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Effects of *Alexandrium minutum* exposure on nutrition-related processes and reproductive output in oysters *Crassostrea gigas*

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ABSTRACT

This study assessed the effects of an artificial bloom of the toxin-producing dinoflagellate, *Alexandrium minutum*, upon nutrition related processes and reproductive output of the Pacific oyster, *Crassostrea gigas*. Oysters were exposed to *A. minutum*, Paralytic Shellfish Toxins (PST) producer and compared to a control batch of oysters fed *Isochrysis galbana* clone Tahitian (T.*Iso*). The experiment was performed in June 2008, when oysters were found ripe. Several physiological variables of oysters, such as PSTs accumulation, digestive gland and histological observations as well as spermatozoa quality, were measured at the end of the exposure. Results indicate that the digestive gland was greatly impacted upon *A. minutum* exposure. Monoacylglycerol and diacylglycerol contents as well as phospholipids (mainly phosphatidylcholine) drastically decreased in the digestive gland of oysters exposed to *A. minutum* compared to control oysters. At the same time, many oysters exposed to the harmful microalga presented a strong inflammatory response in different tissues of the digestive gland: in intestine as well as in digestive ducts and tubules. Spermatozoa in oysters exposed to *A. minutum* were morphologically and functionally modified compared to spermatozoa of control oysters. Indeed, spermatozoa were less motile and had lower ATP content in oysters exposed to *A. minutum*. Meanwhile, spermatozoa produced by control oysters showed higher percentage of mortality and relative DNA content than those produced by *A. minutum* exposed oysters. Finally, the characteristics of the mitochondria of spermatozoa also appeared to be modified upon *A. minutum* exposure. The results of this study suggests that an exposure of oysters to *A. minutum*, reducing energy status and motility of spermatozoa associated to morphological changes at the cellular and sub-cellular levels, can have consequences on spermatozoa fertility and reproduction success.

1. Introduction

Among harmful algae, *Alexandrium* species have worldwide distribution, and some species have caused many PST-related events (Kim et al., 2005). The dinoflagellate *Alexandrium minutum* was observed in several countries in the world (North America, Australia, Taiwan, New Zealand and Jamaica for example) and in Europe, such as Spain, Ireland, Italy, Greece and France (Lilly et al., 2005; Ignatiades et al., 2007; Ranston et al., 2007). Moreover, this species was known to produce Paralytic Shellfish Toxins (PSTs). Several commercial bivalve species, such as oysters, are known to accumulate PSTs by feeding on PST-producing microalga (Oshima et al., 1990; Bricelj and Shumway, 1998). Mode of action of PSTs involved a reversible and highly specific block of ion transport by the sodium channel and thus of the action potential in excitable membranes (nerve and muscle fibers) (Narahashi, 1988; Bricelj and Shumway, 1998). Aductor-muscle paralysis of oysters, *Crassostrea virginica* (Gmelin, 1791), exposed to cultured *Alexandrium fundyense* (Balech, 1985) was observed by Hégaret et al. (2007).

Alexandrium exposure is also known to have a negative impact on filtration and ingestion in bivalves (Bardouil et al., 1993; Wildish et al., 1998; Lassus et al., 1999; Li et al., 2001; Navarro et al., 2008). Inhibition of clearance was observed as the initial

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feeding response of *Crassostrea gigas* (Thunberg, 1793) exposed to *A. tamarense* (Lebour, 1925) and *A. fundyense* (Wildish et al., 1998). Similarly, Lassus et al. (1999) observed that exposing oysters, *C. gigas*, to cultured *A. minutum* (Halim, 1960) induced significant inhibition of shell-valve activity and clearance, filtration, and biodeposition rates.

Exposure of bivalves to harmful microalga can also affect digestion and energy allocation. Li et al. (2002) assessed the effects of *A. tamarense* on the energy budget, quantified as scope for growth (SFG), of the mussel, *Perna viridis* (Linnaeus, 1758), and the manila clam, *Ruditapes philippinarum* (Adams and Reeve, 1850). This study demonstrated that increase in PST burden was associated with significant reduction of SFG in both clams and mussels, primarily because of decreases in absorption efficiency (Li et al., 2002). This reduction in SFG can hypothetically be linked to modification of digestive metabolism (Fernández-Reiriz et al., 2008). Also, a recent study (Haberkorn et al., in press) demonstrated that reserve lipids in digestive glands of *C. gigas* are modified upon exposure to *A. minutum*. Free fatty acid, monoacylglycerol, and diacylglycerol contents, as well as the ratio of reserve lipids (triacylglycerol, ether glycerides and sterol esters) to structural lipids (sterols), decreased significantly in digestive glands of oysters exposed to *A. minutum* compared to oysters fed a nutritious, control diet of *Isochrysis* sp. (Haberkorn et al., in press).

Some recent studies have described inflammatory responses (aggregation/infiltration of hemocytes in organs and/or hemocytes in diapedesis through epithelia) in different tissues of bivalve species exposed to several harmful microalga (Galimany et al., 2008a,b,c; Hégaret et al., 2009). Mussels (*Mytilus edulis*, Linnaeus, 1758) exposed to *A. fundyense* showed an inflammatory response consisting of diapedesis of hemocytes into the alimentary canal and hemocyte migration into the connective tissue between the gonadal follicles (Galimany et al., 2008a). These findings suggest that harmful-algal exposure can elicit activation of defense or repair mechanisms in response to resulting tissue lesions in bivalves.

