

## REVIEW

**The importance of ATP in the immune system of molluscs**

VE Coyne

*Department of Molecular and Cell Biology, University of Cape Town, Rondebosch, 7700, South Africa**Accepted March 8, 2011***Abstract**

Molluscs rely on an innate immune system for defence against infection. Invading organisms are phagocytosed by circulating hemocytes and neutralised by a combination of hydrolytic enzymes and the production of reactive oxygen species. Phagocytosis, the central feature of the molluscan innate immune system, is an energy-demanding process as a consequence of actin polymerisation which requires a constant supply of ATP. Adenylate kinase, shown to couple local ATP supply and actomyosin assembly in macrophage cell lines, may play a similar role in molluscan hemocytes. Hemocyte conformational changes and chemotaxis are regulated via protein kinase C which is phosphorylated in response to bacterial lipopolysaccharide. ATP utilisation in protein kinase cascades is thought to be quite significant. The vast majority of the ATP required to support cellular functions in animal cells is generated via the mitochondrial electron transport chain. Disruption of this process with antimycin A resulted in a 50 % decrease in ATP levels in *Haliotis midae* hemocytes which also exhibited reduced phagocytic activity. Phosphoarginine and arginine kinase are thought to play a role in invertebrates that is similar to phosphocreatine and creatine kinase in vertebrates, by supplying additional ATP during periods of high energy demand. The energetically-expensive processes associated with the molluscan innate immune system, such as chemotaxis, phagocytosis, formation and acidification of the phagolysosome, are severely compromised by abiotic stresses such as elevated water temperature and pollutants, reflecting the fine balance that must be maintained to ensure sufficient ATP is available for both general metabolism and the immune response.

**Key Words:** ATP; Mollusca; immune response; energy demand**Introduction**

The phylum Mollusca includes at least 100,000 living species, making it one of the most diverse groups of animals with members occurring in almost every ecosystem on earth. Not only does this group of animals colonise both terrestrial and aquatic habitats, they occur in extreme environments such as hot thermal vents and cold seeps. Many molluscan species are considered seafood delicacies, while others are an important source of bioactive compounds, used to manufacture jewellery and are an intermediate host of human pathogens. Since aquaculture of commercially important molluscan species has grown worldwide, and is likely to extend to additional species as a source of pharmaceuticals, it is becoming increasingly vital that the molluscan immune system

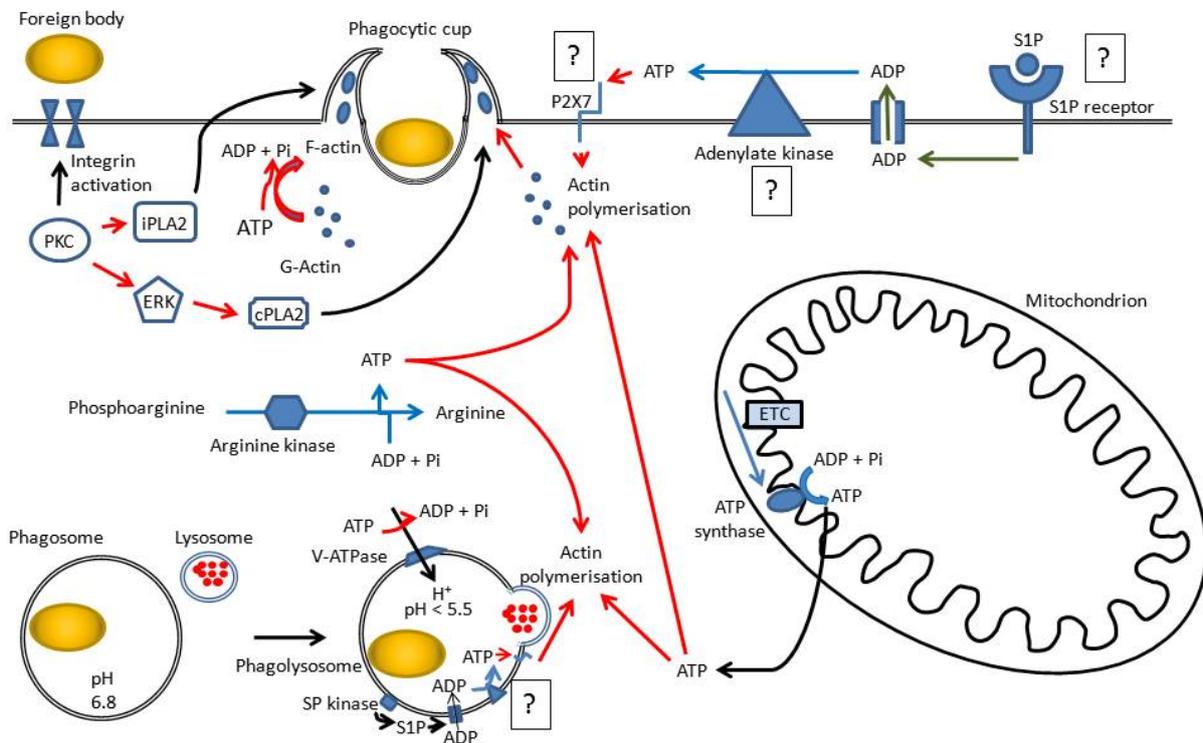
is characterised with regard to its structure, function and regulation. It is well documented that high density monoculture results in increased stress and enhanced susceptibility to infectious disease in farmed molluscs (Malham *et al.*, 2003; Harding, 2004; Reddy-Lopata *et al.*, 2006; Hooper *et al.*, 2007; Gestal *et al.*, 2008; Costa *et al.*, 2009). Additionally, it is becoming increasingly evident that climate change and environmental pollution significantly affect the health status of molluscs (Cherkasov *et al.*, 2007; Anestis *et al.*, 2008; Ivanina *et al.*, 2008).

