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Article

Lipid Profiles and Fatty Acid Positional Distribution in Farmed *Hippocampus abdominalis*: Characterization by Untargeted Lipidomics and Enzymatic Hydrolysis

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Abstract

This study analyzed the lipid profiles of *Hippocampus abdominalis* via untargeted lipidomics coupled with enzymatic hydrolysis, and further compared its lipid content, composition, molecular species, and fatty acid positional distribution with those of the currently farmed *Hippocampus erectus*. Triglyceride (TG) was the dominant lipid class in both species, while *H. abdominalis* exhibited higher phospholipid (PL) levels, with elevated docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in TG. DHA was evenly distributed at the *sn*-1,3 and *sn*-2 positions in *H. abdominalis* but concentrated at *sn*-1,3 in *H. erectus*, whereas EPA localized at *sn*-1,3 in both species. *H. abdominalis* also had higher DHA in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (mainly at *sn*-1) and abundant alkoxy ether PLs. This work fills research gaps in lipid profiling of these commercially cultured seahorses, characterizing interspecific differences in lipid molecular species and fatty acid positional distribution in key lipids (TG, PC, PE), and provides a fundamental basis for seahorse species identification and functional lipid development.

Keywords: the farmed seahorse; lipid profile; untargeted lipidomics; fatty acid positional distribution; *Hippocampus abdominalis*

1. Introduction

Seahorses are rare marine bony fishes with substantial culinary and medicinal potential [1,2]. Currently, seahorses in major Asian regions are primarily traded as traditional medicinal materials, while a small fraction are marketed as ornamental marine fishes [3]. Accordingly, aquaculture has emerged as a crucial solution to meet the edible and medicinal demand for seahorses [4]. Notably, *Hippocampus abdominalis* and *H. erectus* have been commercially cultivated on a global scale [5,6].

Numerous studies have demonstrated that seahorses not only contain beneficial nutrients such as proteins and minerals [7,8] but also rich in unsaturated fatty acids [9,10], including omega-3 long-chain unsaturated fatty acids such as docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) [11]. These compounds exhibit significant biological activities in combating cardiovascular and cerebrovascular diseases as well as neurodegenerative disorders [12]. To date,

there have been literature reports on the lipid profiles of *H. erectus* [13], while no literature has yet reported the lipid profiles of *H. abdominalis* that has been successfully cultivated on a large scale. Thus, the analysis of *H. abdominalis* lipid profiles and the comparison with those of other seahorse species have become an urgent issue to address.

Notably, current lipid analysis of seahorse has primarily focused on lipid profiling, with few studies investigating the fatty acid positional distribution of their major molecular species. Importantly, the positional structure of triglyceride (TG) and phospholipid (PL) can affect the *in vivo* absorption and metabolism of lipids, which in turn influences their biological activity and function [14,15]. Furthermore, the enzymatic method is the most effective and rapid approach for identifying the structure of common lipid molecules [16]. Thus, identifying the fatty acid positional distribution of major lipids in seahorses is also a key topic in seahorse lipid analysis.

In this study, an analytical strategy integrating untargeted lipidomics and immobilized enzyme hydrolysis was used to systematically characterize the lipid profiles and the fatty acid positional distribution of major lipid molecular species in two major cultured seahorse species, *Hippocampus abdominalis* and *Hippocampus erectus*. By examining interspecific differences in lipid content, composition, and structure, this research fills a vital knowledge gap on seahorse nutrition and enables a deeper understanding of their health functions and reliable lipid profiling.

2. Materials and Methods

2.1. Materials

Two seahorse species, designated as *Hippocampus abdominalis* and *H. erectus* respectively, were donated by Weihai Yinze Biotechnology Co., Ltd., a Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and national aquatic wildlife protection law-compliant facility licensed for large-scale seahorse artificial breeding. The seahorses used in our research are in full compliance with the basic principles specified for seahorses intended for scientific research purposes under CITES Appendix II. Rearing conditions for both species were strictly standardized and monitored, including dissolved gas concentrations, light intensity, water temperature, and tank hygiene. The Juveniles were fed *Artemia nauplii*, while adults were given a diet of opossum shrimp. Upon maturity, 100 individuals of each species (1:1 male-to-female ratio, which is consistent with the roughly equal proportion of males to females in consumption, as well as the typical ratio observed under aquaculture conditions.) were randomly sampled from separate same-batch culture tanks. Following guidelines approved by the Institutional Animal Care and Use Committee (IACUC), seahorses were humanely euthanized via overdose anesthesia with tricaine methanesulfonate (MS-222). Euthanized specimens were rinsed thoroughly with 0.22 μm -filtered seawater to remove surface mucus and exogenous impurities, then blotted dry. Each seahorse species was further assigned to three subgroups and subjected to frozen transportation at $-20\text{ }^{\circ}\text{C}$ to the laboratory. Subsequently, the samples were freeze-dried with a Scientz-18N freeze dryer (Ningbo Xinzhi Co., Ltd., China), ground into a fine powder, and processed for subsequent lipid extraction and analysis.

2.2. Reagents

Chloroform, methanol, hydrochloric acid, and sodium hydroxide used in this study were all of reagent grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). n-Hexane, isopropanol, acetonitrile, and ammonium formate were of chromatographic grade and purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Thin-layer chromatography (TLC) silica gel plates were provided by Yantai Huayang New Material Technology Co., Ltd. (Yantai, China), and the triglyceride (TG) assay kit (Cat. No.: A020-1-1) was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.3. Lipid Extraction and Purification

The extraction of total lipids from seahorses was performed with appropriate modifications based on the Folch method [17]. Briefly, a specified mass of dried *H. abdominalis* and *H. erectus* powder was mixed with 10 volumes (m/v) of extraction solvent (chloroform: methanol = 2:1, v/v), respectively. Butylated hydroxyanisole (BHA) was added to the chloroform-methanol extraction solution at a concentration of 100 µg/mL to prevent lipid oxidation. After multiple processing steps, the lower organic phase was collected and concentrated under reduced pressure to remove organic solvents, yielding total seahorse lipids. Subsequently, column chromatography was performed to isolate the triglyceride (TG), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) fractions. Specifically, 10 g of total lipids were loaded onto a silica gel column (packed with 60 g of silica gel; internal diameter = 2 cm). Pure dichloromethane was initially adopted as the eluent, and triglycerides eluted at the third column volume (CV). The eluted fractions were subsequently identified via thin-layer chromatography (TLC). After the complete collection of all neutral lipid fractions, phospholipid components were eluted using a dichloromethane/methanol mixture (5:1, v/v), from which phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were sequentially isolated. The purities of all the aforementioned substances, as determined by an Evaporative Light Scattering Detector (ELSD), were all above 90%. These samples were then frozen and stored at -20 °C for subsequent analysis.

