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A simplified gas chromatographic fatty acid analysis by the direct saponification/methylation
procedure and its application on wild tuna larvae

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Abstract A method for the direct preparation of fatty acid methyl esters was simplified for fatty acid analysis of a single fish larva using gas chromatography (GC). The method included the isolation of a larval trunk and drying in a glass vial, followed by saponification of all the contents without prior lipid extraction. Thereafter, the fatty acids released were methylated by trimethylsilyldiazomethane. This method has advantages over another method, direct acid-catalyzed transesterification, because both the saponification and methylation at room temperature can reduce loss of unsaturated fatty acids and formation of artifacts unavoidable in acidic reaction at high temperature. GC of the products showed that the simplified method can yield methyl esters without artifacts interfering analysis. More than 50 fatty acids were determined, which is twice as many as that previously analyzed by high-performance liquid chromatography. Observation of consistent small impurities in GC of blank tests allowed the accurate determination of fatty acids by correcting the peak areas. Dry matter weights (<3 mg) and the total fatty acid contents displayed a linear relationship. Fatty acid analysis of wild larvae of bluefin tuna, yellowfin tuna, and skipjack tuna collected from the waters around Japan ($n = 100$) revealed that the EPA level in bluefin tuna collected from the Japan Sea was significantly higher than that in the three species collected from Nansei Islands. The simplified direct saponification/methylation method will be a powerful tool to investigate growth and survival of individual larval tuna and other fish species.

44 **Abbreviations**

45 ARA arachidonic acid

46 DHA docosahexaenoic acid

47 EPA eicosapentaenoic acid

48 GC gas chromatography

49 HPLC high-performance liquid chromatography

50 HUFA highly unsaturated fatty acid

51

Introduction

Fatty acids play important roles in marine fish larvae. Long-chain highly unsaturated fatty acids (HUFA), such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (ARA), mediate the regulation of membrane fluidity, neural tissue development and sensory behavior, eicosanoid synthesis, flatfish pigmentation, stress modulation, gene regulation, and immune system regulation (Izquierdo & Koven, 2011). Low supply of HUFA causes abnormal development of marine fish larvae and leads to high mortality during larviculture (National Research Council, 2011). Furthermore, deficiency of DHA results in poor development of the central nervous system in larval fish (Masuda, Takeuchi, Tsukamoto, Sato, Shimizu, & Imaizumi, 1999), which induces late ontogeny of schooling behavior, i.e., an essential antipredator behavior for wild fish at larval and early juvenile stages (Nakayama, Masuda, Shoji, Takeuchi, & Tanaka, 2003). Fatty acids also function as a source of metabolic energy. Marine fish use saturated and monounsaturated fatty acids, such as palmitic acid (16:0) and oleic acids (18:1n-9), as energy sources (Sargent, Tocher, & Bell, 2003). Therefore, fatty acid composition and content are useful indicators for revealing qualitative and quantitative information on the nutritional status and survival potential of fish larvae.

In the last decade, more than 125 papers have been published that describe the fatty acid profiles of fish larvae. Most of the studies dealt with laboratory-reared larvae, and fatty acid analysis was usually performed for pooled larvae obtained from rearing tanks (Boglino, Gisbert, Darias, Estévez, Andree, Sarasquete, & Ortiz-Delgado, 2012; Matsunari, Hashimoto, Oda, Masuda, Imaizumi, Teruya, Furuita, Yamamoto, Hamada, & Mushiake, 2013; Izquierdo, Scolamacchia, Betancor, Roo, Caballero, Terova, & Witten, 2013; Hachero-Cruzado, Rodríguez-Rua, Román-Padilla, Ponce, Fernández-Díaz, & Manchado, 2014; Turkmen, Castro, Caballero, Hernandez-Cruz, Saleh, Zamorano, Regidor, & Izquierdo, 2017). However, such analysis cannot provide information on the individual variance of the larval population, because

body weight of the larvae was lower than the minimum detection limit if higher resolution of fatty acid components is necessary. To overcome this problem, development of a modified technique is needed.

Previously, we accomplished the fatty acid analysis of a single larva using reversed-phase high-performance liquid chromatography (HPLC) (Ando, Haba, Soma, Hiraoka, & Takatsu, 2007; Hiraoka, Takatsu, & Ando, 2014). Highly sensitive fluorescent detection and convenient derivatization procedure allowed the analysis of the isolated tissue such as eyes, head, and body trunk of the wild point-head flounder larva (Hiraoka, Takatsu, & Ando, 2014). On the other hand, gas chromatography (GC) of fatty acid methyl esters is also effective because of much higher resolution of fatty acid peaks. Several species of fish larvae were individually subjected to fatty acid analysis using GC with flame ionization detection coinciding with the HPLC analysis (Perga, Bec, & Anneville, 2009; Grote, Hagen, Lipinski, Verhey, Stenevik, & Ekau, 2011; Diebel, Parrish, Grønkjær, Munk, & Nielsen, 2012; Paulsen, Clemmensen, & Malzahn, 2014; Peters, Diekmann, Clemmensen, & Hagen, 2015; Teodósio, Garrido, Peters, Leitão, Ré, Peliz, & Santos, 2017). This fact indicates sufficient sensitivity of GC for fatty acid analysis of a single larva. The key to successful analysis of a single larva apparently depends on how to minimize fatty acid losses, contaminations, and artifacts during the preparation of methyl esters. The routine method that involves the cumbersome solvent extraction of total lipids and their purification from tissues, is not suitable for the analysis of a single larva. A more convenient method (Meier, Mjøs, Joensen, & Grahl-Nielsen, 2006) was applied for the analysis of a single larva of reared cod (Folkvord, Koedijk, Grahl-Nielsen, Meier, Olsen, Blom, Otterlei, & Imsland, 2017), wherein each larva was directly treated with HCl-methanol at 90°C for 2 h without prior lipid extraction and purification.

In the present study, we introduce another method for the preparation of methyl esters. Direct saponification/methylation method developed by Aldai et al. (Aldai, Murray, Nájera Troy,

& Osoro, 2005; Aldai, Osoro, Barron, & Nájera, 2006) and validated by Juárez et al. (Juárez, Juárez, Aldai, Avilés, & Polvillo, 2010) was reported to be more beneficial than the acid-catalyzed transmethylation, because this method does not require high temperature that is used in the HCl-catalyzed method, does not produce methoxy artifacts nor decomposition products from cholesterol, and does not alter olefinic bond position nor geometric configuration. Additionally, this method is not sensitive to the moisture present in the tissue samples (Juárez, Povillo, Contò, Ficco, Ballico, & Failla, 2008). Because the original method, which was previously applied to seafood required 1 g of tissue as a single sample, we modified and simplified the method for the analysis of a single larva. The modified method was applied for the analysis of three species of wild tuna larvae caught around Japan after checking its reliability.

