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EDITED BY
Siu Gin Cheung,
City University of Hong Kong, Hong Kong SAR, China

REVIEWED BY
Ichiro Tayasu,
Research Institute for Humanity and Nature, Japan
Kyung-Hoon Shin,
Hanyang University, Republic of Korea

*CORRESPONDENCE
Yunkai Li
ykli@shou.edu.cn
Feng Wu
fwu@shou.edu.cn

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Effects of ethanol storage and lipid extraction on stable isotope compositions of twelve pelagic predators

Yongfu Shen[1], Mboglen David[1], Yi Gong[1,2,3,4], Feng Wu[1,2,3,4]* and Yunkai Li[1,2,3,4]*

[1]College of Marine Sciences, Shanghai Ocean University, Shanghai, China, [2]The Key Laboratory of Sustainable Exploitation of Oceanic Fisheries Resources, Ministry of Education, Shanghai, China, [3]National Engineering Research Centre for Oceanic Fisheries, Shanghai Ocean University, Shanghai, China, [4]The Key Laboratory of Oceanic Fisheries Exploration, Ministry of Agriculture and Rural Affairs, Shanghai, China

Stable isotope analysis (SIA) has proven to be a powerful tool in reconstructing diets and characterizing trophic relationships for pelagic predators. Ethanol has been a common preservative solution for biopsy samples from remote areas and archived collections. It is still under debate whether the effects of ethanol (ET) would bias the trophic interpretation of the stable isotope values. Further, lipid extraction (LE) is becoming more popular as a general treatment for standardization prior to SIA, particularly for investigating intra and interspecific variation of sympatric species, because lipids have lower $\delta^{13}C$ values. In this study, the long-term (up to 448 days) effects of treatment ET and combined treatments ET and LE (ET+LE) on stable carbon and nitrogen isotope values ($\delta^{13}C$ and $\delta^{15}N$, respectively) of twelve pelagic predators from the open ocean were evaluated. Results showed that compared with control values, $\delta^{15}N$ values displayed a positive change ($\delta^{15}N_{mean}$ offset was 0.71 ± 0.56‰) but $\delta^{13}C$ values had variable results ($\delta^{13}C_{mean}$ offset was 0.42 ± 0.64‰) among all species following treatment with ET during the first 28 days and then remained stable throughout the experiment. Compared with treatment LE results, no difference was observed in $\delta^{13}C$, $\delta^{15}N$ values, and C/N ratios through time following treatment ET+LE. These results indicated that treatment ET may have species-specific effects on stable isotope values, and the shifts from treatment LE could counter the changes caused by treatment ET. In addition, after 28 days of preservation, the values following treatment ET were similar to those following treatment LE in low C/N species (C/N<3.5), which suggested ethanol may also affect some of lipid contents from muscle tissues. Nevertheless, further research is needed to focus on the mechanisms that control changes in stable isotope composition in tissues stored in ethanol. Given the effects on pelagic predators, muscle tissue samples stored in ethanol from the open ocean or a museum after LE treatment could be used to develop SIA.

KEYWORDS

ethanol storage, lipid extraction, pelagic predator, muscle, stable isotopes

Highlight

1. Ethanol may have species-specific effects on stable isotope values within a short period.
2. Lipid extraction could counter the changes caused by ethanol.
3. Ethanol may affect some of lipid contents from muscle tissues.
4. Muscle tissue samples stored in ethanol from the open ocean or museum following LE could be used for stable isotope analysis.

Introduction

Understanding the trophic ecology of pelagic predators is essential as they can profoundly regulate the structure of marine communities (Baum et al., 2003). Studies using stable isotopes (SIA) to access foraging behaviors and migration patterns of pelagic predators are increasing (Li et al., 2016; Gallagher et al., 2017; Bird et al., 2018; Wyatt et al., 2019; Prieto-Amador et al., 2022). These applications are based on the premise that the stable carbon and nitrogen isotope ratios ($\delta^{13}C$ and $\delta^{15}N$, respectively), fractionate systematically throughout the food web as predators consume preys (Peterson and Fry, 1987; Boecklen et al., 2011). Specifically, $\delta^{13}C$ values are generally used to determine the consumer's original dietary carbon source, whereas $\delta^{15}N$ values can provide knowledge of trophic relationships since ^{15}N relatively enriched in consumer tissues (Peterson and Fry, 1987; Post, 2002).