2.4. Analysis of Fatty Acid Compositions

TG were quantified using a commercial assay kit (Cat. No. A020-1-1, Nanjing Jiancheng Bioengineering Institute). Phospholipids (PL) were determined via the molybdenum blue colorimetric method [18]. Briefly, sample solutions were prepared with chloroform-methanol (2:1, v/v) as the solvent, followed by digestion with perchloric acid. The inorganic phosphorus content in the samples was then measured, and phospholipid content was calculated by multiplying the measured inorganic phosphorus content by a conversion factor of 25.

The total lipids, as well as the samples separated by silica gel column chromatography, were individually placed into methylation reagents (hydrochloric acid: methanol = 1:5, v/v), homogenized, and reacted in a metal bath at 90 °C for 3 hours, with homogenization performed every 30 minutes during the reaction. After cooling, n-hexane was added for extraction. Once the mixture was allowed to stand and separate into layers, 1 mL of the supernatant was collected and dried by nitrogen blowing. The dried sample was then re-dissolved in 50 µL of n-hexane and used for subsequent gas chromatography analysis [19].

The GC system was set as follows: An Agilent 7820A Gas Chromatograph with a capillary column (AE. FFAP; 30 m × 0.32 mm × 0.25 µm film thickness); The gas chromatography detector employed was a Flame Ionization Detector (FID). The detector and injector temperatures were maintained at 250 °C and 240 °C, respectively. The column temperature program was programmed as follows: initial temperature of 170 °C, ramped up to 240 °C at a rate of 3 °C/min, and then held isothermally at 240 °C for 35 minutes. High-purity nitrogen was used as the carrier gas, with a constant flow rate of 1.0 mL/min. The total runtime for a single sample analysis was 48.5 minutes [20].

2.5. Ultra High Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS) Analysis

For lipid profile analysis, a high-resolution orbitrap mass spectrometer (UPLC-Q-Orbitrap) coupled with an ACQUITY UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 µm) was used. The system included a UPLC module from Waters Corporation (Milford, Massachusetts, USA) and an electrospray ionization (ESI) source from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Mobile phase A consisted of acetonitrile/water (60:40, v/v) containing 10 mmol/L ammonium formate, and mobile phase B was isopropanol/acetonitrile (90:10, v/v). The gradient elution program was as follows: initial conditions (100% A); linear increase to 32% B over 1.5 min; further increase to

85% B by 15.5 min; rapid ramp to 97% B within 0.1 min, held for 2.4 min; then decreased to 32% B within 0.1 min, held for 1.9 min. Curtain Gas (Cur) was set to 35 psi; the flow rates of both Gas 1 (GS1) and Gas 2 (GS2) were configured to 55 psi; Ion Source Voltage/Float (ISVF) was adjusted to 5500/-4500 V; Ion Source Temperature (TEM) was maintained at 350 °C; Declustering Potential (DP) was set to 80 V; Entrance Potential (EP) was set to 10 V; and Collision Cell Exit Potential (CXP) was set to 15 V. Quantitative analysis of total ion current (TIC) chromatograms was performed using Lipid Search Launcher 4.2.21 software (Thermo Fisher Scientific) [21].

2.6. Determination of Fatty Acid Positional Distribution

To determine the positional distribution of fatty acids in TG, PC and PE, 2 mg of the sample was placed into each centrifuge tube separately. Then 40 mL of 95% ethanol was added, followed by the addition of Novozym 435 lipase. The hydrolysis was terminated after 12 hours of reaction, followed by the separation of immobilized enzymes from enzymatic hydrolysates. The resulting mixture was heated at 95 °C for 10 minutes under a nitrogen atmosphere to inactivate residual enzyme activity, and then dried and concentrated in a nitrogen environment prior to high-performance thin-layer chromatography (TLC) separation. The TG enzymatic hydrolysates were treated by the solvent system of petroleum ether, diethyl ether and acetic acid at a volume ratio of 85:15:1 (v/v/v), and TG (R_f=0.87) and MG (R_f=0.25) were obtained. The PC and PE enzymatic hydrolysates were treated by the solvent system of chloroform, methanol and water at a volume ratio of 65:25:4 (v/v/v), respectively, and PC (R_f=0.56), PE (R_f=0.84), LPC (R_f=0.27) and LPE (R_f = 0.33) were obtained. Finally, the target bands were scraped off and subjected to fatty acid composition analysis.

2.7. Statistical Analysis

Triplicate experiments were conducted for both the quantitative analysis of lipid molecules and the determination of lipid class composition, while triplicate measurements were performed for fatty acid component analysis. All data in the figures are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test to identify significant differences between groups. Differences were considered statistically significant at $p < 0.05$ and marked with different letters. Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA), heatmap analysis, and volcano plot generation were carried out using the free online data analysis platform Metware Cloud (<https://cloud.metware.cn>).

3. Results

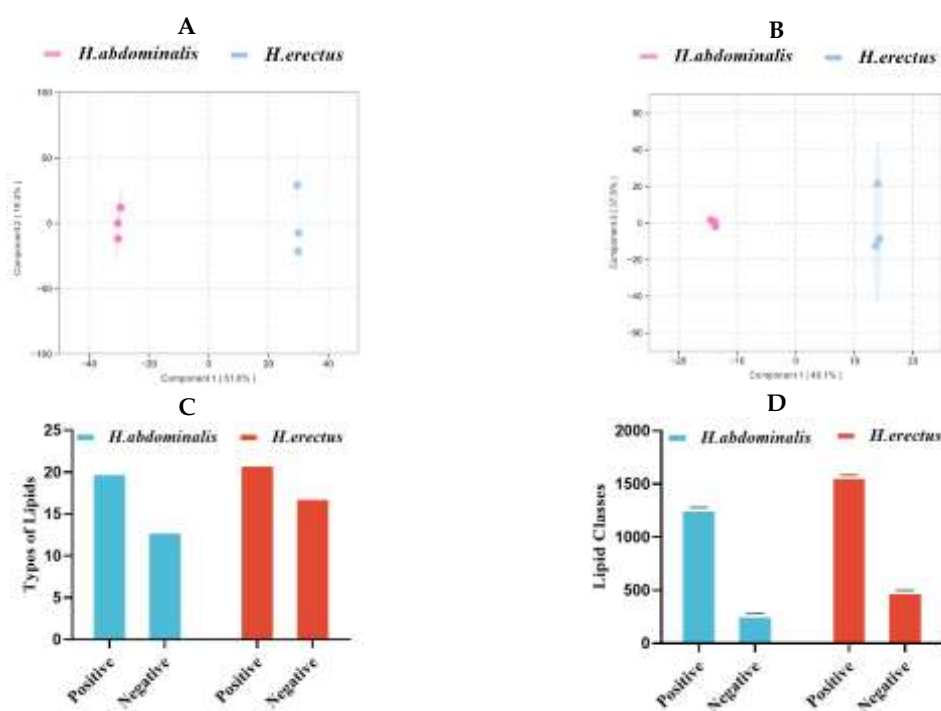
3.1. Lipid Content and Lipid Class Composition