Although Pacific bluefin tuna (*Thunnus orientalis*), yellowfin tuna (*T. albacares*) and skipjack tuna (*Katsuwonus pelamis*) are important species in aquaculture for marine foods (Mourete & Tocher, 2009), complete knowledge on the biochemical components of wild tuna larvae is not understood to date. Morais et al. (Morais, Mourente, Ortega, Tocher, & Tocher, 2011) suggested adequate provision of DHA is essential for normal development of farmed Atlantic bluefin tuna larvae (*T. thynnus*). We conducted field surveys in the two different spawning grounds for Pacific bluefin tuna around Nansei Islands (Ashida, Suzuki, Tanabe, Suzuki, & Aomura, 2015; Ohshimo, Tawa, Ota, Nishimoto, Ishihara, Watai, Satoh, Tanabe, & Abe, 2017) and Japan Sea (Okochi, Abe, Tanaka, Ishihara, & Shimizu, 2016), and collected larvae of Pacific bluefin tuna in both areas, yellowfin tuna and skipjack tuna around Nansei Islands in 2016. To the best of our knowledge, the present study is the first to reveal the individual variances of fatty acid profile for the wild caught Scombridae larvae. This paper reports and discusses the modified procedure of fatty acid analysis, its performance, and the analytical results in the tuna larvae.

Materials and Methods

Materials

Methyl myristate, methyl palmitate, methyl oleate, methyl *cis*-11-eicosenoate, methyl *cis*-13-docosenoate, and methyl tricosanoate (each >99%) were obtained from Sigma-Aldrich (St. Louis, USA). Trimethylsilyldiazomethane (10% solution in hexane) was obtained from Tokyo Chemical Industry (Tokyo, Japan). Other reagents and solvents were obtained from Wako Pure Chemical Industries (Osaka, Japan). All the reagents and solvents were of analytical grade, except for hexane, diethyl ether, and methanol, which were used after distillation in the laboratory.

Larvae of Pacific bluefin tuna ($n = 41$), yellowfin tuna ($n = 14$), and skipjack tuna ($n = 14$) were collected using a ring net (2 m diameter and 0.34 mm mesh size) or tucker trawl net (1 m square and 0.5 mm mesh size) in the waters around Nansei Islands during June 19–28, 2016. Pacific bluefin tuna larvae ($n = 31$) were also collected using the ring net from the Japan Sea off Tottori Prefecture during June 28–August 4, 2016. In both of the samplings, the net was towed at the sea surface and the tucker trawl net was towed 10–30 m depth for 10 min each time aboard Research Vessel ‘Shunyo-Maru’. Larvae were sorted on board as soon as possible, and stored individually in plastic cases at - 25°C. The larvae were photographed individually using a digital camera mounted on a dissecting microscope in the laboratory. Standard length and body height were measured on the digital images imported to a computer using ImageJ software (<https://imagej.nih.gov/ij/>). Each larva was dissected for removing head and digestive tract from body (Hiraoka, Takatsu, & Ando; 2014), and stored at - 25°C along with glazing water. DNA and morphological analyses confirmed the tuna species. Standard length, body height, and dry weight of the trunk are shown in Table 1, along with the analytical results.

Determination of dry matter weight

The body without the head and digestive tract, isolated from a single larva, was transferred to a pre-weighed screw-capped glass vial (1.5-mL volume). The trunk was dehydrated in 99.5% ethanol (50 μ L), followed by blowing through a stream of nitrogen. The muscle was further dried *in vacuo* in the same vial until a constant weight was reached.

Direct saponification/methylation of a single larva

Methyl tricosanoate in toluene (5.425 μ mol/mL corresponding to 2 mg/mL, 1 μ L) and 1 M KOH in 95% ethanol (50 μ L) were added into the vial containing the dried trunk. The trunk was directly saponified overnight in the dark at 23°C after being chopped with a needle. The trunk became pulpy during the reaction. The reaction was stopped by adding 2 M HCl (50 μ L) to acidify the saponification products. Solvents and excess HCl were evaporated by passing through a stream of nitrogen at 23°C until KCl was crystalized.

Fatty acids released in the vial were methylated in a mixture of toluene (80 μ L), methanol (20 μ L), and a 10% solution of trimethylsilyldiazomethane in hexane (50 μ L) at 23°C for 15 min. The reaction was stopped by adding acetic acid (5 μ L), and all solvents were again removed by passing through a stream of nitrogen.

Purification of methyl esters

Fatty acid methyl esters were purified by column chromatography using the silica gel 60 (Merck, Darmstadt, Germany) with hexane/diethyl ether (90:10, v/v) for elution. The silica gel (50 g) taken into a glass beaker was preliminarily washed in methanol and diethyl ether (each 150 mL $\times$ 3 times), dried in air, activated at 110 °C, and stored in a desiccator. The column made from a Pasteur pipette with a cotton plug was cleaned by eluting in 0.1 M KOH-ethanol, ethanol, and diethyl ether in the respective order (each 3 mL) and dried in air just before packing the silica gel in 1-cm height. The methyl esters dissolved in 100 μ L of hexane/diethyl ether (90:10, v/v)

were applied on the silica gel column and eluted with 1.5 mL of the same solvent.

GC

GC was carried out using a GC-4000 gas chromatograph (GL Sciences, Tokyo, Japan), equipped with a flame ionization detector and a capillary column of FAMEWAX (30 m × 0.32 mm i.d., 0.25 µm film thickness; Restek, Bellefonte, USA). Column temperature was programmed from 90°C (0 min) to 170°C at 20°C/min, then to 240°C at 4°C/min, and finally maintained at 240°C for 25 min. The injector and detector temperatures were maintained at 240°C. The carrier gas was helium, used at a linear velocity of 33.5 cm/s at 170°C (85 kPa); and the split ratio was 25:1. The purified methyl esters were re-suspended in 50–200 µL of hexane, which depended on larval size. In blank analysis with no larva, samples were dissolved in 20 µL of hexane. One µL of the solutions were injected in the splitless injection mode held for 1 min. The peak areas were measured in a Shimadzu C-R3A integrator (Shimadzu, Kyoto, Japan).

