

Article

Behavioral and Physiological Requirements for Artificial Shelters in Juvenile Sea Cucumbers *Apostichopus japonicus*

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Simple Summary

This study investigated the behavioral and physiological requirements of the sea cucumber *Apostichopus japonicus* for artificial shelters. We found that feeding but not foraging was the behavioral requirement for the sea cucumbers with a high shelter requirement. At the physiological level, sea cucumbers with a high shelter requirement exhibited a greater demand for food digestion and harbored a more diverse gut microbiota. In addition, sea cucumbers with a high shelter requirement did not rely on shelters for stress alleviation in the absence of external handling stress. This study clarified the behavioral and physiological requirements of sea cucumbers on shelters, and enriched our understanding of the shelter dependence of sea cucumbers.

Abstract

Shelters can enhance the growth efficiency of sea cucumbers, while the preference of sea cucumbers for shelters varies among individuals. Therefore, this study investigated the behavioral and physiological requirements of the sea cucumber *Apostichopus japonicus* for artificial shelters. In this experiment, we considered sea cucumbers that spent more than 80% of their time (2880 s) inside the shelter as the sheltered sea cucumbers and those that spent less than 20% of their time (720 s) inside the shelter as the non-sheltered sea cucumbers. We found that mouth tentacle grasping times in the sheltered group were significantly lower than in the non-sheltered group, while foraging selections of both groups were not significantly different. This indicates that feeding is the behavioral requirement for the sheltered group instead of foraging. The height of the intestinal crease was significantly shorter in the sheltered group than in the non-sheltered group. Further, the defecation rate and 5-HT content in the intestinal tract of the non-sheltered group were significantly lower than those of the sheltered group. This indicates that the sheltered group has a greater demand for food digestion than the non-sheltered group. Compared with the non-sheltered group, the sheltered group showed higher relative abundances of Gammaproteobacteria and Bacteroidia in the gut microbiota. The thermal tolerance was significantly worse in the sheltered group. Furthermore, there was no significant difference in movement distance after mechanical disturbance between the two groups. Cortisol content showed no significant difference either. These indicate that the sheltered sea cucumbers do not require shelters for stress relief in the absence of external handling stress. This study clarified the behavioral and physiological requirements of sea cucumbers on shelters and enriched our understanding of the shelter dependence of sea cucumbers.

Keywords: sea cucumbers; requirements; artificial shelters; behavior; physiology



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1. Introduction

Shelters are an important habitat for both terrestrial and marine animals [1,2]. A shelter is a habitable covered living space providing protective environment such as contact surfaces, shading, and internal safety space. Shelters provide several benefits for animals [3], including anti-predation [4] and reproduction [5]. For instance, different quantities of shelters have different effects on the survival of mud crabs *Scylla paramamosain* (Estampador, 1949) [6]. Shelters are not only important for the survival but also have the benefits for behavior and physiology of animals [6–8]. For example, shelters can reduce the conflicts between juvenile scalloped spiny lobster *Panulirus Homarus* (Linnaeus, 1758) and crucifix crab *Charybdis feriatus* (Linnaeus, 1758), as well as increase the survival rate of aquaculture [9]. A shelter promotes foraging behavior of sea cucumbers at high temperatures [10]. The shelter affects the survival, behavior and physiological functions of animals by providing hiding places [11], alleviating stress [12], and altering digestive enzyme activity [13]. Despite the well-documented benefits of shelters, how shelters are related to animal behavior and physiological regulation remains largely unexplored. The distribution of redfin snapper *Lutjanus erythropterus* (Bloch, 1790) is the highest at 30.4% in shelter areas [14]. Similarly, shelter availability has been shown to promote aggregation and habitat use across diverse aquatic species [15,16]. The aggregation rate of sea cucumbers increases from 1.03% to 16.42% in shelter areas [3]. These indicate that individuals differ in their requirements for shelters due to individual differences. Therefore, studying individual difference is an important way to reveal the shelter requirements of animals.

The sea cucumber *Apostichopus japonicus* (Selenka, 1867), especially in the juvenile stage, is a typical shelter-dependent marine species in shallow waters [11,17,18]. In the present study, under aquaculture conditions, obvious individual differences were observed in the preference of *A. japonicus* for shelters, as reflected by variations in shelter occupancy and residence time among individuals during behavioral observations. The clear behavioral differentiation, together with its strong dependence on shelters and relatively simple benthic lifestyle, makes *A. japonicus* an ideal experimental model for investigating the internal behavioral and physiological requirements associated with shelter use. Further, sea cucumbers have important nutritional and medicinal values [19]. Thus, it is crucial to clarify the demands for shelters to enhance the production efficiency of juvenile sea cucumbers.

Behavior and physiology are important to understand the difference in requirements between more shelter-dependent and less shelter-dependent sea cucumbers. Foraging and feeding are the behavioral processes, by which sea cucumbers find and consume food [20]. The efficiency of both behaviors has a large impact on the nutrient intake of sea cucumbers [21]. These performances are valuable to reveal the behavioral need for shelter between the sheltered and non-sheltered sea cucumbers. Defecation behavior refers to the process of the sea cucumber expelling the part that cannot be digested and absorbed [22]. Gut structure and digestive enzyme activities are important for the digestive ability of sea cucumbers [23,24]. The gut microbiome composition can indicate diseases in sea cucumbers [25,26]. Cortisol and 5-hydroxytryptamine (5-HT) are crucial for regulating the physiology and behavior of sea cucumbers [27]. Cortisol alleviates the stress behavior of sea cucumbers, and 5-HT affects intestinal peristalsis [28]. In addition, CT_{max} and handling stress were used to assess the resistance of sea cucumbers [29,30].

This study aimed to investigate the behavioral and physiological requirements of juvenile sea cucumbers associated with shelter use through controlled ex situ experiments. Juvenile *A. japonicus* originating from aquaculture facilities were exposed to artificial shelters. Their behavioral responses (residence time, foraging, and defecation behavior) and physiological traits (gut structure, digestive enzyme activities, and gut microbiota composition) were quantified and compared between sheltered and non-sheltered individuals.

