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Larval calcification and growth of veligers to early pediveliger of the queen conch *Strombus gigas* in mesocosm and laboratory conditions

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Abstract
The goals of this study were to evaluate growth, development, and calcification process of veligers of *Strombus gigas* grown in natural conditions in mesocosm versus laboratory conditions. In this study, larvae bred in mesocosm conditions had a good growth rate (33.3 ±12.40 μm.day^{-1}) when fed with natural phytoplankton in natural flowing seawater versus a lower growth rate of 8.8±5.20 μm.day^{-1} for larvae reared in laboratory conditions and fed with a monoalgal diet of *Nannochloropsis oculata*. Physicochemical parameters did not explain the difference on larval growth in both culture systems according to the principal component analysis done. Raman microspectrometry carried out on conch larvae grown in mesocosm and lab conditions allowed us to emphasize the biosynthesis of calcium carbonate species and their structure type evolution (amorphous, aragonite, or calcite structure) as a function of the breeding time by detecting and identifying in the spectra the characteristic Raman bands of CO_3 chemical groups and lattice vibrations. This analytical method seems to indicate that crystalline $CaCO_3$ structures are not detected in the recorded spectra on larvae reared in laboratory conditions during the early stages of the shell construction of 1 to 8 days. For this reason, light and current flow have also been considered in the discussion that can help to explain the differences found in this study. Contrarily, the Raman spectra acquired on larvae grown in mesocosms exhibit characteristic bands of aragonite (CO_3 double peak at 697–701 cm^{-1} and lattice vibrations at 170 and 220 cm^{-1}) from the third day of breeding. The best shell growth and shell calcification pointed out in larvae grown in mesocosm compared to laboratory conditions are probably due to the nutrient amounts present in the food available in mesocosm.

Keywords Shell calcification · Gastropod · Aquaculture · Raman spectrometry · Aragonite · Calcite

Introduction

The queen conch, *Strombus gigas*, is one of the six species of conch distributed throughout the Caribbean, southern Florida, and Bermuda (Berg Jr 1976). This large marine gastropod is a valuable mollusk of significant commercial importance in this region (Theile 2001; Aiken et al. 2006; Oxenford et al. 2008). The high market demand and lucrative export trade have resulted in heavy

fishing pressure which, coupled with its vulnerable life history, has caused substantial reductions in conch populations in the past 40 years. Thus, this species was listed as a CITES II species (Conservation of International Trade of Endangered Species of Wild Fauna and Flora) in 1992. Various programs to develop aquaculture techniques to enhance production were developed (Davis 1994). The production of conch seeds was proposed as a basis for restoring depleted natural populations of *S. gigas*. Studies of specific larval preferences of feeding schedule for the success of larviculture are limited. Results of survival rates varied between 6 and 17% (Hensen 1983; Heyman et al. 1989; Davis et al. 1993; Aldana Aranda and Brito Manzano 2017). Most of aquaculture larvae of *S. gigas* were fed with monoalgal culture (Aldana Aranda and Patino Suarez 1998; Davis 2000) and with larval culture temperature ranging between 26 and 31°C leading to an average growth of 13–40 μm.day^{-1} and a survival rate varying from 25 to 82% (Pillsbury 1985; Aldana Aranda and Torrentera Blanco 1987; Davis et al. 1993; Garcia Santaella and Aldana Aranda 1994; Aldana Aranda and Patino Suarez 1998; Aldana Aranda and Brito Manzano 2017). Stoner et al. (1992) and Barile et al. (1994) reported that larvae of *S. gigas* are sensitive to temperature variations. Davis et al. (1993) then Brito Manzano and Aldana Aranda (2004) reported optimal growth of these larvae at 27°C and 28°C, respectively. To maximize production in hatcheries, it is necessary to understand the environmental preferences of the larvae. Therefore, a mesocosm system is an ideal apparatus for conducting ecological research with marine invertebrate larvae (Davis et al. 1996). Determination of larval growth and survival under field conditions can provide more accurate information on growth, survival, metamorphosis, and calcification processes compared to results achieved in laboratory (Davis et al. 1996). According to the literature on calcification of the shell of mollusk larvae, this period corresponds to the crystallization of carbonates from amorphous to aragonite forms (Jardillier et al. 2008; Weiss et al. 2002; Hasse et al. 2000; Marxen et al. 2003; Collin and Voltzow 1998). Optimization of these parameters can result in improved growth and survival rates, a reduction in the larval breeding period, and a subsequent reduction of the production costs. This study was proposed to analyze the calcification process from the planktonic phase to early pediveliger phase of the queen conch *S. gigas* reared in field conditions (mesocosm) compared to larvae reared in laboratory conditions.

The aim of our study was to determine growth, development, and calcification process for young larvae of the queen conch *S. gigas* reared in field conditions (mesocosm) compared to larvae reared in laboratory conditions.

Materials and methods

One fertilized egg mass was used for each experiment. These egg masses were collected in the Yucatan Peninsula, Mexico (22° 21′ N and 89° 49′ W). They were collected under female conch to ensure species identity and egg freshness. They were subsequently transported to the laboratory where epibionts and sand particles were removed before cleaning with filtered and UV-sterilized seawater. Egg masses were placed on a 300-μm mesh and kept immersed in a 25-L aquarium with seawater filtered through 2-μm cotton filters and UV sterilized.

