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SHORT COMMUNICATION

Cytotoxic activity by the mussel *Mytilus galloprovincialis* and the Venus clam *Chamelea gallina* in the Adriatic sea in 2007

D Malagoli, L Casarini, F Fiori[1], E Ottaviani

Department of Animal Biology, University of Modena and Reggio Emilia, Modena, Italy
[1]COOP M.A.R.E. Soc. Coop. a.r.l., Cattolica (RN), Italy

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Abstract

Given the ecological and economic importance of bivalve molluscs, the evaluation of their welfare is one of the primary aims for both biologists and people working in shell fishing. After a three year-long period monitoring the cytotoxic activity exerted by the hemolymph from the mussel *Mytilus galloprovincialis*, we have concluded that cytotoxicity represents a useful parameter to evaluate the status of the immune activity and therefore the health of mussels in a specific period of the year. During 2007, we compared the mussel cytoxicity with that of the Venus clam *Chamelea gallina* from contiguous areas of the Northern Adriatic Sea. Our observations indicate that the cytotoxicity of the hemolymph of the two species follows a similar course during the year, suggesting that cytotoxic activity is primarily determined by the life/reproductive cycles.

Key words: *Mytilus galloprovincialis*; *Chamelea gallina*; mussel; Venus clam; cytotoxicity; immunity; mollusc health

Introduction

In view of their feeding behavior and their localization along the coasts or in internal waters, the identification of biomarkers able to represent reliably the welfare of bivalve molluscs is one of the primary goals for both biologists and people interested in the economic/environmental importance of these species (Baršienė *et al.*, 2006; Pellacani *et al.*, 2006). Bivalves are usually considered as bio-indicators, and their immune system is frequently chosen as the main target of these studies (Ballarin *et al.*, 2003; Canesi *et al.*, 2007a, b; Malagoli *et al.*, 2007b, 2008; Novas *et al.*, 2007a, b). As a result, the understanding of the parameters on which mollusc health relies has improved and led to a better evaluation of the real impact of exceptional situations connected with environmental catastrophes (Laffos *et al.*, 2006). Since 2005, we have been studying the seasonal variations in the cytotoxic activity of the hemolymph from *Mytilus galloprovincialis* grown in mussel farms in the Northern part of Adriatic sea, in order to understand whether this activity could be a useful parameter to evaluate the health of a specific mussel population (Malagoli *et al.*, 2006, 2007a). From these studies, we have concluded that cytotoxicity in *M. galloprovincialis* hemolymph is subject to cyclic modifications during the year (Malagoli *et al.*, 2006, 2007).

To verify whether our considerations could also be extended to other bivalves, we here present data concerning the fluctuations of cytotoxic activity in both *M. galloprovincialis* and the Venus clam, *Chamelea gallina*, during 2007. Moreover, to assess the impact of shell fishing on the health of molluscs, we have compared results collected from clams in areas in which the fishing was closed with those obtained from animals where fishing was regularly undertaken.

Corresponding author:
Davide Malagoli
Department of Animal Biology
via Campi 213/D
41100 Modena, Italy
E-mail: davide.malagoli@unimore.it

Materials and Methods

Animals

Specimens of the bivalve mollusc, *Mytilus galloprovincialis*, (minimum length 60 mm, except for September) were obtained monthly from local fishermen in the Cesenatico area (FC, Italy). Immediately after collection, 40 animals were utilized to obtain the hemolymph as described below. A similar procedure was applied for

Chamelea gallina (minimum length 25 mm), but the clams were sampled only four times in the year 2007: January (winter), May (spring), September (summer) and November (autumn). The *C. gallina* specimens came from the Riccione area (RN, Italy), about 23 miles from mussel farms in Cesenatico. For each single period, 2 lots of 120 clams/each were subjected to hemolymph withdrawal. Of the 2 lots, one came from a restricted area in which any kind of fishing was forbidden from August 2006 and for the whole period of the project (i.e. 15 months), while the second lot was collected about 1 mile away where clam fishing was regularly undertaken.

Hemolymph preparation and cytotoxicity assay

The detailed procedure for the hemolysis assay in mussels has already been described elsewhere (Malagoli and Ottaviani, 2005), but there were some differences in procedure for *M. galloprovincialis* and *C. gallina*. Briefly, the mussel hemolymph was collected by exerting gentle aspiration with a sterile syringe inserted between the mussel valves and filtered into sterile tubes (0.2 μm filter porosity). For *C. gallina*, the valves were opened with a razor blade, and the hemolymph was aspired with a syringe and subjected to centrifugation (13,000x*g* for 5 min). The supernatant was then carefully recovered in order to leave the pellet containing circulating cells and debris undisturbed. Filtration was not used with *C. gallina*, since the collected volumes per animal where lower than 500 μl and therefore not compatible with the size of the syringe filters. The filtered/recovered hemolymph from each animal was kept separately and never pooled.

In order to evaluate the hemolytic activity of the hemolymph from the two species, the cytolysis of human A positive erythrocytes following incubation for 1 h at 25 °C (Hubert *et al.*, 1997; Malagoli and Ottaviani, 2005) and subsequent centrifugation at 3000x*g* for 5 min at 4 °C was quantified using a spectrophotometer (Abs 541 nm). Samples with an optical density (OD) exceeding the fixed threshold OD level of 0,5 where considered cytotoxic (Malagoli and Ottaviani, 2005). For each lot of molluscs, the percentage of cytotoxic animals was then calculated. The experiments were repeated twice and in duplicate for each animal.