Quantification of fatty acids

Quantities of fatty acids were calculated from the GC results without gravimetry. The following formula was used to quantify each fatty acid (M_{FA}) in a sample:

$$M_{FA} \text{ (nmol)} = [(A_{FA}/A_{23:0}) - (A_{FA,blank}/A_{23:0,blank})] \times M_{23:0} \times F_{FA} \quad [\text{Eq. 1}]$$

where A_{FA} indicates peak area counts for the fatty acid, $A_{23:0}$ indicates peak area counts for internal standard (23:0), $A_{FA,blank}$ and $A_{23:0,blank}$ indicate the corresponding values observed in the blank analysis with no larva, $M_{23:0}$ indicates the number of moles of the internal standard (5.425 nmol), and F_{FA} indicates response correction factor experimentally determined for different chain-length fatty acids with the above GC system. Plots of peak area ratios in relation to molar ratios of standard fatty acid methyl esters (0.25–15 nmol, $n = 10$) mixed to 23:0 methyl ester (5 nmol in 100 µL of hexane) gave the following slope (m_{FA}) of the regression line through the origin, coefficient of determination (R^2), and F_{FA} ($= 1/m_{FA}$) for the fatty acid quantifications:

202 $m_{14:0} = 0.7362$ $R^2 = 0.9927$ $F_{14:0} = 1.358$ for C14-15 fatty acids,
 203 $m_{16:0} = 0.8408$ $R^2 = 0.9957$ $F_{16:0} = 1.189$ for C16-17 fatty acids,
 204 $m_{18:1n-9} = 0.9187$ $R^2 = 0.9974$ $F_{18:1n-9} = 1.088$ for C18-19 fatty acids,
 205 $m_{20:1n-9} = 0.9899$ $R^2 = 0.9984$ $F_{20:1n-9} = 1.010$ for C20 fatty acids,
 206 $m_{22:1n-9} = 1.0007$ $R^2 = 0.9985$ $F_{22:1n-9} = 0.999$ for C21-22 fatty acids,
 207 $m_{23:0} = 1.0000$ $R^2 = 1.0000$ $F_{23:0} = 1.000$ for C23-24 fatty acids.

208 The amount of total fatty acids (nmol) and total fatty acid content (nmol/mg) were calculated by
 209 summarizing M_{FA} and dividing it by dry weight of the sample, respectively. Fatty acid
 210 composition was expressed as mol% of each fatty acid in the total fatty acids.

211

212 **Statistical analysis**

213 The results obtained were presented as means $\pm$ standard deviations (SD). Differences between
 214 mean values were analyzed by one-way analysis of variance (ANOVA), followed by a multiple
 215 comparison test (Tukey-Kramer), where appropriate. Differences were reported as statistically
 216 significant at $P < 0.05$.

217 Bray-Curtis similarity matrix and permutational multi-variable ANOVA (PERMANOVA)
 218 (Anderson, 2001; McArdle & Anderson, 2001) were used to investigate how fatty acid profiles
 219 varied between species (pacific bluefin, yellowfin and skipjack tuna) and area (around Nansei
 220 Islands and Japan Sea). Non-metric multidimensional scaling (MDS) analysis was performed to
 221 visualize differences of fatty acid composition among species and sampling area. For these
 222 analysis, statistical software R version 3.3.1 (R Core Team, 2016) with the “vegan” packages
 223 were used with data of 44 fatty acids present in proportions >0.1 mol%.

224

225 **Results**

226 **Performance of the preparation method**

In this study, we minimized the concentrations and volumes of the reagents and solvents accordingly for a single larva, which has around 0.1–0.3 mg of dry weight. We also simplified the method by omitting the extraction steps involving the non-saponifiable matters and fatty acid products inserted between the saponification and methylation in the original procedure (Aldai, Murray, Nájera Troy, & Osoro, 2005; Aldai, Osoro, Barron, & Nájera, 2006). Hence, drying of muscle sample, saponification, and methylation could be carried out in a single vial.

GC analysis showed the occurrence of more than 50 fatty acid components, confirming that the modified method can yield methyl esters (Fig. 1a). Detection limit of the fatty acid methyl ester was 50 fmol under the GC conditions ($S/N = 3$). Artifacts of trimethylsilyl esters of fatty acid can be formed during methylation with trimethylsilyldiazomethane in the absence of benzene and methanol (Park, Albright, Cai, & Pariza, 2001). Toluene and methanol were added for initiating methylation to prevent artifacts in the modified method. GC of tuna larva showed no fatty acid-like unusual peaks.

Several minor peaks appeared in the chromatogram obtained in the blank test, wherein all processes were conducted without any larva (Fig. 1b). Some of them overlapped with the shorter-chain saturated fatty acid peaks. However, the peak areas maintained constant values in repeated blank tests using common reagents and solvents. This result indicated that these peaks were not caused from inconsistent contaminations, but from consistent impurities in the reagents and solvents. Commercial trimethylsilyldiazomethane was reported to contain impurities with trimethylsilyl that would interfere with the fatty acid analysis by GC (Park, Albright, Cai, & Pariza, 2001). The peaks found in Fig. 1b probably included these impurities. However, each peak area was less than 2% in comparison to the internal standard 23:0 (5.425 nmol).

Repeatability was checked by replicated analyses ($n = 6$) in the muscles of a juvenile tuna (4.16 ± 0.17 mg of dry muscle). Coefficient of variation was less than 5.57% for the fatty acid composition (Fig. 2a). These values were comparable to previous findings observed in the

internal validation of the original method (Juárez, Juárez, Aldai, Avilés, & Polvillo, 2010).

Plotting the observed fatty acid contents vs. dry weights of developing muscle ($n = 11$) taken out from a tuna individual showed a linear relationship in the range up to 3 mg of dry muscle (Fig. 2b); correlation coefficient (R) was 0.991.

Fatty acid composition of wild tunas larvae

The fatty acid contents in the dry weight of the larval trunk ranged from 147.4–219.7 nmol/mg (Table 1). No significant difference in fatty acid content was observed among the tuna larvae. The most abundant fatty acids were 16:0 and DHA, accounting for approximately 25 mol% and 22 mol% of total fatty acids, respectively. Other major fatty acids (>5 mol%) were 18:0, 18:1n-9, and EPA. ARA was found at the proportions of 1.48–1.86 mol% of total fatty acids.

The MDS plot of fatty acid profiles showed clear separation of Pacific bluefin tuna caught in Japan Sea from others caught in around Nansei Islands (Fig. 3). Skipjack tuna showed wide variation while yellowfin tuna relatively aggregated. These fatty acid profiles were significantly different between species and sampling area (Fig. 3; PERMANOVA, $P = 0.001$).

Five major fatty acids (except 16:0) showed significant differences in the proportions among the species and/or locations (Table 1; Fig. 4). It is remarkable that 18:1n-9 and EPA were significantly higher in bluefin tuna of the Japan Sea than in all the species of Nansei Islands. EPA remarkably increased with the increase in standard length; which was contrary to 18:1n-9 that decreased with the increase in length (Fig. 4). ARA and DHA in all species were almost stable throughout the larval stage. The molar ratios of DHA/EPA ranged from 2.40–3.29 in the four tuna varieties (Table 1), and the ratio of Pacific bluefin tuna in the Sea of Japan was significantly lower than the other specimens. The DHA/EPA ratios of Pacific bluefin tuna in the Nansei Islands waters were almost stable, however the ratios of Pacific bluefin tuna in the Sea of Japan, yellowfin tuna and skipjack tuna decreased with size.