Larval cultivation systems

Larvae were reared in two different culture systems: laboratory and field-like conditions in a mesocosm.

Laboratory conditions

Larvae were reared in laboratory within 10-L cylindrical containers (in triplicates) in a static system with aeration at a density of 50 larvae.L^{-1}. Larvae were fed at equal amounts of fresh concentrates of the unicellular algae *Nannochloropsis oculata* at a concentration of 1000 cell.mL^{-1}. Larvae are fed daily maintaining same concentration of *N. oculata* throughout experiment, regardless of increasing larval size (Garcia Santaella and Aldana Aranda 1994; Brito Manzano et al. 1999; Brito Manzano and Aldana Aranda 2004). Larvae were reared at 28 ±0.3°C in a Diurnal Plant Growth Chamber (SRI21D SHEL LAB) to control the temperature and the photoperiod (12h/12h) (Brito Manzano and Aldana Aranda 1998). Every 2 days, 30 larvae were randomly collected from each replicate to measure growth and development. Each day, veligers were transferred to new containers with fresh seawater filtered through 2-μm cotton filters and UV sterilized through the experiment.

Field conditions

Larvae were reared in a mesocosm system placed into the field (Fig. 1). In this study, we tested a mesocosm system manufactured of fiberglass. The mesocosm is composed of three cylindrical chambers, each with a volume of 215-L capacity. Each chamber had two windows with a mesh of 100 μm that allows the passage of phytoplankton. The mesocosm system was placed in Xcaret Park, Quintana Roo (20° 34′ N and 87° 47′ W) in the Yucatan peninsula (Mexico). The site where the mesocosms were placed is protected against waves and strong water currents. Thus, mesocosms had an internal water circulation, with a constant flow of seawater coming directly from the natural ecosystem. Flow rate was not recorded.

During the experiment, 24-h veligers obtained in the lab were placed at a density of 50 larvae.$liter^{-1}$ in mesocosms and fed by natural phytoplankton passing through the nylon mesh. Based on Brito Manzano and Aldana Aranda (2004), 10 larvae were extracted daily from each of the three replicates of the larval cultures (n=30). Larvae from days 1, 3, 5, 7, 9, and 11 were used for larval development and growth observations. The Raman calcification observations were made for larvae from day 1 to days 8 of development. The sample corresponding to 7-day-old larvae reared in laboratory conditions could not be analyzed. The same situation for the larvae 4-day old from nesocosmos were not analyzed. Temperature (°C), dissolved oxygen (mg.L^{-1}), and salinity (ppm) were recorded every day at 8 am and 3 pm with a Datasonde 3" Hydrolab in both culture systems. Organisms grew under in situ temperature and light conditions.

Larval growth

Larval growth was assessed by recording increments in the shell length axis. Larvae were measured using a compound microscope with a calibrated ocular micrometer to the nearest 0.10 μm. Under a light microscope, larval shells were cut into the suture line of the body and the last spire to measure the thickness of the shells. Larvae were then positioned on a copper metal support, critical-point dried, and sputter-coated with gold before observation on scanning electron microscope (Philips XL-30 ESEM). The larval shell thickness was measured for larvae of 2, 5, 7, and 10 days reared in the mesocosms and lab conditions.

Differences between means were tested using one-way analysis of variance (ANOVA), and post hoc Tukey's tests were used to discriminate differences among effects of reared systems.

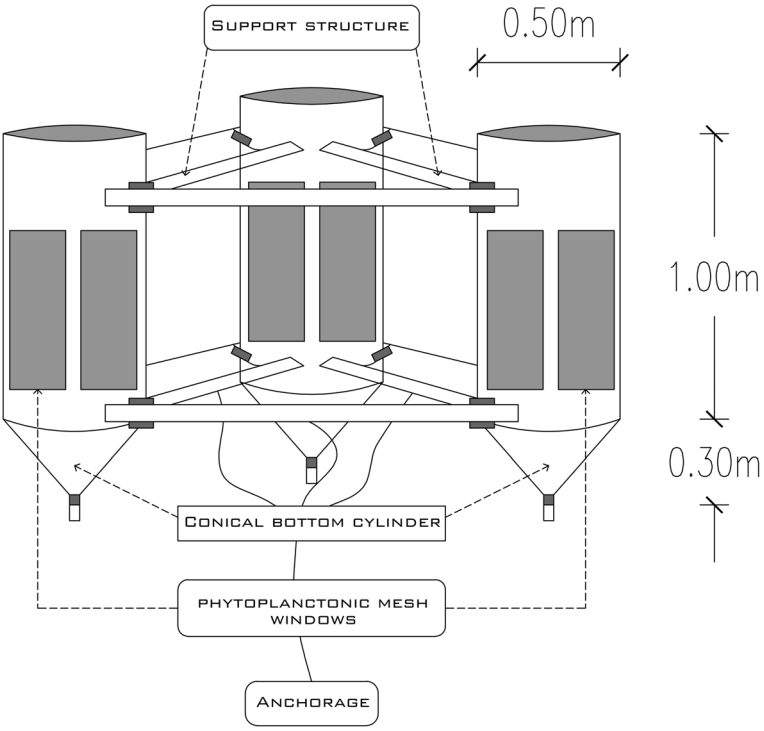

Fig. 1 Field cultivation system (mesocosm) manufactured of fiberglass with 3 chambers. Each chamber had two windows with a mesh of 100 μm that allows the passage of phytoplankton. Total volume of each chamber is 215 L

A regression linear model of daily rate growth was used to calculate a growth curve. Growth rate was assessed by slope coefficients comparison for each one of culture systems. Normality and homoscedasticity of data for each indicator was checked prior to analysis using a Kolmogorov–Smirnov's and a Levene's test, respectively (Sokal and Rohlf 1995). The minimum significance level was set at $p < 0.05$.

