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REVIEW

Bivalve immune responses and climate changes: is there a relationship?

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Abstract

Global climate changes (GCCs) are predicted to occur in the next hundred years through increases in temperature, water acidification and changes in seawater salinity. Increasing atmospheric CO_2 concentrations are considered to be the main responsible for GCCs. Climate changes can pose risks for aquatic ecosystems, mainly for marine coastal areas that are ecologically and economically important. In this context, increasing effort has been addressed to the evaluation of effects of variations in abiotic factors, such as temperature, salinity and pH, on biological responses of marine organisms, as deviation from the optimum for these factors may result in deleterious consequences for the physiological performance of animals. In a climate change scenario, the present review focuses on the effects of variations in some important environmental factors - mainly temperature, salinity and pH - on immune parameters of bivalves.

Key Words: climate changes; bivalves; immune parameters; temperature; salinity; acidification

Introduction

Global climate changes (GCCs) are predicted to occur in the next hundred years through increases in temperature, water acidification and changes in seawater salinity (IPCC, 2007). Metaphorically speaking, we are cooking our planet (Fig. 1). It is commonly accepted that increases in anthropogenic emissions of carbon dioxide (CO_2) in the atmosphere are the main responsible for GCCs. Indeed, increased CO_2 levels are postulated to affect average air and ocean temperatures, to cause widespread melting of snow and ice and to rise average sea level (IPCC, 2007). Pre-industrial atmospheric CO_2 levels were approximately 280 ppm; at present, CO_2 levels are increased to over 380 ppm, mostly as a result of anthropogenic activities (Feely *et al*., 2004). The capability of oceans to uptake CO_2 may influence seawater carbonate chemistry, with a consequent decrease in pH values, concentration of carbonate ions and the related calcium carbonate ($CaCO_3$) saturation state of seawater (Orr *et al*., 2005). Nowadays, it is assumed that seawater acidification due to the continued release of CO_2 into the atmosphere has already caused a reduction in ocean pH values of about 0.1 units with respect to the pre-industrial levels, and reductions from 0.3 to 0.5 pH units are predicted within the end of 21st century (Caldeira and Wickett, 2005; Raven *et al*., 2005; IPCC, 2007). Increasing atmosphere CO_2 levels may cause a pH reduction of 0.7 units by 2300 (Caldeira and Wickett, 2003).

With regard to temperature, it is demonstrated that mean global temperature has increased by about 0.7 °C in the last century, and further increases are expected during the present century (IPCC 2007; Mann *et al*., 2008). Indeed, global temperature is hypothesised to increase of about 1.8 to 4.0 °C by the end of the 21st century (IPCC, 2007). In any case, warming is expected to occur heterogeneously, with land warming faster than oceans, high latitudes warming faster than mid-latitudes, and winters warming more than summers (IPCC, 2007).

There is also concern about future alterations in seawater salinity values, mainly in estuarine and coastal areas (Booij, 2005; Kay *et al*., 2006). Indeed, global warming should be associated with changes in the hydrological cycle at a large scale: increases in precipitations could occur at high latitudes and near the tropics, whereas decreases could be recorded in sub-tropical and mid-latitude regions (Fenoglio *et al*., 2010). As a consequence, many areas of our planet will be subjected to increases either in drought or in flooding. In particular, frequent flood events are hypothesised to lead to prolonged and frequency-increased periods of reduced salinity, in estuarine areas in particular (Bussell *et al*., 2008).

In a GCC scenario, increasing concern is addressed to the well-being of living organisms.

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Indeed, it is suggested that GCCs could affect population dynamics and distribution of livestock parasites, causing increases in disease incidence and production loss (Morgan and Wall, 2009). Generally, when an organism is subject to stressful conditions it can cope with stress modifying its physiological, biochemical and behavioural responses. At immunological level, responses comprise a complex network of specific and non-specific humoral and cell-mediated components. In this context, information concerning direct effects of GCCs on animal immune functions are not available in the literature, to our knowledge at least. Conversely, data about the effects of individual environmental factors on immune parameters are abundant. Taking into account that it is not possible to summarize all information available, in the present review we have summarized results of the most recent studies concerning the influence of environmental factors - temperature, salinity and pH in particular - on immune parameters of molluscs, discussing them in a possible scenario of GCCs.

