Responses of marine mussel *Mytilus galloprovincialis* (Bivalvia: Mytilidae) after infection with the pathogen *Vibrio splendidus*

Maria Giovanna Parisi, Maria Maisano, Tiziana Cappello, Sabrina Oliva, Angela Mauceri, Mylene Toubiana, Matteo Cammarata

ABSTRACT

Bivalve molluscs possess effective cellular and humoral defence mechanisms against bacterial infection. Although the immune responses of mussels to challenge with pathogenic vibrios have been largely investigated, the effects at the site of injection at the tissue level have not been so far evaluated. To this aim, mussels *Mytilus galloprovincialis* were herein *in vivo* challenged with *Vibrio splendidus* to assess the responses induced in hemolymph and posterior adductor muscle (PAM), being the site of bacterial infection. The number of living intra-hemocyte bacteria increased after the first hour post-injection (p.i.), suggesting the occurrence of an intense phagocytosis, while clearance was observed within 24 h p.i. A recruitment of hemocytes at the injection site was found in mussel PAM, together with marked morphological changes in the volume of muscular fibers, with a recovery of muscle tissue organization after 48 h p.i. A concomitant impairment in the osmoregulatory processes were observed in PAM by an initial inhibition of aquaporins and increased immunopositivity of Na⁺/K⁺ ATPase ionic pump, strictly related to the histological alterations and hemocyte infiltration detected in PAM. Accordingly, an intense cell turnover activity was also recorded following the infection event. Overall, results indicated the hemolymph as the system responsible for the physiological adaptations in mussels to stressful factors, such as pathogenicity, for the maintenance of homeostasis and immune defence. Also, the osmotic balance and cell turnover can be used as objective diagnostic criteria to evaluate the physiological state of mussels following bacterial infection, which may be relevant in aquaculture and biomonitoring studies.

1. Introduction

Bivalve molluscs, besides their fundamental role within the marine ecosystem (Newell, 2004), are of substantial economic value in the aquaculture industry. Indeed marine molluscs, including the Mediterranean mussel *Mytilus galloprovincialis* (Lamarck 1819), a very popular shellfish with a wide geographical distribution worldwide (Branch and Stephani, 2004), are commonly included in human diet because of their exceptional nutritional value (Bongiorno et al., 2015; Cappello et al., 2018). Mussels are routinely used as good bioindicators in ecotoxicological studies (Cappello et al., 2017a, 2017b; Giannetto et al., 2015, 2017; Maisano et al., 2017) and, due to their filter-feeding habit, are also known to accumulate large numbers of bacteria from the surrounding waters. Albeit mussels possess effective humoral and cellular defence mechanisms against bacterial infection (Canesi and Pruzzo, 2016), some bacteria can be pathogenic to the bivalve host, affecting its physiological performance and in some cases leading to host death (Travers et al., 2015), thus constituting a potential risk also for seafood consumers.

The immune system of molluscs is constituted by cellular and humoral components, operating in a complementary manner to neutralize invading organisms (Galloway and Depledge, 2001). Cellular response is mediated by hemocytes that circulate in hemolymph vessels and throughout soft tissues, and kill microbes through phagocytosis and citotoxic reactions. During pathogenetic events, invaders are detected via hemocyte-bound recognition factors, with the production of cytokines that mediate the recruitment of additional hemocytes and release of humoral components as lysosomal enzymes and anti-microbial peptides (Allam and Raftos, 2015; Carballal et al., 1997). Hemocytes are also directly engaged in pathogen and parasite killing via the oxidative
burst reaction, which involves the production of oxygen metabolites, with high bactericidal and antiviral activities (Pruzzo et al., 2005).

Bacteria particularly pathogen to mussels are those belonging to the genus *Vibrio*, which are endemic and highly abundant in marine and estuarine ecosystems. Pathogenic vibrios infect larval, juvenile and adult stages of molluscs (Beaz-Hidalgo et al., 2010; Wu et al., 1995), and are commonly associated with massive mortality events of wild or cultured bivalves (Travers et al., 2015), thus representing a serious threat also for aquaculture industry. To date, numerous in *vitro* and in *vivo* studies have been conducted in mussels *M. galloprovincialis* challenged with pathogenic vibrios. In mussels infected by *V. splendidus*, a down-regulation in lysozyme was observed, along with circulating hemocytes expressing mytilin-B and lysozyme, which instead accumulated at the site of injection. Conversely, the opposite responses were documented in mussels after challenge to *V. anguillarum* (Li et al., 2008; Parisi et al., 2009), probably due to differential interaction of living bacteria with the immune mechanisms of mussels (Venier et al., 2011).

