Article

Impact of transportation and rehydration strategies on the physiological responses of clams (*Ruditapes philippinarum*)

Shijie Bi[1], Changhu Xue[2], Cheng Sun[3], Lipin Chen[2], Zhongkai Sun[2], Yunqi Wen[2], Zhaojie Li[2], Guidong Chen[2], Zihao Wei[2*], Hongying Liu[1, 4*]

[1] College of Food Science and Technology, Hebei Agricultural University, Baoding 071000, China; bishijie825@sina.com (S.B);
[2] College of Food Science and Engineering, Ocean University of China, Qingdao 266003, China; oucxuech@163.com (C.X); CChenlp@163.com (L.C); 2990183597@qq.com (Z.S); wenyq0715@163.com (Y.W); lizhaojie@ouc.edu.cn (Z.L); guidong6385@ouc.edu.cn (G.C);
[3] Yantai Hefeng Aquatic Products Technology Co. Ltd, Yantai 264000, China; 1057314747@qq.com (C.S);
[4] Ocean College of Hebei Agricultural University, Qinhuangdao 066000, China.

* Correspondence: liu0660001@163.com (H.L); Tel: +86-0335-3150004; weizihao@ouc.edu.cn (Z.W); Tel: +86-14 0532-82032597

Abstract: With the extension of the post-catch circulation time, a series of changes had taken place in the soft tissue of the live clams, resulting in the decline of its quality. This study investigated the quality changes of clams (*Ruditapes philippinarum*) in cold chain and was mainly focused on the rehydration process, which included gradient heating rehydration (GR) and sudden heating rehydration (SR). It was found that the GR had a better effect on the quality of clams than the SR. The GR stage clams showed a higher survival rate, glycogen content and adenylate energy charge (A.E.C) value than the SR stage clams. Conversely, the GR stage clams showed lower lactic acid content and K-value than the SR stage clams. The results indicated that the gradient heating rehydration was beneficial to the quality of clams. The transportation and rehydration strategies benefited both producers and shellfish merchants to save total cost.

Keywords: clams; physiological status; stress; cold chain; free amino acid

1. Introduction

Clams are one of the major economic shellfish in the world with a production of 4,139,157 t in 2018. In the world, export share of clams occupies 1.12% among total exports of aquatic products [1]. The *Ruditapes philippinarum* (*R. philippinarum*) clams are the typical representative of mudflat shellfish and they are one of the most successful shellfish species commercialized for human consumption [2]. Furthermore, a large quantity of clam products including live *R. philippinarum* are exported from China to Japan and other southeast Asian countries [3].

Nowadays, the cold chain is widely used in the circulation of aquatic food products. However, live shellfish are affected by the transport time, temperature, humidity, oxygen and other natural or anthropogenic stress during the circulation process [4]. These stressors cause undesirable changes in the physiological conditions of shellfish and seriously reduce their quality. Therefore, from the perspective of the supply chain connection, it is necessary to systematically research the impact of harvest, depuration and transport for the decline of live shellfish quality. It is of great significance to explore the key quality indicators of live shellfish and their changing rules, and find the scientific method of anhydrous transportation to keep high-quality live shellfish during the circulation process.

In previous study, the most of the researches were focused on the purification stage. Anacleto et al. studied that the clams were anhydrous transportation at 4 °C and 22 °C, respectively, after depuration until the mortality rate reached 50% [5]. They found that the bacteria reduced after depuration, and the *Vibrio spp*. was less affected. Meanwhile, the survival rate, condition index (CI), nucleotides, glycogen and other physiological reactions were also analyzed [6]. They obtained the best semi-dry transport conditions to maintain good physiological conditions and high quality of clams, and the best transport conditions were that the clams should be performed at low temperatures (4 °C). Furthermore, another study reported that the effects of depuration on the element concentration in bivalves [7].

Of course, there were a few studies investigated the quality changes in shellfish between harvest and rehydration. For instance, Chen et al. investigated the physiological changes of scallops (*Chlamys farreri*) during semi-anhydrous living preservation [8]. They found that oxygen supply was less important for optimizing semi-anhydrous living-preservation. In addition, temperature gradient change was often used to reduce the stress of waterless aquatic live products transport through temporary cultivation [9]. But the impact of different heating rates on clam quality during the rehydration process was rarely investigated. Therefore, in order to deliver high quality clams to the market, the quality changes in clams during the transportation and rehydration process required further study.

In this study, the survival rate, glycogen content, lactic acid content, nucleotides, and free amino acids of *R. philippinarum* during the cold chain process were evaluated. In particular, the influence of gradient heating rehydration and sudden heating rehydration on the clam quality during the rehydration process were investigated. The objective of this study was to investigate the quality changes of *R. philippinarum* during the cold chain process and provided theoretical support for the development of a consistent market for high-quality clams.

2. Materials and Methods

2.1 Samples collection

Living and fresh one-year-old shellfish-clams (*R. philippinarum*) were caught from a farm located in Jiaozhou Bay, Qingdao City, China (35° 35' ~ 37° 09' N, 119° 30' ~ 121° 00' E). All experimental clam samples in a count of 100 kg each group were uniform in size (shell lengths were 29.6 ± 1.6 mm, shell widths were 21.52 ± 1.6 mm, shell heights were 21.2 ± 1.69 mm and weights were 4.42 ± 0.79 g). All clams were harvested in September 2019. The clams were immediately transferred to the laboratory with ice (4 °C) within 40 min after collecting.

2.2 Simulated transport conditions

R. philippinarum clams harvested from Jiaozhou Bay in Qingdao City. After transported to the laboratory, simulated transport of the clams was carried out at 4 °C and 100% moisture for 48 h. Subsequently, the clams were divided into two groups for rehydration (the rehydration system as shown in Fig. 1), in one group, the clams were directly placed in 15 °C seawater, while in the other group, the clams were placed in 4 °C seawater and then the temperature was gradually increased by 3 °C per hour to achieve the experimental temperature (15 °C). The rehydration time was maintained for 12 h after the temperature arrived 15 °C. Samples were collected in the harvest stage (HA), transportation stage and rehydration stage. Thereinto, the transport process included transport to the laboratory (T-Lab), simulated transport for 24 h (T-24) and 48 h (T-48) stage, and the rehydration stage was divided into the gradient heating rehydration (GR) stage and sudden heating rehydration (SR) stage. At each sample collection, 100 clams were used to evaluate the survival rate, and other 150 live clams were immediately dissected, treated with liquid nitrogen and then stored at -80 °C for further analysis.

