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Effects of on-demand feeding on sea urchin larvae (*Paracentrotus lividus*; Lamarck, 1816), development, survival and microalgae utilization

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Abstract

This study compared the growth of sea urchin *Paracentrotus lividus* larvae cultured using two different rearing methods: a variable method based on a variable amount of feed (microalgae) and seawater exchange (30% or 50%) established according to the phytoplankton concentration in the larval cultures and a fixed method characterized by a fixed amount of feed and seawater exchange. Three microalgae diets, *Isochrysis* sp. (Tahitian strain, T-Iso), *Chaetoceros gracilis* and a 50:50 mixed diet, were tested with both rearing methods. Larval development and survival were assessed at the 6-arm pluteus stage (P6), competence (Cp) and metamorphosis (Mt). Data showed that the variable method reduced the requirements for phytoplankton and seawater exchange. Indeed, through the optimization of feed rations, it was possible to reduce the production of debris and settled phytoplankton, minimizing the need for water exchanges. Higher larval survival resulted at Cp and Mt stages for those reared with the variable method as opposed to the fixed one. Survival and development were also influenced by the tested dietary treatments: at Mt stage, the mixed diet resulted in a higher larval survival (63.3 ± 8.9%) than T-Iso (19.7 ± 12.1%) and *C. gracilis* (23.4 ± 15.1%) ($P < 0.05$). These results suggest that the use of the variable method improves the larval survival and development and also it reduces resource consumption (phytoplankton, seawater use and work effort), which in turn could potentially improve the hatchery production of *P. lividus*.

Keywords: sea urchin, *Paracentrotus lividus*, larviculture, survival, development

Introduction

Echinoderms, like sea cucumbers and sea urchins, are commercially relevant resources worldwide. In Europe, France is the most important market of sea urchins, as *Paracentrotus lividus, Psammechinus miliaris* and *Sphaerechinus granularis* are the main exploited and commercialized species (Grosjean 2001).

Paracentrotus lividus (Lamarck) plays an important role in the Mediterranean coastal ecosystems. Its life cycle is characterized by two stages: larval planktonic and adult benthic. During the planktonic stage, the larva, echinopluteus, is able to swim and consume phytoplankton. In the benthic stage, the organism mostly eats macroalgae. Its grazing activity influences the structure of the macroalgae communities: low densities of the organism often result in the substantial growth of macroalgal forests, while high densities overconsume erect algae and cause formation of barrens, bare rocks with encrusting algae (Boudouresque & Verlaque 2007; Agnetta, Badalamenti, Ceccherelli, Di Trapani, Bonaviri & Gianguzza 2015).

Paracentrotus lividus is also relevant for commercial purposes as it is the most consumed sea urchin in Europe (Carboni, Vignier, Chiantore,

Tocher & Migaud 2012). Due to the high market demand for its gonads in both Mediterranean and non-Mediterranean areas (Pais, Chessa, Serra, Ruiu, Meloni & Donno 2007), natural populations are exposed to over fishing in many European coastal areas. This often causes a sharp decline in the abundance of wild stocks (Boudouresque & Verlaque 2007; Pais *et al.* 2007; Addis, Secci, Manunza, Corrias, Niffoi & Cau 2009).

Scientific research aims to bridge the gap between supply and demand of aquaculture products (Pearce 2010). Adults with excellent quality gonads could be available for the market throughout the whole year, while juveniles bred in controlled conditions could be employed in restocking activities (Carboni 2013).

Nowadays, the larval planktonic stage represents one of the key commercial bottle necks for echinoculture (Carboni *et al.* 2012; Carboni, Kelly, Hughes, Vignier, Atack & Migaud 2014). The planktonic stage lasts for about 25 days, during which the echinopluteus goes through a series of morphological transformations until they achieve competence. The competent larva metamorphoses into a benthonic organism when it finds a suitable substrate. Under culture conditions, a high larval mortality rate can represent significant limitations for the production of juvenile sea urchins (Carboni *et al.* 2012, 2014).

With the aim of reducing larval mortality and improving hatchery productivity, previous studies focused on the identification of suitable diets, generally based on microalgal species and on the optimization of feed rations (Pedrotti & Fenaux 1993; Kelly, Hunter, Scholfield & McKenzie 2000; Cárcamo, Candia & Chaparro 2005; Liu, Kelly, Cook, Black, Orr, Zhu & Dong 2007; Carboni *et al.* 2012). Indeed, evident negative effects on larval development and survival have been demonstrated to be produced by low feed rations (Boidron-Metairon 1988; Strathmann, Fenaux & Strathmann 1992; Fenaux, Strathmann & Strathmann 1994; Kelly *et al.* 2000; Sewell, Cameron & McArdle 2004; Azad, Pearce & McKinley 2011) and high ones (Kelly *et al.* 2000; Azad *et al.* 2011).

