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Hepatopancreas cell cultures from mud crab, *Scylla paramamosain*

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Abstract Hepatopancreas is an important digestive and endocrine organ in crustacean. However, there are few reports on cell cultures from crabs. Here, the cell cultures of hepatopancreas from *Scylla paramamosain* was studied in vitro. Both the primary cell culture and subculture were grown in Leibovitz' L-15 medium, M199 medium, or a specially designed medium for *S. paramamosain* (MSP). The results showed that hepatopancreas cells in vitro grew in compact clusters in 2–3 d. Four types of cells could be identified. They were embryo cells, fibrillar cells, resorptive cells, and blister-like cells, respectively. Some of these cells could be subcultured for three generations. The MSP supported the best survival of these hepatopancreas cells, while M199 medium was the least effective of these three media. Fetal bovine serum and crab muscle extracts as supplements stimulated growth, but the crab hemolymph inhibited cell growth. Taken together, MSP is an appropriate medium for hepatopancreas cell cultures from *S. paramamosain* and can support cultures through several passages.

Keywords Hepatopancreas · Cell culture · Subculture · *Scylla paramamosain*

Introduction

In crustacean, the hepatopancreas is a digestive organ with diverse functions, including secreting enzyme, storing nutrients, metabolism, detoxification, etc. Many researches have shown that hepatopancreas is another site of vitellogenin (Vg) synthesis besides the ovary (Yano and Chinzei 1987; Fainzilber et al. 1992). Hepatopancreas is even the dominant site of Vg synthesis in some reports on crustaceans (Pateraki and Stratakis 2000; Tsuisui et al. 2000; Yang et al. 2000; Tseng et al. 2001; Okuno et al. 2002; Jayasankar et al. 2002; Tsang et al. 2003). Vg, the precursor of vitellin, provides nutrients such as amino acid, fat, carbohydrate, vitamin, phosphorus, and sulfur during embryogenesis process (Chang and Shih 1993; Shimizu and Saikawa 1996). Furthermore, Vg also accelerates growth and differentiation of oocytes.

On the other hand, the hepatopancreas of crustaceans is also an important endocrine organ secreting methionine enkephalin, β-endorphin, insulin, vasoactive peptide, and somatotropin (Fingerman et al. 1993). Endocrine hormones including serotonin, glucagon, substance P, gastrin, and proctolin were also found in the hepatopancreas of crabs (Huang et al. 2005). In *Scylla paramamosain*, hepatopancreas is of considerable size and can be easily recognized and obtained. The earlier studies on crab hepatopancreas were focused on microstructure, endocrinology, and enzymology (Li and Li 1998; Huang et al. 2005; Novikov et al. 2007; Hotard and Zou 2008).

The methods of cell culture for shrimps have been established. Cell culture systems of *Penaeus semisulcatus* (Al-Mohanna and Nott 1987), *Penaeus monodon* (Chen et al. 1986; Hsu et al. 1995), *Penaeus stylirostris*, *Penaeus vannamei* (Nadala et al. 1993), etc. have been established. However, there are hardly any reports on culturing hepatopancreas cells from crabs (Ballard et al. 1993; Sashikumar and Desai 2008).

Some previous studies found that L-15 was suited for *Penaeus monodon*, *P. stylirostris*, and *P. vannamei* (Chen et al. 1986; Nadala et al. 1993; Hsu et al. 1995). Rosenthal

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and Diamant (1990) reported that M199 was appropriate for *P. semisulcatus*. Toull (1999) reported that L-15 and M199 are two effective media for crustaceans. Based on a series of pre-experimental designs, an MSP was designed for cell cultures from *S. paramamosain*. The MSP contained some organic additives such as lactalbumin hydrolysate (Itami et al. 1999), crab muscle extracts, and crab hemolymph (Luedaman and Lightner 1992; Owens and Smith 1999).

There are few reports on cell culture from crabs. *S. paramamosain* is a commercial species in China. The development of cell culture from *S. paramamosain* will provide a model for studying the cell biology of *S. paramamosain* under standardized conditions. In order to find an appropriate medium for *S. paramamosain*, three media (MSP, L-15, and M199) were investigated. A series of hepatopancreas cell cultures and subcultures from *S. paramamosain* was set up in this report.

Materials and Methods

Animals. The experimental animals, *S. paramamosain* in the developing stage, were obtained from the local seafood market (length 6.0±0.2 cm, width 9.8±0.5 cm, and weight 90±3 g). They were temporarily reared in a cement pool for 2 d before sacrificed.

