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Induction of settlement, growth and survival of juveniles of *Paracentrotus lividus*

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ABSTRACT

The effect of different treatments on the settlement success, survival and growth of sea urchin juveniles has been evaluated. Two experiments were performed for assessing the ability of substrates or seawater to induce settlement. In the first test 500 competent larvae were inoculated in transparent plastic containers conditioned for ten days with: *Tetraselmis marina*, *Cyilindrotheca closterium*, *Nitzschia* sp., Maërl beds, 100 μm filtered natural seawater and 1 μm filtered and UV disinfected seawater. Maximum settlement values were obtained with *C. closterium*, *Nitzschia* sp. films and UV treated seawater; and significant differences were only detected between *T. marina* films and the other treatments. In the second test the following treatments were performed in order to investigate the effects of filtered seawater on settlement: 0.22 μm filtered and autoclaved seawater (negative control), 1 μm filtered and UV treated seawater and *C. closterium* (positive control). Settlement success was significantly higher for *C. closterium* than for autoclaved seawater. Survival and growth of juveniles were monitored for 32 days on these treatments: *T. marina*, *C. closterium*, *Nitzschia* sp., Maërl beds and 100 μm filtered natural seawater. Growth of juveniles could be adequately described by linear fitting and maximum growth rate was found for *C. closterium* film. There were no significant differences between treatments in terms of mortality at days 21 or 32. In general, the results showed that *C. closterium* biofilms provide good settlement, growth and survival of sea urchin juveniles which would allow improving general performance of these phases of culture compared to the other diets tested.

1. Introduction

Sea urchins have a commercial interest due to the gastronomic value of their gonads. In particular, *Paracentrotus lividus* is the most consumed sea urchin species in the European Union and Spain is the main producer in the region (Ouréns, 2013). *P. lividus* is a species of sea urchin distributed throughout Mediterranean Sea and in the North-eastern Atlantic Ocean (Boudouresque and Verlaque, 2013). The growth of this species is moderate since individuals with a diameter of 2 cm test are generally considered to average 2 years old and individuals with a test diameter of 4 cm to be 4 to 5 years as stated by Boudouresque and Verlaque (2013). Other factor affecting the dynamics of a sea urchin population is that gonadal growth depends on the test diameter, so the reproductive contribution and the gonad index of larger specimens are greater than those of smaller sea urchins (Boudouresque and Verlaque, 2013; Marsh et al., 2013). This added to the size at maturity of *P. lividus* - 2.0–2.8 cm according to Ouréns (2013) - suggests that this fishery is susceptible to overexploitation. In fact, an overall decline in world production of sea urchins has been reported (Andrew et al., 2002). In France, the second country in terms of consumption of sea urchin roe, there has been a sharp drop in catches from 1945 to the present related to overfishing, pollution and diseases (Andrew et al., 2002). The catch statistics in Galicia (NW Spain) do not indicate any overall trend, although some cases of overexploitation have been found, which have suggested the need for appropriated strategies to the management of this resource (Fernández-Boán et al., 2012; Ouréns, 2013).

Two strategies are currently used in some countries: gonad enhancement, by optimizing the gonad growth at sea or in land-based facilities (James and Siikavuopio, 2015), and stock enhancement, by releasing juveniles produced in hatcheries to increase natural recruitment (Agatsuma, 2013). The sea urchin rearing involves obtaining gametes, fertilization, larval culture, induction to settlement and growth from juvenile to adult (Cellario and Fenaux, 1990; Fenaux et al., 1985; Hinegardner, 1969). The closed-system echinoculture has not been completely successful due to technical and economic difficulties (Unuma et al., 2015). The induction to settlement, metamorphosis and survival of post-settlement juveniles are the most critical stages for

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aquaculture of *P. lividus* (De La Uz et al., 2013; Grosjean et al., 1998). Conspecific adults, macroalgae (such as calcareous algae or kelp), microalgal and bacterial biofilms, specific chemicals and turbulence, have been tested as cues for settlement (Gaylord et al., 2013; Mos et al., 2011).

The effect of diet on growth and survival of post-settlement juveniles is key to improve sea urchin culture. The most widely used farming technique at this stage involves obtaining a microbial film (basically diatoms) on a corrugated plastic support (Unuma et al., 2015). Two requirements must be met by the ideal film: a) high settlement rate of competent larvae, and b) low mortality and elevated growth of the post-settlement juveniles (Azad et al., 2010; Grosjean et al., 1998; Mos et al., 2011). The natural microbial films vary markedly throughout the year and the success of metamorphosis, growth and survival of post-settlement juveniles depend upon their composition (Rahim et al., 2004). For this reason some authors have investigated the effects of unialgal diets in order to stabilize and optimize the performance at this stage (Onitsuka et al., 2014; Xing et al., 2007).

The aim of this study was to evaluate: a) different cues (unialgal films, mixed films and a combination of a mixed film on a coralline alga) as inducers of metamorphosis of competent larvae, and b) growth and survival of post-settlement juveniles on these substrates. The final goal was to find a substrate which allowed to improve the performance at the most critical stages of cultivation of *P. lividus*.

