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# Beneficial effects of carpet shell clam (*Ruditapes decussatus*) depuration during short periods of conditioning in shellfish hatchery: Role of the temperature and phytoplankton on reduction and diversity of vibrios

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ABSTRACT

Broodstock conditioning in hatcheries is the step previous to spawning and its optimization may be a key to the success of larval cultures. Cleaning and brushing of the broodstock and the utilization of antibiotics to reduce their vibrios load are used regularly as routine prophylactic measures prior to spawning induction. The development of protocols to reduce these bacteria using cheap and harmless techniques is of utmost importance for commercial bivalve production in hatcheries. With this aim, we have evaluated initially different conditionings (A–D) during short periods (a total of four weeks): first two weeks under gradient temperature (increasing +0.3 °C day$^{-1}$ from 14.5 °C to 20 °C), without (A) or with phytoplankton (B), and constant temperature (20 °C) without (C) or with phytoplankton (D). Afterwards, all conditionings were kept at 20 °C and fed for two more weeks. Furthermore, broodstock optimal feeding time was re-evaluated during a second trial series. In all conditionings, bacterial loads were determined in terms of marine heterotrophic bacteria (MHB) and presumptive vibrios (PV). Broodstock under the optimal short period of conditioning (conditioning C) obtained the best gonadal development and a significant reduction in PV load at a lower expense. A total of 61 PV were isolated from all conditionings and identified by sequencing the 16S rRNA gene. Splendidus clade was dominant in the samples coming from natural beds. Diversity of vibrios changed throughout the conditionings in the hatchery favoured by exogenous factors whose effect was mainly observed at the end of the trials: Splendidus clade was also dominant in the broodstock conditioned at gradient temperature and Mediterranei and Harveyi clades were prevalent at constant temperature. Moreover, the percentage of transformation to D-larvae was estimated and the vertical transmission of vibrios from broodstock to eggs and D-larvae was suggested. Implementation of conditioning C reduces considerably the *Vibrio* load of clams without using antibiotics, and thus it represents a novel, cheap, environmental friendly and harmless methodology that can be easily transferred to commercial hatchery.

*Statement of relevance:* Routine prophylactic measures for bivalve broodstock arriving hatchery facilities include its cleaning/brushing and the frequent use of antibiotics to reduce its bacterial load, particularly *Vibrio*, as step previous to spawning induction. The depuration protocol proposed reduces significantly *Vibrio* load without using antibiotics and represents a novel, cheap, environmental friendly and harmless methodology that can be easily transferred to commercial hatcheries.

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## 1. Introduction

Carpet shell clam (*Ruditapes decussatus*) constituted one of the most important bivalve species in European shellfish aquaculture (4128.73 T and 45 million €) (FishStatJ, FAO). Bivalve spat from hatchery is currently the only sustainable alternative for the support of aquaculture activities (da Costa et al., 2013; Ojea et al., 2008). The first step in hatchery culture is broodstock conditioning, previously collected from wild beds, in tanks where maturation is induced by artificial means, i.e. manipulating the seawater temperature and supplying adequate feed. Therefore, the production season can be extended and the gamete development of broodstock undergoing gametogenesis accelerated (Helm and Bourne, 2004). Different studies, reviewed by da Costa et al. (2013), demonstrated that the conditioning of *R. decussatus* broodstock at a high

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constant temperature (20 °C) or by a gradual increase in temperature from natural environment to the conditioning temperature (20 °C) constitutes two good alternatives to obtain sexually mature adults.

Monitoring bacteria entry routes should be the first step in the prevention of bacterial proliferation in bivalve cultures and the subsequent detrimental effects (Dubert et al., 2015a). Broodstock constitutes one of the most important bacterial sources for larval cultures due to the vertical transmission of bacteria from adults to larvae (Sandaa et al., 2008; Schulze et al., 2006). Microbiota may include opportunistic pathogens harmless to broodstock but harmful to larvae. Indeed, members of the genus *Vibrio*, including known larval pathogens, have been isolated in shellfish hatcheries from the gonad of broodstock (Lodeiros et al., 1987; Prado et al., 2014b; Riquelme et al., 1995; Sainz-Hernandez and Maeda-Martínez, 2005).

Routine prophylactic measures for bivalve broodstock arriving hatchery facilities include cleaning and brushing broodstock prior to spawning induction (Helm and Bourne, 2004). In addition to that, the use of antibiotics to reduce bacterial load of bivalve broodstock is very frequent in experimental and commercial broodstock conditioning (González-Araya et al., 2012; Holbach et al., 2015). This causes additional expense and efficiency of antibiotic treatments for ubiquitous pathogens is seriously questioned (Berthe, 2005). The use of antibiotics in shellfish hatcheries without an effluent treatment system is highly unadvisable, since these hatcheries constitute a potential source of antibiotic residues and resistant bacteria to the surroundings (Dubert et al., 2015b). Consequently, bivalve hatcheries have to develop protocols to reduce bacterial load in broodstock using cheap and harmless techniques.

