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Effect of five benthic diatoms on the survival and development of *Paracentrotus lividus* post-larvae in the laboratory

Valerio Zupo[a,b,*], Francesca Glaviano[b], Davide Caramiello[b], Mirko Mutalipassi[a]

[a] Stazione Zoologica Anton Dohrn, EMI Dpt. Benthic Ecology Centre, 80077 Ischia (NA), Italy
[b] Stazione Zoologica Anton Dohrn, RIMAR Dpt. MaRe Unit, 80121, Villa Comunale, Napoli, Italy

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ABSTRACT

The Mediterranean sea urchin *Paracentrotus lividus* Lamark, 1816 is a resource as food and model organism for scientific research. Increasing market demand, commercial fisheries and destructing harvesting methods produced a dramatic reduction of natural stocks along the Mediterranean coasts. The techniques for in vitro fertilization and growth of larvae and adults are well known but various bottlenecks still hinder the industrial maturity of its aquaculture. In particular, the transition from planktonic larvae to benthic juveniles, as well as their correct development and survival, still represent critical phases, since larval and post-larval experiences are the primary determinant of juvenile fitness. Thus, techniques improving survival and settlement rates in the first two months after fertilization could considerably improve the effectiveness of echinoculture practices. This study was conducted to determine, for the first time, the effect of strictly benthic diatoms on *P. lividus* post-larval performances, as compared to other micro- and macro-algae. In addition, three larval dietary treatments were applied, to confirm previous findings on the efficacy of algae and formulated diets. In particular, survival and ability to produce competent larvae for settlement were tested in groups subjected to three algal diets (*Isochrisis galbana*, *Rhodomonas baltica* and *Dunaliella tertiolecta*), all integrated with a formulated food. Furthermore, six post-larval feeding regimes, i.e., the benthic diatoms *Cocconeis scutellum parva*, *Diploneis* sp., *Navicula incerta*, *Cylindrotheca closterium* and *Nanofrustulum shiloi*, compared with *Ulva rigida* along with a negative control (absence of food), were tested on larvae deriving from the most effective treatment. Our results confirmed the efficacy of *Dunaliella tertiolecta* integrated with a formulated food, consistently producing about 60% survival. Furthermore, the most adhesive benthic diatom, *Cocconeis scutellum parva*, sustained settlement (about 63%) and good survival in the first two months of life of sea urchins.

1. Introduction

The Mediterranean sea urchin *Paracentrotus lividus* is object of an increasing interest due to its economic value for fisheries, as well as for its importance as a model organism for scientific investigations (Faimali et al., 2017) in various fields of Evo-Devo research, toxicology tests and embryology studies (Ruocco et al., 2017). In addition, its natural populations, often associated to seagrass meadows and vegetated benthic communities (Boudouresque and Verlaque, 2007; Soualili and Guillou, 2009), are key components of coastal food webs. Increased fishery pressure (Pais et al., 2007) is producing a decline of natural stocks (Addis et al., 2009; Pais et al., 2007) due to amplified market demand (Carboni, 2013; Mos et al., 2011). Several authors suggested aquaculture as the natural answer to this problem of resource sustainability (Yamabe, 1962), and the need to reach perfectly devised echinoculture techniques has been highlighted (Buitrago et al., 2005).

As a matter of fact, previous investigations defined the settings needed to improve the techniques of in vitro fertilization (Paredes et al., 2015), larval survival according to various diets (Azad et al., 2011; Carboni et al., 2012; Liu et al., 2007), settlement (Cárcamo et al., 2005; Hannon et al., 2017), metamorphosis (Gosselin and Jangoux, 1996; Huggett et al., 2006; Pearce and Scheibling, 1991), first post-larval growth (Grosjean et al., 1998; Rahim et al., 2004; Shimabukuro, 1991), as well as gonadal maturation and quality of gametes (Mercurio and Sugni, 2016). Thanks to these investigations clear data are available about the techniques of fertilization, the microalgae promoting higher larval survival, the factors involved in the settlement and diets allowing for a fast development of adult sea urchins (Buitrago et al., 2005; Kelly et al., 2000; Pedrotti and Fenaux, 1993). Thus, the culture of *P. lividus* in the laboratory is possible and is currently achieved for experimental purposes. However, we still miss important details to make this echinoculture economically feasible in large scale (Grosjean et al., 1998),

* Corresponding author at: Stazione Zoologica Anton Dohrn, EMI Dpt. Benthic Ecology Centre, 80077 Ischia (NA), Italy.
E-mail address: valerio.zupo@szn.it (V. Zupo).