Discussion

Validation of GC analysis

Previously we used reversed-phase HPLC for fatty acid analysis of a single fish larva (Hiraoka, Takatsu, & Ando; 2014). The analysis of fluorescent 9-anthrylmethyl ester derivatives could give fatty acid peaks with detection limit of 65 fmol ($S/N = 3$) (Ando, Haba, Soma, Hiraoka, & Takatsu, 2007). GC under the present conditions showed a similar detection limit (50 fmol, $S/N = 3$). This result confirmed a view that conventional GC equipped with flame ionization detector is usable for fatty acid analysis of a single fish larva.

The most disadvantageous point of the HPLC method was low resolution of fatty acids. Although EPA and DHA could be separated from other fatty acids (Ando, Haba, Soma, Hiraoka, & Takatsu, 2007), peak resolutions (R_s) among ARA, 20:4n-3, 22:5n-6 and 22:5n-3 were no more than 0.6–0.7, which are about one half of complete resolution ($R_s = 1.5$). Peaks of 18:3n-3 and 18:2n-6 overlapped 18:3n-6 and 16:1, respectively. Positional isomers of monounsaturated fatty acids were also inseparable. The HPLC analysis gave only 22 peaks for fish oil fatty acids. In this study, GC analysis gave more than 50 peaks including the above biologically important fatty acids and their isomers (Fig. 1a; Table 1). This analysis enables comparison of fatty acid profiles of fish larvae with those reported for other life stages and other species of fish.

Direct preparation of methyl esters without lipid extraction is suitable in case of small biological tissues, such as those from a single larva. There are two types of direct methods (Juárez, Povillo, Contò, Ficco, Ballico, & Failla, 2008): 1) acid-catalyzed transmethylation; and 2) base-catalyzed saponification, followed by methylation. The former method was employed in the most recent analyses of reared cod larvae individuals (0.07–9 mg, dry weight) (Folkvord, Koedijk, Grahl-Nielsen, Meier, Olsen, Blom, Otterlei, & Imsland, 2017) and pooled Baltic herring samples (0.4–0.5 g, wet weight) (Lind, Huovila, & Käkälä, 2018). However, the acid-

catalyzed method forms artifacts from tissue components during long-time reaction at high temperature (Carrapiso & Garcia, 2000; Aldai, Murray, Nájera Troy, & Osoro, 2005; Aldai, Osoro, Barron, & Nájera, 2006; Juárez, Juárez, Aldai, Avilés, & Polvillo, 2010). In fact, we observed many GC peaks interfering with C22-C24 fatty acids, when methyl esters were prepared from juvenile tuna muscle by heating it in 2M HCl-methanol at 90°C for 2 h (chromatogram not shown). These peaks were not removed by column chromatography.

Formation of artifacts is an essentially inevitable disadvantage of direct acid-catalyzed method.

In contrast, the present saponification/methylation method did not show such artifact peaks (Fig. 1a). Alkaline KOH-ethanol can react with tissue components under conditions different from those of acidic methanol. Saponification does not require high temperature. While the reaction was carried out at 60°C for 1 h in the original method (Aldai, Murray, Nájera Troy, & Osoro, 2005; Aldai, Osoro, Barron, & Nájera, 2006), our method employed cold saponification at 23°C for 18 h. This cold saponification probably lowered formation of artifacts from tissue components and loss of polyunsaturated fatty acids caused by isomerization and oxidation. Minor peaks found in blank test were not artifacts but impurities independent of tissue components (Fig. 1b). The improvements for minimizing the use of reagents, solvents, and glass apparatuses and for simplifying the process seem to result in low levels of impurity peaks in GC analysis. In this study, fatty acid analysis was achieved for 0.04 mg of dry muscle isolated from a 3.320 mm-standard length larva. This level is a half of the minimum weight previously analyzed for cod larvae by the acid-catalyzed method. The improved direct saponification/methylation method is superior to acid-catalyzed method for accurate GC analysis of fatty acids in samples with low mass, such as individual larval fish.

This is the first report that the direct saponification/methylation method can be used for preparation of fatty acid methyl esters from fish larvae. Fish larvae vary in weight according to the species and days posthatch, which differ from the analysis of seafood specifying a quantity

of starting material. However, good repeatability of the seafood analysis (Juárez, Juárez, Aldai, Avilés, & Polvillo, 2010) held for the analysis of much smaller muscle from tuna (Fig. 2a). A linear relationship (Fig. 2b) indicated efficient applicability of the modified method to a wide variety of fish larvae. In this respect, the saponification/methylation method is again superior to acid-catalyzed method, where an increase sample size led to a decrease in recovery of fatty acids (Abdulkadir, & Tsuchiya, 2008). The procedure of the present study is accurate and suitable for fatty acid analysis of a single larva.

Fatty acid composition

The method modified and simplified in this study successfully indicated the characteristics of fatty acid contents for wild caught pacific bluefin, yellowfin and skipjack tuna, especially two different spawning areas for Pacific bluefin tuna. In the present study, fatty acid components of three tuna species in the Nansei area and two nursery areas of bluefin tuna were significantly different. The polyunsaturated fatty acid composition of reared Atlantic bluefin tuna larvae tended to reflect their dietary composition (Ortega & Mourente, 2010; Betancor, Ortega, de la Gándara, Tocher, & Mourente, 2017). In the field, previous studies reported the different feeding habits among two tunas and skipjack tuna (Uotani, Matsuzaki, Makino, Noda, Inamura, & Horikawa, 1981; Uotani, Saito, Hiranuma, & Nishikawa, 1990; Kodama, Hirai, Tamura, Takahashi, Tanaka, Ishihara, Tawa, Morimoto, & Ohshimo, 2017). These previous studies indicate that the differences among the three species in the Nansei area and bluefin tuna in the Sea of Japan and Nansei area could be influenced by differences in prey fatty acid composition. Further testing of particulate organic matter or zooplankton fatty acid components should be evaluated in the future.