Muscle is the common biopsy tissue, mostly used in genetic studies since it can be easily assessed and is increasingly used for other new biochemical prospective studies such as trophic ecology studies based on SIA (Boecklen et al., 2011; Kiszka, 2014). Compared with most neritic species, trophic studies of pelagic predators often involve a delay between sample collection and laboratory processing. Although freezing is the preferred method of samples preservation due to the limited effects on organisms' $\delta^{13}C$ and $\delta^{15}N$ values (Kaehler and Pakhomov, 2001), this method is inconvenient for samples collected from remote areas or archived samples (Hobson et al., 1997; Carabel et al., 2009). Ethanol (ET) is the most commonly used preservative to store fish tissues. Since the effects of treatment ET on muscle $\delta^{13}C$ and $\delta^{15}N$ values of marine organisms were reported to be variable and inconsistent among species, conducting SIA directly using treatment ET samples was not recommended (Barrow et al., 2008; Boecklen et al., 2011; Kim and Koch, 2012). In addition, lipid extraction (LE) was recommended as a standardization step prior to SIA since lipids were ^{13}C-depleted relative to proteins and carbohydrates and could potentially cause the $\delta^{13}C$ values of organisms to be negatively biased relative to their diet with increasing lipid content (Wessels and Hahn, 2010; Elliott and Elliott, 2016; Li et al., 2016; Bennett-Williams et al., 2022). However, there was less evidence to indicate whether treatment LE affected the stable isotope values of treatment ET samples. Thus, biases introduced by combining treatments ET and LE (ET+LE) must be weighed against the magnitude of effects expected to be documented.

In this context, prior to SIA, treatments ET and ET+LE were performed on muscle tissue of twelve pelagic predators from tuna longline fisheries to 1) investigate the temporal and species-specific effects of treatments ET and ET+LE on $\delta^{13}C$, $\delta^{15}N$ values, and C/N ratios; and 2) determine the relationship between treatments ET and LE in stable isotopic effects and whether treatment ET samples could be used in further SIA.

Materials and methods

Sampling methods

The muscle tissues of 63 individuals from twelve fish species were sampled from pelagic longline fisheries operating in the Atlantic (32° 9′S~18°32′S, 5°19′W~7°13′E) in October 2015. For each individual, fork length (FL) was measured to the nearest cm, and a white muscle sample was excised from the dorsal area and immediately frozen at -50°C before being transferred back to the lab (Table 1).

Sample preparation

Each muscle sample was equally divided into seven subsamples. The first one was handled immediately (referred to as "Control"). The others were preserved in 95% ethanol (referred to as "ET"), and were assigned to different periods (7, 28, 56, 112, 238, 448 days). At the assigned date of analysis, each subsample was freeze-dried at -55°C for 48 hours using a Christ Alpha 1-4 LD plus Freeze Dryer (Martin Christ; Osterode am Harz, GER) and homogenized using a Retsch Mixer Mill MM 400 (RETSCH; Haan, GER). Then, in the 2:1 chloroform/methanol mixture, half of each subsample was lipid extracted (referred to as "ET+LE", ET +LE for 0 days could be regarded as "LE"). The mixture was vortexed for 1 min and left undisturbed overnight at room temperature, then centrifuged for 10 min and decanted. This process was repeated three times, and the samples were re-dried overnight at 80°C to eliminate excess solvent. And then a 1.0~2.0 mg powdered muscle tissue from each subsample was prepared for SIA (Li et al., 2016).

Stable isotope analysis

Approximately 1.0-1.5 mg of prepared sample were weighed into 0.3 mg tin capsules and analyzed using an IsoPrime 100 isotope ratio mass spectrometer (IsoPrime Corporation; Cheadle, UK) and a vario ISOTOPE cube elemental analyzer (Elementar Analysensysteme GmbH; Hanau, Germany) with analytical error at Shanghai Ocean University Stable Isotope Laboratory.

