Changes of fatty acids in phosphatidylcholine on sperm membrane during *Macrobrachium rosenbergii* sperm transit through spermatic duct and lipid analysis in spermatic vesicles

Piyaporn Surinlert¹, Somluk Asuvapongpatana¹, Nopparat Srakaew², Thanya Daungchinda³, Mitsutoshi Setou⁴, Wattana Weerachatanukul^{1*}

¹Department of Anatomy, Faculty of Science, Mahidol University, Bangkok Thailand;

²Department of Zoology, Faculty of Science, Kasetsart University, Bangkok, Thailand;

³Aquacultural Technology Research Unit, Thaksin University, Phatthalung, Thailand;

⁴Hamamatsu University School of Medicine, Department of Cell Biology and Anatomy,

Shizuoka, Japan

*Corresponding author should be addressed to:

Assoc. Prof. Wattana Weerachatanukul

Department of Anatomy, Faculty of Science, Mahidol University,

272 Rama VI Rd, Bangkok, Thailand 10400

Email: wattana.wee@mahidol.ac.th

Abstract

Sperm maturation taking place in male accessory tracts is a complex series of biomolecular dynamics of both lipids and proteins on sperm plasma membrane. In this study, we compared fatty acid (FA) compositions on a single banded phosphatidylcholine (PC) in *Macrobrachium rosenbergii* sperm collected from testis (Tsp) and vas deferens (Vsp) to give an insight of membrane lipid dynamic during sperm maturation. Saturated (SAT, predominantly C16:0) and monounsaturated fatty acids (MUFA, predominantly C18:1, *n*9) were apparently higher in Vsp whereas the polyunsaturated fatty acids (PUFA, mainly C20:5, *n*3 and C22:6, *n*3) were evidently lower. Higher level of PUFA in the Vsp was also supported by merocyanine staining followed by flow cytometric analysis. Imaging mass spectroscopy (IMS) gave both comparative and quantitative data of the paired FA within an intact PC molecule while it also provided FA distribution within the PC band. The levels of lipids, particularly cholesterol (CHO) and sphingomyelin (SM), as well as PUFA and other FA species in the spermatid vesicles (SV) were found to be opposite to those of Vsp. We thus believe that an alteration of lipid compositions in sperm membrane is partly modulated via SV transport in vas deferens, which is considerable a part of sperm maturation process in *M. rosenbergii*, similar to that reported for mammalian epididymal sperm maturation.

Key words: membrane lipids, spermatid vesicles, fatty acids, sperm maturation, *M. rosenbergii*

1. INTRODUCTION

During spermatogenesis, expression of newly synthesized biomolecules and their subsequent trafficking to the sperm membrane are limited due to elimination of most cytoplasmic organelles. Therefore, the sperm plasma membrane is considerably non-dynamic in their lipid and protein compositions at the phase of spermatogenesis (Eddy, 2006; Gadella et al., 2014). Sperm plasma membrane is thus not functionally mature; as a consequence, testicular sperm remain infertile. Sperm fertilizing ability is acquired after they undergo a post-testicular sperm maturation, a process that is well described in mammalian epididymis. The process is actively involved in sperm membrane modifications, both in lipid and protein compositions (Girouard et al., 2008; Christova et al., 2004; Wolfe et al., 1998). In some species of marine invertebrates, it is originally believed that the sperm simultaneously acquire fertilizing ability immediately after their production following a “broadcast” spawning from the testis (Ohlendieck and Lennarz, 1996; Vacquier, 1998). However, the recent evidence in the broadcast spawning animals, for instance, abalones and *Penaeus monodon*, addresses the essence of sperm modification post-testicularly in order for them to gain fertilizing capability (Alfaro et al., 2003, Vanichviriyakit et al., 2004; Suphamungmee et al., 2008). In *Macrobrachium rosenbergii* and some other crustaceans, some differences in reproductive biology should also be noted. During mating, the male spermatophore is attached to female “open thelycum”. Females then ruptures the spermatophore to release sperm co-incidentally with the egg spawning. The fertilized eggs are not broadcasting into surrounding environment but rather attached to the abdominal swimmerets (Lynn and Clark, 1983).

