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Genomic abnormalities affecting mussels (*Mytilus edulis-galloprovincialis*) in France are related to ongoing neoplastic processes, evidenced by dual flow cytometry and cell monolayer analyses

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ABSTRACT

In the context of the abnormal mass mortality of mussels in France since 2014, Flow CytoMetry (FCM) was used in 2015 and 2016 to study the DNA content and cell cycle characteristics of hemic circulating cells collected from 2000 mussels. The mussels were sampled from 12 wild and cultivated blue mussels stocks distributed along the French Atlantic coast from the south Brittany to Pertuis Charentais areas. During these surveys, various genetic abnormalities were frequently detected, and ploidy characteristics revealed contrasting profiles that corresponded to respective contrasting sanitary status, i.e. healthy mussels with high cytogenetic quality (HCQ) *versus* diseased mussels with low cytogenetic quality (LCQ). In the present work, FCM and hemocytology cell monolayer techniques were combined in order to determine the putative causes of the observed genetic abnormalities that were significantly associated with mortality levels. FCM and cell monolayer approaches permitted the definition of new threshold values delimiting HCQ mussels from LCQ ones. FCM histograms of mussels from the HCQ group showed one single or a largely dominant population of diploid (2n) nuclei and a large majority of normal hemocytes. Hemolymph cell-monolayer analyses showed predominantly acidophil granulocytes characterized by nuclei of normal size and a large cytoplasm with numerous granulations. In contrast, FCM histograms for the LCQ group showed, in addition to the normal diploid (2n) nuclei, populations of nuclei that displayed aneuploidy patterns in a broad ploidy range, including diploid-triploid (2–3n), tetraploid-pentaploid (4–5n) and heptaploid-octaploid levels (7–8n). The corresponding hemolymph cell-monolayer showed cellular features characteristic of disseminated neoplasia disease with frequent abnormal anaplastic cells that exhibited noticeable numbers of mitotic figures with both normal and aberrant chromosomes segregation patterns. These neoplastic cells were a rounded shape with a reduced, granulation-free cytoplasm and large (11–12 µm) to very large (up to 21 µm) round or ovoid nuclei that correspond to the 4–5n and 7–8n nuclei previously detected by FCM analyses. These characteristics suggest that the genetic abnormalities detected by means of FCM were related to an ongoing neoplastic process that is affecting blue mussels in France, at least since the onset in 2014 of the mortality that heavily impacted French blue mussels stocks.

1. Introduction

In France, farming of *Mytilus* spp. consisting of the blue mussel *Mytilus edulis*, the Mediterranean mussel *M. galloprovincialis* and hybrids of both species is an important industry with production varying during the last decade from 71,000 to 79,000 metric tons per year (FAO, 2014). However, the production of French mussels has decreased dramatically since 2014 because of sudden and unfamiliar mass mortality (90–100%) of both juvenile and adult mussels cultivated at various French Atlantic areas, including south and north Brittany, Bourgneuf Bay and Charentais Sound (Béchemin et al., 2015). Mortality of both naturally occurring and cultured blue mussels has been reported in many other locations around the world (Tremblay et al., 1998; Myrand et al., 2000; Fuentes et al., 2002). The extent of mortality can be highly variable and several stress-related factors have been investigated, including pathogens, pollution, temperature, food depletion and reproduction, leading to a preliminary conclusion that none of these factors alone can explain the cause. Although blue mussels are generally considered to be resilient to environmental disturbances and pollutants; several studies have shown that the exposure of mussels to environmental contaminants results in DNA damage. Damage includes abnormalities in DNA content and structure, as well as the progressive

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development of circulating aneuploid-polyploid cells in the hemolymph, in particular the development of a fatal leukemia-like cancer disease called hemic (or disseminated) neoplasia (Elston et al., 1992; Bihari et al., 2003; Vassilenko and Baldwin, 2014). This malignant disease has been reported in 15 species of marine bivalves with a worldwide distribution that includes the cockle *Cerastoderma edule*, four species of oysters, six species of clams and five species of mussels (Farley et al., 1986; Elston et al., 1992; Villalba et al., 1995, 2001; da Silva et al., 2005; Delaporte et al., 2008; Galimany and Sunila 2008; Le Grand et al., 2010; Diaz et al., 2011). The disease is characterized by proliferation of anaplastic circulating cells with enlarged and pleiomorphic nuclei, a high nucleus-to-cytoplasm ratio and frequent presence of mitotic figures (Elston et al., 1992). In late stages, anaplastic cells completely replace normal hemocytes and penetrate various tissues such as connective tissue, gonads, the mantle and foot (Barber, 2004; Carballal et al., 2015). This disease is progressive and commonly fatal causing significant mortality and decrease in market harvests of economically important species (Bower, 1989; Elston et al., 1992; Barber, 2004, Carballal et al., 2015).

In the context of the abnormal outbreaks of mass mortality of blue mussels in France since 2014, it was hypothesized that mortality was probably linked to poor cytogenetic quality, particularly in terms of ploidy variations and cell cycle DNA alterations affecting circulating cells in the hemolymph. These genetic abnormalities can be reliably studied by Flow CytoMetry (FCM) methods (Elston et al., 1990; Moore et al., 1991; Reno et al., 1994; Da Silva et al., 2005, Vassilenko and Baldwin, 2014). FCM was used in a previous study to investigate the DNA content and cell cycle characteristics of hemic cells collected from various wild and cultivated mussels stocks that had, at our best knowledge, no history of mortality before 2014 and that showed much higher mortality levels after that time (Benabdelmouna and Ledu, 2016). In that survey, different thresholds of genetic abnormality (GA%) were established and appeared to be highly predictive of the final mortality levels. Interestingly, ploidy characteristics of hemic cells revealed contrasting profiles, which corresponded to respective contrasting sanitary status, apparently healthy *vs* diseased mussels. Normal healthy mussels were shown to be of high cytogenetic quality (HCQ) and contained nearly entirely diploid cells in their hemolymph while abnormal diseased mussels had low cytogenetic quality (LCQ) and contained, in addition to normal diploid cells, a broad continuum of aneuploid-polyploid cells that could either be linked to genotoxic effects of unknown origin and/or most probably to ongoing hemic neoplasia disease.

