



## Effect of Peri-Vitelline Fluid from Horseshoe Crab Embryo in Enhancing the Early Gonadal Development in Red Tilapia

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**ABSTRACT:** Peri-vitelline fluid (PVF) from horseshoe crab embryos has been found to contain molecules that stimulate growth and differentiation of specific organs in vertebrate models. Using red tilapia fingerlings, we have made an attempt to study the early development of gonads using PVF. From four different concentrations of PVF tested (100ul, 200ul, 300ul and 500ul), the best concentration was screened based on highest specific growth and successful spawning initiation in red tilapia fingerlings. A concentration of 200ul PVF injected intramuscularly to the red tilapia fingerlings showed high significance in terms of gonad enhancement and Gonado-somatic index for both sexes as compared to controls within 7 days of the study. Results suggest that PVF from horseshoe crab have the ability to enhance the gonadal maturity in red tilapia. This study discusses the impact of PVF treatment on growth performance, spawning and gonadal enhancement and their future applications.

**Key words:** Horseshoe crab, red tilapia, peri-vitelline fluid, gonad, lectin, spawning

### INTRODUCTION

The horseshoe crab, described as the world's oldest living fossil has significant economic importance in pharmaceutical, clinical and food industries. These remarkable 'living fossils' have unique blood cells (amebocytes) that are used to test human vaccines for bacterial contamination. The ameobocytes of horseshoe crab is white but if it comes into contact with bacteria would instantly coagulate around the bacteria and attack it and turns blue (Levin and Bang, 1968). Besides the importance of amoebocytes as a rapid diagnostic reagent (Limulus Amebocyte Lysate, LAL), there are many other important factors hidden within horseshoe crab. The egg of the mysterious horseshoe crab is also filled with valuables. During early developmental stages of the horseshoe crab embryo, the inner egg membrane is formed (Nagai *et al.*, 1999). The fluid which is filled between the inner egg membrane and embryo is known as peri-vitelline fluid (PVF), which contains important primitive types of proteins (Sekiguchi, 1988). Many attempts have been previously made to purify and characterize the amino acid sequences in PVF of the horseshoe crab (Sugita and

Sekiguchi, 1979; Shishikura and Sekiguchi, 1984; Nagai *et al.*, 1999). These studies have identified proteins such as hemagglutinins and hemocyanin and suggested that these proteins may have an important role in embryogenesis (Sugita and Sekiguchi, 1979; Shishikura and Sekiguchi, 1984a). Moreover, three glycoproteins with potent agglutinin-binding activity have also been isolated from the PVF of *Tachypleus gigas*, (Muller, 1785) embryo (Shishikura and Sekiguchi, 1984b). Attempts have also been made to verify the applications of PVF for various bio-medical uses. It has been observed that the PVF from horseshoe crabs have helped in proliferation of beta cells (  $\beta$ -cells) that could be made useful for insulin production in human beings (Parab *et al.*, 2004).

Studies on chick developing embryo showed that the PVF contained certain biologically active molecules that influence the early vertebrate embryonic development and differentiation of specific organs such as brain and heart (Ghaskadbi *et al.*, 2008). The partially purified fluid from this study showed that a molecule Lectin, helps in cardiac development in chick embryos.

Hence, it is clear from the study of Ghaskadbi *et al.* (2008) that the PVF of horseshoe crab contains peptide(s) capable of positively influencing differentiation of specific organs.

Such peptides are likely to be present in minute quantities and may be as proteins (Nagai *et al.*, 1999). Considering the activity of such peptides and proteins present in the PVF of horseshoe crabs on early organ development and differentiation in vertebrates, we have attempted to see the efficacy of such molecules in early gonadal development of red tilapia [*Oreochromis mossambicus* (Peters, 1852) X *Oreochromis niloticus* (Linnaeus, 1758)]. This research was conducted at aquaculture research hatchery at University Malaysia Terengganu.

