

## Review

## MicroRNA-mediated stress response in bivalve species

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## ARTICLE INFO

Edited by Professor Bing Yan

## Keywords:

Bivalve  
Environmental pollutant  
Hemocytes  
MicroRNA  
Stress

## ABSTRACT

Bivalve mollusks are important aquatic organisms, which are used for biological monitoring because of their abundance, ubiquitous nature, and abilities to adapt to different environments. MicroRNAs (miRNAs) are small noncoding RNAs, which typically silence the expression of target genes; however, certain miRNAs directly or indirectly upregulate their target genes. They are rapidly modulated and play an essential role in shaping the response of organisms to stresses. Based on the regulatory function and rapid alteration of miRNAs, they could act as biomarkers for biotic and abiotic stress, including environmental stresses and contaminations. Moreover, mollusk, particularly hemocytes, rapidly respond to environmental changes, such as pollution, salinity changes, and desiccation, which makes them an attractive model for this purpose. Thus, bivalve mollusks could be considered a good animal model to examine a system's response to different environmental conditions and stressors. miRNAs have been reported to adjust the adaptation and physiological functions of bivalves during endogenous and environmental stressors. In this review, we aimed to discuss the potential mechanisms underlying the response of bivalves to stressors and how miRNAs orchestrate this process; however, if necessary, other organisms' response is included to explain specific processes.

## 1. Introduction

In aquatic ecosystems and aquaculture, there are multiple types of potential stress: physical (temperature, dissolved oxygen, light, and sound), chemical (water quality, pollution, metabolic waste, and diet), and biological [microorganisms (pathogenic and nonpathogenic), macro-organisms (parasites), stocking density, lateral swimming space requirements), and procedural (handling, hauling, stocking, disease treatment, feeding methods (manual and automated)] (Burgos-Aceves et al., 2020; Kumar et al., 2015). The stress response is a complex process controlled by multiple systems, including the nervous, immune, circulatory, and endocrine systems (Abdel-Mageid et al., 2020a; Abo-Al-Ela, 2018a, 2018b). When stress response is triggered, a cascade of physico-biochemical changes and neural and neuroendocrine responses

are developed to facilitate adaptation to stressors (Kumar et al., 2015). Although stress response is critical to cope with biotic and abiotic stressors, in many cases, particularly chronic stress, such response hinders the normal physiological status and organisms' health (Guo et al., 2015; Lacoste et al., 2001b).

MicroRNAs (miRNAs) are endogenous noncoding RNA molecules, which are 17–22 nucleotides in length and control almost all known biological processes. The miRNA machinery system is an important regulator of gene expression at transcriptional and post-transcriptional levels by inhibiting or enhancing messenger RNA (mRNA) translation (Abo-Al-Ela and Burgos-Aceves, 2020; Bizuayehu and Babiak, 2014). miRNAs are rapidly modulated in response to endogenous or exogenous changes (Burgos-Aceves et al., 2018a, 2018b). A study on Atlantic cod, *Gadus morhua* L., demonstrated that miRNAs are the novel biomarkers of

**Abbreviations:** Akt, Akt serine/threonine kinase; ATPGD1, ATP gras domain containing protein 1; cAMP, cyclic-AMP; CAT, catalase; CREBL2, cAMP-responsive element binding protein-like 2; HIF1, hypoxia-inducible factor 1; HSP, heat shock protein; IL6, interleukin 6; miRNAs, microRNAs; mRNA, messenger RNA; MXR, multidrug resistance system; NF-κB, nuclear factor kappa light-chain enhancer of activated B cells; NRF2, nuclear factor erythroid 2 related factor 2; PI3K, phosphatidylinositol 3'-kinase; PTEN, phosphatase and TENsin homolog or phosphatidylinositol-3,4,5-trisphosphate 3 phosphatase; RAS, rat sarcoma signaling; RFP1, replication factor A protein 1; ROS, reactive oxygen species; SAM, S-adenosylmethionine synthetase; SOD, superoxide dismutase; Stat3, signal transducer and activator of transcription factor 3; T-AOC, anti-oxidation competence; VWDE, von Willebrand factor D and EGF domain containing protein.

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Received 31 July 2020; Received in revised form 25 September 2020; Accepted 29 September 2020

Available online 7 October 2020

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environmental rearing temperatures: miRNAs related to stress (miR-155) and inflammation (miR-21) were upregulated in fish reared at 9 °C; however, the growth-related miRNA-206 was elevated in fish reared at 4 °C (Magnadóttir et al., 2020).