The isotope compositions of the samples were expressed as $\delta^{13}C$ and $\delta^{15}N$ notation using the following equations:

$$\delta^{13}C = \frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{standard}} - 1$$

$$\delta^{15}N = \left[\frac{(^{15}N/^{14}N)_{sample}}{(^{15}N/^{14}N)_{standard}}\right] - 1$$

TABLE 1 Biological information of samples in this study.

Common name	Scientific name	Code	Number	FL/cm
Yellowfin tuna	*Thunnus albacares*	YFT	5	70~105
Bigeye tuna	*Thunnus obesus*	BET	9	23~110
Albacore tuna	*Thunnus alalunga*	ALB	5	33~100
Skipjack tuna	*Katsuwonus pelamis*	SKJ	5	39~67
Blue shark	*Prionace glauca*	BSH	7	59~73
Shortfin mako shark	*Isurus oxyrinchus*	SMA	6	57~93
Blue marlin	*Makaira nigricans*	BUM	5	85~156
Swordfish	*Xiphias gladius*	SWO	5	71~147
Indo-Pacific sailfish	*Istiophorus platypterus*	SFA	5	100~110
Moonfish	*Lampris guttatus*	LAG	5	/
Wahoo	*Acanthocybium solandri*	WAH	3	50~95
Escolar	*Lepidocybium flavobrunneum*	LEC	3	38~45

where $^{13}C/^{12}C$ and $^{15}N/^{14}N$ are the atomic ratios of ^{13}C and ^{15}N in the sample or standard, respectively, and δ is the measure of the heavy-to-light isotope in the sample, expressed in parts per thousand (‰). The standard reference materials for C and N were Pee Dee Belemnite carbonate (VPDB) and air (Air-N_2), respectively. Reference standards USGS 24 (-16.049‰) and USGS 26 (53.7‰) were used to quantify ^{13}C and ^{15}N stable isotope values, respectively. Every tenth sample was run in triplicate of Organic Analytical Standard (Protein (-26.98‰ and 5.96‰)) to correct for linearity and instrument drift, and a blank sample was run every ten samples to clear off residual gases. The analytical errors were approximately 0.20‰ for both $\delta^{13}C$ and $\delta^{15}N$ values. All C/N ratios were calculated based on atomic mass.

Statistical analysis

Paired Student's t-test was performed on each treatment (ET and ET+LE) on $\delta^{13}C$, $\delta^{15}N$ values, and on C/N ratios to assess the effect of each period of preservation on those parameters. Then we used analysis of variance (ANOVA) to assess the effect of time of preservation on stable isotope values, and used exponential relationships to determine when preservation alters stable isotope values. The difference in $\delta^{13}C$ and $\delta^{15}N$ values between the day 0 and each time ($\delta_{0\ day}$- $\delta_{x\ days}$) was calculated for each sample (hereafter referred to as the $\delta^{13}C$ and $\delta^{15}N$ offsets), and Analysis of Variance (ANOVA) was then performed to compare the differences. Finally, a paired Student's *t*-test was performed to compare the differences between treatments ET and LE samples. All statistical analyses were performed in SPSS 22.0 and/or R 4.1.0.

Results

The means of the $\delta^{13}C$, $\delta^{15}N$, and the C/N ratios for all treatments of the different periods are shown in Table S1. Our results showed that treatment ET produced species-specific effects on the $\delta^{13}C$, $\delta^{15}N$ values, and C/N ratios during 28 days of the experiment and then these values remained stable throughout the experiment. Meanwhile, stable isotope values and C/N ratios of all the species did not change following treatment ET+LE (Figure 1). Paired t-tests revealed no significant difference in $\delta^{13}C$, $\delta^{15}N$ values, and C/N ratios between treatments LE and ET+LE for all twelve species at each period (Figure 1).