This approach was designed to elucidate the internal mechanisms underlying shelter dependence and to provide a scientific basis for improving survivability and production efficiency in intensive sea cucumber aquaculture.

2. Materials and Methods

2.1. Sea Cucumbers

Juvenile sea cucumbers randomly selected from a local aqua-farm in Wafangdian, Dalian (121.73° E, 39.85° N) were transferred to the Key Laboratory of Mariculture & Stock Enhancement in the North China's Sea, Ministry of Agriculture and Rural Affairs at Dalian Ocean University (121.62° E, 39.45° N). Individuals were temporarily maintained in indoor tanks (70 L; length × width × height: 70 × 50 × 40 cm), supplied with aerated seawater under controlled laboratory conditions, and were fed a blended diet consisting of sea mud and commercial feed (5:1, Anyuan Industrial Co., Ltd., Dalian, Liaoning, China). Seawater was changed every day. All replacement seawater was pumped from the Yellow Sea of China into a reservoir for storage and subsequently used for the experimental tanks. The sea cucumbers were maintained in captivity for one week to eliminate the delayed effects caused by transportation stress. According to the measurement results of the water quality analyzer (YSI Incorporated, Yellow Springs, OH, USA), the seawater temperature was 14.51 ± 0.49 °C, the salinity was 29.14 ± 0.43 ‰, and the pH was 8.02 ± 0.03 .

2.2. Experimental Design

This experiment investigated the differences in behavior and physiology between sheltered (group M) and non-sheltered (group L) sea cucumbers. PVC tubes were used to mimic the natural crevices that natural shelters provide. Their shape, rigidity, and enclosed cavity provide standardized and repeatable artificial shelter conditions [11,13]. In this experiment, PVC tubes were also used as the artificial shelters. Each sea cucumber was placed in one pipe shelter (PVC pipe, length × diameter: 50 × 30 mm [11]) for 1 h (3600 s), and the time at which the sea cucumber left the shelter was recorded. In this experiment, we considered sea cucumbers that spent more than 80% of their time (2880 s) inside the shelter as the sheltered sea cucumbers (group M) and those that spent less than 20% of their time (720 s) inside the shelter as the non-sheltered sea cucumbers (group L) (Figure 1A). A total of 120 valid individuals per group were retained for subsequent analyses. The two groups of sea cucumbers were cultured separately in individual tanks (68 × 47 × 35 cm). A total of 120 sea cucumbers were used for each group, with 30 sea cucumbers placed in one tank. We mixed sea mud and feed (Anyuan Industrial Co., Ltd., China) in a ratio of 5:1 and were fed as food. Water was changed every day.

Two groups of sea cucumbers were cultured for 3 days to eliminate the retention effect caused by the artificial shelters. Foraging selection, grasping times of mouth tentacles, and defecation rate were measured in both groups M and L. We performed pepsin-like protease activity, gut morphological analysis, and intestinal microbiological 16S rRNA gene sequencing of sea cucumbers. In addition, movement distance after mechanical disturbance, CT_{max} , 5-HT, and cortisol levels were measured. All the methods of behavioral and physiological parameters were described as follows.

2.3. Foraging Selection

The experimental setup was according to Yang et al. [30]. Foraging selection analysis was performed in a device (length × width × height: 18 × 10 × 10 cm, Figure 1B), which was divided equally into the food zone (FZ) and the non-food zone (NZ). FZ contained 0.5 g of sea mud and food (the mixture consisted of 10 mL of fresh seawater and 0.1 g of feed from Anyuan Industrial Co., Ltd., China). Each sea cucumber (2.06 ± 0.33 g for

group M, 1.95 ± 0.25 g for group L; no significant difference between groups M and L) was carefully placed in the center of the device, and it was then given the option to choose between two areas (Figure 1B). The camera (FDR-AXP55, Sony Corporation, Tokyo, Japan) above the selection device was used to record the foraging behavior of the sea cucumber for 120 min. The experiment ended with separate counts of residence time for sea cucumbers in FZ and NZ. Timing was started when half of the body of the sea cucumber entered the area. Foraging selection experiments were performed with eight different sea cucumbers per group ($N = 8$).