Several miRNAs and their gene targets are conserved and have similar function across species; however, their function is necessarily not the same among different species. Note that few miRNAs are specific to certain organisms (Ha et al., 2008). The low conservation between vertebrate and invertebrate miRNA decelerated the progress of research on miRNAs in invertebrate species (Biggar and Storey, 2018). For aquatic invertebrates, such as crustaceans, many conserved miRNAs have been examined, particularly those involved in the disease and immune responses (Huang et al., 2012; Song et al., 2014; Tan et al., 2013). In marine bivalves, certain progress is accomplished, e.g., a considerable number of the conserved miRNAs was identified in *Crassostrea gigas* and *C. hongkongensis* that involved osmotic stress (Zhao et al., 2016). However, a lot more remains to be explored in mollusks.

Thus, miRNA stress response is conserved across phylogeny (Biggar and Storey, 2018). Research is ongoing to annotate and identify miRNAs that regulate physiological functions in bivalves. This should allow understanding and identifying different pathways involved in certain physiological processes, e.g., the let-7 family of miRNA showed a high conservation in vertebrates and invertebrates (Lee et al., 2007). Moreover, miRNA and mRNA networks have been profiled in the marine mussel *Mytilus galloprovincialis*, which suggested that the highly conserved miRNAs let-7 and miR-100 family plays an essential role in many metabolic pathways (Yu et al., 2020). miRNAs regulate the induction of estivation in terrestrial snails. Of these miRNAs, ola-miR-2001-5p, ola-miR-1989-5p, ola-miR-745b-3p, ola-miR-723-5p, ola-miR-281-5p, ola-miR-190-5p, ola-miR-12-5p, and ola-miR-2a-3p showed a strong upregulation. These miRNAs were involved in regulating the cell survival mechanisms that constituted anti-apoptosis, tumor suppression, and muscle maintenance responses. Furthermore, miR-2 upregulation has been suggested as a conserved invertebrate response to cellular stresses under harsh environmental conditions (Hoyeck et al., 2019).

Mollusks are an important bioindicator because they reflect levels of environmental contamination (Capillo et al., 2018; Pagano et al., 2017). Bivalve mollusks are exposed to environmental fluctuations, particularly coastal zones, and they have to adapt to survive (Freitas et al., 2019, 2020a, 2020b; Stara et al., 2020). Hemocytes are primarily involved in the immune response in such aquatic invertebrates: they exert active phagocytosis and mediate the expression of immune genes after immune stimulation (Burgos-Aceves and Faggio, 2017). Moreover, hemocytes are involved in biomineralization and shell formation (Huang et al., 2018). Hemocytes mediate physiological responses of bivalve mollusks against environmental stressors. The open circulatory system of mollusks allows the continuous exposure of hemocytes to the external environment (Faggio et al., 2016; Pagano et al., 2016; Torre et al., 2013). *In vitro* approaches using hemocytes as cell models are efficient at determining the effects of different environmental contaminants in ecotoxicological studies (Ladhar-Chaabouni and Hamza-Chaffai, 2016). Molluscan hemocytes can respond to a stressful stimulus within 2 min in which time their numbers can double or increase by three- or four-fold. The increase in hemocyte numbers continues with continued stress and is not attributed to the proliferation of hemocytes. Moreover, increases in hemocyte counts drop after a return to optimal conditions. Fluctuations in cell counts are considered to be attributed to the disappearance or return of circulating eosinophilic granulocytes (Renwrandt et al., 2013). Seawater acidification and environmental concentrations of caffeine may affect the physiological conditions and functionality of bivalve hemocytes (Munari et al., 2020). Thus, hemocyte numbers can reflect the magnitude of the body's response to endogenous or exogenous stressors in individual organisms at any given time (Renwrandt et al., 2013).

Bivalve hemocytes are frequently used for monitoring the

genotoxicity of water pollutants (Klobučar et al., 2003; Pavlica et al., 2001). Recently, many miRNAs, including miR-87, miR-281, miR-723, miR-745, miR-1542, miR-1989, miR-1994, miR-2176, miR-6833, and miR-7428, showed a potential implication in stress survival in mollusks (Hoyeck et al., 2019). Although genomic advances allow the understanding of several molecular mechanisms, our knowledge of miRNA functions in stress-responsive adaptations is far from complete: the function of several miRNAs remains unclear.