Media and supplements. L-15 and M199 powder (Thermo Scientific HyClone, Waltham, MA) were dissolved in ultra pure water and diluted to 1 L. The osmolarity was adjusted with D-Hanks (NaCl, 19 g/L; $NaH_2PO_4.12H_2O$, 0.134 g/L; $NaHCO_3$, 0.35 g/L; KCl, 0.4 g/L; KH_2PO_4, 0.06 g/L; and HEPES, 2.38 g/L). pH was adjusted to 7.2 with 0.5 mol/L NaOH. Penicillin and streptomycin were routinely used in the media (100,000 U/L). The composition of the MSP is shown in Table 1.

The *S. paramamosain* muscle extracts (ME) were prepared by homogenizing 50 g crab muscle and 200 mL D-Hanks and were then minced to 1-mm^3 pieces. The muscle fragments were disrupted by ultrasonic treatment (amplitude 10% digital sonifier model 450, Branson) for 1 h and then heated at 60°C for 30 min. After centrifugation at 2,000 g for 1 h at 4°C, the supernatants were collected, sterilized by filtration through a 0.22-μm membrane, and then stored at 4°C.

In preparing *S. paramamosain* hemolymph (SH), one of the pleopods was cut, and the hemolymph was collected into a centrifuge tube with 3 mL anticoagulant (8.19 g/L NaCl; 18 g/L glucose; 5.76 g/L citric acid; 6.71 g/L sodium citrate; 2.92 g/L ethylenediaminetetraacetic acid). About 5–6 mL hemolymph could be collected from a developing crab. After centrifugation at 700 g for 30 min at 4°C, the supernatants were collected, sterilized by filtration through a 0.22-μm membrane, and stored at 4°C.

Table 1. The components of the medium for *Scylla paramamosain*

Components	Content
L-15	1.37 g/L
NaCl	22.00 g/L
$Na_2HPO_4.H_2O$	0.20 g/L
$NaHCO_3$	2.20 g/L
$MgSO_4.7H_2O$	3.00 g/L
$MgCl_2.6H_2O$	5.11 g/L
KCl	4.00 g/L
$CaCl_2$	0.68 g/L
HEPES	2.38 g/L
Glucose	5.00 g/L
Yeast extract	2.00 g/L
Lactalbumin hydrolysate	0.25 g/L
Sodium pyruvate	1.10 g/L
L-Glutamine	3.00 g/L
Glycine	3.00 g/L
Choline chloride	0.10 g/L
Penicillin	100,000 U/L
Streptomycin	100,000 μg/L
Phenol red	0.02 g/L

The proportions of various components were determined by a series of pre-experimental designs; pH was adjusted to 7.2 with 0.5 mol/L NaOH

Cell culture and subculture. The goal of this study is to initiate studies on cell cultures from *S. paramamosain*. The methods of cell cultures from shrimps were used as described by previous reports (Chen et al. 1986; Hsu et al. 1995). The animals were anesthetized for 20 min at 0°C and disinfected by 1:30 iodophor and sterile seawater for 15 min. The animals were dissected, and the hepatopancreas tissue was quickly taken out. The tissue was rinsed with D-Hanks (containing antibiotic) nine times and then minced into 1-mm^3 pieces, and the tissue fragments were rinsed again. Equivalent volume of medium was added. Tissue fragments were disrupted by aspiration with a fine-tipped pipette 50 times and then filtered through a sieve of 200 mesh. The filtrate was homogenous and was inoculated into a 25-cm^2 culture flask (Corning, Lowell, MA). After allowing the cells to attach for 30 min, 5 mL media was added. Cultures were incubated at 27°C and replaced one half medium every 2 d. One hundred microliters of filtered tissue homogenate and equal volume of 2× trypan blue were mixed for dying and smearing. The dissociative cells' initial survival rate was determined by trypan blue dye exclusion analysis.

In order to assay the viability of cells in different media, after 3 d, cells in the culture flask were treated with 0.25% pancreatin for 1–3 min. Cells dissociated were examined with a microscope. As attached cells gradually tended to

detach, pancreatin solution was abandoned, and media was added again. The suspended cells were centrifuged and then extracted DNA and RNA (using E.Z.N.A SQ DNA/RNA/Protein Kit, Omega, Irvine, CA) for testing the ratios of RNA/DNA using nanophotometer (NanoDrop ND-1000, Wilmington, DE).

In order to subculture, after 5 d, 0.25% pancreatin was used to resuspend cells. The suspension solutions were divided into two parts and were inoculated into new culture flasks with fresh medium. Subculture was repeated at 5-day intervals.