## MATERIALS AND METHODS

### A. Horseshoe crab egg collection and artificial incubation

Fertilized eggs of the Malaysian horseshoe crab (*Carcinoscorpius rotundicauda*, Latrielle, 1802) were collected directly from the nest made on the breeding ground of Setiu at Terengganu (Eastern coast of Peninsular Malaysia; Lat 5°42'60" N; Long 102°42'0" E, Srijaya *et al.* (2010); see for details of location). The fertilized eggs collected from the nest were kept for incubation at a constant temperature of 27±1° C and salinity 20±2 ppt as described previously by Srijaya *et al.* (2011). During the initial 15 days, the fertilized eggs were dark green in color which then changed to light greenish-yellow during the 20-35 days of incubation. At this stage the eggs were swollen and transparent. Due to the accumulation of embryonic fluid (PVF) between the outer envelope and embryo, the size of the eggs increased considerably.

### B. Perivitelline Fluid Collection

After 30-35 days of incubation, the developing eggs were collected and thoroughly cleaned with sterile, chilled seawater. The eggs were then transferred to a sterile plastic eppendorf tube (1.5ml) with a minute hole at its base. These tubes were placed in a sterile intact 2ml collection tube. The vitelline membrane of the egg was pierced using a 22gauge sterile needle under highly aseptic conditions and immediately the eppendorf tube placed inside the collection tube was centrifuged at 5000 rpm for 10min at 4° C as described previously by Ghaskadbi *et al.* (2008). The PVF eluted inside the collection tube was pipette, aliquoted and stored at -20° C until usage.

### C. Experimental design

Fingerlings of red tilapia were used for the experimental trials. Fingerlings of approximately 6 gram in weight were sorted from the rearing tanks. The initial weight of the fingerlings was taken and they were introduced into the glass aquarium tanks of 550 l capacity. The temperature of the aquarium tanks were maintained at 28±1° C using digital heater (Model-D-38300, 300W-Italy). During the experimental period the fingerlings were fed with powdered feed (ASEAN Marine Fish Feed with 43% protein) at a ratio of 12-15% of their body weight, thrice a day. Water quality was maintained throughout the experimental period by daily exchange of 40% water. The quality of water was monitored with the help of a master test kit (AquariumPharmaceuticals, INC) where nitrite (NO<sub>2</sub><sup>-</sup>), pH and ammonia were approximately kept at 0.2 mg/l, >7 mg/l and 0.25mg/l, respectively.

The initial experimental attempt was to find out the optimum concentration of PVF for promoting growth and spawning in red tilapia fingerlings. Fingerlings were tested with four different doses of PVF, ranging from 100ul, 200ul, 300ul and 500ul. Control group were kept without any PVF treatments. Each group was taken in duplicate and in each tank 10 fish were taken. All the experimental groups were maintained under similar culture conditions to avoid any experimental error. The Frozen PVF was thawed before injecting into the fingerlings. Using sterile surgical syringes (22gauge) the desired concentration of PVF was injected intramuscularly into the fingerlings. The fishes were then kept under observation for a period of 15 days to determine their growth variation and spawning activity between the control group and treated group.

The next experimental attempt was to determine the effect of PVF on the gonadal enhancement of red tilapia fingerlings. For this, the optimized concentration of PVF from the first trial was tested (200ul) and the control groups were not given any PVF treatments. A total of 15 fingerlings were used for this experiment and both groups were taken in duplicate. The development of gonad was evaluated at the end of 7<sup>th</sup> day. However, before sacrificing, the individual weight of each fish from all the groups was taken using a single pan electronic balance (precision 0.01 g). The parameters like gonadal weight and gonadosomatic index (GSI) were also calculated where GSI was deduced according to the formula as described previously by Pradeep *et al.* (2012).

Gonadosomatic index (GSI) =  $\frac{\text{Weight of gonad(g)} \times 100}{\text{Weight of fish(g)}}$

Specific growth rate (SGR) was determined as:

$\text{SGR} = \frac{[\ln \text{WF} - \ln \text{WI}]/t \text{ (days)}}{100}$ ,

Where,  $t$  is duration of the experiment.

