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Influence of diet assemblage on *Ostrea edulis* broodstock conditioning and subsequent larval development

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ABSTRACT

In contrast with the Japanese oyster *Crassostrea gigas* and the Manila clam *Ruditapes philippinarum*, *Ostrea edulis* seed production in the hatchery has been reported to be erratic, with sudden and unexplained larval and post-metamorphosis mortalities. Fecundity and initial larval quality have been related to broodstock conditioning, but effects on larval development and metamorphosis remain poorly understood. In addition, molluscan larval mortalities have been often associated with bacterial contamination and flow-through techniques may help to overcome this problem. Both aspects have been considered in the present work. *O. edulis* broodstock were conditioned at 19 °C and fed three different microalgal diets. Two were single-species diets: *Rhodomonas salina* (R_s) or *Thalassiosira weissflogii* (T_w) and the third was a combination of both species (R_sT_w: 50/50 in equivalent cell volume). Mean fecundity, expressed as mean number of larvae released by oysters fed different diets, was 0.16, 0.28 and 0.39 million, respectively; whereas, mean larval size at release differed significantly from 174 to 181 μm. Moreover, when broodstock were fed combined assemblage (R_sT_w), larval release occurred more consistently. Larvae were subsequently fed two different diets over an 11-day period: *Chaetoceros gracilis* solely (C_g) or a bi-specific assemblage (T: *Isochrysis affinis galbana* plus C_g). Larval growth ranged from 5.5 to 7.4 μm d^{-1} for larvae fed C_g and was generally higher (8.1 μm d^{-1}) in larvae fed the mixed diet TC_g. On day 11, larval survival and competence ranged from 50 to 75% and 40 to 70% respectively, these results being closely related to broodstock nutrition. On day 18 the larval settlement ranged from 1 to 60%. When analyzing overall performance, from fecundity to settlement, best results were obtained with broodstock, fed the bi-specific diet (R_sT_w), which released numerous larvae over a short period with satisfactory larval development and high metamorphosis, and these larvae also fed bi-specific diet, TC_g.

1. Introduction

From the 70s to the 80s two successive diseases affected *Ostrea edulis* production in Brittany (main area in France for its culture) and the population dropped from 20,000 t to 1000–1500 t y^{-1} nowadays (Buestel et al., 2009). Despite several attempts to control marteiliosis and bonamiosis in natural surroundings (Grizel, 1985) or eradicate its effects through modified husbandry (Le Bec et al., 1991; Robert et al., 1991), introduction of exotic flat oysters (*e.g. Ostrea puelchana*: Pascual et al., 1991), and genetic improvement (Naciri-Graven et al., 1999), the flat oyster population has never recovered.

This situation was quite similar for most countries in Europe (Laing et al., 2005) and, in this context, except in some limited free disease areas (*e.g.* Scotland, North Ireland, Norway, Denmark) flat oyster farming consists in improving oyster growth before the fateful limit of 3 years old or equivalent size and, accordingly, *O. edulis* production in Europe is constrained.

However, progress has been made in breeding for diseases resistance including new genetic tools (Lallias et al., 2009; Morga et al., 2011). Currently, a selective breeding program is, accordingly a possibility to enhance flat oyster farming. Such targeted genetic orientation, however, will not be feasible until the difficulty inherent to a lack of fully reliable methods in hatchery for this species is overcome. Indeed in the hatchery, unexplained mortalities have often been reported during larval rearing on day 8 and post-settlement (anonymous, 2004; Bédier, 2004; Laing et al., 2005). Hatchery methods are now relatively well known for many mollusks (*e.g. Crassostrea gigas*: Utting and Spencer, 1991, *Ruditapes philippinarum*: Helm and Pellizzato, 1990, *Mercenaria mercenaria*: Castagna and Kraeuter, 1981). Despite indisputable know-how, mainly due to pioneer works (Walne, 1974), the state of the art in hatchery rearing of *O. edulis* remains clearly insufficient to support reliable seed production, probably because of a lack of updated, detailed knowledge of the biology of this species. Compared to oviparous and dioecious species such as *C. gigas* and *R. decussatus/philippinarum*, the flat oyster is larviparous. Fecundity and initial larval quality have been related clearly to broodstock conditioning, mainly food delivery (Helm et al., 1991; Millican and Helm;, 1994; Utting and Millican, 1997) but further effects on larval development and metamorphosis are poorly

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known. Maternal effects on metamorphosis have been reported with *Ostrea chilensis* which incubates larvae for a very long period (Wilson et al., 1996); whereas in *O. edulis*, such parental effects have been shown to affect only larval growth and survival (Berntsson et al., 1997). The present work will contribute to this scope by focusing on the effects of food on *O. edulis* broodstock conditioning and subsequent larval development, including metamorphosis.