Although membrane lipids have received much less attention compared to membrane proteins counterparts, they play many significant roles during fertilization. Membrane phospholipids are platforms for bilayered membrane organization, both for the classical mosaic model and for microdomain organization model. A vast variety of phospholipids that make up >40% of membrane lipids including saturated- and non-saturated subtypes are known to be involved in membrane organization and the subsequent packing order – from highly fluidic to gel to crystalline. For instance, an increase in the contents of **unsaturated fatty acids** in sperm membrane has led to a higher membrane fluidity during sperm maturation (Haidl et al., 1997; Lenzi et al., 2000; Gadella et al., 2000; Rejraji et al., 2006). Glycolipids such as sulfogalactosylglycerolipid (SGG) make up of ~10 mole% in sperm membrane lipids and play a significant role in germ cell development during spermatogenesis (Inuoe et al., 2009) as well as in gamete interaction process (White et al., 2000; Weerachatanukul et al., 2007). Among neutral lipids, cholesterol (CHO) is known to function as a “key switch” for controlling membrane fluidity – its sequestering has led to a subsequent signal transduction during sperm capacitation (Visconti et al., 1999; Cross, 2004).

Lipid dynamics during sperm maturation in mammalian epididymis relies on lipid vesicular transport, referred to as “epididymosomes” (Cooper, 1998; Frenette et al., 2001; Girouard et al., 2008). As observed under TEM, they are made up of multiple layers known as “multilamellar vesicles” (~50-500 nm in size) which are secreted by epithelial cells along the entire epididymis (Frenette et al., 2001; Girouard et al., 2008; 2011). The major compositions of these epididymosomes are glycoproteins and lipids. The former are extensively **studied and many of them are** well characterized, while the latter are much less characterized. During sperm membrane modification, a number of proteins that are related to sperm fertilizing ability are

transferred via epididymosomes. The well-described examples shown to be transported via epididymosomes are carbonylanhydrase enzyme family proteins including bovine P25b, hamster P26h, human P34H (Frenette et al., 2001; Girouard et al., 2008; 2011) and cathepsin enzyme family (Girouard et al., 2009; Sae-wu et al., 2012). For lipid compositions, epididymosomes are enriched in sphingomyelin (SM), cholesterol, and saturated fatty acids (Rejraji et al., 2006). In addition, they have an unusual high cholesterol/phospholipid ratio, which is drastically different from that of regular membrane lipid ratio (Cross, 1998). Therefore, epididymosome has been implicated to play a crucial role in cholesterol transport towards sperm plasma membrane during sperm maturation and capacitation process. In this study, we characterized monoacyl and diacyl fatty acid compositions in the plasma membrane of sperm from variable regions of male reproductive tract as well as in the exosomes collected from vas deferens of *M. rosenbergii*. The dynamical alteration of sperm membrane lipids and their mechanisms are suggested and believed to be part of sperm maturation process in this shrimp species.

2. MATERIALS AND METHODS

2.1. Prawns, chemicals and sample collections

Freshwater prawns, *Macrobracium rosenbergii*, were purchased from commercialized livestock at Ayuttaya province, Thailand. The prawns were acclimatized in 3 × 2 metre-plastic tanks with aeration. The water temperature was kept at 27 °C and fed with food pellets. Most of the chemicals were obtained from Sigma Chemicals (St. Louis, MO). Chloroform and methanol were obtained from Merck (Whitehouse Station, NJ). Lipid standards including PE, PC, PI, CHO

and SM were purchased from Sigma while the polar lipids, SGC and GM1, were from Avanti Polar lipids (Avanti Polar lipids, Inc., Alabaster, Alabama).

