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Changes in Lipids During the Ovary Maturation Process of *Balanus rostratus*

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***Balanus rostratus* is a large cold-water acorn barnacle distributed around the northern coast of the Pacific Ocean. In Mutsu Bay, Aomori, Japan, *B. rostratus*, which adhere naturally to scallop shells, are cultured as food. However, current culture methods do not generate sufficient supplies to satisfy market demand. Knowledge of the physiology of *B. rostratus* reproduction is important for the development of more efficient aquaculture methods. Previous studies have suggested that fatty acids and their metabolites play an important role in barnacle reproduction and development; however, few studies have analyzed lipids, particularly during ovary maturation. Here we analyzed lipid content, lipid class, and fatty acid composition of *B. rostratus* ovary throughout the year. The clutch in the present study was observed once per year at the end of November. The lipid content increased as the ovary underwent maturation. The proportion of triacylglycerol increased with increasing lipid content. The proportions of myristic acid, arachidonic acid, EPA and DHA significantly decreased in December. By contrast, the proportion of these fatty acids in lipid extracted from larvae was high relative to lipid extracted from *B. rostratus* ovary in December. These findings suggest that these fatty acids are transferred from the ovary to the larvae. Our novel findings on lipid metabolism during ovary maturation in *B. rostratus* indicate the importance of lipids during reproduction. This information may be useful in establishing methods for the aquaculture of *B. rostratus*.**

Key words: *Balanus rostratus*, lipids, fatty acids, EPA, DHA

INTRODUCTION

Balanus rostratus (Cirripedia, Crustacea) is a large, cold-water acorn barnacle that is distributed around the northern coast of the Pacific Ocean. Although this crustacean is considered to be a fouling organism for fishermen trying to culture scallops, it has recently been recognized as a valuable food resource. So far, however, there have been few attempts to establish aquaculture techniques. In particular, data on the ecology and physiology of *B. rostratus* are needed to establish efficient aquaculture methods.

In Japan, *B. rostratus* seem to live mainly on the pacific coast of the north Touhoku district, but they also live in the gulfs of Sagami and Ise, and the Seto Inland sea (Hiro, 1939; Utinomi, 1960; Yamaguchi, 1977). *Balanus rostratus* has one breeding season per year. In Mutsu Bay, *B. rostratus* is known to breed in November and produce offspring in December. Maturation of the reproductive organs have been classified into four stages (1: early development; 2: late development; 3: maturity; 4: retrogression) by histological analysis. In Mutsu Bay, the ovary of *B. rostratus* starts to develop in December, reaches the second stage in April, and matures in October (Kado et al., 2009). Kado et al. showed that the dry weight of the ovary and the ratio of carbon to nitrogen (C/N) reach a maximum in autumn every year, and suggested that the C/N ratio reflects the lipid content in the ovary. However, the relationship between ovary maturation and lipid content has not been documented.

Fatty acids are major elements of lipids. Fatty acids and their metabolites play important roles in barnacles as hatching factors, energy sources for eggs and larvae, and cold-tolerance factors (Holland et al., 1985; Tooke and Holland, 1985; Waldock and Holland, 1978). In addition, seasonal changes in fatty acid composition have been reported to be related to cold-tolerance in *Elminius modestus* and *B. balanoides* (Tooke and Holland, 1985). We previously studied the fatty acid composition of *B. rostratus,* showing that palmitic acid (PA), palmitoleic acid, stearic acid (SA), oleic acid, 11-octadecanoic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the major fatty acids comprising *B. rostratus* lipids. In addition, EPA accounted for the highest proportion of fatty acids in *B. rostratus* lipids (Yamada et al., 2017). In the present study, we have focused on and analyzed seasonal changes in the lipid and fatty acid composition of the ovary in *B. rostratus* in order to obtain

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physiological information to contribute to the development of aquaculture methods.

MATERIALS AND METHODS

Materials

Balanus rostratus was purchased directly from a fisherman in Mutsu, Aomori, Japan. This species is simultaneously-hermaphrodite, and almost all individuals have both a bloated seminal vesicle, and a matured ovary or a clutch of fertilized eggs (egg lamellae) in November. Similar to other acorn barnacle (balanomorph) species, *B. rostratus* is thought to mate by inserting its penis into adjacent individuals, but direct observation of mating in this species has not been reported. For the present study, the mature ovary or fertilized clutch lying free in the mantle cavity (Anderson, 1994) was readily obtained by the dissection of *B. rostratus* harvested from Aomori in late November. Fatty acids were purchased from Cayman Chemical (Ann Arbor, MI). Dipalmitoyl phosphocholine, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine, glyceryl tripalmitate, DL-a-palmitin and 1-palmitoyl-sn-glycero-3-phosphocoline were purchased from Sigma-Aldrich (St. Louis, MO).

Dissection and GSI

We analyzed *B. rostratus* that had been cultured for three years in Mutsu Bay. For dissection, a nipper blade was placed in the groove of the shell, the shell was then divided into halves, and the ovary or clutch was readily removed. We calculated the gonadosomatic index (GSI) by the weight of ovary divided by the summed weight of prosoma, thorax, cirri, muscle (lateral depressor muscle) and ovary.

