Use of response surface methodology to determine optimum diets for *Venerupis corrugata* larvae: effects of ration and microalgal assemblages

Alejandra Fernández-Pardo^a, Fiz da Costa^{b*}, Diego Rial^c, Susana Nóvoa^a, Dorotea Martínez-Patiño^a, José Antonio Vázquez^d

^aCentro de Cultivos Mariños, Consellería do Mar, Xunta de Galicia, Ribadeo (Lugo), Spain

^bNovostrea Bretagne, Route du Vieux Passage, Banastère, 56370 Sarzeau, France

^cCIIMAR Interdisciplinary Centre of Marine and Environmental Research, Laboratory of Ecotoxicology and Ecology, University of Porto, Rua dos Bragas, 289, 4050-123 Porto, Portugal

^dGrupo de Reciclado e Valorización de Residuos (REVAL), Instituto de Investigacións Mariñas (IIM-CSIC), C/Eduardo Cabello, 6, CP 36208, Vigo, Spain

***Corresponding author:** Fiz da Costa, E-mail address: fizdacosta37@gmail.com

Abstract

Microalgal quantity and quality are major factors affecting bivalve larval growth. Effects of ration using bi-specific diets assemblages with varying proportions of the flagellate *Isochrysis galbana* and the diatom *Chaetoceros neogracile* were evaluated on *Venerupis corrugata* larval development. Response surface methodology was used to

determine the optimal ration and better proportions of flagellate and diatom in the microalgal diet at different phases of larval development (early larva ($< 190 \mu\text{m}$, 10 days), 12 days, early pediveliger (15 days) and young postlarvae (26 days)). Maximum growth was obtained on day 10 for a diet of $22.5 \text{ cells } \mu\text{L}^{-1}$ of *I. galbana*, which suggests that *C. neogracile* was not ingested or digested by early larvae. On day 12 a maximum in the experimental domain was predicted for a diet of $22.5 \text{ cells } \mu\text{L}^{-1}$ of *I. galbana* and the lowest concentration of *C. neogracile* tested ($30 \text{ cells } \mu\text{L}^{-1}$), as *C. neogracile* reduced growth of the larvae at higher concentrations. On day 15, maximum growth was found at $42.6 \text{ cells } \mu\text{L}^{-1}$ of *C. neogracile* and $27.9 \text{ cells } \mu\text{L}^{-1}$ of *I. galbana*. During metamorphosis and early postlarval growth optimal ration was $70 \text{ cells } \mu\text{L}^{-1}$ of a bi-specific diet in similar proportions to that previous stage of development. This novel approach to study bivalve larval nutrition allows the quality and quantity of the optimal diet to be determined.

Keywords

Clam, larvae, response surface methodology, ration, microalgae

Abbreviations

b_0 = constant coefficient of the model

b_i = coefficient of linear effect of the model

b_{ij} = coefficient of interaction effect of the model

b_{ii} = coefficients of squared effect of the model

I = *Isochrysis galbana*

I_{max} = Concentration of *I. galbana* that maximizes clam growth

C = *Chaetoceros neogracile*

51 C_{max} = Concentration of *C. neogracile* that maximizes clam growth

52 EFA = Essential fatty acid

53 F_{den}^{num} = Theoretical values to $\alpha=0.05$ with the corresponding degrees of freedom for

54 numerator (num) and denominator (den)

55 G = Growth of the clam

56 G_{max} = Maximal growth

57 n = Number of variables

58 R_{adj}^2 = Adjusted determination coefficient

59 V_0 = natural value in the center of the domain

60 V_c = codified value of the variable

61 V_n = natural value of the variable to codify

62 ΔV_n = increment of V_n per unit of V_c

63 X_i and X_j = independent variables

64

65 **Statement of relevance**

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67 This study is really relevant to the field of bivalve larval production because it defines

68 the quality and quantity of the optimal diet for larvae of the clam *Venerupis corrugata*.

69 The information provided in this paper has great applicability for clam larval production

70 in commercial hatcheries.

71

72 **Highlights**

73 • Response surface methodology is a powerful tool for nutritional studies in bivalve

74 larvae.

- Early larval nutrition (until at least day 10) is dominated by the ingestion of small microalgae.
- Larger larvae (> 190 µm) efficiently ingested and digested *Chaetoceros neogracile*.

1. Introduction

Clam production is of great economic interest along the Spanish coasts (Albentosa and Moyano, 2009). Its production only partially fills the existing gap of Spanish domestic market demand, with the rest being filled by imports from Italy, the United Kingdom, France and Portugal (Jiménez-Toribio et al., 2003). Among clam species, *Venerupis corrugata* (= *V. pullastra*) is of great importance in Galicia due to its commercial value and it is also commercially exploited in Portugal, France and Italy (Joaquim et al., 2014). However, in recent years decreases have been observed in natural populations. Data provided by the Statistical Service of Consellería do Mar de Galicia show that captures dropped from 2,700 t in 1998 (which represented 58% of total clam captures) to 1,129 t in 2013 (30% of clam captures). This progressive decline could be due to the high sensitivity of this species to salinity variations, failure in recruitment and over-fishing. For this reason, the production of hatchery seed for restocking throughout the year has become essential to alleviate the dependence on unreliable natural spatfall and ensure the sustainability of *V. corrugata* fisheries.

