Fish & Shellfish Immunology 51 (2016) 104–115

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Exposure to the toxic dinoflagellate *Alexandrium catenella* modulates juvenile oyster *Crassostrea gigas* hemocyte variables subjected to different biotic conditions

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ARTICLE INFO

Article history:
Received 19 October 2015
Received in revised form
8 February 2016
Accepted 12 February 2016
Available online 13 February 2016

Keywords:
Crassostrea gigas
Alexandrium
Harmful algal blooms
Hemocyte
Microbial challenge

ABSTRACT

The Pacific oyster *Crassostrea gigas* is an important commercial species cultured throughout the world. Oyster production practices often include transfers of animals into new environments that can be stressful, especially at young ages. This study was undertaken to determine if a toxic *Alexandrium* bloom, occurring repeatedly in French oyster beds, could modulate juvenile oyster cellular immune responses (i.e. hemocyte variables). We simulated planting on commercial beds by conducting a cohabitation exposure of juvenile, "specific pathogen-free" (SPF) oysters (naïve from the environment) with previously field-exposed oysters to induce interactions with new microorganisms. Indeed, toxic *Alexandrium* spp. exposures have been reported to modulate bivalve interaction with specific pathogens, as well as physiological and immunological variables in bivalves. In summary, SPF oysters were subjected to an artificial bloom of *Alexandrium catenella*, simultaneously with a cohabitation challenge.

Exposure to *A. catenella*, and thus to the paralytic shellfish toxins (PSTs) and extracellular bioactive compounds produced by this alga, induced higher concentration, size, complexity and reactive oxygen species (ROS) production of circulating hemocytes. Challenge by cohabitation with field-exposed oysters also activated these hemocyte responses, suggesting a defense response to new microorganism exposure. These hemocyte responses to cohabitation challenge, however, were partially inhibited by *A. catenella* exposure, which enhanced hemocyte mortality, suggesting either detrimental effects of the interaction of both stressors on immune capacity, or the implementation of an alternative immune strategy through apoptosis. Indeed, no infection with specific pathogens (herpesvirus OsHV-1 or *Vibrio aesturianus*) was detected. Additionally, lower PST accumulation in challenged oysters suggests a physiological impairment through alteration of feeding-related processes. Overall, results of this study show that a short-term exposure to *A. catenella* combined with an exposure to a modified microbial community inhibited some hemocyte responses, and likely compromised physiological condition of the juvenile oysters.

1. Introduction

Infectious diseases have caused recurrent losses in shellfish stocks that have decreased the profitability of the aquaculture industry over the past decades [1–3]. Specific, pathogenic microorganisms have been involved in major diseases of bivalves, including the herpesvirus OsHV-1μVar that is associated with

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http://dx.doi.org/10.1016/j.fsi.2016.02.017
1050-4648/

recent, massive mortalities of Pacific oyster spat and juveniles [4–11], and several bacterial species and strains from the *Vibrio* genus, particularly *Vibrio aesturianus* [12–15].

The Pacific oyster *Crassostrea gigas* is the most exploited bivalve species, with a worldwide production estimated over 1.9 million tons in 2013 [16–21]. Oyster farming practices usually include numerous transfers of oysters at all life stages, especially of spat and juveniles grown in hatchery and nursery systems before being transferred to oyster farming areas in open seawater [22]. Along with these farming practices, oysters must adapt to new abiotic and biotic environments. Biotic changes include interactions with new micro-organisms, potentially pathogenic or toxic, which can enter oysters *via* filtration and feeding processes.

In addition to pathogens, phytoplankton biotic interactions can change during and after transplanting. Natural phytoplankton constitute a main component of the oyster diet, but deleterious effects can occur when harmful algae are present. Dinoflagellates are the most represented group causing harmful algal blooms (HABs), with species in the Genus *Alexandrium* producing paralytic shellfish toxins (PSTs) and/or spirolides, both neurotoxic. *Alexandrium* spp. exposures can alter physiological processes and tissue integrity of bivalves [23–31].

Experimental exposures to *Alexandrium* spp. were reported to modulate host-pathogen interactions in oysters [32–34], possibly by altering immune function, as suggested by the alteration of hemocyte characteristics [33]. Several other studies also reported effects of *Alexandrium* spp. exposure upon bivalve hemocytes [35–37]. Hemocytes, present in the tissues and in the circulating hemolymph, are the cellular mediators of immune responses in bivalves, which also include humoral factors. Hemocytes are involved in phagocytosis or encapsulation to achieve pathogen degradation through release of hydrolytic enzymes and oxidative compounds [38,39].

Alexandrium catenella recurrently blooms along the French Mediterranean coast [40,41] where major oyster farming activities occur. Considering the effects of *Alexandrium* spp. upon host-pathogen interactions and upon physiological and immunological variables, we hypothesized that exposure to *A. catenella* (producing PSTs) could compromise immune status of juvenile oysters (supposedly more sensitive than adults and commonly victims of massive mortality events). In addition, to assess if *A. catenella* exposure could render juvenile oysters more susceptible to opportunistic infection, spat produced under controlled conditions were put in contact with field-exposed oysters to introduce microorganisms from the environment.