For sperm collection, mature males with blue claw appearance were anaesthetized in ice and parts of reproductive tracts including testis (Tsp) and vas deferens (Vsp) were carefully dissected. They were cut into small pieces and briefly homogenized by Douce's homogenizer. The sperm were filtered through a 45- μ m mesh (Endecotts LTD., London, England) to eliminate debris and gelatinous masses and re-suspended in PBS. The sperm suspension was centrifuged at 500 $\times$ g and the pellets were collected. Sperm samples in each part of male reproductive tracts were counted on hemacytometer (Scientific, Horsham, PA), resuspended at 2×10^6 sperm/ml and processed for lipid extraction and merocyanine, MC540, staining. The supernatant from vas deferens was also collected and subjected to centrifugation (12,000 $\times$ g, 4°C for 15 min) to eliminate cell debris followed by ultracentrifugation at 200,000 $\times$ g (Beckman Coulter, Fullerton, CA) to collect spermatid exosomes (Girouard et al., 2011). The pellets were resuspended in buffer and kept at -20°C for lipid extraction and transmission electron microscopy (TEM).

2.2. Lipid extraction

Approximately 2×10^6 Tsp and Vsp were collected and subjected to lipid extraction using the modified Bligh-Dyer method (Bligh and Dyer, 1959). In addition, approximately 1 mg dryweight of spermatid duct vesicles (collected from spermatid fluid as mention in session 2.7) were also subjected to lipid extraction. Briefly, chloroform, methanol and water were added at the final ratio of 1:2:0.8 (v/v/v). The monophasic mixtures were incubated with the sperm (25°C, 2 h) to allow efficient lipid extraction. Then, chloroform and water were added to a final ratio of

1:1:0.9 (v/v/v) and allowed to stand until separation of chloroform and aqueous phases was visualized. The lipid suspension was collected from chloroform phase and then dried under a nitrogen stream in the heat block at 37°C. Sperm lipids were stored at -20°C until use.

2.3. Sperm lipid profiling by high performance thin layer chromatography (HPTLC)

Lipid profiles of sperm, isolated SV and lipid standards were applied onto 10×10 cm HPTLC silica plate (Merks, Darmstadt, Germany) and resolved in chloroform/methanol/water (65:25:4, v/v/v) (Furimsky et al., 2005) as a solvent system. The resolved lipids were stained with 0.03% Coomassie Brilliant Blue G-250 in 30% methanol/100 mM NaCl and de-stained with 30% methanol in 100 mM NaCl. Sulfoglycolipid was stained with 0.016% Azure A in 1 mM H₂SO₄. Lipid standards including sulfogalactosylceramide (SGC), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), cholesterol (CHO) and sphingomyelin (SM) were co-chromatographed with the sperm lipid samples.

2.4. Trans-esterification, gas chromatography and mass spectrometry (GC-MS)

Lipid classes separated by HPTLC were identified according to the standard and the well-resolved PC band was scraped out from the silica plates. The scraped powder was subjected to lipid extraction. Each lipid class was trans-esterified to achieve fatty acid methyl esters (FAMES) using a modified acid catalytic method (Jostensen and Landfald, 1997; Christie et al., 2003). Briefly, dry lipids were added with toluene and 2% sulfuric acid in methanol. The single nanodecanoic acid (C19:0) was also added into lipid suspension as an internal standard and

incubated at 80°C for 1 h. Thereafter, 2% (w/v) KHCO_3 and hexane:diethylether (1:1, v/v) with 0.1% BHT (butylated hydroxytoluene, a protective agent for lipid oxidation) were added into the suspension. The upper phase was collected into new glass tube. The remaining solution was re-extracted then pooled together. The samples were evaporated under a nitrogen stream at 37°C. The obtained FAMES were analyzed by capillary gas chromatography (GC) to yield the mass spectrum on the Agilent 6980 and 5973MSD with a DB-23 J&W column using helium as the carrier gas. The separation was performed at the temperature of 60°C for 1 min and then increased to 140°C for 3 min, 190°C for 3 min and then 220°C for 8 min (Jostensen and Landfald, 1997). Quantification data were compared with the known retention time (R_f) of lipid standards and their mass spectra. Statistical comparison between the group of samples were performed by a Student's *t*-test.