The criteria for selecting a suitable algal diet for bivalve larvae are based upon morphology (especially size), ease of culture, the absence of toxicity and ability of the larvae to trap, ingest, digest and assimilate the algae (Marshall et al., 2010). *Isochrysis galbana* and *Chaetoceros neogracile* are two microalgal species widely used as larval feed in bivalve hatcheries due to their easy culture (Coutteau and Sorgeloos, 1992;

Robert and Trintignac, 1997). The flagellate *I. galbana* is rich in the essential fatty acid (EFA) 22:6n-3 (docosahexaenoic acid: DHA); whereas, the diatom *C. neogracile* is rich in 20:5n-3 (eicosapentaenoic acid: EPA) and also contains greater quantities of 20:4n-6 (arachidonic acid: AA) than *I. galbana* (Volkman et al., 1989). In addition, the combination of a flagellate and a diatom is commonly used as bivalve larval diet (Robert and Trintignac, 1997). High growth rates were observed in *Ruditapes philippinarum* larvae when fed a mixture of *I. affinis galbana* (Tiso), recently renamed *Tisochrysis lutea* (Bendif et al., 2013), and *Chaetoceros calcitrans* (Laing and Utting, 1994; Laing et al., 1990). Moreover, the combination of *T. lutea* and the diatom of genus *Chaetoceros* led to the best growth in *Crassostrea gigas* (Rico-Villa et al., 2006) and *Ostrea edulis* larvae (Gonzalez-Araya et al., 2012).

Several studies have investigated the effect of food quantity on bivalve larval growth (e.g. Beiras and Pérez-Camacho, 1994; Liu et al., 2010; Marshall et al., 2014; Rico-Villa et al., 2009). However, the effect of food ration for *V. corrugata* larvae has never been studied. Moreover, our knowledge in larval nutrition of *V. corrugata* is limited because data are scarce. Novoa et al. (2002) showed however that *V. corrugata* can incorporate the EFA AA when delivered in gelatin-acacia microcapsules. Fernández-Reiriz et al. (2011) highlighted that *V. corrugata* larvae fed *Tetraselmis suecica*, a diet deficient in DHA, exhibited low growth and high mortality. However, to date, no standard larval diet for this clam species has been established.

Response surface methodology is a collection of statistical and mathematical techniques useful for optimizing processes (Baş and Boyacı, 2007). The model defines the effect of independent variables on a response of interest with the aim of optimizing this response. Moreover, this methodology generates an empiric mathematical model which describes the process. In marine bivalves, it has been applied to larvae in

ecotoxicological studies (Deruytter et al., 2013; Hrs-Brenko et al., 1977) or to investigate the effect of environmental variables on embryonic and larval development (Tettelbach and Rhodes, 1981). To the best of our knowledge, this methodology has not been currently assigned to bivalve nutritional studies.

The aim of this study is to determine the optimal diet ration with accurate proportions of *I. galbana* and *C. neogracile* for *V. corrugata* larval development. For this, a novel experimental design in nutritional studies with bivalve larvae is proposed in order to explore the relationships between the concentrations of *I. galbana* and *C. neogracile* and larval growth at different times of culture.

2. Materials and Methods

2.1. Microalgal culture

The microalgae *Chaetoceros neogracile* and *Isochrysis galbana* were grown in a continuous culture system in 400 L polyethylene bags held in plastic mesh frames, based on that used by SeaSalter Shellfish Company Ltd. (Farrar, 1975). Stock cultures were part of the culture collection of the Centro de Cultivos de Ribadeo-CIMA and were kept in an isothermal chamber in 20 mL tubes at 19 ± 1 °C. Inocula were transferred to 250 mL Erlenmeyer flasks and then cultured in 2 L and 6 L glass carboys at a temperature of 19 ± 1 °C under continuous illumination at $180\text{--}220 \mu\text{photons m}^{-2} \text{s}^{-1}$. Seawater was 1- μm filtered, autoclaved and enriched with sterilized Algal-1 medium (supplied by Nutrición Avanzada, S.A., A Coruña, Spain). Microalgae inoculation of the continuous system was performed with 6 L glass carboys at late-exponential phase and previously checked under a microscope to avoid including any contamination and ensure the purity of the culture. Culture bags were illuminated by natural and artificial

