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Comparative Studies on the Energy Metabolism in Spermatozoa of Four Closely Related Species of Sea Urchins (Genus *Echinometra*) in Okinawa

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ABSTRACT—Sea urchins of the genus *Echinometra* are abundant on Okinawa reef flats in southern Japan. The Okinawan *Echinometra* is designated into four sympatric and closely related species: A, B, C, and D (Ea, Eb, Ec, and Ed). The sperm head size and shape gradually changes to become longer and more slender according to the following order: Ea, Eb, Ec, and Ed. To obtain information regarding speciation in Okinawan *Echinometra*, this study examined comparatively the energy production system of spermatozoa of Ea, Eb, Ec, and Ed. All spermatozoa contained cholesterol and several kinds of phospholipids. Glycogen, glucose, and triglyceride were present at extremely low levels. After incubation in seawater, a decrease in the level of phosphatidylcholine (PC) was observed in all spermatozoa concomitantly with activation of motility and respiration. The hydrolysis of PC correlated with the activity of phospholipase A_2. Interestingly, the amount of PC consumed, the respiratory rate, and the phospholipase A_2 activity in spermatozoa of Ea and Eb were approximately two-fold higher than those of Ec and Ed. Ultrastructural studies showed that lipid bodies within mitochondria were present in the midpieces of all species of spermatozoa. They became small or disappeared after incubation in seawater. Thus, the results obtained strongly suggest that spermatozoa of Ea, Eb, Ec, and Ed all use PC located in the lipid bodies as a substrate for energy metabolism. Also, it seems likely that energy production activities in Ea and Eb spermatozoa are stronger than those in Ec and Ed. The properties of energy metabolism in different species of sea urchin may be related to their habitat.

Keywords: Sea urchin spermatozoa, *Echinometra* species, energy metabolism, ultrastructural studies, speciation.

INTRODUCTION

The sea urchin *Echinometra mathaei* which was first described by Blainville (1865), occurs in abundance on shallow reefs throughout the tropical to warm Indo-Pacific region. *E. mathaei* is well known as a species exhibiting extensive morphological variation in test shape and spine color. Therefore, the genus *Echinometra* in Okinawa Island, southern Japan, had been considered as a single species of *E. mathaei*. However, Okinawan *Echinometra* were recently recognized as four separate but closely related species, tentatively designed *Echinometra* species A, B, C, and D (Ea, Eb, Ec, and Ed) (Uehara *et al*., 1990, 1991; Arakaki and Uehara, 1991; Matsuoka and Hatanaka, 1991; Metz *et al*., 1991; Metz and Palumbi, 1996). Ea is characterized by white-tipped spines, well-defined bright milled ring, and dark skin on the peristome. Eb is characterized by spines with no white tip, with very faint milled rings, and dark skin on the peristome. Ec is characterized by spines without a white tip, but with a definite *Echinometra* species richly colored variation in spines. Ed is characterized by deep black spines, a faint milled ring, and dark skin around the peristome. Recent studies on their morphological characteristics (Arakaki *et al*., 1998) have suggested that Eb and Ed are the same as *E.*

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mathaei (Nagauni, Japanese) and *E. oblonga* (Himekuro-nagauni), respectively, with the other two species being called Ea (Tumajiro-nagauni) and Ec (Ryukyu-nagauni) (Palumbi *et al.*, 1997; Arakaki *et al.*, 1998). Although these species have been studied extensively (Uehara *et al.*, 1990, 1991; Arakaki and Uehara, 1991; Matsuoka and Hatanaka, 1991), many unsolved questions remain regarding the process of their speciation and the mechanisms that maintain their integrity.

We have demonstrated that the energy production system in sea urchin spermatozoa is related to the phylogeny in the class Echinoidea (Mita and Nakamura, 1998). Energy metabolism in sea urchin spermatozoa is an important consideration when swimming towards the egg. Spermatozoa of sea urchins of Echinoida, such as *Hemicentrotus pulcherrimus* (Mita and Ueta, 1988; Mita and Nakamura, 1993b) and *Paracentrotus lividus* (Mita *et al.*, 1994a) use endogenous phospholipid, particularly phosphatidylcholine (PC), as a substrate for energy metabolism. PC is abundant in sperm midpieces (Mita *et al.*, 1991), which contain several lipid bodies within mitochondria (Mita and Nakamura, 1992). Following initiation of swimming, the lipid bodies become small (Mita and Nakamura, 1992), coincident with a decrease in the levels of PC in the midpieces (Mita *et al.*, 1991). This is strongly suggestive of the PC available for energy metabolism being stored in the lipid bodies. In contrast, the spermatozoa of the orders Arbacioida, Diadematoida, and Clypeasteroida use endogenous triglyceride (TG) instead of PC (Mita, 1991; Mita *et al.*, 1994a, b, 1995). The midpiece of these spermatozoa contain a single mitochondrion and lipid globules, although there are no lipid bodies (Mita and Nakamura, 1993a; Mita *et al.*, 1994c, d, 1995). The lipid globules differ from lipid bodies in that the former are spherical and located in the posterior region between the base of the mitochondrion and the plasma membrane (Mita and Nakamura, 1993a). These spermatozoa obtain energy through oxidation of fatty acids from TG stored in the lipid globules.