Tuna species are considered unique as they contain high levels of DHA and very high DHA/EPA ratios (Mourente & Tocher, 2009). The muscles of the young or adult tunas living

around Japan showed DHA levels ranging from 25–36% (bluefin tuna) (Ishihara & Saito, 1996), 22–36% (yellowfin tuna) (Saito, Ishihara, & Murase, 1996), 24–30% (albacore *Thunnus alalunga*) (Murase & Saito, 1996), and 28–34% (bonito *Euthynnus pelamis*) (Watanabe, Murase, & Saito, 1995) of the total fatty acids. In the present study, the fraction of DHA among three species was approximately 22 mol%, similar to previous studies. DHA/EPA ratios reported for the above tunas ranged from 3.4–5.8 (bluefin tuna), 6.7–9.0 (yellowfin tuna), 3.1–8.1 (albacore), and 3.1–5.4 (bonito). The juvenile and young skipjack tunas (0.194–62.840 g) have DHA levels similar to those of adults (more than 29%); along with DHA/EPA ratios ranging from 2.6–3.7 (Tanabe, Suzuki, Ogura, & Watanabe, 1999). The DHA/EPA ranged from 2.40–3.29 in the present study, furthermore the value of Pacific bluefin tuna in the Sea of Japan was lower than other tunas, and the value decreased with size. The mouth of Pacific bluefin tuna larvae open at three days after hatching, and thus the decreasing DHA/EPA with size could be affected by prey capture behavior. The difference of DHA/EPA of Pacific bluefin tuna between Nansei area and Sea of Japan could be caused by the difference of eggs and maternal organs, such as liver and muscle. To evaluate this hypothesis, analyzing the egg (ovary), liver and muscle of spawning bluefin tuna is needed in the near future. These facts indicate that accurate analysis of the fatty acid components of a single larva could increase our understanding of the growth-survival process in marine fish.

Conclusion

This study accomplished the fatty acid analysis of a single fish larva using GC. The direct saponification/methylation method simplified in this study could yield fatty acid methyl esters without loss of unsaturated fatty acids and formation of artifacts from larval muscle. This method enabled fatty acid analysis by high-resolution GC with ordinary flame ionization detection. There is no necessity to use HPLC low in peak resolution and specific detection

technique, such as mass spectrometry, requiring calibrations for all fatty acids. The simplified direct saponification/methylation followed by GC is probably the most accurate method to detail fatty acid profile of a single fish larva. At present, this method has the highest sensitivity for fatty acid analysis of a single larva. While the gas chromatograph of this study equipped only split/splitless injection system and used helium as the carrier gas, on-column injection and hydrogen should further improve quantification and peak resolution of fatty acids. The analysis of wild tunas larvae successfully indicated the characteristics of fatty acid contents for each population and revealed that the concentrations of DHA and EPA during larval developmental stage. The method used in the present study will be a powerful tool to investigate potential capacity for survival of early life stages of wild tuna and other marine fish.

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Conflict of Interest

The authors have no conflicts of interest directly relevant to the content of this article.

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Figure Legends

Fig. 1 Typical gas chromatograms of fatty acid methyl esters prepared by direct saponification/methylation method. (a) Products of a single wild Pacific bluefin tuna larva, and (b) products in a blank test using no larva. *Phthalic acid di-ester identified in other tuna samples by mass spectrometry ($m/z = 149$).

Fig. 2 Reproducibility and applicability of the direct saponification/methylation method for the preparation of fatty acid methyl esters. (a) Mean proportions, standard deviations, and coefficient of variations (numbers above bars) of major fatty acids obtained from replicated preparations ($n = 6$) of methyl esters from a tuna muscle sample, and (b) content of total fatty acids as a function of the dry weight of developing tuna muscle ($n = 11$ muscle samples from a tuna individual).

Fig.3 Non-metric multidimensional scaling (MDS) plot of Pacific bluefin tuna caught in Japan sea (crosses), in around Nansei Islands (shaded circles), yellowfin tuna (open squares) and skipjack (open triangles) using 44 fatty acids present in proportions >0.1 mol% (two-dimensional stress = 0.18).

Fig. 4 Plots of six major fatty acid proportions (mol% of total fatty acids) (a-f) and DHA/EPA ratios (g) vs. standard lengths of wild tuna larvae.