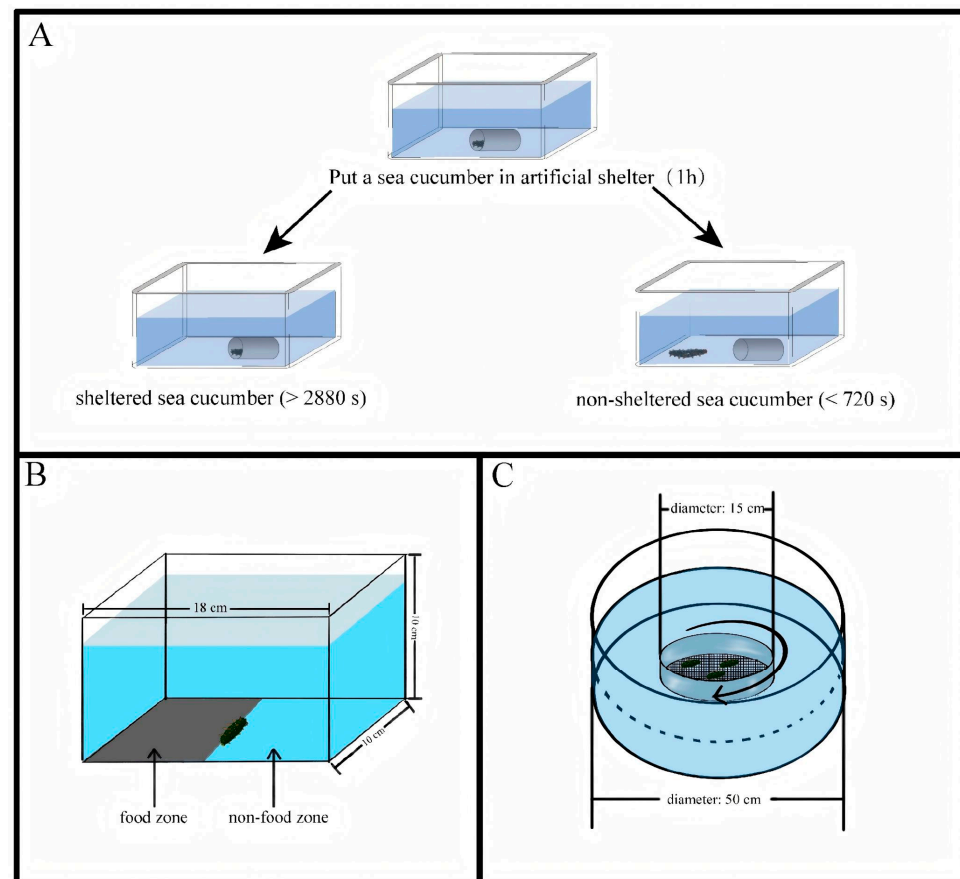


Figure 1. Experimental setup. Experimental design (A), foraging behavior (B), and mechanical disturbance schematic diagram (C).

2.4. Grasping Times of Mouth Tentacles

Sea cucumbers assigned to group M (1.93 ± 0.23 g) and group L (2.14 ± 0.29 g), which did not differ significantly in body weight, were individually placed into an experimental tank (length $\times$ width $\times$ height: $180 \times 140 \times 45$ mm). A video camera (FDR-AXP55, Sony Corporation, Tokyo, Japan) was placed above the experimental setup. Feeding behavior of *A. japonicus* was recorded for 60 min. The number of mouth tentacle extensions and contractions in sea cucumbers was counted over 60 min. We further calculated the average frequency of the mouth tentacles retraction. The experiment was repeated eight times in each group ($N = 8$).

2.5. Defecation Rate

The two groups of sea cucumbers were cultured separately in individual tanks ($68 \times 47 \times 35$ cm). A total of 60 sea cucumbers were used in each group for the experiment, with 20 sea cucumbers being raised in one tank. Feces produced by the sea cucumber within 24 h were collected and rinsed with clean water. After drying in an oven at 65°C for 24 h,

feces were weighed using an electronic balance (ACS-FS102, Zhuheng Electronics Co., Ltd., Wuhan, China) [30]. The experiment was repeated three times in each group ($N = 3$). Their feces were collected. We calculated the defecation rate (DR) using the following formula:

$$DR = W_f \div (W_t \times T)$$

where W_f is the dry weight of feces, W_t is the total wet weight of sea cucumbers, and T is the feeding time ($T = 1$ day).

2.6. Pepsin-like Protease Activity

Sea cucumber intestines were dissected using sterile instruments. For each group, intestines from three individuals were pooled to form one composite sample. The samples were quickly frozen in liquid nitrogen and then stored at -80°C . Protease activity was measured using an ELISA (Epoch, BioTek Instruments Inc., Winooski, VT, USA), following the guidelines of the pepsin assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Three independent pooled samples were prepared for each group ($N = 3$).

2.7. Gut Morphological Analysis

Sea cucumber intestines were dissected using sterile instruments. After dissection, gut tissue samples from sea cucumbers were fixed in Bouin's solution (1:5:15 mixture of glacial acetic acid, formaldehyde, and saturated picric acid) following Ding et al. [24]. Gut morphological analyses were performed on histological sections to evaluate intestinal fold (crease) structure, including fold height and general epithelial organization. Three replicates of the sea cucumber gut from each group were performed for the gut morphological analysis ($N = 3$).

2.8. Intestinal Microbiological 16S rRNA Gene Sequencing

Three sea cucumbers were randomly selected from each of the two groups for the gut microbiota structure analysis [31]. Intestinal sampling was performed after a 48 h fasting. The guts of sea cucumbers were collected using sterile instruments. Each pooled sample consisted of 2 sea cucumber guts mixed and placed into a 1.5 mL tube, resulting in three pooled samples per group ($N = 3$). The samples were flash-frozen in liquid nitrogen and stored at -80°C for later analysis.

Total genomic DNA was extracted from gut content samples using a commercial DNA extraction kit (TIANamp Stool DNA Kit, TIANGEN Biotech Co., Ltd., Beijing, China) following the manufacturer's protocol. DNA concentration and quality were assessed using a NanoDrop spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE, USA) and agarose gel electrophoresis (Bio-Rad Laboratories, Hercules, CA, USA).

The V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified using universal primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') [32,33]. PCR amplification was performed under the following conditions: initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s, with a final extension at 72°C for 10 min.

PCR products were purified, quantified, and pooled in equimolar concentrations. Sequencing libraries were constructed according to the standard Illumina protocol and sequenced on an Illumina MiSeq platform using paired-end reads (2×300 bp) [34].

Raw paired-end reads were processed following a standard bioinformatics pipeline. Adapter and low-quality sequences were removed using Trimmomatic (v0.33), applying a 50 bp sliding window with an average quality score threshold of 20 [31,35]. Primer sequences were trimmed using Cutadapt (v1.9.1), allowing a maximum mismatch rate of

20% and a minimum overlap of 80% [36]. Paired-end reads were merged using USEARCH (v10), with a minimum overlap length of 10 bp, a minimum overlap similarity of 90%, and a maximum of five mismatched bases [37]. Chimeric sequences were detected and removed using the UCHIME algorithm (v8.1) [38].

High-quality sequences were clustered into operational taxonomic units (OTUs) at a 97% sequence similarity threshold using USEARCH (UPARSE pipeline) [37]. Rare OTUs with a relative abundance below 0.005% of the total sequence count were filtered out following recommended practices to reduce spurious diversity [35]. Taxonomic assignment was performed using a naive Bayesian classifier implemented in QIIME2 [39], with a confidence threshold of 0.7, against the SILVA rRNA gene database (release 138) [40]. Alpha diversity indices (Chao1, ACE, Shannon, and Simpson) and beta diversity were calculated, and principal coordinate analysis (PCoA) based on Bray-Curtis distances was used to visualize differences in microbial community structure between groups. Group-level differences in microbial community composition were further tested using permutational multivariate analysis of variance (PERMANOVA, Adonis) and analysis of similarities (ANOSIM) [41].

