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Immunological assays of hemocytes in molluscan bivalves as biomarkers to evaluate stresses for aquaculture

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Abstract: As an important aquaculture section, molluscan aquaculture has traditionally accounted for about 60% of total marine aquaculture production worldwide. The molluscan aquaculture species are majorly bivalves, including oysters, clams, scallops, and mussels. Challenges in molluscan aquaculture include diseases, environmental stresses, coastal pollutions, and seed quality and quantity. This mini review summarized current research updates on immunological assays of hemocytes of molluscan bivalves against the biotic (*e.g.* bacteria, viruses, or protozoan parasites) and abiotic stresses (*e.g.* temperature shock, fluctuated salinity, or environmental toxins). As the frontline of immune system, hemocytes play a significant role against these stresses. The immunological assays of hemocytes could be used as effective biomarkers to evaluate the effects of biotic and abiotic stresses in aquaculture operation and breeding programs.

Key words: molluscan bivalves, immunological assays, hemocyte, stress, biomarker

Introduction

Molluscan aquaculture is a US$23.9 billion industry worldwide for seafood production (FAO, 2018). Sustainability of molluscan aquaculture industry faces many challenges including seed quality and quantity, environmental stresses and climate changes, diseases, natural and genetic resources, best practices of operations, and regulatory scrutiny (Dumbauld *et al.*, 2009). Accordingly, efforts have been made on overcome these challenges through genetic breeding for stock enhancement (Hulata, 2001), disease diagnosis and control by use of probiotics (Hoseinifar *et al.*, 2018), natural resources conservation management (Beck *et al.*, 2011), improvement of water quality through land use management, employment of best practices, and extensive education programs.

Immune system in molluscan bivalves was firstly reported in *Mytilus californianus* about hemolymph agglutinins (Tyler, 1946), which were observed later in butter clams, *Saxidomus giganteus* (Johnson, 1964), and eastern oysters, *Crassostrea virginica* (Tripp, 1966). Hemocytes in bivalves have been studied extensively since the 1970s and summarized in two review publications (Hine, 1999; Anisimova, 2013). In recent years, the molecular mechanisms and signal pathways of bivalve immune system have been becoming research focus to understand the immune protective strategies from various pathogens and environmental stresses (Song *et al.*, 2010).

This mini review summarized the immunological assays of hemocytes from aquaculture molluscan bivalves in response to biotic and abiotic factors, and it is expected that these hemocyte assays could be used as effective biomarkers for disease diagnosis, evaluation of environmental stresses, and breeding tools.

Immune System in Molluscan Bivalves

Immune system in vertebrates includes a series of collectively effective defenses against diseases and pathogen invasions. The first defense is the ***physical barriers***, such as the skin, which can prevent from colonization with other organisms and move

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inhaled materials using the mucociliary apparatus (ciliated epithelial cells and mucus-secreting cells). The second defense is ***innate immunity*** which is a primitive nonspecific immunity to against any pathogens that enter the body rather than targeted specific invaders. The third defense is a complex, specific, and long-lasting ***adaptive immunity*** which relies on the accumulated memory cells after exposure to pathogens.

Molluscan bivalves possess an open circulatory system. Bivalves pump hemolymph into the open body cavity (hemocoel), circulates in hemolymph vessels and sinuses as well as throughout soft tissues, thus the hemolymph can bath the internal organs and deliver nutrients and gases. It is believed that bivalves use their hemocytes and humoral proteins in circulatory system to provide internal defensive functions against various pathogen and environmental stresses (Bayne, 1983).

1. Physical barriers

The external shells in molluscan bivalves are the most important physical barrier to protect their soft tissues and organs and prevent from predators, parasites, harmful substances, and environmental changes. For example, the northern quahog (also called hard clam), *Mercenaria mercenaria*, can keep their shells closed without any movement and ejection for days at −1.0 to 1.9℃ seawater (Loosanoff, 1939); the Pacific oyster, *Crassostrea gigas*, can close their shells tightly at 4℃ air dry for 47.8 days (50% lethal time) (Kawabe *et al*., 2010); scallops can swim away by flapping their shells to escape predators or environmental stresses. Additionally, bivalves can use their gills and labial palps to select food particles and wrap un-selected particles with mucus for ejection (Shumway *et al.*, 1985; Ward and Shumway, 2004).