Specifically, the effects of treatment ET on $\delta^{13}C$ values were variable through time until reaching an asymptote, where the direction and magnitude of change varied among taxa. Compared with control values, positive shifts were observed in *P. glauca*, *I. oxyrinchus*, *M. nigricans*, *X. gladius*, *I. platypterus*, *L. guttatus*, *A.solandri* and *L. flavobrunneum* (Table S1), while substantial variation was detected in $\delta^{13}C$ offsets among these species (Figure 2). There were no changes in $\delta^{13}C$ values of tuna species following treatment ET (Table S1), but a mean negative change occurred in *K. pelamis* ($P<0.05$). The largest offsets were observed in *L. flavobrunneum* (2.36 ± 0.64‰). Treatment LE caused positive shifts in $\delta^{13}C$ values across all species except for tuna species (Figure 1; Table S1) and the $\delta^{13}C$ offsets of *L. flavobrunneum* were utmost (4.86 ± 0.16‰), which were similar to the treatment ET. Paired t-test results between treatments LE and ET+LE showed limited shifts in $\delta^{13}C$ values among all twelve species at each period (Figure 1; Table S1), and the $\delta^{13}C$ offsets displayed little individual variation (Figure 2).

For $\delta^{15}N$ values, both treatments ET and LE caused a positive shift for all the species during a short period (Figure 1; Table S1), with *L. flavobrunneum* showing the largest $\delta^{15}N$ offsets (ET: 2.15 ± 0.56‰, LE: 1.45± 0.17‰, Figure 2). The $\delta^{15}N$ values showed no changes among all the species at each period between treatments LE and ET +LE, which was consistent with the effects of $\delta^{13}C$ values (Figure 1). The C/N ratios of *P. glauca* and *I. oxyrinchus* in either treatment ET (ranged from 2.63 to 3.11) or LE (ranged from 2.97 to 3.25) were generally higher than control (ranged from 2.37 to 2.80, Table S1). On the contrary, the other teleost species displayed negative shifts in C/N ratios following both treatments, LE and ET (Table S1). Furthermore, no difference in C/N ratios was found between treatments LE and ET +LE (Figure 1). In addition, stable isotope values and C/N ratios of muscles following treatment ET were different from the treatment LE

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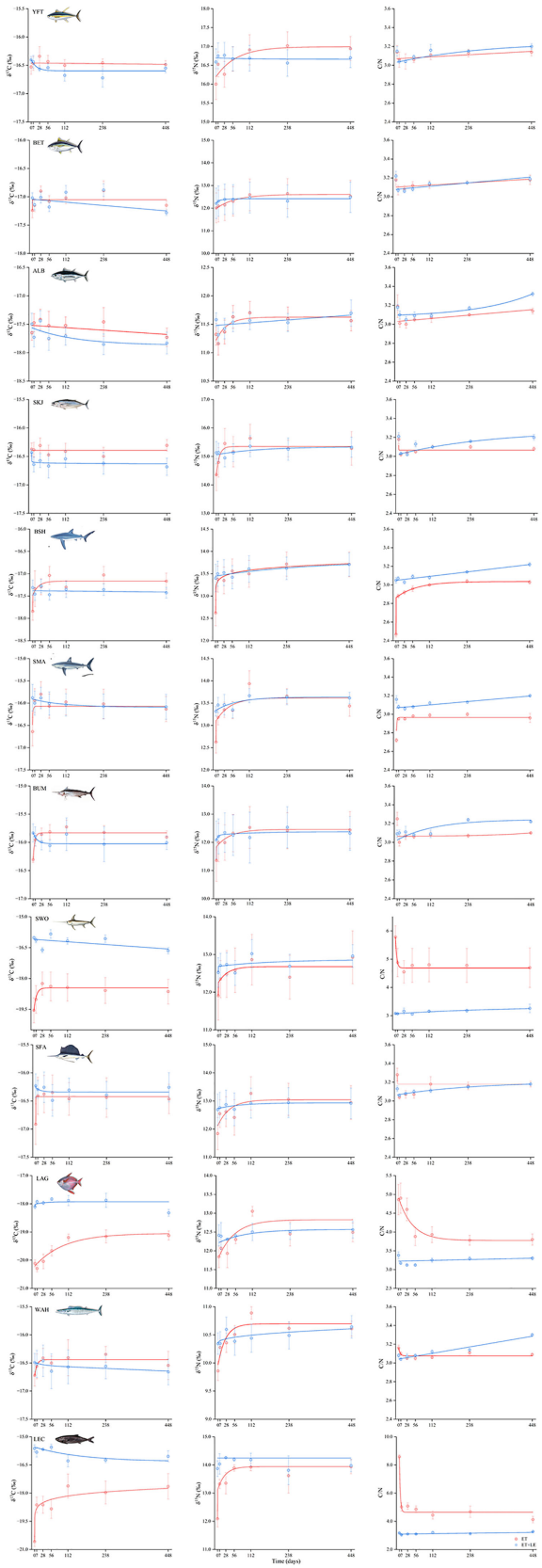