Linear Discriminant Analysis Effect Size (LEfSe) was applied to identify bacterial taxa with significantly different relative abundances between groups M and L. LEfSe is a biomarker discovery method specifically developed for high-dimensional microbiome data, which combines non-parametric statistical testing with effect size estimation. Briefly, taxa showing significant differences among groups were first detected using the Kruskal-Wallis test, followed by pairwise Wilcoxon rank-sum tests to assess biological consistency, and linear discriminant analysis (LDA) was then used to estimate the effect size of each differentially abundant taxon. This method was chosen, because it is robust to non-normal data distributions and enables identification of biologically meaningful microbial biomarkers [42]. An LDA score threshold of 4.0 was used to identify discriminative features.

The abundance of microbial communities was represented by the Chao1 and ACE, and diversity was measured by Shannon and Simpson. The Shannon index (H') was calculated as follows [43]:

$$H' = - \sum_{i=1}^S p_i \ln(p_i)$$

where S is the total number of taxa and p_i represents the relative abundance of taxon i . This index accounts for both species richness and evenness, with higher values indicating greater diversity.

The Simpson diversity index was calculated as follows [44]:

$$D = 1 - \sum_{i=1}^S p_i^2$$

which reflects the probability that two individuals randomly selected from a sample belong to different taxa and is more sensitive to dominant taxa.

Statistical analyses and data visualization were performed using R software (1.3.1093.0), and differences in microbial composition between groups were assessed using appropriate multivariate statistical methods.

2.9. 5-HT and Cortisol Levels

Three sea cucumbers were selected randomly from each group to determine their 5-HT and cortisol content. At the end of the experiment, three individuals from each group were randomly selected and dissected longitudinally. Coelomic fluid was collected individually from each sea cucumber. The samples were flash-frozen in liquid nitrogen and stored at

−80 °C for the following analysis. Enzyme-linked immunosorbent assay (ELISA) was used to determine the levels of 5-HT and cortisol in accordance with the instructions of the 5-HT and cortisol detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Each experiment was repeated three times using different sea cucumbers ($N = 3$).

2.10. Thermal Tolerance

The critical thermal limit (CT_{max}) is the highest temperature, beyond which an organism is unable to maintain a response to external stimuli [45]. The CT_{max} experiment was performed according to the method of Hu et al. [46] with some modifications. Eight healthy *A. japonicus* were randomly selected from groups M and L, respectively. One sea cucumber was placed in a temperature-controlled tank ($775 \times 470 \times 375$ mm) with a constant temperature system (Huixin Co., Dalian, China). The seawater temperature was raised by 2 °C every hour starting from 20 °C until it reached the lethal temperature. The temperature, at which the sea cucumber did not respond to tactile stimulation for 5 s, was recorded as CT_{max} [46]. Thermal tolerance experiments were performed with eight different sea cucumbers per group ($N = 8$).

2.11. Movement Distance of Sea Cucumbers After Mechanical Disturbance

The movement distance analysis was performed using an experimental setup (length $\times$ width $\times$ height: $180 \times 140 \times 45$ mm). Mechanical disturbance refers to the shaking of a plastic sieve in water to simulate the situation encountered in aquaculture [47] (Figure 1C). Sea cucumbers (1.93 ± 0.13 g for group M, 1.92 ± 0.11 g for group L, no significant difference between groups M and L) were carefully placed in the center of the experimental setup after 5 min of mechanical disturbance [30]. The movement behavior of *A. japonicus* over 120 min was recorded by a camera (FDR-AXP55, SONY, Japan) placed above the experimental setup. Video to Picture software (version 5.3.0.0) was used to intercept 721 pictures from the 2 h video, and the interval between each picture was 10 s. We combined 721 images into one stack using the software ImageJ (version 1.8.0), and extracted the coordinates in the middle of the sea cucumber body in each image using a plug-in manual tracking. Eight different sea cucumbers per group were used for movement behavior analysis ($N = 8$). According to Yang et al. [30], the movement distance (d) was calculated as follows:

$$d = \sum_{i=1}^n \sqrt{(x_{(i+1)} - x_i)^2 + (y_{(i+1)} - y_i)^2} * k \quad (1)$$

where k is the scale of the sea cucumber in the picture, which was determined using the “set scale” in the “analyze” plug-in of the ImageJ (version 1.8.0); (x_i, y_i) is the coordinate in the middle of the sea cucumber body in the i image/images; and n is the total number of images during the movement.

2.12. Statistical Analysis

The Shapiro–Wilk test was used to check for normal distribution, and the Bartlett test was used to check for uniformity of variance. One-way ANOVA was used to analyze the foraging selection, cortisol levels, and movement distance. We used t -test to analyze the grasping times of mouth tentacles, defecation rate, intestinal crease height, crease width, muscle layer thickness, and 5-HT. Mann–Whitney U test was used to compare the CT_{max} differences between groups M and L due to the non-normal data distribution.

R studio (1.3.1093.0) software was used for all statistical analyses. Differences were considered statistically significant when the p value was less than 0.05 ($p < 0.05$).

3. Results

3.1. Foraging Selection

There was no significant difference in the residence time in the food zone between groups M and L, as determined by one-way ANOVA ($F = 0.294$, $p = 0.596$, Figure 2A).