The sea urchins *Echinometra* species belong to the order Echinoida. We have already reported that spermatozoa of Ea obtain energy from endogenous PC (Mita *et al.*, 1993b). However, little is known about energy metabolism in spermatozoa of other *Echinometra* species. To obtain further information regarding speciation in Okinawan *Echinometra*, the energy production systems of spermatozoa

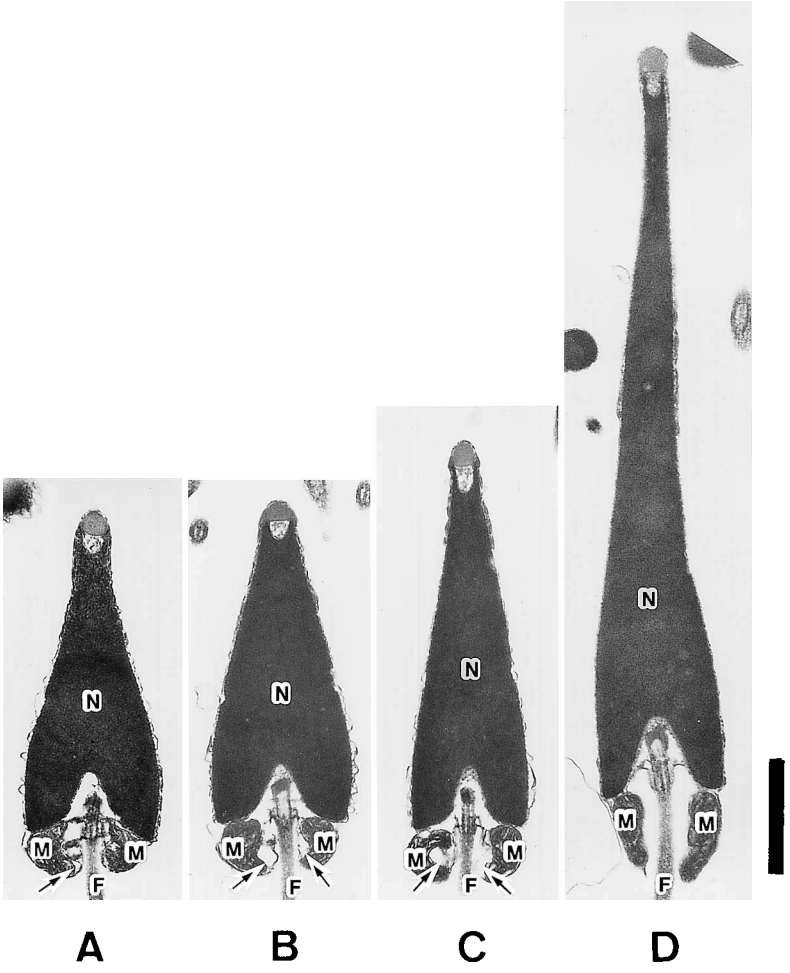

Fig. 1. Longitudinal sections through spermatozoa of *Echinometra* species, Ea (A), Eb (B), Ec (C), and Ed (D). Arrows show lipid bodies. F, flagellum; M, mitochondrion; N, nucleus. Scale bar is 1 μm.

from Ea, Eb, Ec, and Ed were analyzed. The midpieces of the spermatozoa from these four species were also observed ultrastructurally to determine whether the spermatozoon contains lipid bodies or lipid globules.

MATERIALS AND METHODS

Animals

The four species of sea urchins, Ea, Eb, Ec, and Ed, were collected at Sesoko Island, Okinawa, during their breeding season. Spermatozoa were obtained by forced spawning induced by injection of 0.5M KCl into the coelomic cavity. Semen was always freshly collected as "dry sperm" and kept undiluted on ice. The number of spermatozoa was counted using a hemocytometer.

Incubation of spermatozoa

Dry sperm were diluted 100-fold in artificial seawater (ASW) consisting of 458 mM NaCl, 9.6 mM KCl, 10 mM $CaCl_2$, 49 mM $MgSO_4$, and 10 mM Tris-HCl at pH 8.2, and incubated at 27°C. When required, each sample was centrifuged at 3,000 × g for 5 min at 0°C.

Analysis of Lipids

All the total lipids were extracted from the spermatozoa according to the method of Bligh and Dyer (1959) and analyzed by high-performance thin-layer chromatography (HPTLC), according to the method of Macala *et al.* (1983) with some modifications as described previously (Mita and Ueta, 1988). The amounts of PC, phosphatidylserine (PS), phosphatidylethanolamine (PE), cardiolipin (CL), cholesterol (CH), TG, and free fatty acid (FA) in spermatozoa were determined from the standard curves of the respective authentic lipids.

Assays of glycogen and glucose

Before and after incubation of dry sperm in ASW for 1 hr at 27°C, spermatozoa were homogenized with 0.6 M perchloric acid. The homogenate was used for determination of glycogen using an enzymatic method (Keppler and Decker, 1984). The acidified homogenate was centrifuged at 10,000 x g for 10 min at 4°C, and the supernatant was used for estimation of glucose after neutralization to pH 6.5–7.0 with 1 N KOH. Glucose was measured enzymatically according to the method of Kunt *et al.* (1984).