Also, according to Galimany et al. (2008a,b,c) and Hégaret et al. (2009), the digestive gland is generally the organ most-affected by HAB exposure. Thus, as the structure of the digestive gland is often observed to be drastically modified, it appears pertinent to study the structural components of cell membranes in the digestive gland. Phospholipids and sterols are essential structural components of all cell membranes and may be used as markers of membrane modifications. Phospholipid alterations may occur through the oxidation of these labile molecules. Moreover, inflammatory responses are known to be induced by lipid oxidation in vertebrate models (reviewed in Fantone and Ward, 1982). In mussels (*M. edulis*) exposed to *A. fundyense*, increased ceroidosis in tissues was probably attributable to lipid peroxidation generated by ingestion of the harmful alga (Galimany et al., 2008a). Concurrently, an inflammatory response was observed in the mussel alimentary canal (Galimany et al., 2008a). These findings suggested a possible link between changes in digestive-gland lipids and inflammatory response in the same organ.

Beyond the direct effects of HABs described above, it is unknown how HAB exposure affects other physiological functions, such as reproduction. Relationships between food quality and quantity, energy storage, and reproduction are now well established in *C. gigas* (Soudant et al., 1999; Berthelin et al., 2000; Royer et al., 2008; Rico-Villa et al., 2009). These observations suggest that HAB exposure, by affecting nutrition-related functions, may impact reproduction processes and gamete output. Indeed, it is well-established that reserve lipids play an important role in gamete production and larval development (Soudant et al., 1996, 1998). Consequently, the effects of harmful algae on digestive-gland lipids, as demonstrated by Haberkorn et al. (in press), may further affect gametogenic processes, reproduction output and larval development.

Most studies concerning the effects of harmful algae on reproductive cycles of bivalves are focusing on embryonic and larval development (Yan et al., 2001, 2003; Springer et al., 2002; Leverone et al., 2006; Padilla et al., 2006; Shumway et al., 2006). Some studies have observed alterations induced by noxious compounds on quality of gametes produced by oysters (Nice, 2005; Ringwood et al., 2004; Yurchenko et al., 2009), but there are no studies addressing the effects of harmful algae on gametes produced by HAB-exposed bivalve broodstock. Spermatozoa quality, and especially spermatozoa motility, influence successful fertilization in free-spawning invertebrates (Nice, 2005). A positive relationship between ATP content and spermatozoan motility has been reported in carp *Cyprinus carpio* (Perchec et al., 1995). Other sperm characteristics (viability, acrosomal integrity, mitochondrial membrane potential, and DNA integrity) in relation to fertilizing capacity can be accurately and rapidly measured using flow cytometry coupled with fluorescent markers (Gillan et al., 2005; Cordelli et al., 2005). For example, mitochondrial membrane potential measured with JC-1 was shown to be reasonably predictive of *in vitro* fertilisation rates (higher in a group with high mitochondrial membrane potential) (Kasai et al., 2002).

The purpose of the present study was to determine the effects of an artificial bloom of the toxin-producing dinoflagellate, *A. minutum* (strain AM89BM) on nutrition-related processes and reproduction of *C. gigas*; to do so, the digestive gland was assessed for histopathological condition, toxin accumulation, lipid composition, and amylase activity. Further, spermatozoa quality was measured in oysters (*C. gigas*) after 4 days of exposure to *A. minutum* or *Isochrysis* sp. (clone Tahitian T.*Iso*) as a nutritious, non-toxic control.

2. Materials and methods

2.1. Biological material

2.1.1. Oysters

Diploid Pacific oysters, *C. gigas*, used in the experiment were obtained from an oyster producer at île de Kerner (Morbihan, France). Mean total oyster fresh weight was 11.9 ± 1.2 g and mean shell length was 62.6 ± 2.7 mm.

2.1.2. Algal culture

A. minutum (strain AM89BM – isolated in Bay of Morlaix, France, in 1995) was grown in 10-l batch culture using autoclaved seawater filtered through a 1-μm filter and supplemented with L1 nutrient enrichment (Guillard and Hargraves, 1993). Cultures were incubated at 16 ± 1 °C and 100 μmol photon m^{-2} s^{-1}, with a dark:-light cycle of 12:12 h. *A. minutum* was harvested after 12 days, still in exponential growth phase under our conditions. At this stage, this strain produced 1.3 ± 0.1 pg saxitoxin equivalent (STX equiv.) per cell (measured by the method of Oshima, 1995).

Isochrysis galbana, clone Tahitian (T.*Iso*), cultures were obtained from the Argenton hatchery (Ifremer, France). Cultures were produced in 300-l cylinders containing 1-μm filtered seawater enriched with Conway medium at 24 ± 1 °C, air–CO_2 (3%) mix aerated, and with continuous light. T.*Iso* was harvested in the exponential growth phase (4–5 days) for the feeding experiments.

2.2. Experimental design of A. minutum exposures

This experiment was performed on mid-June 2008. To proceed, 120 oysters were placed randomly in six 15-l tanks (20 oysters per tank). Oysters were acclimated for 10 days with a continuous flow of 14 ml min^{-1} of seawater (filtered through a 0.5-μm filter) with T.*Iso* at 5 × 10^5 cells ml^{-1} at 16 ± 1 °C. After acclimation, oysters were fed continuously for 4 days at 14 ml min^{-1} with 5 × 10^5 cells ml^{-1} of T.*Iso*