FIGURE 1
Time series data of mean±SE of $\delta^{13}C$ and $\delta^{15}N$ value and C/N ratios in twelve pelagic predators' muscle stored ethanol (red) and combined ethanol storage and lipid extraction (blue).

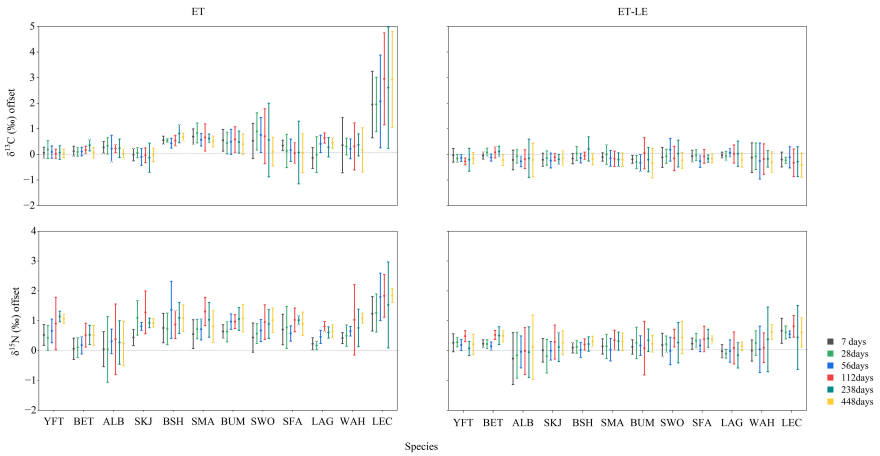

FIGURE 2
The $\delta^{13}C$ and $\delta^{15}N$ offsets for all treatments (ET, ET+LE) in muscle tissue across each species.

samples on the 7th dayand/or the 28th day of preservation ($P<0.05$), and then simulation results were observed throughout the experiment, except for the $\delta^{13}C$ values of *X.gladius*, *L.guttatus*, and *L.flavobrunneum*.

Discussion

SIA has the potential to significantly improve our understanding of predator's trophic ecology, but it is dependent on the removal of known biases caused by the treatment method. Understanding the effects of treatments ET and ET+LE on predator muscle tissue stable isotope values is valuable for accurately interpreting data in food-web studies. Removing these biases is essential for acquiring standardized stable isotope values among different species, and a reasonable adjustment could be helpful for the use of the valuable biopsy samples obtained from remote areas and archived collections (Kim and Koch, 2012; Li et al., 2016; Bennett-Williams et al., 2022). In this study, the results for twelve pelagic predators suggested that both treatments ET and ET+LE caused inconsistent effects in $\delta^{13}C$, $\delta^{15}N$ values, and C/N ratios during the 28 days of preservation and then could remain stable throughout the experiment, yielding new insights into the effects of these treatments.

Post et al. (2007) considered the increases in tissue $\delta^{13}C$ values to be positively correlated to the amount of lipids. In this study, twelve species were affected inconsistently with different $\delta^{13}C$ offsets following treatments either ET or LE suggesting potentially different lipid content and species-specific metabolic pathways (Javornik et al., 2019). Von Endt (1994) found that a variety of lipids in the ethanol storage media, especially some nonpolar lipids such as triglycerides, could be extracted by ethanol. Consequently, ethanol and the 2:1 chloroform/methanol mixture extraction of ^{13}C-depleted and carbon-rich lipids from tissues may lead to changes in $\delta^{13}C$ values. Meanwhile, our results showed the $\delta^{13}C$ values could remain stable after the initial change, which was similar to previous researches (Le Bourg et al., 2020; Sarakinos et al., 2002). A possible mechanism is that protein lysis in muscle and integration of C from the preservative liquid into the samples may occur in a short period (Sarakinos et al., 2002). However, $\delta^{13}C$ values of treatment ET+LE in muscle were similar to the LE, suggesting that ^{13}C-depleted compounds such as lipids have been removed, potentially counteracting the changes in $\delta^{13}C$ values caused by the treatment ET.