In order to identify the cultures, the genomic DNA from parental tissue and cultured cells were extracted. The genomic DNA was extracted using a phenol-chloroform-sodium dodecyl sulfate method. DNA was stored at 4°C until required. Three oligonucleotide primers, S391 (5′-ACG ATGAGCC-3′), UBC456 (5′-GCGGAGGTCC-3′), and UBC457 (5′-CGACGCCCTG-3′) (Klinbunga et al. 2000; Jin et al. 2004), which are specific for Portunidae, were selected. The amplification reaction was carried out in a 25-μL reaction volume containing 0.8 μM of each primer, 30 ng of sample DNA, 1× buffer [10 mM Tris HCl (pH 8.8), 50 mM KCl, 0.08% Nonidet P40], 2.5 mM of $MgCl_2$, 0.2 μM dNTPs, and 0.1 U *Taq* DNA polymerase. Random amplification of polymorphic DNA (RAPD)-polymerase chain reaction (PCR) was performed in a Hybaid thermocycler for 7 min at 94°C followed by 45 cycles consisting of denaturation at 94°C for 60 s, annealing at 37°C for 60 s, and extension at 72°C for 90 s. The final extension was carried out at the same temperature for 10 min. The resulting products were electrophoretically analyzed through 1.5% agarose gels, stained with ethidium bromide, and visualized using a UV transilluminator. DL 2000 DNA marker was used (TaKaRa, D501A, Dalian, China).

Results

Effect of different media on cell growth. The initial survival rate. The initial survival rates of hepatopancreas cells are shown in Table 2. The results of trypan blue dye exclusion test showed that with MSP, hepatopancreas cells had the highest initial survival rate reaching up to 84%. With L-15 medium, hepatopancreas cells had survival rates of 70% to 72%. The M199 medium showed an initial survival rate of about 70%, similar to the results of L-15. The MSP got a better initial survival rate than L-15 and M199 medium. Therefore, MSP is more appropriate for tissue dissociation.

Cells viability. Cells viability was tested via assaying ratios of RNA/DNA after 3 d cultivated. The ratios of RNA/DNA are shown in Fig. 1*a*. In MSP, RNA/DNA ratio was the highest reaching up to 7.01, while the M199 and L-15 medium showed ratios about 3.70 and 2.34, respectively. High RNA/DNA ratio can support robust protein synthesis activities and are therefore the indicator of the high cell viability. To sum up, MSP showed a better cell viability than L-15 and M199 medium. There were significant differences on cell viability between MSP, M199 medium, and L-15 medium ($P<0.05$).

Cells attachment efficiency and survival time. Another parallel group for recording the cells attachment efficiency and survival time was continually cultivated until the attachment efficiency was below 30%. The cells attachment efficiency and survival time are shown in Table 2. The best medium for hepatopancreas cell cultures was MSP. Hepatopancreas cells grew in compact clusters in 2–3 d, and exceeding 70% attachment efficiency was attained (Fig. 2). The attachment efficiency was even more than 90% when 20% mixture of fetal bovine serum (FBS) and ME were added. The survival time was up to 13 d. The attachment efficiency decreased significantly when using L-15 medium and M199 medium; cells attachment efficiencies were from 50% to 70%. However, using L-15 medium, survival time of cells was longer than that of M199 medium.

Effects of different supplements on cell growth. MSP was selected as a basic medium as described above; and different supplements, whose effects on cell growth are shown in Table 3, were added in different proportions. There were significant differences on cell growth using the following supplements. When the ratio of FBS:ME was equal to or larger than 1, the attachment efficiency was more than 90%. When FBS was used exclusively, cells

Table 2. Effect of different media on the initial survival rate, cell attachment efficiency, survival time, and numbers of passages

Media	The initial survival rate (%)	Cell attachment efficiency	Survival time (survival rate higher than 30%; days)	Number of generations
M199	67–72	++	4–5	1
L-15	70–72	++	5–7	1–2
MSP	70–84	+++	10–13	3

Media: penicillin and streptomycin were routinely used in the media; +: 0–50% attachment efficiency; ++: 50–70% attachment efficiency; +++: 70–90% attachment efficiency; ++++: >90% attachment efficiency

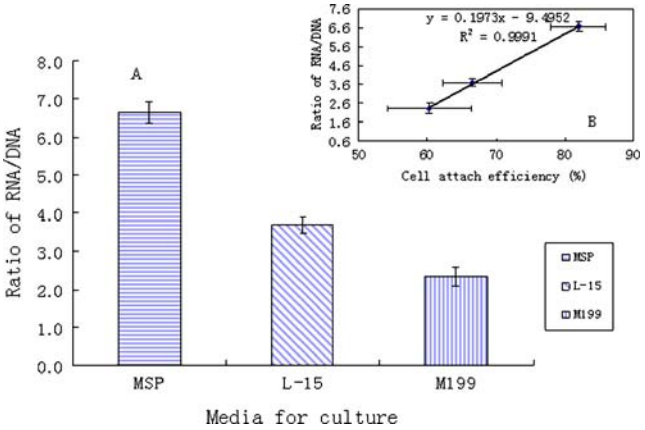

Figure 1. *a* RNA/DNA ratio in hepatopancreas of cells in vitro after 3 days culture. *b* The relationship between RNA/DNA and cell attachment efficiency.

attached in high quantity (70–90%). Using ME exclusively also achieved a similar result as FBS (70–90%). However, when SH was added, the attachment efficiency remarkably decreased. As the SH proportion increased, the attachment efficiency rapidly descended. These results showed that FBS was essential for *S. paramamosain* cell cultures, and ME of crab could improve cell attachment markedly. When mixing FBS and ME, a maximum effect was achieved. Using SH exclusively, only a few cells attached.