In order to maintain a good quality of water, free of debris and settled materials, larval production was traditionally performed using static systems characterized by gentle aeration and complete water exchange (Kelly *et al.* 2000; Liu *et al.* 2007; Dworjanyn & Pirozzi 2008; Azad *et al.* 2011; Carboni *et al.* 2012; Carboni, Hughes, Atack, Tocher & Migaud 2013) or partial water exchange (Pedrotti & Fenaux 1993; Pedrotti & Lemée 1999; Cárcamo *et al.* 2005; Privitera, Noli, Falugi & Chiantore 2011), carried out every 2 or 3 days. As an alternative larviculture system, a flow-through system characterized by a 100% water exchange daily and a Banjo filter (40-μm mesh size) to prevent larvae loss can be very beneficial to larval survival (Carboni *et al.* 2014). Furthermore, some studies revealed that routine maintenance operations like siphoning and bubbling in traditional static systems and sieving and filtration in alternative flow-through systems can be stressful and could damage the larvae in suspension (Strathmann 1978; Russell 2000; Carboni *et al.* 2014).

In the present study, we tested the hypothesis that an alternative static breeding system characterized by a low level of physical disturbance could improve the survival and development of *P. lividus* larvae.

Materials and methods

Adult sea urchins (test diameter >40 mm) were collected from a 5-m depth at the 'Penisola del Sinis-Isola di Mal di Ventre' Marine Protected Area (39°89′N 8°41′W, western Sardinia, Italy) and transported to the laboratory in thermal insulated and refrigerated containers.

More than one individual was used to increase the egg fertilization rate in reproductive trials (Evans & Marshall 2005), and ten specimens (5 males and 5 females) were used for gamete strip spawning. Gametes were obtained after the dissection of adult sea urchins. Each individual was hemisected with metal scissors, and the mouth and the stomach were removed. Male gonads were collected and kept dry at 4°C, while female gonads were kept in a beaker containing filtered (0.47 μm) natural seawater (NSW). Four drops of diluted (5:100) sperm were added to the eggs and the solution was gently stirred in order to facilitate fertilization.

A sample was taken from the starting solution, and the number of fertilized eggs was determined as result of 5 replicates, using a tubular plankton chamber and a Leica MZ8 Stereo Microscope. The fertilization rate was verified by the presence of the fertilization membrane (Grosjean, Spirlet, Gosselin, Vaitilingon & Jangoux 1998; Liu *et al.* 2007; Azad *et al.* 2011).

Embryos were reared under static conditions at a density of 20 mL^{-1} until they reached the echinopluteus stage (approximately 40 h after fertilization took place). Echinoplutei were reared in previously filtered (0.47 μm) NSW, 36.5 ± 0.2 g L^{-1} salinity, constantly kept in motion by motor-driven rotation without aeration and in continuous light with OSRAM Natura type 50 cm below the water's surface. The temperature was maintained at 19.0 ± 2.0°C.

Experimental design

Two different larval breeding methodologies were tested, here defined as *variable* and *fixed*. For each method, three microalgal diets were tested: two monospecifics, *Isochrysis* sp. (Tahitian strain: T-Iso), *Chaetoceros gracilis* (Cha) and a mixture of both species (mix). Larvae were stocked at a density of 1.5 mL^{-1} into 5 L cylindrical tanks made of white plastic material. There were five replicates for each treatment in a total of 30 tanks.

In the fixed method, the larvae were fed every 3 days with a fixed amount of feed adjusted according to larval development. This corresponded to 8000 cells/ml during the first 10 days post fertilization (DPF) and 16 000 cells mL^{-1} after 10 DPF. In the mixture combined, the two species were in equal quantities (50:50 ratio). The number of algal cells was determined using a Leica DMRB microscope and a Neubauer counting chamber (Guillard & Siereki 2005). Before each feeding, a partial (50%) NSW exchange was done to siphon out settled materials and dead cells; a 100-μm mesh size filter was used to avoid larval loss.