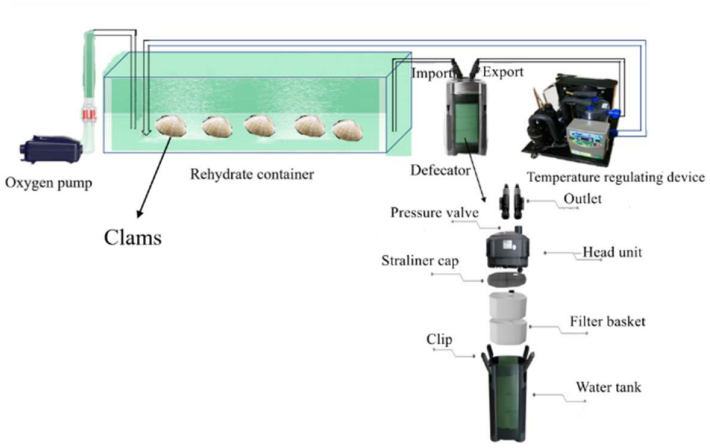

Figure 1. The rehydration system (circulation water)

2.3 Survival rate

The survival rate was determined by a method that was similar to the report by Gonc et al. [10]. Briefly, 100 clams were inspected at a specific time (8: 00 a.m.) every day. The clams were placed in 15 °C seawater for 1 h. The survival rate of the clams was determined by observing their valves. If the clam shells were open, tapped them with a glass rod and observed the clam's response. Subsequently, if the clam shells were not closed for a long time, the clam was considered dead. The survival rate was calculated as follows:

$$\text{Survival rate (\%)} = (\text{live animals}/\text{total animals}) \times 100 \quad (1)$$

2.4 Glycogen

The glycogen content was measured by the anthrone-sulfuric acid method following the protocol of the glycogen analysis kit (Beyotime; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) [8]. Briefly, the fresh soft tissue was rinsed by normal saline, then drained with filter paper and weighed. The samples with the alkaline solution at a rate of 1:3 (w/v) were homogenized and placed in a boiling bath for 20 min (before boiling water bath, the end of the tube was tied with preservative film to prevent water evaporation, and film was made a small hole with a needle to make the gas expanded and contracted in cold and hot.) and then cooled them with running water. An additional boiling bath for 5 min was conducted after the kit's chromogenic reagent was added. After cooling, the glycogen content was determined at 620 nm wavelength with a WFJ 7200 spectrophotometer (Unico Ltd., Shanghai, China). Glycogen concentration was calculated by the absorbance at 620 nm of samples with an equivalent glucose standard. Each analysis was performed in triplicate.

2.5 Lactic acid

The lactic acid level was analyzed with a commercial lactic acid analysis kit (Beyotime; Nanjing Jiancheng Bioengineering Institute, Jiangsu, China), which was reported by Chen et al. [8]. The sample with the normal saline were mixed at a rate of 1:9 (w/v) and centrifuged at 4000 rpm for 10 min. Supernatant (0.02 mL) was taken and set into 1 mL of lactate dehydrogenase and 0.2 mL of chromogenic reagent, then mixed them and set in 37 °C water bath for 10 min. Finally, 2 mL of reaction terminator was mixed and measured the absorbance at 530 nm wavelength with a WFJ 7200 spectrophotometer (Unico Ltd., Shanghai, China). The lactic acid level was calculated with the external standard from the analytical kit. Each analysis was performed in triplicate.

2.6 Nucleotides

The content of the nucleotides was determined with a method reported by Bi et al. and Zhang et al. [11,12]. Briefly, 5.00 g of samples were homogenized in 15 mL of 5% cold perchloric acid for 30 s and placed in a HYC-310 refrigerator (Haier, Qingdao, China) at 4 °C for 30 min. The homogenate was centrifuged at 4 °C and 5000 rpm for 10 min, and the supernatant was maintained at 4 °C. Once again, the precipitate was treated with the aforementioned procedure to obtain additional supernatant, and then two supernatants were combined. The processed samples were neutralized to pH 6.70 with 10 M and 1 M potassium hydroxide, then diluted to the final volume of 50 mL with Milli-Q purified water (Millipore, Burlington, MA, USA), filtered (0.22 μm pore size) and analyzed with a 1260 high-performance liquid chromatography system (HPLC; Agilent Technologies Inc., Santa Clara, CA, USA). A 10-μL portion of the filtrate was injected onto a CAPCELL PAK C18 SG120 S5 column (4.6 mm × 150 mm; Shiseido Co., Ltd., Tokyo, Japan). The temperature of column oven was 40 °C. The mobile phase was 20 mM acetic acid, 20 mM citric acid and 40 mM triethylamine. The mobile phase was neutralized to pH 4.80 with 10 M and 1M potassium hydroxide. The samples were detected at 260 nm and the flow rate of mobile phase was 0.8 mL/min. The nucleotide standard, adenosine 5′-triphosphate (ATP, 99%), adenosine 5′-diphosphate (ADP, 99%), adenosine 5′-monophosphate (AMP, 99%), inosine 5′-monophosphate (IMP, 99%), adenosine (AdR, 99%), adenine (Ad, 99%), xanthine (Xt, 99%), inosine (HxR, 99%) and hypoxanthine (Hx, 99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The identity and quantity of the nucleotides were evaluated by comparison with the retention times and peak areas of each nucleotide standard. Each analysis was performed in triplicate.