Culture and subculture of hepatopancreas cells. Morphology of hepatopancreas cells. There were four types of cells which could be identified. They were embryo cells (E-cells), fibrillar cells (F-cells), resorptive cells (R-cells), and blister-like cells (B-cells; Fig. 2). On the early stage of cell culture, some cells at mitotic division state could be observed. These cells had a round nucleus, with a diameter of 30–38 μm. They did not show lipid when stained with Sudan III and Oil red O. They were recognized as E-cells. F-cells were strongly basophile with a diameter of 40–50 μm, and the whole cell was deep blue after dyed with hematine. R-cells with a diameter of 30–50 μm and the cell were red after dyed with Sudan III. B-cells were the biggest cells with irregular shape among these four cells and with a diameter of 75–85 μm. The cell types and morphology are similar with that of other crustaceans described by previous reports from Al-Mohanna et al. (1985, 1987) and Li and Li (1998).

Subculture of hepatopancreas cells. Under different media, hepatopancreas cells in vitro performed different survival time. Some of these cells could be subcultured for three generations. In this study, it was observed that L-15 and M199 media were not appropriate for *S. paramamosain*. Most cells degenerated after the first generation. However, under MSP, the cell survival time was longer. A high attachment efficiency of the first generation and the second generation was obtained (Figs. 2 and 3*A*). The cells degenerated gradually until the third generation. The biggest B-cells and cells at mitotic division state were gradually disappearing. The residence cells had smaller radius (Fig. 3*B*). A large area of cells were deep blue after dyed with hematine, and some of the cells were red after dyed with Sudan III. The results were similar to that of hepatopancreas cell culture from *Scylla serrata* (Sashikumar and Desai 2008). It suggested that B-cells and E-cells were harder to survive in vitro. R-cells and F-cell were of longer survival time.

Results of genomic deoxyribonucleic acid analysis. The RAPD-PCR results are shown in Table 4 and Fig. 4. It generated five, four, and four RAPD bands from primers S391, UBC456, and UBC457, respectively. It showed that parental tissue and cultured cells from *S. paramamosain*

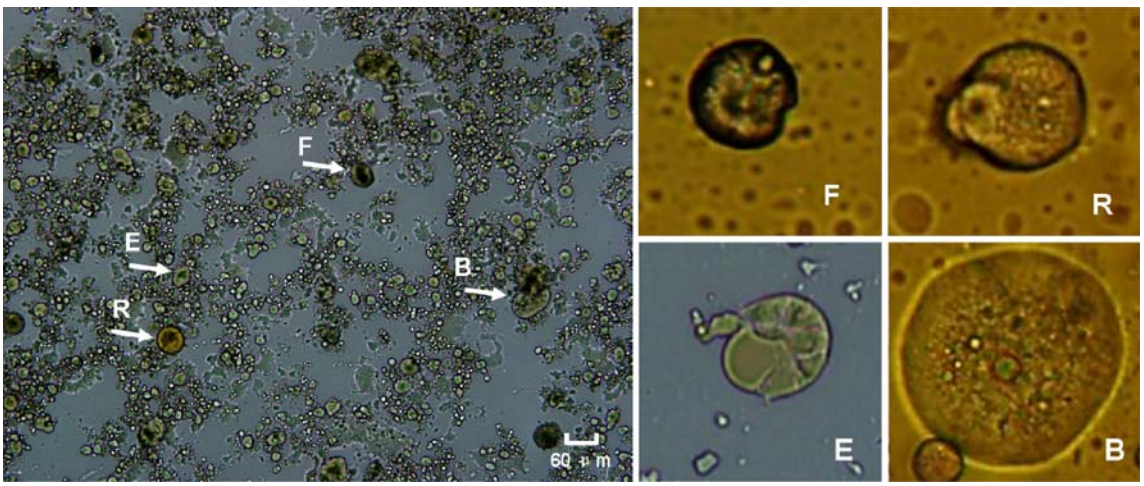

Figure 2. Hepatopancreas cells were cultured in medium for *Scylla paramamosain* for 3 d and examining by inverted microscope (Leica) and beion image system. The *arrows* points to four kinds of cell types: *E*, embryo cells; *F*, fibrillar cells; *R*, resorptive cells; *B*, blister-like cells.