2.5. Determination of unsaturated fatty acid by merocyanine 540 staining

To determine the composition of polyunsaturated fatty acid in the sperm membrane, freshly extracted-live sperm were stained with MC540 and further subjected to flow cytometry. A solution of MC540 (Sigma-Aldrich) was added into Tsp and Vsp suspensions at a final concentration of 10 $\mu\text{g/ml}$ in the dark condition. The mixtures were incubated at 4°C for 30 min and then washed with PBS twice. The fluorescent signals from the sperm cells were observed under a FV10i Olympus confocal microscope. The image acquisition conditions were an excitation of the samples with krypton laser line, line-by-line scanning, and collecting the emitted fluorescence by a 590 band-pass filter. Kalman scanning mode was also applied to eliminate any interference of an autofluorescence in the samples. To quantitatively analyze the staining of MC540, the fluorescence of merocyanine stained sperm were further measured with a

BD FACSCantoTM Flow Cytometer (BD Biosciences, Foster city, CA). All fluorescent data were collected from 30,000 events in a logarithmic mode and analyzed with a BD FACSDiva version 6.1.1 software.

2.6. MALDI imaging mass spectrometry from lipid blotting

HPTLC resolved lipids were transferred onto PVDF membrane, stained with primuline and visualized by UV light as described by Naoko Goto-Inoue et al. (2008). Briefly, the resolved lipids in the silica plate were dipped in the blotting buffer (isopropanol: 0.2% CaCl₂: Methanol at the ratio of 40:20:7, v/v/v) for 20 sec. The dipped plate was immediately covered with a stack of PVDF-, Teflon-membranes and glass fiber, respectively. This membrane assembly was pressed together with TLC plate on the thermal blotter (ATTO, Tokyo, Japan) at 180 °C for 30 sec.

To analyze diacyl fatty acid compositions by MALDI-IMS, the PVDF membrane was attached onto a MALDI sample plate with electrified double-adhesive tape. The matrix, 2,5-dihydroxybenzoic acid (DHB) (Bruker Daltonics, Bremen, Germany), was dissolved at 50 mg/ml in 50% methanol and then dropped onto PVDF membrane. The excess matrix was blotted away by a Kimwipe paper (Crecia, Tokyo, Japan) and blew by cold air to accelerate crystallization. The PVDF membrane was analyzed using a QSTAR Elite high-performance, hybrid quadrupole time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA) in a positive ion mode. Ionization was performed with a 337-nm pulse N₂ laser. The mass spectra were externally calibrated with standard peptides including 10 pmol/ul bradykinin and angiotensin II mixtures. The ion imaging mass spectrometry was automatically performed in the raster scan mode. The ion images were constructed using a BioMap software (Norvatis, basel, Switzerland). The lipid

species were identified by tandem mass analysis and compared with the existing bioinformatic data.

2.7. Transmission electron microscopy of spermatic exosomes isolated from vas deferens

Spermatic fluids were collected from the entire length of vas deferens and sperm were removed by sieving and centrifugation as mentioned earlier. The collected fluid was further centrifuged ($12,000 \times g$, 4°C , 15 min) to eliminate cellular debris and any sperm residuals in the fluid. The supernatants were then subjected to ultracentrifugation ($200,000 \times g$, 4°C , 2 h) to collect spermatic vesicles (SV), followed the method described by Jariyapong et al. (2013). The pellets of an ultracentrifuged SV were resuspended in 10 μl PBS and plated onto the 200-mesh, carbon-coated, nickel grids (Electron Microscopy Sciences, Hatfield, PA). The samples were allowed to stand (3 min, room temperature) and blotted off the excess vesicles. They were washed with distilled water, stained with 2% uranyl acetate and observed under a FEI transmission electron microscope operated at 80 kV.

3. RESULTS

3.1. Lipid profiles of *M. rosenbergii* sperm and their alterations in male reproductive tracts

The male reproductive system of the giant fresh water prawn consists of paired testis, vas deferens, and terminal ampoules or spermatophores (Fig. 1A). Vas deferens projects from middle portion of the testis and then proceeds ventrally to join with spermatophores situated at the base