2. Innate immunity

The innate immune in molluscan bivalves is believed to achieve through humoral innate immunity, which involves in molecules (*e.g.* proteins) in the body humors to stop the growth of pathogens or clump them together, and cellular immunity of hemocytes, which involves in phagocytes to ingest and degrade pathogens (Bayne, 1983).

Humoral immunity can be achieved through antimicrobial peptides, which is an evolutionarily conserved component of innate immunity in all classes of life and represent the main form of invertebrate systemic immunity. In bivalves, the identified humoral factors together with their immune functions included atrial natriuretic peptides (ANPs) in hemolymph and heart of the eastern oyster, *Crassostrea virginica* (Vesely *et al*., 1993), catecholamines in the giant scallop, *Placopecten magellanicus* (Pani and Croll, 2000), lectins in the pearl oyster, *Pinctada maxima* (Flower *et al.*, 1985), and the giant clam, *Hippopus hippopus* (Puanglarp *et al.*, 1995), and hemagglutinins in the eastern oyster (Li and Flemming, 1967) and the northern quahog (Tripp, 1992). The profile of total protein, ions, and sugars composition in hemolymph have been documented in *Mya arenaria* and connected with their immune functions (Sunila and Dungan, 1992; Rees *et al.*, 1993).

Hemocyte immunity is the fundamental immune feature and usually achieved by recognition of foreign substances and subsequent ingestion (Pila *et al.*, 2016). The immunological assays of hemocyte in molluscan bivalves were stated as follows in next Section.

3. Adaptive immunity (also called acquired immunity)

Adaptive immunity is a more sophisticated system to recognize and destroy specific invaders based on cellular memory. The process of this defensive reaction normally uses specific antigens which are activated by exposure to pathogens. Therefore, it is antigen-specific functions through cell-mediated system. Adaptive immunity system uses an immunologic memory to learn about the pathogen and enhance the immune response, accordingly. This system is more effectively and specifically to the pathogens, but usually much slower to respond to threats and infections than the innate immunity.

Generally, adaptive immunity is considered to exist only in vertebrates. However, in recent years, adaptive immunity has been identified in invertebrates and even bacteria, such as the CRISPR/cas9 system which can recognize and destroy the invaded virus RNA sequence (Zhang *et*

al., 2012). For bivalves, the first evidence of antiviral immune priming was just reported in the Pacific oyster (Lafont *et al.*, 2020) against the herpes-like virus Ostreid herpesvirus 1, a major viral disease triggers the Pacific oyster mortality syndrome (Segarra *et al.*, 2010). The injection of various nucleic acids showed the capability to trigger oysters to protect them against a subsequent viral infection. Additionally, specific genes in adaptive immunity pathway in abalones were found to be up/down regulated when exposure to thermal shock and/or hypoxia (He *et al.*, 2017; Zhang *et al.*, 2019) and in the Pacific oyster when exposure to environmental stresses (Guo *et al.*, 2015).

Immunological Assays of Hemocytes in Molluscan Bivalves

Hemocytes in bivalves have been reported to participate a variety of physiological and immune functions, such as wound repair, shell formation and healing, nutrient transport and digestion, excretion, and internal defense (Anderson and Good, 1976; Song *et al.*, 2010; Pila *et al.*, 2016). When exposure to invasive pathogens, hemocytes can encapsulate them and subsequently destroy them via enzyme activity and oxygen metabolite release defense the invading microbes (Song *et al.*, 2010). A comprehensive review has summarized the morphology and functionality of hemocytes in bivalves for further reading (Anisimova, 2013).

In this publication, a literature search was performed about bivalve hemocyte functions under different stresses (**Table 1**). Briefly, the hemocyte assays in bivalves are as follows.

1. Hemocyte morphology and cell types

Based on the morphological characteristics such as cell sizes and cytoplasmic inclusions, the hemocytes in bivalves are classified into two types – granulocyte and hyalinocyte (agranulocyte). For some species, a third type of hemocytes with different characteristics was reported with different names. So far, two reviews have made comprehensive summaries on bivalve hemocyte cells types for further reading (Hine, 1999; Anisimova, 2013). The methodologies for hemocyte morphological observation include light microscopy, transmission electron microscopy, flow cytometry, and monoclonal antibody (Noël *et al.*, 1994).