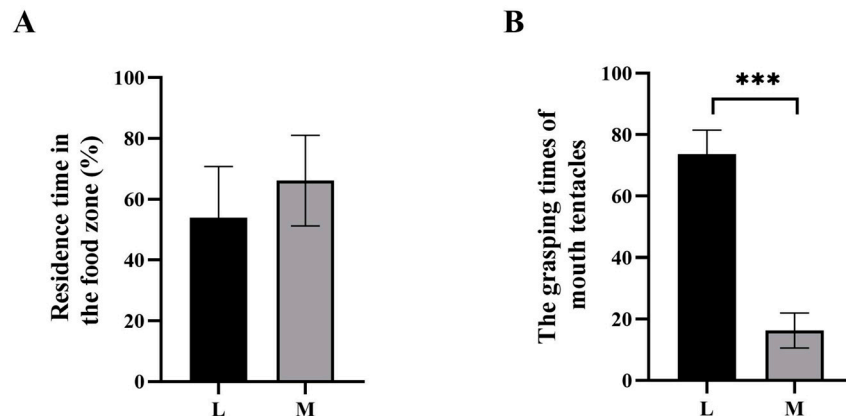


Figure 2. Residence time in the food zone (A) and grasping times of mouth tentacles (B) of *Apostichopus japonicus* (mean $\pm$ SE, $N = 8$). Note: Asterisk indicates significant differences between the non-sheltered group (L) and sheltered group (M) (***) refers to $p < 0.001$).

3.2. Grasping Times of Mouth Tentacles

Compared with group L, significantly less times of mouth tentacle grasping by sea cucumbers were found in group M, as determined by t -test ($t = 5.906$, $p < 0.001$, Figure 2B). The frequency of tentacle grasping of group L (73.63 ± 7.84) was 4.5 times higher than that of group M (16.25 ± 5.73).

3.3. Defecation Rate

The defecation rate was significantly higher in group M (0.22 ± 0.02) than that in group L, as determined by t -test (0.17 ± 0.01 , $t = 2.318$, $p = 0.004$, Figure 3).

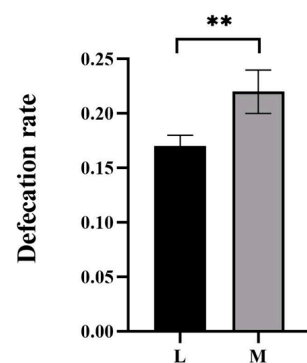


Figure 3. Defecation rate of *Apostichopus japonicus* (mean $\pm$ SE, $N = 8$). Note: Asterisk indicates significant differences between the non-sheltered group (L) and sheltered group (M) (**) refers to $p < 0.01$).

3.4. Pepsin-like Protease Activity

There was no significant difference in the pepsin-like protease activity observed between group M and group L, as determined by one-way ANOVA ($F = 0.091$, $p = 0.770$, Figure 4).

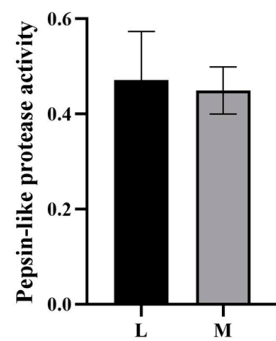


Figure 4. Pepsin-like protease activity of *Apostichopus japonicus* (mean ± SE, $N = 3$). Groups L and M represent the non-sheltered group and sheltered group, respectively.

3.5. Gut Morphological Analysis

Crease height was significantly shorter in group M than that in group L, as determined by t -test on intestinal biopsies ($t = 1.317$, $p = 0.028$, Figure 5A). But there was no significant difference in crease width ($p = 0.447$, Figure 5B) and muscle layer thickness ($p = 0.067$, Figure 5C) of sea cucumbers between groups M and L, as determined by t -tests.

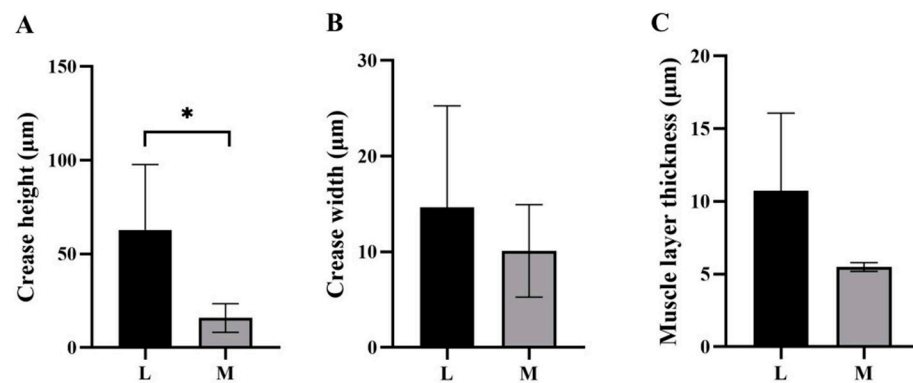


Figure 5. Intestinal crease height (A), crease width (B), and muscle layer thickness (C) of *Apostichopus japonicus* (mean ± SE, $N = 3$). Note: Asterisk indicates significant differences between the non-sheltered group (L) and sheltered group (M) (* refers to $p < 0.05$).

3.6. Microbial Composition

Ace richness ($p = 0.0204$), Chao1 richness ($p = 0.0206$), Shannon–Wiener diversity index ($p = 0.0038$), and phylogenetic diversity ($p = 0.0026$) were significantly lower in group L than those in group M, as determined by t -tests. But there was no significant difference in the Simpson diversity index ($p = 0.2804$) and coverage ($p = 1.000$) between groups L and M, as determined by t -tests (Table 1). Coverage indexes of groups M and L were closed to 0.99, indicating a high sequence detection rate. The sequencing results accurately represented the composition and diversity of the gut microbiota in the studied samples.

Table 1. Alpha diversity of intestinal microflora in *Apostichopus japonicus* ($N = 3$, mean ± SE). Groups L and M represent the non-sheltered group and sheltered group, respectively. Note: Different letters indicate significant differences ($p < 0.05$).