As shown in Supplementary Figure. S1, the lipid extraction yield of *H. abdominalis* (76.4 mg/g dry weight) was higher than that of *H. erectus* (69.1 mg/g dry weight), indicating that *H. abdominalis* exhibited a higher lipid content. The fatty acid content of the total lipids of *Hippocampus abdominalis* and *Hippocampus erectus* were analyzed using gas chromatography (GC) through comparison with the mixed standard chromatogram (Figure. S2), with the results summarized in Table 1. For *H. abdominalis*, 16 fatty acids with carbon chain lengths ranging from C14 to C22 were identified, with major components including C16:0, C16:1, C18:0, C18:1, C20:5n-3 (EPA), and C22:6n-3 (DHA). Saturated fatty acids (SFA) accounted for 38.9% of its total lipids (predominantly C16:0 at 23.1%, C18:0 at 9.6%, and C14:0 at 3.6%), while 10 unsaturated fatty acid (UFA) species contributed 61.1% (monounsaturated fatty acids (MUFA) at 29.9%, mainly C18:1 at 20.8% and C16:1 at 7.4%;

Table 1. Relative abundance and content of fatty acids in two *Hippocampus* species.

Fatty acids	<i>H. abdominalis</i>		<i>H. erectus</i>	
	Relative abundance (%)	Contents (mg/g total lipids)	Relative abundance (%)	Contents (mg/g total lipids)
C14:0	3.59±0.04	18.18±0.08	7.64±0.06	39.89±1.9
C16:0	23.07±0.1	116.76±0.43	27.37±0.09	159.61±1.04
C16:1	7.39±0.02	37.42±0.34	8.46±0.06	49.33±0.45
C17:0	1.76±0.01	8.93±0.06	2.14±0.04	12.47±0.21
C18:0	9.64±0.11	48.79±0.9	11.01±0.06	64.19±0.59
C18:1	20.79±0.01	105.24±0.76	15.62±0.08	91.08±0.16
C18:2n-6	2.91±0.06	14.72±0.33	1.68±0.07	9.82±0.39
C18:3n-6	0.41±0.01	2.1±0.06	1.05±0.04	6.15±0.26
C18:3n-3	2.21±0.02	11.21±0.17	0.6±0.04	3.51±0.21
C20:2	-	-	3.16±0.09	18.45±0.59
C20:0	0.39±0.01	1.96±0.04	1.04±0.06	6.05±0.36
C20:4n-6	2.86±0.02	14.47±0.22	-	-
EPA	8.33±0.1	42.14±0.18	8.03±0	46.85±0.2
C22:0	0.42±0.01	2.11±0.09	1.16±0.04	6.78±0.26
C22:2	0.17±0	0.85±0.02	1.36±0.08	7.93±0.44
DHA	14.37±0.06	72.71±0.69	9.66±0.11	56.3±0.41
∑SFA	38.87±0.06	196.73±1.41	50.36±0.14	288.99±2.22
∑MUFA	28.19±0.12	142.65±1.25	24.08±0.07	140.41±0.29
∑PUFA	31.26±0.03	158.19±1.02	25.56±0.11	149.01±0.51
∑PUFA n-3	24.91±0.09	126.05±0.65	18.29±0.12	106.66±0.39
∑PUFA n-6	6.18±0.06	31.29±0.55	2.74±0.04	15.97±0.26
∑UFA	59.44±0.15	300.84±2.27	49.64±0.14	289.42±0.45
EPA+DHA	22.69±0.09	114.84±0.53	17.69±0.1	103.15±0.22

Values are mean ± SEM (n = 3).



