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RESEARCH ARTICLE

Genesis of Hematopoietic Tissue and Its Relation with Hemocytes of *Litopenaeus Vannamei* (Boone, 1931) (Crustacea: Decapoda)

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ABSTRACT

The *Litopenaeus vannamei* is a prime aquaculture species and has a worth market value all over the world. In this study, we investigated the genesis and morphology of hematopoietic tissue (HPT) and types of hemocyte based on its morphology and cytochemical feature using histological and histochemical methods. The results revealed that HPT in *L. vannamei* is not visible histologically in mysis and early two post larvae (P_1 & P_2), and emerges in P_3 which locates in epigastric region. The HPT showed a continuous propagation of cells in successive post larval stages. Four types of cells can be identified in the HPT from adult specimen and mitotic activity is visible in the HPT. The hemocytes have been differentiated into five types and some of these types are co-related with the HPT cells. The cytochemical studies suggested that type I and II cells of hemocytes are PAS positive while scanty presence of prophenoloxidase was observed in type I cells. Similarly the type I cells are Sudan Black B positive and rest of the cells showed weak activity against lipid detective stain. This is the first effort towards genesis of HPT and its relation with circulating hemocytes in *L. vannamei.*

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INTRODUCTION

Until now it is believed that hematopoietic tissue (HPT) is the source of hemocyte production in crustaceans. The HPT surrounded by connective tissues and positioned on dorsal and dorsolateral sides of stomach, in Penaeid shrimps it is also reported to be present in maxillipeds (Zhang *et al*., 2006). Comparing with a rapid progress of research in the defensive role of hemocytes in crustaceans, the knowledge on HPT is limited. Although the hematopoietic tissue has been identified and described in some crustacean species, such as *Penaeus monodon* (Van de Braak *et al.*, 2002) and *Fenneropenaeus chinensis* (Zhang *et al*., 2006).

Hemocyte morphology is continuously being given importance and recently several studies have been conducted on invertebrate species (Salimi *et al*., 2009; Donaghy *et al*., 2010; Zhao *et al*., 2010; Di *et al*., 2011; Cavalcanti *et al*., 2012; Pandy *et al*., 2012). Hemocytes in shrimps play profound role in wound repair, defense mechanism against parasites, viruses and bacteria. The importance of hemocytes made investigators interested in their classification and it remained the subject of interest by centuries (Heng and Lei, 1998). Until now the classification of crustacean hemocytes is not uniform (Matozzo and Marin 2010b), because different techniques were used for hemocyte classification, such as that of cytochemistry, morphology, biological functions (Hose *et al.*, 1990; Gargioni and Barracco, 1998). Recently, monoclonal antibodies (mAbs) reacting with various proteins of hemoctyes have been obtained in *Penaeus monodon* (Van de Braak *et al*., 2000) which were produced to classify several types of *P. monodon* hemoctyes (Winotaphan *et al*., 2005).

Many types of crustacean hemocytes are different because of diverse criteria used in various techniques. Two types of hemocytes in *C. maenas* have been classified. In *Panulirus homarus*, Manjula *et al*. (1997) discussed four types of hemocytes (Pro-hyalinocytes, hyalinocytes, eosinophilic granulocytes and chromophilic granulocytes), however in blue crab, *Callinectes sapidus* three types of cells were illustrated (Clare and Lumb 1994), and eleven types of hemoctyes were described in American lobster, *H. americanus* based on morphological

features (Battison *et al*., 2003). In *F. chinensis*, Zhang *et al*. (2006) described three types of cells based on Wright-Giemsa staining and TEM observation (hyaline hemocytes, small granular hemocytes and large granular hemocytes). Kakoolaki *et al*. (2010) discussed three types of cells in *Fenneropenaeus indicus* similar as in *F. chinensis*. Moreover, Matozzo and Marin (2010b) classified hemocytes into three basic types by different assays in *Carcinus aestuarii*.