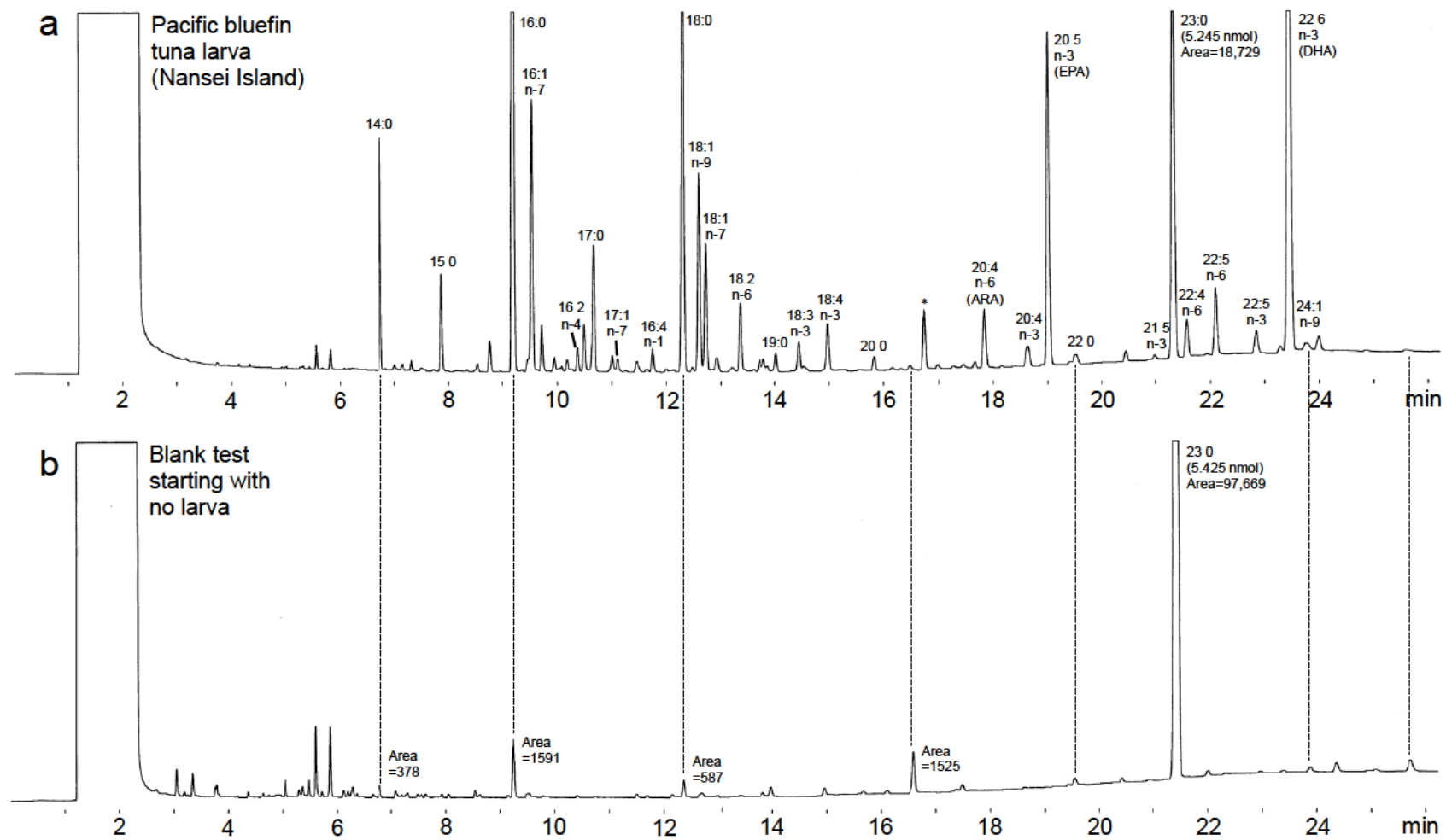


Fig. 1

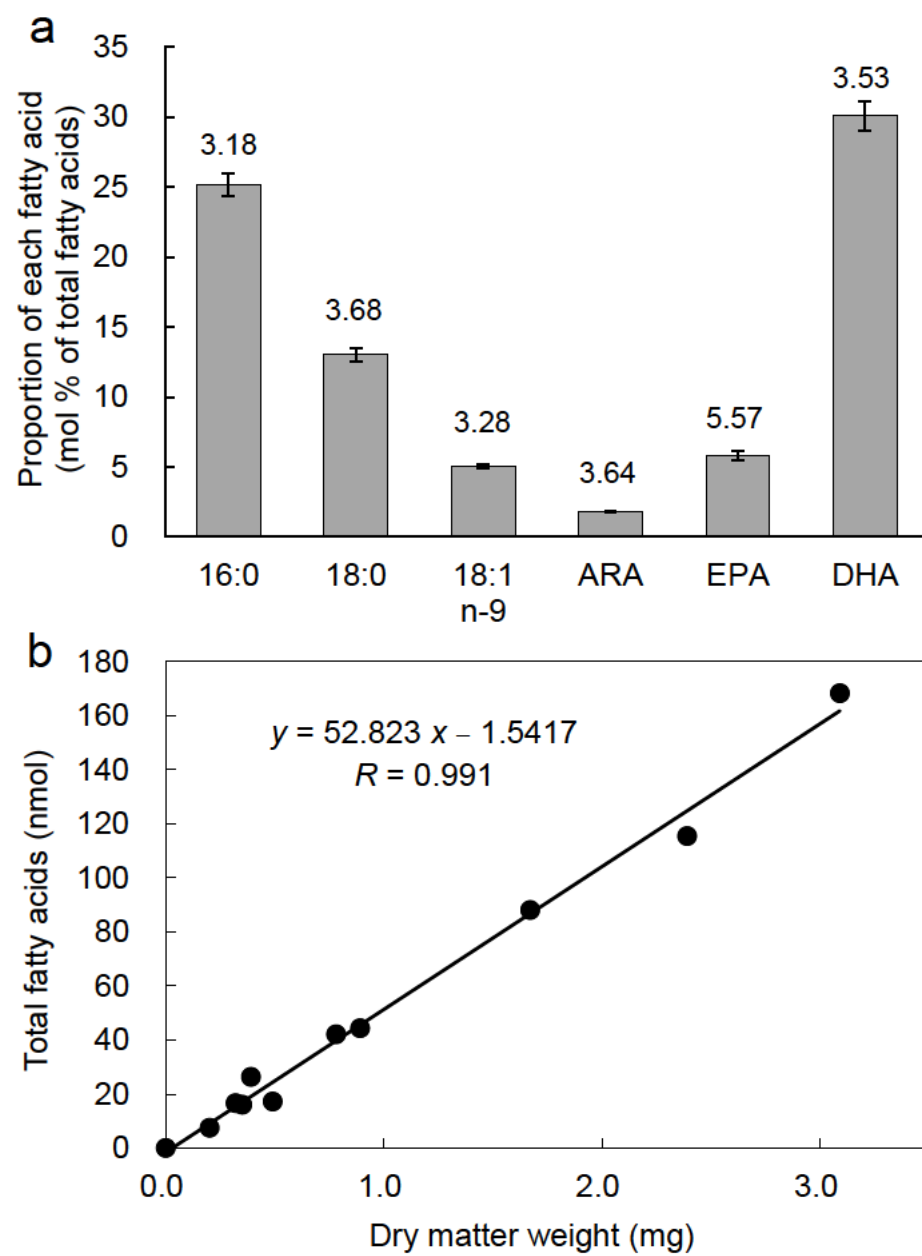


Fig. 2

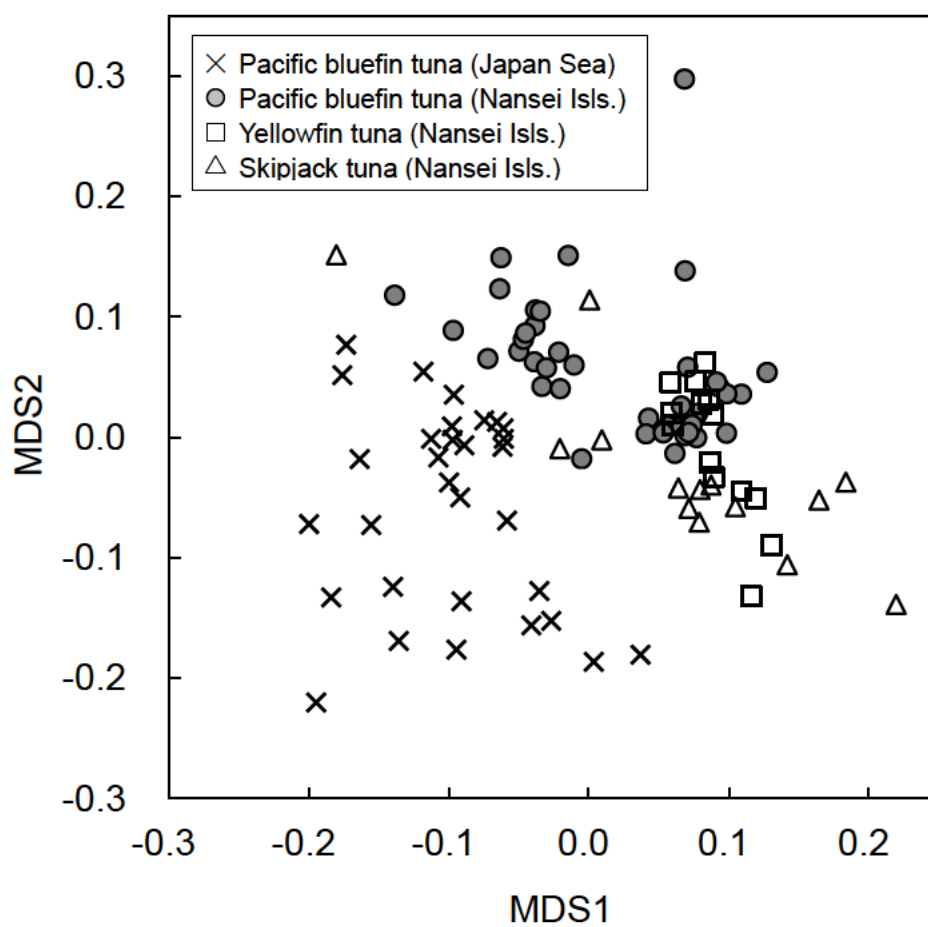


Fig. 3