Sample	Operational Taxonomic Units	Community Richness		Community Diversity		Coverage
		Chao1	ACE	Simpson	Shannon	
Group L	1407.00 ± 50.64	469.21 ± 50.76 a	469.98 ± 51.14 a	0.99 ± 0.01	7.61 ± 0.06 a	0.99
Group M	4726.00 ± 184.89	1575.45 ± 184.85 b	1577.58 ± 184.39 b	0.99 ± 0.01	8.72 ± 0.12 b	0.99

Relationships between different groups were assessed using principal coordinates analysis (PCoA) based on both unweighted and weighted UniFrac distances. The unweighted UniFrac PCoA showed a clear separation between groups M and L (Figure 6A), with the first two principal coordinates explaining 35.66% (PC1) and 21.30% (PC2) of the total variation, respectively, accounting for 56.96% of the cumulative variation. This result indicates the differences in microbial community composition between the two groups. Consistently, the weighted UniFrac PCoA further revealed distinct clustering of groups M and L (Figure 6B), with PC1 and PC2 explaining 66.02% and 11.77% of the variation, respectively, accounting for 77.79% of the cumulative variation, suggesting significant differences in microbial community structure.

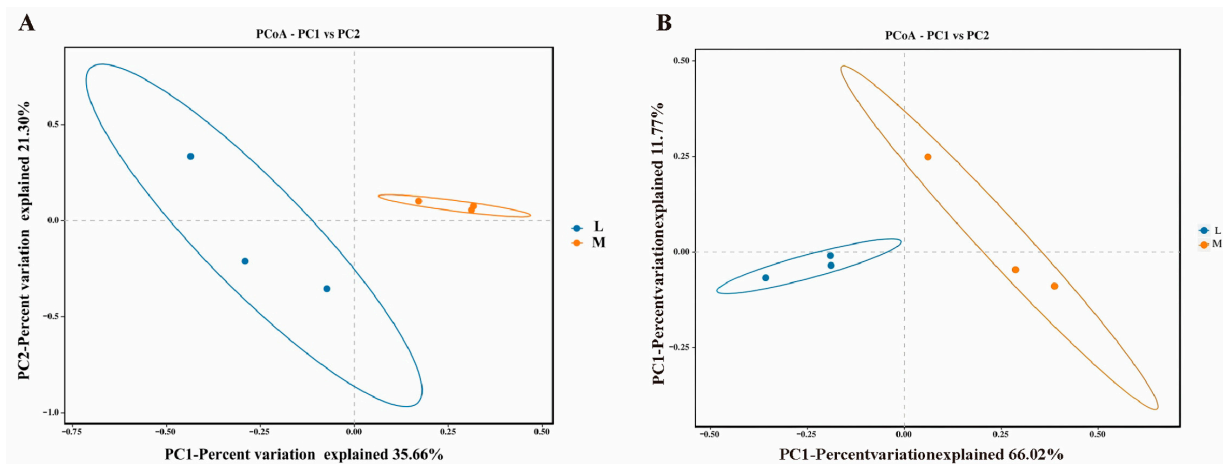


Figure 6. Principal coordinate analysis (PCoA) of gut microbial community in *Apostichopus japonicus* based on the unweighted UniFrac distance (A) and the weighted UniFrac distance (B). The horizontal (pc1) and vertical (pc2) axes represent the two selected principal axes, where the percentages represent the principal explained values for the differences in sample composition. Relative distance refers to the ratio of the horizontal and vertical axes. Groups L and M represent the non-sheltered group and sheltered group, respectively.

The dominant bacterial classes differed between the two groups. In group L, Alphaproteobacteria, Gammaproteobacteria, and Bacteroidia together accounted for over 60% of the intestinal microbiota. Alphaproteobacteria alone represented 36.65% of the community in group L, which was roughly 3.88 times higher than its proportion in group M (9.45%). In contrast, group M was dominated by Gammaproteobacteria, Bacteroidia, and Bacilli. Verrucomicrobiae accounted for 9.45% in group L, about 5.53 times higher than in group M (1.71%) (Figure 7).

Significant differences in the gut microbiota between groups L and M were identified using LEfSe analysis (LDA score > 2.0, $p < 0.05$). At the kingdom level, Bacteria was more abundant in group L than in group M. At the phylum level, Verrucomicrobiae was significantly higher in group L. At the class level, Verrucomicrobiales and Alphaproteobacteria were enriched in group L. At the order level, Rhodobacterales was more abundant in group L. At the family level, Halieaceae, Rubritaleaceae, and Rhodobacteraceae showed significantly higher proportions in group L. At the genus level, *Persicirhabdus* and *Rubritalea* were more enriched in group L (Figure 8). At the phylum level, Clostridia was lower in group L. At the class level, Actinobacteria and Vicinamibacteria showed reduced proportions in group L. At the order level, Vicinamibacteriales and Oscillospirales were less abundant in group L, and at the genus level, *Streptococcus* was significantly decreased.

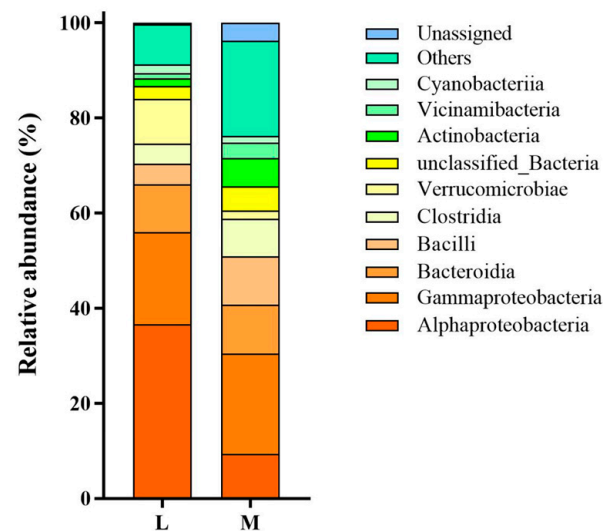


Figure 7. Relative abundance of gut microbiota at class level in different groups of *Apostichopus japonicus*. Groups L and M represent the non-sheltered group and sheltered group, respectively.