A principal component analysis (PCA) among shell length and water temperature, dissolved oxygen, and salinity was used to determine the effect of these parameters on growth in both culture systems. All statistical analyses were carried out in InfoStat student version 2012e.

Larval development

Every day, ten larvae were randomly collected from each replicate of both systems. Larvae were placed on a microscope slide to be anesthetized using a solution of 3mM $MgCl_2$ in seawater (Enriquez Diaz et al. 2015). Developmental characteristics of larvae were analyzed by light microscopy with examination of the number of lobes composing the velum, number of shell whorls, tentacles, and eye stalks. According to Brito Manzano et al. (1999), developmental characteristics were numbered chronologically, and then the incidence percentage in the developing larvae was calculated.

Scanning electron microscopy investigation

Larvae were fixed in 2.5% glutaraldehyde in a 0.2 M cacodylate buffer at pH 7.2 for 2 h, which was made isosmotic to seawater by adding sodium chloride. Samples were then rinsed with an isosmotic buffer, dehydrated through an ascending acetone series, critical-point dried, and sputter-coated with gold before observation (Glauert 1975; Enriquez Diaz et al. 2015). Larvae were observed and shell thickness was measured in a high-resolution scanning electron microscope (HR-SEM).

Calcification analysis by Raman microspectrometry

Raman microspectrometry was used to reveal the presence of carbonate anions and calcium carbonate in the samples and to identify the allotropic phases of calcium carbonate present in the samples. Analyses of calcification were performed on 2-, 3-, 4-, 5-, 6-, and 8-day-old larvae reared in lab and on 2-, 3-, and 5-day-old larvae from mesocosms. The analyses were carried out using a HR800 Horiba microspectrometer. Raman point spectra, in the wavenumber range 200–1300 cm^{-1}, were acquired on the samples in dispersion in distilled water (Himmel et al. 2009). The spectra acquisition conditions were the following: The exciting line was the 532-nm wavelength delivered by a Nd:YAG laser operating with a light power of 150 mW on the sample. An 1800 lines/mm dispersing grating was used leading to a spectral resolution of 3 cm^{-1}.

Laser exciting line focusing and Raman scattering collection were carried out by means of a 10-magnification objective lens leading to an analyzed area of 7 μm diameter (Himmel et al. 2011). In order to identify the crystalline $CaCO_3$ phases present in the samples, standard spectra were acquired on pure calcite and aragonite samples. In order to have a perfect calibration of the spectrometer, the emission line of mercury, located at 485.3cm^{-1} in our acquisition conditions, and the Raman spectra of the samples are simultaneously recorded. For the standard analysis, aragonite as geological crystal was purchased from Begenat Company and calcite standard is high purity $CaCO_3$ (>99.9%) powder purchased from Sigma-Aldrich.

Results

Parameters of culture conditions

During the experiment, average temperatures of seawater were 29.7±0.3°C and 30.0±0.3°C, lab and mesocosm conditions, respectively. Averages of dissolved oxygen were 6.8±0.5 $mg.L^{-1}$ for larvae reared in laboratory conditions and 7.0±0.4 $mg.L^{-1}$ for larvae reared in mesocosms. No significant difference was observed between the two culture systems.

Development

Larval development characteristics are shown in Fig. 2 a and b for larvae reared in field and laboratory conditions, respectively. At hatching, 100% of the larvae in both culture systems presented a typical veliger larva with two lobes and 1.5 turns of conch spiral. For the 3-day-old larvae, in mesocosm 100% of them presented 4 lobes and a shell with 2 spires. The larvae cultivated in the laboratory reached this stage only at day 5. In mesocosms, after 5 days of

development, 80% of the larvae showed 4 lobes and 2.5 turns of spires in their shell, an obvious foot with pigmentation and the formation of the right peduncle. Larvae reared in laboratory did not present a similar stage before day 7. After 11 days of experiment, larvae in mesocosm had 6 lobes and three spires in their shell, both right and left peduncles, and their foot was already pigmented. Larvae reared in laboratory conditions at the same age had a significantly lower development (Fig. 2).

Growth

Shell development, average of shell length, shell thickness, and growth rates are shown for the two treatments. Average shell length of larvae at hatching was 300±30μm. For late veligers (11-day-old larvae), average shell length was significantly lower under laboratory conditions with 407±9μm (growth rate of 8.85 μm.day^{-1}) compared to larvae reared in mesocosms (600 ±38 μm, with a daily growth rate of 33±12 μm.day^{-1}) (Fig. 3). The ANOVA test showed significant differences on growth ($p \leq 0.05$). Principal components analysis between growth and temperature and dissolved oxygen for larvae reared in laboratory and mesocosm did not explain an effect on larval growth in both culture systems analyzed (PC1 46.8% and PC2 34.4%).