Fig. 1 A metaphorical image of global climate changes.

Effects of temperature on mollusc immune parameters

Environmental factors including temperature, salinity, oxygen, food availability, and contaminants can affect immune parameters of molluscs (Fisher, 1988). In particular, temperature is one of the most studied abiotic factors being able to affect many physiological processes in animals, mainly ectotherms. Numerous studies have demonstrated that temperature can influence markedly immune responses of molluscs, even if temperature effects differ among species.

We have recently demonstrated that high temperatures affect some important functional responses of hemocytes in the clam *Chamelea gallina* (Monari *et al.*, 2007). In that study, clams were kept for 7 days at 20, 25 and 30 °C and total hemocyte count (THC), phagocytosis, lysozyme activity (in both hemocyte lysate and cell-free hemolymph), activity and expression of the antioxidant enzyme superoxide dismutase (SOD) (in both hemocyte lysate and cell-free hemolymph) were measured. The highest temperature increased significantly THC in *C. gallina* (Fig. 2). Data concerning effects of temperature on THC in bivalves are contradictory. For example, hemocyte number of *Mytilus galloprovincialis* was positively correlated with water temperature, the lowest values being found in winter and the highest in summer (Carballal *et al.*, 1998). Conversely, in the same mussel species, increased temperature (exposure to 25 °C for 24 h) did not exert any significant effect on the number of circulating immunocytes (Malagoli *et al.*, 2007). In the oyster, *Crassostrea virginica*, THC was lowest in July and August, when the highest water temperatures were recorded (Fisher and Oliver, 1996). The effects of acute temperature challenge (from 17 °C to 11, 23 and 28 °C for 72 h) on THC were also evaluated in the scallop, *Chlamys farreri* (Chen *et al.*, 2007). In that study, just after 1h of acute temperature stress, THC increased significantly from 3.7×10^{7} cells ml^{-1} to an average of 5.3×10^{7} cells ml^{-1} in scallops transferred to 11, 23 and 28 °C. After 72h, THC of scallops held at 11 °C remained significantly higher than those of the other treatment groups. Significantly higher THC values were also found in clams (*Ruditapes philippinarum*) kept at the highest temperature (21 °C) (Paillard *et al.*, 2004). Monari *et al.* (2007) have hypothesised that the increased number of hemocytes found in *C. gallina* kept at 30 °C could be a consequence of a mobilisation of cells from tissues to hemolymph, in order to respond to bacteria. Indeed, although bacteria were not inoculated in clams, a great number of bacteria surrounding hemocytes were observed in hemocyte cultures from 30 °C-exposed animals (Monari *et al.*, 2007). Likewise, inoculation of both *Marteilia refringens* and *Vibrio tapetis* caused a significant increase in circulating hemocyte number in *Mytilus galloprovincialis* (Carballal *et al.*, 1998) and *R. philippinarum* (Oubella *et al.*, 1994), respectively. In a recent study, Perrigault *et al.* (2011) have investigated the effects of temperature on defence factors in the clam *Mercenaria mercenaria*. Clams were maintained at 13, 21 and 27 °C for 4 months, and cellular and humoral defence parameters were assessed after 2 and 4 months. THC exhibited variations according to temperature value and sampling time: THC increased significantly after 2 months at 27 °C, but it decreased significantly after 4 months at the same temperature (Perrigault *et al.*, 2011).