(1) Granulocytes

Granulocytes were found to be the major hemocyte type in bivalves. Granulocytes were usually characterized with cytoplasmic granules and have a low nucleus: cytoplasm ratio. Depending on the granular features granulocytes can be sub-categorized as eosinophilic granulocytes which contain cytoplasmic large eosinophilic granules and stain with acid stains (such as eosin with pink color), basophilic granulocytes which contain small granules and stain with alkaline stains (such as methylene blue with blue color). In several studies, granulocytes were also divided and named as small and large granulocytes. The nuclei of granulocytes are usually uninucleate or binucleate with eccentric, spherical, or occasionally ovoid morphology and stains as dark blue with DNA staining by Giemsa.

(2) Agranulocytes (Hyalinocytes)

Agranulocytes were also named as hyalinocytes in many publications based on microscopic observations. As the name reflects, these cells are characterized with few or without visible cytoplasmic granules in cytoplasm and have relative larger nucleus. Based on the cell size, agranulocytes can be classified into large hyalinocytes (agranulocytes) and small hyalinocytes (agranulocytes, also called blast-like cells in several publications) with a central ovoid or spherical nucleus surrounded by a rim of scant cytoplasm lacking organelles (Bachère *et al.*, 1988).

(3) Other types

Besides the granulocyte and agranulocyte types, other hemocyte types were observed in bivalve species. For example, cells had the general appearance of granulocytes, low nucleus: cytoplasmic ratio and round nucleus, but had few or no granules. These cells were regarded as fibrocytes in the eastern oyster and the northern quahog (Foley and Cheng, 1972) and later were considered as degranulated granulocytes following phagocytosis (Mohandas and Cheng, 1985). Depending on cell characterizations, different names have been used to describe these hemocytes by different authors with no systematic rules (Hine, 1999).

Table 1. The immunological assays of hemocytes in molluscan bivalves under challenges of environmental and biotic stresses