## 2. MicroRNA modulation during stressors and xenobiotics

### 2.1. Osmoregulation and osmotic stress

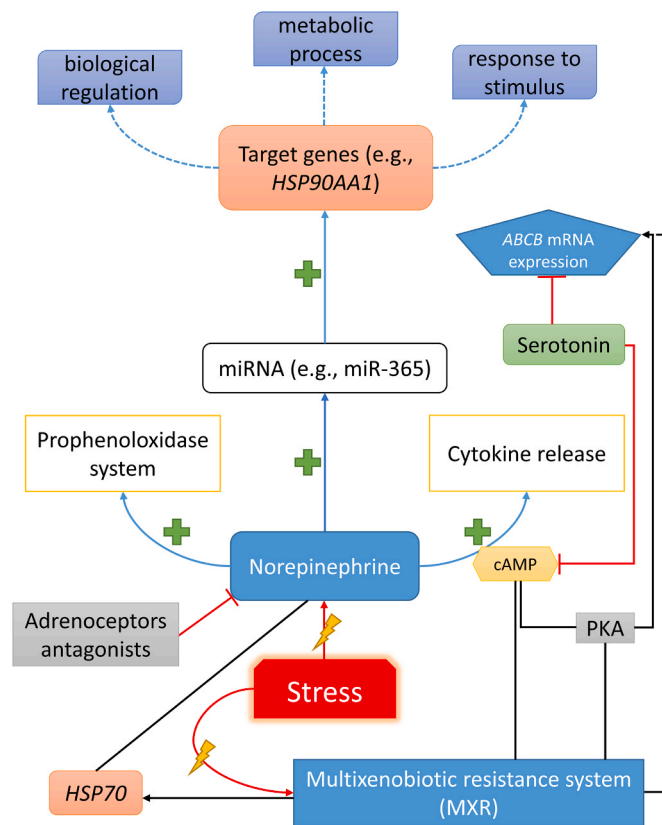
The marine rocky intertidal zone is an area connecting the ocean and land. This area is characterized by harsh environmental conditions, including rapid changes in temperature, pH, salinity, oxygen, tidal levels, and food availability. This fluctuating environment lead to changes in the transcriptome that is linked to processes of cell division, metabolism, respiration, and stress responses (Gracey et al., 2008).

#### 2.1.1. Desiccation

Desiccation is a stress factor that affects the bivalves that dominate the intertidal zone. Oysters, *Crassostrea gigas*, attempted to adapt to this environment and miRNAs were reported to play an essential role in this respect. To illustrate, cgi-miR-365, which is involved in the networks of biological regulation and metabolic process, is rapidly induced after desiccation stress in oysters and their hemocytes (Chen et al., 2017). Moreover, the expression of heat shock protein (HSP) 70 and *CgHSP90AA1* are strongly upregulated, respectively, at different phases in the tidal cycle in the California ribbed mussel, *Mytilus californianus*, and after desiccation stress in the oyster (Chen et al., 2017; Gracey et al., 2008). *CgHSP90AA1* is modulated in relation to the expression of cgi-miR-365 in which the upregulation of cgi-miR-365 is accompanied by a high expression of *CgHSP90AA1* and increases in norepinephrine. Moreover, the expression of *CgHSP90AA1* is induced by norepinephrine in oyster hemocytes (Chen et al., 2017). Typically, miRNAs negatively regulate their target mRNAs (Abo-Al-Ela and Burgos-Aceves, 2020); however, certain miRNAs show evidence of stimulating gene expression post-transcription (Vasudevan, 2012). Similarly, *CgHSP90AA1* has a putative binding site of cgi-miR-365, indicating a direct positive interaction (Chen et al., 2017). It is possible that norepinephrine increases *HSP70* gene promoter activity through the  $\alpha$ -adrenergic signaling pathway in oyster hemocytes (Fig. 1) (Lacoste et al., 2001a, 2001c).

#### 2.1.2. Low or high salt stress

Salt stress is another component of intertidal environments. Gill tissues are vital for environmental adaptation, particularly during osmolality fluctuations. They act as a primary interface between the external environment and hemolymph or cytoplasm in marine mollusks (Hosoi et al., 2007). Zhao et al. (2016) examined the effects of low salinity on miRNA modulation in the gills of two *Crassostrea* species: *C. gigas* and *C. hongkongensis*. Two differentially expressed miRNAs, upregulated chk-miR-3205 and downregulated chk-miR-2353, were identified in *C. hongkongensis*, and a total of six differentially expressed miRNAs (scaffold43364\_10952, cgi-miR-92, and cgi-miR-1984 were upregulated, and cgi-miR-183, cgi-miR-2353, and cgi-miR-184-3p were downregulated) were identified in *C. gigas* (Zhao et al., 2016). The annotation of differentially expressed miRNA target genes suggested a similar gene function in both *C. gigas* and *C. hongkongensis* after osmotic stress. The Gene Ontology enrichment analysis revealed that targeted genes are implicated in essential biological processes such as cellular component movement, microtubule-based processes, intracellular signal transduction, and catabolic and metabolic processes of purine nucleoside (Zhao et al., 2016). Note that miR-2353 was downregulated in both species; however, its function is still unclear. chk-miR-2353 appeared to target the cAMP-responsive element binding protein-like