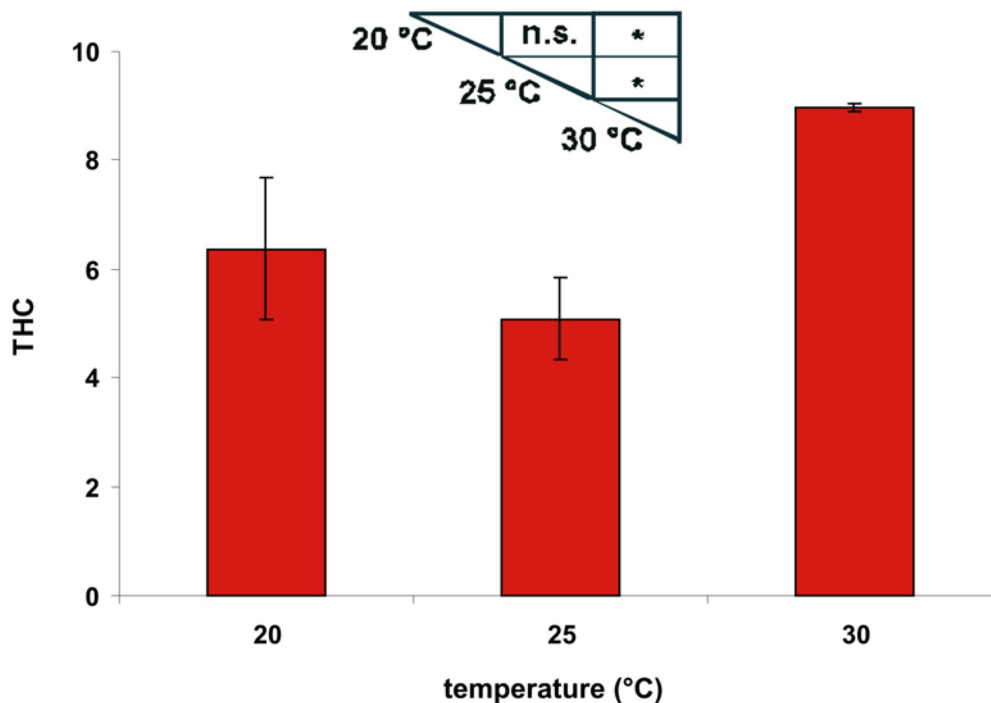

Fig. 2 Effects of temperature on THC, expressed as number of hemocytes (x10^6)/ml hemolymph, in *Chamelea gallina*. Values are means ± SEM (n = 3). Inset: significance of comparisons between experimental groups. *p<0.05, n.s.: not significant (from: Monari *et al.*, 2007).

Monari *et al.* (2007) have also observed a significant inhibition of phagocytic activity in clams, *C. gallina,* kept at 30 °C. Likewise, maintenance of *C. virginica* at 28 °C for 7 days caused a significant decrease in phagocytic activity (Hégaret *et al.*, 2003). In the same species, a significant increase in hemocyte phagocytic activity was recorded in oysters held at 20 °C for 68 days, compared to those held at 10 °C, but activity reduced in oysters kept at 25 °C (Chu and La Peyre, 1993). In *M. galloprovincialis*, Carballal *et al.* (1997) demonstrated that the *in vitro* capability of hemocytes to engulf foreign particles was lower at 10 °C than at 20 °C and 30 °C. In the same species, Malagoli *et al.* (2007) observed that higher temperature (25 °C, 24 h) did not significantly influence the percentage of phagocytic immunocytes. Malham et al. (2009) have recently demonstrated that oysters (*Crassostrea gigas*) held at 12 °C had significantly higher phagocytic hemocytes than oysters held at 21 °C. In an *in vitro* study, incubation of hemocytes at 40, 50 and 60 °C for 4 h caused cell mortality, while percentages of aminopeptidase- and esterase-positive cells were significantly lower after hemocyte incubation at 50 and 60 °C (Gagnaire *et al.*, 2006). In the same study, incubation of oysters for 4 h at 60 °C caused a significant decrease in both hemocyte phagocytic activity and percentage of esterase-positive cells. The authors suggested that the temperature-induced decrease in enzymatic and phagocytic activities of *C. gigas* hemocytes was mainly due to increased cell mortality (Gagnaire *et al.*, 2006). Although those temperatures were particularly high, it should be highlighted that oysters face high temperatures (40 °C) during the summer period in Marennes-Oleron Bay (Charente-Maritime, France), where the study was performed (Gagnaire *et al.*, 2006). In *C. farreri*, the percentage of phagocytic hemocytes was significantly lower in scallops kept at 28 °C than in those placed at 11 °C and 23 °C after 1-h stress application, and significantly decreased over the whole stress application, from 13.9 % initially to 6.3 % at the end of the experiment (Chen *et al.*, 2007). Recently, higher phagocytic activity was observed after 2 and 4 months in *M. mercenaria* held at 21 °C, as compared to levels recorded at 13 °C and 27 °C (Perrigault *et al.*, 2011).