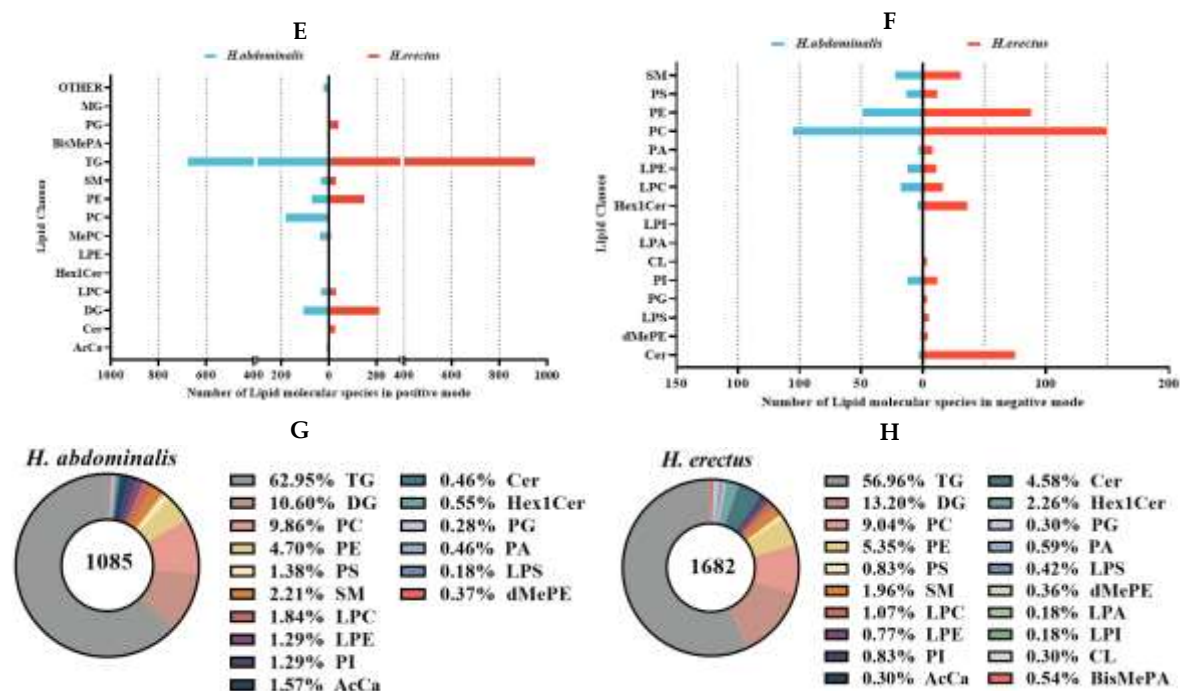


Figure 1. Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) of lipidomics data from *H. abdominalis* and *H. erectus* in positive mode (A) and negative mode (B); Types of lipids (C) and number of lipid classes (D) of the two *Hippocampus* species under different ion modes; Number of lipid molecules in different lipid classes in positive mode lipidomics (E) and negative mode lipidomics (F); The lipid classes and the relative ratio of lipid species number in *H. abdominalis* (G) and *H. erectus* (H). BisMePA for Bis(methoxyethyl)phosphatidylethanolamine, Cer for Ceramide, CL for Cardiolipin, dMePE for Dimethylethanolamine Phosphatidylethanolamine, Hex1Cer for Monoglucosylceramide, LPA for Lysophosphatidic Acid, LPC for Lysophosphatidylcholine, LPE for Lysophosphatidylethanolamine, LPI for Lysophosphatidylinositol, LPS for Lipopolysaccharide, PA for Phosphatidic Acid, PI for Phosphatidylinositol, SM for Sphingomyelin polyunsaturated fatty acids (PUFA) at 31.3%, dominated by DHA at 14.37% and EPA at 8.3%. For *H. erectus*, the major fatty acids were C16:0 (27.4%), C18:1 (15.6%), C18:0 (11.0%), C16:1 (8.5%), and C14:0 (7.6%), with SFA, MUFA, and PUFA accounting for 50.4%, 24.1%, and 25.6% of total lipids, respectively. The UFA content of *H. abdominalis* (59.4%) was higher than that of *H. erectus* (49.6%), and the total DHA + EPA content of both species was higher than that of *H. kelloggi*.

3.2. Differences in TG Molecular Species Between Two Seahorse Species and Fatty Acid Positional Distribution in TG

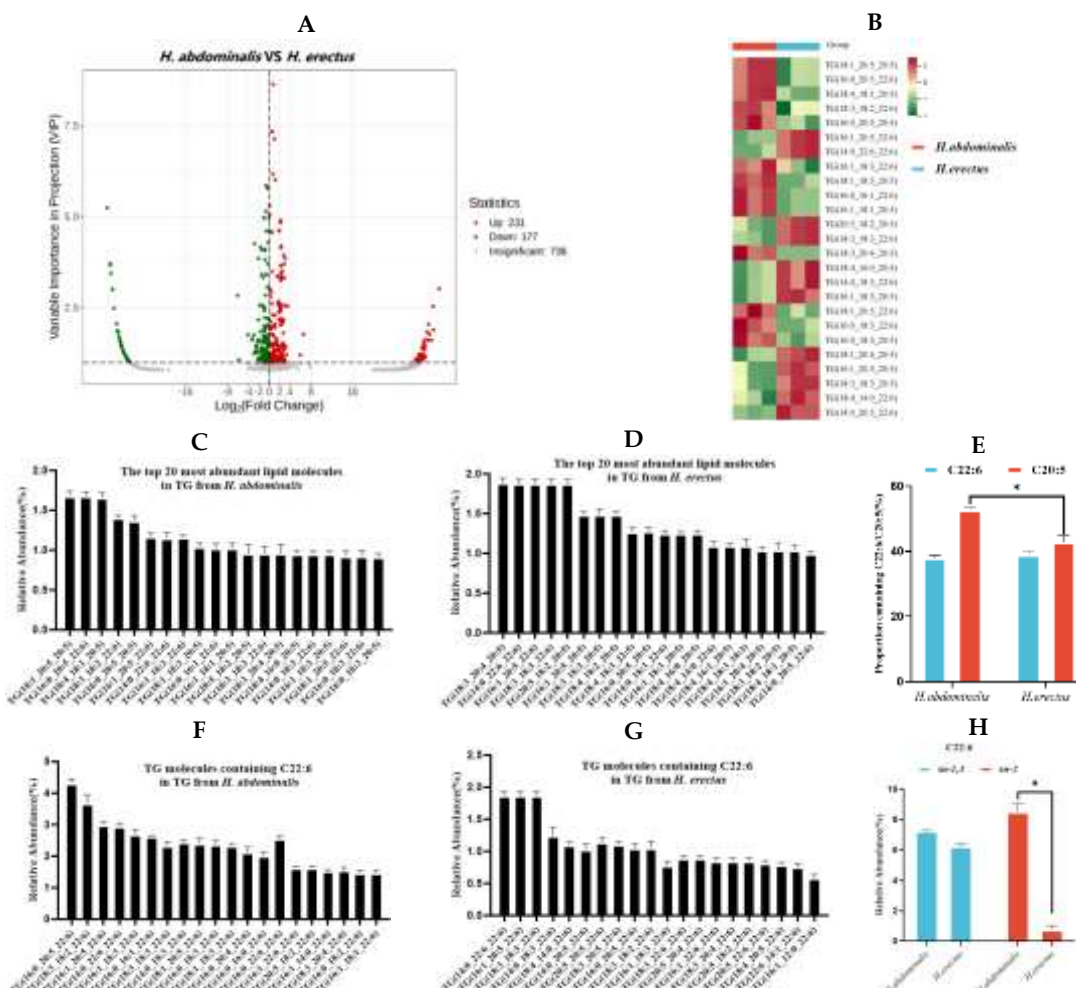
According to Figure. S3 TG was the major component of seahorse lipids, with *H. erectus* (26.7%) having a slightly lower TG content than *H. abdominalis* (29.5%). Phospholipids (PL) accounted for 9.2–12.3% of total lipids, with *H. abdominalis* showing a higher PL content than *H. erectus*. Then the lipid components of the two seahorse species were analyzed by means of lipidomics. Preliminary findings indicated that there were differences between them through comparison with the Total Ion Chromatogram (TIC) profiles under the two modes (Figure. S4–S5). OPLS-DA results (Figure. 1A and B) revealed significant differences in lipid molecular characteristics between the two species. In positive ion mode, the number of lipid types was comparable between them, but *H. erectus* had more lipid types in negative ion mode. From the perspective of lipid classes (Figure. 1D–H), *H. erectus* exhibited significantly more lipid classes in both ion modes. After data integration, TG was identified as the predominant lipid class, representing 63.0% of lipids in *H. abdominalis* and 57.0% in *H. erectus*. Notably, *H. abdominalis* lacked LPA, LPI, CL, and BisMePA, with PC (9.9%) and PE (4.7%) as the primary PL components; *H. erectus* had a lower PC proportion (9.0%) but a higher PE proportion (5.4%).

