ISJ 6: 44-48, 2009 ISSN 1824-307X

SHORT COMMUNICATION

Seasonal changes in functional parameters of the hemolymph of *Mytilus galloprovincialis*

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Accepted April 7, 2009

Abstract

In bivalves, many functional parameters show seasonal changes in relation to both abiotic (such as temperature and salinity) and biotic factors (such as gonad maturation, food availability). Available data indicate that also immune parameters can show seasonal fluctuations in the marine mussel *Mytilus* spp.. In this work we report data on hemocyte lysosomal membrane stability (LMS) and phagocytic activity, as well as on soluble lysozyme activity, in the hemolymph of mussels (*Mytilus galloprovincialis*) collected over a 24 month period in the Adriatic Sea (2006-2007). The results indicate that all the parameters measured show seasonal fluctuations over the year, with lysozyme activity showing the largest changes. Lowest LMS values were observed in early winter and early autumn, whereas maximal values of phagocytic activity were observed in winter and increasing serum lysozyme activities were recorded in autumn. The observed seasonal fluctuations are discussed in relation to both abiotic (temperature) and biotic (changes in endogenous modulators) factors.

Key Words: *Mytilus*; hemocytes; lysosomal membrane stability; phagocytosis; lysozyme; immune parameters; seasonal variation

Introduction

Bivalves (such are mussels, clams and oysters) possess both cellular and humoral defence mechanisms that co-operate to kill and eliminate invading bacteria (Mitta *et al*., 2000; Canesi *et al*., 2002). Hemocytes are responsible for cell-mediated immunity through phagocytosis and various cytotoxic reactions, such as the release of lysosomal enzymes and antimicrobial peptides, and the production of oxygen metabolites (Mitta *et al*., 2000, Canesi *et al*., 2002).

In the recent years, we have investigated the immune responses of *Mytilus galloprovincialis* to different stimuli, from bacterial challenge to exposure to endogenous modulators and heterologous cytokines, in both *in vitro* and *in vivo* studies (Canesi *et al*., 2001, 2003, 2004, 2005, 2006a, b; Betti *et al*., 2006). Although a number of assays can be utilized in order to evaluate the immune function in invertebrates (Ballarin *et al*., 2008), the phagocytic activity is generally considered as one of the most important parameters, especially in the bivalve *Mytilus* spp. where active phagocytes represent the main circulating cell type (Carballal *et al*., 1998; Ottaviani *et a*l., 1998; Wottoon *et al*., 2003). However, another hemocyte parameter, lysosomal membrane stability, evaluated in live hemocytes by the Neutral Red Retention time (NRR) assay, has emerged as an extremely sensitive indicator not only of cellular stress due to environmental perturbations (Lowe *et al*., 1995), but also of the functional status of immunocytes (Hauton *et al.*, 2001; Pruzzo *et al*., 2005). In fact, the majority of *Mytilus* hemocytes are endowed with an extremely developed lysosomal vacuolar system which is involved not only in digestion of engulfed foreign particles, but that also contains a number of hydrolases that are secreted for extracellular degradation of components from invading microorganisms, such as bacterial cell wall (Canesi *et al*., 2002). In addition, lysosomal production of oxygen radicals has been demonstrated (Winston *et al*., 1996).

In mussels, LMS response to a variety of extracellular stimuli has been long widely investigated in a number of studies so that recorded changes can be related to a different functional status of the cell; in particular, moderate lysosomal

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destabilisation indicates activation of lysosomal membrane fusion processes related to endo/exocytosis, whereas larger decreases in LMS correspond to increasing cellular stress conditions that can lead to irreversible cellular damage and autophagy (Moore *et al*., 2006). Changes in both LMS and phagocytosis are induced by immune stimuli, like bacterial challenge or exposure to cytokines (Canesi *et al*., 2001, 2003; 2005; Betti et al., 2006). Moreover, we have identified the natural estrogen 17β-estradiol as an endogenous modulator of these parameters, as well as of lysosomal enzyme release and oxyradical production (Canesi *et al*., 2004, 2006b).

In bivalves, many functional parameters show seasonal changes in relation to both abiotic (such as temperature and salinity) and biotic factors (such as gonad maturation or food availability). These factors may affect also the immune function. In *Mytilus* spp., only a few studies have been focused on seasonal variations in immune parameters (Carballal *et al*., 1997, 1998; Malagoli *et al*., 2006, 2007; Novas *et al*., 2007). In this work, we report data on hemocyte LMS and phagocytic activity, as well as on soluble lysozyme activity in mussels, *Mytilus galloprovincialis*, collected over a 24 month period in the Adriatic Sea in 2006-2007.