SPECIES	STRESS*	IMMUNOLOGICAL ASSAYS OF HEMOCYTES**	REFERENCE
Crassostrea gigas	Spatial and temporal	Hemolymph microbiota status	(Lokmer *et al.*, 2016)
Crassostrea gigas	T and infection	Hemolymph microbiota status	(Lokmer and Wegner, 2015)
Crassostrea gigas	Seasonal changes	Persistence, seasonal dynamics, and pathogenic potential of *Vibrio*	(Wendling *et al.*, 2014)
Crassostrea gigas	Herpesvirus	Antiviral activity of hemolymph protein against herpesviruses	(Green *et al.*, 2014)
Crassostrea gigas	Hypoxia	Regulation of an isoform of AMP-activated protein kinase	(Guevelou *et al.*, 2013)
Crassostrea gigas	Bacteria	Antimicrobial peptides analysis in hemolymph	(Defer *et al.*, 2013)
Crassostrea gigas	Air dry, hypoxia	Changes of pO_2, pCO_2, pH, and osmolality in hemolymph	(Kawabe *et al.*, 2010)
Crassostrea gigas	Virus infection	Antiviral activity in hemolymph	(Olicard *et al.*, 2005)
Crassostrea gigas	Disturbances, salinity, and T	Catecholamine changes in the hemolymph	(Lacoste *et al.*, 2001)
Crassostrea gigas	T, TBT, and trace metals	The alteration of spontaneous hemocyte aggregation	(Auffret and Oubella, 1997)
Crassostrea gigas	Repeated bleeding	THC, phagocytosis, hemolymph pH, and pO_2	(Jones *et al.*, 1993)
Crassostrea gigas	*Vibrio anguillarum*	Agglutination activity after challenge to *Vibrio*	(Olafsen *et al.*, 1992)
Crassostrea gigas	Virus – T3-coliphage	Hemocyte neutralizing activity	(Bachere *et al.*, 1990)
Crassostrea madrasensis	*Vibrio alginolyticus*	THC, serum protein, lysozyme activity	(Ittoop *et al.*, 2010)
Crassostrea rhizophorae	Seasonal change	Carbonic anhydrase to reflect environmental contaminations	(dos Santos *et al.*, 2017)
Crassostrea rhizophorae	Virus infection	Evaluation of antiviral activity in hemolymph	(Carriel-Gomes *et al.*, 2006)
Crassostrea virginica	Cadmium	Apoptosis	(Sokolova *et al.*, 2004)
Crassostrea virginica	Heat shock to 28℃	Changes of hemocyte cell types and viability	(Hegaret *et al.*, 2003)
Crassostrea virginica	*Perkinsus marinus*	Function of hemocytes to kill *Perkinsus marinus*	(Volety and Fisher, 2000)
Crassostrea virginica	Fungicide	Phagocytosis, reduced pyridine nucleotides, and ROS	(Baier-Anderson and Anderson, 2000)
Crassostrea virginica	Salinity	Atrial natriuretic peptides (ANPs) in the circulation hemolymph	(Palmer *et al.*, 1994)
Crassostrea virginica	T, salinity, food, *Dermo*	Hemolymph lysozyme activity and protein profile	(Chu and Lapeyre, 1989)
Crassostrea virginica	TBT	Kinetic regulation of chloride-ion and osmotic-pressure in hemolymph	(Bokman and Laughlin, 1989)
Crassostrea virginica	Repeated bleeding	Hemolymph protein and parasitism infection of *Haplosporidium nelsoni*	(Ford, 1986b)
Crassostrea virginica	*Haplosporidium nelsoni*	Hemolymph proteins from resistant and susceptible oysters	(Ford, 1986a)
Crassostrea virginica	Starvation, salinity, T	Protein and carbohydrate levels in the hemolymph	(Fisher and Newell, 1986)
Crassostrea virginica	Cercariae	Encapsulation of cercariae by hemocytes	(Font, 1980)
Crassostrea virginica	Bacteria	Clearance of enteric bacteria from the hemolymph	(Hartland and Timoney, 1979)
Crassostrea virginica	*Bacillus megaterium*	Aminopeptidase activity in hemocytes and hemolymph	(Yoshino and Cheng, 1976)
Crassostrea virginica	*Minchinia nelsoni* (MSX)	Hemolymph enzyme activities during oyster-MSX interaction	(Douglass and Haskin, 1976)
Crassostrea virginica	*Micrococcus lysodeikticus*	Hemocyte lysozyme capability	(Mcdade and Tripp, 1967)
Ostrea edulis	*Bonamia ostreae*	Apoptosis of hemocytes	(Gervais *et al.*, 2018)
Ostrea edulis	Salinity, T	Expression of HSP/C70 or metallothionein genes in hemocytes	(Corporeau and Auffret, 2003)
Ostrea edulis; C. gigas	*Bonamia ostreae*	Enzymatic activities – a commercial kit for detection of 19 enzymes	(Xue and Renault, 2000)
Ostrea edulis	*Bonamia ostreae*	Hemolymph function against *Bonamia ostreae*	(Cochennec *et al.*, 1992)
Pinctada fucata martensii	Post-operative care	Hemolymph hemagglutination activity	(Sano *et al.*, 2017)
Pinctada fucata martensii	Diseased oyster fluids	Hemocyte viability and survival of infected pearl oysters	(Morizane *et al.*, 2002)