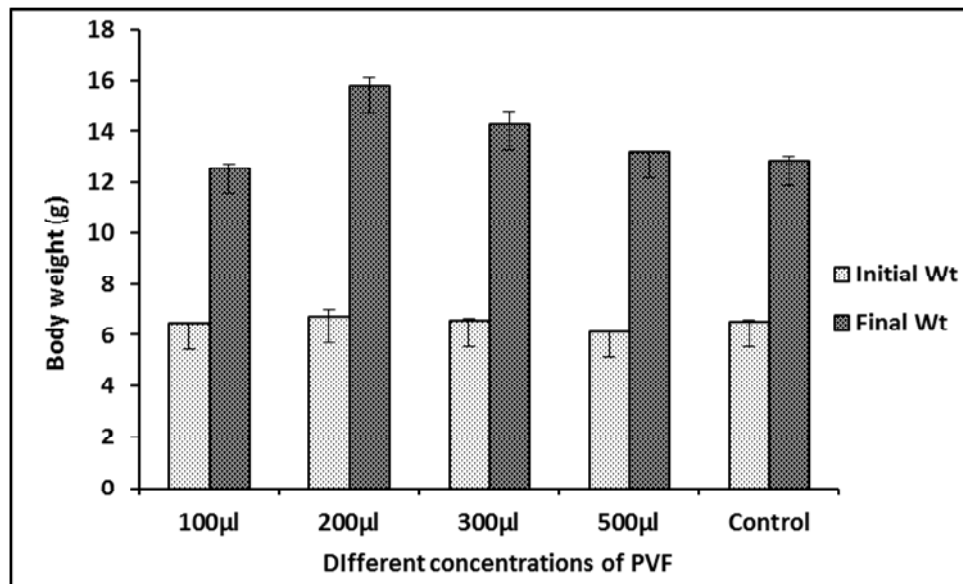
#### D. Statistical analysis

Standard statistical procedures were used for data processing using the statistical package SPSS, 20.0 and data were expressed as a mean  $\pm$  SD. Comparison of total weight and specific growth rate between different concentrations of PVF and control was made using one-way ANOVA, followed by tukey test. Levene's test was used to determine the homogeneity of variances. The data for the factors GSI and gonad weight among treatment and control were analyzed using one-way

ANOVA. Differences were considered significant when  $P < 0.05$ .

## RESULTS AND DISCUSSION

Comparison of the mean initial and final body weight of the red tilapia fingerlings for a total of 15 days (Trial 1) injected with various concentrations of PVF are given in Fig. 1. When the results of the final weight between the various treatment was statistically analyzed, the concentration of 200ul showed some significance over 100ul and control ( $P > 0.05$ ), but not with the other 2 groups. Though the specific growth rate were higher in PVF treated groups (200ul, 300ul and 500ul) as compared to control group, none were statistically significant. The specific growth rate obtained from various concentrations; 100, 200, 300, 500ul and control tested in the present study was 4.4, 5.7, 5.1, 5.1, 4.5; respectively.



**Fig. 1.** Mean initial and final weight of the red tilapia fingerlings injected with different concentrations of PVF.

(Note: All fishes in 500ul PVF concentration were found dead and thus the standard deviation was not able to provide.)

The concentration 500ul was found to be lethal for the red tilapia fingerlings, as all the experimental fish were found dead in one of the replicate and out of 10 fishes, only 2 were left in the other replicate. A concentration of 200ul PVF was found very effective in inducing the red tilapia fingerlings to spawn (Table 1). Although, 300ul PVF also showed some spawning success, the results were not as consistent as found in 200ul concentration.