light under a photoperiod regime of 18:6 h of light:darkness in a greenhouse. The artificial illumination was provided by vertical “daylight” fluorescent lamps (Philips TL-D) at 180-220 $\mu\text{photons m}^{-2} \text{s}^{-1}$. Incoming water was sterilized by pasteurization at 75 °C for 30 min. Continuous aeration was provided to prevent the algae from settling. Moreover, CO₂ addition allowed pH maintenance between 7 and 8, which was daily measured in the outflow of the system. Seawater was maintained at 21 $\pm$ 1 °C and enriched with the following medium prepared in distilled water: EDTA (C₁₀H₁₄N₂Na₂O₈·2H₂O) 40 g L⁻¹, K₂PO₄H 16 g L⁻¹, NO₃K 80 g L⁻¹, Fe₂S₃O₁₂ 1.24 g L⁻¹, MnSO₄+H₂O 124 mg L⁻¹, ZnSO₄+H₂O 17.6 mg L⁻¹, CuSO₄+5H₂O 1.6 mg L⁻¹, CoSO₄+H₂O 1.6 mg L⁻¹, NaMo₄+2H₂O 0.8 mg L⁻¹, thiamine 100 mg L⁻¹, cyanocobalamin 5 mg L⁻¹ and biotin 1.25 mg L⁻¹. Culture medium was added constantly (1 mL L⁻¹ of algal culture); whereas, sodium sulphate was supplied to the diatom culture twice a week. Algae were harvested at exponential growth phase and maintained in this phase during 2 months under a dilution rate of 0.125 day⁻¹. Each species used in this experiment was harvested and daily controlled under a light microscope. Algal concentration was determined in a Bürker-Turk counting chamber.

2.2. Broodstock collection and larval culture

Adult specimens of *Venerupis corrugata* were collected in a natural bed in an intertidal zone of Cangas in Ría de Vigo (Galicia, NW Spain) and transferred cooled at 4 °C to the Centro de Cultivos de Ribadeo-CIMA. Broodstock were maintained in 200 L rectangular tanks at 18 $\pm$ 1 °C in a flow through at ambient salinity of 32-33 ppt, with a continuous supply of a mixture of *I. galbana*, *Diacronema lutheri* (= *Pavlova lutheri*), *Tetraselmis suecica* and *Chaetoceros* sp. in equal proportions (equivalent number of

cells), representing a ration of 6% of dry meat weight in dry algal weight per day. A “natural” (not induced) spawning was collected in a 45 µm-mesh and transferred to 500 L larval culture tanks with aerated and filtered UV-irradiated seawater at 18 ± 1 °C for incubation. No food was supplied during embryo incubation. From day 2 onwards, larvae were daily fed a mixture of *I. galbana* and *C. neogracile* (1:1) at a rate of 40 cells μL^{-1} as an initial ration. On day 5, the experiment with different diets was initiated. Larvae were randomly split into 50 L tanks (batch culture) at an initial density of 5 larva mL^{-1} . The temperature was set at 18 ± 1 °C and controlled by a thermostat. Seawater was completely renewed every two days using 1-µm filtered and UV-sterilized seawater. Salinity throughout the larval rearing was 32 ± 1 ppt. Food was added daily, in the morning, to each tank. Larvae from each diet were collected every two days with nytex screens and shell length was achieved on 100 randomly selected individuals per tank using a binocular microscope (Nikon Labophot-2) connected to an image analyzer (NIS-Elements BR 3.0 Nikon). On day 19, pediveliger larvae were ready to settle and they were transferred to sieves in the same larval rearing tanks with a down-welling system to undergo settlement. On day 26, the experiment ended.

2.3. Experimental design and statistical analysis

The growth of clam (G , in µm) as a function of two microalgae concentration, *C. neogracile* (C) and *I. galbana* (I) was studied using a rotatable second order design following the characteristics reported by Box et al. (2005) and Akhnazarova & Kafarov (1982). It is based on the combination of five levels of the two studied variables (in coded values): -1.41, -1, 0, 1 and 1.41 to generate 8 run experiments and 5 replica experiments in the center of the experimental domain (0,0) in order to assure a full

statistical analysis with reduced risk of type I and type II errors. Thus, the replicates were made in a single experimental point (center of the experimental domain). The conditions of the independent variables studied were: C in the range (30-65 cells μL^{-1}) and I in the range (5-40 cells μL^{-1}). The encoding procedure of the variables was performed by the following formulas:

Codification	Decodification
$V_c = (V_n - V_0) / \Delta V_n$	$V_n = V_0 + (\Delta V_n \times V_c)$
V_n = natural value of the variable to codify V_c = codified value of the variable V_0 = natural value in the centre of the domain ΔV_n = increment of V_n for unit of V_c	

Both expressions of the independent variables, codified and natural values, in each experimental run are summarized in Table 1. Orthogonal least-squares calculation on factorial design data was used to obtain (Box et al., 2005), empirical equations describing the clam growth (G) as dependent variable related to C and I nutrition effects. The general form of the polynomial equations is:

$$G = b_0 + \sum_{i=1}^n b_i X_i + \sum_{i=1}^{n-1} \sum_{j=2}^n b_{ij} X_i X_j + \sum_{i=1}^n b_{ii} X_i^2 \quad (1)$$

where G represents the clam growth response to be modelled, b_0 is the constant coefficient, b_i is the coefficient of linear effect, b_{ij} is the coefficient of interaction effect, b_{ii} the coefficients of squared effect, n is the number of variables and X_i and X_j define the independent variables (C and I). The statistical significance of the coefficients was verified by means of the Student t-test ($\alpha=0.05$); goodness-of-fit was established as the

adjusted determination coefficient (R_{adj}^2) and the model consistency by the Fisher F test ($\alpha=0.05$) using the following mean squares ratios:

the model is acceptable when

$$F1 = \text{Model} / \text{Total error}$$

$$F1 \geq F_{den}^{num}$$

$$F2 = (\text{Model} + \text{Lack of fitting}) / \text{Model}$$

$$F2 \leq F_{den}^{num}$$

$$F3 = \text{Total error} / \text{Experimental error}$$

$$F3 \leq F_{den}^{num}$$

$$F4 = \text{Lack of fitting} / \text{Experimental error}$$

$$F4 \leq F_{den}^{num}$$

F_{den}^{num} are the theoretical values to $\alpha=0.05$, with the corresponding degrees of freedom for numerator (num) and denominator (den). All fitting procedures, coefficient estimates and statistical calculations were performed on a Microsoft Excel spreadsheet.