Analysis of fatty acid composition

Isolated PC, PS and PE on a thin-layer chromatograph (TLC) plate was subjected to methanolysis by heating with 5% HCl in methanol at 85°C for 2 hr as described previously (Mita and Ueta, 1988, 1989). Fatty acid methylesters were extracted with *n*-hexane, followed by N_2 blow evaporation. The residues were dissolved in a small amount of *n*-hexane and analyzed using a GC-8A gas-liquid chromatograph (GLC) (Shimadzu Instruments, Kyoto) equipped with a flame ionization detector and a coiled column. The column was packed with 15% EGSS-X (GL Sciences Inc., Tokyo) and operated at 190°C. Each fatty acid was also analyzed as trimethylsilyl derivatives by GLC (Mita and Ueta, 1992).

Assay of phospholipase A_2 activity

Dry sperm were washed with 0.21 M mannitol, 0.07 M sucrose, and 10 mM Tris-HCl at pH 7.5, and homogenized with 10 mM $CaCl_2$, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 50 mM Tris-HCl at pH 7.5. The homogenate was incubated with 4.75 kBq 1-palmitoyl-2-[1-^{14}C]linoleoyl-PC (1.9 GBq/mmol) for 1 hr at 27°C in a total volume of 0.4 ml. The lipids were extracted from each sample according to the method of Bligh and Dyer (1959). The radioactivity in the FA fraction obtained by TLC was measured using liquid scintillation spectrometry. The protein content of the homogenate was measured according to the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

Oxygen consumption

Oxygen consumption in a sperm suspension was measured polarographically with an oxygen consumption recorder (MD-1000, Iijima Electronics Mfg. Co., Aichi, Japan). Twenty-five microliters of dry sperm were incubated in 2.5 ml ASW in a closed vessel of the

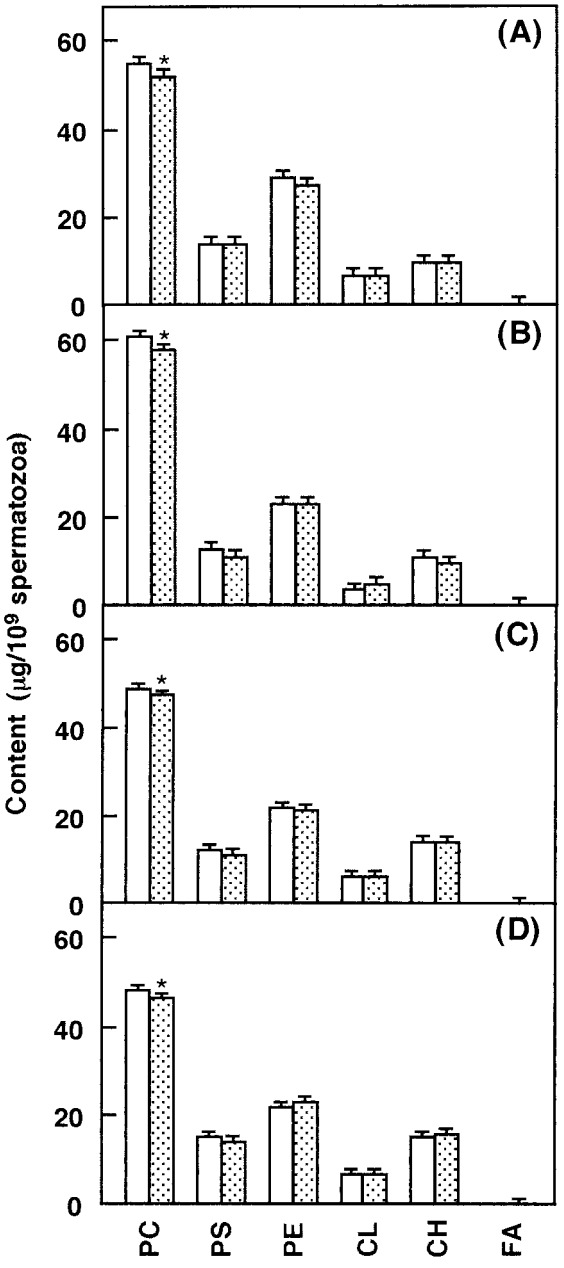

Fig. 2. Changes in the lipid levels after incubation of four species of *Echinometra* spermatozoa in ASW. Before (clear) and after (dotted) 100-fold dilution and incubation of dry sperm of Ea (A), Eb (B), Ec (C), and Ed (D) in ASW for 1 hr at 27°C, lipids were extracted and analyzed by TLC. Each value is the mean of three separate experiments. Vertical bars show SEM. *$P < 0.1$.

oximeter at 27°C. Total oxygen consumption was calculated from the respiratory rate and incubation period, as described previously (Mita and Yasumasu, 1983).

Electron microscope observation

The spermatozoa were prefixed in 2.5% glutaraldehyde-ASW solution. The fixed spermatozoa were rinsed with cold ASW and post-fixed with 1% OsO_4 in ASW. Samples were washed in distilled water, and then immersed in saturated aqueous uranyl acetate for block staining. After dehydration in a graded series of ethanol solutions, the specimens were embedded in epoxy resin and ultrathin sections were cut on a Reichert Ultracut ultramicrotome. After staining with lead citrate, these were examined using a Hitachi 7000 electron microscope.