The mean bodyweight, gonad weight and GSI for both the sexes of PVF treated (200ul) and control group

without the PVF treatment after 7 days are given in Table 2. The average total weight gained by both sexes was almost similar and a significant variation was not visible. Testes of PVF treated group was elongated with well-developed testicular organ, which showed motile spermatozoa, whereas testes were rather thin and flat with somewhat watery spermatozoa for the control treatment without PVF (Fig. 2). Highly significant difference was seen with testes weight and GSI of the PVF treated group with that of the controls ( $P > 0.001$ ).

**Table 1.** Effect of different PVF concentrations in spawning the red tilapia fingerlings

Treatments	Spawning	
	Replicate 1	Replicate 2
100ul	NS	NS
200ul	All 5 females spawned (S)	All 4 females spawned (S)
300ul	5 out of 6 females spawned (S)	2 out of 5 females spawned (S)
500ul	(NS)	(NS)
Control	(NS)	(NS)

(Note: S = Spawning; NS = No spawning.)

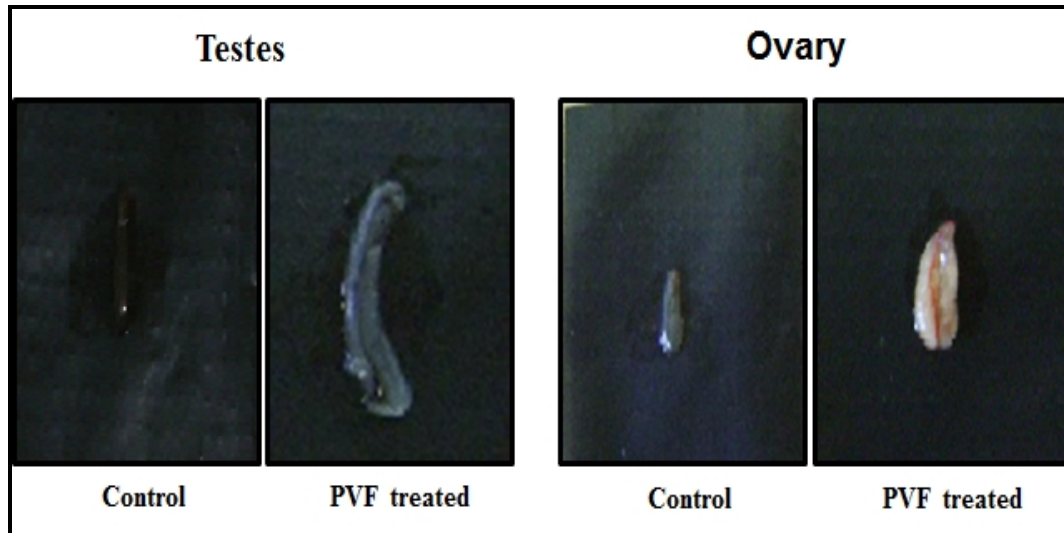
**Table 2:** Summary of the growth parameters of the male and female control and PVF treated red tilapia fingerlings for 7 days.

Parameters <sup>1</sup>	Control	Treatment
<b>Male</b>		
Total Weight (g)	7.5±0.16 <sup>a</sup>	7.72±0.03 <sup>a</sup>
Gonad Weight (g)	0.006±0.001 <sup>a</sup>	0.02±0.0 <sup>b</sup>
GSI	0.08±0.01 <sup>a</sup>	0.27±0.01 <sup>b</sup>
<b>Female</b>		
Total Weight (g)	7.5±0.11 <sup>a</sup>	7.53±0.021 <sup>a</sup>
Gonad Weight (g)	0.02±0.0 <sup>a</sup>	0.04±0.002 <sup>b</sup>
GSI	0.24±0.014 <sup>a</sup>	0.51±0.021 <sup>b</sup>

(<sup>1</sup>Mean (±SE), a common superscript in rows indicates values which do not differ significantly (P>0.05)).