2.4. Fatty acid analysis of microalgae

Microalgal samples (80×10^6 cells) were collected during all larval culture (diets were temporally replicated, $n = 3$). Samples were filtered through pre-combusted GF/F glass fiber filters at 450°C and washed with 3 % ammonium formate to remove salt. Lipids were extracted in 6 ml chloroform–methanol (2:1, v/v) according to Folch et al. (1957), sealed under nitrogen, and stored at -20 °C. Fatty acids (FA) of microalgae were quantified relative to total lipids. The saturated FA 23:0 was added as an internal standard for FA quantitative measurements. Samples were evaporated under nitrogen and transesterified with 1 ml of $\text{BF}_3\text{-MeOH}$ (10 %) for 10 min at 100 °C (Metcalf and Schmitz, 1961). After cooling, FA methyl esters were extracted with hexane. FA composition and quantification were determined using gas chromatography (Variant,

CP-3800), equipped with a fused silica capillary column (JW SCIEN, 30 m length, 0.25 mm i.d., 0.25 μ m film thickness), with a cool on-column injector at 63 °C. The carrier gas was H₂, at an initial pressure of 80 kPa. The oven was programmed to stay at an initial temperature of 60 °C for 2 min, increase from 60 to 160 °C at a rate 50 °C min⁻¹, stay there for 2 min, then increase from 160 to 170 °C at 1.5 °C min⁻¹, from 170 to 185 °C at 2 °C min⁻¹, from 185 to 240 °C at 3 °C min⁻¹, and finally remain at 240 °C.

FAs were identified by comparing their retention times with those of standards. A response factor was calculated for each FA in order to perform quantitative analyses.

3. Results

3.1. Fatty acid composition of microalgae

The strain *I. galbana* showed a high proportion of 18:4n-3 (28%), 14:0 (15%), DHA (14%) and 18:1n-9 (10%), whereas *C. neogracile* was richer in EPA (21%), 14:0 (18%) and 16:1n-7 (16%, Table 2). AA was significantly higher in *C. neogracile* than in *I. galbana* (0.7 vs. 0.1%, respectively; Table 2). *C. neogracile* stored more total FA than *I. galbana* (2.4 vs. 1.6 pg cell⁻¹; Table 2).

3.2. Larval growth

The nutritive capacity of two microalgae (*C. neogracile*, *I. galbana*) and their combined effect on clam growth at different times of culture was studied by means of response surface methodology. Growth curves for each diet treatment are shown in Figure A (Supplementary material). The design and responses (experimental and predicted) of the 2-factor rotatable design are summarized in Table 1. Data from clam

growth were converted into second-order polynomial equations as a function of two independent variables (C and I). Consequently, the polynomial model describing the correlation between the response and the variables followed the general form defined by equation [1] (Table 3). A remarkable proportion of the variability in larval clam growth (>74%) can be successfully explained by the second order equations at three different times of the culture, which indicates a good agreement between the observed and theoretical values. The consistency of equations was high in three cases (on days 12, 15 and 26, all Fisher F tests were significant) and good for assessment on day 10 (only $F1$ and $F2$ were significant). These models can therefore be considered good predictors for clam growth in the range of microalgae concentration studied.

The results of the multivariate analysis showed that the statistical significance of coefficients was dependent on the time of culture selected. For instance, on days 15 and 26 the quadratic parameters for both variables (C and I) were negative and significant ($p<0.05$), while on days 10 and 12 growth was only affected by I and C linear terms respectively and for both days by the I quadratic term. The coefficient of interaction among variables ($C \times I$) was only significant on day 15. Figure 1 displays the theoretical growth surfaces at different times of clam cultivation corroborating the observed variability in the responses.

By individually analyzing each response we can establish the following conclusions:

- 1) On day 10, the only microalgae effective is I and the highest clam growth ($G_{max}=189.1 \mu\text{m}$) is achieved at $29.8 \text{ cells } \mu\text{L}^{-1}$ of *Isochrysis* regardless of *Chaetoceros* proportion (C had no effect on growth response). These theoretical

optima values summarized in Table 4 were in all cases calculated by numerical derivative and finding local maxima (Wardhani et al., 2010).

2) On day 12, both microalgae showed significant effects on clam growth, C linearity and I by quadratic way. Thus, value of $G_{max}=216.2 \mu\text{m}$ was obtained when clams were fed with I_{max} of $22.5 \text{ cells } \mu\text{L}^{-1}$ (Table 4).

3) On day 15, all linear and quadratic terms were significant for both microalgae. The convex surface showed a well-defined maximum growth ($243.7 \mu\text{m}$) at $42.6 \text{ cells } \mu\text{L}^{-1}$ of C and $27.9 \text{ cells } \mu\text{L}^{-1}$ of I (Table 4).