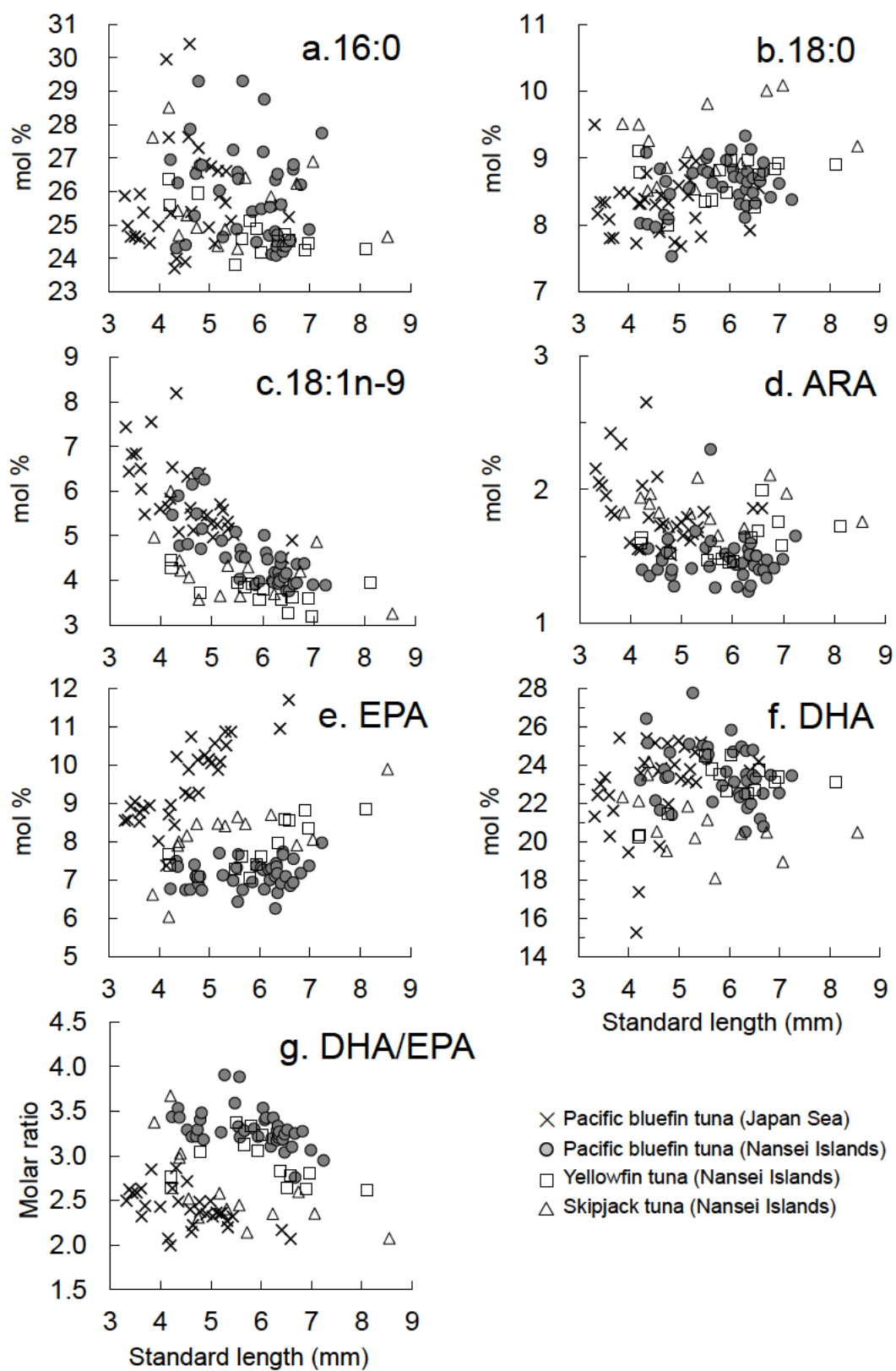


Fig. 4

Table 1. Fatty acid content and fatty acid composition of wild tuna larvae collected from the waters around Japan