Similarly, highly significant result was observed (P<0.001) in ovary weight and GSI between PVF treated group and control groups at 7<sup>th</sup> day (Table 2). The ovaries of PVF treated were nearly 2 times heavier than the ovaries of their control counterpart. The ovaries of the PVF treated females showed numerous developing oocytes, while the ovaries of the control were very thin and string-like. There were no signs of

developing oocytes in the control group as observed in the treatment group (Fig. 2). In the present study, we have demonstrated for the first time that the crude PVF collected from the Malaysian horseshoe crab (*C. rotundicauda*) can enhance the growth, early gonadal maturation and spawning in red tilapia fingerlings. The most prominent observed effect was related to enlarged gonads.



**Fig. 2.** Effect of PVF on the testes and Ovary of the red tilapia fingerlings.

Among all tested concentrations of PVF, 200ul concentration was very effective for enhancing the growth, gametogenesis and spawning.

The embryo of the horseshoe crab inside the PVF goes through four molts before they are hatched out as trilobite larvae (Sekiguchi *et al.*, 1982). The protein components of PVF of horseshoe crab embryo (*T. tridentatus*) was first reported by Sugita and Sekiguchi (1979). Peri-vitelline fluid of horseshoe-crabs has many important proteins such as hemagglutinins and hemocyanin, which has a substantial role in the process of embryogenesis (Sugita and Sekiguchi, 1979; Shishikura and Sekiguchi, 1984 a, b). Lectin proteins were also isolated and characterized from the PVF of horse shoe crabs including; *T. tridentatus* Nagai *et al.* (1999) and *T. gigas* (Ghaskbadi *et al.*, 2008). These lectins were proposed to play an important role in completing early embryonic development by interacting with endogenous glycoproteins or *N*-acetylhexosamines.

The lectin isolated from, *T. gigas* with a relative molecular mass of  $2.7 \times 10^4$ , made of 221 amino acid residues, were found to enhance the cardiac development enhancing activity in the chick embryo (Ghaskbadi *et al.*, 2008). This study has revealed that the lectin isolated from PVF of horseshoe crabs is capable of stimulating various aspects of embryonic development and specific organ enhancement (brain and heart) in chick embryo. This clearly indicates that the PVF contains molecules (peptides) that stimulate growth and differentiation of specific organs. More

likely such peptides may be present as particular protein in minute amount (Nagai *et al.*, 1999). Our study using red tilapia also has proven this concept, by showing the enhancement of its gonad during the early developmental period. Since our study was only a preliminary trial, we haven't attempted to characterize the molecules or peptides responsible for the gonad enhancement. However, from the clearly proven results of Ghaskbadi *et al.* (2008) using chick embryo, it could be hypothesized that certain proteins/peptides present in the PVF are also capable of stimulating gonad enhancement in red tilapia.

Morphological analysis and comparison of gonads in terms of weight and GSI from each sexes of the treated group with that of the non PVF treated control revealed that PVF treatment using 200ul concentration has substantially increased the gonad size and GSI index. Similar to our results, Ghaskbadi *et al.* (2008) also has observed cardiac enhancement in majority of the chick embryos tested, while the rest of the features like neural tube, notochord and gut from the treated embryos were comparable to the controls when a 20 ng concentration of PVF Fraction VII was applied. The hypertrophy of gonadal structures and early spawning in red tilapia fingerlings suggests that there was an unusually large amount of sex hormone in circulation. In fishes, both spawning and gonadal development are synchronized through the hypothalamus-pituitary-gonadal and hepatic axis, because these organs produce substances influencing each other, leading to successful reproduction (Zohar *et al.*, 2009).

We suspects that the factors in PVF (similar to neuro-peptides and neuro-secretions) have positive feed back on the red tilapia brain, especially the hypothalmo-hypophysial-pituitary-gonadal and hepatic axis. This might have triggered the downstream activation like the release of tropic hormones, gonadotropin (GtHs) in particular, which induce the production of sex steroids like estrogens and progestogens in female and androgens in male, regulating ultimately the various reproductive events (Evans and Claiborne, 2006; Lal and Dubey, 2011). In chick embryo also PVF fraction VII has showed significant influence on the brain development and activities (Ghaskbadi *et al.*, 2008).