Collection of larvae

To examine whether the EPA and DHA fatty acids that accumulate during ovary maturation are utilized during the reproductive period or transferred to larvae, we extracted lipids from larvae collected within a day of hatching, and analyzed the fatty acid composition. Barnacles with a clutch of fertilized eggs were purchased from fisherman in Mutsu bay just after the breeding season. They were kept in a 30-L tank with running sand-filtered seawater. Hatched larvae were transferred to another tank with an overflow, and then collected by pipette within a day of hatching. Each sample for analysis contained about 100 mg of larvae (approx. 4~8 × 10^3).

Lipid extraction and analysis of lipid class

To examine the relationship between ovary maturation and lipid, we analyzed the lipid content and lipid classes population in *B. rostratus* ovary. The ovary was frozen in liquid nitrogen and homogenized using a mortar. Lipid was extracted from a 2-g sample of ovary by the Bligh-Dyer method, the solvent was removed, and the lipid weight was measured. Lipid classes were analyzed by silica gel thin-layer chromatography (TLC). First, 10 μg of lipid extract was applied to a Chromarod (LSI Medience, Tokyo, Japan). Next, chloroform/methanol/water (42:24:2.5) was drawn 5 cm into the Chromarod. After drying the Chromarod, hexane/diethyl ether (50:30) was drawn 10 cm up the Chromarod. Lipids were detected and measured by TLC-FID (LSI Medience). Dipalmitoyl phosphocholine, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine, glyceryl tripalmitate, DL-a-palmitin and 1-palmitoyl-sn-glycero-3-phosphocoline were used as lipid standards.

Gas chromatography/mass spectrometry (GC/MS)

We examined seasonal changes in the fatty acid proportion of ovary lipids by GC/MS. Triacylglycerol (TAG) and phospholipid (PL) were esterified by incubation with methanol containing 5% hydrochloric acid at 50°C for 30 min. Analysis of fatty acid methyl esters was performed on a gas chromatography (Agilent Technologies 6890N)/mass spectrometry (Agilent Technologies 5975B) system using with a DB-23 gas chromatography column (60 m × 0.25 mm i.d. and 0.15-μm film thickness [Agilent technologies]). Helium (carrier gas) was passed through the column at a constant linear velocity of 40.0 mL/min, and the split ratio was 50. The initial oven temperature was maintained at 50°C for 1 min, increased to 175°C at a rate of 25°C/min, increased to 230°C at a rate of 4°C/min, and then maintained for 5 min. The temperatures of the inlet, interface, and ion source were 250°C, 280°C and 230°C, respectively. Elec-

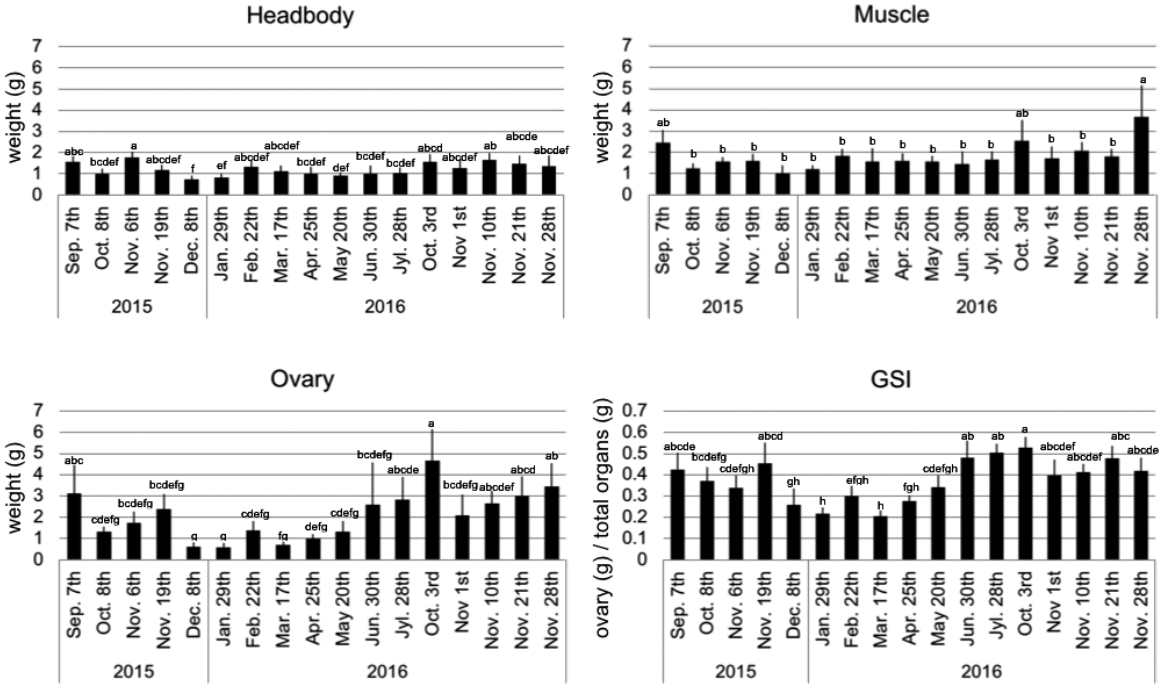

Fig. 1. Weight of organs and GSI in *B. rostratus*. Values are the mean ± s.d. of five samples. Values marked by different letters were significantly different ($P < 0.05$).

tron impact (EI, 70 eV) was used as the ionization mode. MS data were analyzed with Agilent GC/MSD ChemStation software. We measured the area of all fatty acids whose area was more than 3% of the largest one observed. MS fragment data were matched against entries in NIST atomic spectra database 2.0.