Larval shell thickness was affected by the culture conditions, being 40% higher for larvae reared in the field, with an average of 3.7±0.8 μm compared to 2.3±0.4 μm obtained in laboratory conditions. Thickness of shells for larvae reared in field varied from 1.2 to 5.5 μm and for those reared in lab condition 1.0 to 3.0 μm. For larvae of 11-day-old, average of thickness was 4.2±0.9 μm in mesocosms, while for larvae in the laboratory conditions the thickness was 2.5±0.7 μm (Table 1).

Raman spectra analysis of aragonite and calcite

Figure 4 a presents the spectra collected in the 150–850 wavenumber range of the crystalline standards calcite and aragonite. Calcite spectrum Raman spectrum exhibits only two bands at 171 and 291 cm^{-1} and a single band at 708 cm^{-1} whereas aragonite spectrum exhibits numerous lattice vibration bands in the range of 150–300 cm^{-1} and a double peak at 697–701 cm^{-1}.

The two typical spectra presented in Fig. 4b were recorded on larvae reared in laboratory conditions at 2 and 8 days after hatching. The Raman bands characteristic of crystalline $CaCO_3$ phases are not detected.

The Raman spectra acquired on conch larvae grown in natural environment and sampled at 2, 3, and 5 days after hatching are shown in Fig. 4c. At day 3, it can be noticed that the Raman characteristic bands of the aragonite crystalline $CaCO_3$ phase (double peak at 697–701 cm^{-1}and aragonite lattice vibration bands at 170–220 cm^{-1}) are clearly detected, the bands being more visible on the spectra acquired at the periphery of the larval shells.

Discussion

The queen conch is one of the six species of Strombidae widely distributed in the Caribbean although being the most exploited. Since 1970 a decline of conch populations due to overfishing has been observed (Appeldoorn 1994). In order to solve this problem, various

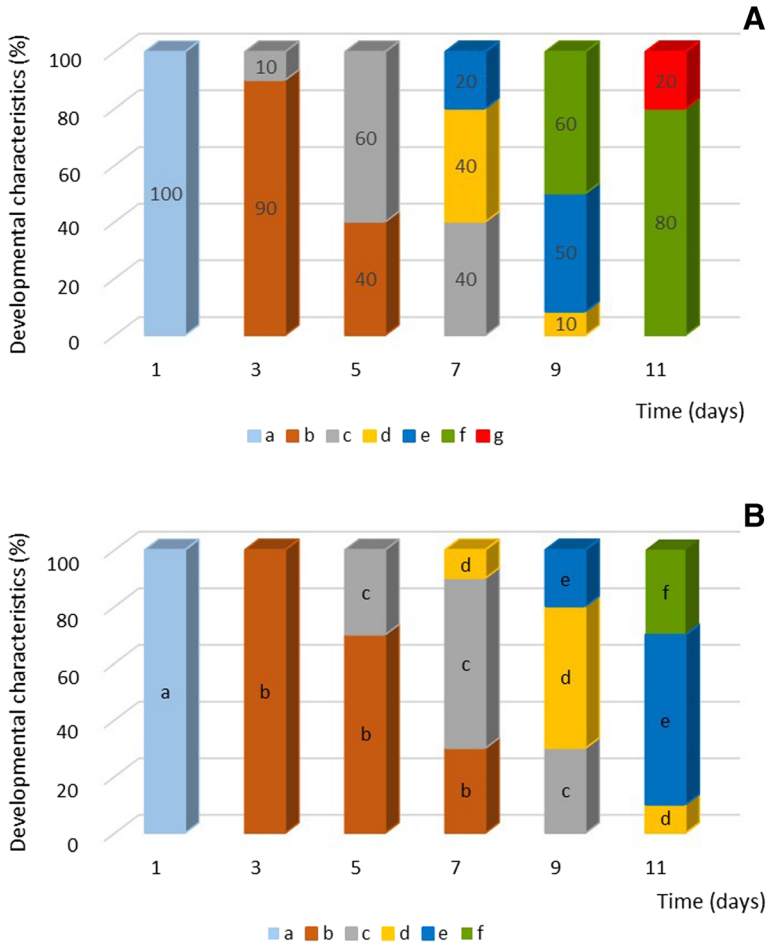

Fig. 2 Developmental stages and characteristics of *Strombus gigas* grown in two systems (A, mesocosm and B, laboratory). Larval stages are indicated by low case letters: (a) hatching, 1.5 shell whorl, 2 velar lobes; (b) early veliger, two shell whorls, 4 velar lobes; (c) early veliger, two shell whorls, 4 velar lobes, right ocular eyestalk start; (d) early veliger, two shell whorls, 4 velar lobes, right ocular eyestalk, right ocular tentacle start; (e) late veliger, two shell whorls, 4 velar lobes, right ocular eyestalk and tentacle, left ocular tentacle start; (f) late veliger, three shell whorls, 6 velar lobes, right ocular eyestalk and tentacle, left ocular peduncle, left ocular tentacle start, adult heart; and (g) early pediveliger, three shell whorls, 6 velar lobes, both ocular eyestalk and both ocular tentacles, adult heart, foot