Table 3. Effects of different supplements on cell attachment efficiency

Supplements	2:0	2:1	1:1	1:2
FBS + ME	+++	++++	++++	+++
FBS + SH	−	++	++	+
ME + SH	+++	++	+	+
SH + ME	+	−	−	−

Supplements: the supplements account for 20% total media; +: 0–50% attach efficiency; ++: 50–70% attach efficiency; +++: 70–90% attach efficiency; ++++: >90% attach efficiency

ME muscle extracts, *SH Scylla paramamosain* hemolymph

shared identical RAPD bands in sizes and numbers. Therefore, we came to a conclusion that the cultures mentioned above were derived from the parental tissue, rather than the protistan parasite that was growing in the cultures.

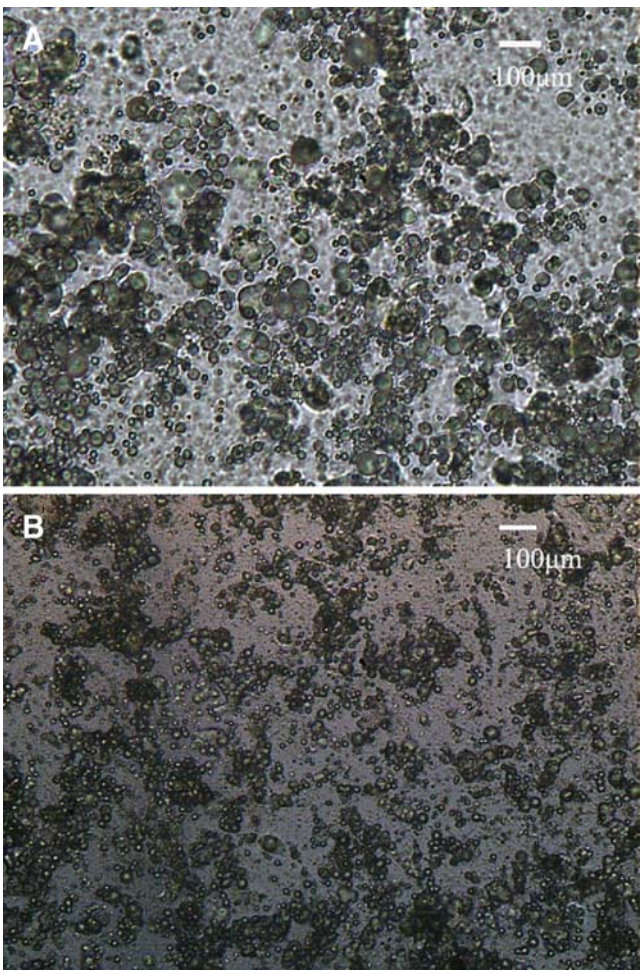

Figure 3. Hepatopancreas cells were subcultured two to three generations in medium for *Scylla paramamosain* and examined by inverted microscope (Leica) and beion image system. *A* The second generation. *B* The third generation.

Table 4. Sequences of oligonucleotide primers, sizes, and number of random amplification of polymorphic DNA bands using Primers S391, UBC 456, and UBC 457

Primer	Sequence	Size range and number of bands	
		Parental DNA	Cultures DNA
S391	ACGATGAGCC	100–2,000 (5)	100–2000 (5)
UBC456	GCGGAGGTCC	600–2,000 (4)	600–2000 (4)
UBC457	CGACGCCCTG	400–2,000 (4)	400–2000 (4)

Discussion

In this study, our results have shown that the MSP was significantly superior to L-15 medium and M199 medium for hepatopancreas cell cultures. As L-15 and M199 media were not created for crustacean cells, they may have components unsuitable for crustacean cell growth or may be missing ingredients essential for crustacean cells. In contrast, MSP was specially developed for *S. paramamosain* hepatopancreas cells, and *S. paramamosain* hepatopancreas cell culture could be established and subcultured successfully for several generations in MSP. Thus, in vitro culture of *S. paramamosain* hepatopancreas cells in MSP can facilitate the study of the physiological roles of hepatopancreas cells in *S. paramamosain* by well-established biochemistry and molecular biology approach.

