Immune response of blue mussels (*Mytilus edulis*) in spawning period following exposure to metals

M. Fraser[1,2] P. Rault,[3] M. Fortier,[1] P. Brousseau,[1] M. Fournier,[1] C. Surette,[4] C. Vaillancourt[1,2]

[1]INRS - Institut Armand-Frappier, Laval, QC, Canada; [2]BioMed Research Centre, Université du Québec, Laval, QC, Canada; [3]Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, Plouzané, France; [4]Université de Moncton, Département de chimie et de biochimie, Moncton, NB, Canada

Introduction

Mytilus edulis are sensitive to environmental contamination like metals, such as silver (Ag), lead (Pb), cadmium (Cd) and mercury (Hg), which are bioavailable for this species.[1-3] It is well documented that these metals can disrupt the immune responses in bivalves.[4-6] During spawning, important physiological systems are involved, such as serotonin and dopamine,[7-11] which in turn could modulate the immune system.[12,13] Indeed, bivalves reduce their metabolic activity after spawning which is reflected by a decrease of the phagocytosis activity.[14-18] It is expected that during the period of reduced immune activity (*i.e.*, after spawning) mussels are more vulnerable to contaminations and diseases.[14-16,18,19] The aim of this pilot study was to determine if spawning modulates immune responses of *Mytilus edulis* exposed to metals (Ag, Cd, Hg and Pb).

Materials and Methods

Mussels

Mytilus edulis were collected from the *Baie de Plaisance* located in the *Îles de la Madeleine* region (QC, Canada) (47°29'N, 61°87'W), an area clear from industrial and urban activities. Upon arrival at the laboratory, the mussels were transferred and maintained in tank filled with artificial seawater (15°C, salinity 31-1 psu) and fed twice a week with a concentrate of phytoplankton (Phytoplex , Reef solution, Laval, QC, Canada). Mussels were sacrificed one week before (W-1) spawning, during (W0) spawning, two and three weeks after spawning period (W2 and W3 respectively). The spawning period was determined by the release of gametes in the water. Hemolymph was collected from the posterior adductor muscle of each mussel using a 3 mL syringe and immediately used for analysis.

Metal exposure

Mussel hemocytes were exposed *in vitro* to increasing metal concentrations (n=3 by metal): one metal at a time during 21 hours (Ag, Cd, Hg and Pb; 10^{-9} to 10^{-3} M). Metals used are reagent grade: cadmium chloride ($CdCl_2$), lead chloride ($PbCl_2$), mercuric chloride ($HgCl_2$) and silver nitrate ($AgNO_3$) (Sigma-Aldrich, ON, Canada).

Cell viability

The viability of hemocytes was evaluated by flow cytometry using the Guava PCA flow cytometer (Guava Technologies, CA, US) and the Viacount kit (Guava Technologies) according to the supplier's instructions. Briefly, an aliquot of hemolymph was mixed with Viacount and 1000 events were recorded.

Phagocytosis of hemocytes

Phagocytosis was assessed by flow cytometry according to Brousseau *et al.*[20] Briefly, hemocytes were incubated with latex fluorescent beads (Yellow-green Fluoresbrite, Polysciences®). The number of engulfed beads in each hemocyte was determined using a FACSCalibur (Becton-Dickinson, CA, USA) flow cytometer. Fluorescence emission was read in FL1 (λ=530 nm), with at least 10 000 events in the region of interest were recorded. Results were analyzed with the Cell Quest Pro software (Becton-Dickinson, CA, USA) to determine the percentage of hemocytes that engulfed one bead and more (phagocytic activity) or three beads and more (phagocytic efficiency).

Statistical analysis

Data are presented as mean standard error of the mean (SEM). The results were tested for normality by the Kolmogorov-Smirnov test. Variance homogeneity was verified by Levene's test. Significant differences were determined using ANOVA or Kruskal-Wallis. SPSS 20.0 was used for all statistical analyses (IBM Corp., 2011, IL, USA). Half maximal inhibitory concentrations (IC_{50}) were determined with GraphPad Prism 5.0a (GraphPad Software Inc., 2007, CA, USA).

Results and Discussion

Phagocytic capacity and efficiency of hemocytes from *Mytilus edulis* during spawning period are 60% lower than those from mussels after the spawning period (Figure 1). This observation is consistent with previous studies.[14,18,19] Li *et al.* suggest a trade-off of the energy to the spawning process to explain the effects observed since glycogen and protein levels in the mantle of *Pacific oysters* also decreased during this period.[14] As suggested by Cartier *et al.*, it is possible that the increased level of sex steroid hormones during spawning period plays a suppressive role on the immune system.[19,21,22] This possibility was verified in the common carp, where 17β-estradiol, progesterone and 11-ketotestosterone were shown to inhibit phagocytosis.[20] More studies should be done to explain how the immune system of *Mytilus edulis* could be affected by spawning. Since sex can also influence the immune and endocrine systems of bivalves,[23,24] it should be included in such studies.