The K-value and adenylate energy charge (A.E.C. value) were the index of freshness, which were calculated based on the following equations [6,13]:

$$K\ (\%) = [(Hx+HxR)/(ATP+ADP+AMP+Hx+HxR)] \times 100 \quad (2)$$

$$A.E.C.\ (\%) = [(ATP+0.5ADP)/(ATP+ADP+AMP)] \times 100 \quad (3)$$

2.7 Free amino acids

Free amino acids were extracted with a method reported by Bi et al. and Zhong et al. [14,15]. Briefly, 5.00 g of samples were homogenized in 10 mL of 0.02 M dilute hydrochloric acid for 30 s and treated with an ultrasound for 5 min. The homogenate was centrifuged at 4 °C and 5000 rpm for 10 min, and the supernatant was maintained at 4 °C. The precipitate was homogenized in 10 mL of 0.02 M dilute hydrochloric acid for 30 s and centrifuged (5000 rpm, 4 °C) for 5 min, after which the two supernatants were mixed. The processed samples were diluted to the final volume of 25 mL with Milli-Q purified water (Millipore, Burlington, MA, USA). After, 2 mL of 5% sulfosalicylic acid was added to 2 mL of diluent and the mixed diluent was centrifuged again (10000 rpm, 4 °C) for 10 min. Subsequently, the supernatant was filtered through a 0.22 μm filter membranes-aquo system and analyzed with a L-8900 automatic amino acid analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan). Each analysis was performed in triplicate.

2.8 Statistical analysis

The data on the nucleotides, free amino acids, survival rate, lactic acid content and glycogen content of the clams were processed by a one-way ANOVA analysis of variance and Duncan's multiple range test. Statistical analysis was performed by SPSS 20.0 (SPSS Inc., Chicago, IL, USA). A p-value of less than 0.05 was considered statistically significant. Heatmap, principle components analysis (PCA) and correlation analysis were conducted by MetaboAnalyst 4.0 [16]. All experiments were performed in triplicate, and the experimental data were expressed as mean ± standard deviation.

3. Results

3.1. Survival rate

The change in the survival rate of the clams during the experiment is shown in Figure 2A. The mortality rarely occurred initially. The survival rate was 100% in the harvest and transport stage. From the rehydration stage, acceleration of mortality was observed during the GR and SR stage. A significant difference in survival rate was observed during the rehydration period. The clams had a higher survival rate in the GR stage than that in the SR stage, with 95% and 76%, respectively.

It was known that bivalves could survive for a long time without water if they were kept in a cold and moist environment, which reduced the bivalve's metabolism and the moist environment made the clams consequently decrease the excretion products. Hence, the clams kept a higher survival rate in the transport process in this study. Anacleto et al. also found that the survival rate of clams was 100% after 48 h of simulated transport (4 °C) [6]. Furthermore, this study indicated that the different heating rates affected the survival rate of the clams. It could be that sudden change of temperature could stress the clams, which could reduce the immunity of the clams and lead to the death of clams. The results showed that the rehydration process had an effect on the survival rate of clams, and the sudden change of temperature had a tendency to reduce the survival rate of clams. Zeng et al. studied that low-temperature acclimation of crucian carp to the dormant state at cooling rates of 1 °C /h and 5 °C /h and they found that under the condition of 1 °C /h, crucian carp could live without water for up to 24 h [17]. Therefore, gradient heating rehydration could reduce the environmental stress in the process of low temperature transportation, further reduce the mortality of live shellfish and prolong the survival period.

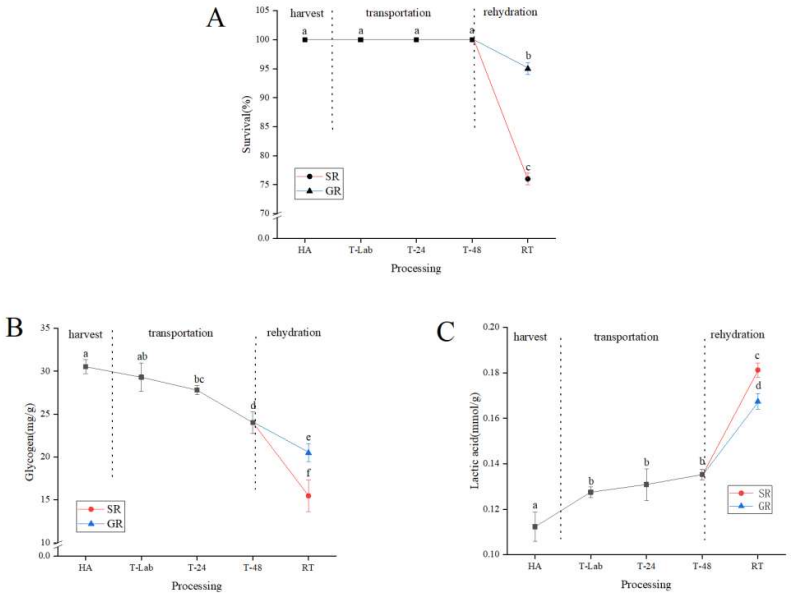

Figure 2. Survival rate (%), Glycogen content and Lactic acid content of *R. philippinarum* subjected to different treatments

3.2. Glycogen

The glycogen content in this study is shown in Figure 2B. At the beginning of the experiment, the glycogen content was 30.53 ± 0.08 mg/g. As time went on, the glycogen content showed a downward trend. Furthermore, the glycogen content in the GR stage showed a higher value than that in the SR stage (20.49 ± 1.05 mg/g and 15.47 ± 1.84 mg/g, respectively).