Results and Discussion

The monthly evaluation of the hemolytic activity of *M. galloprovincialis* hemolymph confirmed that significant fluctuations in the percentage of cytotoxic animals occur during the year (Fig. 1A). The comparison of these data with those already collected in 2005 and 2006 confirmed that higher values are seen during the summer (Malagoli *et al.*, 2007a). The major discrepancies observed between data collected in 2007 and results in 2005 and 2006 (Malagoli *et al.*, 2007a) concerns the findings for January (nearly 100 % of animals positive in 2007) and September (nearly 0 % of animals positive in 2007) (Fig. 1A). While for the data collected in January we have no indication of exceptional situations, the countertendency observed in September is most probably related to the introduction of new mussels (length < 20 mm). In that

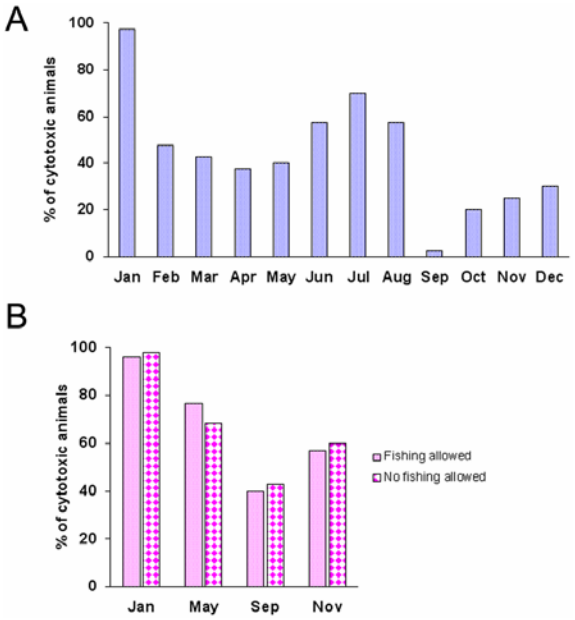

Fig. 1 Course state of *Mytilus galloprovincialis* and *Chamelea gallina* cytotoxicity of contiguous areas throughout 2007. **A**) Course state of mussel grown in mussel farms of Cesenatico; **B**) Course state of Venus clams coming form fishing or no fishing areas of Cattolica. Each histogram represent for every month the result of one out of a set of four independent experiments.

period, hemolymph had to be taken from animals of significantly different size and age compared to the specimens usually sampled during the rest of the year. This result suggests the hypothesis that molecules responsible for hemolytic activity in *M. galloprovincialis* are less abundant in young animals.

To our knowledge, there are no data in the literature concerning the cytotoxic activity of the hemolymph of *C. gallina*. Consequently, the data we present here cannot be compared with other information related to this model. However, as with the mussel, we have also observed in the clam a fluctuation in the number of cytotoxic animals during the year (Fig. 1B). No differences were seen between clams from restricted and open fishing areas (Fig. 1B), and there were no differences between the characteristics of the water in the two areas during the year. Therefore, normal fishing activity does not appear to have an impact on the health of the population.

As far as the comparison between the *M. galloprovincialis* and *C. gallina* is concerned, it is worth noting that despite an always higher percentage of cytotoxic animals in the *C. gallina* population, the trend in the clams over the year is very similar to that observed for *M. galloprovincialis* (Fig. 2). This last observation points to a possible similarity between the time course of cytotoxic activity in bivalves from contiguous areas.

Fluctuations in biological activity during the year are not new for mussels (Cao *et al.*, 2007; Novas *et*

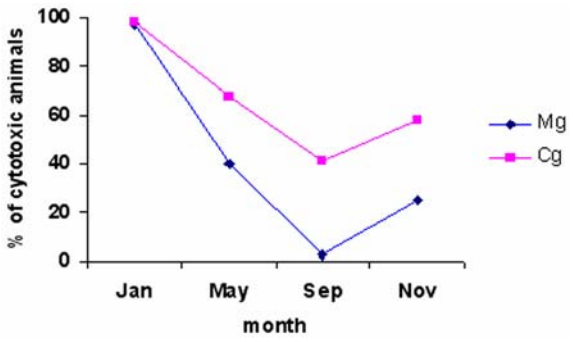

Fig. 2 Comparison between the course of hemolymph cytotoxic activity of *M. galloprovincialis* (Mg) and *C. gallina* (Cg) during the year.

al., 2007b). In particular, variations in immune reactivity or the production of immune-related molecules have been recorded (Barcia and Ramos-Martinez, 2008). Various stress factors have been linked to alterations in molluscan immune responsiveness, particularly of the cellular component, i.e. the hemocytes (Pampanin *et al.*, 2002; Ballarin *et al.*, 2003; Canesi *et al.*, 2006; Cao *et al.*, 2007; Novas *et al.*, 2007b; Malagoli *et al.*, 2007b, 2008). However, cytotoxic activity is part of the humoral component of the immune system and is of protein origin (Hubert *et al.*, 1997). This means that cytotoxicity is not susceptible to rapid modification (i.e. within hours) as a consequence of a sudden alteration in an environmental parameter, because the rate of protein turnover and biosynthesis are the main causes of fluctuations in cytotoxic activity in the hemolymph. It remains to be established whether the component determining the time course of cytotoxicity is primarily environmental or rather connected to the mollusc life-cycle. From the present and previous data (Malagoli *et al.*, 2006, 2007a), it seems probable that environmental conditions may modify a state that is based on the life/reproductive cycle.

Thus, the cytotxic activity of the hemolymph has to be recommended as a valid parameter to determine the status of immune surveillance and welfare, especially for regular checks of mollusc welfare. Indeed, for this purpose, the evaluation of cytotoxcity seems more reliable than rapidly changing parameters such as phagocytosis and lysosomal membrane stability, the levels of which may be influenced by manipulations and fishing procedures (Pampanin *et al.*, 2002; Ballarin *et al.*, 2003; Malagoli *et al*., 2007b, 2008).

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