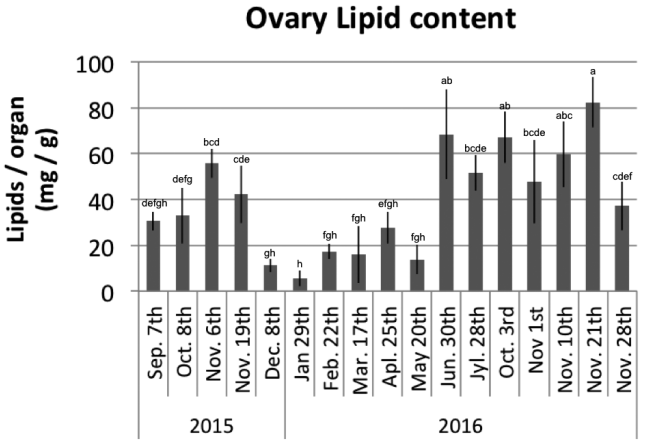

Fig. 2. Lipid content of *B. rostratus* ovary. Values are the mean ± s.d. of the ovary from five individuals. Values marked by different letters were significantly different ($P < 0.05$).

Liquid chromatography/hybrid quadrupole time of flight mass spectrometry (LC/QTOFMS)

To examine fatty acid metabolism, we analyzed the concentration of free fatty acids in *B. rostratus* ovary by LC-QTOFMS. Fatty

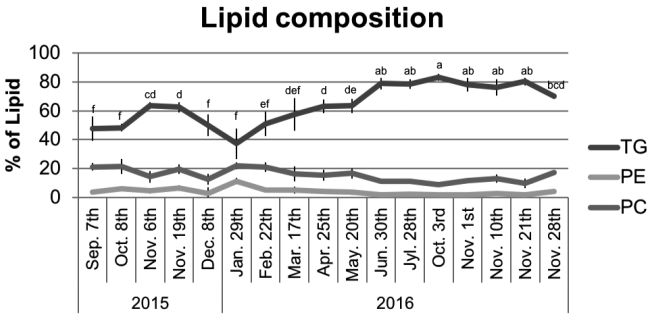

Fig. 3. Lipid composition of *B. rostratus* ovary. Values are the mean ± s.d. of the ovary from five individuals. TG, triacylglycerol; PE, phosphatidyl ethanolamine; PC, phosphatidyl chorine. Values marked by different letters were significantly different ($P < 0.05$).

Table 1. Fatty acid composition of *B. rostratus* ovary as a percentage of total fatty acid. Values are the mean ± s.d. of five samples in units of % of total fatty acids. Values marked by different letters were significantly different ($P < 0.05$).