Previous research have provided evidence that factors like cytokines, growth factors, regulatory peptides, reactive oxygen and nitrogen species synthesized and released by a variety of testicular and ovarian cells could influence the reproductive activity greatly and could also modulate the action of endocrine hormones delivered to gonads from the hypothalmo-hypophysial axis (Lal and Dubey, 2011). Several growth factors which are polypeptide proteins, have been known to regulate the proliferation of germ cells in the mammalian gonads (Guraya 1995, 1998, 1999). Moreover, the peptide growth factors are known to be involved inductive events in the early amphibian embryo by either modulating the gene expression or by serving to constrain the pathways (Asashima *et al.*, 1999). However, such kind of peptide growth factors that is capable of regulating these events in teleostean fish is still scarce.

Previously, cell differentiating factors capable of insulin production was identified from the PVF of fertilized eggs of horseshoe crab (*T. gigas*) (Parab and Chatterji, 2003). Study by Ghaskbadi *et al.* (2008) showed that the partially purified PVF have positive effect on cardiac development by increasing the number of cells constituting the heart and by modulating the expression of several cardiac development regulatory genes in chick embryos. They suggested that the property of PVF can be exploited for use in vitro heart regeneration. A recent study has observed that peri-vitelline fluid (PVF) could maintain the CD 34<sup>+</sup> phenotype in human bone marrow-derived stem cells for prolonged duration and increases the total number of CD 34<sup>+</sup> cells in culture (Mirshahi *et al.*, 2010).

Taken together with all these studies and the current study, we hypothesis that the growth factors from PVF might be having an effect on cell proliferation in spermatogonial and oogonial stem cells. This was evident in the present study, where the red tilapia fingerlings treated with 200ul of PVF have nearly 2 times larger gonads than their control counterparts. Moreover, successful spawning tendency observed in

the fingerlings revealed that the spermatogonial and oogonial cells were functional and fertile. In aquaculture aspects, the results from the present study is very encouraging as PVF treatment could be a viable option for overcoming the reproductive dysfunctions in many fishes of commercial interest. However, further detail investigation is required to isolate and characterize the particular factors responsible for the enhancement of gonads in fish. Overall, the present study if taken in a wider perspective implies that the growth factors in PVF can be exploited for regenerative stem cell biology.

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#### REFERENCES

- Asashima, M., Kinoshita, K., Arizumi, T. and Malacinski, G.M. (1999). Role of activin and other peptide factor in body patterning in the early amphibian embryo. *Inter. Rev. Cytol.* **191**: 1-52.
- Evans, D.H. and Claiborne, J.B. (2006). *The physiology of fishes*. Third edition. Taylor and Francis, CRC Press. 601pp.
- Ghaskadbi, S., Patwardhan, V., Chakraborty, M., Agrawal, S., Verma, M.K., Chatterji, A., Lenka, N. and Parab, P.B. (2008). Enhancement of vertebrate cardiogenesis by a lectin from peri-vitelline fluid of horseshoe crab embryo. *Cell. Mol. Life Sci.* **65**(20): 3312-3324.
- Guraya, S.S. (1995). The comparative cell biology of accessory somatic (or sertoli) cells in the animal testis. *Inter. Rev. Cytol.* **160**: 164-221.
- Guraya, S.S. (1998). *Cellular and molecular biology of gonadal development and maturation in mammals; fundamentals and biomedical implications*. Narosa, New Delhi: Springer-verlag, Heidelberg.
- Guraya, S.S. (1999). *Comparative testicular biology in animals; in structure, functions and intercellular communication*. Lebanon: USA science publishers.
- Lal, B. and Dubey, N. (2011). Intra-testicular nitric oxide regulates steroidogenesis in fish. *Indian J. Sci. Tech.* 9th ISRP Issue, Vol. **4** No. **8**.
- Levin, J. and Bang, F.B. (1968). Clottable proteins in Limulus. Its localization and kinetics of its coagulation by endotoxin. *Thromb. Et Diath. Haem.* **7**: 12-17.