Reagents

The phospholipids, CH, TG, and FA standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 1-Palmitoyl-2-[1-^{14}C]linoleoyl-PC was obtained from DuPont-New England Nuclear (Wilmington, DE, USA). All reagents and solvents were of analytical grade. HPTLC and TLC plates (silica gel 60) were obtained from Merck (Darmstadt, Germany).

RESULTS

It has been demonstrated that sperm size and shape vary among the four species of *Echinometra* (Arakaki *et al.*, 1998). To elucidate the differences between each type of spermatozoa, thin sections of Ea, Eb, Ec, and Ed spermatozoa were observed ultrastructurally by electron microscopy. Each spermatozoon consisted of a head, a midpiece, and a tail (Fig. 1). Confirming the observations in report by Arakaki and coworkers (1998), their heads changed gradually to longer and more slender according to the following order: Ea, Eb, and Ec. Those of Ed were very different from the others, being very long and slender. The sperm head was mostly occupied by the nucleus, and the head tip consisted of an acrosomal granule and a subacrosomal fossa (Fig. 1). It seems likely that the size of the sperm head depends on the shape of the nucleus, since the nucleus of Ed was very long and slender (Fig. 1D). In contrast, there were only small differences in the size and shape of the acrosomal granule and the subacrosomal fossa in the sperm head tip among the four species of *Echinometra*.

Previous studies have shown that sea urchin spermatozoa obtain energy for motility from an endogenous lipid (Rothschild and Cleland, 1952; Mohri, 1957; Mita and Yasumasu, 1983). It has been also reported that lipids in sea urchin spermatozoa of the order Echinoida are composed of CH and several kinds of phospholipids (Mita and Ueta, 1988; Mita and Nakamura, 1993b). Similar phospholipids

Table 1. Comparison of fatty acid composition of phosphatidylcholine in four species of *Echinometra* spermatozoa

Fatty acid	Percentage			
	Ea	Eb	Ec	Ed
14:0	8.0±1.7	2.9±1.1	4.1±2.5	5.0±2.0
15:0	tr.	tr.	tr.	tr.
16:0	34.6±1.9	31.6±3.9	31.6±2.8	40.4±2.6
16:1	tr.	4.5±2.2	8.5±4.4	tr.
18:0	15.2±4.5	14.1±1.3	10.5±1.4	14.5±1.1
18:1	6.8±1.7	11.7±0.9	10.9±0.5	tr.
20:1	tr.	tr.	tr.	tr.
20:4 (n-6)	28.8±1.7	20.6±4.2	19.1±2.5	28.9±0.7
20:5 (n-3)	7.3±0.5	12.9±2.3	12.1±1.3	11.2±2.5
Saturated	57.1±3.0	48.9±5.0	46.7±4.1	59.9±1.9
Monoenoic	6.8±1.7	17.5±3.1	20.4±5.3	tr.
Polyenoic	36.1±1.9	33.6±6.4	32.9±4.4	40.1±1.9

Each value is the mean±SEM obtained in three separate experiments. tr., trace amount (less than 0.1%).

Table 2. Comparison of fatty acid composition of phosphatidylserine in fou species of *Echinometra* spermatozoa

Fatty acid	Percentage			
	Ea	Eb	Ec	Ed
14:0	13.4±2.0	3.7±0.8	7.8±1.7	9.9±5.3
15:0	tr.	tr.	tr.	tr.
16:0	3.4±0.5	6.3±1.4	6.9±2.0	14.8±2.9
16:1	tr.	tr.	tr.	tr.
18:0	37.2±2.4	35.9±1.9	37.1±2.2	30.4±2.4
18:1	tr.	tr.	tr.	tr.
20:1	19.0±2.0	15.3±0.6	17.9±0.3	15.0±1.6
20:4 (n-6)	22.6±2.3	23.6±1.0	22.0±0.7	22.2±3.6
20:5 (n-3)	4.4±0.5	13.5±2.2	8.3±0.5	6.5±1.6
Saturated	54.0±4.5	46.0±4.1	51.8±0.7	56.4±6.7
Monoenoic	19.0±2.0	15.9±1.2	17.9±0.3	15.0±1.6
Polyenoic	27.0±2.6	38.1±4.0	30.3±1.0	28.6±5.2

Each value is the mean±SEM obtained in three separate experiments. tr., trace amount (less than 0.1%)

Table 3. Comparison of fatty acid composition of phosphatidylethanolamine in four species of *Echinometra* spermatozoa

Fatty acid	Percentage			
	Ea	Eb	Ec	Ed
14:0	16.1±0.6	17.0±1.4	17.3±2.0	17.3±1.9
15:0	tr.	3.1±0.5	2.3±0.3	3.1±0.1
16:0	34.8± 0.5	31.4±0.1	29.9±0.1	33.4±1.7
16:1	tr.	tr.	tr.	tr.
18:0	12.2±0.9	11.3±0.4	13.1±0.4	10.2±1.5
18:1	tr.	tr.	tr.	tr.
20:1	tr.	tr.	tr.	tr.
20:4 (n-6)	28.3±1.3	22.5±1.7	23.3±0.9	25.1±0.4
20:5 (n-3)	8.6±1.3	14.7±1.2	14.0±0.6	10.9±1.2
Saturated	63.1±2.3	62.7±2.3	62.7±1.4	63.9±0.9
Monoenoic	tr.	tr.	tr.	tr.
Polyenoic	36.9±2.6	37.3±2.3	37.3±1.4	36.1±0.9

Each value is the mean±SEM obtained in three separate experiments. tr., trace amount (less than 0.1%).

and CH were also detected in the spermatozoa of Ea, Eb, Ec, and Ed. Among the phospholipids, PC, PS, PE, and CL were identified in these spermatozoa (Fig. 2). PC was present at high concentrations in the dry sperm of each species. TG and cholesterol ester were not detectable, although FA was observed at trace levels.