Moreover molluscan larval mortalities have often been associated with bacterial contamination, specifically to vibrios (Elston, 1984; Estes et al., 2004; Thompson et al., 2003). Widely described for Pectinidae (Lambert et al., 1999; Riquelme et al., 1996), and cupped oysters *Crassostrea* spp. (Elston and Leibovitz, 1980; Elston et al., 2008; Jeffries, 1982; Sugumar et al., 1998), vibriosis has recently been shown to affect *O. edulis* as well (Prado et al., 2005) confirming previous results on the same species (Jeffries, 1982). It is well known that scallops are very sensitive to vibrio infection (Nicolas et al., 1996) which led to the use of antibiotics, as a preventive measure, to limit larval mortalities (Robert et al., 1995). Such practices are not sustainable, and Norwegian researchers have partly overcome this problem by developing 5000-L flow-through larval rearing technique (Magnesen et al., 2006). To study the ecophysiological requirements of mollusks with low fertility (1–2 million oocytes in *O. edulis*) *vs.* 10–20 in *P. maximus* in hatchery conditions (Le Pennec et al., 1998) a 5-L container was designed, and a new, flow-through larval-rearing configuration was developed for *O. edulis*.

2. Material and methods

2.1. Microalgae and diet composition

Four different microalgal species were used in the present study: two large species for broodstock conditioning, *Rhodomonas salina* (R_s: volumetric size ≈ 200 μm^3, dry weight 60 pg cell^{-1}, strain CCAP 978/24) and *Thalassiosira weissflogii* (T_w: 950 μm^3, 250 pg cell^{-1}, CCAP 1077/5) and two small species for larval rearing, *Isochrysis affinis galbana* (T: 40 μm^3, 12 pg cell^{-1}, CCAP 927/14 also named T-Iso) and *Chaetoceros gracilis* (C_g: 80 μm^3, 25 pg cell^{-1}, UTEX LB2658).

O. edulis broodstock conditioning was assessed using three microalgal diets in duplicate: two single-species, *R. salina* with a daily ration of 10^9 cells oyster^{-1}or *T. weissflogii* (0.25×10^9 day^{-1} oyster^{-1}) and the bi-specific combination of *R. salina* plus *T. weissflogii* (50/50 equivalent cell volume). Because feeding in the inhalant chamber occurs in *O. chilensis* during motherhood (Chaparro et al., 2001, 2006), 10% of T-Iso supplemented all diets during the second month of conditioning.

O. edulis larval development was achieved using mono (*C. gracilis*) or bi-specific diets (T-Iso plus *C. gracilis*) delivered to maintain continuously 25 cells (±5) per each larva, as recommended for *C. gigas* larvae (Rico-Villa et al., 2009); whereas unfed larvae were used as a negative control. Additionally in a single experiment, a batch of larvae was also fed T-Iso to elucidate its role in larval development excluding metamorphosis.

2.2. Broodstock conditioning

Three hundred flat oysters from the same location were dredged from the Bay of Brest (Brittany, France) in March 2007. After scrubbing shells free of fouling organisms and debris before stocking in experimental structures at the Ifremer experimental station of Argenton (Brittany, France), oysters were pre-conditioned to experimental temperature (19 °C) by a daily gradual increase of 1 °C during 10 days. Seawater temperature was maintained at 19 °C by means of regulated thermal floodgates. Thereafter 50 2 year-old oysters were distributed homogeneously (70 g ± 20 mean whole weight, 1.7 g ± 0.6 mean meat dry weight, 70 mm ± 8 mean length) in each duplicate tank (2 m length × 0.5 m width × 0.2 m depth) per experimental condition. Continuous flow of 1-μm filtered-seawater was delivered from the top at a constant flow rate of 40 L h^{-1} (40% renewal h^{-1}). Daily rations of 6% dry weight microalgae (mg) per oyster meat (g) were provided to broodstock by peristaltic pumps that mixed the algae with filtered seawater at the inlet of each tank. All tank outlets were secured with a 100-μm mesh sieve to prevent larvae from escaping. *O. edulis* broodstock conditioning continued over a 4 month-period from February to May 2007.