4) Finally, another theoretical convex surface was obtained on day 26 with optimum growth ($411.1 \mu\text{m}$) showing similar values ($I_{max}= 22.5$ and $C_{max}= 47.5 \text{ cells } \mu\text{L}^{-1}$) to those obtained on day 15 (Table 4).

4. Discussion

Factorial design is a dedicated statistical tool to determine optimal values of independent variables and empirical predictive equations performing a reduced number of experiments. The use and validity of this approach are in agreement with the findings of previous works studying the growth of different marine organisms. Thus, the increase of rotifer (*Brachionus plicatilis*) production using a combination of lactic acid bacteria was optimized by means of a first order factorial design (Planas et al., 2004). The joint effect of different environmental variables (irradiance, temperature and salinity) on the kinetic parameters of *Protoceratium reticulatum* growth was also evaluated by a similar design (Paz et al., 2006). The influence of salinity, temperature and inoculum size on regulating dinoflagellate *Alexandrium minutum* life-cycle (planozygote and resting-cyst

formation) was successfully studied using that target statistical approach (Figuerola et al., 2011).

The differences observed on the significant parameters of the equations (Table 3) could be due to the fact that the culture times selected were representative of each phase that define the conventional sigmoid curve of the clams and microorganism growing under batch conditions (initial, exponential and plateau). Thus, the nutritive needs in each clam phase are different and change as a function of the metabolic dynamic of the culture (Matias et al., 2011). Nevertheless, in the four cases presented, the quadratic effect of *I* was always significant and the optima values for *I* and *C* were very similar (Table 3).

Bivalve larval production in hatcheries is undoubtedly related to the quality and the quantity of the supplied microalgae (Helm and Bourne, 2004). The choice of the microalgal species to be used as feed for larvae is of utmost importance. Nutritional value of microalgae for bivalve larvae depends on several criteria such as size, shape, availability in the water column, digestibility, biochemical profile and culture productivity (Brown et al., 1989; Robert et al., 2004). In the present study, *V. corrugata* larvae were fed a combination of *Isochrysis* and *Chaetoceros* species, which has been reported to be successful for bivalve larval rearing (Gonzalez-Araya et al., 2012; Matias et al., 2014; Rico-Villa et al., 2006). We observed great differences in larval growth related to the dietary concentration of *Isochrysis* and *Chaetoceros* in each larval developmental stage. This can be explained because the food value of a given microalga depends on both the mollusk species and growth stage considered (Brown et al., 1997; Knauer and Southgate, 1999).

On day 10, larval growth was unaffected by *C. neogracile* concentration, whilst increasing rations of *I. galbana* up to 29.8 cells μl^{-1} maximized growth. One possible

explanation for the lack of response for increasing *C. neogracile* concentrations can be microalgal size, since it is an important determinant of ingestion rate in bivalve larvae (Rico-Villa et al., 2006). *C. neogracile* has a volume of $77 \mu\text{m}^3$ (5.3 μm of relative diameter) compared to $45 \mu\text{m}^3$ in *I. galbana* (4.5 μm of relative diameter) (Robert et al., 2004). Retention of particles by bivalve larvae is dependent on mouth size and gut diameter, which increase as larvae grow (Raby et al., 1997). However, ingestion size ranges can be also species-dependent in larvae of different bivalve species with a similar size (Raby et al., 1997; Marshall et al., 2010). In fact, 5 and 13 day-old *Mytilus edulis* larvae (150 and 170 μm in length) could not ingest particles smaller than 1 μm or larger than 9 μm in diameter (Riisgard et al., 1980). Moreover, a preferential selection of different size microalgae was described depending on larval size. Small larvae (150 μm) fed on cultured algal mixtures (size range from 1 to 11 μm) preferred 1 μm algae, whereas larger larvae preferentially ingested 11 μm algae (Baldwin, 1995). *Crassostrea gigas* larvae between day 2 and day 8 ingested significantly less *C. neogracile* than *Tisochrysis lutea* (*I. affinis galbana* clone T1so), which has similar size and nutritional value than *I. galbana* (da Costa et al., 2015). *Ruditapes philippinarum* larvae exhibited the highest ingestion rates with particles ranging from 1.4 to 2.0 μm (Tezuka et al., 2009). This observation can explain the lack of response at increasing concentrations of *C. neogracile* when supplied to *V. corrugata* larvae. In fact, Matias et al. (2014) reported that *Ruditapes decussatus* larvae cannot use *Chaetoceros calcitrans* during early development.

Moreover, the presence of lateral spines in *C. neogracile* may also interfere with larval feeding in smaller larvae of the clam *V. corrugata*, as suggested by Ragg et al. (2010) in *Perna canaliculus* larvae fed *C. calcitrans*. Larvae of the Catarina scallop *Argopecten ventricosus-circularis* did not ingest *C. calcitrans* and *Chaetoceros muelleri*

until day 6 and 7, respectively, due to the presence of extracellular spines (Lora-Vilchis and Maeda-Martinez, 1997). Rose and Baker (1994) also suggested that the poor growth and delayed metamorphosis of pearl oyster *Pinctada maxima* larvae fed *C. neogracile* may be partially attributed to the presence of elongated spines in this diatom. In the case of clams, Matias et al. (2014) suggested that the poor growth observed in *R. decussatus* larvae until day 13 when fed the monospecific diet *C. calcitrans* was due to the presence of silica rods.