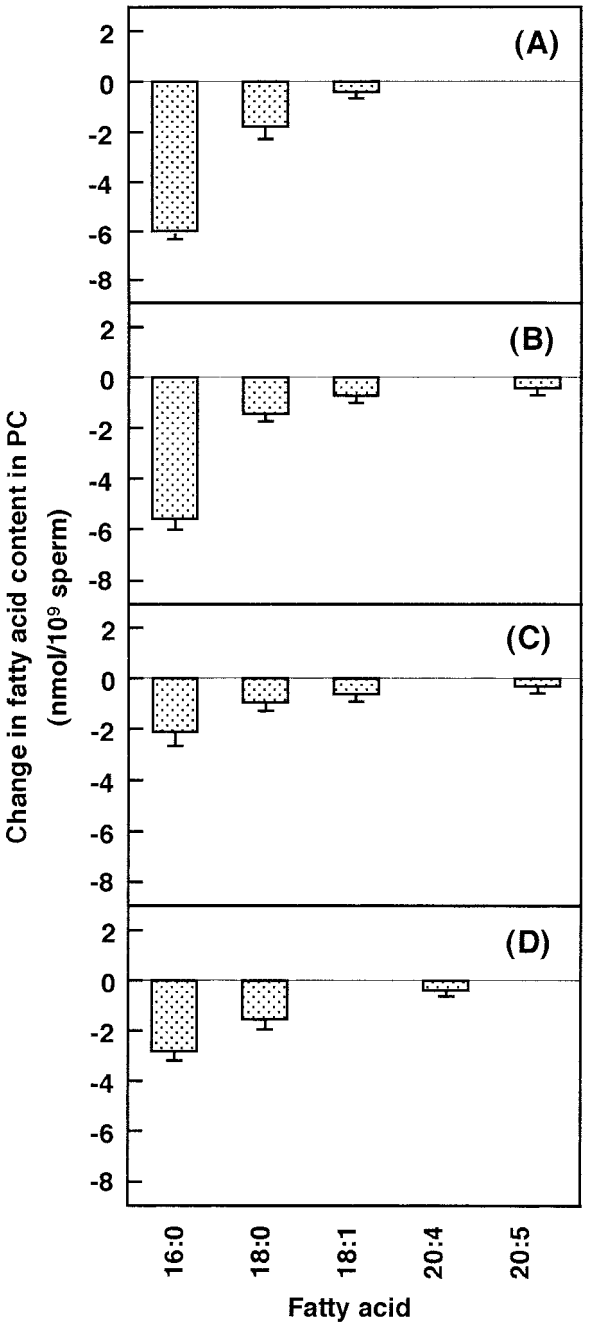

Fig. 3. Changes in the levels of fatty acid moieties in phosphatidylcholine after incubation of four species of *Echinometra* spermatozoa in ASW. The fatty acid composition was calculated from the absolute value of PC and its fatty acid composition in Ea (A), Eb (B), Ec (C), and Ed (D) spermatozoa before and after incubation for 1 hr at 27°C. Each value is the mean of three separate experiments. Vertical bars show SEM.

Fatty acid components of PC (Table 1), PS (Table 2), and PE (Table 3) were compared among the four species of *Echinometra* spermatozoa. In general, saturated fatty acids constituted more than 50% of total fatty acid moiety of PC, PS, and PE in these spermatozoa. Polyenoic fatty acids were present at approximately 30–40%, with arachidonic (20:4) and eicosapentaenoic (20:5) acids being predominant. The PC in spermatozoa of Ea, Eb, and Ec was composed of mostly palmitic (16:0) (32–40%), stearic (18:0) (10–15%), oleic (18:1) (7–12%), arachidonic (20:4) (19–29%), and eicosapentaenoic acids (20:5) (7–13%), except that there was only trace level of oleic acid in Ed (Table 1). PS mostly contained myristic (14:0) (4–13%), palmitic (16:0) (3–15%), stearic (18:0) (30–37%), eicosamonoenoic (20:1) (15–19%), arachidonic (20:4) (22–24%), and eicosapentaenoic acids (20:5) (4–13%) (Table 2). These fatty acid components in PS were almost the same in Ea, Eb, Ec, and Ed. Fatty acid moieties of PE from the four species of spermatozoa were mostly myristic (14:0) (16–17%), palmitic (16:0) (30–35%), stearic (18:0) (10–13%), arachidonic (20:4) (22–28%), and eicosapentaenoic acids (20:5) (9–15%) (Table 3).