A total of 408 significantly different TG lipid species were identified between the two seahorse species (Figure. 2A), with 231 upregulated and 177 downregulated in *H. abdominalis* relative to *H. erectus*. Differential TG species screened by $|\log_2 \text{fold change}| > 1$ and VIP score > 1 were visualized via volcano plots (Figure. 2B), and 25 of the top 20 most abundant TG species in each seahorse showed significant interspecific differences. *H. abdominalis* had 683 detected TG molecular species, while *H. erectus* had 958 (Figure. 1E). The most abundant TG species in both seahorses almost all contained C20:5 or C22:6 (Figure. 2C and D). The proportion of C22:6-containing lipid species was approximately 38% in both, while higher levels of C22:6-containing lipids (e.g., TG(C16:0/C20:5/C20:6) at 4.3%) and slightly higher levels of C20:5-containing lipids (e.g., TG(C18:1/C20:5/C20:5) at 3.9%) than *H. erectus* (dominant C22:6-containing the proportion of C20:5-containing species *w-* as significantly higher in *H. abdominalis* (52.6%) than in *H. erectus* (42.9%) (Figure. 2E). *H. abdominalis* had substantially lipid TG(C14:0/C22:6/C22:6) at 1.9%; dominant C20:5-containing lipid TG(C18:3/C20:4/C20:5) at 3.6%) (Figure. 2F, 2G 2I, 2J). Enzymatic hydrolysis results (Table S1, Figure. 2H and K) showed that in *H. abdominalis*, C22:6 was distributed at both *sn*-1,3 (7.2%) and *sn*-2 (8.5%) positions of TG, while in *H. erectus*, C22:6 was predominantly at *sn*-1,3 positions (6.2%). C20:5 was concentrated at *sn*-1,3 positions in both, with a higher content in *H. abdominalis* (7.2%) than in *H. erectus* (3.3%).

3.3. Differences in PL Molecular Species Between Two Seahorse Species

For PL components, 44 differential PC species were identified between the two seahorses (Figure. 3A), with 42 upregulated and 2 downregulated in *H. abdominalis*. Ten differential PE species were detected (Figure. 3B), all upregulated in *H. abdominalis*. Volcano plots visualized these differential species (Figure. 3C). Among the top 10 most abundant

PC and PE species in each seahorse, 15 PC and 15 PE species showed significant interspe-



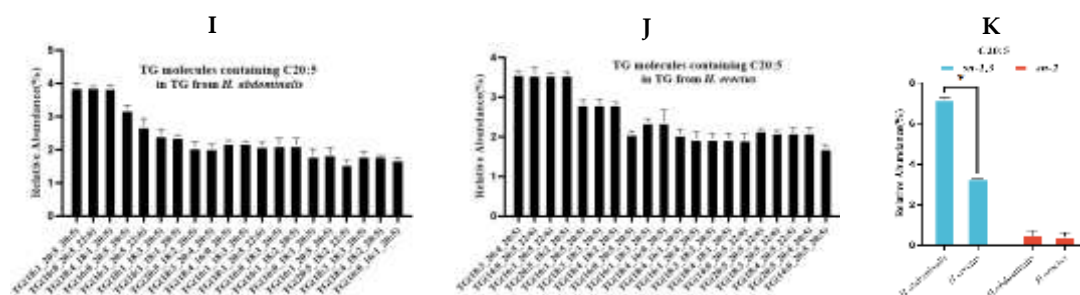
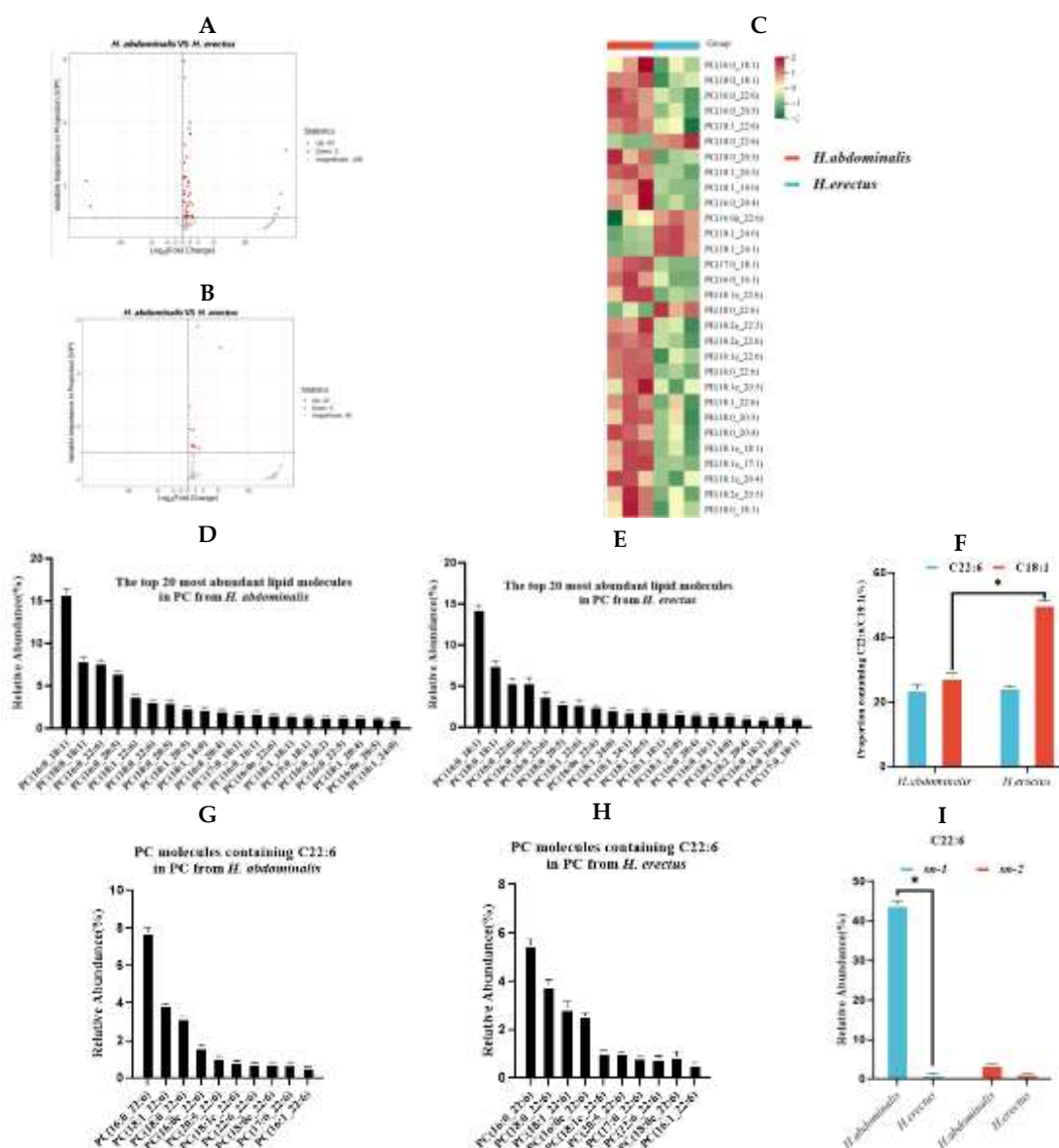