In the variable method, the amount of feed was established every 3 days and adjusted according to larval consumption. The first feed supply (2 DPF) was characterized by a fixed quantity (8000 cells mL^{-1}), while the subsequent administrations were established after measuring the residual phytoplankton concentration in larval tanks. Feed was not given when cells were abundant in the rearing tanks (more than 8000 and 16 000 cells mL^{-1} before and after 10 DPF, respectively). In the mixed diet treatment, T-Iso and *C. gracilis* were counted and supplied separately. No feed was supplied when the microalgae concentration within the tanks was higher than 4000 and 8000 cells/ml, before and after 10 DPF, respectively, for both species. The amount of NSW exchange was established according to the phytoplankton concentration inside the tanks. A 30% exchange was done when the microalgae concentration resulted higher than 12 000 cells mL^{-1}, within 10 DPF, and 24 000 cells mL^{-1}, after 10 DPF. A 50% exchange was done when the microalgae concentration resulted higher than 16 000 cells mL^{-1}, within 10 DPF, and 32 000 cells mL^{-1}, after 10 DPF.

Phytoplankton cultures

We chose to use *Isochrysis* sp. and *C. gracilis* as feed for sea urchin larvae because they were previously used in numerous other studies (Bustos, Olave, Troncoso & Godoy 1992; Zamora & Stotz 1994; Miller & Emlet 1999; Cárcamo 2004; Azad *et al.* 2011; De La Uz, Carrasco, Rodríguez & Anadón 2013; Paredes, Bellas & Costas 2015). They were provided by the Agency for Agricultural Research in Sardinia (AGRIS) and sourced from the Culture Collection for Algae and Protozoa (CCAP: Oban, Scotland).

Cultures were maintained in batch lines at 25°C, exposed to a 16 h L/8 h D photoperiod and supplied with gentle aeration. The 30 g L^{-1} salinity seawater was pre-filtered (1-μm filter paper), enriched with modified Guillard f/2 and autoclaved at 121°C for 30 min.

The phytoplankton was supplied to the larvae during the exponential growth phase.

Larval development and survival

Larval development was evaluated by observation of larval structures (number of arms, presence and size of the rudiment) under the microscope, and development stages were defined according to previous studies (Liu *et al.* 2007; Carboni *et al.* 2012). For these observations, a minimum of 10 randomly sampled larvae were used. The different development stages were considered achieved when at least 75% of the sampled larvae were considered to be at that stage. Competence for settlement was considered achieved when the rudiment was equal in size or larger than the stomach (Carboni *et al.* 2012).

Larval survival was assessed volumetrically, and the mean value of each measurement was then used to calculate the number of larvae in the tanks. Survival at each development stage was

finally expressed as percentage of the initial number of larvae stocked.

Metamorphosis tests were done when larvae reached competence. A stock of 50 larvae was transferred into a 50-mL volume beaker containing filtered NSW and a 50 × 50 mm PVC layer colonized by the macroalgae *Ulvella lens*. This macroalgae was used as a metamorphosis-inducing factor for many organisms in previous studies (Taniguchi, Kurata, Maruzoi & Suzuki 1994; Daume, Huchette, Ryan & Day 2004; De Viçose, Viera, Huchette & Izquierdo 2012). PVC layers were set up according to the method described in Daume *et al.* (2004).

The number of metamorphosed individuals was counted at 24, 48 and 72 h post exposure to settlement media; Mt was considered achieved when at least 75% of the larvae were metamorphosed.

Statistical analysis

Data were analysed by Statistica 6.1 StatSoft, Inc. (2004). The amount of feed supplied and seawater exchanges, as well as the effects of rearing systems and algal diets on larval development and survival, were assessed using a two-way analysis of variance (ANOVA). Shapiro Wilk's W test was used to verify the normality of the data distribution and Levene's test was used to verify the homogeneity of variances. The General Linear Model (GLM) was used when normality and homogeneity were significant and also when the experimental design was unbalanced. Tukey's honestly significant difference (HSD) test was used to evaluate all pair-wise treatment comparisons ($P < 0.05$).

Results

Water exchange and feeding regime

The amount of NSW employed for the total experimental period significantly differed ($P < 0.01$) for the two rearing methods studied and for the different diets used. Indeed, 23.8 ± 0.7 L (mean ± SE) of NSW were consumed in the fixed method, while 9.6 ± 0.7 L were employed for the variable method. For the variable method, a lower amount of NSW was required when T-Iso was used as the larval feed when compared to the Cha treatment (Fig. 1).

Furthermore, the variable method required a significantly ($P < 0.001$) lower amount of *C. gracilis* in both the Cha ($40.0 \pm 0.0 \times 10^6$) and the mixed diet ($20.0 \pm 0.0 \times 10^6$) treatments, than the fixed method (respectively $552.0 \pm 32.0 \times 10^6$ and $284.0 \pm 27.0 \times 10^6$ cells). A lower ($P < 0.01$) consumption of T-Iso resulted in the mixed diet treatment when using the variable method ($123.0 \pm 20.9 \times 10^6$) than when the fixed method was used ($284.0 \pm 27.1 \times 10^6$). However, a 24.6% increase ($P < 0.01$) of T-Iso was observed when this microalgae was used as a monospecific diet. This was due to longer rearing cycle required by the variable method (38 days) compared to the fixed one (26 days; Fig. 2).