When the dry sperm of Ea, Eb, Ec, and Ed were diluted 100-fold in ASW and incubated for 1 hr at 27°C, the PC content decreased significantly following initiation of flagellar movement, but other phospholipids remained at constant levels (Fig. 2). During incubation in ASW for 1 hr, approximately 3.0, 3.0, 1.5, and 1.8 μg of PC were consumed per 10^9 spermatozoa of Ea, Eb, Ec, and Ed, respectively. In contrast, the relative percentages of fatty acid components of PC in four species of spermatozoa hardly changed after incubation in ASW for 1 hr (data not shown). Based on the net content of PC shown in Fig. 2, changes in the levels of fatty acid moieties in PC during incubation for 1 hr were calculated. The PC composed of palmitic (16:0) and stearic acids (18:0) was particularly consumed during incubation (Fig. 3).

Glycogen was present in Ea, Eb, Ec, and Ed spermatozoa, but only at extremely low levels (approximately 1 μg/10^9 sperm). Glucose was present at trace levels. After incubation in ASW for 1 hr, there was little change in the glycogen and glucose levels in these spermatozoa (data not shown).

Table 4. Comparisons of oxygen consumption and phospholipase A_2 activity in four species of *Echinometra* spermatozoa

Echinometra sp.	Oxygen consumption (μmol O_2 uptake/hr/10^9 spermatozoa)	Phospholipase A_2 activity (nmol PC hydrolyzed/hr/mg protein)
Ea	0.23±0.02	54±6
Eb	0.25±0.02	62±7
Ec	0.11±0.01	27±2
Ed	0.14±0.01	33±3

Each value is the mean±SEM obtained in three separate experiments.

Since oxygen is required for oxidation of the lipid, the amount of O_2 consumed by spermatozoa was measured after dilution in ASW. Approximately 0.23, 0.25, 0.11, and 0.14 μmol O_2 was consumed during incubation of 10^9 spermatozoa of Ea, Eb, Ec, and Ed for 1 hr, respectively (Table 4). The respiratory rates of Ea and Eb were significantly (two-fold) higher than those of Ec and Ed.

Previous studies have shown that hydrolysis of PC in *H. pulcherrimus* spermatozoa occurs *via* the action of phospholipase A_2 (Mita and Ueta, 1988, 1990; Mita *et al.*, 1990). The phospholipase A_2 activity was measured in spermatozoa of Ea, Eb, Ec, and Ed. The homogenates of dry sperm from the four species of *Echinometra* were incubated with 1-palmitoyl-2-[1-^{14}C]linoleoyl-PC for 1 hr at 27°C, followed by

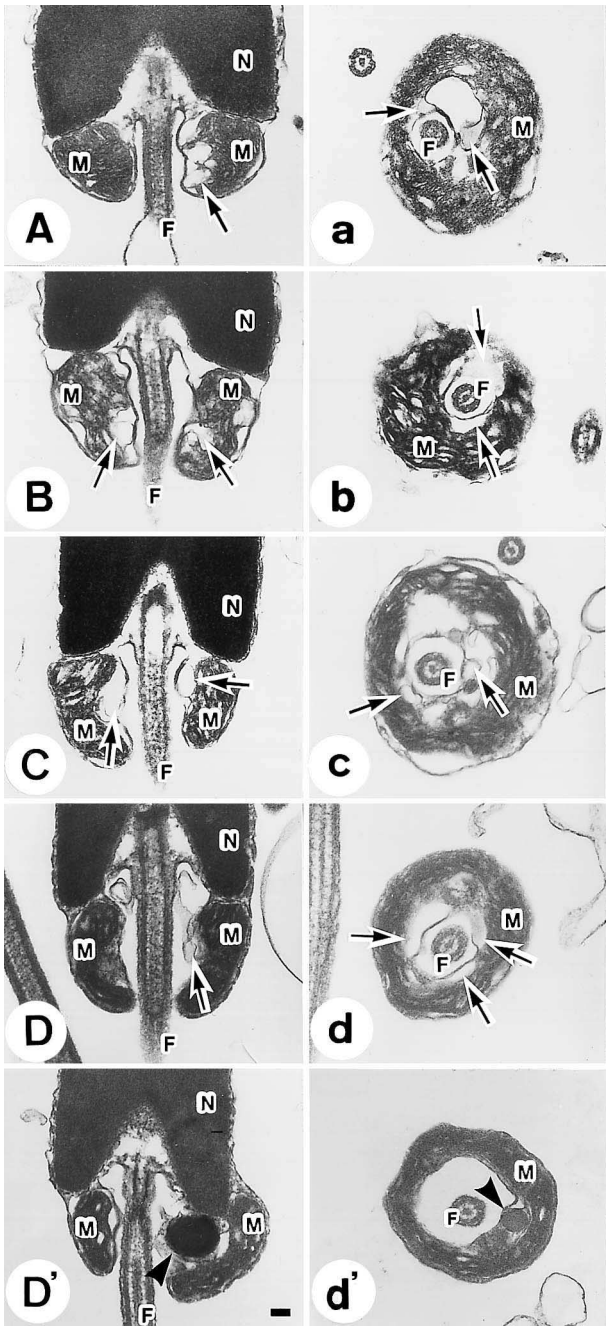

Fig. 4. Longitudinal and transverse sections through the mitochondrial region of *Echinometra* species, Ea (A, a), Eb (B, b), Ec (C, c), and Ed (D, d, D', d') spermatozoa in semen. D' and d' were examples that inclusion bodies were observed in Ed spermatozoa. Arrows and arrow heads show lipid bodies and inclusion bodies, respectively. F, flagellum; M, mitochondrion; N, nucleus. Scale bar is 0.1 μm.