Figure 2. Analysis of differential triglycerides in *H. abdominalis* and *H. erectus*. The volcano plot (A). Differential lipid species were selected out by using the criteria of P Value $|\log_2\text{FoldChange}| > 1$, VIP score > 1 in the volcano plot ($n=3$). The lipid thermograms (B) of 25 triglycerides in two kinds of *Hippocampus* were screened by relative content and significance. Relative abundances of the top 20 TG lipid molecular species in TG from *H. abdominalis* (C) and *H. erectus* (D) under positive mode; The ratio of the number of TG molecular species containing C22:6 and C20:5 from the two seahorse species (E); The relative abundance of top 20 lipid molecules containing C22:6 in TG from *H. abdominalis* (F) and *H. erectus* (G); Fatty acids intra-positional composition of C22:6 in TG of the two *Hippocampus* species (H); The relative abundance of top 20 lipid molecules containing C20:5 in TG from *H. abdominalis* (I) and *H. erectus* (J); Fatty acids intra-positional composition of C20:5 in TG of the two *Hippocampus* species (K). Values are expressed as mean \pm standard error of the mean (SEM). The number of independent biological replicates is $n = 3$ for each group.



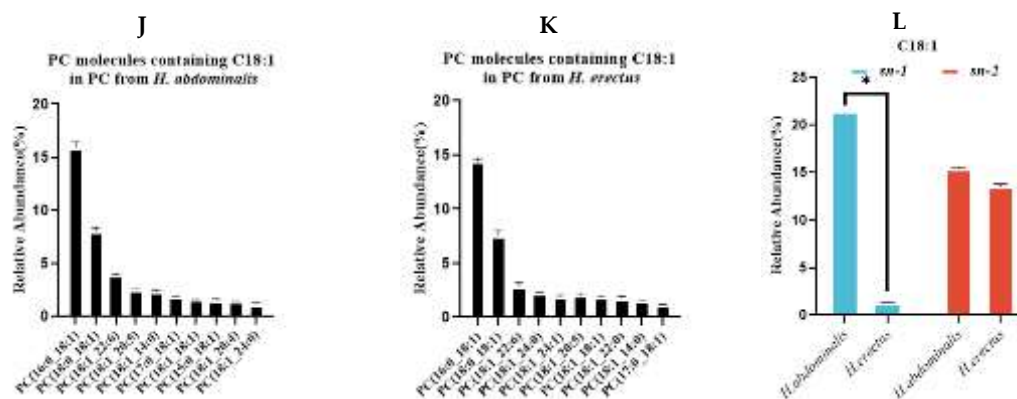


Figure 3. Analysis of differential PC (A) and PE (B) in *H. abdominalis* and *H. erectus*. Differential lipid species were selected out by using the criteria of P Value $|\log_2\text{FoldChange}| > 1$, VIP score > 1 in the volcano plot ($n=3$). The lipid thermograms (C) of 15 kinds of PC and 15 kinds of PE in the two kinds of *Hippocampus* were screened by relative content and significance. Relative abundances of the top 20 PC lipid molecular species in PC from *H. abdominalis* (D) and *H. erectus* (E) under positive mode; The ratio of the number of PC molecular species containing C22:6 and C18:1 from the two seahorse species (F); The relative abundance of top 20 lipid molecules containing C22:6 in PC from *H. abdominalis* (G) and *H. erectus* (H); Fatty acids intra-positional composition of C22:6 in PC of the two *Hippocampus* species (I); The relative abundance of top 20 lipid molecules containing C18:1 in PC from *H. abdominalis* (J) and *H. erectus* (K); Fatty acids intra-positional composition of C18:1 in PC of the two *Hippocampus* species (L). Values are expressed as mean \pm standard error of the mean (SEM). The number of independent biological replicates is $n = 3$ for each group cific differences, with most being more abundant in *H. abdominalis*. *H. abdominalis* had 51 PE molecular species, compared to 90 in *H. erectus* (Figure. 1F).

3.4. Molecular Species and Positional Distribution of Fatty Acids in PC

The most abundant PC species in both seahorses almost all contained C22:6 or C18:1 (Figure. 3D–E). The proportion of C22:6-containing PC species was slightly lower in *H. abdominalis* (23.8%) than in *H. erectus* (24.5%), while the proportion of C18:1-containing PC species was significantly lower in *H. abdominalis* (27.5%) than in *H. erectus* (50.2%) (Figure. 3F). *H. abdominalis* had higher levels of C22:6-containing PC species (e.g., PC(C16:0/C22:6) at 7.8%) and slightly higher levels of C18:1-containing PC species than *H. erectus* (PC(C16:0/C22:6) at 5.5%) (Figure. 3G–H, 3J–K). The most abundant PC species in both as PC(C16:0/C18:1), with relative contents of 15.9% (*H. abdominalis*) and 14.4% (*H. erectus*). Enzymatic hydrolysis results (Table S2, Figure. 3I and 3L) showed that PC in *H. abdominalis* had high DHA content (44.0% at *sn-1* position), while DHA content in *H. erectus* was approximately 1% at both *sn-1* and *sn-2* positions. C18:1 in *H. abdominalis* was primarily at *sn-1* (21.4%), while in *H. erectus*, C18:1 content at *sn-1* (15.1%) and *sn-2* (13.6%) were similar.