**Fig. 1.** Norepinephrine mediates the stress response in bivalve hemocytes. Stress induces norepinephrine release that, in turn, stimulates downstream pathways. Norepinephrine regulates the prophenoloxidase system via  $\alpha$ - and  $\beta$ -adrenergic receptors, activating immune cells, and mediating cytokine release mediated by cyclic-AMP (cAMP). Adrenoceptor antagonists can suppress the action of norepinephrine. A strong link was found between serotonin, cAMP, dependent protein kinase (PKA), and the expression of the ATP-binding cassette transporter gene *ABCB*. Serotonin has a negative feedback on cAMP levels, PKA activities, and *ABCB* mRNA expression. The *ABCB* promoter region has several putative PKA-related regulatory elements. The transcriptional regulation of the *ABCB* is mediated through the phosphorylation activity of the cAMP-PKA. *ABCB* encodes the P-glycoprotein that is involved in the multixenobiotic resistance system (MXR). The MXR helps aquatic organisms to cope with and adapt to polluted environments by preventing cellular accumulation of potentially harmful xenobiotics. MXR mediates cAMP/PKA activities and many genes, including *ABCB* involved in stress response in bivalves. Moreover, norepinephrine can induce the expression of certain microRNA (miRNAs) related stress, such as miR-365 during desiccation stress in oysters, and thus mediates the expression of several genes, such as heat shock protein (*HSP*) 90AA1, which is involved in metabolic processes, biological regulation, and response to stimulus.

2 (CREBL2) and the ATP grasp domain containing protein 1 (ATPGD1) genes. Oysters primarily use intracellular-free amino acid metabolism pathways to adjust their euryhaline adaptation (Zhao et al., 2016). As a member of this pathway, ATPGD1 catalyzes beta-alanine degradation, which in turn maintains osmotic equilibrium under hypo-osmotic stress conditions. This was demonstrated by the significant upregulation of ATPGD1 on the seventh day of hypo-osmotic stress (Meng et al., 2013). chk-miR-3205 was reported to target replication factor A protein 1 (RFP1), hygromycin phosphotransferase, von Willebrand factor D, and EGF domain containing protein (VWDE) genes during osmotic stress response. RFP1 is involved in the stress response and apoptosis (Morga et al., 2012; Zhao et al., 2012), and VWDE was putatively linked to both stress and immunity (Buckley and Rast, 2015; Prado-Alvarez et al., 2009). Together, these properties allow oysters to adapt to osmotic changes by adjusting intracellular concentrations of osmolytes and

regulating cell volume.

Once again but in crustaceans, the miRNAs of gills participate in adaptation and the response to stressors. Chen et al. (2019) demonstrated that various miRNAs can be modulated during low salt stress when certain miRNAs were induced in response to different concentrations of salts. For example, let-7c was downregulated in the three low salinity treatments, of 10, 15, and 20 ppt; however, miR-276b-3p was upregulated at 15 and 20 ppt (Chen et al., 2019). Let-7c regulates the proliferation and osteodifferentiation of adipose-derived mesenchymal stem cells under oxidative stress (Zhou et al., 2019), and it mediates the proliferation and migration of heat-denatured dermal fibroblasts by targeting *HSP70* (Jiang et al., 2016). Let-7c-3p regulates autophagy under oxidative stress (Li et al., 2020). Moreover, Chen et al. (2019) suggested that several genes that play essential roles in the desaturation, cholesterol biosynthesis, fatty acid elongation (i.e., cytochrome b5 reductase), and catalyzation of the dismutation of reactive oxygen species (ROS) into  $H_2O_2$  or oxygen are modulated by miRNAs. These expression-mediated miRNAs enable aquatic organisms to adjust the permeability of and gas exchange in gill membranes, which, in turn, maintains the osmoregulation of the hemolymph. Furthermore, miR-2788b was the most abundant miRNA and the only potential regulatory miRNA of S-adenosylmethionine synthetase (SAM) in the gills of *Portunus trituberculatus* under low salinity (Lv et al., 2016). SAM catalyzes the formation of S-adenosylmethionine from methionine and ATP (Horikawa et al., 1990), and it has a key role in the plant response to salt stress (Espartero et al., 1994).