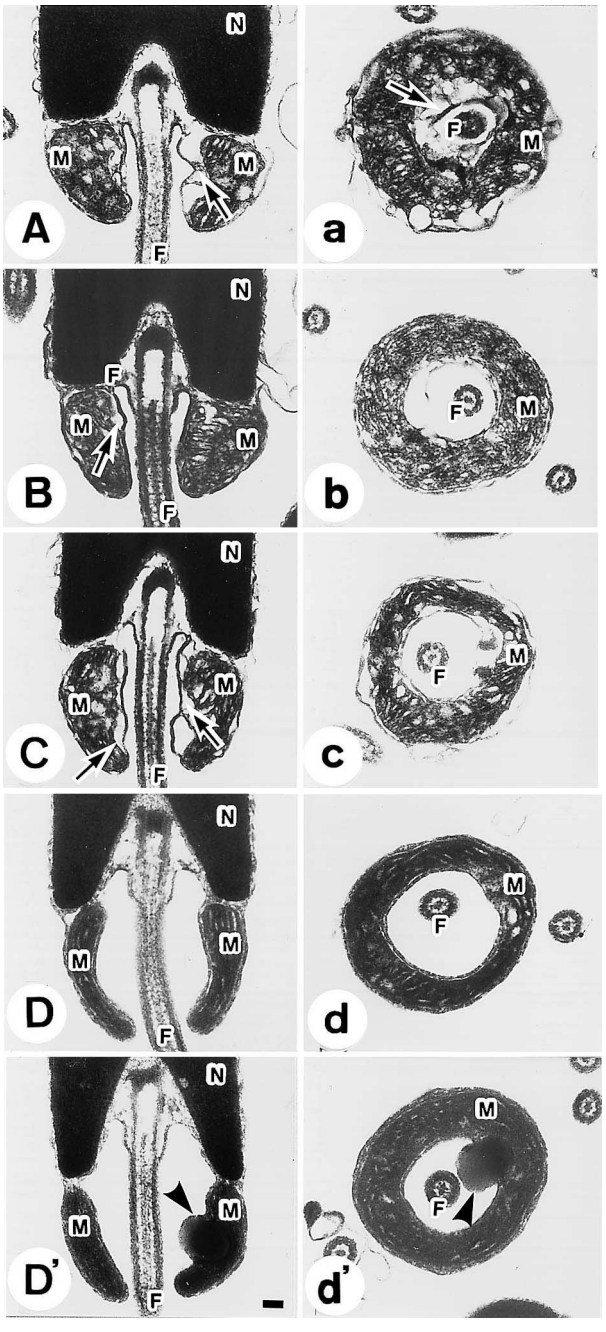

Fig. 5. Longitudinal and transverse sections through the mitochondrial region of *Echinometra* species, Ea (A, a), Eb (B, b), Ec (C, c), and Ed (D, d, D', d') spermatozoa after incubation in ASW for 1 hr at 27°C. D' and d' were examples that inclusion bodies were observed in Ed spermatozoa. Arrows and arrow heads show lipid bodies and inclusion bodies, respectively. F, flagellum; M, mitochondrion; N, nucleus. Scale bar is 0.1 μm.

extraction and separation of FA by TLC. Approximately 54, 62, 27, and 33 nmoles of PC were hydrolyzed for 1 hr by phospholipase A_2 in 10^9 spermatozoa of Ea, Eb, Ec, and Ed spermatozoa, respectively (Table 4). Thus, the phospholipase A_2 activities of Ea and Eb were significantly (two-fold) higher than those of Ec and Ed.

In the next experiment, sperm midpieces were observed ultrastructurally to determine whether the spermatozoon contains lipid bodies or lipid globules. The lipid bodies were present within mitochondria of the sperm midpieces in Ea (Fig. 4A and a), Eb (Fig. 4B and b), Ec (Fig. 4C and c), and Ed (Fig. 4D and d). The lipid bodies observed in a region between the mitochondrial outer and inner membrane were irregular in profile and had a low electron density. After incubation of spermatozoa for 1 hr at 27°C in ASW, the lipid bodies became small or disappeared completely (Fig. 5). Also, spherical inclusion bodies were observed in a few Ed spermatozoa within the mitochondrial matrix (Fig. 4D' and d'). After incubation in ASW, some of inclusion bodies became irregular in the surface (Fig. 5D' and d'). In contrast, spermatozoa of the four species of *Echinometra* did not possess the lipid globules which have been observed in sea urchin spermatozoa of the order Arbacioida, Clypeasteroida, and Diadematoida (Mita *et al*., 1993a, 1994c, d, 1995).