authors have developed rearing techniques to restore some overfished areas. Even though the effort of conch larviculture, the lack of consistency in culture conditions is still persistent resulting in a large variation of growth, larval development and survival rate (Aldana Aranda et al. 1989; Garcia Santaella and Aldana Aranda 1994; Aldana Aranda and Patino Suarez 1998; Davis 2000). Most of larvae reared assays are conducted in laboratory conditions. Only Davis et al. (1996) conducted conch larval development in natural conditions. However, to obtain successful results in the hatchery, it is necessary to know particularly the natural diet. Over the last decades, several microalgae species have been tested as food in conch larvae aquaculture. (Aldana Aranda and Patino Suarez 1998). Most of hatchery reared conch larvae using monoalgae food. To provide a better-balanced nutrition and obtain more efficient

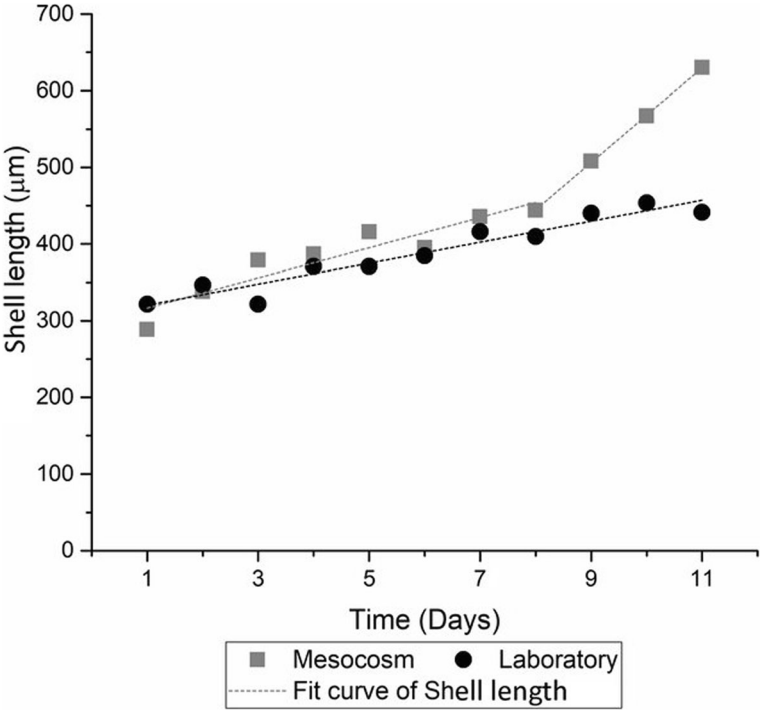

Fig. 3 Average and SD of shell length of *Strombus gigas* larvae, grown in laboratory and mesocosm conditions

growth, it is better to provide a carefully selected mixture of microalgae as food for mollusk larvae than a single microalgae diet. An optimum growth rate was obtained when the conch

Table 1 Average and SD of shell length and shell thickness of queen conch, *Strombus gigas* larvae cultivated in laboratory and field conditions in mesocosms. $p \leq 0.05$ significant difference

Larval age (days)	Shell length (μm)					Thickness of shell larvae (μm)			
	Larvae at mesocosms		Larvae in lab		*p*	Larvae at mesocosms		Larvae in lab	
	Average (μm)	SD (μm)	Average (μm)	SD (μm)		Rank	Average ± SD	Rank	Average ± SD
1	290.33	23.12	308.63	36.85	0.2480				
2	330.70	21.71	334.27	28.53	0.5880	1.2–4.2	2.7±1.1	1.0–1.9	1.4±0.9
3	383.27	29.41	337.57	39.68	0.0001				
4	385.11	18.17	364.63	10.67	0.0001				
5	409.97	24.47	361.77	37.71	0.0001	1.7–5.1	3.4±1.2	1.1–2.9	2.2±0.8
6	398.32	34.71	373.43	14.96	0.0008				
7	429.47	21.41	376.37	36.82	0.0001	2.5–5.2	4.1±0.9	1.3–3.0	2.4±0.7
8	444.00	29.66	387.17	17.65	0.0001				
9	508.00	36.59	393.97	27.46	0.0001	2.6–5.3	4.1±1.1	1.4–3.0	2.4±0.9
10	576.37	55.43	397.65	12.11	0.0001				
11	600.27	73.84	406.81	9.38	0.0001	2.8–5.5	4.2±0.9	1.5–3.0	2.5±0.7
Growth rate.day^{-1}	33.29	12.4	8.85	5.20					