of the 5th walking legs. Upon collecting the sperm, their extracted membrane lipids were initially analyzed by HPTLC to compare individual lipid subclasses in the different portions of male reproductive tracts. HPTLC chromatograms in Figure 1B revealed that sperm membrane lipids were composed mainly of three major groups of lipids including phospholipids, sphingolipid and neutral lipids. Following densitometric analysis, the most abundant phospholipid subclass was phosphatidylcholine (PC), made up of ~34% of total lipids on the chromatograms. The other subclass included phosphatidylethanolamine (PE, ~8%). The major sphingolipid detected was sphingomyelin (SM), and the neutral lipid that moved most rapidly among all lipids was cholesterol (CHO). A gradual increase in the amount of PC, PE, and SM was observed in Vsp. In fact, SM and PC increased by ~20% and ~11%, respectively, compared to those in Tsp. Inversely, the amount of CHO was reduced by ~12% in Vsp. We could not detect the presence of glycolipids in any types of *M. rosenbergii* sperm using Azure A staining method (Fig. 1B), even though glycolipids are known to be one of the major lipids in mammalian sperm (Lenzi et al., 2000, Furimsky et al., 2005).

3.2. Changes of fatty acid patterns in phosphatidylcholine subclass

In most of the previous studies, changes of the FA patterns were usually analyzed in the total sperm lipid extracts. We analyzed here the change of fatty acid chains within a single lipid subclass, PC – the most abundant phospholipid in *M. rosenbergii* sperm membrane. Mass distribution patterns of FA in the form of FAME were shown in Figure 2 and their quantitative data were in Table 1. It was revealed that PC in Tsp was made up chiefly of saturated palmitic acid, PA (C16:0) and stearic acid, SA (C18:0). The amount of PA significantly increased in Vsp whereas SA was grossly unchanged. Monounsaturated fatty acids (MUFA) was mainly

composed of oleic acid, OA (C18:1, *n*9), and its level was rather consistent in both Tsp and Vsp. Inversely, the level of vacenic acid, VA (C18:1, *n*7) was higher in Vsp. The level of most polyunsaturated fatty acids (PUFA) appeared to decrease in Vsp with eicosapentaenoic acid, EPA (C20:5, *n*3) significantly ($P<0.05$) decreased by fourfold, from 8.68% in Tsp to 2.40% in Vsp. Docosahexaenoic acid, DHA (C22:6, *n*3) and linoleic acid, LA (C18:2, *n*6) also slightly decreased in Vsp. On the other hand, arachidonic acid, AA (C20:4, *n*6) increased from 2.52% in Tsp to 4.77% in Vsp. In the overall analysis of the 3 types of FAME, the amount of total SAT and MUFA apparently increased in Vsp, while the total PUFA significantly decreased (Fig. 2A).

3.3. On-membrane fatty acid analysis by imaging mass spectroscopy (IMS)

Static patterning of FAME analysis shown above was the results of cumulative analysis of FA in PC. The picture of how the corresponding pairs of FA in PC molecules are assembled would give an insightful information of the dynamic changes of lipids in sperm membrane. We utilized the powerful IMS analysis directly on the resolved lipids blotted onto PVDF membrane (Inoue NG et al., 2008) to achieve the patterns of paired FA in PC species. Initially, the amount and pattern of PC was visualized by primuline staining prior to IMS analysis (Fig. 3A). We selected 2 pairs of FA in PC as the representatives of the major SAT/MUFA and PUFA for their comparative purpose. The pairs included 1) C16:0/C18:1 and C18:1/C20:4 and 2) C18:0/C18:2 and C18:0/C22:6. IMS scanning patterns revealed that the lipid mass to charge ratio (m/z) of 782.5 represented paired FA of C16:0/C18:1 with Na^+ adduction (Fig. 3C) and the m/z of 808.5, 830.5 and 856.5 represented a pair of C18:0/C18:2+ Na^+ , C18:1/C20:4 + H^+ and C18:0/C22:6 + Na^+ , respectively (data not shown). As translated the IMS scanning patterns of these paired FA species into the pseudo-color bands, it was clear that the SAT/MUFA (red) was distributed

towards the inferior portion of PC band, while the PUFA (green) was at the superior portion of the PC band (compared with primuline staining in panel A). In addition, the IMS patterns of PC band also indicated the variable amount (through the band intensities) of the paired FA species, thus directly allowed the comparison between the distributions of FA in each sample analyzed. The apparent increased in the band intensity of C18:1/20:4 was evident in Vsp compared to that in Tsp, which was well corresponded to those FA profiles shown in table 1.

