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Effects of microalgal diet on growth, survival, biochemical and fatty acid composition of *Ruditapes decussatus* larvae

Jose Andrés Aranda-Burgos, Fiz da Costa *[,1], Susana Nóvoa, Justa Ojea, Dorotea Martínez-Patiño

Centro de Investigacións Mariñas, Consellería do Medio Rural e do Mar, Xunta de Galicia, Muelle de Porcillán s/n, 27700 Ribadeo, Lugo, Spain

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ABSTRACT

Successful bivalve larval growth and survival depend on stored reserves provided by diet. The aim of this study was to evaluate the effects of microalgal diet on growth, survival, together with biochemical and fatty acid composition during larval development in grooved carpet shell (*Ruditapes decussatus*). Four mono- and multi-species diets were tested: (1) *Isochrysis galbana*, *Pavlova lutheri* and *Chaetoceros muelleri* (1:1:1, IPC); (2) *I. galbana*, *P. lutheri* and *C. muelleri* (1:1:2, IP2C); (3) *I. galbana* and *P. lutheri* (1:1, IP); (4) *C. muelleri* (C). Our results showed that feeding regime greatly influenced larval growth and survival, as well as biochemical and fatty acid composition. Higher growth and survival rates were observed in the IPC, IP2C and C diets, which all include *C. muelleri*. Despite the fact that the larvae fed with IP exhibited the lowest length at settlement and survival, they stored more total lipids than any of the diets including *C. muelleri*. The IP and IPC diets exhibited a higher percentage of total lipids, whereas IP2C and C showed a higher proportion of proteins. Lipid and carbohydrate concentrations in these diets negatively affected growth and growth and survival of the larvae, respectively. Protein content of the larvae was however positively related to larval survival, whereas lipid content of the larvae negatively affected growth. Fatty acid (FA) profile of neutral lipids (NL) and polar lipids (PL) of 22-day-old larvae clearly reflected the FA proportion of each diet. Dietary DHA and DHA in NL of the larvae were negatively related to larvae growth. DHA incorporation into PL in larvae fed with DHA-deficient diets was compensated by an enrichment in EPA, 18:0dma, 22:5(n-3) and 22:2 non-methylene-interrupted dienoic FA. According to these results, the presence of *C. muelleri* in the diet seems to promote better growth and survival of *R. decussatus* larvae.

1. Introduction

The grooved carpet shell *Ruditapes decussatus* is distributed along the coastal and estuarine areas from the North Sea to Senegal and along the Mediterranean Sea, where it lives buried or semi-buried in sand or mud, mainly in the intertidal zone, although it also inhabits subtidal zones (Vela and Moreno, 2005). It is the most appreciated native clam species in Spain, and it is also harvested in France, Italy, Portugal and Tunisia. Its capture production accounted for 1349 t in 2009 (FAO, 2010). The same source also recorded an aquaculture production of 2052 t and a market value of approximately $20 million. *R. decussatus* is the clam species preferred by consumers due to its delicate taste and meat texture, also being the most expensive clam species in Europe. Its market value can reach up to 4-fold the price of any other clam species (Fernández et al., 2000). The Galician coast (NW Iberian Peninsula) is well known for its shellfish production, which has great economic and social importance (Beiras and Albentosa, 2004). Nevertheless, culture of this clam is clearly limited by the availability of natural seed (Ojea et al., 2008). Therefore, hatchery production is of the utmost importance as a way to reinforce natural recruitment in order to restock natural beds which are threatened due to over-fishing.

Nutrition is one of the dominant factors influencing bivalve larval growth and survival, and has been extensively reviewed in Marshall et al. (2010). Live microalgae are traditionally used as food for bivalves in mollusk hatcheries (Pernet et al., 2003). The criteria for selecting a suitable algal diet for bivalve larvae must be based on form, mobility, size, toxicity and the ability of the larvae to trap, ingest, digest and assimilate the algae (Marshall et al., 2010). Persoone and Claus (1980) identified the microalgae *Isochrysis galbana*, *Isochrysis* sp. (T-ISO), *Pavlova lutheri*, *Tetraselmis suecica*, *Pseudoisochrysis paradoxa*, *Chaetoceros calcitrans* and *Skeletonema marinoï* as important strains for bivalve feeding.