The white leg shrimp *Litopenaeus vannamei* is the most potential commercial aquaculture species all around the world. A number of experiments in multi dimensional aspects of this species have been carried out. Its hemocytes were classified into three basic types by using transmission electron microscope (Heng and Lei, 1998), but to the best of our knowledge no record is available on cytochemical studies of hemocytes and genesis of hematopoietic tissue of *L. vannamei* including the co-relation between hematopoietic tissue and circulating hemocytes.

The present investigation is the first attempt to reveal the genesis of the hematopoietic tissue (HPT) and co-relation between circulating hemocytes and HPT cells. Furthermore, the hemocytes were classified on the basis of Wright-Giemsa and Giemsa staining, and cytochemical features were determined by Sudan Black B, Periodic acid Schiff and Prophenoloxidase, respectively.

MATERIALS AND METHODS

Animal: Mysis and post larvae (P_1-P_3) of *L. vannamei* were obtained from a shrimp hatchery in Zhanjiang, China in 2009. The adult *L. vannamei*, 12.25±0.45cm in body length and 15.3±1.2g in body weight were purchased from a market of aquatic product in Qingdao, China and acclimatized in an aerated, recirculating seawater aquarium (17±1°C, salinity 15%) in the laboratory. Shrimps were fed with live polychaet and sea water was renewed twice in a day before the experiment.

Histology of HPT: For histological observations of mysis and post larvae, specimens of each stage were first fixed in Bouin's solution for 24 h, and then dehydrated directly in ascending series of ethanol 30 min each after washed three times with 70% ethanol, furthermore cleared in xylene two times 15 min each. The serial sections in transverse, longitudinal and horizontal for each specimen were obtained at 5-6 µm thickness using a rotary microtome. Slides were then stained by hematoxylin and eosin (H&E) dye, examined and captured images by a Nikon E80i microscope. For adult *L. vannamei*, cephalothorax was cut apart and dissected to obtain the dorsal HPT, and fixed in Bouin's solution for 24 h. Others are the same as mentioned above.

Cytochemical assays of hemocytes

Hemocyte harvesting: The haemolymph were collected using a syringe containing equal volume of anticogulant (2.5% glutaraldehyde in 0.1M phosphate buffer at pH 7.4) from the ventral sinus of each shrimp. The smears of hemocyte monolayer were prepared and fixed for 4 min at 4℃. The berief staining protocol is given below:

Giemsa's dye: Hemocytes after smears were stained for 10 min in a 10% Giemsa aqueous solution and washed with distilled water. Slides were observed and pictured by a Nikon E80i microscope. **Wright-Giems:** The smears were allowed to semi-dry and rinsed three times for 5 minutes each in 0.1M phosphate buffer (PBS, pH 7.2). Then several drop of Wright's stain were added on the smears with equal volume of 1/15 M PBS (pH 6.8) and replaced by Giemsa stain after 1-2 min The smears were stained in Giemsa solution for 15 min, rinsed in deionised water and observed under a Nikon E80i microscope. **Periodic acid Schiff:** The cells were given 15 min rinse thrice in PBS (pH7.4) and stained with Periodic acid Schiff (PAS). **Sudan Black B staining:** The fixed hemocytes were washed in PBS (pH 7.4) and treated for 3 min with 50% ethanol and immersed for 15 min in a saturated solutin of Sudan Black B in 70% ethanol. Slides were then immersed in 50% ethanol, rinsed with distilled water and observed. **Pro-phenoloxidase:** The hemocyte monolayer was washed three times for 15 min each with 1 M PBS (pH 7.4). The slides were incubated in 0.1% L-DOPA (dihydroxyphenylalanine) prepared in 0.1 M PBS (pH7.4) with 2% sodium chloride for 90 min at 30°C.

RESULTS

Genesis of hematopoietic tissue: The hematopoietic tissue (HPT) does not emerge in mysis and in the first two post larval (P_1 and P_2) stages (Fig. 1A-B). The first occurrence of HPT was noted in P_3 stage where it lies on the epigastric region, surrounding cardiac chamber of stomach (Fig. 1C). The cell propagation of HPT is continuous process in post larval stages and lobular structure was primitively distinguished in P_4. Since then, the HPT morphology in post larval stages only expands in thickness and length rather than any worth noting change (Fig. 1 E-G).

