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# Microalgae diet for juveniles of *Spondylus limbatus*

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## Abstract

The effects of mono- and bi-microalgal diets on shell length, organic tissue, and survival of *Spondylus limbatus* juveniles were tested in two separate feeding experiments. The first examined nine single species of microalgae: *Chaetoceros gracilis*, *C. muelleri*, *Isochrysis* aff. *galbana* (T-ISO), *Tetraselmis chuii*, *T. maculata*, *Pavlova lutheri*, *Dunaliella salina*, *Rhodomonas* sp., and *Nannochloropsis* sp. An extra treatment maintaining the juveniles in a long line culture was at Ayangue Bay, Province of Santa Elena, Ecuador. Single diets of *P. lutheri*, *C. muelleri*, and *C. gracilis* showed the best combined growth of survival performance in juveniles, and were therefore selected for the bi-algal diet experiment design with the inclusion of *I. galbana* T-ISO due to its recommended use in bivalve culture. A second experiment consisted in selecting the best three microalgae species that resulted from the first experiment and combined each other in binary treatments at 1:1 ratio. All bi-algal diets in combination with *P. lutheri* showed good result in growth (shell and biomass), yield, and survival. However, single *P. lutheri* mono-algal diet showed equal or better performance index than the other bi-algal combined diets, demonstrating the pertinence in using this microalga as food for *S. limbatus* juveniles.

**Keywords** Bivalve culture · Mollusks · Algal diets · Spondylids · Eastern Tropical Pacific · Juvenile diets

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## Introduction

In aquaculture, the production of healthy and robust juveniles is an important issue to assure success. The use of adequate diets for satisfactory growth and survival is a key factor in culture technologies (Lucas and Southgate 2012). In bivalve mollusks cultures, live microalgae provide nutritive elements to satisfy their metabolic needs for growth and reproduction, and to date, artificial or other natural feeds have not proven suitable to replace live microalgae in bivalve mollusk cultures (Hemaiswarya et al. 2011; Mamat and Alfaro 2014).

There are several microalgae species cataloged for use in bivalve culture (Coutteau and Sorgeloos 1992; Helm and Bourne 2004; Marshall et al. 2010; Brown and Blackburn 2013), and several microalgal diets have been established for different bivalve species for culture. However, the ability of organisms to trap, ingest, digest, and assimilate microalgal species of variable morphologies and size, to meet nutritional requirements among bivalve species, even within the same genus, has made it difficult to generalize formulations of microalgal diets (Coutteau and Sorgeloos 1992; Ponis et al. 2003; Aranda-Burgos et al. 2014). Thus, screening for microalgal species with high nutritional value is a priority for research of mollusk cultures in hatcheries.

A microalgal diet that comprises several species theoretically may give better yields than mono-algal diets; however, factors associated with cost and logistics in hatchery production suggest the use of microalgae combinations, with no more than 2 species to form a diet. Thus, new cultured species require considerable background and experimental research to identify the best diets for cost-effective stock production.

The spiny rock scallop, *Spondylus limbatus* (Sowerby II, 1847), is a pectinoid distributed along the Eastern Tropical Pacific coast from the Baja California Peninsula and the Gulf of California, Mexico, to Tumbes, Peru (Coan and Valentich-Scott 2012). This species has played an important economic, political, and cultural role in coastal communities of the Eastern Tropical Pacific for thousands of years (Cudney-Bueno and Rowell 2008). Although the importance of spondylids in America remained dormant after the conquest of the Spanish empire, in the 1980s, the spondylids, particularly *S. limbatus*, became important again for gastronomy and jewelry crafting, which led to the decline of natural banks. Currently, the different species of *Spondylus* in Mexico and Ecuador are under special protection, promoting the interest for developing technologies for their aquaculture production and ecological restoration (Lodeiros et al. 2016).

The collection of wild juveniles to culture is not feasible since they are cemented to hard substrates, making spat extraction difficult or impractical. Hence, experimental studies focused on spat production, and some of them were made in broodstock maintenance and spawning induction, larvae culture, and postlarvae production under laboratory conditions (Soria et al. 2010; Loor et al. 2016). However, further research is needed to optimize the growth of *S. limbatus* juveniles of adequate size to be used as spat for grow out in the sea (Lodeiros et al. 2016).

Since microalgal cultures are one of the most important and largest investments in aquaculture activities, and since information about microalgal diets for juveniles of *Spondylus* species is non-existent, the objective of our study was to evaluate mono-algal and bi-algal diets for the culture of juveniles of this species.