Glycogen was the main storage form of shellfish energy. When the shellfish were stressed by surrounding environment, the glycogen could be decreased to support en-

ergy. The glycogen content was related to the reproductive period, nutritional status, feeding, stress and temperature of shellfish [18,19]. It had been reported that glycogen was strongly associated with oyster fatness and meat color [20]. Other studies had shown that in hypoxic water, the massive death of bivalve shellfish was related to the degradation of glycogen [21]. In addition, the researchers had shown that the energy required to maintain the clam's life function as the shortage of food intensified, such as quantitative respiratory rate, must be provided by the catabolism of body components present at the beginning of starvation. Glycogen was the corporal component that was consumed in the greatest proportion with regard to the initial value. Albentosa et al. indicated that when the clams were starved for some days, the glycogen utilization indices reached values of 60-70% of initial content, this being greatly superior to that shown by lipids (30-50%, according to the species) or proteins (20-40%) [22]. Our study indicated that the glycogen utilization indices reached values of 67% and 51% of initial content respectively after the GR and SR process. It was clearly demonstrated that clams seemed to use their metabolic reserves during the cold chain. Compared to the glycogen content of the GR and SR stages showed that the sudden change of temperature could intensively consume energy in clams.

In our experiment, the glycogen content was decreased, which could be explained as follows. In order to adapt to the stress of environment, more polysaccharides needed to be consumed by the clams. It indicated that under the stressful conditions, glycogen levels were used for maintaining needs and reflecting the bivalve capacity to withstand further stress. Similar conclusion was reported by Patrick et al. [23].

Our study indicated that the glycogen contents could decrease with the extension of time as the shortage of food intensified. Glycogen content was also influenced by the temperature change rate, and the quick change of temperature could lead to a large downward trend of glycogen content. The sudden change of temperature could cause the stress of oyster under the stress respond, leading to glycogen degradation and quality decrease.

3.3. Lactic acid

The lactic acid content in this study is shown in Figure 2C. The results showed that the lactic acid content was 0.11 ± 0.01 mmol/g at the initial stage. As time went on, the lactic acid content showed an increasing trend. Moreover, the lactic acid content during the GR stage showed a larger up-regulation trend than those at the SR stage (0.18 ± 0.01 mmol/g and 0.16 ± 0.01 mmol/g, respectively).

Studies had reported that the lactic acid level of bivalve molluscs could increase when they suffered the adverse environmental stress [24]. The lactic acid content of clams increased sharply at the harvest and transport stages. It could be that after leaving water, the living environment of clams was relatively harsh, and the oxygen content was low. The clams mainly underwent anaerobic respiration, which produced a large amount of lactic acid. At the same time, the excretory system of the clams was also affected, which caused rapid increase in the lactic acid content of the muscle [25]. In the rehydration stage, the lactic acid content in the GR stage showed a larger up-regulation trend than that in the SR stage. It could be that when the clams re-entered the watery environment, they were suffered the stress of environment. Meanwhile, the clams in the SR stage also suffered the drastic stress of temperature. Due to the large temperature changed, the living environment was relatively difficult, so the lactic acid content was higher than that in the GR stage.

Compared to the change curve of lactic acid content with the change curve of glycogen content, it could be seen that the change of lactic acid content was negatively correlated with the change of glycogen content. During the experiment process, the glycogen content was decreased and the lactic acid content was increased.

3.4. Nucleotides, K-value, and A.E.C. value

The ATP and related compounds contents of *R. philippinarum* are shown in Figure 3A. Throughout the experiment, the ATP and ADP contents changed significantly ($p < 0.05$), and the ATP contents were the highest in all nucleotides. The results indicated that the clams maintained a positive physiological state throughout the experiment. In the HA stage, the ATP and ADP contents were lower than those in the T-Lab stage, which could be that the clams suffered stress response in the period of harvest, and recovered in the process of transport to the laboratory. In the T-24 stage, the ATP content reached the highest level (103.18 ± 3.41 mg/100 g), and the ADP content was 45.87 ± 2.41 mg/100 g. Subsequently, the ATP and ADP contents went down to 53.17 ± 1.53 mg/100 g and 37.69 ± 3.03 mg/100 g in the T-48 stage, respectively. The ATP and ADP contents increased during the rehydration process, and they had higher levels in the GR stage than those in the SR stage. The results indicated that the gradient change of temperature was beneficial to restoring the physiological state of the clams during the rehydration process. Furthermore, AMP and IMP were the main nucleotides in crustaceans and most fish species [26,27]. Throughout the experiment, the AMP and IMP contents were similar except the T-24 stage. The highest AMP and IMP contents were 26.62 ± 2.98 mg/100 g and 25.51 ± 0.91 mg/100 g, respectively. It was reported that excessive accumulation of HxR and Hx could lead to the decline of shellfish quality [28,29]. In this study, HxR and Hx had the lowest contents in all the evaluated nucleotides and their contents changed little throughout the experiment.

ATP-related compounds were the main components of muscle nucleotides in the aquatic animals. They were actively involved in muscle metabolism and provided energy to physiological processes [10,30]. Furthermore, the ATP-related compounds determined the freshness of aquatic animals [31]. The ATP degradation pathway in the musculature of shellfish had been widely studied. Wang et al. suggested that the main pathway of ATP degradation in oysters was ATP → ADP → AMP → IMP → HxR→ Hx. Previous research had suggested that squid, shellfish and other mollusks could not contain AMP deaminase, therefore, they could not produce IMP [32]. However, Anacleto et al. showed that the main pathway of ATP degradation in *R. philippinarum* was ATP → ADP → AMP → IMP → HxR → Hx. The pathway of ATP degradation was confirmed in our present study [6].

The ATP-related compounds in clams not only indicated the physiological status of clams but also influenced the sensory quality, as there was a close relationship between some nucleotides and flavor. Especially, the IMP was the main constituent of umami, which could enhance the umami and contribute to the palatability, delicacy and fresh flavor of shellfish. The degradation process of ATP to IMP was relevant to the endogenous autolytic enzyme activity [33]. In the whole experiment, the IMP content was almost constant, achieving a balance between conversion and degradation. Furthermore, the AMP was also considered as a flavor enhancer. It contributed to the saline taste as well as sweetness and inhibited the bitter taste. In addition, the AMP and IMP had the synergistic effect. When IMP had a low concentration, the AMP not only presented umami but also enhanced sweetness. With regards to the HxR and Hx, they were the main products of nucleotide degradation and important off-flavor contributors in the clam. The degradation from IMP to HxR and Hx was related to the bacterial growth and enzymatic activity. The ratio of the sum of HxR and Hx to the total sum of the ATP, ADP and AMP, expressed as a percentage, was known as the K-value. It could also be considered as a stress indicator for the clams [6]. In our study, the change of HxR and Hx contents indicated that the quality of clams changed little during the simulated transport.