Pinctada fucata martensii	Disease individuals	Hemocyte morphological changes	(Maeno *et al.*, 2001)
Pteria hirundo	*Vibrio* sp.	Hemocyte morphological and functional changes	(Vieira *et al.*, 2017)
Argopecten ventricosus	*Vibrio alginolyticus*	Antibacterial activity in the hemolymph	(Luna-Gonzalez *et al.*, 2007)
Chlamys farreri	Starvation	THC, ROS, acid phosphatase in hemolymph	(Xu *et al.*, 2008)
Chlamys farreri	T, salinity, air dry	Catecholamines in hemolymph	(Chen *et al.*, 2008)
Euvola (Pecten) ziczac	Pesticide	Cholinesterase activities of hemocytes	(Owen *et al.*, 2002)
Mytilus californianus	Diatom (Domoic Acid)	Changes in hemolymph pH, pCO_2, and pO_2	(Jones *et al.*, 1995)
Mytilus californianus	Tissue implantation	Hemocyte cell type, phagocytosis, and implant rejection	(Bayne *et al.*, 1979)
Mytilus coruscus	TiO_2	THC, viability, phagocytosis, lysosome, MN; MMP, ROS	(Wang *et al.*, 2019)
Mytilus edulis	Carbon nanofibers	Gene expression of hemocytes	(Barrick *et al.*, 2019)
Mytilus edulis	Heavy metals	Transportation of heavy metals in the circulatory system	(Devoid *et al.*, 2007)
Mytilus edulis	Air dry, hypoxia	Hemolymph acid-base status – pCO_2, ammonia, pO_2, and pH	(Booth *et al.*, 1984)
Mytilus edulis	Air dry	Changes of pH and pO_2 in hemolymph	(Jokumsen and Fyhn, 1982)
Mytilus edulis	Freshwater	Changes of the electrolyte contents	(Khlebovich *et al.*, 1981)
Mytilus galloprovincialis	Crude oil and dispersant	ROS, viability, phagocytosis, MXR, ACI	(Katsumiti *et al.*, 2019)
Mytilus galloprovincialis	Zinc pyrithione	THC	(Katalay *et al.*, 2019)
Mytilus galloprovincialis	Hypoxia	Cell type, ROS, viability	(Andreyeva *et al.*, 2019)
Mytilus galloprovincialis	Bacteria challenge	Bacterial adherence and association with hemocytes	(Zampini *et al.*, 2003)
Mytilus galloprovincialis	Estrogens	Vitellogenin levels in hemolymph	(Riffeser and Hock, 2002)
Mytilus galloprovincialis	BaP, 4NQO	DNA damage of hemocytes	(Bihari *et al.*, 1990)
Perna viridis	TiO_2, hypoxia	Cell type and number, THC, viability	(Wang *et al.*, 2014)
Perna viridis	Acute salinity	Hemocyte osmolality change	(McFarland *et al.*, 2013)
Perna viridis	Bacteria	Identification of a potent serine protease inhibitor	(Khan *et al.*, 2008)
Anadara senilis	Fluctuating Salinity	Osmotic pressure and ionic concentrations of hemolymph	(Djangmah *et al.*, 1979)
Laternula elliptica	Heat (10℃ for 48 hr)	Expression of HSP70 in thermally stressed hemocytes	(Park *et al.*, 2007)
Macrocallista nimbosa	Salinity	Viability, ROS, lysosome activity, phagocytosis	(Jauzein *et al.*, 2013)
Mercenaria mercenaria	QPX disease, T	ROS, phagocytosis, lysozyme activity	(Perrigault *et al.*, 2011)
Meretrix meretrix	$CdCl_2$, BaP	Identification of a small HSP gene and expression	(Li *et al.*, 2013)
Mya arenaria	Heavy metals	Phagocytosis, cell number, and viability	(Brousseau *et al.*, 1999)
Mya arenaria	Spatial and temporal change	Prevalence of leukemia in hemolymph	(Craig *et al.*, 1989)
Mya arenaria	*Bacillus megaterium*	Lipase activity in hemolymph and hemocytes	(Cheng and Yoshino, 1976)
Ruditapes decussatus	Dinoflagellate algae	Genotoxicity in gills and hemolymph	(Florez-Barros *et al.*, 2011)
Ruditapes philippinarum	Heat and cold shock	Identification of stress-immune response genes in hemocytes	(Menike *et al.*, 2014)
Ruditapes philippinarum	*Vibrio*	Cell count, viability, lysozyme activity, assay of bacterial infection	(Allam *et al.*, 2000)
Ruditapes philippinarum	*Vibrio*	Total protein and leucine aminopeptidase in hemocytes	(Oubella *et al.*, 1994)
Tapes philippinarum	Spatial and temporal change	Hematocrit, phagocytosis, lysozyme activity	(Matozzo *et al.*, 2003)
Tapes philippinarum	TBT	Superoxide dismutase, lysozyme activity	(Matozzo *et al.*, 2002)
Tridacna gigas	Repeated sampling	Total protein, ions composition, glucose, glycerol	(Rees *et al.*, 1993)

* T: temperature; TBT: tributyltin; BaP: benzo[a]pyrene; 4NQO: 4-nitroquinoline-N-oxide; TiO_2: titanium dioxide.
**THC: total hemocyte count; ROS: reactive oxygen species in cells; MN: mitochondrial number; MMP: mitochondrial membrane potential; MXR: multi-xenobiotic resistance; ACI: actin cytoskeleton disruption.