## 2.2. Temperature changes stress

Changes in room temperature are considered a stress factor, especially during both high and low temperatures. Heat stress can mediate the expression of certain immune-related miRNAs that enhance the environmental adaptation of oysters. Moreover, an immune challenge modulated the expression of immune-related miRNAs and ultimately modulated the oxidation-reduction (redox) reaction, phagocytosis, and apoptosis (Zhou et al., 2014). The expression of cgi-miR-1984 was significantly increased, while scaffold631\_909 was significantly downregulated in heat-stressed oyster hemocytes challenged with *Vibrio splendidus* compared with those in the bacteria group (Zhou et al., 2014). Interestingly, cgi-miR-1984 was involved in the response to stress that was induced by low salinity (Zhao et al., 2016). miR-1984 seems to be a mollusk-specific miRNA (Zhou et al., 2014), and miR-184 and miR-10 are abundant and highly expressed in mollusk hemocytes, suggesting their role as key mediators in maintaining the physiological function of hemocytes. Note that mir-10c was increased under heat stress in genetically improved farmed tilapia, *Oreochromis niloticus* (Bao et al., 2018).

## 2.3. Heavy metal stress

Heavy metals (such as mercury, cadmium, copper, arsenic, chromium, and lead) contamination is problematic in certain geographic areas (Liu et al., 2019; Merly et al., 2019; Safiur Rahman et al., 2019). Therefore, heavy metals should be regularly monitored in fresh and marine ecosystems. They can occur at detectable concentrations without affecting the health of aquatic organisms and act as a potential health hazard for marine life and seafood consumers (Merly et al., 2019). The high concentration of such metals disrupts the normal physiological process, causes tissue damage, inhibits growth and reproduction, and modulates early development in aquatic animals including bivalves (Cherkasov et al., 2006; Ghazy et al., 2017; Khan et al., 2018; Saidov and Kosevich, 2019). Mollusks exhibit a high concentration of heavy metals, followed by crustacean and fish (Liu et al., 2019). Oysters are the hyperaccumulators of zinc and copper, whereas scallop bivalves are the hyperaccumulator of cadmium (Wang and Lu, 2017). Marine bivalves are often used to monitor the coastal metal pollution (Mandich, 2018;

Wang and Lu, 2017).

The blood clam, *Tegillarca granosa*, was exposed to the toxic levels of cadmium to identify the regulatory function of miRNAs during heavy metals stress. Five miRNAs were significantly induced, and 11 were significantly repressed during cadmium stress in *T. granosa* hemocytes (Bao et al., 2014). These miRNAs were putatively linked to genes implicated in regulating the stress response induced by heavy metals. Among the differentially expressed miRNAs, Tgr-nmiR-21, Tgr-nmiR-8, and Tgr-miR-2a were significantly downregulated with the highest fold-changes by more than six-fold, and Tgr-miR-33-5p, Tgr-miR-10a-5p, and Tgr-miR-184b were significantly upregulated by more than five-fold (Bao et al., 2014). Of these miRNAs, miR-21 generally targets genes with a potential impact on melanomagenesis; moreover, its upregulation results in the evasion of apoptosis, genetic instability, and increased oxidative stress (Melnik, 2015). However, the overexpression of miR-21 provides partial protection from H<sub>2</sub>O<sub>2</sub>-induced ROS activity by interacting with the nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) (Wei et al., 2014). Moreover, miR-21 is possibly involved in the pathogenetic mechanisms underlying heavy metal exposure and albuminuria (Kong et al., 2012). The miR-21 expression was negatively correlated with total anti-oxidation competence, superoxide dismutase, and catalase concentrations in gastric cancer patients (Tu et al., 2014). In general, miR-21 and let-7 demonstrated crosstalk during stress, ultimately activated several pathways such as NF-κB and RAS signaling pathways (Saibiyasachi and Yong, 2012), which allow bivalves to cope with stress (Fig. 2). There are similar pathways that include other potential miRNAs; however, the knowledge of their function and pathways remains incomplete.

To address the function of these miRNAs, ionocytes are specialized

branchial epithelial cells that regulate the maintenance of osmotic homeostasis (Bizuyehu and Babiak, 2014). Similar to heavy metal stress, the miR-8 family regulates osmoregulation in zebrafish embryos. miR-8 enables the precise control of ion transport in ionocytes during the early developmental stages before gill formation (Flynt et al., 2009). Moreover, miR-33-5p promotes osteoblast differentiation (Wang et al., 2016), and miR-33 and miR-10a-5p are associated with stress factors (Hao et al., 2016; Jovasevic et al., 2015; Sun et al., 2018). miR-10a-5p and miR-184b were regulated to maintain normal physiological function during a bacteria challenge and heat stress in oyster hemocytes and vertebrates (Hao et al., 2016; Zhou et al., 2014). Thus, in bivalves, heavy metal stress can modulate osmoregulation.

