor. Our results showed that *S. prismatica* can do without AA from the diet due to its ability to obtain it from other fatty acids. A study with fatty acid profiles in the algae diet combination and the larvae during the ontogenic larval development is necessary to elucidate this hypothesis.

Curiously, when comparing monoalgal phase I with bialgal I, we obtained a shorter cycle with the first. This behaviour is justified in the fact that the monoalgal experiment was carried out at higher temperature than the bialgal one (26 ± 1.5°C and 24.5 ± 0.5°C, respectively, see Table 2), resulting in a phase I of only 6 days until reaching the early umbonated stage (2 days less than the phase I bialgal). This result translates into an apparent no need for EPA (in great quantities) to efficiently complete this phase. We suggest studies of temperature evaluation in the larval culture of *S. prismatica* accompanied by a biochemical analysis in the larvae are necessary to deepen this hypothesis.

On the evaluation of settlement substrates, the treatment that ensure greater fixation of postlarvae was the ground shell of greater surface, reaching values 5 times higher than other tested substrates, including the same ground shell of smaller pieces, or other materials with greater surfaces (polycarbonate and sandpaper). Settled larvae on sandpaper showed greater growth, probably because of the lower intraspecific competition, reaching 2.4 mm in dorsoventral length. The use of small particles (200–350 μm) enables individualized oyster spat, which is desirable because it allows a better handling of spat, and a better presentation of the product (Roman, 1987; Vasquez et al., 2013). With this in mind, it was noted that shell pieces with larger surface area supported the settling of various spat, with an average of about 4–8 settled postlarvae per particle (estimations from photographic records), which does not allow us to meet our objective of obtaining individualized spat that ensure better performance in the field.

The polycarbonate was not accepted by the pediveliger since, apparently, the composition of the substrate surface did not satisfy the specific needs of the animals despite the particle size. A similar result was observed with the sandpaper, because in spite of its rough surface, settling on it was not significantly better. Finally, the substrate made of ground rubber with particles (2–3 mm^2), although the settling percentage was low (4.2%), the settling was done in a more unitary way, which would facilitate the individualization of the spat, leading to a greater number of appropriate seeds.

As suggested above, the mixed diets are necessary to optimize growth and survival in the larval development of *S. prismatica*. Accordingly, two alternatives are proposed for feeding the early larval stages, using the classic combination of microalgae *T. lutea* and *Ch. gracilis*, with a higher proportion of *T. lutea* in the first 6 days (3:1), and then add *Ch. gracilis* in proportion 1:1 in the intermediate days of larval development until early umbonated larval stage (8–10 PF), and 1:3 in the rest of the larval development. Another alternative that can be evaluated in future researches is to incorporate *P. lutheri* in a bialgal diet with *T. lutea* (1:1), at least to

complete phase I (6–8 days), and then to incorporate Cg in a trialgal diet in similar proportions until day 10th when the ratio should be changed to 1:1:3 (PI:TI:Cg).

Although a settlement of 20% pediveliger larvae of *S. prismatica* was obtained on shell pieces of the same species, the final yield was low, so it is considered that technology of the settlement phase has not been optimized. Experiments are recommended to obtain substrates suitable, together with the induction of metamorphosis by physical factors (temperature, light, turbidity, etc.), as well as chemical (use of neurotransmitters), on different substrates, including the use of ground rubber and substrates with materials of greater specific weight, with restricted movement under the regular conditions of the water flow in bivalve cultures. Also, studies of the size-dependent substrates are recommended to optimize this important phase in the production of spat of *S. prismatica*.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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