The molluscan innate immune system, which includes both a cellular and humoral component, has been well described by Sokolova (2009). Circulating hemocytes are integral to the molluscan immune system, being responsible for infiltration, aggregation, encapsulation, cytotoxic reactions and phagocytosis of foreign particles (Hooper *et al.*, 2007; Gestal *et al.*, 2008). Ingested pathogens are neutralised by a hemocyte-mediated oxidative burst which generates reactive oxygen species (ROS) that extensively damage the macromolecular

*Corresponding author:*

Vernon Coyne  
Department of Molecular and Cell Biology  
University of Cape Town  
Rondebosch, 7700, South Africa  
E-mail: [vernon.coyne@uct.ac.za](mailto:vernon.coyne@uct.ac.za)

## Hemolymph



**Fig. 1** Diagrammatic summary of ATP involvement in phagocytosis of a foreign body by a generalised molluscan hemocyte. Energy-requiring processes are depicted by red arrows, while energy-generating processes are represented by blue arrows. The boxed question marks indicate processes that occur in macrophages and which may hypothetically occur in molluscan hemocytes as a consequence of the similarity between the cell types and conservation of function. PLA2, phospholipase A2; cPLA2, Ca<sup>2+</sup>-dependent phospholipase A2; iPLA2, cytosolic, Ca<sup>2+</sup>-independent phospholipase A2; ERK, extracellular signal-regulated kinase; PKC, protein kinase C (ERK and PKC regulate phagocytosis in macrophages by inducing arachidonic acid production through activation of iPLA2 and cPLA2 isoforms; García-García and Rosales, 2002); P2X7, P2X7 receptor (expressed by monocytes, including macrophages); S1P, sphingosine-1-phosphate (a phosphorylated lipid produced by sphingosine kinase (SP kinase) which is proposed to open a channel or transporter, allowing ADP to enter the phagosome lumen and be converted to ATP by adenylate kinase (▲). The ATP binds to the luminal domain of P2X7( ) causing the receptor to transmit a signal across the membrane to activate the actin-assembly machinery on the cytoplasmic side of the phagosome; model proposed by Kuehnel *et al.*, 2009a); ETC, electron transport chain.

components of the pathogenic organism (Goedken and De Guise, 2004; Tiscar and Mosca, 2004; Sokolova, 2009; Donaghy *et al.*, 2010). The oxidative burst is catalysed by membrane bound NADPH oxidase that converts molecular oxygen to superoxide anion which is dismutated to hydrogen peroxide (Buggé *et al.*, 2007). Hydrogen peroxide is a source of other toxic ROS such as hydroxyl radicals and singlet oxygen, and is a substrate for myeloperoxidase which it converts to hypochlorous acid (Murphy and DeCoursey, 2006; Terahara and Takahashi, 2008; Buggé *et al.*, 2007). In addition to the toxic effect of ROS, phagocytosed microorganisms are attacked by a variety of lysosomal enzymes, such as lysozyme,  $\beta$ -glucuronidase, acid and alkaline phosphatase,

lipase and aminopeptidase, following the formation of the phagolysosome (Gestal *et al.*, 2008; Sokolova, 2009).

It is not the aim of this review to describe the innate immune system of molluscs in detail, but rather to consider the importance of ATP as an essential component of the molluscan immune system (Fig. 1).

### Energy production in animal cells

Animal cells derive energy from mitochondria through oxidative phosphorylation, a process in which electrons are passed along a series of carrier molecules called the electron transport chain (ETC), generating the vast majority of the ATP required to

support cellular functions (Watabe and Nakaki, 2007). The electrons are initially generated from NADH and FADH<sub>2</sub> and ultimately transferred to molecular oxygen (Miyazaki *et al.*, 2003). The mitochondrial outer membrane is freely permeable to small molecules and ions, while the convoluted, invaginated inner membrane is rich in cardiolipin, is impermeable to most small molecules and ions, including H<sup>+</sup>, and includes the enzymes responsible for oxidative phosphorylation, the co-factor coenzyme Q (ubiquinone Q), the F<sub>0</sub>F<sub>1</sub>-ATP synthase and some carrier proteins (Sas *et al.*, 2007). The matrix, bordered by the inner membrane, contains the mitochondrial DNA, chaperones, peptidases and a variety of enzymes that function in different metabolic pathways, including the citric acid cycle, fatty acid oxidation and the urea cycle. The mitochondrial ETC consists of several enzyme complexes and cofactors (designated complexes I-IV, the last enzyme is often referred to as complex V); I: NADH ubiquinone reductase, II: succinate ubiquinone reductase, III: ubiquinol cytochrome c reductase, IV: cytochrome c oxidase (COX), V: F<sub>1</sub>F<sub>0</sub>-ATP synthase (Sas *et al.*, 2007). Energy generated by the passage of electrons down the ETC creates a proton gradient across the membrane that drives ATP synthase to produce ATP from ADP. The synthesized ATP is used for energy-requiring reactions in the matrix and exported to the cytoplasm by the adenine nucleotide translocator in exchange for cytosolic ADP (Miyazaki *et al.*, 2003).