It has been demonstrated that temperature can affect other important immune functions of bivalves. In *C. gallina*, significantly increased lysozyme activity was observed in hemocyte lysate from clams kept at 25 °C, and in cell-free hemolymph from animals held at 20 and 30 °C (Monari *et al.*, 2007). A relationship between lysozyme activity in hemocyte lysate and cell-free hemolymph was observed at 20 °C, enzyme activity being significantly reduced in hemocytes and increased in cell-free hemolymph. This was probably due to the high phagocytic activity of hemocytes from clams held at 20 °C. Interestingly, animals maintained at the highest temperature (30 °C) also showed increased cell-free hemolymph lysozyme activity,

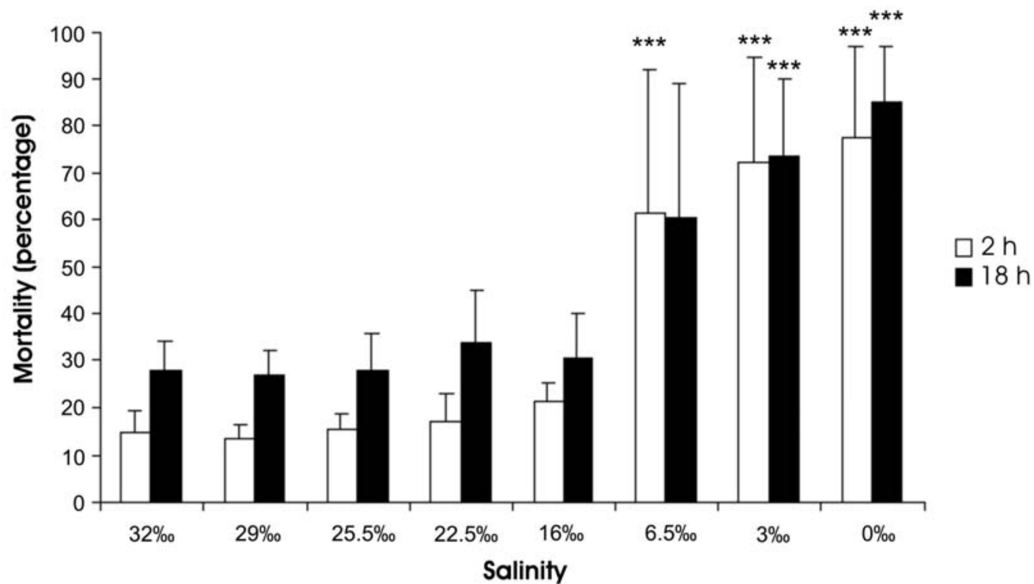

Fig. 3 Hemocyte mortality percentage of oysters after an *in vitro* 2 h or 18 h incubation period at different salinities. Values are mean of four replicates. Bars represent standard deviation. ***p<0.001 (from Gagnaire *et al.*, 2006).

even if their phagocytic activity was low. Clams kept at 30 °C might have been induced to release lysozyme in order to respond to the high number of bacteria in the hemolymph. In the surf clam, *Mactra veneriformis*, maintenance of animals at 10 °C decreased THC, lysozyme activity and Neutral Red Retention NRR times, but increased the phagocytic activity. In the same study, the highest temperature tested (30 °C) significantly increased THC, whereas it decreased phagocytic activity, lysozyme activity and NRR times (Yu *et al.*, 2009). The cytotoxicity of *M. galloprovincialis* hemolymph (evaluated by the cytolysis of human A-positive erythrocytes) was significantly reduced after maintenance of animals at 25 °C for 96 h (cytotoxic activity reduced significantly from 75 % in controls to 16 % in stressed animals) (Malagoli and Ottaviani, 2005). That study demonstrated that high temperature reduced cytotoxic responses in mussels, making them more vulnerable to pathogen aggression. In *M. mercenaria*, unstimulated reactive oxygen species (ROS) production was also significantly affected by temperature, with higher levels at 13 °C with respect to 21 and 27 °C, at both sampling times (2 and 4 months) (Perrigault *et al.*, 2011). In the same study, higher lysozyme activity was observed after 4 months in clams maintained at 13 °C as compared to those held at 21 °C and 27 °C. A significant decrease in hemocyte Mn-SOD and Cu/Zn-SOD activities was also recorded with increasing temperature (Monari *et al.*, 2007). In cell-free hemolymph, the highest Mn-SOD activity was recorded at 30 °C, whereas the Cu/Zn-SOD activity showed no significant changes in clams maintained at the three temperatures tested (20, 25 and 30 °C).