Rearing method effects on larval development and survival

Our results show that larval development and survival are significantly influenced by diets and rearing methodology. In fact, the variable method resulted in a significantly ($P < 0.001$) higher lar-

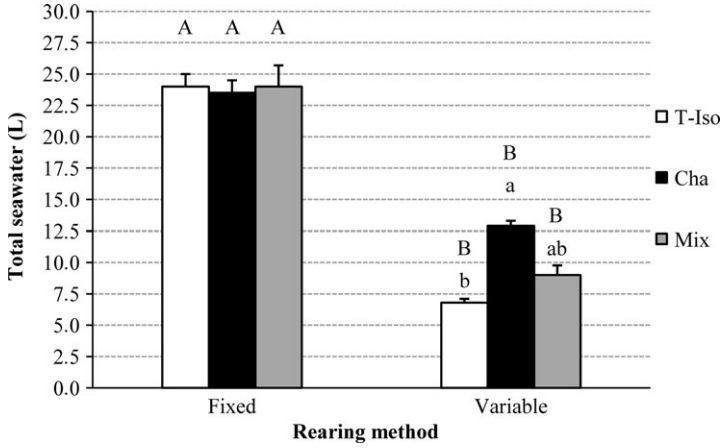

Figure 1 Total seawater (L) consumed during the whole larval rearing. *Isochrysis* sp. (Tahitian strain, T-Iso), *Chaetoceros gracilis* (Cha), 50:50 mixture of the same species (mix). Capital superscripts indicate significant differences between rearing methods, and lowercase superscripts indicate significant differences among diets. Values are expressed as mean ± SE ($n = 5$).

val survival at the Cp stage than the fixed method. Also the larvae which were fed T-Iso achieved significantly ($P < 0.01$) higher survival rates compared with the larvae fed with the Cha treatments, respectively, 88.5 ± 4.4 (mean ± SE) and 43.8 ± 10.1 (Fig. 3).

Feeding T-Iso resulted in a significantly ($P < 0.01$) faster development up to the P6 stage regardless of the method used. At 14 DPF, in fact, 84.9 ± 7.3% (mean ± SE) of the larvae-fed T-iso achieved this stage, while only 28.7 ± 10.9% and 46.9 ± 16.6% of the larvae-fed Cha and mix diet, respectively, achieved this stage. Similar results were observed for the variable method where 92.6 ± 6.0% of the larvae-fed T-Iso achieved P6, while 9.2 ± 5.8% and 55.9 ± 8.9% of the larvae were at this stage when fed with Cha and mix diet respectively. By 17 DPF, all the larvae fed T-Iso in the variable methods were observed to be at the P6 stage, while 88.0 ± 7.4% of them was at this stage under the fixed rearing regime. Nonetheless, larvae fed T-Iso were significantly more advanced ($P < 0.01$) than larvae fed with Cha regardless of the rearing method used. Interestingly, a significant difference ($P < 0.001$) between the fixed and the variable methods was observed in the mix diet treatment at 17 DPF. Indeed, significantly more larvae achieved P6 when reared under variable conditions than under the fixed method (Fig. 4).

Competence was achieved by all surviving larvae in the variable method by 29 DPF. However, larvae fed the mix diet achieved competence significantly faster ($P < 0.01$) than those reared under fixed conditions. In the former method, 34.6 ± 10.2% were considered competent to settle

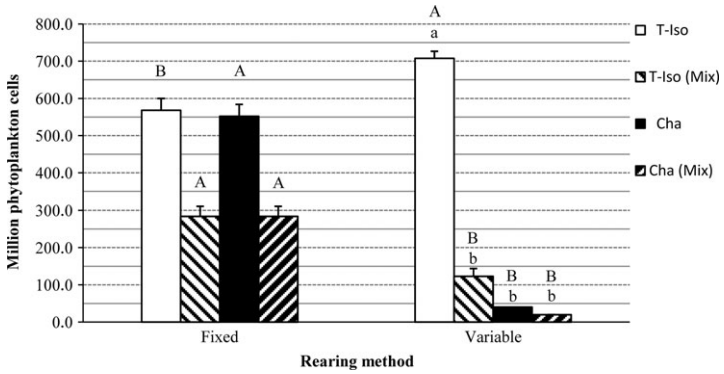

Figure 2 Total phytoplankton (million of cells) supplied during the whole larval rearing. *Isochrysis* sp. (Tahitian strain T-Iso), *Chaetoceros gracilis* (Cha), *Isochrysis* sp. [T-Iso (Mix)] and *C. gracilis* [Cha (Mix)] in the mixture. Capital superscripts indicate significant differences between rearing methods, and lowercase superscripts indicate significant differences among diets. Values are expressed as mean ± SE ($n = 5$).