2. Material and methods

2.1. Algal isolation, DNA extraction, PCR amplification and sequencing

Unialgal strains were isolated from seawater samples taken in the Ría de Vigo (*Tetraselmis marina*, *Cylindrotheca closterium* and *Nitzschia* sp.). In order to confirm the taxonomical identity of the algal strains used in this study, exponentially growing cultures (1 ml) of *T. marina* (GenBank accession no. KY045847), *C. closterium* (GenBank accession no. KY045848), and *Nitzchia* sp. (GenBank accession no. KY045849) were centrifuged for 3 min at 12,000, in a benchtop Eppendorf centrifuge, pellets rinsed in milliQ water, centrifuged again and DNA extracted using Chelex® 100 (Bio-Rad) following the extraction procedure of Richlen and Barber (2005). For amplification of 18S rDNA gene, primers EUK A(5′- AAC CTG GTT GAT CCT GCC AGT-3′), and EUK B (5′-TGA TCC TTC TGC AGG TTC ACC TAC-3′) (Medlin et al., 1988) were used. PCR amplifications were performed in a thermo-cycler (Eppendorf AG, USA). For this amplification, 1 μl of the extracted DNA was used as template and the PCR was run as follow: 3 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 55 °C, 3 min at 72 °C, and a final incubation for 10 min at 72 °C. PCR reaction was checked by agarose gel electrophoresis (1.5% TAE, 50 V) and SyBR Safe DNA gel staining (Invitrogen, California, USA). The PCR products were purified with ExoSAP-IT (USB Corp., Cleveland, Ohio, USA). Purified DNA was sequenced with Sanger ABI 3730xl at GATC Biotech, Germany (http://www.gatc-biotech.com).

2.2. Larval culture

Adult specimens of *P. lividus* were caught by diving in the subtidal area of Illa de Toralla (Ría de Vigo, Galicia) and maintained in tanks with open circuit. Gametes were obtained by dissection of adults and the eggs were fertilized. Larval culture was performed per duplicate in polypropylene cylindrical-conical tanks of 100 l. Seawater was renewed three times per week, initial density was 1 larva/ml, the temperature was maintained at 19 ± 1 °C and a diet of 40–80 equivalents/μl of *Isochrysis galbana* was added and composed equally by *Tetraselmis suecica*, *Tisochrysis lutea*, *Chaetoceros neogracile* and *Phaeodactylum tricornutum*. A more detailed description of larval culture can be found in a previous work of Paredes et al. (2015). Two tests were performed and larval culture lasted 23 days in the first experiment and 31 days in the second one.

2.3. Induction of settlement

Tests were performed for assessing the ability of algal films and seawater without added inoculum to induce the settlement and metamorphosis of competent larvae. The most adequate moment for starting the assays was pointed out by a significant proportion of competent larvae according to their morphology and tactile behavior (Gosselin and Jangoux, 1998).

In the first test, unialgal and mixed films were prepared in transparent plastic containers. 1.5 l of 1 μm filtered UV-irradiated seawater enriched with F/2 medium was added to each container ten days prior the onset of competent larvae. Each treatment was performed per triplicate with a non-axenic culture of: a) unialgal strains (*T. marina*, *C. closterium* and *Nitzschia* sp.), b) mixed cultures of microalgae from 100 μm filtered seawater collected in an intertidal area (hereinafter Natural), and c) the microalgal community present over a Maërl bed composed mainly of the coralline alga *Phymatolithon calcareum* and d) a treatment without inoculum (hereinafter seawater treated with UV). The microalgal species *C. closterium*, *Nitzschia* sp. and *T. marina* were selected for their ability to adhere and form biofilms. The mixed culture -Natural- was selected as a positive control. *P. calcareum* is a coralline red algae which serve as a substrate for the development of epiflora and may be a inducer of metamorphosis for competent larvae (Pearce and Scheibling, 1990). The containers were kept at 20 °C and photoperiod of 24 h (cool-daylight fluorescent, 3000 lx).

In the second test, the capacity of competent larvae to settle exposed to: a) 0.2 μm filtered and autoclaved (120 °C/10 min) seawater; b) 1 μm filtered seawater treated with UV, and c) a film of *C. closterium*, was evaluated. The same containers and conditions described above were used except for the former, in which autoclaved seawater was introduced at the beginning of the test and no F/2 medium was added.

In both experiments, seawater of the containers was changed before introducing the competent larvae (1 μm filtered and UV disinfected except for autoclaved seawater) and containers were located in semi-darkness conditions (20–200 lx, photoperiod 14:10 h light:darkness). 500 competent larvae were introduced into each container and the percentage of settlement was determined by direct counting after 48 h. Since then, postlarvae were maintained in open circuit with 1 μm filtered UV disinfected seawater.

2.4. Growth and survival of juveniles

The juveniles reached the exotrophic stage about 5–10 days after the beginning of the metamorphosis (Vaïtilingon et al., 2001). Post-settlement juveniles were removed from the containers 7 days post-settlement (DPS), pooled and distributed in each container (260 individuals per container). Five treatments were performed per triplicate: unialgal cultures of *Cylindrotheca closterium*, *Nitzschia* sp. and *Tetraselmis marina*; and mixed cultures of microalgae from 100 μm filtered seawater or present above Maërl. Containers were maintained in the conditions indicated above (open circuit, 20–200 lx, 14:10 h L:D).

The growth of juveniles obtained in the first experiment of settlement success was assessed for 32 DPS. The test diameter was measured on 7, 14, 21, 26 and 32 DPS in at least 20 individuals using a Nikon SMZ1500 stereomicroscope (Nikon corp., Tokyo, Japan) coupled with image analysis software (NIS-Elements BR, Nikon corp.). Survival was determined on 21 and 32 DPS, although could not be established reliably in Maërl beds.

An ANOVA test followed by Tukey post hoc test was performed (SPSS Statistics 19) to assess differences in percentage of settlement of competent larvae and survival of post-metamorphic juveniles.

The growth in test diameter of newly settled juveniles was described using: a) a linear regression to data from 7 to 32 DPS and b) daily growth rates (*DG*) (μm/day) calculated as the increase in length divided