As shown in Figure 3B and 3C, the changes in the K-value and A.E.C. value during the cold process were illustrated. The K-value was the lowest (5%) and the A.E.C. value was the highest (77%) during the T-24 stage. When the clams rehydrated, the K-value was significantly lower in the GR stage than that in the SR stage. Correspondingly, the A.E.C. value was significantly higher in the GR stage than that in the SR stage.

It was known that A.E.C. value of invertebrates strongly varied depending on the importance of natural or anthropogenic stressors [34]. The more stressed an animal was

exposed to, the more energy it consumed, thus reducing its A.E.C. level. Furthermore, it had been suggested that A.E.C. value could be used as a recovery indicator for shellfish [35]. Previous studies recommended that when the A.E.C. value was higher than 75%, the shellfish were active, if the shellfish with A.E.C. value less than 75% but higher than 50% were recoverable, and the shellfish with A.E.C value lower than 50% were unrecoverable [11]. In our study, the A.E.C. value was all greater than 50%, which indicated that the clams could recover from the stress statues during the simulated transport period. In addition, the A.E.C value was greater in the GR stage than that in the SR stage, which indicated that the clams in the GR stage had a better physiological state.

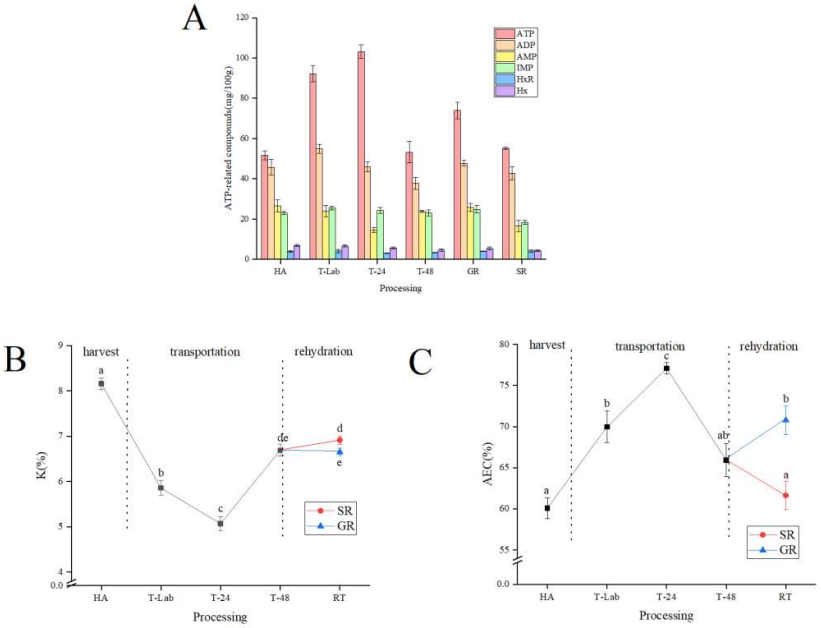

Figure 3. Nucleotides contents, K-value and A.E.C. value of *R. philippinarum* subjected to different treatments

3.5. Free amino acids

The free amino acid contents in this study are shown in Table 1. Twenty-one free amino acids were detected in the clams. The total free amino acid contents in T-24, T-48, GR and SR stages were significantly different ($p < 0.05$) from those in the HA and T-Lab stages. They were significantly higher ($p < 0.05$) in the GR stage than those in the SR stage. Taurine was the dominant free amino acid composition in clams [31]. In this study, the concentrations of taurine in the HA stage were similar to those in the T-Lab and T-24 stages but significantly higher than those in the T-48, GR and SR stages. In addition, Asp, Glu, Gly and Ala were also the major free amino acids in clams. Glu and Asp were umami amino acids, while Ala and Gly were sweet amino acids [26]. These amino acids provided clams with the thick seafood flavor. The ratios of these four free amino acids (Asp, Glu, Gly and Ala) to the total free amino acids in the experiment were 49.46, 50.95, 48.77, 47.65, 50.20 and 45.59%, respectively. The highest proportion was in the T-Lab stage and the lowest was in the SR stage. Glu had not significantly changed ($p > 0.05$) during all the stages. Asp was significantly lower ($p < 0.05$) in the T-24, T-48 and SR stages than that in the HA stage, and it was significantly higher ($p < 0.05$) in the GR stage than that in the SR stage. In the HA stage, the concentrations of Gly and Ala were significantly different ($p < 0.05$) compared with those in the SR and GR stages. Furthermore, other sweet amino acids, Thr, Ser, Lys and Pro were also found in this study. The bitter amino acids, Arg, His, Ile, Leu, Cys, Phe, Tyr, Val and Met. The ratios of bitter amino acids to the total free amino acids in the experiment were 9.70, 9.46, 9.44, 10.71, 9.55 and 11.79%, respectively. The highest proportion was in the SR stage.

One source of free amino acids was the conversion of ketoacids to amino acids by transamination. This process was used to replace the amino acids lost in excretion and provide the amino acids needed to adjust to the changes in ambient tension. In order to identify the important free amino acids in the intracellular osmotic pressure of shellfish, Hosoi et al. studied the changes of free amino acid content in the mantle of *Crassostrea gigas* when the salinity of aquaculture seawater suddenly decreased or increased. The results showed that most of the free amino acids showed significant synchronous decline within 2-8 h in the process of low osmosis adaptation, indicating that the non-selective effluent of free amino acids was the main reason for the decline of free amino acids. Significant increases in glycine, alanine, b-alanine, proline, arginine and taurine were found in the process of high osmotic adaptation [36]. However, Potts did not believe that free amino acids were an excretory product and suggested that the loss of free amino acids was the same as the loss of Na^+ and K^+, that is, by spreading them to the outside of epithelial cells. The above indicated that a large number of amino acids in shellfish were excreted (or leaked) into the environment under low osmotic pressure, and these amino acids were the major source of amino acid metabolism [37]. Thus, the loss of amino acids meant that they could not be used to synthesize proteins, and they could not be oxidized for energy. Therefore, with the drastic changes in environmental conditions, the type and content of free amino acids in shellfish could affect the quality of shellfish during the cold chain process.