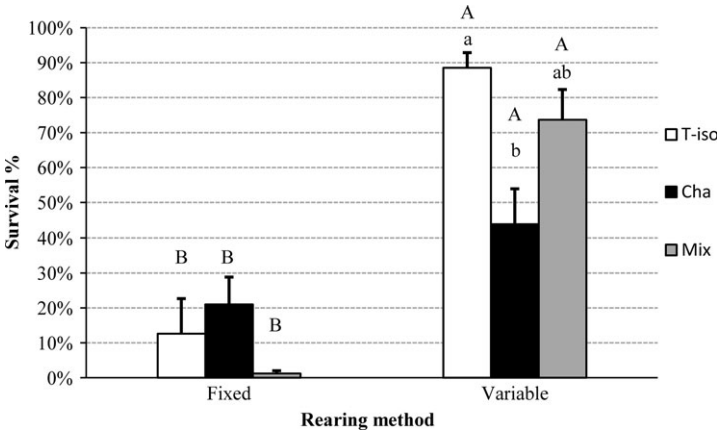

Figure 3 Larval survival with variable and fixed rearing method at the Competence stage (Cp). *Isochrysis* sp. (T-Iso), *Chaetoceros gracilis* (Cha), mixture of T-Iso and *C. gracilis* (mix). Capital superscripts indicate significant differences between rearing methods, and lowercase superscripts indicate significant differences among diets. Values are expressed as mean ± SE ($n = 5$).

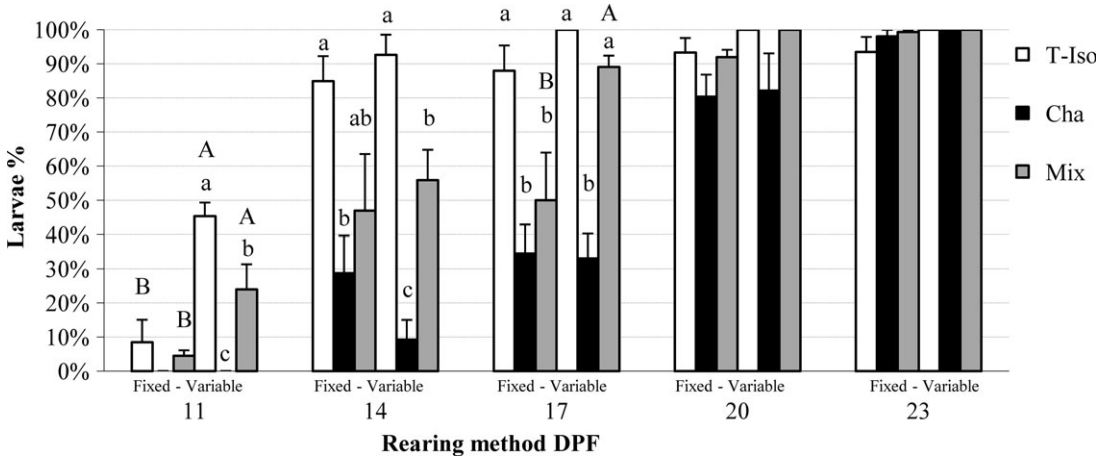

Figure 4 Larval development at the 6-arm stage (P6). Days post fertilization (DPF), *Isochrysis* sp. (T-Iso), *Chaetoceros gracilis* (Cha), mixture of T-Iso and *C. gracilis* (mix). Capital superscripts indicate significant differences between rearing methods, and lowercase superscripts indicate significant differences among diets. Values are expressed as mean ± SE (n = 5).

at 17 DPF, while none of them were ready in the latter. By 20 DPF, 99.0 ± 1.0% and 9.6 ± 8.2% achieved competence in the variable and fixed methods respectively. At 23 DPF, all larvae fed the mixed diet in the variable method achieved competence, while only 9.6 ± 8.2% were found to be at this stage in the fixed method. Between 23 and 29 DPF, however, 44.8 ± 22.4% of the larvae fed this diet in the fixed method achieved the competence stage. No significant differences were observed between the two tested rearing methods when larvae were fed the other diets, with the only exception of larvae fed Cha at 20 DPF where, once again, larvae under the variable method outperformed those in the fixed one. Within the variable method, significant differences were nonetheless observed between diets. A faster development occurred in larvae fed with the mixed diet than T-Iso at 20 DPF (99.0 ± 1.0% and 41.0 ± 19.2%, respectively), 23 DPF (100% and 58.2 ± 13.7%, respectively) and 26 DPF (100% and 72.0 ± 10.2%, respectively) (Fig. 5).