Structure of HPT in Adult: The lobular shape of HPT surrounded by spongy connective tissues (Fig. 2A) does not show remarkable difference with the post larval stages excluding the difference in size and cell structure of HPT. In adult, the HPT lengthens till antennal gland and cell type of HPT can be histologically identified by four types. The type I cells named A type were deeply stained. Their nuclei are bigger than that of other cells. The second type cells (B type cells) have comparatively light stain and the nuclei are on either side of the cell. The third type (C type cells) has almost round shape and the nuclei are similar as the hemocyte of B type. Cells of the last type (D type) are pear shaped in structure and having light stain (Fig. 2B). Moreover, the mitotic activities in the adult HPT was noted and are shown in the Fig. 2B.

Hemocyte morphology and cytochemical assay: The circulating hemocytes have different morphological features when stained with Wright-Giemsa and Giemsa method, and can be distinguished into five distinctive types in *L. vannamei*. In type I cells (Fig. 3 A-D), the nucleus occupies the largest part of the cell and cytoplasm can hardly be demonstrated. In type II cells, the nucleus is more or less round in shape and the cytoplasm unevenly covers the nucleus (Fig. 3 E-H) than the type I cells. The

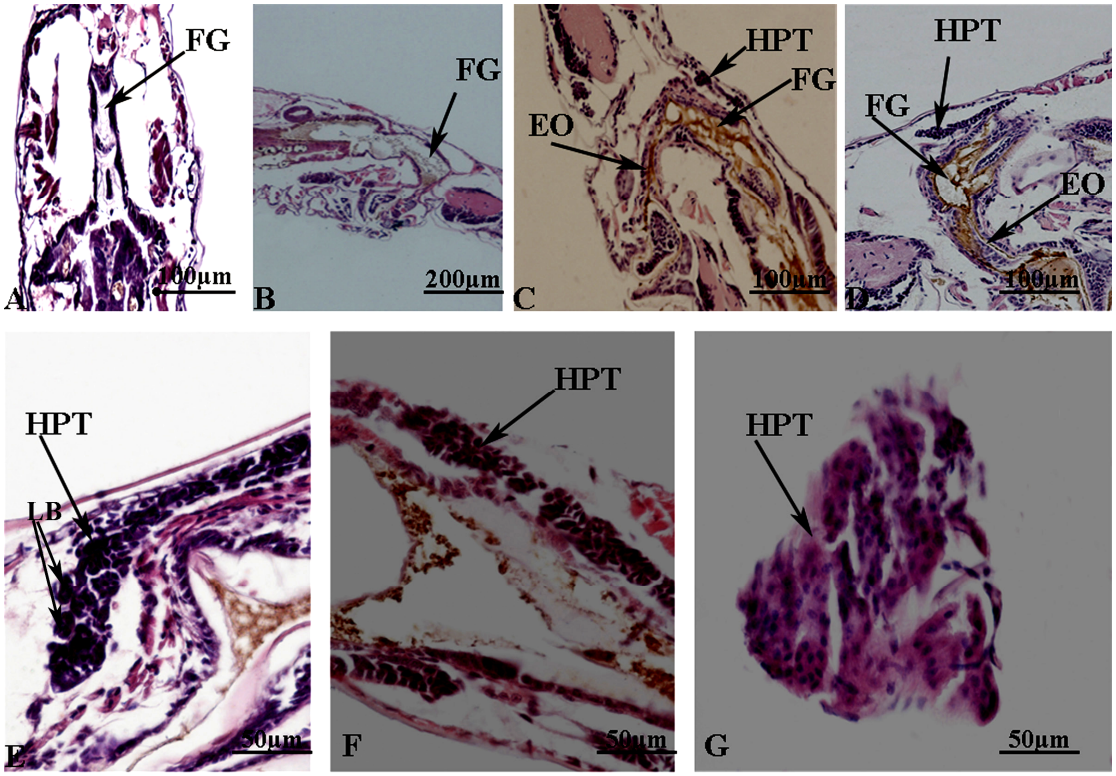

Fig. 1: Histological observations on genesis of HPT in *L. vannamei*. A-B. Horizontal section of mysis and longitudinal section of post larval (P_2) stage respectively, showing absence of HPT structure. C. Longitudinal section of P_3, showing the HPT. D Longitudinal section of P_4, showing HPT. E-F. Horizontal section of P_{10}, showing the position of the HPT. G. Transverse section of HPT in P_{10}, showing the thin sheet like structure of HPT. EO: Esophagus; FG: Foregut; HPT: Hematopoietic tissue; LB: Lobule.

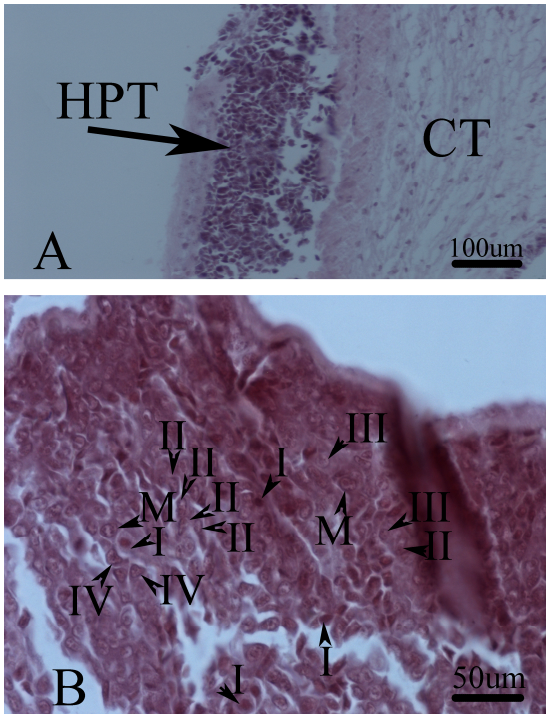

Fig. 2: Histological structure of HPT in adult *L. vannamei*. A. Horizontal section showing HPT cells and connective tissue in adult. B. The transverse section of HPT showing four types of cells which are sibling with circulating hemoctyes of *L. vannamei*. CT: Connective tissue; HPT: Hematopoietic tissue; M: Mitotic division; I, II, III and IV are types of cells, respectively.

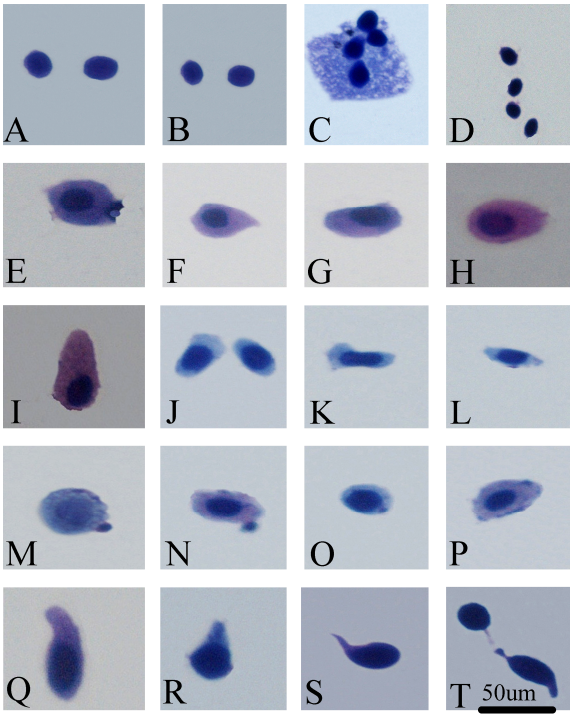

Fig. 3: Classification of hemocytes stained with Giemsa and Wright-Giemsa in adult *L. vannamei*. A-D. The type I cells. E-H. The type II cells. I-L. The type III cells. M-P. The type IV cells. Q-T. The type V cells. Scale bar = 50µm. The Fig. E, F, G, H, I, N, P, Q, S and T are stained with Wright Giemsa.