Food value is determined largely by biochemical composition (lipid, carbohydrate and protein). The viability of the larvae may be limited by the accumulation and/or utilization of energy substrates, either lipids as in *Ostrea edulis* (Helm et al., 1973; Holland and Spencer, 1973) or proteins during settlement as in *Crassostrea gigas* (Barlett, 1979) and *O. edulis* (Rodriguez et al., 1990). Carbohydrates may play a role in the optimal utilization of other reserves during metamorphosis, such as proteins, as in *Crassostrea virginica* and *C. gigas* (Haws et al., 1993). In

* Corresponding author at: Ifremer, Station Expérimentale d'Argenton, 12 Presqu'île du Vivier, 29840, Argenton en Landunvez, France. Tel./fax: +33 2 98 89 29 59.
E-mail address: fiz.da.costa.gonzalez@ifremer.fr (F. da Costa).
[1] Present address: Ifremer, Laboratoire des Sciences de l'Environnement Marin (UMR 6539, LEMAR) 29280 Plouzané, France.

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the starved larvae of *R. decussatus* neutral lipids have been identified as the most abundant lipid constituents and the principal source of energy, followed by protein, with very little energy contribution from carbohydrate (Matias et al., 2011). Essential fatty acids (EFAs), particularly the omega-3 fatty acids eicosapentanoic acid (20:5(n-3), EPA) and docosahexanoic acid (22:6(n-3), DHA), are important for growth and development (Langdon and Waldock, 1981) because they are major membrane components (Hendriks et al., 2003) and possible modulators of membrane function (Palacios et al., 2005). Moreover, the omega-6 fatty acids docosapentanoic acid (22:5(n-6), DPA) and arachidonic acid (20:4(n-6), AA) have been identified as potential modulators for growth and survival of the larvae (Pernet et al., 2005) and post-larvae in bivalves (Milke et al., 2008). Nevertheless, these EFAs must be supplied exogenously due to the very limited and/or absent capability of their synthesis in bivalves (Chu and Greaves, 1991; Laing et al., 1990; Waldock and Holland, 1984).

It is widely accepted that mixed diets better meet the nutritional requirements of bivalve larvae than unialgal diets. A combination of two or three highly nutritional microalgal species, including a suitably sized diatom and a flagellate, invariably provide improved rates of larval growth and development than single species diets (Helm et al., 2004). *I. galbana* is a worldwide used food supply for cultured suspension-feeding bivalves, and *Isochrysis* sp., *P. lutheri* and *Chaetoceros* sp. are the most common species used to feed the larvae, early juvenile and broodstock (during hatchery conditioning) of bivalve molluscs, due to their good nutritional properties (Brown, 2002).

In spite of the *R. decussatus*'s great commercial importance, studies on its nutritional requirements are very scarce. Currently, there is little information on diets to feed spat (Albentosa et al., 1996, 1997; Fernández-Reiriz et al., 1998, 1999; Pérez-Camacho et al., 1998) and larvae (Matias et al., 2011), but to our knowledge no previous study has dealt with the fatty acid composition of larvae fed with mono- and multi-species diets. Therefore, the aim of the present work was to determine the effects of the most widely used microalgal diets (mono- and multi-species diets) on growth and survival, as well as biochemical and fatty acid composition in *R. decussatus* larvae.

2. Materials and methods

2.1. Microalgae culture

The microalgae *I. galbana*, *P. lutheri* and *C. muelleri* 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). Inocula of these algae were part of an own culture collection of our institute and were kept in an isothermal chamber in 20 mL tubes under axenic conditions. 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 180–220 μphotons m^{-2} s^{-1}. Seawater at ambient salinity (32–33 ppt) 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 μphotons m^{-2} 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, CO_2 addiction allowed pH maintenance between 7 and 8. Seawater at ambient salinity 32–33 ppt was maintained at 21 ± 1 °C, and was enriched with the Solution C medium, which includes a mixture of mineral salts, nitrate and phosphate. Culture medium was added constantly (1 mL per L of algal culture), whereas the supply of sodium silicate, in the diatom culture, was added twice a week. Algae were harvested when the culture had reached the exponential growth phase and algal culture is maintained in this phase during 2 months under a dilution rate of 12.5 day^{-1}. Each species used in this experiment is harvested using a small pipe with a tap at the bottom of each bag and inspected daily under a light microscope. Before being used as food, algal cells were counted with a Bürker–Turk counting chamber and algal mixtures prepared for their distribution to larval cultures.