In mussels infected by *V. anguillarum*, disruption in energy metabolism and induction of oxidative stress were revealed (Ji et al., 2013), whereas immune stress, osmotic disruption and reduced energy demand were observed in *V. harveyi*-challenged *M. galloprovincialis* (Liu et al., 2014). Interestingly, autophagic processes were induced by *V. tapetis* in hemocytes of *M. galloprovincialis*, as a result of protective mechanisms towards damaged cell components (Balbi et al., 2018). More recently, a dose-dependent decrease in hemocyte lysosomal membrane stability and bactericidal activity of whole hemolymph, were observed in *M. galloprovincialis* in response to *in vitro* and *in vivo* challenge with the emerging marine pathogen *V. coralliilyticus* (Li et al., 2014). Interestingly, autophagic processes were induced by *V. tapetis* in hemocytes of *M. galloprovincialis*, as a result of protective mechanisms towards damaged cell components (Balbi et al., 2018). However, the effects of bacterial infection at the sites of injection at tissue level have not been so far specifically investigated.

The cell-mediated innate response can be identified also at histological level, as diffuse infiltrative hemocytosis in severe systemic infections or as focal accumulation of hemocytes at sites of pathogen invasion (Carella et al., 2015). As osmoconformers, molluscs have a osmoionic plasticity regulated by the hemolymph that induces changes in cell volume and composition of ionic contents for adaptation to the external environment (Hosoi et al., 2005). However, little is known about the mechanisms of response in mussels to maintain homeostasis at sites infected by pathogens. Cell proliferation and apoptosis are two opposed and balanced processes responsible for the maintenance of the correct histology and physiology of organs and tissues. Apoptosis plays important roles in many mollusc physiological processes, including normal tissue and organ development, homeostasis and immune defence (Sokolova, 2009). Nevertheless, the knowledge on the modulation of mollusc apoptosis induced by pathogens is still lacking.

Therefore, the present study was designed in order to evaluate the responses of mussel *M. galloprovincialis* to *in vivo* challenge with *V. splendidus* in the posterior adductor muscle (PAM), the site of bacterial infection. PAM is the main muscular system in bivalve molluscs, and its impairment may negatively impact on the life functions of molluscs (Cappello et al., 2017a). After the infection event, the behaviour of circulating hemocytes in the mussel hemolymph and the histology of PAM were assessed, as well as the occurring osmoregulatory processes (by the water channel proteins aquaporins and the Na⁺/K⁺ ATPase pump) and cell turnover (by the proliferating cell nuclear antigen, PCNA, and Caspase-3 as biomarker of apoptosis).

### 2. Material and methods

#### 2.1. Mussels and bacterial preparation

Adult marine mussels *Mytilus galloprovincialis* (6–7 cm shell length) were purchased from the Les Compagnons de Maguelone marine farm (Palavas, France). In the laboratory, specimens were acclimatized in tanks filled with 50 L of oxygenated seawater at 20 °C, for 3 days prior to bacterial injection.

**Table 1**

<table>
<thead>
<tr>
<th>Description</th>
<th>Supplier</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>Primary antibody Na⁺/K⁺ ATPase</td>
<td>Mouse (M)</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Water Channel Aquaporin (AQP)</td>
<td>Rabbit (P)</td>
<td>Sigma-Aldrich, St Louis, MO, USA</td>
</tr>
<tr>
<td>Active Caspase 3 (CASP3)</td>
<td>Rabbit (P)</td>
<td>MBL International, Woburn, MA</td>
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<tr>
<td>Proliferating Cell Nuclear Antigen (PCNA)</td>
<td>Rabbit (P)</td>
<td>Abcam, Cambridge, UK</td>
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<tr>
<td>Secondary antibody Peroxidase-conjugated anti-rabbit IgG</td>
<td>–</td>
<td>Sigma-Aldrich, St Louis, MO, USA</td>
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<tr>
<td>Peroxidase-conjugated anti-mouse IgG</td>
<td>–</td>
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<tr>
<td>FITC-conjugated anti-rabbit IgG</td>
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<td>TRITC-conjugated anti-mouse IgG</td>
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**Table 1** Details of the antibodies used in this study.