Free amino acid could be treated as not only markers for clams to cope with environmental stress, but also an important flavor substance in mollusk and crustacean aquatic products. Free amino acid had been reported to be the main flavor substance in shellfish in China, Australia and Japan [32,38,39]. Hayashi et al. studied the effect of aquatic products flavor with free amino acids and evaluated the taste changes by using the deficiency reduction experiment and addition experiment system. It was found that only four free amino acids (Ala, Arg, Glu and Gly) affected the characteristic flavor of aquatic products [40]. In addition, the free amino acid contents directly affected the freshness of the shellfish [40].

In our study, the total contents of free amino acid were decreased in the transportation stage and increased in the GR stage. However, the SR stage had the lowest total free amino acid value, it could be indicated that when the clams re-entered the water, the free amino acid was influenced by the change rate of temperature. The gradient heating could increase the free amino acid contents and the sudden heating could decrease the free amino acid contents. Furthermore, the main pleasure flavor free amino acids (sweet and umami amino acids) also decreased in the transportation stage and increased in the GR stage, while the SR stage had the lowest pleasure flavor free amino acid value. In contrast, the bitter free amino acid contents had the highest value in the SR stage. The results indicated that the free amino acid could be influenced by the cold chain process, and the gradient heating stage had a better influence on the free amino acid contents than the sudden heating stage.

Principal component analysis (PCA) was used to evaluate the difference among 21 sorts of free amino acids on the clams. Figure 4 showed that the top two principal components (PC1 and PC2) accounted for 58.8% and 25.8% of the total variability, respectively, which represented 84.6% of the total variance. According to the score values of PC1, the free amino acid of clams at HA, T-Lab and T-24 stages could be well distinguished from SR stage. Moreover, the free amino acid of clams at SR stage could be well distinguished from the GR stage according to the score values of PC2. Heat map visualization could directly provide intuitive visualization of the free amino acid contents of the clams. Figure 5 showed the relative differences in free amino acid concentrations. They were indicated by color, with red and blue represented higher and lower concentration, respectively.

Table 1. The free amino acid content in different treatment groups

FAA(mg/100g)	HA	T-Lab	T-24	T-48	GR	SR
Tau	511.17±13.41c	507.19±1.99bc	512.77±13.89c	494.76±8.11a	491.47±7.52ab	488.81±4.02a
Asp	81.69±2.09c	83.88±0.37c	51.56±2.89b	52.63±2.65a	76.46±2.6c	39.89±1.85b
Thr	5.6±0.59a	4.85±0.15a	7.47±0.2b	5.97±0.23a	6±0.11ab	6.18±0.2ab
Ser	4.42±0.17c	4.1±0.17bc	3.61±0.04a	9.52±0.85ab	9.89±0.41e	8.55±0.2d
Glu	132.37±3.5a	137.64±0.77a	130.09±17.9a	146.92±7.93a	147.78±3.2a	138.6±5.9a
Sar	0.85±0.04a	1.35±0.25ab	0.89±0.01a	1.53±0.43ab	1.07±0.08ab	1.91±0.03b
Gly	330.25±9.4cd	340.25±4.22d	331.22±8.32cd	288.69±9.36c	299.11±1.69a	280.99±11.5b
Ala	114.6±2.09c	118.37±0.27d	117.54±2.68cd	105.31±19.23b	96.91±1.86a	130.68±3.56e
Val	7.42±0.05a	7.3±0.23a	7.55±0.11a	8.2±0.55a	7.58±0.23a	8.39±0.25b
Met	1.52±0.12a	1.77±0.01a	2.98±0.61b	2.91±0.32a	2.57±0.05ab	3.21±0.10b
Ile	3.41±0.08a	3.36±0.1a	3.81±0.04a	4.08±0.62a	3.77±0.01a	3.67±0.17a
Leu	4.13±0.1ab	3.4±0.21a	4.43±0.1c	5.41±0.96d	4.31±0.02bc	5.99±0.07e
Tyr	2.52±0.05a	2.65±0.26ab	2.79±0.07ab	3.19±0.46b	2.68±0.01ab	3.57±0.1c
Phe	0.9±0.05bc	0.77±0.02ab	1.1±0.12d	1.2±0.53cd	0.63±0.03a	1.67±0.12e
NH_3	4.1±0.02ab	3.51±0.06a	5.3±0.17bc	5.67±0.19ab	5.8±0.03c	3.72±0.04a
Orn	4.63±0.28a	3.73±0.9a	3.62±0.8a	3.74±0.41a	3.75±0.01a	3.33±0.1a
Lys	11±0.77ab	9.87±0.5ab	7.63±0.4a	12.62±1.21ab	11.51±0.4b	12.45±0.79b
His	4.05±0.13ab	4.04±0.13ab	4.08±0.18b	4.58±0.22a	4.42±0.15c	4.49±0.1c
Car	1.56±0.67a	0.93±0.13a	1.15±0.31a	1.19±0.02a	1.23±0.03a	0.98±0.01a
Arg	94.3±2.5b	93.16±1.46b	87.62±2.38a	93.15±6.19a	86.95±3.79a	99.34±1.74c
Pro	11.85±0.47c	2.92±0.12a	5.38±0.09b	3.7±0.13a	5.21±0.05b	2.89±0.26a
Total	1332.34	1335.04	1292.59	1263.55	1302.87	1211.54

[1] The concentration data were presented as mean ± SD.
[2] Different small letters within the same row denote significant differences between means at $p < 0.05$.