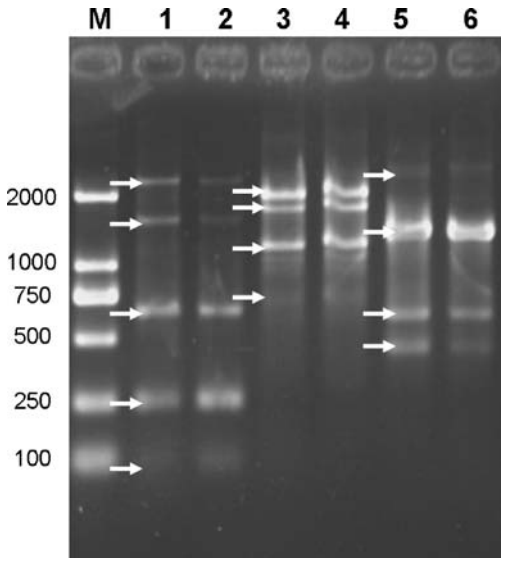

Figure 4. Random amplification of polymorphic DNA (RAPD) patterns of *Scylla paramamosain* generated from RAPD-polymerase chain reaction using primers S391 (*lanes 1* and *2*), UBC456 (*lanes 3* and *4*), UBC 457 (*lanes 5* and *6*), and 2,000-bp DNA marker was used. *Arrowheads* indicate species-diagnostic RAPD bands described in Table 4.

In our effort to search for the supplements supporting in vitro culture of *S. paramamosain* hepatopancreas cell, we found that FBS was essential for cell cultures of *S. paramamosain*, and that ME of crab could improve cell attachment markedly, while SH inhibited cell attachment efficiency noticeably. Our results are in disagreement with that reported for *S. serrata* by Sashikumar and Desai (2008), which showed that FBS was not an effective additive and would hamper cell viability. Since *S. serrata* and *S. paramamosain* were closely related species, it's interesting to notice that FBS has a promoting effect on *S. paramamosain* cell culture, while an inhibitory effect on *S. serrata* cell culture. There are several possible explanations for this discrepancy. Firstly, FBS is a natural supplement, and its ingredients were not ascertained. Many factors such as the place of production, manufacturer, and produce batch of FBS may impact the culture effect. Secondly, hepatopancreas cells from different development phases may have different nutrition requirements. *S. paramamosain* used in our experiments were in developing stage (weight 90±3 g), while *S. serrata* in the report of Sashikumar and Desai (2008) was juvenile (weight 13.83±0.60 g). Since the hepatopancreas is a detoxification organ, different developmental stage of hepatopancreas may possess varying detoxification capacity. Previous reports have shown that crustacean in late stages of development have higher detoxification capacity than that of early stages (Cheng et al. 2000). It is possible that some unknown inhibitors in FBS may not be tolerated by hepatopancreas cells from the juvenile crabs, while it would not affect culture of hepatopancreas cells from adult crabs. Similar results have already been reported for the effect of crustacean hemolymph on crustacean cell culture. Luedaman and Lightner (1992) have reported that lobster hemolymph could promote *P. stylirostris* and *P. vannamei* cell culture, while Tong and Miao (1996) reported that Squilla hemolymph inhibited *Penaeus chinensis* cell culture. FBS and hemolymph are both nutriments whose ingredients are not ascertained. It has been reported (Valk et al. 2004) that in addition to subjects that accelerate growth, inhibiting subjects such as endotoxin were also contained in FBS and hemolymph. Therefore, it is worth to further study and explore the ingredients of FBS and hemolymph in detail for a substitute whose components are clear.

Many studies have shown a correlation between the RNA/DNA ratio and growth of the organism (Bullow 1970; Haines 1973; Dortch et al. 1983; Steinhart and Eckmann 1992; Itami et al. 1999; Toull 1999; Caldarone et al. 2003; Rosa and Nunes 2004; Islam and Tanaka 2005; Tanaka et al. 2008). This study is the first to use the ratio of RNA/DNA to evaluate the cell viability in crab cell cultures. The results showed that cell viability with MSP was notably higher than that of M199 and L-15. The ratio of RNA/DNA had a positive relationship with attachment efficiency (Fig. 2*B*).

Our data provided basic information for hepatopancreas cell culture from *S. paramamosain*. The hepatopancreas is involved in digestion, metabolism, gonadogenesis, and endocrine system. Cell culture techniques could be applied to above-mentioned areas of research. Besides, cell culture techniques of crustacean are also needed for understanding the mechanism of pathogenicity and improving tools for diagnosis and cure of diseases caused by environmental degradation in recent years. Further work will be extended to culture cells derived from other tissues of *S. paramamosain*. The ultimate purpose is to establish a cell culture system of *S. paramamosain.*

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References

Al-Mohanna S. Y.; Nott J. A. R-cells and the digestive cycle in *Penaeus semisulcatus* (Crustacea: Decapoda). *Mar. Biol.* 95: 129–137; 1987.

Al-Mohanna S. Y.; Nott J. A.; Lane D. J. W. Mitotic E- and secretory F-cells in the hepatopancreas of the shrimp *Penaeus semisulcatus* (Crustacea: decapoda). *J. Mar. Biol. Assoc. U. K.* 65: 901–910; 1985.