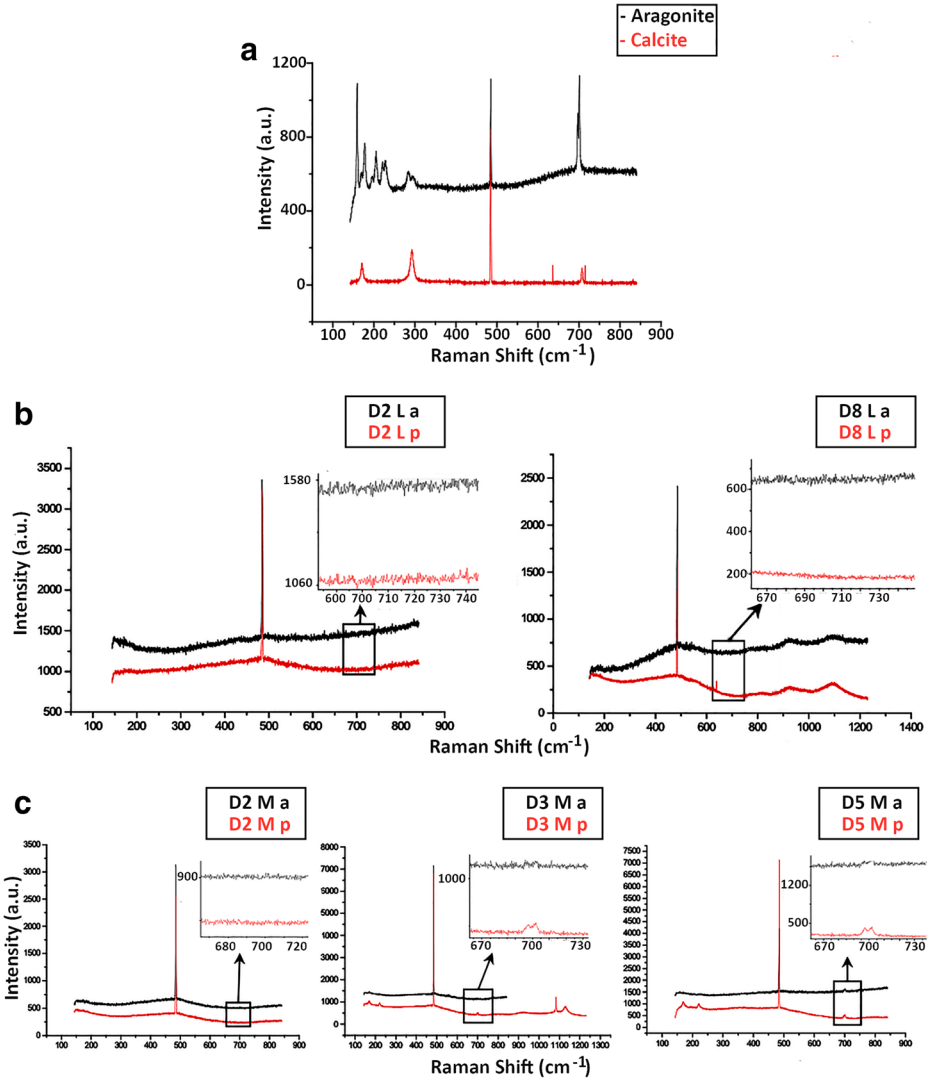

Fig. 4 (A) Spectra collected in the 150–850 wavenumber range of the crystalline standards calcite and aragonite. A single band at 708 cm^{-1} is observed in the case of calcite with two bands at 171 and 291 cm^{-1}. Aragonite spectrum exhibits numerous bands in the range of 150–300 cm^{-1} and a double peak at 697–701 cm^{-1}. (B) and (C) Raman spectra acquired on larvae of *Strombus gigas* grown in lab conditions (L) and in mesocosms in the field (M) and sampled at 2 (2d), 3 (3d), 5 (5d), and 8 (8d) days after hatching. The letters a and p refer to the spectra acquired at the apex and at the periphery of the shell, respectively. It can be noticed that the CO_3 (715 and 1085 cm^{-1}) and lattice (150–300 cm^{-1}) vibrations are not detected

veligers fed mixture of algae (Ballantine and Appeldoorn 1983; Davis and Hesse 1983; Pillsbury 1985; Aldana Aranda and Torrentera Blanco 1987).

However, the nutritional requirements of bivalve and/or gastropod larvae are poorly defined. Feeding experiments have shown that carbohydrate and polyunsaturated fatty acid (PUFA) levels are major factors for growth of oyster larvae (Epifanio 1979; Marshall et al. 2010) and *Haliotis* larvae (Gordon et al. 2004, 2006) and for the larval settlement, post-larval

growth, and survival of juvenile of *Haliotis discus hannai* (Maia et al. 1995; Floreto et al. 1996; Gordon et al. 2004). Diatoms are a rich source of eicosapentaenoic acid (EPA, 20:5n-3) considered as highly nutritious in terms of requirements for essential PUFAs. Diatoms offer high levels of lipids and PUFAs, especially the essential PUFA 20:5(n-3) (Dunstan et al. 1996; Brown et al. 1997).

Few studies have used mesocosm systems to investigate the effects of nutrition on the life span of marine invertebrate larvae in their natural environment (Brooke and Mann 1996). Davis et al. (1996) used mesocosms to rear conch larvae fed with natural phytoplankton from the Bahamian waters. They obtained the first fully metamorphosed larvae on day 13, and 95% of the veligers were competent for metamorphosis at day 16. These results suggest that quality of food may be more important than quantity. Thus, the nutritional value of natural phytoplankton cells may be superior to cultured algae, and that natural food items must be used to establish growth rates likely to occur in the field. In this study, larvae reared in mesocosm conditions with natural phytoplankton had a good growth rate (33±12 $\mu m.day^{-1}$) versus a growth rate of 9±5 $\mu m.day^{-1}$ for larvae reared in laboratory conditions and fed with a monoalgal diet of *Nannochloropsis oculata.* After 2 days, the growth rate showed a significant difference for the two culture systems used.