After achieving the competence stage, a delay was observed before competent larvae could undergo metamorphosis. A significant difference was observed in the percentages of successfully metamorphosed larvae for the different dietary treatments and rearing methods. Indeed, the fixed method did not yield any metamorphosed larvae while 35.5 ± 8.4% of larvae successfully metamorphosed under the variable method. Furthermore, higher survival rates resulted for larvae fed with mix (63.3 ± 8.9%) than with T-Iso (19.7 ± 12.1%) and Cha (23.4 ± 15.1%) treatments ($P < 0.05$; Fig. 6).

Discussion

The alternative rearing system tested in this study (variable method) improved survival and development rates in *P. lividus* larvae up to metamorphosis. Indeed, by giving a variable amount of microalgae, it was possible to optimize the feed ration, according to larval consumption. Moreover, a visible decrement in the production of debris and settled phytoplankton on the bottom of the tanks was observed, and this led to less maintenance of larval tanks and therefore reduced larval handling and water replacement.

Our results clearly show faster larval development and higher survival with the variable method *versus* the fixed one. More specifically, larvae fed with a variable amount of feed reached competence faster than those reared with the fixed method. Moreover, regardless of the dietary treatment applied, all larvae reared with the variable method achieved the metamorphosis stage. Instead, when a fixed amount of feed was supplied, larval development decreased progressively, and regardless of the feed used, no larvae reached metamorphosis. More importantly and regardless of the method employed, a lower larval survival rate at metamorphosis compared to competence and a delay between competence and successful

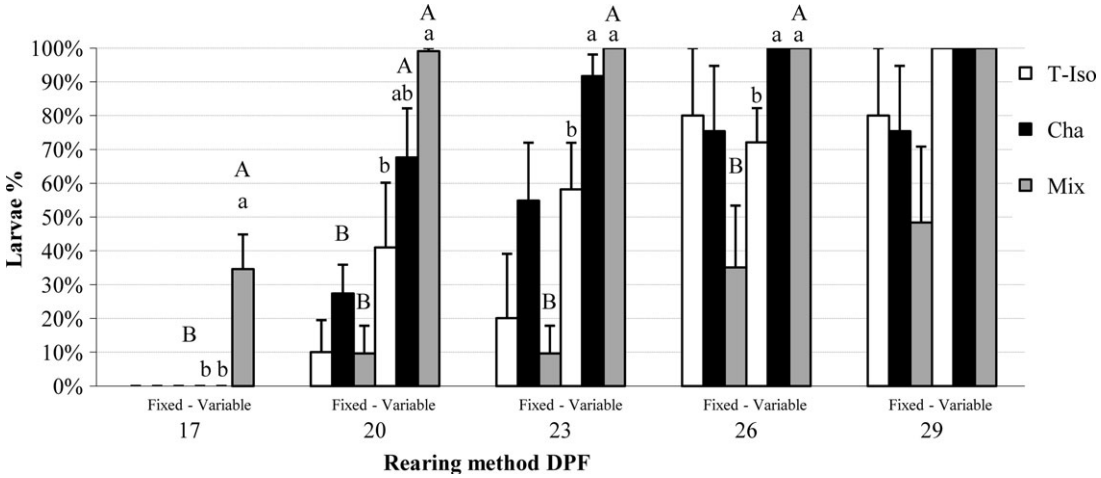

Figure 5 Larval development percentage at the competence stage (Cp). Days post fertilization (DPF), *Isochrysis* sp. (T-Iso), *Chaetoceros gracilis* (Cha), mixture of T-Iso and *C. gracilis* (mix). Capital superscripts indicate significant differences between rearing methods, and lowercase superscripts indicate significant differences among diets. Values are expressed as mean ± SE (n = 5).