2. Total hemocyte number and proportions of different types

The total hemocyte number and relative proportions of different types were the most direct measurement of hemocyte immune responses. Although variations in hemocyte number may exist among individuals, any significant changes would be more likely linked to the metabolic condition changes. Counting of different types of hemocyte numbers could be accomplished by microscopic observation, but this method is time consuming with the process of making slides, staining, and counting. Flow cytometer is a fast and accurate method to count single-cell suspension such as sperm (Yang *et al.*, 2016) and can distinguish different types of hemocytes based on side scatter (SSC, measurement of cell granularity) and forward scatter (FSC, measurement of cell size) (Ashton-Alcox and Ford, 1998). Therefore, flow cytometry is becoming the mostly used approach for measuring the changes of hemocyte cell types.

3. Hemocyte viability

Hemocyte viability is a measure of the proportion of alive cells to evaluate the overall hemocyte health. Viability assays can be assessed based on cellular metabolism, enzyme activity, or cell membrane integrity. The widely used approach was double fluorescence staining with membrane permeable nuclear dyes, such as SYBR, and the membrane impermeable dyes, such as propidium iodide (PI), and detected by use of fluorescence microscopy or flow cytometry (Allam *et al.*, 2002). Alternatively, because cell membrane damage can cause release of cytosolic contents into the extracellular space including the enzyme lactate dehydrogenase (LDH), measurement of the extracellular LDH has also been used as an effective assay for hemocyte viability (Chu *et al.*, 2002).

4. Hemocyte apoptosis and cell cycle

Hemocyte apoptosis is a fundamental biological process in immune system for defensive functions (Sokolova, 2009). Hemocyte proliferation in cell number due to cell division in a sample can be used as an indicator to evaluate the cell health status. Therefore, apoptosis and cell cycle have been used as important assays for hemocytes in bivalves, such as in the eastern oyster against cadmium exposure (Sokolova *et al.*, 2004) and in the flat oyster *Ostrea edulis* against parasite *Bonamia ostreae* (Gervais *et al.*, 2018). Apoptosis and cell cycle assays could be performed by flow cytometry or genomic sequences.

5. Phagocytosis

Phagocytosis is the most fundamental role for hemocytes in bivalves to defense invasive pathogens, such as bacteria (Canesi *et al.*, 2002), and involves in collaboration of humoral defense factors such as agglutinins. Hemocytes can recognize, bind, and phagocytize the microbes, and the encapsulated microbes would be eventually degraded by cellular enzymes and oxidization to decrease the number of microbes.

Phagocytosis of hemocytes on foreign substances was firstly observed in the Pacific oyster (Feng, 1965), and has been reported to accomplish majorly by granulocyte hemocytes (Pipe, 1990), especially the eosinophil granulocytes (Hine, 1999; Anisimova, 2013; Pila *et al.*, 2016). The commonly used method to evaluate phagocytosis is to incubate fluorescence labelled beads (*e.g.* The Bangs Laboratories, Inc., https://www.bangslabs.com/) or actual microbes at certain temperature for a period of time, and the quantification of hemocytes with phagocytic beads can be performed by use of direct microscopic examination, fluorometric evaluation, or flow cytometry.

For bivalves, phagocytosis has been studied in many species (**Table 1**). The phagocytosis process involves in humoral defense factors such as agglutinins and lysosomal enzymes, and the surface-bound factors play a significant role in the bacteria-hemocyte interactions leading to the phagocytosis. Phagocytosis in bivalves can be affected by the environmental temperatures and other seasonal factors, but underlying factors influencing phagocytosis are still not completely understood (see a comprehensive review in Canesi *et al.*, 2002).