Predicted miRNA target analyses have revealed a set of miRNAs that target genes involved in the stress response to heavy metals during cadmium stress in hemocytes (Bao et al., 2014). Of the regulated miRNAs, Tgr-nmiR-21 targets the cation diffusion facilitator proteins that belong to the family of cation efflux transporters, which possibly play a regulatory role in metal homeostasis and tolerance (Blaudez et al., 2003). Similarly, Tgr-nmiR-8, Tgr-miR-10, and Tgr-miR-67 target heavy metal-transporting proteins such as glutamine synthetase, metal-transporting ATPase, disintegrin, metalloproteinase, and GTPase-activating-like protein (Bao et al., 2014), and they were modulated during cadmium stress in plants (Williams et al., 2000). The primary responses in blood clam hemocytes are related to genes that regulate sulfur acquisition and assimilation (Bao et al., 2014); moreover, sulfur-containing metabolites are involved in heavy metal homeostasis and detoxification (Ernst et al., 2008). Similarly, the transsulfuration pathway is mediated by copper treatment in the hemocytes of the mussel, *Perna canaliculus*, which demonstrated decreases in methionine and cysteine (Nguyen et al., 2018). Mitogen-activated protein kinase

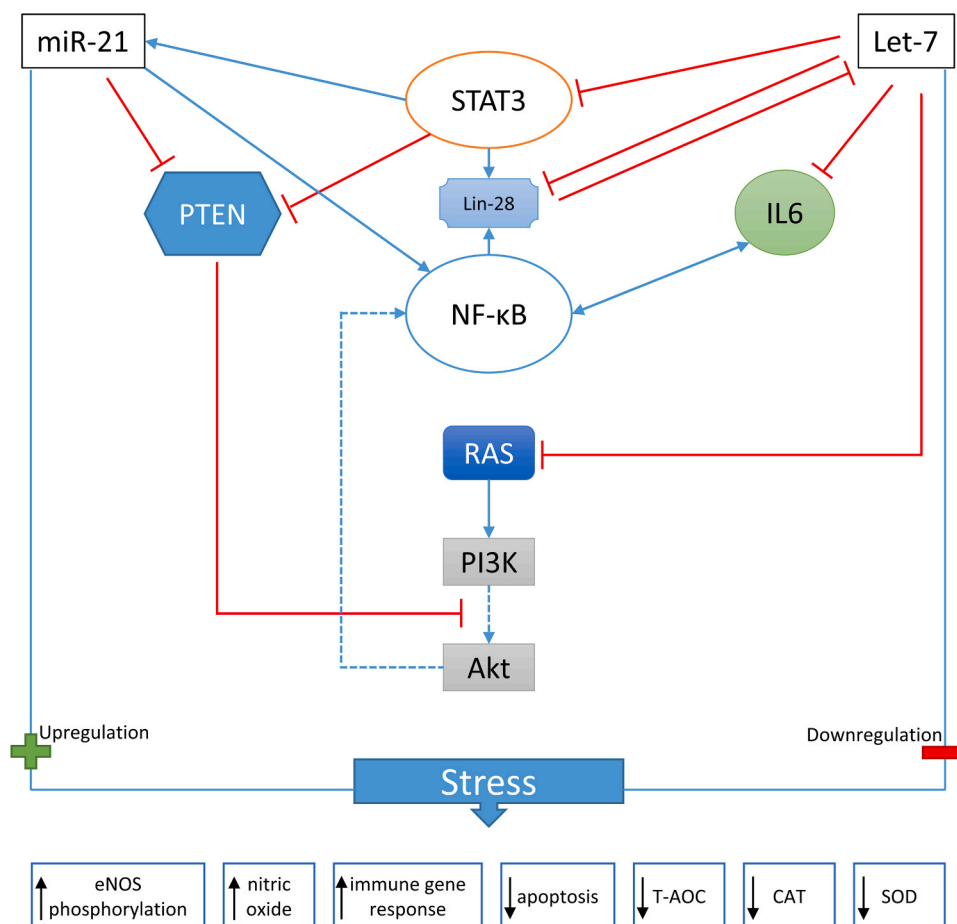


Fig. 2. Proposed schematic representation of miR-21 upregulation and let-7 downregulation in bivalve mollusks under stress. Some of these results were adopted from similar reports on mammals. Akt: Akt serine/threonine kinase; CAT: catalase; IL6: interleukin 6; NF-κB: nuclear factor kappa light-chain enhancer of activated B cells; PI3K: phosphatidylinositol 3'-kinase; PTEN: phosphatase and TENsin homolog or phosphatidylinositol-3,4,5-trisphosphate 3 phosphatase; RAS: rat sarcoma signaling; SOD: superoxide dismutase; Stat3: signal transducer and activator of transcription factor 3; T-AOC: anti-oxidation competence.

signaling was a target for miRNAs (Bao et al., 2014), which mediates resistance to acute thermal stress and heavy metal stress (Kefaloyianni et al., 2005; Kim et al., 2004). Thus, sulfur compound biosynthesis and metabolic processes and mitogen-activated protein kinase signaling are primarily involved in heavy metal detoxification, of cadmium in particular, in *T. granosa*.