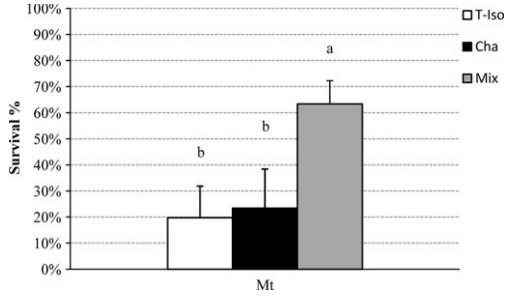

Figure 6 Larval survival at the Metamorphosis stage (Mt). *Isochrysis* sp. (T-Iso), *Chaetoceros gracilis* (Cha), mixture of T-Iso and *C. gracilis* (mix). Fixed method is not shown due to 0% survival. Lowercase superscripts indicate significant differences among diets. Values are expressed as mean ± SE (n = 5).

metamorphosis were observed. This could indicate that the use of the rudiment size, equal or larger than the stomach, may not be the best morphological indicator of larval readiness for settlement, as reported by numerous authors (Fenaux *et al.* 1994; George, Lawrence & Lawrence 2004; Carboni *et al.* 2012).

The variable method involved supplying a feed ration directly proportional to the larval consumption, thereby minimizing the phytoplankton in excess. In traditional static systems, the fixed feed ration causes a high amount of debris and phytoplankton to settle on the bottom of the tanks, so siphoning and water exchange are crucial for maintaining water quality and for achieving successful larval production (Cárcamo 2004; Azad *et al.* 2011). Nevertheless, manual water exchange can be counterproductive as it is stressful and can damage the larvae in suspension (Russell 2000). This was also suggested by Carboni *et al.* (2014), who obtained better results with a flow-through system (characterized by the absence of manual water exchange) than with a traditional static water exchange system. Nonetheless, the flow-through system described by Carboni *et al.* (2014) still required significant amount of microalgae as the continuous water exchange applied increased the risk of feed losses. With our proposed variable method, we have also reduced larval handling and associated potential causes for mortality and, at the same time, reduced the requirements for microalgae and water replacement. The survival rate achieved with the variable method regardless of the diets was significantly higher than that of the fixed method. Moreover, the larval survival is comparable with that achieved by other authors that used the same diets and similar initial stocking density and higher than that obtained by previous studies that employed higher stocking densities (Carboni *et al.* 2012, 2014).

It is possible that this difference is due to the small volumes used in the present experiment. It is known, in fact, that a laboratory scale results in

higher survival rates, but these techniques are not always applicable on a large scale (Fenaux, Cellario & Etienne 1985; Pedrotti & Fenaux 1993; Kelly *et al.* 2000; George *et al.* 2004; Liu *et al.* 2007; Carboni *et al.* 2012). The highest survival of *P. lividus* larvae fed with phytoplankton was obtained by Pedrotti and Lemée (1999) in 1-L volume tanks, with a 95% survival at 18 days post-fertilization with *Cricosphaera elongata*. Using higher culture volumes, Liu *et al.* (2007) and Paredes *et al.* (2015) reported lower survival than Pedrotti and Lemée (1999), respectively, 68–76% in 60 L (*Dunaliella tertiolecta)* and 40% in 100 L (mix of *Tetraselmis suecica*, T-Iso, *C. gracilis*, *Phaeodactylum tricornutum* and *Cylindrotheca closterium*).

In 80-L tanks, Carboni *et al.* (2012) obtained even lower survival rates: 0% with *T. suecica*, ~5% with *D. tertiolecta*, ~6% with *Pleurochrysis carterae* and ~14% with *C. elongata*. These low survival percentages could be due to the high larval stocking densities (4/ml) adopted by Carboni *et al.* (2012), as that investigation focused on the application of the *P. lividus* echinoculture on a commercial scale. Conversely, work by Pedrotti and Lemée (1999), Liu *et al.* (2007) and Paredes *et al.* (2015) used lower densities: 2, 1.5 and 1 larva mL^{-1} respectively. In laboratory experiments, low densities improve larval survival. However, the use of higher stocking densities in commercial settings offsets the lower larval survival at the competence stage by increasing the final output of competent larvae.

In this study, larval development and survival were also influenced by diets. *C. gracilis* resulted in a slower development up to the P6 than T-Iso. According to previous studies, the *I. galbana* diet is generally considered inferior to other microalgae for echinoderm larval rearing (Pechenik 1987; Schiopu, George & Castell 2006) as its use resulted in poorer growth and survival for *P. lividus* larvae (Fenaux, Cellario & Rassoulzadegan 1988) and other marine invertebrates such as *Crassostrea gigas*, *Venerupis philippinarum* and *Pecten maximus* (Marshall, McKinley & Pearce 2010). At the end of the rearing cycle, the larvae fed with the mix diet presented the fastest development and the highest survival at competence when the variable methodology was employed. Indeed, the fact that invertebrate larvae grow better on a mixture of phytoplankton species is established knowledge (Pedrotti & Fenaux 1993), as the lack of biomolecules supplied by one algae species could be provided by the other species in the mix (Strathmann 1971; Pechenik 1987; Schiopu *et al.* 2006).