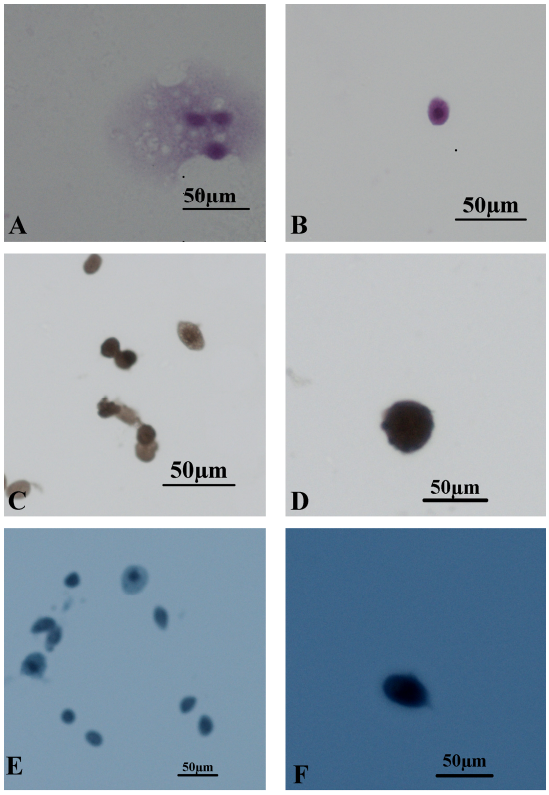

Fig. 4: Histochemical results of hemocytes in *L. vannamei*. A-B. Cells are PAS positive (Type I and II cells). C-D. Scanty presence of prophenoloxidase in type I cells (In general showing type I and IV cells). K-O. (Type I and Type II cells) Weakly positive Sudan black B staining. Scale bar = 50µm.

type III cells are elongated and flat in shape and the cytoplasm is almost unequal to both of the ends of nucleus (Fig. 3 I-L), However, only in a few cells the nucleus is on edge of the cytoplasm as shown in Fig. 3I, whereas in other cells of this category the nucleus is not round in shape and occupy the centre of cytoplasm. The type IV cells have rough cytoplasm containing a small granule along the margins of the cells and the nucleus is not same (Fig. 3 M-P). Type V cells have a protuberance and do not show any proper cytoplasmic structure. However the shape of the protuberance varies among cells, where the nucleus covers the whole cell excluding tiny projection (Fig. 3 Q-R).

Periodic acid Schiff (PAS) staining results reveal that all the hemoctyes are PAS positive however, the type I and II cells showed more activity (Fig. 4 A-B) rather than other cells, predicting the presence of more carbohydrates. The prophenoloxidase activity was mainly observed as scantly black pigment in the type I cells, and rest of cells showed prophenoloxidase negative activity (Fig. 4 C-D). All types of hemocytes are weakly Sudan Black B positive excluding type I, suggesting the presence of lipid in type I cells of *L. vannamei* (Fig. 4 E-F).

DISCUSSION

It is well known that the hematopoietic tissues and hemoctyes have pivotal role in several aspects of immunity, where hemocytes are considered important and HPT can produce hemocytes (Zhang *et al*, 2006). In *L. vannamei*, the hematopoietic tissue HPT emerged firstly in P_3 and propagation of HPT cells continued in successive stages, presuming time of HPT producing hemocytes should begin at P_3 stage. However, Wang *et al*. (2002) reported that hemocytes are visible firstly in nauplius of *Penaeus chinensis*, although type of cells was simple. This work was not enough on genesis of HPT and early initiator of hemocytes in decapod, therefore, we could not predict how to generate hemoctyes at earlier larval stages in *L. vannamei*.