3.5. Molecular Species and Positional Distribution of Fatty Acids in PE

The most abundant PE species in both seahorses almost all contained C22:6 or C18:1 (Figure. 4A–B). The proportion of C22:6-containing PE species was lower in *H. abdominalis* (44.9%) than in *H. erectus* (52.2%), while the proportion of C18:1-containing PE species was higher in *H. abdominalis* (44.9%) than in *H. erectus* (38.4%) (Figure. 4C). *H. abdominalis* had a significantly higher abundance of alkoxy ether phospholipid PE, such as PE(C18:1e/C22:6) (12.2%), PE(C18:2e/C22:6) (7.4%), and PE(C18:1e/C20:5) (4.4%) (Figure. 4D–E, 4G–H). Enzymatic hydrolysis results (Table S3, Figure. 4F and 4I) showed that PE in *H. abdominalis* had high DHA content (40.2% at *sn-1*, 13.72% at *sn-2*), while DHA content in *H. erectus* was below 2.5% at *sn-2*. C18:1 in *H. abdominalis* was relatively evenly distributed between *sn-1* (14.8%) and *sn-2* (10.8%), while in *H. erectus*, C18:1 content was higher at *sn-1* (27.4%) than at *sn-2* (13.2%).

4. Discussion

The present study systematically characterized the lipid composition, molecular species, and fatty acid positional distribution of two seahorse species (*H. abdominalis* and *H. erectus*), revealing significant interspecific differences that may be attributed to variations in their habitats and species-specific metabolic characteristics [22,23]. These differences highlight the superior nutritional value and functional potential of *H. abdominalis* compared to *H. erectus*. In terms of lipid class composition, both seahorse species showed TG as the predominant lipid class, which is consistent with the lipid profile of many deep-sea fish [24]. Notably, the two seahorse species were relatively abundant in the diversity of their DG molecular species, a phenomenon that might be associated with the hydrolysis

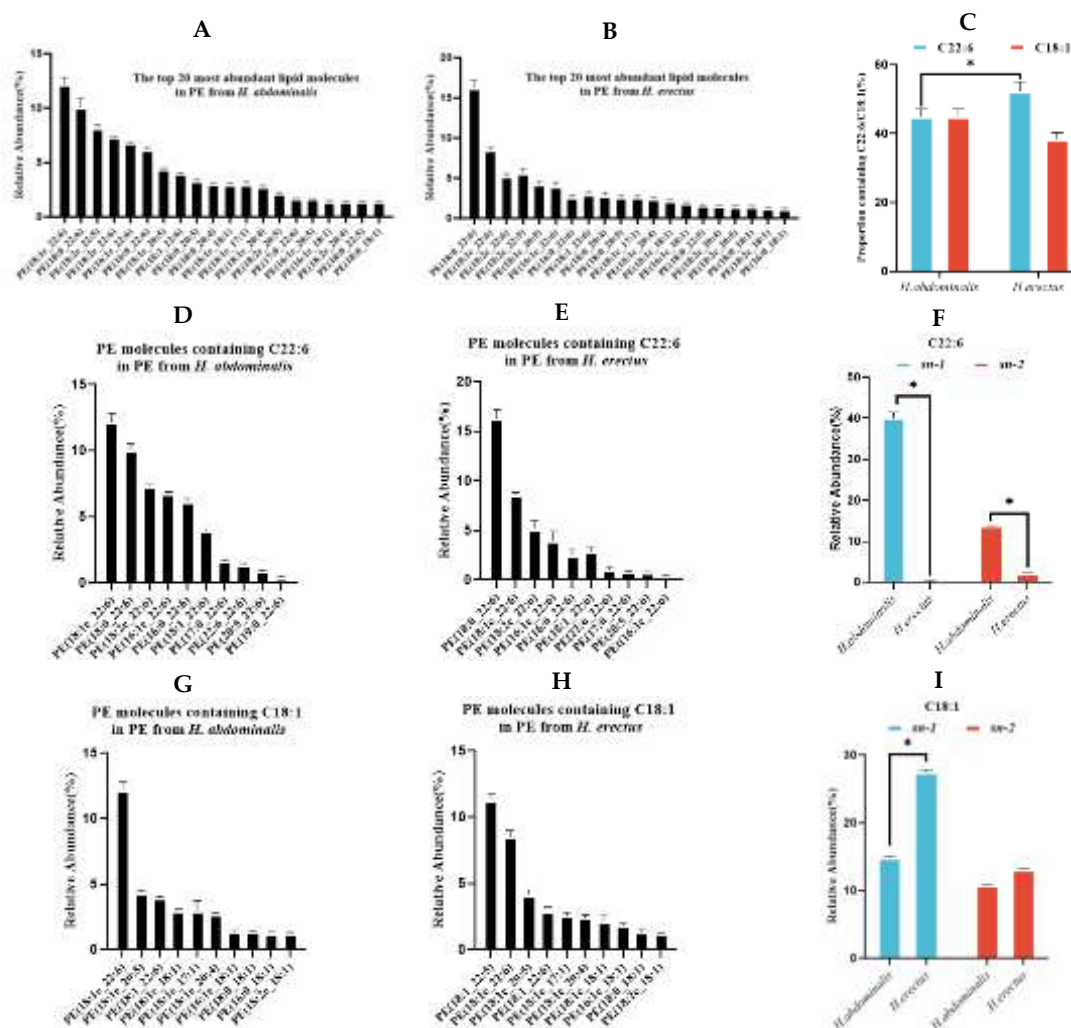


Figure 4. Relative abundances of the top 20 PE lipid molecular species in PE from *H. abdominalis* (A) and *H. erectus* (B) under positive mode; The ratio of the number of PE molecular species containing C22:6 and C18:1 from the two seahorse species; The relative abundance of top 20 lipid molecules containing C22:6 in PE from *H. abdominalis* (D) and *H. erectus* (E); Fatty acids intra-positional composition of C22:6 in PE of the two *Hippocampus* species (F); The relative abundance of top 20 lipid molecules containing C18:1 in PE from *H. abdominalis* (G) and *H. erectus* (h); Fatty acids intra-positional composition of C18:1 in PE of the two *Hippocampus* species (I). Values are expressed as mean \pm standard error of the mean (SEM). The number of independent biological replicates is $n = 3$ for each group of TG catalyzed by TG lipase within the organisms during transportation and storage. Furthermore, The higher UFA content (especially DHA and EPA) in *H. abdominalis* (61.1%) compared to *H. erectus* (49.6%) is a key finding, as PUFAs like DHA and EPA are well-documented for their beneficial effects on human health, including lowering blood lipids and alleviating atherosclerosis [25]. The total DHA + EPA content of both seahorses was higher than that of *H. kelloggi* [26], further confirming seahorses as a valuable source of these essential fatty acids.