# Materials and methods

## Experimental conditions

Experiments were carried out with juveniles cemented in adult *S. limbatus* shell pieces raised at the Centro Nacional de Acuicultura e Investigaciones Marinas of the Escuela Superior Politécnica del Litoral of Ecuador (CENAIM-ESPOL) following protocols described in Loor et al. (2016) and Lodeiros et al. 2016.

Nine microalgae species widely used in aquaculture (Helm and Bourne 2004; Brown and Blackburn 2013; Table 1) were evaluated. All microalgae species were cultured in F/2 Guillard medium, with 0.45-μm filtered and UV-treated seawater and salinity at 34 practical salinity unit (PSU), by increasing culture volumes of 5 ml, 250 ml, and 2 l culture batches up to 50-l carboys, at 20 °C. Cultures were aerated and irradiated with fluorescent light (3.500–5.000 lx). Microalgae cellular dry biomass was determined to feed *Spondylus* juveniles on a weight base. During the exponential growth phase, 20 ml of each culture species was filtrated through a fiberglass Whatman GF/F filter (previously dried at 60 °C/24 h and weighed), using a vacuum Millipore equipment. Algal retentions were washed with 4 ml 0.5 M ammonium formate to remove salts and dried at 60 °C to a constant weight for 48 h.

The cellular concentration was determined using a Neubauer chamber (hemocytometer) and microalgae dry weight was used to determine the cellular biomass of each alga (Table 1). The diet ration (supplied two times a day) was 4 mg $l^{-1}$, which was equivalent to dry microalgal biomass fed to the pectinoid *Nodipecten subnodosus* juveniles (Lora-Vilchis et al. 2004). In all treatments (diets with 3 replicates), microalgal ration was adjusted to microalgae tank concentration evaluated during water exchange.

**Table 1** Size, dry weight, and morphological characteristics of the microalgae used

| Microalgae | Size (μm) | Weight (pg) | Morphological characteristics |
|---|---|---|---|
| Chlorophyta | | | |
| *Dunaliella salina* | 8.5 | 98 | Motile large cells, two flagella |
| *Tetraselmis chuii* | 9.7 | 178 | Ovoid body shape and a distinct curved body when viewed sideways, four-flagellated, which is a thin cell wall formed by extracellular fusion of scales |
| *Tetraselmis maculata* | 13.8 | 259 | |
| *Nannochloropsis* sp. | 2.5 | 13 | Very small, fibrous glycoprotein cell wall |
| Cryptophyta | | | |
| *Rhodomonas* sp. | 12.9 | 112 | Free-swimming, generally ovoid, without cell wall |
| Haptophyta | | | |
| *Isochrysis* aff. *galbana* (Clone T-ISO) | 4.4 | 40 | Two flagella, round-oval-shaped, cell wall covered of polysaccharide scales |
| *Pavlova lutheri* | 3.9 | 53 | Spherical cell with two flagella and cell wall |
| Bacillariophyta | | | |
| *Chaetoceros gracilis* | 4.9 | 62 | Solitary diatom. It is rectangular in shape |
| *Chaetoceros muelleri* | 4.9 | 82 | Rectangular cell, often solitary and lightly silicified |

Experimental units (replicates) were assigned randomly to the treatment and consisted of 50-l conical tanks with 30 l of 1-μm filtered and UV-treated seawater, with a density of 0.5 juvenile L-1 (15 in each tank). The *S. limbatus* shell pieces with juvenile were arranged on a horizontal hard plastic screen placed inside the tank. Water temperature and salinity throughout the experiments were 25.6 ± 2 °C and 34 ± 0.5 PSU, respectively. Tanks were slightly continuously aerated and the culture water was exchanged every 48 h.

All juveniles were acclimated for 2 weeks prior to the start of the experiments with a mixed diet (2:1—*C. gracilis* and *I. galbana* T-ISO) fed *ad libitum* and starved for 2 days at the end of the 2-week period. Each experiment had a duration of 45 days.

An extra treatment was also tested by suspending three pearl nets with 15 juveniles fixed at the base of the basket and suspended at 2 m in depth from a long line at Ayangue Bay, Santa Elena Province, Ecuador (1° 59′ 1.59″ S; 80° 45′ 35.15″ W), called "natural sea diet."