**Vibrio splendidus** LGP32 (50 μL of overnight-cultured inoculum) were grown in 10 mL trypsin-casein-soya (TCS) at 20 °C for 4–6 h, centrifuged for 10 min at 500 × g, and then adjusted to 10⁸ bacteria/mL with phosphate buffered solution isotonic to sea water (PBS-NaCl: 2 mM KH₂PO₄, 10 mM Na₂HPO₄, 3 mM KCl, 600 mM NaCl, pH 7.4), according to 1 Optical Density (OD)₆₀₀ nm = 5 × 10⁸ bacteria/mL (Li et al., 2008).

#### 2.2. Experimental procedure

Four batches of 10 mussels for each sampling time-point were in *vivo* challenged by one injection of approximately 100 μL of bacterial suspension (10⁷ bacteria) into the posterior adductor muscle (PAM), by using a gauge needle fitted to a syringe. For the control, 100 μL of bacteria-free PBS-NaCl was injected to four batches of 10 unchallenged mussels each (referred to as controls). Following the injections, mussels of each group were placed back in separated tanks filled with 20 °C aerated seawater until each withdrawal time. Water exchange, tank cleaning and microalgae were provided daily.

Hemolymph was collected from mussel PAM with a 1 mL syringe containing 100 μL of the anti-aggregate Alsever’s solution, at 1, 3, 6, 9, 12, 24, 48 and 72 h post-injection (p.i.). Hemolymph samples from 10 mussels were pooled and centrifuged (500 × g, 6 min, 4 °C), and the hemocyte pellet was then suspended in PBS–NaCl for the clearance evaluation. At the same time-points, small pieces of mussel PAM were collected for histological and immunohistochemical analyses.

#### 2.3. Clearance measurement

Aliquots of 50 μL of PBS–NaCl serial diluted hemolymph, were spread in duplicate Petri dishes containing either TCS or Luria-Bertani (LB) agarose medium. Agar was used for heterotrophic plate count analyses because this non-selective medium provides better support for stressed cells and produces discrete (larger) colonies overnight. Thus, yellow colonies were considered total *Vibrio* colonies and counted as a colony-forming unit (CFU) after 24 h incubation at 20 °C. Data obtained from at least two consecutive dilutions were expressed as mean ±
Fig. 1. Evaluation of bacterial colonies (CFU) post challenge in the circulating hemocytes of mussel *M. galloprovincialis* infected with *V. splendidus*. Two peaks were detected, at 1 h and 6 h after infection. A first decrease up to 9–12 h and a second up to 24 h were found. The cell number level appears constant from 24 to 72 h. Results are expressed as the mean ± SEM (bars) of CFUs counted in four different assays, each of them measured in duplicate.

Fig. 2. Histological sections of PAM tissues from control and *V. splendidus*-infected mussels, stained with Hematoxylin/Eosin. Compared to control (a), alterations of PAM structure were observed after 1 h from injection, when large white spaces (asterisks) appeared among fibers (F) and fascicles (b), followed by a recovery process starting from 3 h post-infection (c). Hemocytes (arrows) recruitment from 6 h until 48 h (d-h) and a marked hypertrophy were detected (g-i).
standard error of the mean (S.E.M.). Differences were evaluated using Student’s t-test ($p < .05$) for each time point.

2.4. Histological analysis

For histological assessment, mussel PAM tissues were fixed in 4% paraformaldehyde suspended in 0.1M phosphate buffered solution (pH 7.4) at 4 °C, dehydrated in ethanol and embedded in paraffin (Bio-Optica, Italy). Histological sections (4 μm thick) were cut with a rotary automatic microtome (Leica Microsystems, Wetzlar, Germany), and stained with Hematoxylin/Eosin (Bio-Optica, Italy) to evaluate morphological features (Maisano et al., 2017).