Seawater at inlet and outlet of each experimental tank was sampled twice a day (morning and afternoon) and phytoplankton counts were made using an electronic particles counter (Multisizer 3 equipped with a 100-μm aperture tube). Grazing was expressed in number of cells removed from suspension oyster^{-1} d^{-1} or in μm^3 oyster^{-1} d^{-1}. For each experimental condition, fecundity was assessed as the number of released larvae over the considered period.

2.3. Larval rearing

When detected, expelled larvae were counted and measured for length. When a large release of larvae was recorded (≥1 million), larval rearing was set up in a dedicated flow-through cylindrical system inspired by the Cawthron design (King et al., 2005). Made from 3 mm, transparent Polymethyl methacrylate, six 5-l tanks (104 cm height and 9 cm diameter) were self-supported on a 12-mm PVC table of 150 cm length (Fig. 1a). The outlet of each tank was equipped with a 32 mm PVC pipe connected within the tank to a beveled, 100-μm sieve to prevent larvae from escaping. Each microalga was delivered by pumping from a reservoir into each inlet pipe through a 4-mm translucent, flexible line (Fig. 1b). Each larval rearing tank received from the top 1-μm-filtered phytoplankton-enriched seawater through a secondary line with flow controlled by a flow-meter (one per tank: Fig. 1c). In each larval tank, aeration was provided from the bottom and was maintained at 0.5 L min^{-1} using a 4-mm PVC valve (Fig. 1). Lastly the outlet of each cylinder was connected to a 6-mm tube allowing complete tank draining. For each broodstock origin (diet) and when a sufficient number of released larvae were collected, larvae were reared in those flow through units at a density of 5 larvae ml^{-1} with seawater flow maintained at 1.3 L h^{-1} (≈ 6 renewals day^{-1}). Seawater temperature/salinity was maintained at 22 °C/34 ppt according to Robert et al. (1988). Each broodstock diet was split into treatments with three larval diets, *i.e.*, 9 different larval conditions were assessed: **B**R_s**L**S, **B**R_s**L**C_g, **B**R_s**L**TC_g, **B**T_w**L**S, **B**T_w**L**C_g, **B**T_w**L**TC_g, **B**R_sT_w**L**S, **B**R_sT_w**L**C_g and **B**R_sT_w**L**TC_g. Moreover a high number of released larvae, originating from broodstock fed R_sT_w, allowed to set up an additional feeding condition and accordingly a batch of larvae was fed T. Iso alone. Thus, the designation **B**R_s**L**S means that broodstock was fed *R. salina* and larvae from them were starved; whereas, **B**R_sT_w**L**TC_g means that broodstock was fed *R. salina* plus *T. weissflogii*, and larvae from them were fed T-Iso plus *C. gracilis*.

Phytoplankton cell density was assessed twice a day at inlet and outlet of each experimental rearing tank, and adjustments were made to stabilize cell count around each larva at 25 cells (±5) or 1000 μm^3 equivalent T-Iso, values reported as an effective algal-cell density for *C. gigas* larval development (Rico-Villa et al., 2009).

In each rearing tank, larvae were collected by siphoning subsamples and larval shell length was measured on days 0 (released), 3, 7 and 11 using image analysis (Image vision Builder version 6.0). Survival was estimated on day 11 on the entire larval population collected by draining, mixing thoroughly, and counting a sub-sample under the light microscope. The number of larvae ready to set (competent larvae showing presence of large eyespots and active foot) was estimated prior to a selective grading on 200-μm mesh. Pediveligers > 280 μm were distributed at an initial density varying from 0.3 to 0.7 larvae ml^{-1} in 30-L tanks containing plastic disks as cultch (settlement material) (Rico-Villa et al., 2006). They were fed the bi-specific diet (TC_g: 50/50, v/v) thereafter, post-set, were maintained in a flow through system (9 L h^{-1}; 30% h^{-1} seawater renewal) for an additional week. The number of eyed pediveligers selected was, however, limited and we only obtained