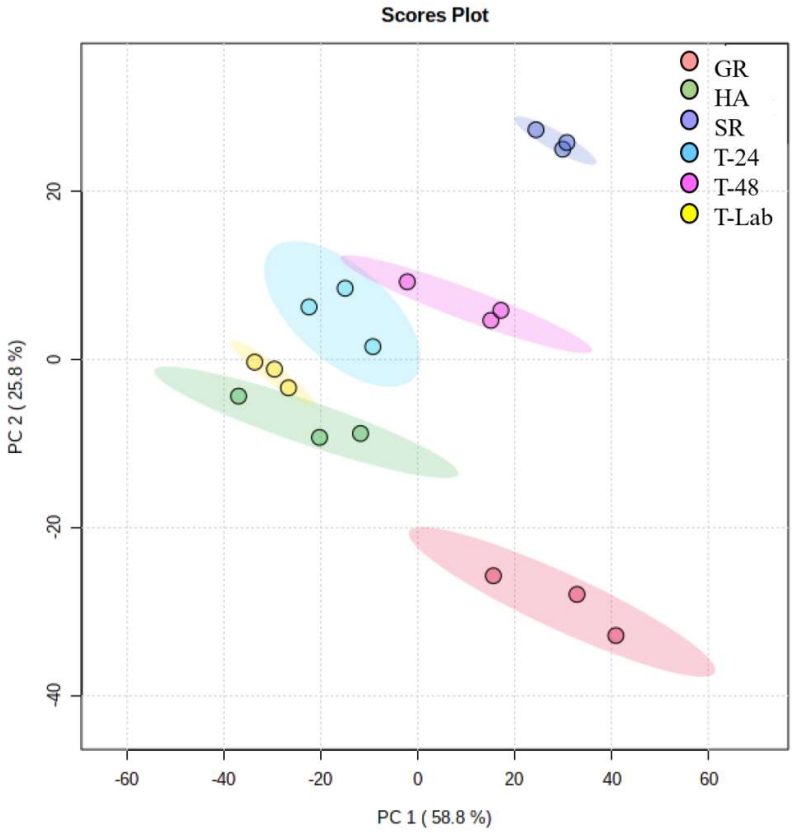

Figure 4. Principal component analysis of the free amino acid profiles of clams during the cold chain

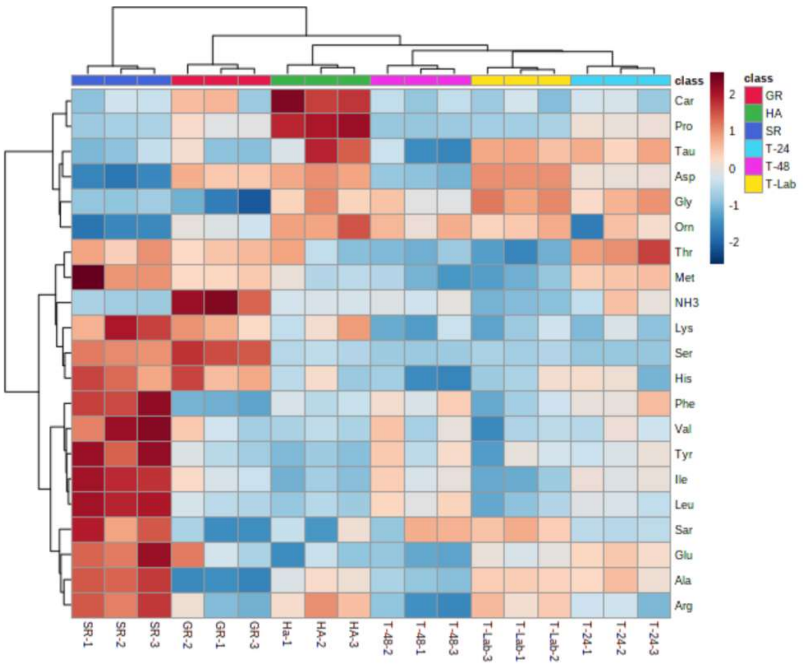

Figure 5. Heatmap explains the different contents of free amino acid in the difference cold chain stage of clams (red indicates free amino acid distributed at a high concentration and blue indicates free amino acid distributed at a low concentration).

4. Conclusions

This was the first report that compared the effect of different heating rates for *R. philippinarum* during the rehydration process. The physiological responses during transport and rehydration were evaluated. The results demonstrated that the survival rate was decreased during the rehydration stage and it had a higher value in the GR stage than that in the SR stage. The glycogen content showed a larger downward trend in the SR stage than that in the GR stage. On the contrary, the lactic acid content showed a larger upward trend in the SR stage than that in the GR stage. In addition, the nucleotides, K-value and A.E.C. value also affected by the cold chain process. Especially in the rehydration process, the SR stage had a higher K-value and a lower A.E.C. value than the GR stage. Above all, the quality of the clams decreased with the extension of time and it was affected by the rehydration condition. Therefore, the suitable rehydration condition was helpful to maintain the quality of clams. In our study, the most effective rehydration condition to sustain the higher quality clams was that placed clams in 4 °C seawater, and the temperature was gradually increased by 3 °C per hour to achieve the final temperature (15 °C). In this study, the quality indicators provided a detailed and accurate sketch of clams under the conditions of the cold chain. Overall, this study could be useful to improve the live circulation value of clams, since the physiological indexes reflected the quality of aquatic food products and the stress-resistant ability of bivalves to natural or anthropogenic stresses. The ideal rehydration condition was described in this study, which presented an effective method to deliver high-quality clams for customers.

Author Contributions: Conceptualization: S.B., Z.L., C.X.; methodology: S.B., C.S., L.C., Z.S., Z.W.; data curation: S.B., G.C.; funding resources: H.L., C.X.; writing - original draft: S.B.; writing-review & editing: S.B., Y.W., Z.W., H.L.; supervision: H.L. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the National Key R&D Program of China (2018YFD0901004), Innovation Team Project of Hebei (Province) Modern Agricultural Industry Technology System (HBCT2018170207), Innovation Center Project of Hebei Agricultural Product Processing Technology (199676183H) and the modern agricultural industry technology system (CARS-49).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data herein presented are available on request from the corresponding author.