- Mirshahi, M., Mirshahi, P., Soria, J., Alam, H. Therwath, A. and Chatterji, A. (2013). Perivitelline fluid of the fertilized eggs of the Indian Horseshoe Crab (*Tachypleus Gigas*, Müller) helps in maintaining the original phenotype of CD 34+ stem cells and enhances colony formation in vitro. In: Horseshoe crab in Asia, Published by: Lambert Academic Publishing, Germany (In press).
- Nagai, T., Kawabata, S., Shishikura, F. and Sugita, H. (1999). Purification, characterization, and amino acid sequence of an embryonic lectin in perivitelline fluid of the horseshoe crab. *J. Biol. Chem.* **274** (53): 37673-37678.
- Parab, P.B. and Chatterji, A. (2003). Isolation of perivitelline fluid of the fertilized eggs of horseshoe crab and use thereof. US patent #20030017212.
- Parab, P.B., Ghaskadbi, S., Patwardhan, V., Mishra, G.C., Chatterji, A. and Parameswaran, P.S. (2004). Cardiac development promoting activity of peri-vitelline fluid of embryos of Indian horseshoe crab, *Tachypleus gigas* (Muller). Patent Number: 0319NF2004.
- Pradeep, P.J., Srijaya, T.C., Papini, A. and Anil, C.H. (2012). Effects of triploidy induction on growth and masculinization of red tilapia [*Oreochromis mossambicus* (Peters, 1852) X *Oreochromis niloticus* (Linnaeus, 1758)]. *Aquaculture.* **344-349**: 181-187.
- Srijaya, T.C., Pradeep, P.J., Mithun, S., Hassan, A. and Chatterji, A. (2010). A new record on the morphometric variations in the populations of Horseshoe crab (*Carcinoscorpius rotundicauda*) obtained from two different ecological habitats of Peninsular Malaysia. *Our Nature.* **8**: 204-211.
- Srijaya, T.C., Pradeep, P.J., Shaharom, F. and Chatterji, A. (2011). A study on the energy source in the developing embryo of the mangrove horseshoe crab, *Carcinoscorpius rotundicauda* (Latreille). *Invert. Reprod. Devel.* 1-10.
- Sekiguchi, K., Yamamichi, Y. and Costlow, J.D. (1982). In: *Physiology and Biology of Horseshoe Crabs: Studies on Normal and Environmentally Stressed Animals*. Bonaventura, J., C. Bonaventura, and S. Tesh, (Eds.). Alan R. Liss, Inc., New York. pp. 53-73.
- Sekiguchi, K. (1988). In: *Biology of Horseshoe Crabs*. Sekiguchi, K. Eds. Science House Co, Ltd, Tokyo. pp. 139-181.
- Sugita, H. and Sekiguchi, K. (1979). Protein components in the peri-vitelline fluid of the embryo of the horseshoe crab. *Develop. Biol.* **73**: 183-192.
- Shishikura, F. and Sekiguchi, K. (1984a). Studies on Perivitelline Fluid of Horseshoe Crab Embryo. I. Purification and Properties of Agglutinin from the Perivitelline Fluid of *Tachypleus gigas* Embryo. *J. Biochem.* **96**(3): 621-628.
- Shishikura, F. and Sekiguchi, K. (1984b). Studies on Perivitelline Fluid of Horseshoe Crab Embryo. II. Purification of Agglutinin-Binding Substance from the Perivitelline Fluid of *Tachypleus gigas* Embryo. *J. Biochem.* **96**(3): 629-636.
- Zohar, Y., Munoz-Cueto, J.A., Elizur, A. and Kah, O. (2009). Neuroendocrinology of reproduction in teleost fish. *Gen. Comp. Endocrinol.* doi:10.1016/j.ygcen.2009.04.017.