The location of HPT, the epigastric region surrounding cardiac chamber of stomach in *L. vannamei* is same as in other species, such as *F. chinensis* (Zhang *et al*., 2006), and *P. monodon* (van de Braak *et al*., 2002). We have observed four types of cells in HPT of *L. vannamei*, some of type A cells in HPT have structural relationships with the type I hemocytes where major part of the cell has been occupied by nucleus. Type B cells were similar to type III hemocytes where cells are flat and the shape of nucleus is not similar. Type C cells were sibling with type IV hemocytes, however the position of nucleus was different. The possible reason may be the differences in period of their maturity. The D type cells were pear shaped that have somewhat morphological similarity to the type V hemocytes. The structure similarity between HPT cells and hemocytes reveals that hemocyte cells originate from hematopoietic tissue. The results are enhancing the previously reported statement that hematopoietic tissue produces the hematocytes. Conventionally three hemocyte types were described in crustaceans, such as hyaline, semigranular, and large granule hemocytes (Heng and Lei, 1998; Zhang *et al*., 2006; Kakoolaki *et al*., 2010; Matozzo and Marin, 2010 a&b). But Battison *et al*. (2003) described the eleven types of hemocytes using morphological criteria. Our results by means of light microscopy suggested that the hemocytes of *L. vannamei* have five basic types. Only one type of cells (type IV) has granules along the margins of the cytoplasm. Furthermore, some of the hemoctyes in *L. vannamei* has pointed extension from one end (type V cells) which are not been discussed in most of the available literature.

The cytochemical studies suggested that glycogen is present in all the hemoctyes while strongly positive in type I and II cells. The presence of carbohydrates in other species has also been reported, such as *Scrobicularia plana* (Wootton and Pipe, 2003) and *C. aestuarii* (Matozzo and Marin, 2010 a&b). The prophenoloxidase activity was observed only in a few cells of hemoctyes that has also been reported in *Crassostrea madrasensis* (Ittoop *et al*., 2006). The activity of Sudan Black B in *L. vannamei* has been detected in most of the cells but the staining showed least presence of lipids in hemocyte cells excluding type 1. However, the presence of lipids in other species of crustaceans has also been investigated, such as *H. americanus, P. interuptus and L. grandis* (Hose *et al*., 1990) showing positive hyalinocytes to Sudan Black B, but our results are in agreement with *C. aestuarii* (Matozzo and Marin, 2010b).

Conclusion: This study reveals that like other species of Penaeidae, the localization of HPT does not differ and the co- relation of cell types in HPT and hemocytes revealed

that HPT is the mother tissue for hemocyte production. The comparative classification of hemoctyes in several groups of decapod species need to be investigated all together in the same experimental conditions by means of different methods, the most important will be the immnocytochemical one.

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REFERENCES

Battison A, R Cawthorn and B Horney, 2003. Classification of *Homarus americanus* hemocytes and the use of differential hemocyte counts in lobsters infected with *Aerococcus viridians* Var. Homari (Goffkemia). J Invertebr Pathol, 84: 177-197.

Cavalcanti MGS, FC Filho, AMB Mendonca, GR Duarte, CCGS Barbos, CMMB De Castro, LC Alves and FA Brayner, 2012. Morphological characterization of hemocytes from *Biomphalaria labrata* and *Biomphalaria straminea*. Micron, 43: 285-291.

Clare AS and G Lumb, 1994. Identification of haemocytes and their role in clothing in the blue crab *Callinectes sapidus*. Mar Biol, 118: 601-610.

Di GL, ZX Zhang, CH Ke, JR Guo, M Xue, JB Ni and DX Wang, 2011. Morphological characterization of the haemocytes of the ivory snail, *Babylonia areolata* (Neogastropoda: Buccinidae). J Mar Biol Assoc UK, 91: 1489-1497.

Donaghy L, HK Hong, C Lambert, HS Park, WJ Shim and KS Choi, 2010. First characterization 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.

Gargioni R and MA Barracco, 1998. Hemocytes of the Palaemonids *Macrobrachium rosenbergii* and *M. acanthurus*, and of the Penaeid *Penaeus Paulensis*. J Morphol, 236: 209-221.