6. Reactive oxygen species (ROS) production

ROS are natural byproducts of the normal metabolism of oxygen in cell signaling and homeostasis. When exposure to environmental

stress, ROS production within cells would increase dramatically because of the damage to cell structures. After phagocytosis, the encapsulated microbes could be degraded by the oxidization process and cause ROS changes.

ROS production can be measured by use of a nonfluorescent analogue 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Eruslanov and Kusmartsev, 2010). After diffusing into the cells, DCFH-DA is hydrolyzed into 2',7'-dichlorofluorescein (DCFH) which would be trapped within the cells. The intracellular DCFH can be oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) by ROS, and measurement of DCF fluorescence at 530 nm can be used to quantify the ROS production by use of a flow cytometer (Lambert *et al*., 2003) or proteomic approach (Sheehan and McDonagh, 2008).

In molluscan bivalves, the production of ROS has been reported in hemocytes of many bivalve species, including oysters, mussels, scallops, and clams against environmental and biotic stresses (Donaghy *et al.*, 2012).

7. Lysosome enzyme activity

Lysosomes are membrane-bound vesicles containing digestive enzymes, such as glycosidases, proteases, and sulfatases, which can digest engulfed foreign microbes. In addition, lysosomes can destroy targeted organelles through autolysis, and be responsible for digesting protein from cell surface presented via endocytosis. Therefore, lysosome enzyme activity is a parameter to evaluate the status of hemocytes after phagocytosis.

In molluscan bivalves, the role of lysosomes following phagocytosis has been studied widely (Cheng, 1983). Lysosome enzyme activities was demonstrated in hemocytes of the north quahog after exposure and phagocytosis of single-cell algae (Moore and Gelder, 1985) and other stresses (**Table 1**). The measurement of lysosomal enzyme activities was usually performed by incubating with specific substrates and quantification of enzymatic products through comparing with negative controls without substrate (Moore and Gelder, 1985). Alternatively, probes linked to the factor controlling lysosomal homeostasis was identified and used as an effective and efficient tool for measuring lysosomal activity in mammalian cells (Ishii *et al.*, 2019), and may be applied for bivalve hemocytes.

8. Molecular pathways for hemocyte immunity

In recent years, molecular signal pathways for hemocyte immunity have been investigated in aquaculture bivalves. The molecular mechanisms for hemocyte immune recognition, signal transduction, and effector synthesis have been reviewed in two recent publications together with humoral immunity (Song *et al.*, 2010; Zhang *et al*., 2019).

Application of Hemocyte Immunological Assays for Aquaculture

Environmental stresses, such as temperature, salinity, dissolved oxygen, pollutions, and red-tide algal toxins, are the challenges for molluscan aquaculture. To overcome these challenges, molluscan bivalves would close their shells as immediate responses and use their hemocyte immune system to respond (**Table 1**). However, with prolonged exposure to environmental stresses, molluscan bivalves could be subsequently susceptible to pathogens, increase disease outbreaks, and eventually suffer heavy mortality. Therefore, immunological responses of hemocytes in molluscan bivalves could show different levels and link to their considerable resilience to adverse environmental conditions. This suggests that, similar to the blood tests as diagnostic tool for health evaluation in human and livestock, hemocyte immunological assays in bivalves could be used as effective parameters to evaluate the impact of the environmental stresses, serve as measuring tools for genetic breeding, and provide diagnosis tools to guidance the operation management.

With the fast development of DNA sequencing technology, genomic tools such as immunological related genes, molecular pathways, and specific up- or down-regulation genes, have been investigated in responses to different environmental stresses. It is expected that combination of organism level, cellular, and molecular immunological assays could provide a full spectrum of immunological assays and serve as tools for improvement of molluscan aquaculture.

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Zhang G., Fang X., Guo X. *et al.*, 2012: The oyster genome reveals stress adaptation and complexity of shell formation. *Nature,* **490(7418)**, 49–54.

Zhang X., Shi J., Sun Y., Habib Y. J., Yang H., Zhang Z., and Wang Y., 2019: Integrative transcriptome analysis and discovery of genes involving in immune response of hypoxia/thermal challenges in the small abalone *Haliotis diversicolor*. *Fish Shellfish Immunol.,* **84**, 609–626.