## Experimental design

### Experiment I: mono-algal diets

The first experiment was designed to compare growth and survival of *S. limbatus* juveniles (mean shell height 6.4 ± 0.59 mm of shell dorsoventral axis) exposed to mono-algal and natural sea diets. Growth was measured by the difference between initial and final shell height and organic tissue biomass. The shell height was measured with Canvas Draw 1.1 software (AC System) using digital micro-images of juveniles and the biomass was determined by dehydration of tissue at 60 °C for 48 h and final heating at 450 °C during 4 h in a muffle furnace. The difference between the dry organism and its inorganic mass was considered as the organic tissue biomass. Growth variables (shell height and biomass) were expressed as relative growth rate ($r$): $r = ((Vf - Vi)/Vi) \times 100\%$ where V is the shell length or biomass, at the end (f) or at the start of the experiment (i).

Initial tissue biomass was estimated by extrapolation from a shell length-tissue biomass lineal relationship determined with 30 juveniles of the spiny rock scallop. All juveniles were marked in the position where they were placed on the plastic screen in all experimental units. Survival was estimated at the end of the experiment by counting live organism, i.e., juveniles that reacted by closing their valves after perturbation with a glass rod.

### Experiment II: bi-algal diets

The second experiment was designed to determine the best binary microalgal diets on growth and survival of juveniles (mean shell height 9.6 ± 0.52 mm), using a combination 1:1 (dry biomass) of the best three mono-algal diets that resulted from the first experiment, i.e., *P. lutheri*, *C. muelleri*, and *C. gracilis*. Mixed algal treatment diets consisted of all possible binary combinations. *I. galbana* T-ISO was added to this group due its common use as component of diets in bivalve cultures (Helm and Bourne 2004; Brown and Blackburn 2013). Experimental conditions and evaluation of binary diets were performed in the same way as experiment I.

## Performance index

In order to choose the best diets (mono-algal, bi-algal, or natural sea diet), we estimated a performance index (PI) based on the proportion increase of organic tissue biomass and survival obtained for each diet. $PI = ((OTf - OTi)/OTi) \times S$; OTf = organic tissue final; OTi = organic tissue initial; and S = survival.

## Statistical analysis

Normality and homogeneity of variance for all treatment variables were analyzed with the Kolmogorov-Smirnov and Levene test. Statistical differences ($P < 0.05$) in growth and survival among treatments were analyzed using a one-way analysis of variance (ANOVA I). In those cases, where the ANOVA showed significant differences, a post hoc Tukey multiple comparisons test was carried out to determine which treatments differed. In the interest of brevity, only post hoc results are provided in this work.

## Results

### Experiment I: mono-algal diets and natural diet

All tested diets produced shell growth and tissue biomass with a survival > 55% among the juvenile spondylids (Fig. 1). However, survival, and particularly growth, was significantly dependent on the algae species tested.

Highest growth in shell height was reported in the natural sea diet with a 37.3 ± 10.05% (95% confidence interval in all cases) increase from initial size, followed by *P. lutheri* and *C. muelleri* that conformed a statistically similar group with 19.1 ± 0.71 and 18.3 ± 9.15%, respectively. The remaining mono-algal diets showed growths below 10% (Fig. 1a).

The increase in organic tissue biomass of juveniles also showed statistical differences among diets. *P. lutheri* produced significantly more biomass (74.7 ± 7.22% of initial biomass) compared to natural sea diet (60.1 ± 5.28%), *C. muelleri* (57.1 ± 8.34%), *T. maculata* (53.8 ± 3.96%), and *C. gracilis* (50.1 ± 10.51%), which were statistically similar, while other mono-algal diets reached less than 40% of the initial juvenile biomass (Fig. 1b).

At the end of the experiment, overall survival among diets was > 55% (Fig. 1c), with statistically significant differences among diets. The lowest survival was observed with the natural sea diet (55.6 ± 10.88%) and the mono-algal diet of *I. galbana* T-ISO (63.4 ± 7.87%), which were a statistically equal group, followed by *Nannochloropsis* sp. diet (76.5 ± 13.93%) and the rest of the mono-algal diets that made up another group statistically equal, with survival from 84 to 92%.

### Experiment II: bi-algal diets

*P. lutheri*, *C. muelleri*, and *C. gracilis* were selected for this experiment of binary diets (Fig. 1) which also included *I. galbana* T-ISO. The binary diets resulted in an increased growth of 15 to 23% (Fig. 2a) in comparison with the mono-algal diets. However, the great variability within tested diets hindered the identification of significant differences.