This study discovered unexpected effects of treatment ET on $\delta^{15}N$ in all species. Such $\delta^{15}N$ enrichment following treatment ET was reported in other fishes and molluscs (Kelly et al., 2006; Sweeting et al., 2006; Syväranta et al., 2011; Liu et al., 2013). Ethanol causing tissue hydrolysis, leaching, and extracting certain constituents containing nitrogen from the muscle tissue in addition to lipids could explain these shifts (Horii et al., 2015; Sarakinos et al., 2002). Furthermore, we discovered that treatment LE resulted in a significant increase in $\delta^{15}N$ values in muscle tissues. It conflicts with previous research showing no effects or a small positive shift (Javornik et al., 2019; Le Bourg et al., 2020), but agrees with Li et al. (2016). Polar solvents, such as chloroform-methanol and the 2:1 chloroform/methanol mixture extraction, are commonly used for lipid extraction since they can extract both nonpolar lipids (e.g., triglycerides) and polar lipid compounds (e.g., phospholipids and free fatty acids) (Schlechtriem et al., 2003; Doucette et al., 2010). Giménez et al. (2017) reported that polar solvents could increase $\delta^{15}N$ values in tissues as a substantial amount of non-lipids, including some hydrophobic essential amino acids, were extracted (Elliott and Elliott, 2016). In addition, the C/N ratios were between 3 and 3.5 in both treatments ET+LE and LE treatments, suggesting lipid extraction was sufficient. No major effects on $\delta^{15}N$ between the two treatments instructed that lipid extraction was sufficient. Thus, sufficient lipid extraction may reduce the variability of treatment ET effects on stable isotope signatures in marine organisms (Lesage et al., 2010; Ruiz-Cooley et al., 2011). Meanwhile, our study showed no time-dependent variation of $\delta^{15}N$ signatures with chemical preservatives. Sweeting et al. (2004) also reported that the most significant effects of ethanol preservation on stable isotope signatures generally occurred within a short period. We observed a decrease in C/N ratios of all teleost species following two treatments because of removing of carbonaceous waste from lipids. (Kaehler and Pakhomov, 2001; Javornik et al., 2019). However, shark species showed the opposite effect, suggesting that the 2:1 chloroform/methanol mixture could also remove urea leading to depletes in ^{15}N (Hussey et al., 2012; Li et al., 2016).

Previous research showed that it is not necessary to account for lipids in aquatic animal samples when lipid content is consistently lower than 5% (C/N< 3.5) (Post et al., 2007). However, in our study, ethanol and the 2:1 chloroform/methanol mixture could significantly affect the stable isotope values of eight species with low lipid content (C/N<3.5), suggesting removing lipids are also necessary for these species. In addition, the stable isotope values following treatment ET were similar to those following treatment LE in nine species after the 28 days of preservation, indicating ethanol could also affect some of lipid contents from muscle tissues, though this effect is not significant in species with high C/N ratios (*X.gladius*, *L.guttatus*, and *L.flavobrunneum*). However, further research is required to understand the mechanisms behind ethanol-induced changes in stable isotope values.

In summary, when using archived samples to SIA, the effects of preservation method should be considered. Our results indicated that ethanol could produce species-specific effects on stable isotope values and C/N ratios in pelagic predators within a short period. For LE samples, we can ignore the effects of ethanol since the shifts from LE could counter the changes caused by ET. In addition, researchers could use low C/N samples (C/N<3.5) following treatment ET after 28 days for stable isotope analysis instead of treatment LE samples, since the ethanol and the 2:1 chloroform/methanol mixture may have similar effects in these species. Treatment ET+LE is thus recommended as the optimal method for obtaining stable isotope values from archived samples.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by Shanghai Ocean University.

Author contributions

YS and YL conceived and designed the experiments. FW provided the tissue samples. YS performed the experiments and analyzed the data with the help of MD, YG and YL. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1118013/full#supplementary-material

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