#### ATP and the molluscan immune system

Immune cells require a constant supply of energy for basic housekeeping and specific immune functions (Buttgereit *et al.*, 2000). The latter includes migration, phagocytosis and cytotoxicity (Krauss *et al.*, 2001). Consequently, immune stimulation results in increased biochemical activity and thus a greater demand for ATP, while processes crucial for specific immune functions are rapidly impaired when immune cells are deprived of energy.

Phagocytosis, the core of the molluscan innate immune system, is an energy-requiring process (Wu *et al.*, 2009). Phagocytosis is initiated when a foreign target is recognised and bound by a hemocyte (Hughes *et al.*, 1991; Wu *et al.*, 2009), resulting in activation of actin-dependent cytoskeletal changes (Kuiper *et al.*, 2008). Actin polymerisation is considered to be primarily responsible for the formation of pseudopod protrusions around the target, as well as events that occur during early phagocytosis, including ruffle formation, membrane delivery, closure of the phagocytic cup and movement of the newly formed vesicles through the cellular cortex (Kuiper *et al.*, 2008). Stockinger *et al.* (2006), using a scintillation proximity assay to study lysosome-phagosome interactions *in vitro*, showed that lysosome phagosome fusion required ATP, actin polymerisation, calmodulin, other cytosolic factors and an absence of Ca<sup>2+</sup>. Indeed, a continuous supply of ATP is required for F-actin polymerisation from G-actin, and to maintain the activity of non-muscle myosin ATPases that assist in actin and

membrane recruitment, and provide motor activity around the phagocytic cup (García-García and Rosales, 2002; Kuiper *et al.*, 2008). In fact, Kuiper *et al.* (2008) propose that phagocytosis poses a formidable challenge to cellular energy homeostasis as a consequence of the significant requirement for ATP to power the actomyosin-based micromechanical events associated with this process.

#### Adenylate kinase

The molluscan innate immune system relies on recognition of conserved structures associated with microorganisms, termed pathogen-associated molecular patterns (PAMPs). Lipopolysaccharides (LPS), an essential component of the outer membrane of Gram-negative bacteria, is a PAMP that elicits a strong immune response in molluscs (Araya *et al.*, 2010; Costa *et al.*, 2009; Hannam *et al.*, 2010). Once hemocytes detect PAMPs, they move towards the attractant by a process called chemotaxis (Hooper *et al.*, 2007). Interleukins and related cytokines are small proteins called chemokines that regulate the effector phase of the immune response (Ottaviani *et al.*, 1995; Tiscar and Mosca, 2004; Hooper *et al.*, 2007). IL-1α-, IL-1β-, IL-2-, IL-6- and TNF-α-like molecules have been isolated from the hemocytes of two fresh water snails *Planorbarius corneus* and *Viviparus ater* (Ottaviani *et al.*, 1995). The study showed that IL-1α, IL-2 and TNF-α played a role in hemocyte motility, phagocytosis and the induction of nitric oxide synthase. Similarly, *Mytilus galloprovincialis* hemocytes were shown to contain IL-8, and recombinant human (rh)IL-8 induced conformational changes and chemotaxis in the mussel hemocytes (Ottaviani *et al.*, 2000). Changes in cell shape induced by rhIL-8 were generated via protein kinase A and C pathways, leading to reorganisation of actin microfilaments. Mouse RAW 264.7 and J774A.1 macrophage cell lines exposed to either serum or sphingosine-1-phosphate (S1P), the inducing component in serum, were found to rapidly accumulate extracellular ATP that coincided with actin assembly (Kuehnel *et al.*, 2009b). The ATP was synthesised by adenylate kinase from ADP released from the cells within 3 sec of exposure to S1P. The ATP pool increased within 5 sec to levels sufficient for activation of the ATP receptor P2X7R which links ATP-dependent signalling to the actin assembly machinery (Kuehnel *et al.*, 2009b). van Horssen *et al.* (2009), using embryonic fibroblasts from knockout mice lacking major ATP-ADP exchange enzymes, showed that adenylate kinase-1 plays a significant role in providing ATP for cytoskeletal functions. Thus, adenylate kinase is a coupling factor between local ATP supply and regulation of actomyosin assembly and other molecular motor behaviour, which is central to cell shape changes and cell motility (Dzeja and Terzic, 2009). Besides a study by Moraga and Tanguy (2000) which showed that adenylate kinase is a molecular biomarker of sensitivity or resistance to the pesticides atrazine and isoproturon in the Pacific oyster *Crassostrea gigas*, there have been no studies to date regarding adenylate kinase from

molluscan hemocytes. Since molluscan granulocytes strongly resemble vertebrate monocytes and macrophages both in structure and function (Sokolova, 2009), it is highly likely that adenylate kinase plays a similar role in the molluscan immune system as that elucidated in mouse macrophage cell lines and thus warrants further investigation.