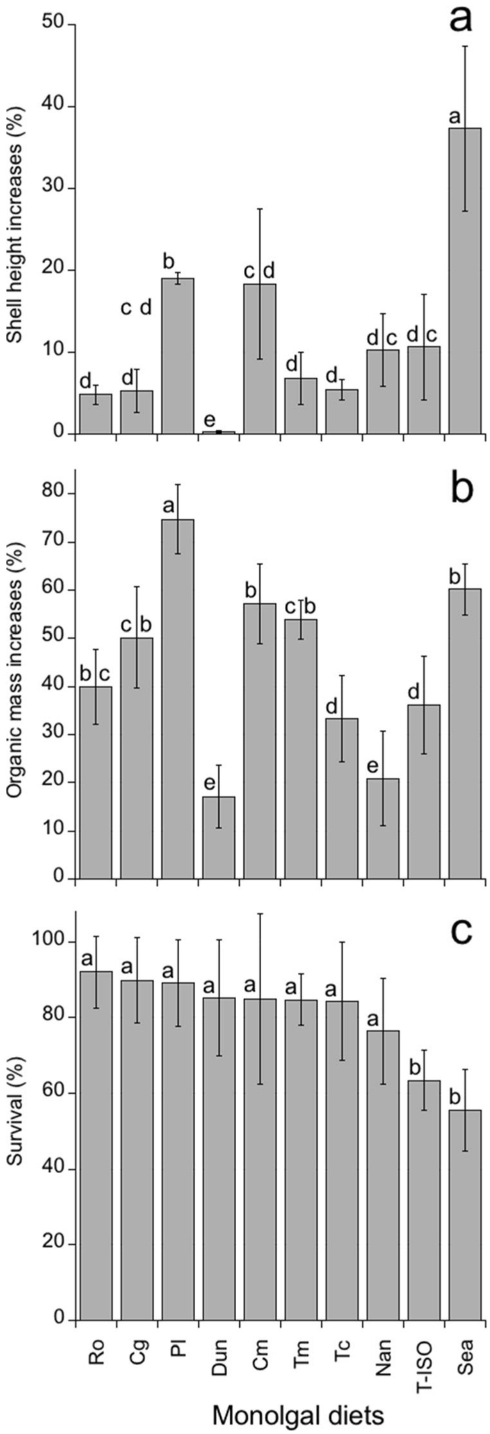

**Fig. 1** Increase (%) from initial shell height (**a**), organic mass (**b**), and survival (**c**) of *Spondylus limbatus* juveniles fed with the mono-algal and natural sea diets. Vertical lines indicate mean ± 95% confidence interval ($n = 3$). *Cg*, *Chaetoceros gracilis*; *Rsp*, *Rhodomonas* sp.; Pl, *Pavlova lutheri*; Ds, *Dunaliella salina*; Cm, *Chaetoceros muelleri*; Tm, *Tetraselmis maculata*; Tc, *Tetraselmis chuii*; Nsp, *Nannochloropsis sp.*; T-ISO, *Isochrysis galbana clone* T-ISO; Sea, natural diet in the sea