2.5. Immunohistochemical analysis

Histological sections of mussel PAM were also used for an immunohistochemical assessment, applying an indirect immunofluorescence method (Brunelli et al., 2011). Briefly, the slides were incubated overnight in a wet chamber at 4 °C with the primary antisera, namely anti-water channel aquaporin (AQP), A5560, Sigma-Aldrich; anti-Na$^+$/K$^+$ ATPase, ab7671, Abcam; anti-PCNA, ab2426, Abcam; and anti-Caspase3 (CASP3), JM-3015-100, MBL Polyclonal Antibody. More details are reported in Table 1.

After a rinse in PBS (137 mM NaCl, 10 mM KH$_2$HPO$_4$, 2 mM KH$_2$PO$_4$, 2.7 mM KCl, pH 7.6) for 10 min, sections were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG secondary antibody (Sigma), or tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (Sigma), diluted 1:100, for 2 h at room temperature. Positive controls for labelling specificity of each peptide were performed by incubating sections with antiserum pre-absorbed with the respective antigen (10–100 g/mL), while negative controls were conducted by omission of the primary antisera.

All observations were made on five randomly selected fields per section using a 40× oil-immersion objective with a motorized Zeiss Axio Imager Z1 epifluorescence microscope (Carl Zeiss AG, Werk Göttningen, Germany), equipped with an AxioCam digital camera (Zeiss, Jena, Germany) for the acquisition of images. Sections were imaged using the appropriate filters for the excitation of FITC (480/525 nm) and TRITC (515/590 nm), and then processed by using AxioVision Release 4.5 software (Zeiss) for the count of immuno-positive cells.

The immunohistochemical data were statistically analysed by one-way analysis of variance (ANOVA) using the GraphPad (Prism 5.0, San Diego CA, USA) software. The Dunnett’s multiple comparison post-hoc test was applied in order to determine significant differences between control and treatment groups. Data were presented as means ± standard deviation (S.D.), and considered statistically significant at $p < .05$. 

Fig. 3. Immunohistochemical detection of AQP in mussel PAM by indirect immunofluorescence method with the primary antisera anti-AQP. The graph (h) indicates the quantification of immunopositive cells in the control (a) and at various time-points of V. splendidus-infected mussels (b–g). Scale bar 20 μm.
2.6. Western blotting

Western blot analysis was performed to determine the antibody specificity against selected target proteins in PAM from *M. galloprovincialis*. PAM extracts and positive control cell or tissue lysates specific for each antibody were run in parallel lines, following antibody suppliers’ recommendations. Protein extracts from PAM tissues were obtained through homogenization in a lysis buffer containing protease inhibitor cocktail, as extensively described in previous works (Cappello et al., 2015; Giannetto et al., 2017). Total protein concentration was determined using the BCA assay (Pierce) and samples of 40μg protein were resolved by SDS-polyacrylamide gel electrophoresis (12%). The separated proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane; blots were blocked for 1 h in 5% bovine serum albumin in TPBS (PBS pH 7.4, 0.1% Tween 20), and incubated overnight at 4 °C with the appropriate primary antibody, the same as reported above, according to the manufacturer’s instructions. After washing, blots were incubated with goat anti-rabbit IgG or goat anti-mouse IgG conjugated to peroxidase (Sigma-Aldrich). Antibody binding was detected by chemiluminescence staining using the Immuno-Star Western C Chemiluminescent Kit (BioRad).

Immunoreaction with each antibody used in this study revealed the known specifically sized immunopositive band both in positive control lysates and mussel PAM extracts. In particular, specific AQP, Na⁺/K⁺ ATPase, PCNA and CASP3 immunoreactive bands of approximately 112, 31, 29 and 17 kDa, respectively, were observed. Representative western blot results are reported in the Supplementary Fig. S1.

3. Results

3.1. Bacterial clearance

The bacterial CFU count is referred to the intra-hemocytes colonies. Following injection, the quantities of living intra-hemocyte bacteria and the kinetics of clearance were considered (Fig. 1). When living *V. splendidus* bacteria were injected into the circulation of the mussel, the number of living intra-hemocyte bacteria dramatically increased already after the first hour p.i., suggesting the occurrence of an intense phagocytosis (121 CFU, *p* = .00011). After a reduction in the number of bacterial colonies as recorded at 3 h post-injection (60 CFU), a peak of living bacteria was observed at 6 h post-injection (275 CFU, *p* = .0008), followed then by a rapid decrease. Very few living *V. splendidus* were detected at 24 h, 48 h and 72 h post-injection.