### 3. Other stressors involve microRNA responses

#### 3.1. Oxidative stress

Oxidative stress is an imbalance between oxidants and antioxidants; when oxidants increase to levels that exceed the defense mechanisms, they damage proteins, nucleic acids, and cell membranes (Birben et al., 2012). The primary player in the oxidative stress is ROS; they include nonradical molecules such as peroxides and singlet oxygen, as well as free radicals such as superoxide anion and hydroxyl radical (Rezayian et al., 2019; Sharma et al., 2012). Imbalance in the ROS level can be generated from endogenous sources such as during cellular biochemical reactions and exogenous sources such as exposures to xenobiotics, pollutants, and heavy (Birben et al., 2012; Sharma et al., 2012).

As discussed in the previous sections, ROS have an important role in the stress response to osmotic stress, temperature, and heavy metals. ROS can prompt or repress miRNA expression, and thereby modulate downstream biological function by targeting specific genes (Lin, 2019). Increasing evidence has demonstrated an interaction between miRNAs and components of redox signaling (Gong et al., 2018; Lan et al., 2018; Zhou et al., 2014). miRNAs can regulate important components of cellular antioxidant machinery such as transcription factors (e.g., NF- $\kappa$ B, p53, c-Myc, and nuclear factor erythroid 2 related factor 2 (NRF2)) and kinases (e.g., IKK and Akt) (Lin, 2019). Oxidative stress modulates the immune function in aquatic organisms and this could affect their health status (Abo-Al-Ela, 2019; Abo-Al-Ela et al., 2017a, 2017b).

Copper induces oxidative stress in mussel hemocytes (Nguyen et al., 2018). Glutathione is an important antioxidant, which acts as a biomarker of oxidative stress (Abdel-Mageid et al., 2020a, 2020b; Rossi et al., 2006). Copper exposure causes a remarkable reduction of glutathione, which is accompanied by increase in ROS. Moreover, the transsulfuration pathway has been identified as a potential primary target pathway that involves the metabolism of cysteine and methionine, which is considered a cysteine source for glutathione (Nguyen et al., 2018). Furthermore, copper increases the number of apoptotic hemocytes in a dose-dependent manner, and this is primarily because of a high percentage of the late apoptotic sub-population. Copper-exposed hemocytes show a remarkable increase in depolarized dead cells. Moreover, copper stress results in an increase in alanine and a decrease in glutamic acid (Nguyen et al., 2018), which has been reported for various cell types driven into apoptosis (Halama et al., 2013; Rainaldi et al., 2008).

A similar investigation in the hemocytes of the white shrimp, *Litopenaeus vannamei*, during copper stress disclosed increased ROS production, thereby inducing oxidative stress and apoptosis in dose-dependent and time-dependent manners. In addition to the expression of copper-zinc superoxide dismutase and catalase, apoptosis-related genes, such as inhibitors of apoptosis protein and caspase-3, and metallothionein, which is a specific biomarker gene of heavy metal pollution, is markedly upregulated. These genes have been suggested to provide protection from copper stress and regulate apoptosis because of superfluous ROS generation (Guo et al., 2017). Several miRNAs were significantly modulated in the white shrimp under copper stress, and the differentially expressed miRNAs were reported to target genes involved in xenobiotic metabolism, immune defense, and apoptosis. The targeted genes involved immune-related genes; detoxification-related genes such as cytochrome p450; glutathione S-transferase and HSP60; and apoptotic-related proteins such as p53 and inhibitor of apoptosis protein, which supports certain miRNAs and their target genes as essential

regulators in intricate adaptive response networks (Guo et al., 2018). Of interest, miR-184 has been demonstrated to putatively target most genes (Guo et al., 2018), in addition to its role during osmotic stress (Zhao et al., 2016; Zhou et al., 2014). Furthermore, miR-183 is modulated during low salinity stress in *C. gigas* (as discussed in Section 2.1.2) (Zhao et al., 2016) and copper stress in white shrimp (Guo et al., 2018). miR-1175a-3p and miR-1175a-3p and novel-miR-46 were the highest-expressed miRNAs during low salinity stress, whereas miR-228 and novel-miR-8 were the lowest-expressed miRNAs during copper stress. These miRNAs are predicted to target immune-related genes. miR-1175-3p targets prophenoloxidase, which is an essential gene in nonself-recognition and function of the innate defense system in invertebrates (Cerenius and Söderhäll, 2004; Söderhäll and Cerenius, 1998); miR-46 targets Spz3, which is a signaling ligand in innate immune response (Boonrawd et al., 2017); miR-228 targets Relish, which is a gene that stimulates the expression of several anti-microbial peptides (Shi et al., 2015); and novel-miR-8 targets the Kazal-type serine proteinase inhibitor, which is an important gene in the immune response, regulating the Toll signal pathway in *Cyclina sinensis* hemocytes and is involved in responses to stress such as heat stress (Ren et al., 2015; Visetnan et al., 2009). Together, this indicates signal crosstalk and regulation between immune function and stress response, indicating that stress is a factor that could facilitate pathogen invasion, thus causing mortalities.