From the Benabdelmouna and Ledu (2016) study, it was apparent that systematically combining FCM and light microscopy methods (especially hemocytology by cell monolayer technique) would be beneficial in order to (i) determine the putative cause of the observed genetic abnormalities, (ii) to characterize at the cellular level the disorder during mussel mortality events, and (iii) determine more precise thresholds of genomic abnormality (GA%) that could provide tools to manage mortality outbreaks. For this purpose, non-invasive FCM analyses were used to preliminarily constitute two groups of blue mussels of cytogenetic quality LCQ and HCQ. Individual mussels were then subjected to hemocytology exams by the cell monolayer technique ("cytospin®") in order to establish a clear relationship between FCM-based cytogenetic quality status and the corresponding cellular and cytological characteristics of hemocytes.

2. Material and methods

2.1. Biological material

Adult mussels used in this study were randomly collected during April 2017 from a wooden pole "bouchot" grow-out facility dedicated to mussel culture and located along the English Channel at Donville les Bains in western Normandy, France (48′52′957N; 00′34′892W). Farmer's mussel seed were originally collected during spring 2016 from natural spatfall at Fouras in the Pertuis Charentais area of the Atlantic coast. At the end of Summer 2016, mussel spat of less than 1 mm shell length that settled on coconut fiber ropes at a mean density of 10,000 individuals per meter were transferred to their final growing zone at Donville les Bains. In April 2017, following a brief mortality event of about 15%, 300 live adult mussels of 3–6 cm shell length were transported to our LGPMM experimental hatchery at LaTremblade where they were maintained in a separate tank alimented through 250-L per hour of unheated and UV-filtered seawater.

2.2. Hemolymph collection

Mussels were anaesthetized in a solution containing 50 g L^{-1} of magnesium chloride until the valves opened. Hemolymph was withdrawn in a non-destructive manner from the adductor muscle of each individual mussel with sterile 1 mL syringe fitted with a 26 gauge needle. For each animal, a volume of approximately 0.2 mL of hemolymph was collected and stored for 5 min in an Eppendorf microcentrifuge tube on ice to prevent clumping. Each hemolymph sample was immediately subjected to FCM and cell monlayer analyses.

2.3. Flow cytometry analysis

FCM analyses were conducted as described in Benabdelmouna and Ledu (2016). In brief, 0.1 mL of hemoplymph collected from each individual mussel was used for nuclei extraction and staining. The samples were first collected in a 1.5 mL Eppendorf tube containing 1 mL of nuclei extraction buffer (5 mM $MgCl_2$, 85 mM NaCl, 10 mM Tris, 0.1% Triton X-100, pH 7) and the nuclei were then collected by filtration through a 30-µm nylon sieve. Samples were simultaneously treated with DNase-free RNase A (50 µg mL^{-1}, Sigma, R4875, Saint-Louis, MI, US) and stained at room temperature for 30 min in the dark with propidium iodide (PI, Sigma, P4170, Saint-Louis, MI, US) at a concentration of 50 µg mL^{-1} in a 2-mL final solution. FCM was performed on a Partec PA II flow cytometer equipped with a 590 nm, 30 mW green laser (Sysmex, Sainte Geneviève des Bois, France). Peak position and cell-cycle estimates were done as described in Benabdelmouna and Ledu (2016). In order to distinguish nuclei in the G2/M phase from doublets of G0/G1 nuclei that have the same DNA content, FL3-area *vs* FL3-width dot-plots were used to gate single nuclei. Thus a region (R1) was drawn on these dot-plot representations to discriminate single nuclei from doublets. After gating them on R1, single nuclei were next plotted on a FL3-area histogram on 1024 linear scale and used to calculate the percentages of nuclei populations according to their DNA content. Selection of the two mussel groups according to their cytogenetic quality status (LCQ *vs* HCQ) was done according to our previous work (Benabdelmouna and Ledu, 2016) that fixed an upper limit of 10% of non-diploid nuclei for a normal HCQ mussel. Beyond this limit, mussels were considered abnormal with a LCQ status.

2.4. Cell monolayer analysis

Hemocytological analyses by cell monolayer technique were conducted for individuals with contrasting FCM profiles constituting the LCQ and HCQ groups. For each of these selected mussels, 40 µL of freshly collected hemolymph taken from the venous sinuses in the adductor muscle were pipetted onto a poly-L-lysine coated glass slides for cyto-centrifugation (4 °C, 1 min, 500 rpm – Universal 16R, Hettich-Zentrifugen, Tuttlingen, Germany). Supernatant was removed; each slide was dried at room temperature and then stained with hematoxylin-eosin and observed under light microscopy (BX50, Olympus, Tokyo, Japan). Digital images were also captured using a Zeiss Axioplan 2 Imaging microscope, and digitized images were prepared for printing in Axiovision software (Zeiss).

Twenty individuals were analyzed; ten each of HCQ and LCQ