3.2. Histomorphology

The PAM of mussels inoculated with PBS-NaCl showed a regular

![Fig. 4. Na⁺/K⁺ ATPase localized by indirect immunofluorescence with the primary antisera anti- Na⁺/K⁺ ATPase in mussel PAM sections. The graph (h) indicates the quantification of immunopositive cells in the control (a) and at the various time-points of *V. splendidus*-infected mussels (b-g). Scale bar 20μm.](image-url)
Histological organization, with fibers forming dense bands, more or less parallel to each other, surrounded by connective tissue. The adductor muscle was well supplied with hemolymph, and hemocytes were visible among fibers and within the connective tissue (Fig. 2a). In mussels injected with bacteria, severe histological alterations of PAM structure were observed. In detail, after 1 h p.i., the adductor muscle lost cellular volume and, in turn, large white spaces appeared both among fibers and among fascicles (Fig. 2b). Following 3 h p.i., a recovery process of the muscle structure occurred (Fig. 2c–f), leading to a marked hypertrophy at the higher time points, namely 24 h, 48 h and 72 h p.i. (Fig. 2g–i).

Within 24 h after the bacterial injection, a massive migration of hemocytes to the location of the injection at the muscular site was observed. Particularly, from 6 h post-injection an intense presence of hemocytes was also noticed both among muscle fibers and among fascicles (Fig. 2d), with a greater presence at 24 h and 48 h (Fig. 2g, h).

3.3. Osmotic balance

In respect to PAM of mussels from control, a drastic and statistically significant inhibition of the immunopositivity to AQP was observed in mussel adductor muscles after 1 h from injection, but at the following time points, an increasing number of immunolabeled cells was noticed. Worthy to note, the immunopositivity to AQP remained slightly below the control value at 6, 24 and 72 h post-injection (Fig. 3a–h).

By using the antibody directed against Na+/K+ ATPase, a significant high number of immunopositive cells was found at each post-treatment time point tested, in respect to control value. A noticeable increase was observed at 48 h and 72 h post-injection, at which the immunopositive regions detected in mussel PAM were almost three and five times higher than those of the control (Fig. 4a–h).

3.4. Cell turnover

The immunohistochemical investigation of PCNA in mussel PAM showed a significant rise of immunopositivity starting from 12 h post-injection in respect to control, with peaks of expression observed at 24 h and 48 h, followed by a decrease recorded at 72 h (Fig. 5a–h).

The immunodetection of CASP3 showed, after a slight increment at 6 h and 12 h p.i., a significant increase of immunopositivity from 24 h to 72 h post-injection, with respect to control and 1 h post-injection group (Fig. 6a–h).

4. Discussion

Phagocytosis is the process by which certain immune cells recognize...
and eliminate non-self-components, such as invading and/or associating microorganisms, and it is one of the most important defensive functions of hemocytes in bivalve molluscs (Bayne, 1990; Ottaviani, 2006). In the present study, after challenging mussel *M. galloprovincialis* to the pathogen *V. splendidus*, it was found that the phagocytosis process was activated during the first hour post bacterial injection, as also reported in a previous study on *in vitro* phagocytosis-associated activities in mussels (Parisi et al., 2009), and *V. splendidus* bacteria were gradually killed up to 24 h from infection. Since phagocytosis occurs thanks to mechanisms of cell recognition prior to the process of ingesting the infectious agent, it is possible that the kinetics of clearance may occur in a variable manner according to the particular *Vibrio*-bivalve partners (Canesi et al., 2002). For instance, it was reported that in the clam *Ruditapes decussatus* infected by *V. tapetis*, clearance occurs in 3 days (Allam et al., 2002), whereas it occurs in 13 and 14 days in the *V. vulnificus*-challenged oyster *Crassostrea virginica* and clam *R. philippinarum*, respectively (Froelich and Oliver, 2013).