Materials and Methods

Chemicals

All reagents were of analytical grade and were purchased by Sigma (St. Louis, MO).

Animals

Specimens of the bivalve mollusc *Mytilus galloprovincialis*, collected in the Cesenatico area (RN, Italy) were purchased monthly from a local fishing company (SEA, Gabicce Mare, PU) for two years (from January to December 2006 and 2007). Analysis were carried out on individuals of 4-5 cm size. After collection, animals (30 mussels) were taken immediately to the laboratory where they were kept in an aquarium for 24 h in static tanks containing aerated artificial sea water (ASW) (1 l/mussel), 36 % PSU, at different temperatures (from 15 to 20 °C, depending on the sampling period to minimize the effect of laboratory conditions).

Hemolymph was sampled from the posterior adductor muscle using a sterile 1 ml syringe with an 18 G1/2" needle. With the needle removed, hemolymph was filtered through sterile gauze and pooled in 50 ml Falcon tubes at 18°C. Hemolymph samples from 8-10 mussels were pooled and utilised for subsequent analyses. Hemolymph serum was obtained by centrifugation of whole hemolymph at 200xg and the supernatant was sterilised through a 0.22 µm pore size filter. All analyses were performed in quadruplicate.

Lysosomal membrane stability (LMS)

LMS was evaluated by the NRR (Neutral Red Retention time) assay as previously described (Canesi *et al*., 2005) according to Lowe *et al*. (1995). Hemocyte monolayers on glass slides were incubated with 30 µl of a neutral red (NR) solution (final concentration 40 mg/ml from a stock solution of NR 40 µg/ml in DMSO); after 15 min excess dye was washed out, 30 µl of ASW was added, and slides were sealed with a coverslip. Every 15 min, slides were examined under an optical microscope and the percentage of cells showing loss of the dye from lysosomes in each field was evaluated. For each time point 10 fields were randomly observed, each containing 8-10 cells. The end point of the assay was defined as the time at which 50 % of the cells showed sign of lysosomal leaking (the cytosol becoming red and the cells rounded). All incubations were carried out at 18 °C.

Phagocytosis assay

Phagocytosis of Neutral Red-stained zymosan was used to assess the phagocytic ability of hemocytes as previously described (Canesi *et al*., 2006b) according to Pipe et al. (1995). Neutral Red-stained zymosan in 0.05M Tris-HCl buffer (TBS), pH 7.8, containing 2 % NaCl was added to each monolayer at a concentration of about 1:50 hemocytes:zymosan diluted in ASW, and allowed to incubate for 30 and 60 min at 18 °C. Monolayers were then washed three times with TBS, fixed with Baker's formol calcium (4 %, v/v, formaldehyde, 2 % NaCl, 1 % calcium acetate) for 30 min and mounted in Kaiser's medium for microscopical examination with a Vanox (Olympus Italy 1.2.1, MI) optical microscope. For each slide, the percentage of phagocytic hemocytes was calculated from a minimum of 200 cells. Data are expressed as % of phagocytizing cells.

Serum lysozyme activity

Lysozyme activity in aliquots of serum was determined as previously described (Pruzzo *et al*., 2005) following Chu and La Peyre (1989). Briefly, lysozyme activity was determined as the ability to lyse a standard suspension of *M. lysodeikticus* (15 mg/100 ml in 66 mM phosphate buffer, pH 6.4) and measured as decrease in absorbance at 450 nm at room temperature. Hen egg-white (HEW) lysozyme was used to construct a standard curve and lysozyme activity was expressed as HEW lysozyme equivalents/mg protein/ml. Protein content was determined according to the Lowry method using bovine serum albumin (BSA) as a standard.

Data analysis

Results are presented as the arithmetical mean ± SD of experiments carried out in quadruplicate. Statistical analysis was performed by using the Mann-Whitney *U*-test with significance at $p<0.05$.

Results and Discussion

Mussels were sampled for 24 months during 2006 and 2007. Since no significant differences in the results obtained were recorded between the two years, only data from Jan-Dec 2007 are reported in Fig. 1.

Figure 1A shows the results obtained for hemocyte LMS: mean annual values of NR Retention times were 117.75 ± 13.6 min. Lowest LMS (about 100 min) were recorded in winter (Jan-Feb) and early autumn (Sept-Oct). With respect to these values, significantly higher LMS were

recorded in spring, with a maximum in May (+39 %; $p<0.05$) and December + 31 %; $p < 0.05$). However, both minima and maxima did not significantly differ from mean annual values.