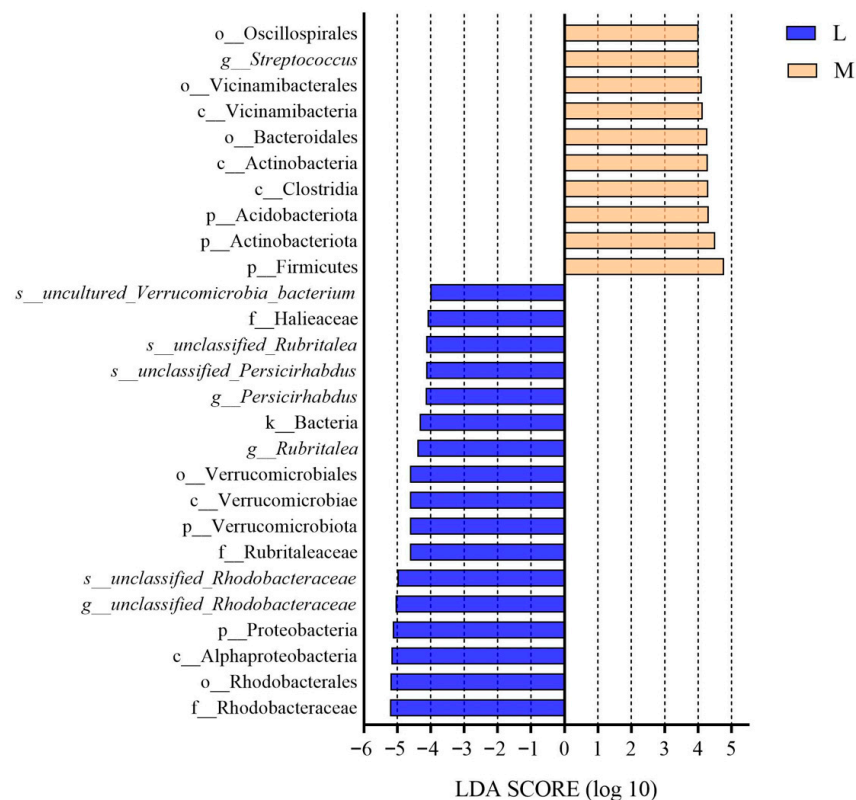


Figure 8. The differential bacteria among different groups were selected by LEfSe analysis. The letters p, c, o, f, g, and s denote phylum, class, order, family, genus, and species, respectively. Groups L and M represent the non-sheltered group and sheltered group, respectively.

3.7. 5-HT and Cortisol Level

5-HT content in sea cucumbers of group M was significantly higher than that in group L, as determined by *t*-test ($t = -4.381$, $p = 0.001$, Figure 9A). But there was no significant difference in cortisol content between groups M and L, as analyzed by one-way ANOVA ($F = 0.114$, $p = 0.753$, Figure 9B).

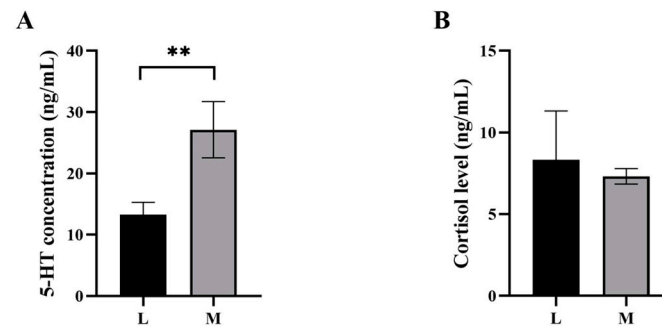


Figure 9. 5-HT concentration (A) and Cortisol (B) of *Apostichopus japonicus* (mean $\pm$ SE, $N = 3$). Note: Asterisk indicates significant differences between the non-sheltered group (L) and sheltered group (M) (** refers to $p < 0.01$).

3.8. Thermal Tolerance

Thermal tolerance in sea cucumbers of group M was significantly lower than that in group L by the Mann–Whitney U test (Mann–Whitney $U = 119.3$, $p = 0.010$, Figure 10A). CT_{max} was 36.75 ± 0.75 °C in group M, while 39.25 ± 0.37 °C in group L.

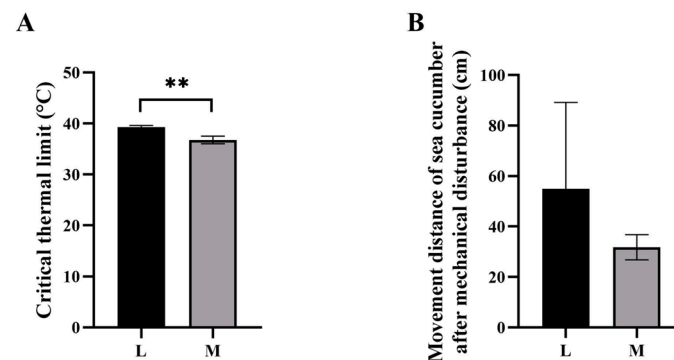


Figure 10. Thermal tolerance (A) and movement distance after mechanical disturbance (B) of *Apostichopus japonicus* (mean $\pm$ SE, $N = 8$). Note: Asterisk indicates significant differences between the non-sheltered group (L) and sheltered group (M) (** refers to $p < 0.01$).

3.9. Movement Distance After Mechanical Disturbance

A 5 min mechanical interference resulted in no significant difference in movement distance between groups M and L, as determined by one-way ANOVA ($F = 0.448$, $p = 0.514$, Figure 10B).

4. Discussion

The present study found significant differences in behavior and physiology between the non-sheltered and sheltered juvenile sea cucumbers. The frequency of mouth tentacle grasping in sheltered sea cucumbers was significantly lower than in the non-sheltered individuals. This result indicates that sheltered juvenile sea cucumbers have higher feeding requirement. Juvenile sea cucumbers reared in a shelter environment, but without direct contact with the shelter, did not show significant differences in feeding behavior compared with those reared without shelter under high-temperature conditions [11]. Juvenile sea cucumbers in direct physical contact with the shelter exhibited significantly higher feeding activity than those raised outside the shelter, as reflected by both their foraging selection and the number of mouth tentacle grasping events [10,11]. This suggests that the feeding activity of juvenile sea cucumbers, as reflected by the frequency of mouth tentacle grasping, can be promoted through the provision of shelters. However, there was no significant difference in foraging selection between the non-sheltered and sheltered sea cucumbers.