All these studies demonstrate that temperature (high temperatures, in particular) influences strongly immune parameters of bivalves. In a scenario of a possible global warming, information available on temperature-induced immunemodulation in invertebrates could provide essential knowledge useful to define early warning systems based on immunomarkers.

Effects of salinity on mollusc immune parameters

As stated above, GCCs can also occur through changes in seawater salinity. Warmer temperatures could increase seawater evaporation and reduce rainfall, concentrating salt in the water. On the other hand, warming could form areas of heavy tropical rainfall, with consequent decreases in seawater salinity, mainly along the coasts. Salinity can influence several metabolic and physiological parameters in aquatic organisms. Salinity was also shown to influence immune parameters in molluscs. Results from both laboratory and field studies have demonstrated a relationship between variations in salinity levels and infection in bivalves (Gauthier *et al.*, 1990; Chu *et al.*, 1993; Reid *et al.*, 2003). In oysters (*Ostrea edulis*) kept for 7 days at differing salinity levels (32, 25 and 16 psu), the highest salinity promoted the growth of the opportunistic bacterial pathogen *Listonella anguillarum*, increased the number of large granulocytes, and decreased the concentration of the microbicidal agent, hydrogen peroxide, in the hemolymph (Hauton *et al.*, 2000). Oysters (*O. edulis*) acclimated at 32, 28 and 25 psu showed a Neutral Red dye retention time in the lysosomal compartment longer than oysters kept at low salinities (16 and 19 psu), suggesting a reduced cell membrane stability (Hauton *et al.*, 1998). In a recent *in vitro* study, a reduction in salinity levels (6.5, 3 and 0 psu) induced high hemocyte mortality (Fig. 3), whereas one day in hyposalinity conditions (15 psu) significantly reduced phagocytic activity in *C. gigas*

(Gagnaire *et al.*, 2006). In the abalone *Haliotis diversicolor*, Cheng *et al.* (2004) found low hemocyte numbers at low salinities, and low hemocyte phagocytic capability at both reduced and elevated salinities. Reid *et al.* (2003) found significantly increased THC values with increasing salinity from 20 psu (control) to 40 psu in *R. philippinarum.* Conversely, in clams (*C. gallina*) kept at 28 psu, THC significantly increased with respect to animals kept at 34 and 40 psu, whereas higher phagocytic activity was recorded in hemocytes from clams kept at 34 psu, with respect to those kept at 28 and 40 psu (Matozzo *et al.*, 2007). In *M. galloprovincialis*, the number of circulating immunocytes increased significantly in mussels exposed for 24 h to 40 psu salinity, whereas the percentage of phagocytic immunocytes did not change significantly (Malagoli *et al.*, 2007). Bussell *et al.* (2008) have demonstrated that mussels (*M. edulis*) kept for two days at reduced salinity (16 psu) had a significant reduction in the number of hemocytes, percentage of eosinophils and the percentage of phagocytic activity, with respect to mussels kept at environmental salinity (32 psu). In that study, the authors suggested that alterations in immune parameters due to low salinity were a consequence of reduced movement of hemocytes caused by the osmotic stress or enhanced infiltration of hemocytes into the connective tissue of differing organs. In a recent study, the effects of hypo-saline conditions on immune parameters of the Akoya pearl oyster, *Pinctada imbricata*, have been evaluated (Kuchel *et al.*, 2010). Both phagocytosis and phenoloxidase activity decreased significantly when oysters were exposed to low salinity (25 psu), whereas THC significantly increased. Also in this case, the authors hypothesised that the reduction in phagocytic activity was a consequence of either osmotic stress or increased infiltration of hemocytes into connective tissue and organs (Kuchel *et al.*, 2010). In the same study, the frequency of circulating granulocytes was significantly higher in oysters kept in hypo-saline conditions, than in control oysters. In addition, total protein content of hemolymph increased significantly when oysters were subject to low salinity (Kuchel *et al.*, 2010). After 96 h, low salinity (25 psu) also reduced hemolymph cytotoxicity in mussels, *M. galloprovincialis*, with respect to controls (35 psu), and only 12 % of the animals showed cytotoxic activity (Malagoli and Ottaviani, 2005).