Previous researches have demonstrated a great diversity in the immune responses of mussels, both at inter and intra-population level, in relation to the microorganism to which they are exposed (Cantet et al., 2012). In mussel *M. galloprovincialis*, widely used as an experimental model species of bacterial infection, it has been demonstrated a recruitment of hemocytes containing antimicrobial peptides (AMPs), including mytilins, towards the site of injection within hours following bacterial challenge, along with a prominent role of mytilins in killing intracellular bacteria after phagocytosis (Mitta et al., 2000a; Parisi et al., 2012; Sonthi et al., 2012). Furthermore, an increase of soluble AMPs in the plasma of mussels suggests the occurrence of secondary systemic responses to microbial challenges (Mitta et al., 2000b; Toubiana et al., 2014). Results from this study showed a phenomenon of recruitment of hemocytes at the bacterial injection site into mussel PAM, together with marked morphological changes in the volume of muscular fibers, especially from 6 h following the infectious event. However, a recovery of the muscle tissue organization in mussels was clearly noticed after 48 h post bacterial injection.

Data reported herein demonstrate also an important involvement of aquaporins (AQPs) in physiological activities and maintenance of the regular structure of the mussel PAM. AQPs are highly conserved transmembrane water channel proteins, ubiquitous in various organs and tissues throughout the body, that play a crucial role in transcellular and transepithelial water movement. Water transport mechanisms have been widely investigated in the gills of fish (Brunelli et al., 2010; Giffard-Mena et al., 2007; Watanabe et al., 2005) and mussels (Scarfì et al., 2006). In skeletal muscle fibers, AQPs are involved in the rapid equilibration of osmotic gradients generated by intracellular accumulation of metabolites (Frigeri et al., 2004). The expression of AQP, belonging to the aquaglyceroporin group, was documented in the sarcolemma of skeletal muscles (Frigeri et al., 2004). In the present work, the
initial loss of cellular volume observed in mussel PAM is supported by the drastic inhibition of AQP, that seems to play, in conjunction with Na⁺/K⁺ ATPase, a key role in the maintenance of the regular hydro-osmotic balance and cellular volume of muscle fibers.

The Na⁺/K⁺ ATPase is an ionic pump mainly involved in the maintenance of cell membrane potential and osmotic balance and, in the skeletal muscle of mammals, it plays also a role in the responses to various stress conditions (Pinoni and Lopez Mananes, 2009). Recently osmoregulatory mechanisms, and therefore processes of volume regulation, were demonstrated to occur in mussel PAM, which exhibited significant changes in the levels of osmolytes in response to environmental perturbations (Cappello et al., 2017a). In bivalve molluscs it was suggested that, in conjunction with the Na⁺/K⁺ ATPase, also the Na⁺ ATPase is involved in regulating the intracellular concentration of Na⁺ in response to the different environmental conditions (Pagliarani et al., 2006, 2008). Therefore in the present study, the increase in Na⁺/K⁺ ATPase immunopositivity might be related with the histological PAM alterations and marked presence of hemocytes observed among bundles of muscle fibers following the bacterial injection.

In molluscs, cell proliferation and apoptotic mechanisms are well documented, both activated by ligand–receptor interaction and cytoplasmic signals. The nuclear antigen of cell proliferation (PCNA) is a protein involved in DNA replication, and plays a key role in the regulation of cell cycle and check-point control (De Domenico et al., 2013; Fasulo et al., 2012; Johnson and O’Donnell, 2005; Kelman, 1997). On the contrary, caspases, a family of proteases described also in bivalves (Sokolova et al., 2004; Sokolova, 2009), are involved in the activation and implementation of the apoptotic programme. Apoptosis in molluscs is involved in larval development process (Gifondorwa and Leise, 2012), and implementation of the apoptotic programme. Apoptosis in molluscs is involved in larval development process (Gifondorwa and Leise, 2012), and implementation of the apoptotic programme.

Conflicts of interest

The authors declare no conflicts of interest in this work.

Acknowledgements

This work was supported by the FFR-Camarata (PJ. RIC. FFABR. 2017 004312 MC) and FFR-Parisi (PJ. RIC. FFABR 2017 161753 MGP) for Scientific Research from the University of Palermo, Italy, and the MC RITMARE Project (SP2-WP4-A23-03; CNR and CONISMA).

References