3.4. Determination of unsaturated fatty acids by MC540 staining

We performed merocyanine (MC) 540 staining (an indicative dye that stains unsaturated FA in a highly fluidic membrane) (Gadella et al., 2000; Muratori et al., 2004) which may correlate the membrane lipid organization in different types of the sperm cells. The results revealed that MC540 stained moderately or intensely in Tsp with a patchy staining pattern around the rim of the main body and the base of anterior spike (Fig. 4A). The overall staining intensity was relatively weaker, particularly at the main body vicinity of Vsp. Quantitative analysis by flow cytometry indicated a missing fluorescent peak at the fluorescent intensities of $\sim 7.0 \times 10^3$ (Fig. 4B, dashed circle), while the additional fluorescent peak at $\sim 2.0 \times 10^2$ was present in Vsp stained with MC540. These flow cytometric results agreed well with the microscopic observations mentioned above.

3.5. Ultrastructure and FAME profiles of spermatid vesicles

We hypothesized that fluid isolated from male reproductive tracts of *M. rosenbergii* should contain epididymosome-like vesicles which serve as the key modulator for trafficking of

macromolecules leading to sperm membrane modification (Dacheux et al., 2005; Sullivan et al., 2005). We initially examined ultrastructure of SV that were isolated from spermatid fluid through ultracentrifugation. Generally, the shape of SV was relatively spherical but variable in their size (Fig. 5A). They showed either unilamellar or multilamellar appearances (insets). The unilamellar vesicles varied from 20 to 100 nm in diameter, whereas the multilamellar vesicles were more heterogeneous in size varying from 50 to 500 nm in diameter.

We further characterized lipid compositions of SV by HPTLC followed by quantitative analysis of each lipid subclass. It was shown that SV comprised three major lipid subclasses of lipids similar to those found in sperm plasma membrane, including CHO, SM and variable types of phospholipids (Fig. 5B). However, an inverse pattern in the amount of those lipid subclasses was notable when compared to that of sperm membrane lipids, namely, SM and PC were apparently reduced (with the relative density of 3.9, 2.2 respectively), while PE was non-measurable or absent in SV. The level of CHO apparently increased in these isolated SV (with the relative density of ~12.5) compared with that of sperm membrane (Fig. 5C).

We also analyzed FAME pattern in SV and the data were shown in Table 1. In general, all PUFA contents apparently increased when compared to Vsp, particularly EPA (Table 1, Fig 2). In contrast, MUFA (particularly OA) and SAT (particularly PA) significantly decreased in SV lipid contents (Table 1). Together, the FA profiles of SV appeared to be opposite to that of Vsp which then suggested the interchangeable lipid contents between sperm and their surrounding SV. With these corresponding alterations of the lipid patterns observed in sperm membrane and SV, we thus believe that there are shuffling process of lipid molecules between these two compartments in the adluminal compartment of male reproductive tract.

DISCUSSION

We have shown here comparative profiles of FA compositions in *M. rosenbergii* sperm and spermatid vesicles (SV). Membrane lipids of *M. rosenbergii* sperm are rather common towards those reported in mammalian sperm such as mice (Furimsky et al., 2005), other murines (Rejraji et al., 2006) and rabbits (Meirav Apel-Paz et al., 2003). However, membrane lipids in mammalian sperm are unique in the way that they are composed of high ratio of glycerophospholipids among various types of other phospholipids and neutral lipids (Mafia et al., 1985; Lenzi et al., 2000; Furimsky et al., 2005). Presence of sperm glycerophospholipids has been reported to be crucial for gamete interaction (White et al., 2000; Weerachatanukul et al., 2001). Alternatively, shrimp sperm do not possess glycerophospholipids in their lipid extracts (neither in the sperm lysates nor SV extracts). It is thus possible that shrimp sperm utilize other kinds of sperm ligand(s), part of which might be surface glycoproteins in concert with other lipid molecules to be imparted in this early fertilization process. Apart from glycolipids, alteration of other lipid subclasses has been documented during sperm residence in epididymis as part of sperm maturation. In murine sperm, the levels of PC, PE and PI compositions are quite stable throughout sperm transit in epididymis, while SM slightly increases but those of PS and CHO are reduced in distal epididymis (Rejraji et al., 2006). In *M. rosenbergii* sperm, changes in the level of many lipid classes and total FAME compositions were also observed when sperm moved towards distal male reproductive tract, similar to those reported in mammals mentioned above. It is worth noted that analysis of FAME compositions are usually performed in total or crude lipid extracts which did not allow the interpretation of in what specific lipid subclass and in what extent that the FA are altered. Our results thus filled this missing gap providing a fundamental