The effects of feeding rations on sea urchin larval development and survival are well documented in the literature. Indeed, supplying an appropriate feed ration promotes growth rate and enhances the size of the larvae and the post-metamorphic survival in different sea urchin species, *P. lividus* (Vaïtilingon, Morgan, Grosjean, Gosselin & Jangoux 2001), *Loxechinus albus* (Cárcamo *et al.* 2005), *Dendraster excentricus* (Hart & Strathmann 1994), *P. miliaris* (Kelly *et al.* 2000), *Strongylocentrotus purpuratus* (Miller & Emlet 1999; Azad *et al.* 2011) and *S. droebachiensis* (Meidel, Scheibling & Metaxas 1999). By using the variable method, this study describes a protocol to further optimize feed rations for optimal larval development and survival. As observed by Azad *et al.* (2011), the larvae of *S. purpuratus* displayed a typical development and the highest survival percentage when fed with a standardized ration (1500–4000 cells mL^{-1}, increasing according to larval developmental stage and stocking density). A low ration (500 cells mL^{-1}), on the other hand, had negative effects on larval development (failure to metamorphosis) and survival. Nonetheless, high feed levels may also have detrimental effects on morphometric larval development and survival. In their study, Azad *et al.* (2011) also observed that larval development and survival of *S. purpuratus* differed according to the microalgal diets used, although their results showed slower development and survival when compared with the results described in this report. Moreover, Jimmy, Kelly and Beaumont (2003) analysed the morphology, the development and the metamorphic rate of *Echinus esculentus* larvae fed with a standard ration (1000, 3000 and 5000 cells mL^{-1} according to the developmental stage) and high ration (3000, 9000 and 15 000 cells mL^{-1}) of *D. tertiolecta*. Indeed, a standard ration promoted larval growth (length, width and rudiment length), a shorter development time (from 21–23 to 16 days), a higher number of larvae metamorphosed and larger juveniles at 5 months post settlement. Similar results have been reported for *P. miliaris* larvae (Kelly *et al.* 2000).

In our work, different amounts of microalgae species were supplied in the tanks. The tanks fed with T-Iso needed more constant and larger amounts of feed than the other tanks, due to their higher cell consumption. On the contrary, larvae fed with Cha were supplied with microalgae only

when the larvae achieved the echinopluteus stage (2 DPF) as a high amount of unconsumed *C. gracilis* was recorded during the whole rearing cycle. In the tanks fed with the mixed diet, a higher amount of *C. gracilis* was consistently observed along with the almost total absence of T-Iso. The different microalgae species consumption observed in this study was probably due to cell dimension, especially during the initial developmental stages. Two pairs of arms echinoplutei consume phytoplankton cells with a volume of 200–400 μm^3 (Carboni 2013). The lack of a cell wall and the small dimension (5 ± 1 μm in diameter) make *Isochrysis* sp. readily digestible by small larval invertebrates (Cordoba-Matson, Arredondo-Vega & Carreón-Palau 2013), while *Chaetoceros* sp. is larger than *Isochrys* sp., with a diameter of 7.3 ± 0.8 μm (Herawati, Hutabarat, Prayitno & Darmanto 2013), and may therefore be less preferable as food if an alternative microalgae species is also offered. This observation highlights the complexity of feed choices for different larval stages and suggests that the nutritional profile of a given microalgae species does not always dictate larval preferences.

Our results demonstrate for the first time that a fixed method characterized by continuous seawater replacement may not be essential for the production of *P. lividus* larvae if there is constant monitoring and replacement of the consumed microalgae cells. Variable amounts of feed improved larval survival and development, avoiding the need for water exchanges and minimizing phytoplankton requirements for the hatcheries. This variable method requires more technical labour and expertise in order to make assessment every 3 days regarding the amount of microalgae to be fed to the larvae, but it has the potential to reduce manual labour, water volumes and phytoplankton requirements in a sea urchin hatchery.

The survival results obtained in this study could be further improved by applying other microalgae diets, which have been demonstrated to promote higher survival and development of *P. lividus* larvae, such as *C. elongata* and *P. carterae*. Nevertheless, further studies would be needed to test the variable method at greater volumes and at a higher stocking density of larvae, in order to verify the applicability of this method to larger scale production systems and for other commercially important marine invertebrate species.

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