Data on hemocyte phagocytic activity are reported in Fig. 1B as % of phagocytosing cells. Mean values (%) were 53.87 ± 5.55. Higher values were observed in winter, with a maximum in January (64 %), followed by a slow decrease in spring-early summer, that reached lowest values in June and September (-27 % and 26 %, respectively, with respect to January; $p<0.05$). However, as observed for LMS data, also for phagocytosis neither minima nor maxima were significantly different from mean annual values.

In Figure 1C data on soluble lysozyme activity are reported. Mean annual values were 147.25 ± 68 mU/mg protein. In this case, larger seasonal differences were observed, with lower values in late winter-early spring (less than 100 mU/mg protein from February to April, with a minimum in March of 84±15 mU/mg protein.). A large, progressive rise was observed from late summer to autumn (up to a +150 % increase in October and November with respect to July; $p<0.05$). Maximal values recorded in October and November were also significantly different from mean annual values (+83 % and +100 %, respectively; $p<0.05$).

Taken together, the results indicate seasonal fluctuations in the parameters measured in the hemolymph of mussels from the Adriatic Sea. Lowest LMS values were observed in early winter and early autumn. On the other hand, maximal values of phagocytic activity were observed in winter and increasing serum lysozyme activities were recorded in autumn. These observations are in line with the fact that decreases in LMS are associated with membrane fusion processes during both endo/phagocytosis and release of lysosomal enzymes by exocytosis. However, when data were analysed by the Spearman rank correlation test, no significant correlation was observed among the different parameters measured (data not shown).

The observed changes in hemolymph functional parameters can be related to both abiotic (such as temperature) and biotic factors (such as reproductive stage and also food availability). In the Adriatic Sea, fluctuations in hemolymph cytotoxicity of *M. galloprovincialis* were reported (Malagoli *et al.*, 2006, 2007), with two peaks at the end of spring and of summer. However, the temperature apparently was not the main parameter affecting cytotoxicity (Malagoli *et al.*, 2007). Moreover, no significant correlation was found between changes in immune parameters recorded in the present work and environmental temperature (data not shown).

Although circulating hemocyte concentration did not show seasonal variations in *M. galloprovincialis* (Carballal *et al.*, 1998), the proportion of different hemocyte subpopulations endowed with different phagocytic activity due to different hemocyte maturation stages may change at different times of the year. Since phagocytosis represents one of the main components of the innate immune response, lower phagocytic activities observed in this work during the summer may be

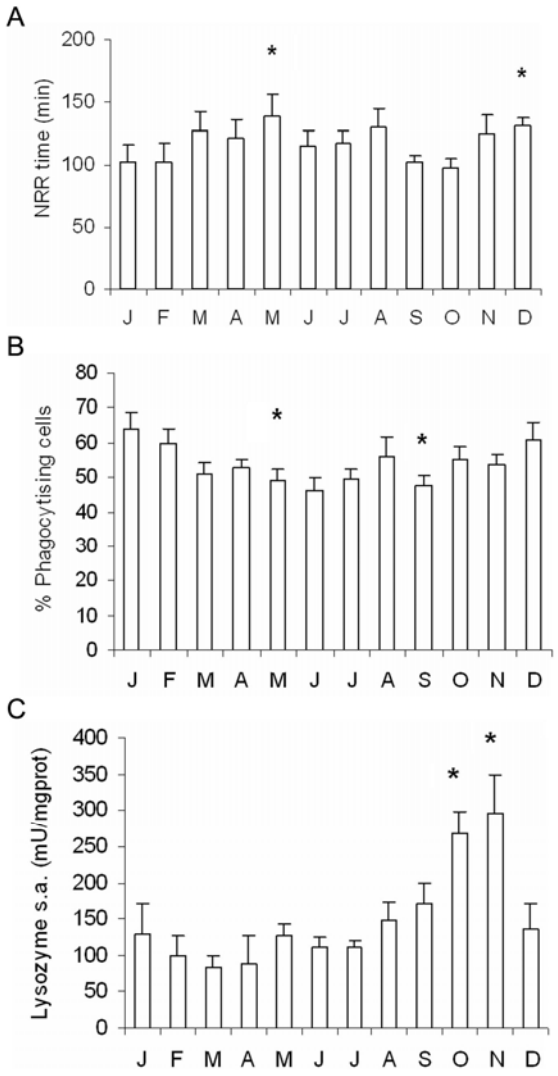

Fig. 1 Seasonal trend of lysosomal membrane stability (LMS) (A) and phagocytic activity (B) in hemocytes and of serum lysozyme activity (C) in hemolymph of mussels from Adriatic Sea. Data, representing the mean ± SD of four replicates were analysed by the Mann-Whitney U test.
A) * = $p<0.05$: May *vs* January and February; December *vs* September and October
B) * = $p<0.05$: January *vs* June and September
C) * = $p<0.05$: July *vs* October and November

responsible for lower immune defence against invading micro-organisms during this period.