### Protein kinase C

As mentioned earlier, conformational changes and chemotaxis were initiated by rhIL-8 via protein kinase A and C pathways in *M. galloprovincialis* hemocytes (Ottaviani *et al.*, 2000). Protein kinase C (PKC) has also been detected in hemocytes from the gastropod snail *Lymnaea stagnalis* and found to be phosphorylated (activated) in response to challenge with bacterial LPS (Walker and Plows, 2003). Besides PKC activation, LPS has been shown to activate both MAPK/ERK kinase (MEK) and extracellular-signal regulated kinase (ERK), resulting in phagocytosis of *Escherichia coli* by *L. stagnalis* hemocytes (Plows *et al.*, 2004). Thus, in accordance to the classical MAP kinase pathway, activated PKC phosphorylates Ras which phosphorylates Raf1, which in turn phosphorylates MEK which subsequently activates ERK (García-García and Rosales, 2002; Plows *et al.*, 2004; Lacchini *et al.*, 2006). In addition to phagocytosing invading pathogenic microorganisms, parasites too large to engulf are encapsulated by hemocytes which adhere to and spread over the invading organism and adjacent cells (Walker *et al.*, 2010). A model proposed by Walker *et al.* (2010) to explain the signalling pathway leading to encapsulation of a foreign body by *L. stagnalis* hemocytes, includes a requirement for phosphorylated PKC and focal adhesion kinase during hemocyte adhesion and spreading. Phosphorylated PKC is also required for activation and engagement of integrin which is integral to hemocyte adhesion and spreading. Because protein kinase cascades amplify signals by serially phosphorylating target kinases, it is likely that ATP utilization in phosphorylation reactions is quite significant (Krauss *et al.*, 2001). Phorbol myristate acetate (PMA), a synthetic analogue of diacylglycerol which can activate PKC (Costa *et al.*, 2009), has been shown to induce a rapid increase in oxygen consumption (oxidative burst) in neutrophils and lymphocytes (Krauss and Brand, 2000). Similarly, PMA induced oxidative activity has been reported in hemocytes from the common periwinkle *Littorina littorea* (Gorbushin and Iakovleva, 2007), the freshwater snail *Biomphalaria glabrata* (Yoshino and Humphries, 2008), the disk abalone *Haliotis discus discus* and the spiny top shell *Turbo cornutus* (Donaghy *et al.*, 2010). Although the exact cause of this phenomenon is unclear, Krauss *et al.* (2001) suggest that activated kinases such as PKC can control energy flow in the mitochondria of lymphocytes. In support of this hypothesis, PMA induction of oxidative activity in hemocytes from *H. discus discus* and *T. cornutus* is thought to originate mostly from the mitochondria since incubation with carbonyl cyanide-p-trifluoromethoxyphenylhydrazone, which uncouples

mitochondrial oxidative phosphorylation, resulted in strong inhibition of the oxidative activity (Donaghy *et al.*, 2010).

### Mitochondrial respiratory chain

A cDNA microarray investigation of gene expression in hemocytes from immune-stimulated *Haliotis midae* identified two ETC genes, namely cytochrome *b* and cytochrome *c* oxidase III, which were differentially expressed in comparison to unstimulated abalone hemocytes (Arendze-Bailey, unpublished). The role of these genes, and thus the electron transport system, in the abalone immune response was investigated by specifically inhibiting cytochrome *b* with antimycin A and measuring hemocyte immune parameters *in vivo* (van Rensburg and Coyne, 2009). Antimycin A inhibits succinate oxidase and NADH oxidase, as well as mitochondrial electron transport between cytochrome *b* and *c1* (Park *et al.*, 2007) by blocking electron flow in the Q-cycle of complex III (Raha *et al.*, 2000). The level of ATP in *H. midae* hemocytes treated with antimycin A decreased by more than 50% of the ATP levels present in control cells (van Rensburg and Coyne, 2009). Furthermore, the study showed that phagocytosis of FITC-labelled *Vibrio anguillarum* was significantly reduced in *H. midae* hemocytes exposed to antimycin A for 2 h as opposed to PBS, indicating the importance of the mitochondrial respiratory chain in the immune response of the abalone due to increased metabolic needs of the cell during immune-stimulation.

### Phosphoarginine and arginine kinase

Although glycolysis and oxidative phosphorylation are the main pathways for energy generation, cells and tissues have other mechanisms for energy production and storage. For example, phosphagen molecules, such as phosphocreatine (PCr), can act as a temporary buffer during high-demand periods (Cloutier and Wellstead, 2010). Since phagocytosis is such an energy intensive process (Kuiper *et al.*, 2008), a PCr buffer is thought to be crucial for coping with a sharp increase in energy demand since the conversion of one molecule of PCr to creatine (Cr) regenerates one molecule of ATP from ADP without contribution from other cellular subsystems (Cloutier and Wellstead, 2010). Indeed, PCr was found to decrease by 40 % in phagocytosing macrophages, indicating that between 1.5 to 2 times more ATP is consumed by macrophages engaged in phagocytosis (Loike *et al.*, 1979). Clearly, ATP generation via this reaction is limited by the amount of available PCr, which can be replenished if the energetic state (ATP/ADP) is favourable. The enzyme creatine kinase catalyses the conversion of PCr to Cr (Kuiper *et al.*, 2008; Cloutier and Wellstead, 2010). The importance of creatine kinase in maintaining PCr levels, and thus a reservoir of phosphate for ATP synthesis in macrophages, is supported by the observation that both actin and creatine kinase-B (CK-B) are simultaneously recruited to the sites of action during the early phases of particle ingestion (Kuiper *et al.*, 2008).