		Lauric acid	Myristic acid	Palmitic acid	Palmitoleic acid	Stearic acid	Oleic acid	11-octadecanoic acid	Arachidonic acid	EPA	DHA
2015	Sep. 7th	1.16 ± 0.05^{abcd}	9.98 ± 0.11^{a}	15.66 ± 0.50^{bcdef}	14.70 ± 0.69^{abc}	4.42 ± 0.23^{c}	4.68 ± 2.45^{ab}	4.76 ± 0.21^{bcd}	2.20 ± 0.21^{cdef}	30.84 ± 1.19^{cde}	3.84 ± 0.26^{abc}
	Oct. 8th	1.02 ± 0.33^{abcd}	8.86 ± 0.92^{ab}	14.72 ± 0.58^{cdef}	13.92 ± 1.14^{abcd}	4.58 ± 2.16^{c}	3.40 ± 0.60^{b}	5.00 ± 0.49^{abcd}	2.24 ± 0.27^{bcdef}	32.47 ± 2.13^{bcde}	4.28 ± 0.27^{ab}
	Nov. 6th	1.08 ± 0.24^{abcd}	9.24 ± 0.21^{a}	15.02 ± 0.44^{cdef}	14.04 ± 0.47^{abcd}	3.26 ± 0.17^{c}	3.98 ± 0.66^{ab}	4.54 ± 0.22^{bcd}	2.16 ± 0.18^{defg}	32.90 ± 0.66^{bcde}	4.50 ± 0.39^{ab}
	Nov. 19th	0.72 ± 0.60^{cd}	8.15 ± 1.33^{ab}	16.33 ± 0.76^{bcd}	13.13 ± 1.47^{abcd}	4.18 ± 0.77^{c}	4.35 ± 0.65^{ab}	5.32 ± 0.64^{abc}	2.28 ± 0.36^{bcde}	34.28 ± 1.21^{bc}	5.02 ± 0.62^{a}
	Dec. 8th	1.03 ± 0.67^{abcd}	4.60 ± 3.04^{c}	21.00 ± 1.74^{a}	8.03 ± 5.80^{de}	19.33 ± 10.35^{a}	5.13 ± 0.25^{ab}	5.27 ± 2.11^{abcd}	0.43 ± 0.75^{h}	19.47 ± 6.61^{f}	1.90 ± 1.31^{cde}
2016	Jan. 29th	0.15 ± 0.30^{d}	2.93 ± 0.90^{c}	17.88 ± 2.24^{b}	5.40 ± 1.85^{e}	14.68 ± 5.02^{ab}	5.73 ± 0.78^{a}	6.70 ± 0.57^{a}	2.35 ± 0.42^{bcde}	26.50 ± 2.80^{ef}	2.88 ± 2.34^{abcd}
	Feb. 22th	1.34 ± 0.39^{abcd}	8.22 ± 0.82^{ab}	16.92 ± 1.16^{bc}	13.80 ± 1.19^{abcd}	7.42 ± 1.50^{bc}	4.02 ± 0.51^{ab}	4.86 ± 0.40^{bcd}	1.90 ± 0.45^{efg}	27.88 ± 2.08^{de}	1.90 ± 1.77^{cde}
	Mar. 17th	0.82 ± 0.65^{bcd}	5.98 ± 3.48^{bc}	17.64 ± 1.84^{b}	11.08 ± 6.71^{cd}	13.06 ± 7.01^{ab}	4.10 ± 0.73^{ab}	5.60 ± 1.54^{ab}	1.56 ± 0.23^{fg}	27.64 ± 4.06^{e}	2.66 ± 0.52^{bcde}
	Apr. 25th	0.98 ± 0.61^{abcd}	9.06 ± 0.77^{a}	14.58 ± 0.72^{def}	17.46 ± 2.11^{a}	3.62 ± 1.83^{c}	4.12 ± 0.90^{ab}	3.54 ± 1.20^{d}	1.64 ± 0.18^{efg}	32.74 ± 2.68^{bcde}	0.70 ± 1.57^{de}
	May 20th	0.68 ± 0.43^{cd}	8.12 ± 1.29^{ab}	15.82 ± 0.52^{bcde}	16.38 ± 0.88^{ab}	8.94 ± 4.16^{bc}	3.06 ± 0.30^{b}	5.06 ± 0.21^{abcd}	1.34 ± 0.36^{g}	26.96 ± 3.29^{e}	0.44 ± 0.98^{e}
	Jun. 30th	2.04 ± 0.58^{a}	10.58 ± 0.77^{a}	13.86 ± 0.71^{ef}	16.60 ± 1.31^{ab}	2.68 ± 0.61^{c}	3.38 ± 1.13^{b}	3.60 ± 0.19^{d}	2.58 ± 0.41^{bcd}	33.08 ± 1.67^{bcd}	1.64 ± 1.51^{cde}
	Jul. 28th	2.00 ± 0.41^{a}	10.26 ± 0.71^{a}	13.54 ± 0.59^{f}	15.94 ± 0.49^{abc}	2.62 ± 0.26^{c}	3.26 ± 0.71^{b}	3.86 ± 0.18^{cd}	2.80 ± 0.50^{bcd}	33.46 ± 2.05^{bcd}	3.10 ± 0.34^{abc}
	Oct. 3rd	1.96 ± 0.34^{ab}	10.38 ± 0.37^{a}	14.46 ± 0.64^{def}	15.76 ± 1.01^{abc}	2.30 ± 0.28^{c}	4.32 ± 0.49^{ab}	3.84 ± 0.19^{cd}	2.80 ± 0.46^{bcd}	32.18 ± 1.40^{bcde}	3.46 ± 0.46^{abc}
	Nov 1st	1.42 ± 0.80^{abc}	9.38 ± 1.42^{a}	14.20 ± 0.32^{def}	14.48 ± 1.45^{abc}	2.62 ± 0.67^{c}	3.74 ± 0.87^{ab}	4.32 ± 0.31^{bcd}	3.08 ± 0.22^{abcd}	34.30 ± 2.50^{abc}	4.00 ± 0.54^{ab}
	Nov. 10th	1.24 ± 0.81^{abcd}	9.02 ± 1.37^{a}	14.74 ± 0.70^{cdef}	13.92 ± 2.32^{abcd}	2.40 ± 0.35^{c}	3.78 ± 0.35^{ab}	4.52 ± 0.87^{bcd}	3.14 ± 0.33^{ab}	37.22 ± 3.82^{ab}	3.60 ± 0.34^{abc}
	Nov. 21th	1.78 ± 0.22^{abc}	9.82 ± 0.79^{a}	15.24 ± 0.44^{cdef}	14.86 ± 1.02^{abc}	2.84 ± 0.49^{c}	4.46 ± 0.42^{ab}	4.20 ± 0.32^{bcd}	2.82 ± 0.58^{abcd}	34.20 ± 1.85^{bc}	3.72 ± 0.36^{abc}
	Nov. 28th	0.20 ± 0.45^{d}	8.04 ± 0.51^{abc}	14.56 ± 1.22^{def}	11.62 ± 1.24^{abcd}	3.08 ± 0.43^{c}	3.48 ± 0.46^{b}	5.38 ± 0.26^{abc}	3.72 ± 0.59^{a}	40.32 ± 3.00^{a}	4.76 ± 0.38^{ab}