The two systems differ greatly, not only in food composition and concentration but also in flow rate and light intensity, which plays a key factor in successful larval rearing. The influence of light on the development of larvae in different species of mollusk has been evaluated by several authors. The light factor may be a physical cue that influences development and settlement of pediveliger larvae. Light has an effect of larval settlement of the scallop, *Nodipecten nodosus* (Bourne et al. 1989) Natural sunlight was tested in these larvae. Three different light levels were examined: (i) dark, the tanks were kept in total darkness; (ii) shadow, the tanks were placed in a location without direct sunlight; and (iii) light, the tanks were kept in areas that were exposed directly to sunlight. The only source of light tested was the sun. There were no statistical differences between treatments; however, settlement rate was slightly higher in the light treatment (41.95%) compared with the shadow (37.06%) and dark (33.33%) treatments (Carvalho et al. 2013). Concerning the embryonic development of the cuttlefish *Sepia lycidas*, the high light intensity has an adverse effect observed in an abnormal development, while the culture kept out of direct sunlight showed a better development (Peng et al. 2019). The effect of artificial light and darkness conditions was studied on larvae of *Chlamys varia* carried out under laboratory conditions. Results showed a slight positive effect of settlement rate in larval culture with higher incidence of light than in dark conditions (La Roche et al. 2005). Similar observations were registered by Burnell (1983), obtaining a higher larval settlement rate in *C. varia* culture with moderate light level by comparison with dark condition.

Gallardo et al. (2013) assessed the effect of different levels of light intensity on the swimming behavior in the larvae of the muricid gastropod *Chorus giganteus*. The highest percentage of pediveligers showed a significant increase in the swimming activity during dark periods, promoting a negative phototropism behavior probably due to a reduced level of protection against light radiation through a lesser development of chromatophores in the velum. However, in the muricid *Concholepas concholepas*, usually the larvae exhibit a higher incidence of movements with sunlight, related to the positive phototactism and development of structures that provide a protection towards the light radiation (Poulin et al. 2002).

Moreover, larvae of the fighting conch *Strombus pugilis* were reared from hatching to settlement under three photoperiods, with light phases of no light, 12 h of light (12L), and 24 h

per day (24L) to test their effect on development, growth, and survival. Under no light conditions, metamorphosis was lower. The settlement period was similar in 24L and 12L conditions. Continuous light had a negative effect on survival rate, while continuous darkness was advantageous.

Effect of flow water was evaluated in the development of post-larvae of *Pecten maximus*, which had a positive effect on the settlement of the larvae (Christophersen and Magnesen 2009). This observation was consistent with data concerning the settlement of *Mytilus trossulus* exposed to greater water flow (Eckman and Duggins 1998). The low flow water conditions in the culture may cause a high concentration of toxic residues harmful to the development of the larvae. On the other hand, in an attempt to induce early spat settlement and improve mussel seed production, the influence of water management, photoperiod, and aeration, on the growth, survival, and settlement of green mussel (*Perna viridis*), was evaluated. A higher rate of settlement and growth of larvae was influenced by a limited light regime and a longer period of darkness (Mero et al. 2019). The dark conditions promoted a higher feed intake; hence, a higher growth rate was obtained in the larvae, while an adequate water flow ensures sufficient aeration necessary to survival and larval settlement.

The embryonic and larval cultures present opportunities to study the biological control of shell ontogenesis mineralization (Wilt et al. 2003; Jackson et al. 2006, 2007). Many mollusks form a calcareous shell, composed of more aragonite than calcite (Ries 2011). On the other hand, Nehrke et al. (2012) observed three polymorphs in the shell of clam *Laternula elliptica*: aragonite, calcite, and vaterite. Shell formation starts with the differentiation of the shell gland from an invagination of ectodermal dorsal cells, in the early trochophore larvae (Jablonski and Lutz 1980). The shell gland evaginates to form the shell of protoconch I, whereas epithelial cells produce a thin organic layer (periostracum), providing the early support for $CaCO_3$ deposition (Jablonski and Lutz 1980).

The shell of marine mollusks is a composite biomaterial made of calcium carbonate intimately associated with organic matrix components secreted by mantle epithelial cells (Wilbur and Saleuddin 1983; Watabe 1998). The organic matrix, which accounts for 0.1–5% of the shell weight, is a mixture of proteins, glycoproteins, lipids, chitin, and acidic polysaccharides driving crystal nucleation, selecting the $CaCO_3$ polymorph and controlling the growth and spatial arrangement of minerals (Falini et al. 1996; Levi-Kalisman et al. 2001). The early steps of shell formation and initial stages of calcification in larval gastropods remain poorly studied and understood (Jablonski and Lutz 1980; Kniprath 1981; Eyster 1986; Bielefeld and Becker 1991; Collin and Voltzow 1998). The onset of mineralization has been studied in few gastropods (Iwata 1980; Bandel 1982; Eyster 1986; Page 1997; Hickman 2001).