Creatine kinase, PCr and Cr occur predominantly in vertebrates, but have also been identified in a polychaete *Chaetopterus variopedatus*, some annelids, echinoderms, hemichordates, urochordates and sea urchin sperm (Wyss and Kaddurah-Daouk, 2000; Pineda and Ellington, 2001; Suzuki *et al.*, unpublished data). Invertebrates have been found to possess a variety of other phosphorylated guanidino compounds and their corresponding kinase, including phosphoarginine (arginine kinase, AK), phosphoglycocyanine (glycocyanine kinase), phosphotaurocyanine (taurocyanine kinase), phospholombricine (lombricine kinase) and phosphohypotaurocyanine (hypotaurocyanine kinase), which play a physiological role that is similar to PCr in vertebrates (Wyss and Kaddurah-Daouk, 2000; Uda *et al.*, 2006). AK is the most widely distributed phosphaen kinase among the invertebrates (Suzuki *et al.*, 2003; Uda *et al.*, 2006). AK genes have been sequenced from a variety of molluscs, including the gastropods *Conus novaehollandiae* (Safavi-Hemami *et al.*, 2010), *Littorina fabalis* (Kemppainen *et al.*, 2010), *Turbo cornutus* (Suzuki *et al.*, 1997), *Haliotis madaka* (Suzuki and Furukohri, 1994) and *Biomphalaria glabrata* (Fukunaga *et al.*, unpublished data), the bivalves *Ensis directus* (Compaan and Ellington, 2003), *Pholas orientalis* (Wong and Chew, 2009b), *Meretrix lyrata* (Wong and Chew, 2009a), *Calyptogena kaikoi* (Uda *et al.*, 2008) and *C. gigas* (Suzuki *et al.*, unpublished data), and the cephalopods *Octopus vulgaris* (Suzuki *et al.*, unpublished data) and *Nautilus pompilius* (Suzuki *et al.*, unpublished data). Clearly, investigation of the role of AK and phosphoarginine in generating sufficient ATP for driving phagocytosis in molluscan hemocytes would be important in furthering our understanding of this energetically expensive process. In support of this point, microarray analysis of gill tissue from *Crassostrea virginica* challenged with the protozoan parasite *Perkinsus marinus* showed increased expression of a putative AK gene in comparison to uninfected control oyster samples (Wang *et al.*, 2010).

### Vacuolar-type ATPases

Once the invading organism has been engulfed by hemocytes and a phagosome has formed, phagolysosome biogenesis is the next step in the phagocytic process where the phagosome fuses with a lysosome containing various hydrolases that degrade the molecular components of the phagocytosed organism (Steinberg *et al.*, 2007). Fusion between the phagosome and lysosome requires actin polymerisation and thus ATP (Stockinger *et al.*, 2006). The internal pH of the phagolysosome becomes acidic in order for the hydrolytic enzymes to function effectively. Acidification of the phagolysosome to a pH of less than 5.5 is accomplished by the action of vacuolar-type ATPases (V-ATPases) which use energy generated from ATP hydrolysis to move protons across the phagolysosome membrane (Garin *et al.* 2001; Steinberg *et al.* 2007; Clarke *et al.* 2010). V-ATPase is a multi-subunit enzyme that occurs in all

eukaryotes. However, there is only one published study of a molluscan V-ATPase to date. Huvet *et al.* (2004) used suppression subtractive hybridisation to investigate differential gene expression in resistant (R) and susceptible (S) *C. gigas* progeny to determine the genetic basis for the S oysters' increased summer mortality rate. One of the cDNA clones characterised from the subtracted library that was significantly up-regulated in R oysters compared to the S progeny possessed sequence similarity to a V-ATPase from *Caenorhabditis elegans*. Interestingly, *v-atpase* mRNA levels in mantle-gonad tissue sampled from R progeny challenged with the bacterial pathogen *Vibrio splendidus* were seven-fold lower than *v-atpase* mRNA levels in samples from unchallenged R oysters, possibly reflecting hemocyte migration from the location of the sampled tissue to the site of bacterial infection (Huvet *et al.*, 2004).

### Stress and ATP

The importance of ATP with regard to the molluscan immune system is reflected by the many studies conducted to elucidate the effect of abiotic conditions on the defence response of various commercially important marine shellfish. Gametogenesis in bivalves is induced by elevated water temperatures which coincide with increased summer mortalities of the Pacific oyster *C. gigas*. This is thought to be due to depression of the immune system as a consequence of depleted energy reserves. Delaporte *et al.* (2006) showed that the adenylate energy charge decreased significantly during gametogenesis in *C. gigas*, and that this coincided with inhibition of key immune parameters such as the phagocytic and adhesive capacity of the oyster hemocytes. Similarly, phagocytosis and hemolymph antimicrobial activity decreased in post-spawning *C. gigas* due to depleted energy reserves as a consequence of spawning which is induced by elevated water temperatures (Li *et al.*, 2010). The polycyclic aromatic hydrocarbon pollutant phenanthrene was shown to have an immunosuppressive effect on the immune system of the Great Scallop *Pecten maximus* by reducing hemocyte membrane stability, phagocytic and cytotoxic activity possibly due to disruption of cell metabolism and ATP production (Hannam *et al.*, 2010). Indeed, elevated water temperature has been shown to enhance the deleterious effect of the trace metal pollutant cadmium on mitochondrial enzymes of *C. virginica*, including the ETC, resulting in an energy deficiency (Ivanina *et al.*, 2008) which would be expected to compromise the animal's immune system.