acids were separated on an InertSustain ODS-3 column (2.0 mm dia. × 250 mm; GL Science Inc.) by gradient elution (water containing 10 mM ammonium acetate/ acetonitrile, 55/45 to 5/95 in 25 min) at a flow rate of 0.2 mL/min. The column temperature was kept at 40°C. Compounds were identified and quantified by LC/QTOFMS (Agilent Technologies) using Agilent Mass Hunter Workstation Software (Agilent Technologies). The flow of the drying gas was 10 L/min, and the temperature was 325°C. The Vcap, fragmentor, and skimmer voltages were 3500, 125, and 65 V, respectively. The pressure of the nebulizer was 30 psig.

Statistical analysis

Statistically significant differences between the experimental groups were identified by one-way ANOVA and Tukey's post-hoc tests using the "R" software (https://www.r-project.org).

RESULTS

Lipid content and lipid classes of *B. rostratus* ovary

The weight of organs and gonadosomatic index (GSI) from September 2015 to November 2016 are summarized in Fig. 1. During the period 7 September 2015 to 28 November 2016, a clutch was observed only on 6 November 2015 and 28 November 2016. Thus, the breeding seasons in 2015 and 2016 appear to have been 6 November to 19 November, and 21 November to 28 November, respectively. The GSI increased from winter to summer, and was significantly higher in summer and autumn (30 June to 3 October, 2016) than in winter and spring (8 December 2015 to 20 May 2016). The GSI remained high until the breeding season.

The lipid content in the ovary was less than 10 mg/g in January 2016, but rose to more than 60 mg/g in June 2016 (Fig. 2). The lipid content in the ovary was significantly higher from 30 June to 21 November 2016 than from 8 December 2015 to 22 February 2016. Subsequently, the lipid content of the ovary remained at more than 40 mg/g until the breeding season in November 2016. The lipid content decreased in the clutch. Alongside the increasing lipid content in ovary, the population of triacylglycerol also increased from 37.3% in January 2016 to 78.8% in June 2016 (Fig. 3). In addition to the lipids displayed in Fig. 3, free fatty acids, diacylglycerol, and monoacylglycerol were also detected by TLC-FID analysis.

Fatty acid proportion of *B. rostratus* ovary

Next, we examined seasonal changes in the fatty acid proportion of ovary lipids by GC/MS. The lipids of *B. rostratus* ovary mainly comprised four saturated acids (lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), and stearic acid (C18:0)), three mono-unsaturated acid (palmitoleic acid (C16:1 n-9), oleic acid (C18:1 n-9), and 11-octadecanoic acid (C18:1 n-7), and three poly-unsaturated fatty acids (arachidonic acid (C20:4 n-6), EPA (C20:5 n-3), and DHA (C22:6 n-3)). The proportion of the ten major fatty acids in *B.*