This suggests that there is no difference in food sensitivity between the two groups of juvenile sea cucumbers, and that enhancement of feeding activity, as measured by mouth tentacle grasping frequency, could not be achieved solely through the provision of shelters. Foraging speed is greatly affected by food sensitivity [48,49]. This explains the phenomenon that the sheltered sea cucumbers do not leave the shelter when food is largely provided outside the shelter in aquaculture. External handling stress affects the foraging selection of sea cucumbers [30]. This indicates that juvenile sea cucumbers not exposed to external stress do not exhibit a shelter requirement for foraging behavior. In this study, the external stress factor applied was mechanical disturbance, which was used to simulate environmental perturbations. Thus, this study clarifies that feeding but not foraging is the behavioral requirement of the sheltered juvenile sea cucumbers.

It was found that the gut crease height of sheltered sea cucumbers was significantly lower than that of non-sheltered individuals. As gut crease height is positively related to the contact surface available for food digestion [50], this suggests that sheltered juvenile sea cucumbers possess a relatively smaller digestive contact area. However, this morphological characteristic does not necessarily indicate intestinal defects. Instead, it may reflect a structurally efficient gut with a reduced digestive surface area. In this context, shelter preference is more likely a behavioral strategy to compensate for potential digestive constraints and to optimize digestive efficiency. Although a moderate prolongation of food retention time in the intestine can promote nutrient absorption, excessively long retention may reduce digestive efficiency. Notably, sheltered sea cucumbers exhibited significantly higher defecation rates and intestinal 5-HT contents compared with non-sheltered individuals. Defecation rate is commonly used as an indicator of digestive activity in sea cucumbers [35], and 5-HT is a key neurotransmitter regulating intestinal peristalsis [51]. Elevated 5-HT levels suggest more enhanced and coordinated peristalsis, which can prevent excessive food retention and help maintain an optimal residence time in the gut [52,53]. Taken together, these results indicate that sheltered juvenile sea cucumbers achieve improved digestive performance through a balance among gut morphology, food retention, and peristaltic regulation, rather than through increased digestive enzyme production. This interpretation is supported by the absence of significant differences in digestive enzyme contents between sheltered and non-sheltered individuals, as well as by previous findings showing enhanced feeding and defecation in sea cucumbers provided with artificial shelters [12].

Compared with the non-sheltered group juvenile sea cucumbers, the individuals in the sheltered group exhibited significantly higher Ace richness, Chao1 richness, Shannon-wiener diversity index, and phylogenetic diversity of the gut microbiota. Increased food intake accelerates the remodeling of gut microbiota and changes the diversity of the gut microbiota in sea cucumbers [54]. Therefore, the observed higher diversity in sheltered individuals may reflect ongoing changes in gut microbial composition, although direct evidence for reconstruction of specific bacterial taxa in this process was not assessed in this study. Rubritaleaceae and Rhodobacteraceae in the non-sheltered sea cucumbers showed significantly higher proportions than those in the sheltered sea cucumbers. Rhodobacteraceae and Rubritaleaceae are related to the algal polysaccharide degradation [55]. The lower abundance of these taxa in sheltered sea cucumbers may suggest potential differences in polysaccharide degradation capacity, which could partly explain the lower frequency of mouth tentacle grasping, although direct measurements of digestive ability were not conducted in this study. Gammaproteobacteria and Bacteroidia were predominant in the sheltered sea cucumbers. Most of the bacteria in Gammaproteobacteria are pathogenic to *A. japonicus* [56,57]. Alphaproteobacteria are the dominant species in healthy sea cucumbers [24]. There were less Alphaproteobacteria in the sheltered sea cucumbers than in the non-sheltered sea cucumbers. Mortality and morbidity were significantly lower in sea cu-

cucumbers in artificial shelters than those without artificial shelters [11]. The results indicate that the provision of artificial shelters can reduce the morbidity and improve survivability of cultured sea cucumbers. Moreover, thermal tolerance of the sheltered sea cucumbers was significantly lower than that of the non-sheltered individuals. This indicates that the sheltered sea cucumbers are more vulnerable than the non-sheltered individuals at high temperature [11]. There was no significant difference in the cortisol content in gut between the non-sheltered and sheltered sea cucumbers. This is not consistent with Tian et al. [58], who highlighted that the cortisol content in the sea cucumbers of the artificial shelter group was higher than that of those of the non-artificial shelter group at high temperature. One possible reason is that the juvenile sea cucumbers were not stressed in the present experiment. Thus, cortisol was probably not largely produced in the present study. Movement distance can directly indicate the movement ability of sea cucumbers. Movement distance of sea cucumbers decreased significantly under environmental stress, and the movement of sea cucumbers under mechanical disturbance was the most affected [30]. However, there was no significant difference in movement distance after mechanical disturbance between the non-sheltered and sheltered juvenile sea cucumbers in this experiment. This reveals that the sheltered juvenile sea cucumbers have a limited requirement to relieve stress in the absence of environmental stresses.

5. Conclusions

The present study demonstrates that sheltering behavior in juvenile *A. japonicus* is closely associated with their physiological condition rather than being a simple habitat preference. Individuals exhibiting shelter dependence showed distinct behavioral and physiological characteristics related to feeding, digestion, thermal tolerance, and immune status, suggesting that sheltering may serve as a behavioral indicator of increased physiological stress or suboptimal physical condition under culture environments. Our results further indicate that the provision of artificial shelters can effectively alleviate these constraints by improving digestive performance, regulating physiological processes, and enhancing stress resistance. These findings highlight the behavioral and physiological requirements of sea cucumbers for shelters, emphasizing the importance of appropriately establishing shelters in aquaculture, and providing new insights into the ecological and physiological mechanisms underlying shelter dependence in *A. japonicus*.

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