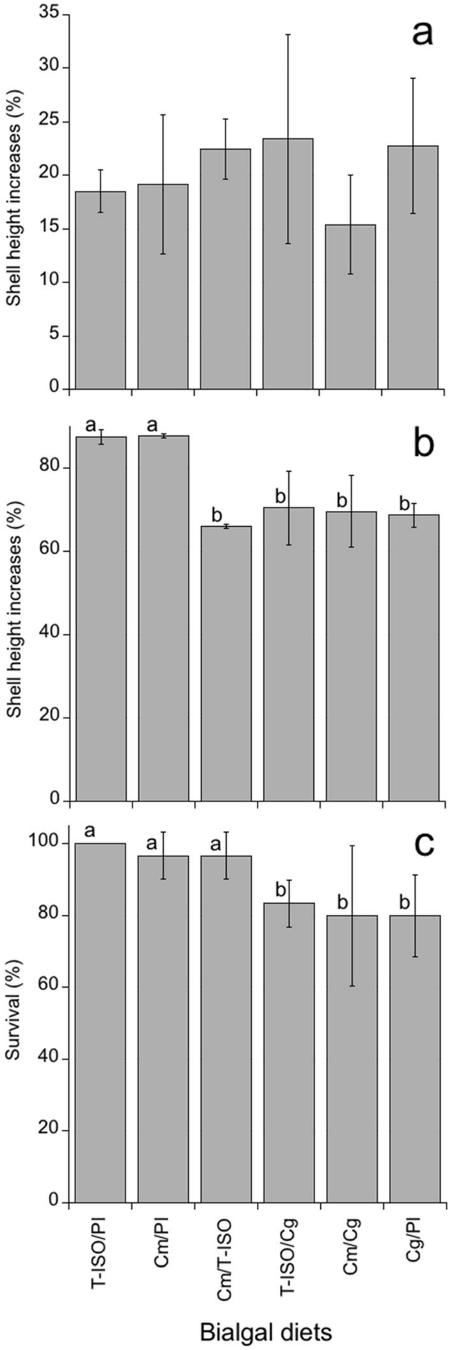

**Fig. 2** Increase from initial shell height (**a**), organic mass (**b**), and survival (**c**) of *Spondylus limbatus* juvenile fed with the bi-algal diets. The vertical lines indicate mean ± 95% confidence interval ($n = 3$). *Cg*, *Chaetoceros gracilis*; Pl, *Pavlova lutheri*; Cm, *Chaetoceros muelleri*; T-ISO, *Isochrysis galbana* clone T-ISO

The highest organic tissue biomass was obtained with the binary combination of *P. lutheri* and *C. muelleri* and *P. lutheri* with *I. galbana* T-ISO (87.7 ± 0.59 and 87.5 ± 1.82%, respectively; Fig. 2b), which were significantly different from the rest of the binary diets (65–70%). The differences among diets in the latter group were statistically non-significant.

The survival in the groups fed with *I. galbana* T-ISO, *P. lutheri*, and *C. muelleri* with any binary combinations formed a statistically higher group than any species binary combined with *C. galbana* to feed the spondylid juveniles.

## Performance index

The highest performance index was obtained with bi-algal diets (≥ 0.50, Fig. 3), particularly with the combination of *P. lutheri* with *C. muelleri* and with *I. galbana* (T-ISO) (0.88 ± 0.03 and 0.85 ± 0.06, respectively), which make up the diets with the highest performance. Likewise, the *P. lutheri* mono-algal diet showed a high index (0.77 ± 0.09), statistically similar to bi-algal diets. The rest of diets, all mono-algal and the natural sea diets, showed a performance