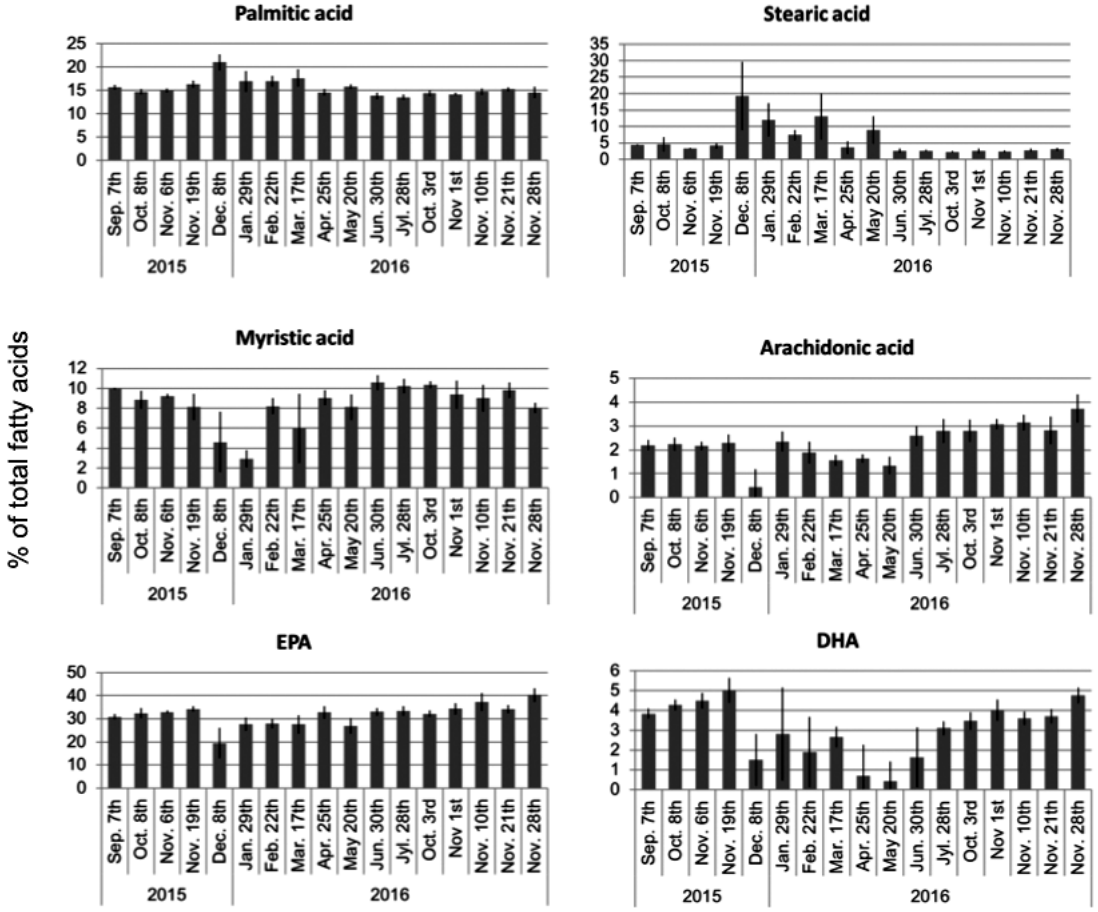

Fig. 4. Fatty acid composition of *B. rostratus* ovary. Values are the mean ± s.d. of the ovary from five individuals. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

rostratus ovary from September 2015 to November 2016 is summarized Table 1. In addition to these ten fatty acids, a very small amount of capric acid (C10:0), 9,12-hexadecadienoic acid (C16:2 n-4), 6,9,12-hexadecatrienoic (C16:3 n-4), arachidic acid (C20:0), 11-eicosenoic acid (C20:1 n-9) and 9-eicosenoic acid (C20:1 n-11) were detected in lipids from some of *B. rostratus* ovary samples. During this study, except for 8 December 2015, the most abundant fatty acid was EPA, although the proportions of palmitic acid and EPA similar on 8 December 2015 (Table 1). The concentrations of myristic acid, arachidonic acid, EPA and DHA decreased significantly in 8 December 2015, whereas those of palmitic acid and stearic acid significantly increased. The proportions of six fatty acids (myristic acid, palmitic acid, stearic acid, arachidonic acid, EPA, and DHA) showed different seasonal changes, as depicted graphically in Fig. 4. For example, the proportion of EPA significantly increased from 27.6% in March 2016 to 40.3% in November 2016, while that of DHA significantly increased from 0.7% in April 2016 to 4.7% in November 2016. By contrast, the proportion of palmitic acid significantly increased from 16.3% on 19 November 2015 to 21.0% in December 2015. The proportion of stearic acid also significantly increased from 4.1% on 19 November 2015 to 19.3% in December 2015.

Free fatty acids concentration in *B. rostratus* ovary

As above, EPA was found to be the most abundant fatty acid. A statistically significant seasonal difference in the

Table 2. Free fatty acid content of *B. rostratus* ovary. Values are the mean ± s.d. of five samples in units of μg/g (free fatty acid/wet weight of ovary). Values marked by different letters were significantly different ($P < 0.05$). EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.