Results here summarised suggest that bivalves experiencing changes in salinity have altered immunesurveillance, and become more susceptible to infection/diseases.

Combined effects of temperature and salinity on mollusc immune parameters

In aquatic environments, differing stressors act jointly. As a consequence, effects of stressors should be evaluated in combination. However, very few studies have investigated the combined effects of environmental parameters on bivalve immune responses, to our knowledge at least. In a recent survey, the combined effects of various temperatures (5, 15, and 30 °C) and salinities (18, 28, and 38 psu) on hemocyte functionality of the clam *R. philippinarum* were evaluated (Munari *et al.*, 2011). In that study, both the extreme temperature and salinity values were chosen considering their occurrence in lagoon shallow waters where clams live. Effects of the resulting 9 experimental conditions on THC, Neutral Red uptake (NRU, indicative of hemocyte pinocytotic capability), hemolymph protein concentration, and lysozyme activity in both hemocyte lysate and cell-free hemolymph were studied. Temperature influenced significantly THC and NRU, whereas salinity and temperature/salinity interaction affected NRU only. Temperature and salinity did not affect significantly hemocyte lysate and cell-free hemolymph lysozyme activity, as well as hemolymph total protein content. Overall results suggested a better physiological condition for animals kept at 15 °C temperature and 18 psu salinity.

Effects of acidification on mollusc immune parameters

A possible consequence of increased atmospheric CO_2 levels is ocean acidification. Indeed, almost one half of the anthropogenically produced CO_2 should be taken up by the oceans, with consequent increases in hydrogen ion and in carbonic acid and bicarbonate ion concentrations, whereas concentration of carbonate ions should reduce (Raven *et al.*, 2005; Bibby *et al.*, 2008). It is well known that calcium-carbonate minerals (e.g., calcite and aragonite) constitute the shells of many aquatic species, such as bivalves. As a consequence, reduction in pH values could have biological implications for such animals, both wild and cultivated, that require carbonate to form their shells or skeletons (Wikfors and Krome, 2009; Range *et al.*, 2011). Some studies have demonstrated the role of mantle cells and circulating hemocytes in shell deposition in molluscs. In particular, Mount *et al.* (2004) reported that mantle-epithelial cells create a protein matrix, while hemocytes deposit calcium-carbonate crystals during shell growth of oysters (*C. virginica*). Due to the functional role of hemocytes in shell deposition, efforts should be addressed to the evaluation of acidification effects on these important circulating cells. In addition, the involvement of mollusc hemocytes in immune responses is well known. Therefore, it can be hypothesised that water acidification can also compromise the immunosurveillance status of animals.

However, only few studies have investigated the effects of acidification on mollusc immunosurveillance. Malagoli and Ottaviani (2005) investigated the effects of low pH keeping *M. galloprovincialis* at 7.3 pH for 96 h. Applied stress reduced significantly hemolymph cytotoxicity, with respect to controls (pH = 8.0), suggesting that changes in the water physical conditions can reduce hemolymph cytotoxicity in mussels, making them more vulnerable to pathogens. In a recent study, effects of hypercapnia on the immune response of *M. edulis* were investigated by exposing mussels for 32 days to acidified (by CO_2) sea water (pH 7.7, 7.5