This study thus investigated the possible interactions between juvenile oysters *C. gigas*, grown in hatchery, and a new biotic environment, defined by (i) an artificial bloom of *A. catenella*, and (ii) a modification of the microbial community induced by cohabitation with oysters previously exposed to the field, a process known to release and transmit pathogens [34,42]. Upon exposure to these biotic changes, hemocyte responses, toxin accumulation, OsHV-1 and *V. aesturianus* burdens (two pathogens monitored during massive mortality events by the French Monitoring Network for Shellfish Farming, RESCO [43,44]) as well as total *Vibrio* loads, were assessed after 4 and 9 days of exposure.

2. Material and methods

2.1. Algal cultures

Tisochrysis lutea (Bendif & Probert) (T-*Iso*) was fed to oysters during acclimation and maintenance stages at 5×10^5 cells mL^{-1}. T-*Iso* was cultured in 300-L cylinders containing seawater enriched with Conway medium [45] at 20 °C with continuous light (200 μmol photons m^{-2} s^{-1}). T-*Iso* was harvested after 3–5 days of growth, at a cell density approaching 1×10^7 cells mL^{-1}.

The dinoflagellate *Alexandrium catenella* (Whedon & Kofoid) strain VGO676, a paralytic shellfish toxin (PST) producer [46], isolated in 2003 from the Thau lagoon (France), was used for toxic algal exposure, and *Heterocapsa triquetra* (Ehrenberg) Stein, strain HT99PZ (isolated from Penzé Bay, France in 1999), was used as a control, non-toxic dinoflagellate. Both strains were provided by the Phycotoxin Laboratory, Ifremer, Nantes (France). Both dinoflagellate cultures were grown in L1 medium [47] at 17 °C with a light:dark cycle of 12:12 h and were harvested during the exponential growth phase at a cell density approaching 5×10^4 cells mL^{-1}.

Algal cell densities were determined by counts using Malassez and Nageotte cells under a light microscope.

2.2. Specific pathogen-free (SPF) oysters

The Pacific oysters, *Crassostrea gigas* (Thunberg), used in this study all came from a single cohort produced in April 2011 in the Argenton Ifremer facilities (France) following a standardized procedure to obtain OsHV-1-free diploid oysters described by Petton et al. [42]. Screening for OsHV-1 DNA was conducted by qPCR (following the standard procedure described in Pépin et al. [48] a first time during D-larval stage and at 3 months of age following thermal challenge, and all tested negative (analyses by IDHESA, Quimper, France). At the beginning of the experiment (September 2011), oysters were 5 months of age, measured 30–40 mm shell height and total wet weight (soft tissues and shell) was 3.2 ± 0.2 g (mean ± SE).

2.3. Field-exposure

On September 1, 2011, a subsample of the SPF oysters was transferred to an oyster farming area in the Bay of Brest, at Pointe du Chateau (48° 20′ 06.19″ N, 4° 19′ 06.37″ W). Although mass mortality events associated with OsHV-1 recurrently occur in this location, low mortality was reported during this period (5–26% mortality in one month of juvenile, SPF oysters, RESCO: http://wwz.ifremer.fr/observatoire_conchylicole/Resultats-nationaux/Resultats-nationaux-2011/Mortalite-par-site-et-par-classe-d-age; B. Petton, pers. com.). No harmful algal blooms were detected during this period (data monitored by VELYGER and RESCO networks, Ifremer). After 2 weeks in the field (i.e. on September 19, 2011), oysters were transferred to the experimental facilities and were used to challenge SPF oysters by cohabitation, as described below.

2.4. Experimental design

On September 15, 2011, 420 SPF oysters were distributed into twelve 15-L tanks (35 SPF oysters per tank) and acclimated for 4 days fed continuously with T-*Iso* at $3–5 \times 10^5$ cell mL^{-1}. At the end of this acclimation period, on September 19, 10 field-exposed oysters per tank, held in a net, were added to six of the 12 experimental tanks. The SPF oysters that were thus maintained in cohabitation with these field-exposed oysters were designated as “challenged”. In the six other tanks, 10 other SPF oysters per tank, held in a net, were added to obtain the same number of oysters in all tanks. The oysters in these tanks were then designated as “unchallenged”. In addition, 3 “challenged” tanks and 3 “unchallenged” tanks were exposed continuously to 1×10^2 cell mL^{-1} of the toxic dinoflagellate *A. catenella*; whereas, the other tanks were exposed to the same concentration of the control, non-toxic dinoflagellate, *H. triquetra.*

Experimental design is summarized in Fig. 1. Four experimental conditions were used: *A. catenella* (toxic algae) exposure and