### Concluding remarks

A great deal of progress has been made in characterising the innate immune system of molluscs over the past decade. Much of our understanding of the molluscan immune system at the molecular level has been extrapolated from information obtained from model invertebrates such as the fruit fly *Drosophila melanogaster* and evolutionary conservation of the innate immune

system in vertebrates. However, many gaps remain regarding our understanding of the role that ATP plays in the molluscan immune system. Our ability to address the importance of ATP in the immune system of molluscs will rely on a systems biology approach which will involve genomic, transcriptomic, proteomic and metabolomic characterisation of the molluscan immune system, which in turn, will require a holistic understanding of the physiology of these animals. Such an approach is essential given the centrality of ATP as the energy currency of the cell and the importance of maintaining a balanced energy budget to ensure survival of the animal in its habitat.

### Acknowledgements

This work was partially supported by funds obtained from the National Research Foundation (grant 71094) and a URC Block Grant obtained from the Faculty of Science, University of Cape Town.

### References

- Anestis A, Pörtner HO, Lazou A, Michaelidis B. Metabolic and molecular stress responses of sublittoral bearded horse mussel *Modiolus barbatus* to warming sea water: implications for vertical zonation. *J. Exp. Biol.* 211: 2889-2898, 2008.
- Araya MT, Markham F, Mateo DR, McKenna P, Johnson GR, Berthe FCJ, *et al.* Identification and expression of immune-related genes in hemocytes of soft-shell clams, *Mya arenaria*, challenged with *Vibrio splendidus*. *Fish Shellfish Immunol.* 29: 557-564, 2010.
- Buggé DM, Hégarret H, Wikfors GH, Allam B. Oxidative burst in hard clam (*Mercenaria mercenaria*) haemocytes. *Fish Shellfish Immunol.* 23: 188-196, 2007.
- Buttgereit F, Burmester GR, Brand MD. Bioenergetics of immune functions: fundamental and therapeutic aspects. *Immunol. Today* 21: 194-199, 2000.
- Cherkasov AA, Overton Jr. RA, Sokolov EP, Sokolova IM. Temperature-dependent effects of cadmium and purine nucleotides on mitochondrial aconitase from a marine ectotherm, *Crassostrea virginica*: a role of temperature in oxidative stress and allosteric enzyme regulation. *J. Exp. Biol.* 210: 46-55, 2007.
- Clarke M, Maddera L, Engel U, Gerisch G. Retrieval of the vacuolar H-ATPase from phagosomes revealed by live cell imaging. *PLoS ONE* 5: e8585, 2010.
- Cloutier M, Wellstead P. The control systems structures of energy metabolism. *J. R. Soc. Interface* 7: 651-665, 2010.
- Compaan D, Ellington W. Functional consequences of a gene duplication and fusion event in an arginine kinase. *J. Exp. Biol.* 206:1545-1556, 2003.
- Costa MM, Prado-Alvarez M, Gestal C, Li H, Roch P, Novoa B, *et al.* Figueras A. Functional and molecular immune response of Mediterranean mussel (*Mytilus galloprovincialis*) haemocytes against pathogen-associated molecular patterns and bacteria. *Fish Shellfish Immunol.* 26: 515-523, 2009.
- Delaporte M, Soudant P, Lambert C, Moal J, Pouvreau S, Samain J-F. Impact of food availability on energy storage and defense related hemocyte parameters of the Pacific oyster *Crassostrea gigas* during an experimental reproductive cycle. *Aquaculture* 254: 571-582, 2006.
- Donaghy L, Hong H-K, Lambert C, Park H-S, Shim WJ, Choi K-S. First characterisation of the populations and immune-related activities of hemocytes from two edible gastropod species, the disk abalone, *Haliotis discus discus* and the spiny top shell, *Turbo cornutus*. *Fish Shellfish Immunol.* 28: 87-97, 2010.
- Dzeja P, Terzic A. Adenylate kinase and AMP signaling networks: Metabolic monitoring, signal communication and body energy sensing. *Int. J. Mol. Sci.* 10: 1729-1772, 2009.
- García-García E, Rosales C. Signal transduction during Fc receptor-mediated phagocytosis. *J. Leukoc. Biol.* 72: 1092-108, 2002.
- Garin J, Diez R, Kieffer S, Dermine JF, Duclos S, Gagnon E, *et al.* The phagosome proteome: insight into phagosome functions. *J Cell Biol.* 152: 165-80, 2001.
- Gestal C, Roch P, Renault T, Pallavicini A, Paillard C, Novoa B, *et al.* Study of diseases and the immune system of bivalves using molecular biology and genomics. *Rev. Fish. Sci.* 16: 133-156, 2008.
- Goedken M, De Guise S. Flow cytometry as a tool to quantify oyster defence mechanisms. *Fish Shellfish Immunol.* 16: 539-552, 2004.
- Gorbushin AM, Iakovleva NV. Functional characterization of *Littorina littorea* (Gastropoda: Prosobranchia) blood cells. *J. Mar. Biol. Ass. UK* 87: 741-746, 2007.
- Hannam ML, Bamber SD, Galloway TS, Moody AJ, Jones MB. Functional immune response in *Pecten maximus*: combined effects of a pathogen-associated molecular pattern and PAH exposure. *Fish Shellfish Immunol.* 28: 249-252, 2010.
- Harding J. Evaluation of the neutral red assay as a stress response indicator in cultivated mussels (*Mytilus* spp.) in relation to post-harvest processing activities and storage conditions. *Aquaculture* 231: 315-326, 2004.
- Hooper C, Day R, Slocombe R, Handlinger J, Benkendorff K. Stress and immune responses in abalone: limitations in current knowledge and investigative methods based on other models. *Fish Shellfish Immunol.* 22: 363-379, 2007.
- Hughes TK, Smith EM, Barnett JA, Charles R, Stefano GB. LPS stimulated invertebrate hemocytes: a role for immunoreactive TNF and IL-1. *Dev. Comp. Immunol.* 15: 117-122, 1991.
- Huvet A, Herpin A, Dégremont L, Labreuche Y, Samain J-F, Cunningham C. The identification of genes from the oyster *Crassostrea gigas* that are differentially expressed in progeny exhibiting opposed susceptibility to summer mortality. *Gene* 343: 211-220, 2004.
- Ivanina AV, Cherkasov AS, Sokolova IM. Effects of cadmium on cellular protein and glutathione synthesis and expression of stress proteins in