knowledge in a single lipid subclass analysis. Through IMS analysis, we could further compare the patterns of paired FA within an intact PC molecule with a single -esterified FA in FAME analysis. An example of the well-correlated data was the quantitative amount of SA (C18:0) and PA (C16:0) which were made up the major compositions of sperm membrane lipids. Higher SA than PA contents in the IMS profiles would suggest paring of SA with other FA species such as DHA, C22:6 or LA, C18:2 (Fig. 3).

To gain full fertilizing ability, sperm plasma membrane has to undergo extensive modification with a final net negatively charged property and higher membrane fluidity (Kawano et al., 2011). Molecular changes in the membrane involve both the alterations of lipid and proteins that constitute membrane architecture (Vanichviriyakit et al., 2004; Gadella et al., 2000; Cross, 2004). We addressed here the importance of lipid alterations that may mark maturation changes of sperm in order for them to gain fertilizing ability. Among SATs, PA (C16:0) and SA (C18:0) presented most extensively in the membranes of Tsp and Vsp. They made up the greatest proportion of membrane lipids of ~45%, the percentage which is consistent with mammalian sperm including human (48-50%) (Avelano et al., 1992; Lenzi et al., 2000). The level of SAT was rather constant when sperm transit along male reproductive tracts (Table 1, Fig. 2). This suggests their pivotal role in keeping proper membrane integrity that is mandatory for the sperm functions. For PUFA subtypes, DHA, C22:6, *n*3 (or omega 3) was the most predominant PUFA found in *M. rosenbergii* sperm, the results that are also consistent with that reports for mature spermatozoa and immature germ cells (Lenzi et al., 2000; Tavilani et al., 2006). DHA is thought to play a major role in the regulation of membrane fluidity in sperm. The level of DHA has shown to be reduced when sperm migrate from seminiferous tubules to cauda epididymis in mice (Ollero et al., 2000). Our result also showed that DHA and the overall PUFA (Fig. 2A)

significantly decreased when sperm moved into vas deferens. The results of MC540 staining and its quantitative analysis also supported the decrease of PUFA in V_{sp}. In addition, the amount of the major neutral lipid, CHO, was also reduced in the distal reproductive tract, which would infer a great impact on sperm maturation and capacitation as extensively described in mammals (Visconti et al., 1999; Cross, 2004).

In mammals, epithelial lining cells of reproductive tracts secreted membrane bound vesicles either epididymosomes or prostasome into the adluminal compartments (Cross and Mahasreshi, 1997, Sullivan et al., 2005). Organ-specific vesicles such as “epididymosomes” have been identified and shown to play a role in membrane modification (Frenette et al., 2010). Our TEM micrographs showed that shrimp reproductive epithelial cells secreted both uni- and multilamellar vesicles which were similar to those of mammals and may be structurally and functionally equivalent to epididymosomes in mammals (Rejraji et al., 2006; Girouard et al., 2008; 2011). It has been shown that alterations of lipid and protein compositions in sperm membrane are partly, if not all, modulated by molecular trafficking between sperm membrane and epididymosomes to exchange their macromolecule components. To the best of our knowledge, we have shown here for the first time the existence of membrane bound vesicles (so called, SV) that were isolated from the spermatid fluid of marine invertebrates. General lipid compositions of SV including phospholipids, sphingolipids and neutral lipids are quite similar to those in mammalian epididymosomes (Girouard et al., 2011). We additionally documented here their comparative changes of lipid subtypes and the FAME compositions with those of the resident V_{sp} sperm. It was apparent that the reduced level of SM and increased level of CHO in SV which was opposite to those of V_{sp} lipid patterns (Fig. 5). As reported for mammalian epididymosomes, we believe that reduction of sperm membrane CHO, increase of sperm SM and

the subsequence changes of FA in PC subclass, may be modulated by shuffling of sperm membrane lipids with their surrounding SV taking place in the adluminal compartment of vas deferens, which thus suggests sperm maturation process in this marine invertebrate species.