Heng L and W Lei, 1998. On the ultrastructure and classification of the hemocytes of Penaeid shrimp *Penaeus vannamei* (Crustacea, Decapod). Chinese J Oceanol Limnol, 16: 333-338.

Hose JE, GG Martin and AS Gerard, 1990. A decapods hemocyte classification scheme integrating morphology, cytochemistry and function. Biol Bull, 178: 33-45.

Ittoop G, KC George, NK Sanil, RM George, KS Sobhana and PC Nisha, 2006. Characterization of haemoctyes of the Indian edible oyster, *Crassostrea madrasensis*. Aquac Res, 37: 1636-1643.

Kakoolaki S, I Sharifpour, M Soltani, MH Ebrahimzadeh, S Mirzargar and M Rostami, 2010. Selected morpho-chemical features of hemocytes in farmed shrimp, *Fenneropenaeus indicus* in Iran. Iran J Fisher Sci, 9: 219-232.

Manjula PL, K Rahman and TJ Abraham, 1997. Hemocyte classification and differential counts in the Indian spiny lobster, *Panulirus homarus* (Linneaus). J Aquacul Trop, 12: 113-121.

Matozzo V and GM Marin, 2010b. First cytochemical study of haemoctyes from the crab *Carcinus aestuarii* (Crustacea, Decapoda). Eur J Histochem, 54: e9.

Matozzo V and MG Marin, 2010a. The role of hemocytes from the Crab *Carcinus aestuarii* (Crustacea, Decapoda) in immune responses: A first survey. Fish Shellfish Immunol, 28: 534-541.

Pandy JP, D Kumar, Sk Roy, PK Mishra, AK Sinha and BC Prasad, 2012. Phagocytosis of insect hemocytes as a new alternative model. Int J Zoo Res, 8: 1-11.

Salimi L, S Jamili, A Motalebi, P Eghtesadi-Araghi and M Rabbani, 2009. Morphological characterization and size of hemocytes in *Anodonta cygnea*. J Invet Pathol, 101: 81-85.

Van de Braak, CBT, MHA Botterblom, W Liu, N Taverne, WPW Van der Knaap and JHWM Rombout, 2002. The role of the hematopoietic tissue in hemocyte production and maturation of the black tiger shrimp (*Penaeus monodon*). Fish Shellfish Immunol, 12: 253-272.

Van de Braak CBT, N Taverne, MHA Botterblom, WPW Van der Knaap and JHWM Rombout, 2000. Characterisation of different morphological features of black tiger shrimp (*Penaeus monodon*) hemocytes using monoclonal antibodies. Fish Shellfish Immunol, 10: 515- 530.

Wang JF, ZF Zhang, DB Zhang, XY Liu and CY Liao, 2002. The genesis of the circulatory system in *Penaeus chinensis*. J Fisher CHINA (in Chinese), 26: 1-6.

Winotaphan P, P Sithigomgul, O Muenpol, S Longyant, S Rukpratanpom, P Chaivisuthangkura, W Sithigorngul, A Petsom and P Menasveta, 2005. Monoclonal antibodies specific to hemocytes of black tiger prawn *Penaeus monodon*. Fish Shellfish Immunol, 18: 189-198.

Wootton EC and RK Pipe, 2003. Structural and functional characterization of the blood cells of the bivalve molluscs, *Scrobicularia plana*. Fish Shellfish Immunol, 15: 249-262.

Zhang ZF, M Shao and KH Kang, 2006. Classification of hematopoietic cells and hemocytes in Chinese prawn *Fenneropenaeus chinensis*. Fish Shellfish Immunol, 21: 159-169.

Zhao LL, Y Xiao-zhen, W Xu-gan, W Jin-feng, W Xiao ya and C Yong-xu, 2010. Study on the morphology and classification of hemocytes in adult *Neomysis japonica* and the immune enzyme activities at different developmental stages in *Neomysis japonica*. J Fisher China (in Chinese) 34: 108-115.