- eastern oysters, *Crassostrea virginica* Gmelin. J. Exp. Biol. 211: 577-586, 2008.
- Ivanina AV, Habinck E, Sokolova IM. Differential sensitivity to cadmium of key mitochondrial enzymes in the eastern oyster, *Crassostrea virginica* Gmelin (Bivalvia: Ostreidae). Comp. Biochem. Physiol. 148C: 72-79, 2008.
- Kemppainen P, Lindskog T, Butlin R, Johannesson K. Intron sequences of arginine kinase in an intertidal snail suggest an ecotype-specific selective sweep and a gene duplication. Heredity DOI: 10.1038/hdy.2010.123, 2010.
- Krauss S, Brand MD. Quantitation of signal transduction. FASEB J. 14: 2581-2588, 2000.
- Krauss S, Brand MD, Buttgerit F. Signaling takes a breath - new quantitative perspectives on bioenergetics and signal transduction. Immunity 15: 497-502, 2001.
- Kuehnel MP, Rybin V, Anand PK, Anes E, Griffiths G. Lipids regulate P2X7-receptor-dependent actin assembly by phagosomes via ADP translocation and ATP synthesis in the phagosome lumen. J. Cell Sci. 122: 499-504, 2009a.
- Kuehnel MP, Reiss M, Anand PK, Treede I, Holzer D, Hoffmann E, et al. Sphingosine-1-phosphate receptors stimulate macrophage plasma-membrane actin assembly via ADP release, ATP synthesis and P2X7R activation. J. Cell Sci. 122: 505-512, 2009b.
- Kuiper JWP, Pluk H, Oerlemans F, van Leeuwen FN, de Lange F, Fransen J, et al. Creatine kinase-mediated ATP supply fuels actin-based events in phagocytosis. PLoS Biol. 6: e51, 2008.
- Lacchini AH, Davies AJ, Mackintosh D, Walker AJ. Beta-1,3-glucan modulates PKC signalling in *Lymnaea stagnalis* defence cells: a role for PKC in H<sub>2</sub>O<sub>2</sub> production and downstream ERK activation. J. Exp. Biol. 209: 4829-4840, 2006.
- Li Y, Qin JG, Li X, Benkendorff K. Assessment of metabolic and immune changes in postspawning Pacific oyster *Crassostrea gigas*: identification of a critical period of vulnerability after spawning. Aquaculture Res. 41: e155-e165, 2010.
- Loike JD, Kozler VF, Silverstein SC. Increased ATP and creatine phosphate turnover in phagocytosing mouse peritoneal macrophages. J. Biol. Chem. 254: 9558-9564, 1979.
- Malham SK, Lacoste A, Gélébart F, Cueff A, Poulet SA. Evidence for a direct link between stress and immunity in the mollusc *Haliothis tuberculata*. J. Exp. Zool. 295A: 136-144, 2003.
- Miyazaki T, Neff L, Tanaka S, Horne WC, Baron R. Regulation of cytochrome c oxidase activity by c-Src in osteoclasts. J. Cell Biol. 160: 709-718, 2003.
- Moraga D, Tanguy A. Genetic Indicators of Herbicide Stress in the Pacific Oyster *Crassostrea Gigas* Under Experimental Conditions. Environ. Toxicol. Chem. 19: 706-711, 2000.
- Murphy R, DeCoursey TE. Charge compensation during the phagocyte respiratory burst. Biochim. Biophys. Acta 1757: 996-1011, 2006.
- Ottaviani E, Franchini A, Cassanelli S, Genedani S. Cytokines and invertebrate immune responses. Biol. Cell 85: 87-91, 1995.
- Ottaviani E, Franchini A, Malagoli D, Genedani S. Immunomodulation by recombinant human interleukin-8 and its signal transduction pathways in invertebrate hemocytes. Cellular and Molecular Life Sci. 57: 506-513, 2000.
- Park W-H, Han Y-W, Kim S-W, Kim S-H, Cho K-W, Kim S-Z. Antimycin A induces apoptosis in As4.1 juxtaglomerular cells. Cancer Lett. 251: 68-77, 2007.
- Pineda AO, Ellington WR. Organization of the gene for an invertebrate mitochondrial creatine kinase: comparisons with genes of higher forms and correlation of exon boundaries with functional domains. Gene 265: 115-121, 2001.
- Plows LD, Cook RT, Davies AJ, Walker AJ. Activation of extracellular-signal regulated kinase is required for phagocytosis by *Lymnaea stagnalis* haemocytes. Biochim. Biophys. Acta 1692: 25-33, 2004.
- Raha S, McEachern GE, Myint AT, Robinson BH. Superoxides from mitochondrial complex III: the role of manganese superoxide dismutase. Free Radic. Biol. Med. 29: 170-180, 2000.
- Reddy-Lopata K, Auerswald L, Cook P. Ammonia toxicity and its effect on the growth of the South African abalone *Haliothis midae* Linnaeus. Aquaculture 261: 678-687, 2006.
- Safavi-Hemami H, Young ND, Williamson NA, Purcell AW. Proteomic interrogation of venom delivery in marine cone snails: novel insights into the role of the venom bulb. J. Proteome Res. 9: 5610-5619, 2010.
- Sas K, Robotka H, Toldi J, Vécsei L. Mitochondria, metabolic disturbances, oxidative stress and the kynurenine system, with focus on neurodegenerative disorders. J. Neurol. Sci. 257: 221-239, 2007.
- Sokolova I.M. Apoptosis in molluscan immune defense. Inv. Surv. J. 6: 49-58, 2009.
- Steinberg BE, Touret N, Vargas-Caballero M, Grinstein S. *In situ* measurement of the electrical potential across the phagosomal membrane using FRET and its contribution to the proton-motive force. Proc. Natl. Acad. Sci. USA 104: 9523-9528, 2007.
- Stockinger W, Zhang SC, Trivedi V, Jarzylo LA, Shieh EC, Lane WS, et al. Differential requirements for actin polymerization, calmodulin, and Ca<sup>2+</sup> define distinct stages of lysosome/phagosome targeting. Mol. Biol. Cell 17: 1697-1710, 2006.
- Suzuki T, Ban T, Furukohri T. Evolution of phosphagen kinase V. cDNA-derived amino acid sequences of two molluscan arginine kinases from the chiton *Liolophura japonica* and the turbanshell *Battillus cornutus*. Biochim. Biophys. Acta 1340: 1-6, 1997.
- Suzuki T, Furukohri T. Evolution of phosphagen kinase. Primary structure of glycoamine kinase and arginine kinase from invertebrates. J. Mol. Biol. 237: 353-357, 1994.
- Suzuki T, Chisa M, Uda K, Ishida K, Mizuta K, Sona S, et al. Evolution and divergence of the genes for cytoplasmic, mitochondrial, and flagellar