		Lauric acid	Myristic acid	Palmitic acid	Palmitoleic acid	Stearic acid	Oleic acid	11-octadecanoic acid	Arachidonic acid	EPA	DHA
2015	Sep. 7th	0.13 ± 0.05^{c}	0.95 ± 0.54^{b}	0.55 ± 1.00^{abc}	0.63 ± 0.50^{abc}	0.16 ± 0.24^{d}	0.73 ± 0.80^{b}	0.03 ± 0.02	0.56 ± 0.73^{ab}	33.77 ± 29.87^{ab}	2.80 ± 3.04^{b}
	Oct. 8th	0.22 ± 0.15^{bc}	1.05 ± 0.72^{b}	0.36 ± 0.44^{bc}	0.46 ± 0.30^{c}	0.14 ± 0.14^{d}	0.56 ± 0.39^{b}	0.08 ± 0.13	0.15 ± 0.15^{abc}	19.49 ± 17.89^{b}	0.82 ± 0.54^{ab}
	Nov. 6th	0.60 ± 0.35^{a}	3.00 ± 1.85^{a}	1.06 ± 0.78^{abc}	1.47 ± 0.86^{a}	0.10 ± 0.17^{d}	1.05 ± 0.55^{ab}	0.01 ± 0.007	0.55 ± 0.45^{abc}	70.06 ± 46.93^{a}	2.72 ± 2.11^{a}
	Nov. 19th	0.12 ± 0.07^{c}	0.77 ± 0.45^{b}	0.71 ± 0.31^{abc}	0.32 ± 0.26^{c}	0.70 ± 0.27^{d}	0.51 ± 0.36^{b}	0.03 ± 0.008	0.01 ± 0.01^{c}	9.70 ± 6.71^{b}	0.37 ± 0.26^{b}
	Dec. 8th	0.19 ± 0.07^{bc}	0.49 ± 0.09^{b}	1.13 ± 0.65^{abc}	0.19 ± 0.16^{c}	10.69 ± 3.59^{a}	0.73 ± 0.50^{b}	0.03 ± 0.004	0.09 ± 0.09^{abc}	3.75 ± 2.46^{b}	0.52 ± 1.07^{ab}
2016	Jan. 29th	0.16 ± 0.08^{bc}	0.33 ± 0.16^{b}	0.80 ± 0.46^{abc}	0.09 ± 0.08^{c}	8.16 ± 4.83^{ab}	0.41 ± 0.26^{b}	0.02 ± 0.005	0.07 ± 0.04^{abc}	4.14 ± 1.23^{b}	0.13 ± 0.12^{b}
	Feb. 22th	0.21 ± 0.05^{bc}	0.70 ± 0.20^{b}	0.79 ± 0.44^{abc}	0.47 ± 0.18^{c}	3.65 ± 2.13^{bcd}	0.60 ± 0.24^{b}	0.02 ± 0.005	0.17 ± 0.07^{abc}	10.78 ± 4.28^{b}	0.26 ± 0.19^{b}
	Mar. 17th	0.21 ± 0.06^{bc}	0.60 ± 0.16^{b}	1.21 ± 0.11^{abc}	0.21 ± 0.12^{c}	9.54 ± 1.66^{ab}	0.37 ± 0.22^{b}	0.03 ± 0.004	0.08 ± 0.05^{abc}	6.87 ± 3.93^{b}	0.06 ± 0.07^{b}
	Apr. 25th	0.38 ± 0.17^{ab}	1.58 ± 0.79^{ab}	1.22 ± 0.42^{abc}	0.79 ± 0.40^{abc}	10.40 ± 6.47^{a}	0.84 ± 0.22^{b}	0.03 ± 0.01	0.27 ± 0.13^{abc}	22.30 ± 6.13^{b}	0.53 ± 0.16^{ab}
	May 20th	0.22 ± 0.16^{bc}	1.03 ± 0.57^{b}	0.81 ± 0.49^{abc}	0.70 ± 0.43^{abc}	5.36 ± 4.69^{abcd}	0.69 ± 0.52^{b}	0.03 ± 0.01	0.21 ± 0.16^{abc}	17.94 ± 14.31^{b}	0.50 ± 0.49^{ab}
	Jun. 30th	0.37 ± 0.18^{abc}	1.99 ± 0.97^{ab}	1.06 ± 0.44^{abc}	0.86 ± 0.34^{abc}	3.78 ± 2.67^{bcd}	1.35 ± 0.85^{ab}	0.04 ± 0.02	0.48 ± 0.19^{abc}	37.51 ± 15.25^{ab}	1.41 ± 0.73^{ab}
	Jul. 28th	0.28 ± 0.11^{bc}	1.25 ± 0.39^{ab}	0.72 ± 0.17^{abc}	0.70 ± 0.25^{abc}	0.67 ± 0.23^{d}	0.89 ± 0.32^{b}	0.02 ± 0.01	0.02 ± 0.01^{bc}	26.85 ± 10.57^{b}	1.39 ± 0.72^{ab}
	Oct. 3rd	0.33 ± 0.22^{abc}	1.16 ± 0.67^{ab}	1.18 ± 0.42^{abc}	0.79 ± 0.57^{abc}	5.44 ± 1.08^{abcd}	0.80 ± 0.37^{b}	0.03 ± 0.01	0.25 ± 0.27^{abc}	14.04 ± 10.73^{b}	0.71 ± 0.54^{ab}
	Nov 1st	0.41 ± 0.21^{ab}	1.69 ± 0.68^{ab}	1.53 ± 0.22^{a}	0.92 ± 0.37^{abc}	8.32 ± 1.15^{ab}	0.96 ± 0.18^{b}	0.05 ± 0.02	0.30 ± 0.13^{abc}	16.68 ± 7.38^{b}	0.65 ± 0.24^{ab}
	Nov. 10th	0.14 ± 0.08^{bc}	0.93 ± 0.24^{b}	1.43 ± 0.33^{ab}	0.53 ± 0.18^{bc}	7.42 ± 1.72^{abc}	1.03 ± 0.54^{ab}	0.07 ± 0.04	0.18 ± 0.04^{abc}	14.69 ± 4.22^{b}	0.49 ± 0.18^{ab}
	Nov. 21th	0.26 ± 0.09^{bc}	0.73 ± 0.39^{b}	0.13 ± 0.29^{c}	0.58 ± 0.29^{bc}	1.02 ± 0.90^{d}	0.61 ± 0.32^{b}	0.02 ± 0.01	0.14 ± 0.08^{abc}	15.98 ± 8.43^{b}	0.58 ± 0.35^{ab}
	Nov. 28th	0.21 ± 0.12^{bc}	1.09 ± 0.48^{b}	1.11 ± 0.68^{ab}	1.34 ± 0.44^{ab}	1.93 ± 0.60^{cd}	2.12 ± 0.75^{a}	0.04 ± 0.02	0.59 ± 0.39^{a}	35.01 ± 11.48^{ab}	1.75 ± 0.81^{ab}

Table 3. Fatty acid composition of *B. rostratus* larva as a percentage of total fatty acid.The unit of value is % of total fatty acids. EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid.