2.2. Broodstock conditioning

Adult specimens of *R. decussatus* were collected by rake in June 2010 in a natural bed in an intertidal zone of Cambados in Ría de Arousa (42°50′N; 08°80′W) (Galicia, NW Spain). Then, clams were transferred cooled at 4 °C to our hatchery facilities. At Centro de Cultivos de Ribadeo-CIMA, broodstock were conditioned in 200 L rectangular tanks at 18 ± 1 °C in an open circuit at ambient salinity of 32–33 ppt, with a continuous supply of a mixture of *I. galbana*, *P. lutheri*, *T. suecica*, *Chaetoceros* sp. and *S. marinoï* in equal proportions, representing a ration of 6% of dry meat weight in dry algal weight per day.

For spawning induction, 90 adult clams (44.8 mm and 18 g of average length and weight respectively) were cleaned and kept dry at 4 °C for 12 h. Then, clams were placed in a tray with UV-sterilized seawater at ambient salinity of 32–33 ppt and without aeration for spawning induction. Individuals were subjected to thermal shock, with temperatures up to 25 °C for 2 h, decreasing to 14 °C for 30 min. A total of 2 cycles were performed. Additional stimulus was provided by adding gametes stripped from one of the conditioned bivalves and microalgae (*T. suecica*). Each spawning specimen was quickly transferred into individual receptacles for the release of sperm or eggs, thus avoiding polyspermy. Once spawning was completed, sperm from several males was pooled and added to the container, at 20 ± 1 °C, with oocytes to obtain synchronous fertilization. After counting, fertilization was performed at a ratio of 50 spermatozoids per oocyte. After fertilization, the eggs were sieved through a 45-μm mesh screen to eliminate excess sperm. Two hours after fertilization, when the embryos have reached the 4-cell stage, embryos were transferred to 500 L larval culture tanks with aerated and filtered UV-irradiated seawater at a temperature of 20 ± 1 °C. The density of the embryos was adjusted to 35 embryos mL^{-1}. No food was supplied during embryo incubation.

2.3. Larval culture

The experimental design used in larval rearing was based on the routine work of a hatchery, i.e. the effect of diet on larval culture was evaluated at an intensive or industrial scale (large volumes and high density) and not at laboratory conditions (small volumes and low density). The experiment started on day 2 post-fertilization (at the stage of early D-veliger larvae). After sieving the larvae through a 60-μm mesh screen, they were transferred to a 5-L beaker. Filtered seawater was added to a known volume. With an automatic pipette set at 50 μL, 5 replicate subsamples were taken of the contents, while agitating the contents of the beaker, with a suitable diameter perforated plunger. Larvae were counted using a light microscope.

Then, D-larvae were transferred to 150 L tanks at an initial density of 10 larvae mL^{-1}. Larvae were randomly split into eight batches for duplicate exposure to four nutritional regimes: diets 1: *I. galbana* (4.8–5.5 μm diameter), *P. lutheri* (5.3–5.8 μm diameter) and *C. muelleri* (4.6–6.4 μm diameter) (1:1:1, IPC); 2: *I. galbana*, *P. lutheri* and *C. muelleri* (1:1:2, IP2C); 3: *I. galbana* and *P. lutheri* (1:1, IP); 4: *C. muelleri* (C). All multi-species diets were prepared in cell volume equal proportions. Food was added daily to each tank at a rate of 40 cells $μL^{-1}$ as an initial ration and 70 cells $μL^{-1}$ as maximum ration at the end of the experiment. The ration provided varied with culture age (Table 1), but was always over larval feeding requirement standards found in the literature (e.g. Rico-