Moreover, the effect of seasonal changes in endogenous modulators (such as neuropeptides, cytokines and sex steroids) should be taken into account. In the hemocytes of *M. galloprovincialis,* physiological concentrations of the natural estrogen 17β-estradiol can stimulate immune parameters, including phagocytosis, both *in vitro* and *in vivo* (Canesi *et al.*, 2004, 2006b). In bivalves, 17β-estradiol has a role in reproduction: seasonal changes in estrogen content have been observed in

different bivalve species in relation to gametogenesis, with increases during gonad maturation and decreases at spawning (Osada *et al*., 2004; Gauthier-Clerc *et al*., 2006). However, in the present study, different extents of gonad ripening were observed in different individuals (males and females) throughout the year; although during acclimation in the laboratory a major gamete emission was observed in early spring, minor spawnings were also recorded at different times of the year. The absence of marked seasonal changes in gametogenesis may be partly related to a relatively constant food availability throughout the year in the Adriatic Sea.

In *M. galloprovincialis* from the northern Spanish coast, basal hemocyte NO production showed lowest values in winter. However, only in mussels collected in winter IL-2 greatly induced NO production probably through an immunoreactive e-NOS protein that was expressed only in this period by the hemocytes (Novas *et al.*, 2007). These data suggest that, in addition to fluctuations in basal values of immune parameters, changes in the inducibility of the immune response over the year should be considered.

Our data give a further insight on the seasonal inter-relationship among immune parameters in *M. galloprovincialis*. Accumulation of this knowledge may greatly help understanding and predicting the immune response against potential pathogens, as well as the possible immunotoxic effects of exposure to environmental contaminants in an economically and ecologically relevant species of bivalves.

Acknowledgements

This work was supported by the EU program IMAQUANIM (FOOD-CT-2005-007103) subcontract CNRS 009252.

References

Ballarin L, Cammarata M, Cima F, Grimaldi A, Lorenzon S, Malagoli D, *et al*. Immune-neuroendocrine biology of invertebrates: a collection of methods. Inv. Surv. J. 5: 192-215, 2008.

Betti M, Ciacci C, Lorusso LC, Canonico B, Falcioni T, Gallo G, *et al*. Effects of tumor necrosis factor alpha (TNFalpha) on *Mytilus* hemocytes: role of stress-activated MAP kinases. Biol Cell. 98: 233-244, 2006.

Canesi L, Pruzzo C, Tarsi R, Gallo G. Surface interactions between *Escherichia coli* and the hemocytes of the mediterranean mussel *Mytilus galloprovincialis* Lam. leading to efficient bacterial clearance. Appl. Environ. Microbiol. 67: 464-468, 2001.

Canesi L, Gavioli M, Pruzzo C, Gallo G. Bacteria-hemocyte interactions and phagocytosis in marine bivalves. Microsc. Res. Tech. 57: 469-476, 2002.

Canesi L, Betti M, Ciacci C, Citterio B, Pruzzo C, Gallo G. Tyrosine kinase mediated cell signaling in the activation of *Mytilus* hemocytes: possible role of STAT proteins. Biol. Cell. 95: 608-618, 2003.

Canesi L, Ciacci C, Betti M, Lorusso LC, Marchi B, Burattini S, *et al*. Rapid effect of 17beta-estradiol on cell signaling and function of *Mytilus* hemocytes. Gen. Comp. Endocrinol. 136: 58-71, 2004.

Canesi L, Betti M, Ciacci C, Lorusso LC, Gallo G, Pruzzo C. Interactions between *Mytilus* hemocytes and different strains of *Escherichia coli* and *Vibrio cholerae* O1 El Tor: role of kinase-mediated signalling. Cell. Microbiol. 7: 667-674, 2005.

Canesi L, Betti M, Ciacci C, Lorusso LC, Pruzzo C, Gallo G. Cell signaling in the immune response of mussel hemocytes. Inv. Surv. J. 3: 40-49, 2006a.