In abalones, primary shell calcification occurs before larval torsion and the fact that protoconch II is completed (Collin and Voltzow 1998; Jackson et al. 2007; Jardillier et al. 2008). At metamorphosis, the change in the biomineralizing secretome allows the formation of juvenile shell in which the protoconch is fully integrated (Bevelander 1988; Jackson et al. 2007). Mineralogical composition was investigated in some species using X-ray micro-diffraction, extended X-ray absorption fine structure (EXAFS), infra-red (IR) spectroscopy, as well as micro-Raman spectroscopy (Medakovic et al. 1997; Hasse et al. 2000; Weiss et al. 2002; Marxen et al. 2003; Nehrke et al. 2012; Ramesh et al. 2018). In marine bivalves, larval prodissoconchs were found to contain a large amount of amorphous calcium carbonate (ACC), progressively transformed into aragonite (Weiss et al. 2002). In the marine gastropod *Haliotis tuberculate*, the mineral phase initially deposited in young larvae was essentially composed of aragonite, and then the presence of amorphous $CaCO_3$ was detected only in early trochophore

stages (Jardillier et al. 2008). Together, these observations supported the role of ACC as a transient precursor phase for mineral deposit in larval shell formation and probably also during adult shell growth (Addadi et al. 2003, 2006; Nassif et al. 2005; Jager and Colfen 2007; Jacob et al. 2008; Ramesh et al. 2018). The abalone *H. tuberculata* is a model organism to study the basic mechanisms of shell formation, particularly because of its shell contains both aragonite and calcite polymorphs. EDX analyses and IR spectroscopy demonstrated that early protoconch was mostly composed of amorphous calcium carbonate, while veliger stages showed a gradual crystallization under the form of aragonite. Post-metamorphic shell and juvenile were essentially made of aragonite (Auzoux-Bordenave et al. 2010).

In our study, the light micrographs acquired from larvae reared in laboratory and in natural conditions show significant differences. In individuals reared in laboratory conditions, from day 2 to day 8, the shells appeared complete without damages whereas in the case of the larvae grown in natural conditions, the shells exhibit strong damages since the 3rd after hatching. The shells are heavily broken in many parts revealing the brittle nature of their constituents. These observations suggested a difference of nature of the shell constituents leading to differences of mechanical behavior: in the case of larvae grown in laboratory conditions, the shells, up to day 8 after hatching, are constituted by a soft elastoplastic material (organic material or composite mineral organic material with low concentration of mineral species) whereas in the case of the larvae grown in marine environment, the shells are constituted of a solid and brittle material (crystalline mineral phase) since day 3 after hatching.

Raman spectra acquired at apex and at the periphery of shells of conch larvae grown in laboratory environment sampled up to 8 days after hatching do not exhibit any characteristic bands of crystallized $CaCO_3$ species whereas the Raman bands characteristic of aragonite structure (double peak at 697–701 cm^{-1} and bands at 170 and 220 cm^{-1}) are visible in the spectra acquired on larvae sampled as early as 3 days after hatching.

These analytical results seem to indicate that in laboratory conditions, larvae did not develop crystalline $CaCO_3$ species during early stages of shell construction (i.e., before 8 days of development), the shells being probably made of organic polymers. On the other hand, in marine environment, crystalline $CaCO_3$ (in aragonite phase only) is produced since the third day of development leading to a solid and brittle shell. The Raman study does not allow us to evidence differences between apex and periphery crystalline structure as far as the probe integrates The differences of growth, shell composition, and crystalline structure pointed out between larvae grown in mesocosm conditions are probably associated to the nutrients present in the natural food composed by various 21 species; 45% diatoms, 28% of cyanophytes, 18% of chlorophytes, and 9% of dinoflagellates (Hernandez et al. 2014; Aldana Aranda et al. 2015)

To date, most of the information about marine larval life come from laboratory experiments as it is difficult to obtain direct assessment of larval life span of marine organisms from oceans. Mesocosm appears to be ideal for conducting ecological research with marine invertebrate larvae in semi-controlled conditions. In this study, larvae of *S. gigas* raised in a mesocosm with natural phytoplankton as food not only demonstrated faster growth but also a better mineralization of the larval shell. Larvae grown in marine environment have a shell made up of a solid crystalline mineral phase which provides a greater resistance to the effect of waves and predation. The results obtained here are promising, and using mesocosm devices for culture of pelagic larvae must continue to be deeper studied for *S. gigas* larvae. Field conditions, as those encountered in mesocosm, provide positive effects in the process of mineralization of the shell of this tropical species.

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The nature of potential conflict of interest is described below:
Due to the disagreement of the study approach, we prefer that Eric Baqueiro and Anastazia Banaszak not be part of the reviewers of this manuscript.
Eric Baqueiro is retired without Institution to work and published scientific information of the Cinvestav that did not belong to him in Development and reproduction of invertebrates. Baqueiro Cardenas Erick PhD. Retired, ebaqueiro@gmail.com
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Declarations

Ethics approval

This article does not contain any studies with animals performed by any of the authors.

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