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due to scarce quality of larvae, low percentages of settlement, slow growth of adults or scarce fattening of gonads (Liu et al., 2007). These lacks make the process hardly adaptable to industrial plants, due to low economic convenience and low efficiency of rearing techniques, which make fisheries still the primary source for marketable individuals, despite a research history of over 100 years on this species (Koehler, 1883).

In particular, various studies reported details on the microalgae favouring a sufficient survival and development of larvae, as well as the factors triggering settlement, but inconsistent results were achieved by commercial hatcheries. Although strain differences may influence the success of cultures, various studies confirm the efficacy of *Dunaliella tertiolecta* for promoting the development of larvae (Basch, 1996; Cameron and Hinegardner, 1974; Carboni et al., 2012; Liu et al., 2007; Strathmann, 1971) as compared to such planktonic microalgae as *Isochrisis* spp., *Chaetoceros* spp. and other unicellular species (Brundu et al., 2016). The positive results promoted by *D. tertiolecta* have been related to its fatty acid profile, in respect to other species of microalgae (Carboni et al., 2012), but also to the easy of ingestion and digestion of its cells (Basch, 1996; Cameron and Hinegardner, 1974; Strathmann, 1971). For this reason, the first aim of the present study has been to test the effect of the addition of synthetic plankton (artificial marine snow), easy to be digested and quickly available for larvae (Liu et al., 2007), to promote consistent results in terms of growth and survival rates.

In addition, previous research indicated that the presence of feeding biofilms (e.g. *Ulvella lens*; Brundu et al., 2016) and some infochemicals produced by adults (De La Uz et al., 2013) stimulate the settlement of larvae. In particular, when reaching competence stage, larvae of *P. lividus* metamorphose and shift to the benthic stages (settlement) in response to infochemicals produced by macroalgae (Dworjanyn and Pirozzi, 2008; Juinio-Méñez and Bangi, 2010), coralline algae (Grosjean et al., 1998) and natural biofilms (Dworjanyn and Pirozzi, 2008; Rahim et al., 2004), as well as in response to the presence of adult individuals (Brundu et al., 2016). Most probably, the algal substrates play both the role of inducers of larval metamorphosis and that of convenient food for settled juveniles (Takahashi et al., 2002), while the presence of adults may indicate feasible sites for feeding and enhanced survival. However, the production of competent larvae and the settlement rates obtained taking advantage of the mentioned substrates are still insufficient to guarantee economically effective procedures. For this reason, we also tested the effect of various benthic diatoms as cues for the settlement and survival of post-larvae in the first months after metamorphosis. In fact, the survival in this very critical period is one of the factors negatively impacting the culture of *P. lividus* and it is considered to be the major bottleneck in echinoculture, seriously limiting production (De La Uz et al., 2013).

Thus, this study was conducted to add information to the complex issue of larval feeding and settlement (Hodin et al., 2016), in order to reduce the level of unpredictability that still characterizes the period when post-larvae acquire exotrophy and develop as functional juveniles. To this end, we compared for the first time the effect of some strictly benthic diatoms, as they are the main producers of natural biofilms (Zupo and Messina, 2007) in the environments typically hosting *P. lividus* (Azad et al., 2010; Zupi and Fresi, 1984), but their contribution to the settlement and survival of post-larvae was scarcely considered (Hannon et al., 2017). In particular, the effect of very adhesive diatoms (e.g., *Cocconeis* spp.), characterizing the benthic communities in the periods of settlement of *P. lividus*, has never been compared to the effectiveness of other, less adhesive diatoms like *Navicula incerta* and *Nanofrustulum shiloi*, while a few studies (Cárcamo et al., 2005; Harris et al., 2003; McBride, 2005) reported the results obtained with such scarcely adhesive diatoms as *Amphora* spp. and *Navicula* spp. (Dworjanyn et al., 2007; Xing et al., 2007). In fact, given the numerical abundance of the above-mentioned diatoms in the benthic substrates (Majewska et al., 2014) hosting settled post-larvae of *P. lividus*, their role in the diet of this species could be determinant to guarantee higher levels of survival and fitness.