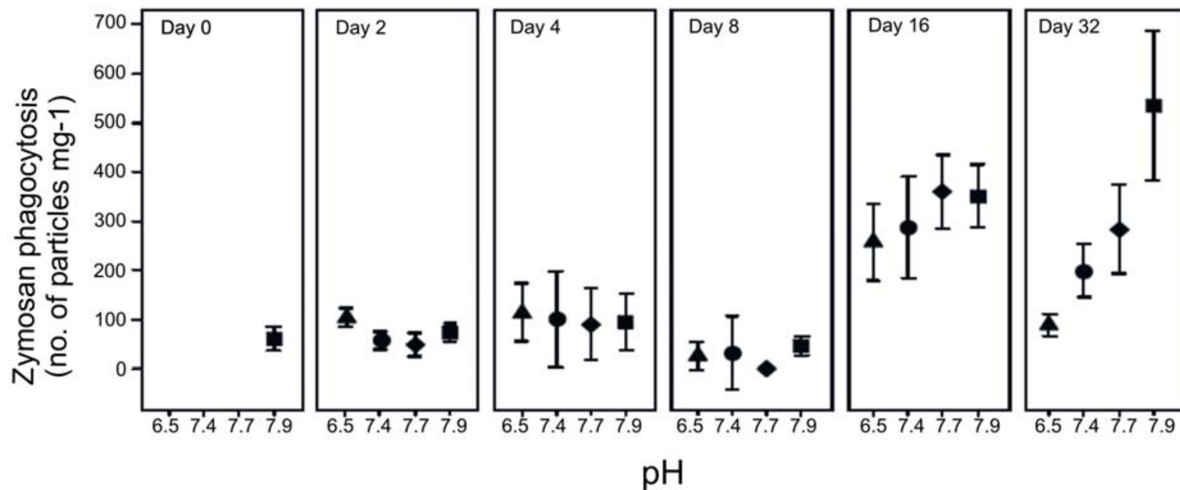

Fig. 4 Number of phagocytosed zymosan by hemocytes of mussels (*Mytilus edulis*) exposed for 32 days to differing pH values (from: Bibby *et al.*, 2008).

and 6.7; control: pH 7.8) (Bibby *et al.*, 2008). In that study, although phagocytic activity increased significantly during the exposure period at all pH values tested, phagocytosis was significantly lower in mussels kept in acidified sea water at the end of the exposure (Fig. 4). Acidified sea water did not have significant effects on the other immune parameters measured (superoxide anion production, total and differential cell counts). The authors stated that although their study did not clarify exactly the mechanisms of actions of reduced pH, one possible explanation of the results obtained was the dissolution of the mussel shell, resulting in elevated levels of Ca^{2+} in the hemolymph. In turn, increased hemolymph Ca^{2+} levels affected cellular metabolism, function and signalling pathways (Bibby *et al.*, 2008).

In another study, adults of the oyster *Saccostrea glomerata* were exposed to a seawater matrix of varying salinity, sulphuric acid and Al^{3+} for 48 h, and the expression of 7 genes involved in immune responses were evaluated by quantitative reverse-transcription polymerase chain reaction (Green and Barnes, 2010). Sulphuric acid was used to reproduce acidification conditions, as this acid is naturally produced by oxidation of acid sulphate soils and released into water ecosystems after rains. In that study, a reduction in salinity from 35 to 15 psu caused a 1.7-fold down-regulation in the expression of peroxiredoxin 6 gene. However, changes in salinity, sulfuric acid and Al^{3+} concentrations did not affect the expression of other target genes. The effects of salinity, sulfuric acid and Al^{3+} on the capability of immune genes to respond to bacteria were also evaluated by exposing oysters to the seawater matrix for 40 h, followed by injection with heat-killed *Vibrio alginolyticus*. A combination of reduced salinity and *V. alginolyticus* injection caused a down-regulation of peroxiredoxin 6 and C1q-like protein, whereas exposure to sulfuric acid and Al^{3+} had no significant effects on the immune gene responses. On the basis of the results obtained, the authors suggested that reduction in salinity, and not acidification, was the main factor affecting immunesurveillance in oysters (Green and Barnes, 2010).

Conclusions

Information summarized in the present review demonstrate, once again, that changes in abiotic environmetal factors influence strongly mollusc immune parameters. However, most of the studies performed up to here have investigated the effects of individual environmental factors, whereas surveys aimed at evaluating the combined effects of differing abiotic factors are poor. As a consequence, taking into account that GCC could occur in the next decades through contemporaneous changes in various environmental factors, efforts should be addressed to the evaluation of the combined effects of abiotic factors on immune responses of animals. This approach could be essential in understanding thoroughly the possible relationship between environmental conditions and immunemodulation mechanisms in invertebrates, and in highlighting the susceptibility of each species to infections.

Considering that invertebrates represent about 95 % of living species and play important roles in ecosystems (aquatic in particular), the knowledge of the mechanisms involved in immunemodulation will be crucial to develop management and conservation programs in future GCC scenarios.

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