Ballard T. A.; Roer R. D.; Dillaman R. M. Long-term culture of integumental explants from premoltblue crab, *Callinectes sapidus*. *J. Tissue Cult. Methods* 15: 11–14; 1993.

Bullow F. J. RNA/DNA ratios as indicators of recent growth rates of a fish. *J. Fish. Res. Board Can.* 27: 2343–2349; 1970.

Caldarone E.; St Onge-Burns J. M.; Buckley L. J. Relationship of RNA/DNA ration and temperature to growth in larvae of Atlantic cod *Gadus morhua*. *Mar. Ecol. Prog. Ser.* 262: 229–240; 2003.

Chang C. F.; Shih T. W. Purification and characterization of vitellin from the mature ovaries of prawn, *Macrobrachium rosenbergii*. *Comp. Biochem. Physiol. B.* 105(3–4): 609–615; 1993.

Chen S. N.; Chi S. C.; Kou G. H.; Liao I. C. Cell culture from tissues of grass prawn, *Penaeus monodon*. *Fish Pathol.* 21: 161–166; 1986.

Cheng Y. X.; Xu Z. L.; Chen Y. Q. Ultrastructyral study of the toxic effect of different content of Zn^{2+} on hepatopancreas in the larvae of Chinese crab, *Eriocheir sinensis*. *Zool. Res.* 21(5): 343–347; 2000. (in Chinese).

Dortch Q.; Roberts T. L.; Clayton J. R.; Ahmed S. I. RNA/DNA ratios and DNA concentrations as indicators of growth rate and biomass in planktonic marine organisms. *Mar. Ecol. Prog. Ser.* 13: 61–71; 1983.

Fainzilber M.; Tom M.; Shafir S. Is there extraovarian synthesis of vitellogenin in Penaeid shrimp? *Biol. Bull.* 183: 233–241; 1992.

Fingerman M.; Nagabhushanam R.; Sarojini R. Vertebrate-type hormones in crustaceans-localization, identification and functional significance. *Zool. Sci.* 10(1): 13–29; 1993.

Haines T. A. An evaluation of RNA/DNA ratio as a measure of long-term growth in fish populations. *J. Fish. Res. Board Can.* 30: 195–199; 1973.

Hotard S.; Zou E. Activity of glutathione S-transferase in the hepatopancreas is not influenced by the molting cycle in the Fiddler Crab, *Uca pugilator*. *Bull. Environ. Contam. Toxicol.* 81: 242–244; 2008.

Hsu Y. L.; Yang Y. H.; Chen Y. C.; Tung M. C.; Wu J. L.; Engelking M. H.; Leong J. C. Development of an in vitro subculture system for the oka organ (Lymphoid tissue) of *Penaeus monodon*. *Aquaculture.* 136: 43–55; 1995.

Huang H. Y.; Ye H. H.; Li S. J. Immunocytochemical localization of endocrine cells in the digestive system of the mud crab, *Scylla serrata*. *J. Xiamen Univ. Nat. Sci.* 44: 94–97; 2005. (in Chinese).

Islam Md S; Tanaka M. Nutritional condition, starvation status and growth of early juvenile Japanese sea bass (*Lateolabrax japonicus*) related to prey distribution and feeding in the nursery ground. *J. Exp. Mar. Biol. Ecol.* 323: 172–183; 2005.

Itami T.; Maeda M.; Kondo M.; Takahashi Y. Primary culture of lymphoid organ cells and haemocytes of kuruma shrimp, *Penaeus japonicus*. *Meth. Cell Sci.* 21: 237–244; 1999.

Jayasankar V.; Tsutsui N.; Jasmani S.; Saido-Sakanaka H.; Yang W. J.; Okuno A.; Hien T. T. T.; Aida K.; Wilder M. N. Dynamics of vitellogenin mRNA expression and changes in hemolymph vitellogenin levels during ovarian maturation in the giant freshwater prawn Macrobrachium rosenbergii. J. Exp. Zool. 293: 675-682; 2002.

Jin S.; Zhao Q.; Wang C.; Chen Y. RAPD genetic markers in six species of marine crab. *Zool. Res.* 25: 172–176; 2004. (in Chinese).

Klinbunga S.; Boonyapakdee A.; Pratoomchat B. Genetic diversity and species-diagnostic markers of mud crabs (genus *Scylla*) in Eastern Thailand determined by RAPD analysis. *Mar. Biotechnol.* 2: 180–187; 2000.

Li F. H.; Li S. J. Studies on the hepatopancreas of larval *Scylla serrata*. *Oceanol. Limnol. Sin.* 29: 29–35; 1998. (in Chinese).

Luedaman R. A.; Lightner D. V. Development of an in vitro parimary cell culture system from the penaeid shrimp, *Penaeus stylirostris* and *Penaeus vannamei*. *Aquaculture.* 101: 205–211; 1992.