2. Material and methods

2.1. Collection and fertilization

Adult sea urchins were collected by scuba divers in the bay of Napoli (Italy) and carried to the laboratory in thermostatic bags, to avoid temperature changes. They were acclimatized in open cycle tanks and then measured using a calliper to select reproducers, i.e., individuals whose diameter (theca, excluding the spines) was higher than 40 mm. In fact, sea urchins in this size range correspond to those investing maximum energy in reproduction (Fernandez and Pergent, 1998). Specimens of the convenient size classes were then sorted according to their sex, observing the differences in the genital papillae under optical microscopy (Arizza et al., 2013).

To obtain gametes for in vitro fertilization, three sea urchins of each gender were injected 1 ml of 0.5 M KCl into the coelom, through the soft derma around the mouthparts, to stimulate the contraction of gonads. The subjects were then vigorously shacked and females were placed with their mouths up, inside a 50 ml beaker, until the gametes were released into filtered (0.22 μm Millipore) seawater to facilitate the collection of oocytes, that were rinsed three times with clean seawater to remove possible organic residuals (Chapman, 1995). Sperms were collected "dry", after the injection of 1 ml of 0.5 M KCl into the coelom of males, using a Pasteur pipette and sucking over the surface of gonopores, to avoid premature activation. The gametes obtained from three individuals were pooled and transferred to 50 ml vessels, to reduce strain influences, prior to start the fertilization process.

Twenty sub-samples of oocytes were collected and added with a drop of sperm suspension. Normal embryo activation was revealed by the elevation of the fertilization membrane within 40–80 s, appearing as a clear circle. Pools of embryos exhibiting percentages of fertilization lower than 95% or showing malformations during the first 48 h of development were discarded. Pools exhibiting viable embryos were stored at a constant temperature of 20 °C in Petri dishes for 48 h, until reaching the prism stage.

2.2. Cultivation of algal items

Alive microalgae used to feed *Paracentrotus lividus* larvae (*Isochrisis galbana*, *Rhodomonas baltica* and *Dunaliella tertiolecta*) have been cultured in the laboratory, starting from strains set in the algal collections of the Stazione Zoologica Anton Dohrn. Microalgae were cultured in aerated 2 L Erlenmeyer flasks containing Guillard's *f/2* medium without silicates (Sigma-Aldrich, Milan, Italy). Axenic cultures were weekly renovated under a laminar flow hood and kept in a thermostatic chamber at 20 °C, with a 12/12 day/night photoperiod. Diatoms used for settlement and survival tests (*Cocconeis scutellum parva*, *Diploneis* sp., *Navicula incerta*, *Cylindrotheca closterium* and *Nanofrustulum shiloi*) were cultivated in the laboratory starting from mother cultures kept at the Benthic Ecology Centre of the Stazione Zoologica Anton Dohrn. Diatoms were cultivated in 14 cm Petri Dishes containing 100 ml of Guillard's *f/2* medium with silicates (Sigma-Aldrich, Milan, Italy). Axenic cultures were renovated every 15 days and kept in a thermostatic chamber at 20 °C, with a 12/12 day/night photoperiod. Macroalgae (*Ulva rigida*) were collected in shallow (1–3 m) coastal areas of the bay of Napoli and immediately transferred to the laboratory. Cotton gauzes were scraped on their surface to remove most epiphytes. They were kept alive in closed cycle tanks containing filtered seawater at 20 °C, with a 12/12 day/night photoperiod.

2.3. Larval rearing and feeding tests

The larvae obtained in various culture plates were pooled into a 1500 ml conical flask after 48 h, to reduce natural variability. The