Fourteen days after spawning, phagocytic capacity and efficiency of hemocytes return to the baseline levels founds before spawning period (Figure 1). In the pacific oysters, a

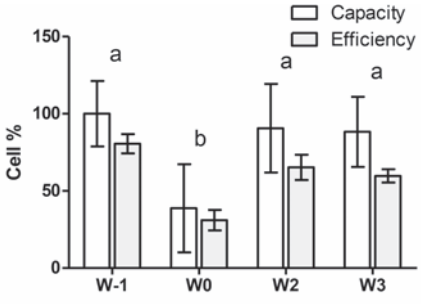

Figure 1. Phagocytic capacity and efficiency of hemocytes from Mytilus edulis according to spawning period. Mussels were sacrificed one week before (W-1) spawning, during (W0) spawning, two and three weeks after spawning period (W2 and W3, respectively).Different letters indicate, for capacity and efficiency, a significant difference (ANOVA, P<0.0001).

Correspondence: Michel Fournier and Cathy Vaillancourt, INRS – Institut Armand-Frappier, Université du Québec, Laval, QC, Canada.
E-mail: cathy.vaillancourt@iaf.inrs.ca; michel.fournier@iaf.inrs.ca

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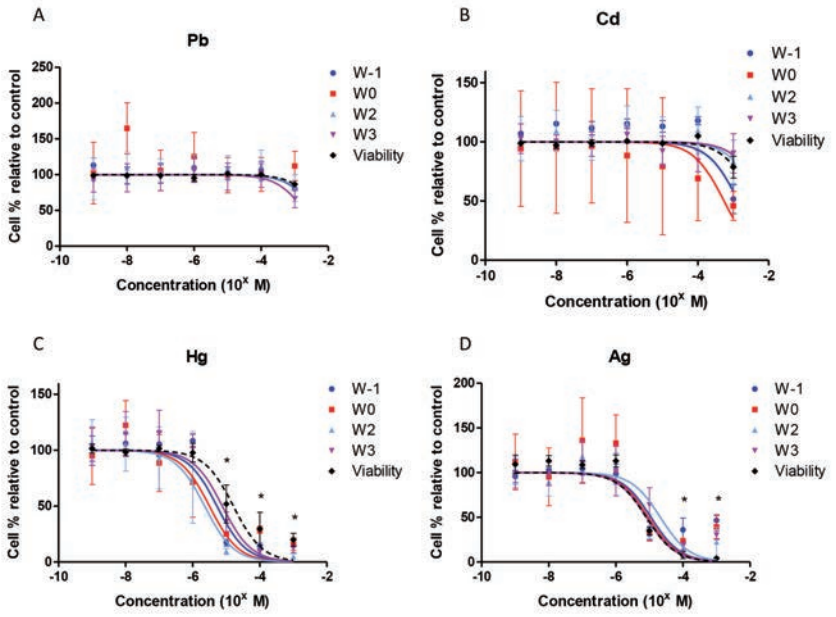

Figure 2. Phagocytic capacity and viability of hemocytes exposed to metals [A: lead (Pb), B: cadmium (Cd), C: mercury (Hg) and D: silver (Ag)] from Mytilus edulis in or out spawning period. Mussels were sacrificed one week before (W-1) spawning, during (W0) spawning, two and three weeks after spawning period (W2 and W3, respectively). Controls were not exposed to metals. To compare difference between concentrations: *ANOVA/Kruskal-Wallis P<0.05.

recovery is observed 8 days after the possible that some mussels sacrificed were

Table 1. Inhibitory concentrations (IC50) of phagocytic capacity of hemocytes exposed to metals in or out spawning period (expressed in 10^x M).

	Pb	Cd	Hg	Ag
W-1	> -3	> -3	-5.±0.2	-5.1±0.2
W0	> -3	-3.±0.7	-5.5±0.3	-5.0±0.5
W2	> -3	> -3	-5.6±0.1	-5.0±0.2
W3	> -3	> -3	-5.1±0.2	-4.7±0.3
Viability	> -3	> -3	-4.8±0.2	-5.1±0.1

Pb, lead; Cd, cadmium; Hg, mercury; Ag, silver. W-1, one week before spawning; W0, during spawning; W2, two weeks after spawning; W3, three weeks after spawning.

spawning.[14] Future experiment should include more time points to better understand the recovery of immune system of *Mytilus edulis* after spawning.

A significant decrease in hemocytes viability exposed to Ag and Hg at 10^{-5} M was observed independently of the spawning period (Figure 2C and D). A decreased in phagocytic capacity was associated.

Spawning did not affect phagocytic capacity following an exposure to Ag, Hg and Pb (Figure 2 A, C, D and Table 1). However, spawning decreased the phagocytic capacity of mussel hemocytes exposed to Cd (Figure 2B and Table 1). Thus, mussels in spawning period exposed to Cd could be more vulnerable to diseases than those not exposed to Cd, this remains to be examined further.[14-16,18,19]

Since spawning period was determined by the observation of gametes in the water, it is still not in spawning process. Future experiments should include histological analysis of the gonads in order to determine the gametogenesis stage and the sex of each mussel. Nevertheless, this preliminary pilot study demonstrates the importance of considering the stage of reproduction of *Mytilus edulis* in studies of the immune system.

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