Nadala E. C.; Lu Y.; Loh P. C. Primary culture of lymphoid, nerve and ovary cells from *Penaeus stylirostris* and *Penaeus vannamei*. *In Vitro Cell. Dev. Biol. Anim.* 29: 620–622; 1993.

Novikov Y. V.; Mukhin V. A.; Rysakova K. S. Properties of chitinolytic enzymes from the hepatopancreas of the red king crab (*Paralithodes camtschaticus*). *Appl. Biochem. Microbiol.* 43: 159–163; 2007.

Okuno A.; Yang W. J.; Jayasankar V.; Saido-Sakanaka H.; Huong D. T. T.; Jasmani S.; Atmomarsono M.; Subramoniam T.; Tsutsui N.; Ohira T.; Kawazoe C.; Aida K.; Wilder N. Deduced primary structure of vitellogenin in the giant freshwater prawn, *Macrobrachium rosenbergii*, and yolk processing during ovarian maturation. *J. Exp. Zool. B Mol. Dev. Evol.* 292: 417–429; 2002.

Owens L.; Smith J. Early attempts at production of prawn cell lines. *Meth. Cell. Sci.* 21: 207–211; 1999.

Pateraki L. E.; Stratakis E. Synthesis andorganization of vitellogenin and vitellin molecules from the land crab *Potamon Potamios*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 125: 53–61; 2000.

Rosa R.; Nunes M. L. RNA, DNA and protein concentrations and amino acid profiles of deep-sea decapod *Aristeus antennatus*: an indication for seasonal variations of nutrition and growth. *Aquat. Living Resour.* 17: 25–30; 2004.

Rosenthal J.; Diamant A. In vitro primary cell cultures from *Penaeus semisulcatus*. In: Perkins F. O.; Cheng T. C. (eds) Pathology in marine science. Academic, New York; 1990.

Sashikumar A.; Desai P. V. Development of primary cell culture from *Scylla serrata*. *Cytotechnology* 56: 161–169; 2008.

Shimizu K.; Saikawa W. Identification and characterization of vitellin from the barnacle, *Balanus amphitrist*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 115: 114–119; 1996.

Steinhart M.; Eckmann R. Evaluating the nutritional condition of individual whitefish (*Coregonus* spp.) larvae by RNA/DNA ratio. *J. Fish Biol.* 40: 791–799; 1992.

Tanaka Y.; Satoh K.; Yamada H.; Takebe T.; Nikaido H.; Shiozawa S. Assessment of the nutritional status of field-caught laval Pacific bluefin tuna by RNA/DNA ratio based on a starvation experiment of hatchery-reared fish. *J. Exp. Mar. Biol. Ecol.* 354: 56–64; 2008.

Tong S. L.; Miao H. Z. Attempts to initiate cell cultures from *Penaeus chinensis* tissues. *Aquaculture.* 147: 151–157; 1996.

Toull J. Y. Crustacean primary cell culture: a technical approach. *Meth. Cell. Sci.* 21: 193–198; 1999.

Tsang W. S.; Quackenbush L. S.; Chow B. K. C. Organization of the shrimp vitellogenin gene: evidence of multiple genes and tissue specific expression by the ovary and hepatopancreas. *Gene.* 303: 99–109; 2003.

Tseng D. Y.; Chen Y. N.; Kou G. H.; Lo C. F.; Kuo C. M. Hepatopancreas is the extraovarian site of vitellogenin synthesis in black tiger shrimp, *Penaeus monodon*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 129: 909–917; 2001.

Tsuisui N.; Kawazoe I.; Ohira T. Molecular characterization of a cDNA encoding vitellogenin and its expression in the hepatopancreas and ovary during vitellogenesis in the kuruma prawn, *Penaeus japonicus*. *Zool. Sci.* 17: 651–660; 2000.

Valk J.; Mellor D.; Brands R.; Fischer R.; Gruber F.; Gstraunthaler G.; Hellebrekers L.; Hyllner J.; Jonker F. H.; Prieto P.; Thalen M.; Baumans V. The humane collection of fetal bovine serum and possibilities for serum-free cell and tissue culture. *Toxicol. In Vitro.* 18: 1–12; 2004.

Yang W. J.; Ohira T.; Tsutsui N.; Subramoniam T.; Huong D. T. T.; Aida K.; Wilder M. N. Determination of amino acid sequence and site of mRNA expression of four vitellins in the giant freshwater prawn, *Macrobrachium rosenbergii*. *J. Exp. Zool.* 287: 413–422; 2000.

Yano I.; Chinzei Y. Ovary is the site of vitellogenin synthesis in kuruma prawn, *Penaeus japonicus*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 86: 213–218; 1987.