Annotated Bibliography

(1) Anisimova A. A., 2013: Morphofunctional parameters of hemocytes in the assessment of the physiological status of Bivalves. *Russ. J. Mar. Biol.,* **39(6)**, 381–391.

This is a comprehensive review publication on shellfish hemocyte immunology. The basic morphology, cell type, cell function and dynamic changes of hemocytes in shellfish bivalves were summarized. The effects of temperature, season, salinity, annual cycle, food quality availability, toxin algae, bacteria and virus, pollutions, and heavy metals on hemocyte morphology, number and function were reviewed and documented.

(2) Donaghy L., Kim B. -K., Hong H. -K., Park H. -S., and Choi K. -S., 2009: Flow cytometry studies on the populations and immune parameters of the hemocytes of the Suminoe oyster, *Crassostrea ariakensis*. *Fish Shellfish Immunol.,* **27(2)**, 296–301.

This publication studied the immunological activities and morphology of hemocytes in the Suminoe oyster, *Crassostrea ariakensis* using flow cytometry and light microscopy. Three types of hemocyte types were identified, including hyalinocyte, granulocyte and blast-like cells. Cell count, survival, mortality, phagocytosis, and reactive oxygen species (ROS) production were evaluated using flow cytometer with different staining methods. It revealed that the granulocytes are most active in the cell phagocytosis and the hyalinocytes showed a certain level of the phagocytosis and oxidative activity, and the blast-like cells did not show any phagocytosis or oxidative activity.

(3) Vieira G. C., da Silva P. M., Barracco M. A., Hering A. F., de Albuquerque M. C. P., Coelho J. D. R., Schmidt E. C., Bouzon Z. L., Rosa R. D., and Perazzolo L. M., 2017: Morphological and functional characterization of the hemocytes from the pearl oyster *Pteria hirundo* and their immune responses

against *Vibrio* infections. *Fish Shellfish Immunol.*, **70**, 750–758.

This paper tested most hemocyte parameters including morphological characterization through light and electron microscopy and flow cytometry. Same as that in Sumino oysters, three types of the hemocytes were identified. Assays of phagocytosis and reactive oxygen species (ROS) production was performed by use of flow cytometer. Furthermore, hemocyte responses with exposure to a *Vibrio* pathogen was evaluated.

(4) Hégaret H., Wikfors G. H., and Soudant P., 2003: Flow cytometric analysis of haemocytes from eastern oysters, *Crassostrea virginica*, subjected to a sudden temperature elevation II. Haemocyte functions: aggregation, viability, phagocytosis, and respiratory burst. *J. Exp. Mar. Biol. Ecol.*, **293(2)**, 249–265.

This paper studied the effects of temperature increase on hemocyte functions of eastern oysters, including aggregation, viability, phagocytosis, and ROS production. This is one of a series of publications on oyster hemocyte functions and detailed protocols were documented for readers to understand the analysis procedure. The results indicated that temperature increase (from 20 to 28 degree) caused no significant change in hemocyte aggregation, decreased the phagocytosis of all hemocyte types, induced significant hemocyte mortality in all hemocyte types, and increased, although not significantly, the ROS production.

(5) Jauzein, C., Donaghy L., and Volety A. K., 2013: Flow cytometric characterization of hemocytes of the sunray venus clam *Macrocallista nimbosa* and influence of salinity variation. *Fish Shellfish Immunol.*, **35(3)**, 716–724.

Salinity is one the most important factor potentially affecting shellfish physiology, especially in the inshore area with river flows. This paper estimated the hemocyte types and cellular parameters (oxidative activity, lysosomal content, phagocytosis capacity) in sunray venus clams, a potential aquaculture species in Florida. After exposure to salinities of 18, 21, 25, 30, 35 and 38 ppt for 7 days, hemocyte samples were collected and analyzed the parameters along with estimation of physiological status of clams, including mortality, valve closure, and filtration activity. It was interestingly found that hemocytes of sunray venus clam appeared as a unique population, both in terms of morphology and intracellular parameters. Clams after transferring to 18 and 21 ppt resulted in valve closure, mortality, and decreased filtration activity. Low salinities highly impacted hemocyte functions as follows: increased cell and lysosomal compartment volumes, decreased phagocytosis capacity, and increased oxidative stress and mortality.