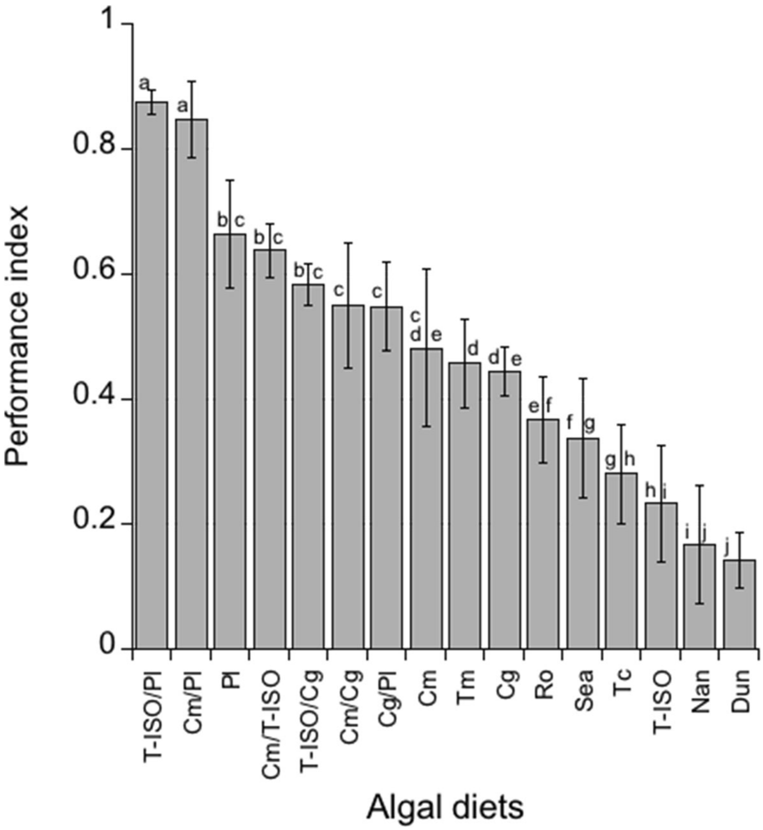

**Fig. 3** Performance index of the *Spondylus limbatus* juveniles fed with mono-, bi-algal, and natural diet (in the sea). Vertical lines indicate mean ± 95% confidence interval ($n = 3$). *Cg*, *Chaetoceros gracilis*; Rsp, *Rhodomonas* sp.; Pl, *Pavlova lutheri*; Ds, *Dunaliella salina*; Cm, *Chaetoceros muelleri*; Tm, *Tetraselmis maculata*; Tc, *Tetraselmis chuii*; Nsp, *Nannochloropsis* sp.; T-ISO, *Isochrysis galbana* clone T-ISO; Sea, natural diet in the sea

index less than 0.5. The mono-algal diets *I. galbana* (T-ISO), *Nannochloropsis* sp., and *D. salina* registered the lowest performance index.

## Discussion

All tested mono-algal diets produced somatic growth (shell and tissue) in juveniles, with average survivals above 55%, showing that any of these algal species could be used as food for *S. limbatus* juveniles. However, microalgae that supported highest growth in height and biomass were, in decreasing order, *P. lutheri*, *C. muelleri*, and *C. gracilis*. The natural sea diet also produced one of the highest growths but had presented the lowest survival, followed by *I. galbana* T-ISO and *Nannochloropsis* sp., while survival with the other microalgae diets reached on average 84%.

We observed a higher shell height growth in *S. limbatus* juveniles when fed *C. muelleri* compared to *C. gracilis*. This can be explained by higher weight of *C. muelleri* (82 pg cell$^{-1}$) than *C. gracilis* (62 pg cell$^{-1}$). These differences may be related to variances in energy content of the cells or slight differences in fatty acids, such as 20:5n-3 (EPA), 20:4n-6 (AA) and other fatty acids, or sterols; this slight difference in biochemical composition of microalgae from the same genus may have an effect in the bivalve growth and survival, as showed in the study of Ponis et al. (2006) which tested the nutritional value of six Pavlovophyceae for oyster and scallop larvae.

The fact that the natural sea diet supported more growth is sustained by the consumption of phytoplankton species and other components of the seston that generated a higher-quality diet (Strohmeier et al. 2009; Bock and Miller 1994; Gran 1996; Hawkins et al. 1996). The mortality observed (more than 50% in 1 month) could be associated to culture conditions of juveniles, which are susceptible to predation and other intrinsic factors associated with suspended culture-like fouling. At the end of the experiment, broken shell valves were observed suggesting predation by crabs, and also abundant fouling on the shell valves. Furthermore, wave action also produces negative effects in pectinoides (Freites et al. 2011; Robinson et al. 2016), although this effect may not have a major influence since juvenile spondylids were fixed to the base of the culture elements, a difference to other scallop species that are cultivated without attachment. Suspended culture in the open sea is not recommended for *S. limbatus* juveniles (20–30 mm shell height) and suggests further research is needed to determine culture strategies to reach a more robust size to avoid predation and fouling effects.

In general, binary diets in combination with *P. lutheri* showed the highest values in results in growth (shell and biomass) and survival in *S. limbatus* juveniles. Furthermore, the *P. lutheri* mono-algal diet showed an equal performance index in comparison with the other bi-algal diets. These results support the use of *P. lutheri* as food source for juveniles of *S. limbatus*.

Although the mono-algal T-ISO diet did not provide good results in the mono-algal diet experiment, its combination with *P. lutheri* produced the highest performance index. This effect can be due to the complementarity of growth factors present in diets, and probably this is more important than the number of calories consumed (Díaz and Martínez 1992; Velasco-Blanco 1997). These results support the selection of these species to prepare combined algae diets for feeding aquatic organisms (Helm and Bourne 2004; Brown and Blackburn 2013). Biochemical composition is a major factor determining the nutritive value of microalgae, particularly the composition of polyunsaturated fatty acid (PUFA) content, specifically eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic (22:6n-3, DHA), and their balance greatly enhances nutritive properties (Pernet and Tremblay 2004; Martínez-Fernández et al. 2006; Batista et al. 2014). The high nutritional value of *P. lutheri* in DHA and particularly EPA could explain the higher performance index as mono-algal diet or in combination diets. In contrast, the lower performance index of *I. galbana* T-ISO diets, in spite of being rich in DHA (8%), is explained by the presence of trace amounts of EPA in this species (see Brown and Blackburn 2013 for biochemical composition of microalgae). However, it was the diet that showed high growth results when combined with *P. lutheri*. It is possible that the synergy or additive effect of this binary diet is due to the balance in other nutritional components and other factors as the size and digestibility related to the cell wall structure and morphology of the algae. In this sense, easily digested algae include naked flagellates such as *P. lutheri* (Le Pennec and Rangel-Dávalos 1985; Martínez-Fernandez et al. 2004).