Canesi L, Ciacci C, Lorusso LC, Betti M, Guarnieri T, Tavolari S, *et al*. Immunomodulation by 17β-Estradiol in bivalve hemocytes. Am. J. Physiol. Integr. Resp. Comp. Physiol. 29: R664-R673, 2006b.

Carballal MJ, Lopez C, Azevedo C, Villalba A. Enzymes involved in defense functions of hemocytes of mussel *Mytilus galloprovincialis*. J. Invertebr. Pathol. 70: 96-105, 1997.

Carballal MJ, Villalba A, Lopez C. Seasonal variation and effects of age, food availability, size, gonadal development, and parasitism on the hemogram of *Mytilus galloprovincialis*. J. Invert. Pathol. 72: 304-312, 1998.

Chu FLE, La Peyre JF. Effect of environmental factors and parasitism on hemolymph lysozyme and protein of American oysters (*Crassostrea virginica*). J. Invert. Pathol. 54: 224-232, 1989.

Gauthier-Clerc S, Pellerin J, Amiard JC. Estradiol-17β and testosteorne concentrations in male and female *Mya arenaria* (mollusca bivalvia) during the reproductive cycle. Gen. Comp. Endocrinol. 145: 133-139, 2006.

Hauton C, Hawkins LE, Hutchinson S. Response of hemocyte lysosomes to bacterial inoculation in the oysters *Ostrea edulis* L. and *Crassostrea gigas* (Thunberg) and the scallop *Pecten maximus* (L.). Fish Shellfish Immunol. 11: 143-153, 2001.

Lowe DM, Fossato VU, Depledge MH. Contaminant-induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis* from the Venice Lagoon: an in vitro study. Mar. Ecol. Prog. Ser. 129: 89-196, 1995.

Malagoli D, Casarini L, Ottaviani E. Monitoring of the immune efficiency of *Mytilus galloprovincialis* in Adriatic sea mussel farms in 2005. Inv. Surv. J. 3: 1-3, 2006.

Malagoli D, Casarini L, Ottaviani E.. Monitoring of the immune efficiency of *Mytilus galloprovincialis* in Adriatic sea mussel farms in 2006: regular changes of cytotoxicity during the year. Inv. Surv. J. 4: 10-12, 2007.

Mitta G, Vandenbulcke F, Roch P. Original involvement of antimicrobial peptides in mussel innate immunity. FEBS Lett. 486: 185-190, 2000.

Moore MN, Icarus Allen J, McVeigh A. Environmental prognostics: an integrated model supporting lysosomal stress responses as predictive biomarkers of animal health status. Mar. Environ. Res. 61: 278-304, 2006.

Novas A, Barcia R, Ramos-Martinez JI. Nitric oxide production by haemocytes from *Mytilus galloprovincialis* shows seasonal variations. Fish Shellfish Immunol., 23: 886-891, 2007.

Osada M, Tawarayama H, Morii K. Estrogen synthesis in relation to gonadal development of Japanese scallop *Patipecten yeasoensis*: gonadal profile and immunolocalization of P450 aromatase and estrogen. Comp. Biochem. Physiol. 139B: 123-128, 2004.

Ottaviani E, Franchini A, Barbieri D, Kletsas D. Comparative and morphofunctional studies on *Mytilus galloprovincialis* hemocytes: presence of two aging-related hemocyte stages. It. J. Zool. 65: 349-354, 1998.

Pipe R, Coles JA, Farley SR. Assays for measuring immune response in the mussel *Mytilus edulis*. In: Stolen J, Fletcher TC, Smith SA, Zelikoff JT, Kaattari SL, Anderson, RS (eds), Techniques in fish immunology: fish immunology technical communications, Immunological and pathological techniques of aquatic invertebrates, vol. 4, SOS publications, Fair Haven NJ, pp 93-100, 1995.

Pruzzo C, Gallo G, Canesi L. Persistence of vibrios in marine bivalves: the role of interactions with hemolymph components. Environ. Microbiol. 7: 761-772, 2005.

Winston GW, Moore MN, Kirchin MA, Soverchia C. Production of reactive oxygen species by hemocytes from the marine mussel *Mytilus edulis*: lysosomal localization and effect of xenobiotics. Comp. Biochem. Physiol. 113C: 221-229, 1996.

Wootton EC, Dyrynda EA, Ratcliffe N.A. Bivalve immunity: comparison between the marine mussel (*Mytilus edulis*), the edible cockle (*Cerastoderma edule*) and the razor-shell (*Ensis siliqua*). Fish Shellfish Immunol. 15: 195-210, 2003.