On day 10, at low *I. galbana* concentrations larval growth was significantly reduced since *V. corrugata* larvae did not ingest or digest *C. neogracile*. The nutritional value of microalgae depends on algal digestibility and biochemical composition (Robert and Trintignac, 1997). Diatoms with a silica frustule have lower digestibility than microalgae with organic cell walls (Robert and Trintignac, 1997). Lora-Vilchis and Maeda-Martinez (1997) reported that the digestion index for *C. calcitrans* and *C. muelleri* by *A. ventricosus-circularis* larvae was lower than those for *T. lutea*.

On day 12, growth of *V. corrugata* larvae decreased linearly when fed increasing rations of *C. neogracile* from 30 to 65 cells μL^{-1} . At this stage, larvae seem to ingest and digest limited quantities of this diatom. We cannot, however, discard that lower *C. neogracile* ration might improve growth. The observed pattern of growth can be explained by the fact that increasing proportions of *C. neogracile* may not meet nutritional requirements of *V. corrugata* larvae. *I. galbana* and *C. neogracile* differs greatly in essential fatty acid profile. *I. galbana* is rich in DHA, whereas, *C. neogracile* is rich in EPA and also contains greater quantities of AA than *I. galbana* (Volkman et al., 1989). Low supply of DHA in diets with increasing proportions of *C. neogracile* may explain the reduction of growth, since the lack of this fatty acid increased mortality in *V. corrugata* larvae when fed *Tetraselmis suecica*, which lacks DHA (Fernández-

Reiriz et al., 2011). In contrast, *R. decussatus* larvae fed *C. muelleri* alone or in combination with *I. galbana* and *Diacronema lutheri* exhibited the highest growth, even when DHA dietary supply was low (Aranda-Burgos et al., 2014).

V. corrugata larvae nearly reached pediveliger stage and metamorphosed on days 15 and 26, respectively (Cerviño-Otero, 2011; Fernández-Reiriz et al., 2011). The convex surface showed a well-defined maximum growth at 42.6 and 47.5 cells μL^{-1} of *C. neogracile* and 27.9 and 22.5 cells μL^{-1} of *I. galbana* on days 15 and 26, respectively. In both cases, *C. neogracile* represents $\approx 70\%$ and *I. galbana* $\approx 30\%$ of the diet when referred to microalgal volume. This is agreement with the observations of Rico-Villa et al. (2006) for *C. gigas* larvae who reported maximized growth when larvae were fed 75% of *Chaetoceros calcitrans* forma *pumilum* (Cp) and 25% of *T. lutea* (T) in a volume base (75Cp/25T) and 50Cp/50T. *R. decussatus* larvae exhibited the highest growth rates when fed the bi-specific diet *T. lutea* and *C. calcitrans* (60/40 cells μL^{-1}) (Matias et al., 2014).

As previously mentioned, late larvae and young postlarvae of *V. corrugata* exhibited the highest growth when *C. neogracile* represented $\approx 70\%$ of the diet referred to microalgal volume. This may suggest a preferential need for EPA and AA in late larval development, metamorphosis, and young postlarval growth in *V. corrugata*. EPA fulfills a role as both an energy source and a precursor of eicosanoids in bivalve larvae (Howard & Stanley, 1999; Marty et al., 1992). The importance of AA in invertebrate species is due to its role in eicosanoid production and stress response (Howard and Stanley, 1999). Aranda-Burgos et al. (2014) highlighted that EPA should be more important than DHA for a good larval performance in *R. decussatus*. These authors also reported that increasing proportion of dietary AA led to higher growth in clam larvae.

AA also plays a role in enhancing growth in *Mytilus galloprovincialis* larvae (Pettersen et al., 2010) and *Placopecten magellanicus* post larvae (Milke et al., 2008).

The best treatments in terms of growth were those supplying algal concentrations of $\approx 25\text{-}30$ cells μL^{-1} of *I. galbana* and 70 cells μL^{-1} of a mixture of *I. galbana* and *C. neogracile* until days 10-12 and 15-25, respectively. This is slightly lower than the cell concentrations reported for the high growth rate of larvae of *Ruditapes philippinarum* (200 cells μL^{-1} of *C. calcitrans*) (Utting and Doyou, 1992), *Pecten maximus* (100 cells μL^{-1} of *C. calcitrans* f. *pumilum* and *Pavlova* sp. AC 250) (Ponis et al., 2006), *C. gigas* (100 cells μL^{-1} of *T. lutea* and *C. calcitrans* f. *pumilum*) (Brown and Robert, 2002), and *Ostrea edulis* (100 to 200 cells μL^{-1} of *I. galbana*) (Beiras and Pérez-Camacho, 1994).