- creatine kinases. *J. Mol. Evol.* 59: 218-226, 2004.
- Suzuki T, Tomoyuki T, Uda K. Kinetic properties and structural characteristics of an unusual two-domain arginine kinase of the clam *Corbicula japonica*. *FEBS Lett.* 533: 95-98, 2003.
- Terahara K, Takahashi KG. Mechanisms and immunological roles of apoptosis in molluscs. *Curr. Pharm. Des.* 14: 131-137, 2008.
- Tiscar PG, Mosca F. Defense mechanisms in farmed marine molluscs. *Vet. Res. Commun.* 28: 57-62, 2004.
- Uda K, Fujimoto N, Akiyama Y, Mizuta K, Tanaka K, Ellington WR, *et al.* Evolution of the arginine kinase gene family. *Comp. Biochem. Physiol.* 1D: 209-218, 2006.
- Uda K, Yamamoto K, Iwasaki N, Iwai M, Fujikura K, Ellington WR, *et al.* Two-domain arginine kinase from the deep-sea clam *Calyptogena kaikoi* - evidence of two active domains. *Comp. Biochem. Physiol.* 151B: 176-182, 2008.
- van Horssen R, Janssen E, Peters W, van de Pasch L, Lindert MMT, van Dommelen MMT, *et al.* Modulation of cell motility by spatial repositioning of enzymatic ATP/ADP exchange capacity. *J. Biol. Chem.* 284: 1620-1627, 2009.
- van Rensburg MJ, Coyne VE. The role of electron transport in the defence response of the South African abalone, *Haliotis midae*. *Fish Shellfish Immunol.* 26: 171-176, 2009.
- Walker AJ, Lacchini AH, Sealey KL, Mackintosh D, Davies AJ. Spreading by snail (*Lymnaea stagnalis*) defence cells is regulated through integrated PKC, FAK and Src signalling. *Cell Tissue Res.* 341: 131-145, 2010.
- Walker AJ, Plows LD. Bacterial lipopolysaccharide modulates Protein Kinase C signalling in *Lymnaea stagnalis* haemocytes. *Biol. Cell* 95: 527-533, 2003.
- Wang S, Peatman E, Liu H, Bushek D, Ford SE, Kucuktas, H, *et al.* Microarray analysis of gene expression in eastern oyster (*Crassostrea virginica*) reveals a novel combination of antimicrobial and oxidative stress host responses after dermo (*Perkinsus marinus*) challenge. *Fish Shellfish Immunol.* 29: 921-929, 2010.
- Watabe M, Nakaki T. ATP depletion does not account for apoptosis induced by inhibition of mitochondrial electron transport chain in human dopaminergic cells. *Neuropharmacology* 52: 536-541, 2007.
- Wu T-T, Chen T-L, Chen R-M. Lipopolysaccharide triggers macrophage activation of inflammatory cytokine expression, chemotaxis, phagocytosis, and oxidative ability via a toll-like receptor 4-dependent pathway: validated by RNA interference. *Toxicol. Lett.* 191: 195-202, 2009.
- Wyss M, Kaddurah-Daouk R. Creatine and creatinine metabolism. *Physiol. Rev.* 80: 1107-1213, 2000.
- Yoshino TP, Humphries JE. Regulation of hydrogen peroxide release in circulating hemocytes of the planorbid snail *Biomphalaria glabrata*. *Dev. Comp. Immunol.* 32: 554-562, 2008.