Date of hatched	Lauric acid	Myristic acid	Palmitic acid	Palmitoleic acid	Stearic acid	Oleic acid	11-octadecanoic acid	Arachidonic acid	EPA	DHA
Dec. 18th. 2017	0	5.5	15.1	11	4	2.8	8.2	4.4	37.9	10
Dec. 19th. 2017	0	1.3	15.8	11.9	3.8	2.9	8.7	2.9	36.3	11

concentration of free fatty acids in ovary was not observed (Table 2).

Fatty acid composition in *B. rostratus* larva

The lipid content of larva was 1.86 ± 0.14%, and the percentage of triacylglycerol in the lipids was 39.1 ± 5.1%. The percentage of EPA in total fatty acids was more than 35%, and that of DHA was more than 10% (Table 3). The proportion of 11-octadecanoic acid in total fatty acids in larva was high as compared with that in ovary. By contrast, lauric acid was not detected in lipids extracted from larva.

DISCUSSION

This study has revealed that *B. rostratus* accumulate triacylglycerol in the ovary with maturation. Both GSI and triacylglycerol increased significantly from 20 May to 30 June 2016 (Fig. 1). A clutch was observed only in late November. The features of ovary maturation and the reproductive cycle observed in our study are consistent with previous findings (Kado et al., 2009). Kado et al. demonstrated that the C/N ratio in ovary increased with maturation of the ovary, and suggested that the C/N ratio might reflect lipid content in ovary. Our study supports their suggestion; that is, the increasing C/N ratio observed by Kado et al. may reflect an accumulation of triacylglycerol in the ovary. The proportions of myristic acid, arachidonic acid, EPA and DHA in lipid of ovary were significantly higher in November 2016 than in December 2015. Lipid content and triacylglycerol of ovary were significantly decreased in December 2015 as compared with November 2015. The proportions of myristic acid, arachidonic acid, EPA and DHA were also significantly decreased in December 2015. By contrast, the proportions of arachidonic acid, EPA and DHA were higher in larva lipids than in the ovary in December 2015. These observations seem to indicate that the triacylglycerol and fatty acids that accumulate during ovary maturation are utilized for development of the embryo and larva in *B. rostratus*. It is conceivable that arachidonic acid, EPA and DHA are needed to produce lipid mediators that play an important role in larva development, as indicated by previous studies on lipid mediators and their functions. For example, Song et al. showed that trihydroxy fatty acids and monohydroxy fatty acids are important for the hatching process in barnacles (Song et al., 1990). Furthermore, Hill et al. identified 8-hydroxyeicosapentaenoic acid (8-HEPE), which is a metabolite of EPA, as a hatching factor in the barnacles *Elminius modestus* (Hill et al., 1988), and *Balanus balanoides* (Hill and Holland, 1992). These fatty acids, which are transferred from ovary to larvae, are thought to be involved in the hatching in *B. roustratus,* as well as in other barnacle species.

In terms of food, *B. rostratus* is one of the most valuable acorn barnacle species (López et al., 2010). Although the abundance of this species has been reported to have increased in the north-western part of the Sea of Japan (Silina and Ovsyannikova, 1998), aquaculture is strongly needed to secure a stable supply of edible barnacles. Aquaculture of the giant barnacle *Austromegabalanus psittacus* "Picoroco", which is an economically important edible species in Chile, similar to *B. rostaratus* in Japan, has been conducted by collecting natural juvenile barnacles (Bedecarratz et al., 2011). Although a few fishermen culture *B. rostratus* in the same way in Aomori, northern Japan, this production is not sufficient to meet a market demand. Techniques of breeding, hatching, and larval settlement in artificial conditions will need to be established to support the increasing economic importance of fisheries of this barnacle. These kinds of techniques have been successful for other barnacles so far. Laboratory cultures of *Balanus amphitrite* larvae (Qiu and Qian, 1997), *Balanus trigonus* larvae (Mishra, et al., 2001), *Megabalanus rosa* larvae (Kado and Hirano, 1994; Okano et al., 1996; Yoshimura et al., 2006), *Megabalanus volcano* (Kado and Hirano, 1994) and *Chinochthamalus scutelliformis* larvae (Yan, 2003) have also been reported. By contrast, little has been reported on laboratory cultures of *B. rostratus.* A reason for this might be that *B. rostratus* reproduces once in a year (Kado et al., 2009). Information on seasonal changes in lipid content, lipid class, and proportions of the 10 major fatty acids reported in this study might encourage the development of a method to control the reproduction of *B. rostratus* and contribute to the establishment of seedling techniques for these barnacles.

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COMPETING INTERESTS

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

H.Y., A.I. and K.T. designed the research; H.Y., M.H., A.S., K.K., and K.T. conducted the research; H.Y., analyzed the data; H.Y., A.I. and K.T., wrote the manuscript; and H.Y. had primary responsibility for the final content. All authors read and approved the final manuscript.

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