DISCUSSION

The results from this study strongly suggest that PC is a common endogenous substrate for energy metabolism in the spermatozoa of the four species of Okinawan *Echinometra*. This finding is consistent with the observations from Ea (Mita and Nakamura, 1993b) and other species of sea urchin spermatozoa of the order Echinoida (Mita and Ueta, 1988; Mita and Nakamura, 1993b; Mita *et al*., 1994b). However, the amount of PC consumed during incubation of these spermatozoa in ASW for 1 hr was only to about 5% in total (Fig. 2). Generally, phospholipids including PC are components of the plasma membrane. It is considered that most of PC is located in sperm plasma membrane. This suggests that a special kind of PC is available for utilization in energy metabolism in sea urchin spermatozoa.

Our study also showed that several lipid bodies were present within the mitochondria in sperm midpieces of the four species of *Echinometra* (Fig. 4). Similar lipid bodies located between the mitochondrial outer and inner membrane have been observed in sea urchin spermatozoa of the order Echinoida (Mita and Nakamura, 1992, 1993b; Mita *et al*., 1994c). These lipid bodies shrink and disappear after incubation in seawater (Fig. 5) (Mita and Nakamura, 1992). These findings suggest that the disappearance of the lipid bodies is correlated with the decrease in the level of PC. Presumably, the lipid bodies within mitochondria of the four species of *Echinometra* spermatozoa are reservoirs of PC that are used as an endogenous substrate.

Based on fatty acid moieties in PC (Table 2), the mass of PC in micro grams was converted to nanomoles using the approximate relation: 1 μg=1.33 nmol. PC consumption was calculated to be approximately 4.0, 4.0, 2.0, and 2.4 nmol per 1 hr in 10^9 spermatozoa of Ea, Eb, Ec, and Ed, respectively. Since PC is composed of two fatty acid moieties, this is equivalent to rates of approximate 8.0, 8.0, 4.0, and 4.8 nmol fatty acids/10^9 spermatozoa, respectively, from PC during incubation for 1 hr. These values well accord with those of actual consumed fatty acid in PC by Ea, Eb, Ec, and Ed spermatozoa, respectively (Fig. 3). It is possible that these fatty acids are utilized to produce energy by way of the β-oxidation and tricarboxylic acid cycle systems. From this, the amount of O_2 required for PC metabolism during incubation for 1 hr was determined to be 0.22 μmol in 10^9 spermatozoa for Ea, 0.22 μmol for Eb, 0.11 μmol for Ec, and 0.13 μmol for Ed. These values are quite consistent with those actually determined for O_2 consumption; 0.23, 0.25, 0.11, and 0.14 μmol O_2 per 1 hr in 10^9 spermatozoa of Ea, Eb, Ec and Ed, respectively (Table 4).

The energy metabolism in the four species of Okinawan *Echinometra* spermatozoa may be divided into two groups, since the activities of the PC metabolism (Fig. 2), the respiration (Table 4) and the phospholipase A_2 (Table 4) in Ea and Eb spermatozoa were two-fold higher than those of Ec and Ed. Previous studies have shown that the activities of sperm motility and respiration depend on intracellular pH (pH*i*) (Christen *et al*., 1982, 1983; Lee *et al*., 1983). Internal alkalization leads to activation of dynein ATPase, consequently stimulation of motility. The PC metabolism in sea urchin spermatozoa is also activated following increase in the pH*i* of spermatozoa (Mita *et al*., 1990). Following dilution in seawater, pH*i* of sea urchin spermatozoa has been shown to rise from 6.8 to 7.5 (Christen *et al*., 1982, 1983). It is assumed that PC metabolism is enhanced, with concomitant activation of respiration and motility. This may suggest that energy for sperm motility is produced more actively in the former than in the latter, which would make it likely that spermatozoa of Ea and Eb can swim faster and/or for a longer distance than those of Ec and Ed. However, it is strange that the properties of energy metabolism in Ed spermatozoa were similar to those of Ec spermatozoa, because the size and shape of the sperm head in Ed are quite different from those in Ec: this study has shown that the nucleus shape is long and slender in Ed sperm head (Fig. 1), but the reason for this morphological difference remains unclear.

The phylogenetic tree among the four species of Okinawan *Echinometra* reveals that Ea is more closely related to Ec than to the other species, and that Eb is more closely related to Ed than to the other species (Matsuoka and Hatanaka, 1991). This genetic information contradicts our results for sperm energy metabolism. It is therefore important that the ecological distribution and habitat preference are considered for the four species of Okinawan *Echinometra* (Uehara, 1990; Nishihara *et al*., 1991). These four species inhabit different habitats showing partial overlap. Ea sea urchins are mainly present in shallow tide pools. Eb are rare, and Ec and Ed are absence in the tide pool. Ec and

Ed occur mostly in burrows at the reef crest exposed to wave action. Eb inhabits the rocky area close to the ocean and at positions that are slightly below those of Ec and Ed. Since spermatozoa of Ec and Ed can use strong wave action, it may be relatively easy for their spermatozoa to meet an egg for fertilization. In contrast, spermatozoa of Ea and Eb should swim faster or for a longer distance than those of Ec and Ed. The properties of energy production in their spermatozoa may therefore be related to the individual habitat. It has also been shown that spermatozoa of sea urchin *Colobocentrotus mertensii* possess very long and slender heads as same as those of Ed (Uehara, 1990). Since *C. mertensii* inhabits dominantly the shore exposed to strong wave, it may be likely that the individual habitat influences the shape of sperm head in sea urchin.

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