The OPLS-DA results demonstrated distinct lipid molecular characteristics between the two species, with *H. erectus* possessing more lipid types, particularly in negative ion mode. This difference may be related to the ionization properties of lipids: triglycerides are effectively ionized in positive ion mode, while phospholipids (with negatively charged phosphate groups) form stable $[M-H]^-$ or $[M+HCOO]^-$ ions in ESI negative mode, facilitating their detection [27,28]. The higher relative proportion of PC in *H. abdominalis* (9.9% vs. 9.0% in *H. erectus*) is noteworthy, as PC is a critical component of cell membranes and plays important roles in lipid metabolism. The analysis of TG molecular species revealed 408 differential species between the two seahorses, with *H. abdominalis* showing higher levels of DHA- and EPA-containing lipids. The enrichment of DHA and EPA in the major TG species of both seahorses, even at moderate overall proportions in total lipids, indicates targeted accumulation of these PUFAs, which is consistent with findings in some marine fish species [29]. Furthermore, we focus primarily on the differences in fatty acids at the *sn*-1,3 and *sn*-2 positions of TG. The rationale behind this focus is that triglycerides in the digestive tract are preferentially hydrolyzed by pancreatic lipase into *sn*-2 monoacylglycerol prior to absorption. The experimental results demonstrated that the positional distribution of fatty acids in TG further amplifies the nutritional superiority of *H. abdominalis* in that DHA is enriched at the *sn*-2 position (8.5%) a position with higher fatty acid absorption efficiency while the EPA content at the *sn*-1,3 positions is significantly higher than that in *H. erectus*. This distinct positional distribution pattern facilitates the more efficient exertion of DHA and EPA's nutritional benefits [30].

For phospholipids, *H. abdominalis* showed upregulation of most differential PC and PE species, despite having fewer total PE molecular species than *H. erectus*. The high abundance of alkoxy ether phospholipid PE in *H. abdominalis* (e.g., PE(C18:1e/C22:6)) is particularly significant, as these lipids possess superior oxidation resistance and are associated with multiple nutritional function [31,32]. The positional distribution of fatty acids in PC and PE also contributes to the higher lipid absorption efficiency of *H. abdominalis* in that DHA is highly concentrated at the *sn*-1 position of PC (44.0%) and PE (40.2%), while the even distribution of C18:1 in PE may further optimize lipid metabolism [33].

Collectively, these results indicate that *H. abdominalis* may possess superior nutritional value to *H. erectus* from the aspect of lipid and thus holds greater potential for the development of lipid-derived functional ingredients and precision nutrition products [34]. The distinct lipid profiles of the two seahorse species furnish a scientific foundation for their targeted application in the food and pharmaceutical industries. Given that the present study is constrained by the lack of quantitative analysis of lipid molecular species, future research could focus on conducting in-depth quantitative investigations to clarify the precise lipid composition of the two seahorse species, as well as evaluating the *in vitro* and *in vivo* biological activities of these target lipids.

5. Conclusions

This study clarified the lipid differences between two farmed seahorse species, *Hippocampus abdominalis* and *Hippocampus erectus*, by comparing their lipid content, composition, molecular species, and fatty acid positional distribution and using untargeted lipidomics combined with enzymatic hydrolysis. Of these lipids, triglyceride (TG) were the major lipid class in both species. Interestingly, *H. abdominalis* had higher levels of phospholipids (PL) than *H. erectus*. Specifically, the content of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in the TG of *H. abdominalis* was higher than that in *H. erectus*. Moreover, DHA in *H. abdominalis* was evenly distributed at the *sn*-1,3, and *sn*-2 positions, while in *H. erectus*, DHA was mainly distributed at the *sn*-1,3 positions. In contrast, EPA was concentrated at the *sn*-1,3 positions in both seahorse species. Additionally, the DHA content in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of *H. abdominalis* was much higher than that in *H. erectus*, and this DHA was mainly concentrated at the *sn*-1 position. Notably, *H. abdominalis* also contained a large amount of alkoxy ether phospholipids. This study addresses the existing research gaps in the lipid profiles of two commercially viable large-scale cultured seahorse species. Specifically, it characterizes the interspecific differences in lipid molecular species, as well as the difference, in fatty acids at distinct positions of the major molecular species

(i.e., TG, PC, and PE. These findings provide a fundamental basis for the species identification and development of functional lipids derived from seahorses.

Supplementary Materials: The following supporting information can be downloaded at: 10.5281/zenodo.18428464, Figure. S1 Lipid Extraction Yield of the Two Seahorse Species (mg/g dry weight); Figure. S2 The mixed standard of the FAME GC chromatograms; Figure. S3 The relative abundance of triglycerides (TGs) and phospholipids (PLs) in *Hippocampus abdominalis* and *Hippocampus erectus*; Figure. S4 Schematic diagram of Total Ion Chromatogram (TIC) of two seahorse species under positive ion mode; Figure S5 Schematic diagram of Total Ion Chromatogram (TIC) of two seahorse species under negative ion mode. Table S1: Fatty acids intra-positional composition in TG of the two *Hippocampus* species; Table S2: Fatty acids intra-positional composition in PC of the two *Hippocampus* species. Table S3: Fatty acids intra-positional composition in PE of the two *Hippocampus* species.

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Abbreviations

The following abbreviations are used in this manuscript:

BisMePA	Bis(methoxyethyl)phosphatidylethanolamine
Cer	Ceramide
CL	Cardiolipin
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
DHA	Docosahexaenoic Acid
dMePE	Dimethylethanolamine Phosphatidylethanolamine
EPA	Eicosapentaenoic Acid
Hex1Cer	Monoglucosylceramide
IACUC	Institutional Animal Care and Use Committee
LPA	Lysophosphatidic Acid
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPI	Lysophosphatidylinositol
LPS	Lysophosphatidylserine
PA	Phosphatidic Acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PL	Phospholipid
SEM	Standard Error of the Mean
SM	Sphingomyelin

TG Triglyceride

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