Our data with *V. corrugata* larvae showed that growth was significantly reduced at low microalgal concentrations, whereas increasing rations favored growth until a limit and thereafter a reduction of growth occurred. Low food availability increases larval energy loss during food searching, while at the same time larvae ingest less (Marshall et al., 2014). Consequently, this may reduce larval growth (Ponis et al., 2003; Tang et al., 2006) due to depletion of endogenous biochemical reserves (Holland and Spencer, 1973). Contrary, excessive feed levels may led to reduced growth rates (Loosanoff et al., 1953) due to rapid saturation of the gut and increased particle rejection by larvae caused by high encounter rates with algae (Gallager, 1988).

In summary, our results clearly show the importance of providing *V. corrugata* larvae with the appropriate diet during each developmental stage. Larvae should be fed *I. galbana* alone until day 10-12 and a mixed diet of *I. galbana* and *C. neogracile* should be provided for larger larvae. This strategy will allow larvae to grow faster and have a higher quality. Since the food is utilized more efficiently, microalgal algal

production costs can be kept to a minimum (Laing and Millican, 1986). In our study, we used the diatom *C. neogracile*, but it may be possible that other diatoms with lower cellular volume, such as *Chaetoceros* sp. “*minus*”, *C. sp. tenuissimus* and *C. calcitrans forma pumilum* (Robert et al., 2004), may be more adapted for feeding early larvae of *V. corrugata*. We cannot discard that the inclusion of *Chaetoceros* at early stages of development improves larval performance in late development, as previously suggested for *R. decussatus* larvae by Matias et al. (2014). Further nutritional studies of *V. corrugata* larval stages including proximate and fatty acids analysis of larvae should be carried out in order to improve (1) our knowledge of clam larval nutrition and (2) larval performance.

5. Conclusions

In this study, an experimental design to determine the effects of two microalgae (*Isochrysis* and *Chaetoceros*) on clam growth was performed. In general, *Isochrysis* showed the most important effects on the growth of early *V. corrugata* larvae. In late larval development and young postlarvae, the best growth was reached at the concentration ranges of 23-30 cells μL^{-1} and 43-48 cells μL^{-1} for *Isochrysis* and *Chaetoceros*, respectively. The predictive capacity of the empirical equations and statistical results obtained in the present proposal revealed the great importance of the factorial design technique in order to define and optimize complex effects among environmental and nutritive factors, specifically in the aquaculture area, in which such approach is not commonly explored.

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Tables

Table 1 Summary of the independent variables in the response surface design with the corresponding experimental (G_{exp}) and predicted (G_p) clam growth at different culture times. X_1 : *Chaetoceros neogracile* concentration (cells μL^{-1}) and X_2 : *Isochrysis galbana* concentration (cells μL^{-1}). Natural values of experimental conditions are in brackets.

Independent variables		10 days		12 days		15 days		25 days	
X_1 : <i>Cht</i> (C)	X_2 : <i>Iso</i> (I)	G_{exp}	G_p	G_{exp}	G_p	G_{exp}	G_p	G_{exp}	G_p
-1 (35.1)	-1 (10.1)	176.6	179.1	199.9	208.7	230.0	231.5	357.3	351.9
1 (59.9)	-1 (10.1)	174.4	179.1	196.9	202.8	223.6	224.8	391.3	394.7
-1 (35.1)	1 (34.9)	185.4	188.4	206.2	208.7	242.7	240.5	388.5	394.7
1 (59.9)	1 (34.9)	186.1	188.4	205.3	202.8	235.0	233.7	336.7	351.9
-1.41 (30)	0 (22.5)	188.7	187.8	220.3	215.9	237.9	238.3	369.7	368.3
1.41 (65)	0 (22.5)	191.5	187.8	206.0	207.5	228.8	228.8	376.6	368.3
0 (47.5)	-1.41 (5)	176.9	173.3	204.8	199.8	227.4	225.4	366.6	378.8
0 (47.5)	1.41 (40)	188.7	186.4	200.7	199.8	235.6	238.1	401.4	378.8
0 (47.5)	0 (22.5)	188.4	187.8	213.4	211.7	239.8	242.0	405.2	411.1
0 (47.5)	0 (22.5)	187.8	187.8	212.3	211.7	243.8	242.0	405.6	411.1
0 (47.5)	0 (22.5)	188.4	187.8	207.8	211.7	240.5	242.0	412.2	411.1
0 (47.5)	0 (22.5)	186.3	187.8	213.3	211.7	244.0	242.0	421.2	411.1
0 (47.5)	0 (22.5)	192.8	187.8	211.7	211.7	242.0	242.0	411.1	411.1

Codification: $V_c = (V_n - V_0) / \Delta V_n$; Decodification: $V_n = V_0 + (\Delta V_n \times V_c)$

V_n =natural value in the centre of the variable to codify; ΔV_n = increment of V_n per unit of V_c ; V_c =codified value of the variable; V_0 = natural value in the centre of the domain

677 **Table 2** Fatty acid composition of the total lipids (wt% of total fatty acids $\pm$ SD, n = 3)
678 of *Isochrysis galbana* and *Chaetoceros neogracile*.