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- 1 **Table 1** Fatty acid contents prepared from the purified band of phosphatidylcholine (PC)
 2 extracted from sperm of testis, vas deferens and spermatid vesicles of *M. rosenbergii*.

	FA Species	Percent Distribution of Individual FA in PC		
		Tsp (%)	Vsp (%)	SV (%)
PUFA	C18:2	10.20 ± 0.56	7.67±0.92 ^a	9.97 ± 0.13
	C20:2	1.15 ± 1.63	0.44±0.76	2.35 ± 0.05 ^b
	C20:4n-6	2.51 ± 3.56	4.77±0.50	6.68 ± 0.08 ^b
	C20:5n-3	8.86 ± 1.95	2.40±4.16 ^a	11.39 ± 0.22 ^b
	C22:6n-3	9.94 ± 0.07	6.29±0.30 ^a	9.70 ± 0.81 ^b
MUFA	C16:1	Nd	2.70±0.40	2.35 ± 0.03
	C18:1n-9	25.60 ± 2.74	30.66±3.96 ^a	22.72 ± 0.26 ^b
SAT	C14:0	1.30 ± 0.63	2.95±0.81	1.60 ± 0.04
	C16:0	23.10 ± 0.17	24.58±0.17 ^a	14.73 ± 0.34 ^b
	C18:0	15.05 ± 1.51	17.65±0.24	16.25 ± 0.16

- 3 Tsp = testis sperm, Vsp = Vas deferens sperm, SV = spermatid vesicles, FA = fatty acid

- 4 ^{a,b} $P < 0.05$, determined by Student's t-test (n=4). Nd = non-detectable

Figure Legends

Figure 1. Male reproductive tract in *M. rosenbergii* and lipid profiles of sperm collected from different part of male reproductive tract. Lipids extracted from testicular (Tsp) and vas deferens (Vsp) sperm were extracted and subjected to Coomassie Blue and Azure A staining (B). The quantitative analysis of band intensities of individual lipid subclasses from the triplicate HPTLC experiments was expressed as mean intensity $\pm$ SD.

Figure 2. Distribution of fatty acids in the purified band of phosphatidylcholine (PC) in testicular and vas deferens sperm. Note a significant decrease (*) of total polyunsaturated fatty acid (PUFA) contents in the Vsp lipid extracts (A) and the significant change of PUFA *n*3 subclass (B).

Figure 3. Imaging mass spectrometric analysis of the paired fatty acids with the PC band on the lipid blots. The representative pairs of SAT/MUFA/PUFA were selected from primuline stained PC (A) and their density plot were compared between Tsp and Vsp (B). The representative mass/charge (*m/z*) pattern of C16:0/18:1 was shown in panel C.

Figure 4 Fluorescent labeling patterns of unsaturated fatty acid using merocyanine (MC) 540 staining. Live Tsp and Vsp were isolated and exposed to MC540. The fluorescent images were

1 acquired by confocal microscopy (A) and further quantitatively analyzed by flow cytometry (B).
2 Note that the fluorescent peak at $\sim 7.0 \times 10^3$ was absent in the Vsp panel. Bar, 10 μm .

3
4 **Figure 5.** Transmission electron micrographs of spermatic vesicles collected from vas deferens.
5 The spermatic vesicles were isolated by ultra-centrifugation, negatively stained and observed
6 under TEM. Note the uni- and multi-lamellar vesicles (insets) with the variable size of about 10-
7 500 nm. (A). Comparative lipid profiles of Vsp and SV after resolving by HPTLC followed by
8 Coomassie Blue staining (B) and further subjected to densitometric analysis (C). Data were
9 expressed as mean $\pm$ SE of the duplicate experiments. Bar, 100 nm.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5