Periods of conditioning will be directly related to reproductive activity of the broodstock, i.e. the conditioning periods will be shorter if the broodstock from the natural environment are ripe. In that case, vibrios load associated to broodstock must be reduced rapidly as step previous to spawning induction to minimize the potential risk of vertical transmission to larval cultures. This is the first study until now, in which we have evaluated the bacterial load of *R. decussatus* broodstock during short periods of conditioning previous to the spawning induction, modifying exogenous factors (temperature and phytoplankton). Cultivable bacteria, including *Vibrio* spp., associated to the gonad of these broodstock were also quantified. Vibrios were isolated, characterized and identified to evaluate the direct influence of exogenous factors on these bacterial populations. The optimal conditioning was based on the best reduction in vibrios load and gonadal developmental stage (GDS) of the broodstock. Moreover, in certain cases, D-larvae transformation rate was determined and vertical transmission suggested.

## 2. Materials and methods

### 2.1. Design of short periods of conditioning and sampling of broodstock

Two series of trials (Fig. 1) were designed in consecutive years to optimize the short periods of conditioning of *R. decussatus* broodstock in a shellfish hatchery for a total of 4 weeks. Ripe gametes could be released by the natural populations of carpet shell clam in Ría de Arousa (NW Spain) between April and August and major spawning efficiency occurs in August–September (Rodríguez-Moscoso and Arnaiz, 1998). Hence, adult specimens were collected in May by rake from a natural bed located in Illa de Arousa (Ría de Arousa, Galicia, NW Spain) in two consecutive years depending of the trial. Clams were kept at 10 °C, transferred to the hatchery of the Centro de Investigacións Mariñas (CIMA) (Ribadeo, Galicia, NW Spain) and conditioned under different exogenous factors (temperature and phytoplankton) for a total of 4 weeks. Broodstock were maintained in 150 L rectangular tanks with an open circuit of sand-filtered (1 μm) and UV-sterilized seawater at ambient salinity of 32–33 ppt. Tanks were kept with aeration under a photoperiod regime of 12:12 h with a continuous renewal of seawater in a ratio of 20 L/h/kg. Gametogenic scale proposed by Wilson and Seed (1974) was used with slight variations to adapt it for *R. decussatus* to assign the sex and gonadal developmental stage (GDS) of each individual after the observation by microscope (20×) of the histological preparations corresponding to the gonad: stage 0 (rest stage), 1 (start of gametogenesis), 2 (advanced gametogenesis), 3 (ripe), 4 (spawning) and 5 (restoration).

#### 2.1.1. First trial

In the first trial series, the bacteriological optimization of the short periods of conditioning was studied modifying the temperature of the tanks and supplying phytoplankton or kept the broodstock in a brief depuration without phytoplankton. Four challenges were performed in duplicate and a total of 560 clams (mean shell length: 43.5 ± 3.3 mm) were distributed in eight tanks (70 individuals per tank; 1.3 kg/150 L) and conditioned for a total four weeks. During the first two weeks (Fig. 1A), clams were conditioned varying combinations of temperature and food (conditionings A–D) following the conditions described by da Costa et al. (2013): gradual increase of temperature (increasing +0.3 °C $day^{-1}$ from 14.5 °C to 20 °C), without (A) or with phytoplankton (B), or at high constant temperature (20 °C) without (C) or with phytoplankton (D). Broodstock corresponding to conditionings B and D were continuously fed with a mixture of phytoplankton based on *Isochrysis galbana*, *Diacronema lutheri*, *Tetraselmis suecica*, *Chaetoceros* sp. and *Skeletonema marinoï* in equal cell concentration

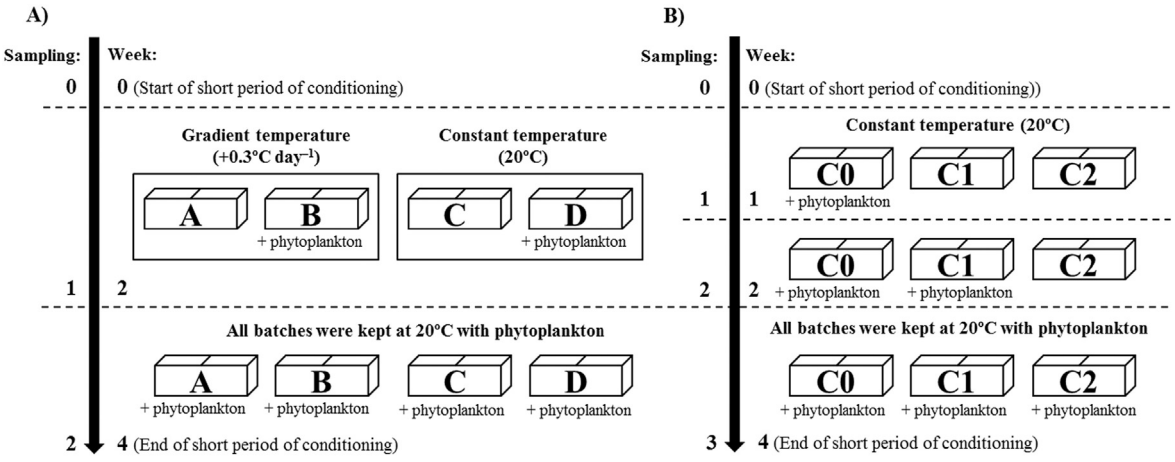


**Fig. 1.** Series of trials designed to optimize the short periods of conditioning modifying only temperature and supplying or not phytoplankton: first (A) and second (B) trials. Trials were performed in duplicate.

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