Common name	Pacific bluefin tuna	Pacific bluefin tuna	Yellowfin tuna	Skipjack tuna
Scientific name	<i>Thunnus orientalis</i>	<i>Thunnus orientalis</i>	<i>Thunnus albacares</i>	<i>Katsuwonus palamis</i>
Location	Japan Sea	Nansei Islands	Nansei Islands	Nansei Islands
Number	<i>n</i> = 31	<i>n</i> = 41	<i>n</i> = 14	<i>n</i> = 14
Standard length (mm)	4.55 ± 0.81	5.80 ± 0.81	5.97 ± 1.04	5.47 ± 1.26
Body height (mm)	0.63 ± 0.26	1.14 ± 0.22	1.42 ± 0.37	0.96 ± 0.52
Dry weight of trunk (mg)	0.12 ± 0.08	0.22 ± 0.11	0.27 ± 0.19	0.16 ± 0.19
Total fatty acids (nmol/trunk) ¹	18.11 ± 12.76	34.13 ± 14.08	44.47 ± 27.98	27.15 ± 28.42
Fatty acid content (nmol/mg) ²	147.4 ± 63.2 ^a	168.9 ± 61.2 ^a	197.3 ± 94.4 ^a	219.7 ± 120.1 ^a
Fatty acid composition (mol%) ³				
14:0	3.43 ± 0.79 ^a	3.50 ± 0.47 ^a	2.94 ± 0.31 ^a	2.93 ± 0.50 ^a
14:1n-5	0.12 ± 0.06 ^a	0.19 ± 0.11 ^b	0.09 ± 0.04 ^a	0.14 ± 0.07 ^{ab}
iso-15:0	0.22 ± 0.05 ^a	0.19 ± 0.03 ^{ab}	0.15 ± 0.04 ^b	0.15 ± 0.04 ^b
15:0	1.16 ± 0.22 ^a	1.52 ± 0.14 ^b	1.50 ± 0.10 ^b	1.51 ± 0.13 ^b
iso-16:0	0.14 ± 0.06 ^a	0.21 ± 0.20 ^a	0.15 ± 0.05 ^a	0.18 ± 0.08 ^a
16:0	25.84 ± 1.54 ^a	25.88 ± 1.41 ^a	24.80 ± 0.69 ^a	25.72 ± 1.23 ^a
16:1n-7+16:1n-9	3.54 ± 0.95 ^a	4.38 ± 1.13 ^b	5.33 ± 0.46 ^b	5.12 ± 0.77 ^b
16:1n-5	0.45 ± 0.23 ^a	0.63 ± 0.36 ^a	0.61 ± 0.23 ^a	0.36 ± 0.28 ^a
iso-17:0	0.25 ± 0.07 ^a	0.34 ± 0.12 ^b	0.29 ± 0.06 ^{ab}	0.24 ± 0.04 ^a
anteiso-17:0	0.21 ± 0.04 ^a	0.22 ± 0.05 ^a	0.23 ± 0.04 ^a	0.19 ± 0.03 ^a
16:2n-4	0.33 ± 0.20 ^a	0.37 ± 0.15 ^a	0.50 ± 0.08 ^a	0.40 ± 0.12 ^a
7-Me-16:1	0.90 ± 0.14 ^{ab}	1.04 ± 0.14 ^b	0.98 ± 0.11 ^{ab}	0.79 ± 0.21 ^a
17:0	1.93 ± 0.17 ^a	2.55 ± 0.17 ^b	2.51 ± 0.23 ^b	2.34 ± 0.24 ^b
17:1n-7+17:1n-8	0.16 ± 0.07 ^a	0.28 ± 0.09 ^b	0.33 ± 0.09 ^b	0.33 ± 0.05 ^b
iso-18:0	0.49 ± 0.18 ^a	0.36 ± 0.07 ^b	0.29 ± 0.04 ^b	0.25 ± 0.07 ^b
16:4n-1	0.30 ± 0.13 ^a	0.40 ± 0.07 ^b	0.44 ± 0.04 ^b	0.44 ± 0.10 ^b
18:0	8.29 ± 0.41 ^a	8.59 ± 0.37 ^a	8.68 ± 0.32 ^{ab}	9.19 ± 0.51 ^b
18:1n-13	0.11 ± 0.06 ^a	0.11 ± 0.03 ^a	0.09 ± 0.03 ^a	0.08 ± 0.03 ^a
18:1n-9	5.85 ± 0.85 ^a	4.55 ± 0.70 ^b	3.77 ± 0.33 ^c	4.23 ± 0.68 ^{bc}
18:1n-7	2.27 ± 0.28 ^{ab}	2.23 ± 0.52 ^a	2.65 ± 0.21 ^{bc}	2.85 ± 0.33 ^c
18:1n-5	0.26 ± 0.12 ^a	0.31 ± 0.11 ^a	0.31 ± 0.11 ^a	0.20 ± 0.14 ^a
18:2n-6	1.64 ± 0.10 ^{ab}	1.54 ± 0.13 ^b	1.58 ± 0.15 ^{ab}	1.83 ± 0.32 ^a
18:2n-4	0.10 ± 0.04 ^a	0.20 ± 0.10 ^b	0.29 ± 0.06 ^c	0.30 ± 0.09 ^c
18:3n-6	0.20 ± 0.24 ^a	0.13 ± 0.06 ^a	0.11 ± 0.05 ^a	0.14 ± 0.08 ^a
19:0	0.33 ± 0.06 ^a	0.50 ± 0.05 ^b	0.44 ± 0.07 ^{bc}	0.41 ± 0.10 ^{ac}
18:3n-3	0.57 ± 0.08 ^a	0.53 ± 0.13 ^a	0.76 ± 0.17 ^b	0.78 ± 0.21 ^b
18:4n-3	1.54 ± 0.40 ^a	1.15 ± 0.16 ^b	1.36 ± 0.17 ^{ab}	1.36 ± 0.21 ^{ab}
18:4n-1	0.01 ± 0.03 ^a	0.01 ± 0.06 ^a	0.14 ± 0.43 ^a	0.00 ± 0.00 ^a
20:0	0.41 ± 0.09 ^a	0.33 ± 0.02 ^b	0.32 ± 0.03 ^b	0.33 ± 0.06 ^b
20:1n-11+20:1n-9	0.21 ± 0.15 ^a	0.14 ± 0.08 ^a	0.17 ± 0.45 ^a	0.15 ± 0.14 ^a
20:1n-7	0.13 ± 0.39 ^a	0.06 ± 0.02 ^a	0.05 ± 0.02 ^a	0.07 ± 0.01 ^a
20:2n-6	0.10 ± 0.04 ^a	0.11 ± 0.02 ^a	0.09 ± 0.01 ^a	0.10 ± 0.04 ^a
20:3n-6	0.16 ± 0.09 ^a	0.14 ± 0.04 ^a	0.14 ± 0.02 ^a	0.16 ± 0.03 ^a
20:4n-6 (ARA)	1.84 ± 0.27 ^a	1.48 ± 0.17 ^b	1.61 ± 0.14 ^{bc}	1.86 ± 0.13 ^{ac}
20:4n-3	0.52 ± 0.12 ^a	0.53 ± 0.11 ^a	0.73 ± 0.20 ^b	0.66 ± 0.15 ^{ab}
20:5n-3 (EPA)	9.56 ± 1.00 ^a	7.13 ± 0.37 ^b	7.87 ± 0.62 ^{bc}	8.12 ± 0.88 ^c
22:0	0.20 ± 0.09 ^a	0.25 ± 0.08 ^a	0.26 ± 0.05 ^a	0.25 ± 0.21 ^a
21:5n-3	0.18 ± 0.05 ^a	0.15 ± 0.04 ^a	0.14 ± 0.02 ^a	0.18 ± 0.04 ^a
22:4n-6	0.72 ± 0.19 ^a	0.89 ± 0.12 ^b	0.94 ± 0.10 ^{ab}	0.85 ± 0.27 ^{ab}
22:5n-6	1.21 ± 0.20 ^a	1.90 ± 0.20 ^b	1.91 ± 0.13 ^b	2.10 ± 0.24 ^b
22:5n-3	0.63 ± 0.26 ^a	0.57 ± 0.09 ^a	0.70 ± 0.16 ^{ab}	0.92 ± 0.25 ^b
24:0	0.01 ± 0.04 ^a	0.08 ± 0.10 ^{ab}	0.18 ± 0.07 ^b	0.10 ± 0.15 ^{ab}
22:6n-3 (DHA)	22.90 ± 2.36 ^{ab}	23.48 ± 1.54 ^b	22.82 ± 1.28 ^{ab}	20.98 ± 1.62 ^a
24:1n-9	0.37 ± 0.21 ^a	0.49 ± 0.30 ^a	0.37 ± 0.09 ^a	0.38 ± 0.16 ^a
Others	0.22	0.36	0.36	0.32
DHA/EPA ratio	2.40 ± 0.21 ^a	3.29 ± 0.21 ^b	2.92 ± 0.26 ^c	2.63 ± 0.45 ^{ac}

Results are means ± SD of individual larvae. Values bearing different superscript letters are significantly different ($P < 0.05$).

¹ Absolute amount of total fatty acids determined for trunk samples.

² Content of total fatty acids based on dry weight of trunk.

³ Mole % of total fatty acids.