*Tetraselmis* spp. strains exhibited a poor food value to *S. limbatus* juveniles even if they are rich in EPA. This may be due to the low digestibility of thick cell wall of *Tetraselmis* (Robert and Trintignac 1997), or toxic metabolites produced by *Tetraselmis* cells (Davis and Guillard 1958). *T. suecica* was also relatively poor food for *Crassostrea virginica* and *Mercenaria mercenaria* spat when used as single diet, but its food value was markedly increased when fed in combination with either (or both) *I. galbana* or (and) *Thalassiosira pseudonana* (Epifanio 1979). Contrary to that, other authors reported higher growth rate when *T. suecica* was fed to *Ruditapes decussatus* seed compared to the other diets tested, such as *I. galbana* (Albentosa et al. 1996). Although there are no ingestion and clearance studies of microalgae in spondylids, all microalgae tested have an acceptable size range (> 3–4 μm) for filtration of bivalves (Gosling 2015), except the *Nannochloropsis* sp., whose size was 2.5 μm. It is possible that species of *Spondylus*, being phylogenetically close to Pectinidae (Serb 2016), have similar gill structures (single row of simple latero-frontal cilia) and low retention efficiency for particles less than 5–7 μm in diameter, in contrast to most studied bivalves which possess well-developed, compound latero-frontal cilia (Beninger and Le Pennec 2016). This functional gill can explain the low-performance index of *S. limbatus* juveniles fed with *Nannochloropsis* sp. diet. Additionally, *Nannochloropsis* species are usually not well digested due to its fibrous glycoprotein cell wall (Lora-Vilchis and Maeda-Martinez 1997; Martínez-Fernandez et al. 2004). Studies on the structural gills of *Spondylus* spp. and the clearance, retention, and digestibility of food items with different algae, along with studies that include their structural cell components and sizes, are necessary to verify the formulated hypotheses.

The *Chaetoceros* spp. tested showed, in combination with *P. lutheri*, a high-performance index. Also, its combination with *I. galbana* T-ISO and as mono-algal diet resulted in an intermediary-performance index. This can be explained by its high biochemical quality, but in counterpart, its silica shell and chain formations possibly reduce its ingestion rate (Lora-Vilchis and Maeda-Martinez 1997).

Although Loor et al. (2016) working with larval diets of *S. limbatus* showed that the mixture of *C. gracilis* and *I. galbana* can be used during its early life cycles (12 days), the results obtained in this study with juveniles show the need to evaluate more microalgae species, in particular *P. lutheri* and *C. muelleri*, to optimize larval culture of *S. limbatus*.

Juveniles fed on *Dunaliella salina* exhibited nearly a nil shell growth. This fact can be explained by the lack of EPA and DHA in this microalga (Zhukova and Aizdaicher 1995). Both EPA and DHA are considered essential fatty acids (EFAs) for bivalve survival and growth (Rico-Villa et al. 2006) because they are major membrane components (Hendriks et al.

2003) and possible modulators of membrane function (Palacios et al. 2005). Moreover, in *D. salina*, the proportion of 18:1n-9 surpassed 50% (Sahu et al. 2013). One study with *Crassostrea gigas* larvae showed that high supplies of 18:1n-9 may cause deleterious effects on bivalve larval survival (da Costa et al. 2016). Langdon and Waldock (1981) showed that *D. tertiolecta*, which contained no fatty acid longer or more unsaturated than 18:3n-3, almost does not promote growth in *C. gigas* spat. Similar results were found when *D. tertiolecta* was fed to scallop larvae, *Argopecten purpuratus* (Nevejan et al. 2003). The microalgae *P. lutheri* (either alone or mixed with other species, particularly with *I. galbana* T-ISO and *C. muelleri*) is recommended for feeding the early life stages of *S. limbatus*. Further research is required to identify specific nutritional requirements of *S. limbatus*, focusing on such components as fatty acids, proteins, carbohydrates, and micronutrients.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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