During hypoxia stress in the mussel, *Mytilus galloprovincialis*, hemocytes were sensitive to hypoxia and showed increased abilities to produce ROS (Andreyeva et al., 2019; Sui et al., 2016). Such prolonged hypoxia could reduce resistance to oxidative stress (Nogueira et al., 2017). Moreover, hypoxia increases glutathione levels in mussel gills (Nogueira et al., 2017). Furthermore, miRNAs exhibit a regulatory function during hypoxia in invertebrates. A large number of miRNAs, including let-7, miR-101, miR-143, and miR-210, are responsive to hypoxia (Wang et al., 2019). Furthermore, both miR-101 and miR-143 can target the key glycolytic enzyme hexokinase in mammals (Xu et al., 2017; Yao et al., 2014) and fish (Soñanez-Organis et al., 2011) under hypoxia. However, in marine invertebrates, hexokinase appears to be targeted by miR-24-3p, miR-252b-5p, and miR-3966 during hypoxic conditions (Wang et al., 2019). It seems that both miR-101 and miR-143 may have other roles during hypoxia in marine invertebrates. The miR-143 expression may be regulated by p53 (Otsuka and Ochiya, 2014) as a stress response gene induced during hypoxia (Felix-Portillo et al., 2016; Wang et al., 2019). The hypoxia-inducible factor 1 (HIF1) transcription factor directly binds to the hypoxia response element of the miR-210 promoter (Lin, 2019). Importantly, HIF-1 $\alpha$  expression is significantly increased in hemocytes and gills of the small abalone *Haliotis diversicolor* under hypoxia and heat stress (Cai et al., 2014).

#### 3.2. Mechanism underlying microRNA responses through the neural-endocrine-immune system

The nervous system and hemocytes are major players in the neuro-endocrine system with which various molecules, such as neurotransmitters, neuropeptides, hormones, and cytokines, interact to regulate immune function and responses to environmental stress (Liu et al., 2018). Norepinephrine is one of the key neurotransmitters in the neural-endocrine-immune system that is involved in maintaining homeostasis in organisms, including bivalves (Chen et al., 2015). Furthermore, it increases during stress and infection (Fig. 1 for more details on the role of norepinephrine in mediating the stress response in bivalve hemocytes (Abo-Al-Ela, 2020; Chen et al., 2017; Zhou et al., 2013, 2011). Norepinephrine interacts with members of the multi-xenobiotic resistance system to mediate responses to stress in bivalve hemocytes (Fig. 1) (Franzellitti and Fabbri, 2013). The neural-endocrine-immune system and adaptation mechanism of oysters are greatly controlled by miRNAs (Chen et al., 2015). Thus, it has been suggested that miRNAs mediate decreases in the late apoptosis and

necrosis rates in oyster hemocytes after neurotransmitter stimulation (Chen et al., 2015).

#### 4. Conclusion and perspectives

Because hemocytes are involved in antioxidant and stress responses and immune defense, this makes them a model for examining systemic responses to endogenous and environmental changes. Moreover, hemocytes are considered bioindicator tools for monitoring environmental contamination. The miRNA machinery system regulates most, if not all, cellular processes. *In silico* analyses to predict target genes of miRNAs have helped to provide an overview of the cellular microenvironment; however, additional studies are required to validate these results and address future issues related to the effect of stressors on both environmental and human health.

Such field studies prompted biologists to try to address how miRNA regulates the adaptation of bivalves under stress. However, a handful of questions require further research: What are the unique miRNAs that could act as biomarkers to specific pollutants and stressors? How do different tissues interact to adapt to particular stressors? Additional research would help to understand the specific mechanisms underlying the rapid response of bivalve, particularly hemocytes, to biotic and abiotic stress and how such cells organize systematic responses in the context of miRNA machinery, particularly since miRNA is most conserved between species. An improved understanding of these issues may be of some help to enhance aquatic health and the monitoring of aquatic environments.

#### Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

#### Author contributions

Abo-Al-Ela wrote and revised the manuscript and designed and prepared the figures. Faggio contributed to the manuscript revision and added valuable insights into the manuscript. Both authors read and approved the manuscript.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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