	<i>Isochrysis galbana</i>	<i>Chaetoceros neogracile</i>
14:0	15.1 $\pm$ 0.7	18.1 $\pm$ 0.3
16:0	9.1 $\pm$ 0.6	5.7 $\pm$ 0.3
18:0	0.5 $\pm$ 0.2	0.6 $\pm$ 0.0
20:0	1.7 $\pm$ 0.1	0.0 $\pm$ 0.0
16:1n-7	5.5 $\pm$ 0.0	15.5 $\pm$ 0.2
18:1n-11	0.0 $\pm$ 0.0	0.5 $\pm$ 0.0
18:1n-9	10.1 $\pm$ 0.5	1.0 $\pm$ 0.0
18:1n-7	1.6 $\pm$ 0.1	0.8 $\pm$ 0.0
18:2n-6	2.8 $\pm$ 0.0	0.3 $\pm$ 0.0
18:3n-3	8.1 $\pm$ 0.7	1.4 $\pm$ 0.0
18:4n-3	27.6 $\pm$ 1.3	0.5 $\pm$ 0.0
20:4n-6	0.1 $\pm$ 0.0	0.6 $\pm$ 0.0
20:5n-3	0.7 $\pm$ 0.0	21.1 $\pm$ 1.4
22:5n-6	1.3 $\pm$ 0.0	0.1 $\pm$ 0.0
22:6n-3	13.6 $\pm$ 0.3	1.4 $\pm$ 0.1
Σ SFA	26.6 $\pm$ 1.4	24.6 $\pm$ 0.5
Σ MUFA	17.5 $\pm$ 0.5	18.7 $\pm$ 0.3
Σ n-9	10.4 $\pm$ 0.5	1.2 $\pm$ 0.0
Σ n-7	7.1 $\pm$ 0.0	16.3 $\pm$ 0.2
Σ PUFA	55.9 $\pm$ 0.9	56.7 $\pm$ 0.8
Σ n-4	1.0 $\pm$ 0.0	22.1 $\pm$ 0.8
Σ n-6	4.7 $\pm$ 0.1	1.4 $\pm$ 0.0
Σ n-3	51.4 $\pm$ 0.9	26.0 $\pm$ 1.7
n-3/n-6	10.9 $\pm$ 0.4	18.9 $\pm$ 1.7
22:6/20:5	20.6 $\pm$ 1.6	0.1 $\pm$ 0.0
22:5/20:4	7.8 $\pm$ 0.9	0.5 $\pm$ 0.0
Total FA (pg cell ⁻¹)	1.6 $\pm$ 0.4	2.4 $\pm$ 0.1

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Table 3 Second order equations describing the effect of the microalgae concentration (C: *Chaetoceros neogracile* and I: *Isochrysis galbana*) on clam growth (G) at different times of culture (used in coded values according to criteria defined in Table 1). The coefficient of adjusted determination (R_{adj}^2) and F-values (F_1 , F_2 , F_3 and F_4) is also shown. S: significant; NS: non-significant.

Parameters	10 days	12 days	15 days	25 days
b_0 (intercept)	188.75	211.72	242.01	411.08
b_1 (C)	-	-2.98	-3.37	-
b_2 (I)	4.66	-	4.49	-
b_{12} (C×I)	-	-	-	-21.44
b_{11} (C ²)	-	-	-4.24	-21.54
b_{22} (I ²)	-3.97	-5.98	-5.17	-16.24
R_{adj}^2	0.736	0.552	0.907	0.783
F_1	17.72 [$F_{10}^2 = 4.10$] ⇒ S	8.40 [$F_{10}^2 = 4.10$] ⇒ S	30.39 [$F_8^4 = 3.84$] ⇒ S	15.41 [$F_9^3 = 3.86$] ⇒ S
F_2	0.32 [$F_2^8 = 19.4$] ⇒ S	0.38 [$F_2^8 = 19.37$] ⇒ S	0.52 [$F_4^8 = 6.04$] ⇒ S	0.44 [$F_3^8 = 8.85$] ⇒ S
F_3	10.49 [$F_4^{10} = 5.96$] ⇒ NS	3.65 [$F_4^{10} = 5.96$] ⇒ S	1.23 [$F_4^8 = 6.04$] ⇒ S	3.24 [$F_4^9 = 5.99$] ⇒ S
F_4	16.81 [$F_4^6 = 6.16$] ⇒ NS	5.41 [$F_4^6 = 6.16$] ⇒ S	1.45 [$F_4^4 = 6.39$] ⇒ S	5.04 [$F_4^5 = 6.26$] ⇒ S

Table 4 Optimum concentration of both microalgae (C_{max} and I_{max}) leading to clams optimum growth (G_{max}) at different rearing time.

	10 days	12 days	15 days	25 days
C_{max}	ND	DL	42.58	47.50
I_{max}	29.77	22.50	27.87	22.50
G_{max}	189.12	216.20	243.66	411.08

ND: non dependent

IL: increase linearly

DL: decrease linearly

Figure 1: Theoretical response surfaces describing the combined effects of the microalgae concentration (C : *Chaetoceros neogracile* and I : *Isochrysis galbana*) on clam growth (G) at different cultivation times. A. On day 10. B. On day 12. C. On day 15. D. On day 26.

750

751 **Figure A (Supplementary material):** Clam growth kinetics fed with the microalgae

752 concentrations described in Table 1.