Acknowledgments: The authors acknowledge the companies which supply the necessary materials for experiments.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. FAO. Fisheries and Aquaculture Information and Statistics Service. **2020**.
2. Yin, X.; Chen, P.; Chen, H.; Jin, W.; Yan, X. Physiological performance of the intertidal Manila clam (*Ruditapes philippinarum*) to long-term daily rhythms of air exposure. *Sci Rep-uk.* **2017**, *7*, 41648.
3. Nie, H.; Jahan, K.; Zhang, W.; Huo, Z.; Yan, X. Physiological and biochemical responses of different shell color strains of Manila clam to low salinity challenges. *Aquacult Rep.* **2020**, *16*.

4. Oliveira, P.; Almeida, Â.; Vânia Calisto, V.I.; Esteves, R.J.; Schneider, F.J.; Wrona, A.M.V.M.; Soares, E.F.; Freitas, R. Physiological and biochemical alterations induced in the mussel *Mytilus galloprovincialis* after short and long-term exposure to carbamazepine. *Water Res.* **2016**.
5. Anacleto, P.; Maulvault, A.L.; Chaguri, M.; Pedro, S.; Nunes, M.L.; Rosa, R.; Marques, A. Microbiological responses to depuration and transport of native and exotic clams at optimal and stressful temperatures. *Food Microbiol.* **2013**, *36*, 365-373.
6. Anacleto, P.; Maulvault, A.L.; Barrento, S.; Mendes, R.; Nunes, M.L.; Rosa, R.; Marques, A. Physiological responses to depuration and transport of native and exotic clams at different temperatures. *Aquaculture* **2013**, *408-409*, 136-146.
7. Freitas, R.; Ramos Pinto, L.; Sampaio, M.; Costa, A.; Silva, M.; Rodrigues, A.M.; Quintino, V.; Figueira, E. Effects of depuration on the element concentration in bivalves: Comparison between sympatric *Ruditapes decussatus* and *Ruditapes philippinarum*. *Estuar Coast Shelf S.* **2012**, *110*, 43-53.
8. Chen, S.; Zhang, C.; Xiong, Y.; Tian, X.; Liu, C.; Jeevithan, E.; Wu, W. A GC-MS-based metabolomics investigation on scallop (*Chlamys farreri*) during semi-anhydrous living-preservation. *Innov Food Sci Emer.* **2015**, *31*, 185-195.
9. Harmon, T.S. Methods for reducing stressors and maintaining water quality associated with live fish transport in tanks: a review of the basics. *Rev Aquacult.* **2009**, *1*, 58-66.
10. Goncalves, A.; Pedro, S.n.; Duarte, A.; Nunes, M.L. Effect of enriched oxygen atmosphere storage on the quality of live clams (*Ruditapes decussatus*). *Innov Food Sci Emer.* **2009**, *44*, 2598-2605.
11. Bi, S.; Chen, L.; Sun, Z.; Wen, Y.; Xue, Q.; Xue, C.; Li, Z.; Liu, H. Physiological responses of the triploid Pacific oyster (*Crassostrea gigas*) to varying salinities of aquaculture seawater. *Aquac Res.* **2021**.
12. Zhang, Q.; Jing, R.; Wang, B.; Cui, P.; Zhou, C.; Yan, T.; Kanyukt, S.; Si, W. Fast Mode Decision Based on Gradient Information in 3D-HEVC. *IEEE Access* **2019**, *7*, 135448-135456.
13. Hu, C.; Xu, G. Mass-spectrometry-based metabolomics analysis for foodomics. *TrAC-Trend Anal Chem.* **2013**, *52*, 36-46.
14. Bi, S.; Chen, L.; Sun, Z.; Wen, Y.; Xue, Q.; Xue, C.; Li, Z.; Sun, C.; Wei, Z.; Liu, H. Investigating influence of aquaculture seawater with different salinities on non-volatile taste-active compounds in Pacific oyster (*Crassostrea gigas*). *J. Food Meas Charact.* **2021**.
15. Zhong, C.; Nakanishi, M.; Geng, J.; Okazaki, E.; Cao, M.; Weng, W.; Osako, K. Comparison of non-volatile taste-active components in fish sauce produced from *lizardfish Saurida wanieso* viscera under different conditions. *Fisheries Sci.* **2015**, *81*, 581-590.
16. Dong, M.; Qin, L.; Ma, L.X.; Zhao, Z.Y.; Du, M.; Kunihiko, K.; Zhu, B.W. Postmortem nucleotide degradation in turbot mince during chill and partial freezing storage. *Food Chem.* **2020**, *311*, 125900.
17. Zeng, P.; Chen, T.; Shen, J. Effects of cold acclimation and storage temperature on crucian carp (*Carassius auratus gibelio*) in a waterless preservation. *Fish Physiol Biochem.* **2013**, *40*, 973-982.
18. Dridi, S.; Romdhane, M.S.; Elcafsi, M.h. Seasonal variation in weight and biochemical composition of the Pacific oyster, Crassostrea gigas in relation to the gametogenic cycle and environmental conditions of the Bizert lagoon, Tunisia. *Aquaculture* **2007**, *263*, 238-248.
19. Hwang, D.; Chen, T.; Shiau, C.; Jeng, S. Seasonal variations of free amino acids and nucleotide-related compounds in the muscle of cultured Taiwanese puffer Takifugu rubripes. *Fisheries Sci.* **2000**, *66*, 1123–1129.
20. Liu, S.; Li, L.; Wang, W.; Li, B.; Zhang, G. Characterization, fluctuation and tissue differences in nutrient content in the Pacific oyster (*Crassostrea gigas*) in Qingdao, northern China. *Aquac Res.* **2020**, *51*, 1353-1364.
21. Uzaki, N.; Kai, M.; Aoyama, H.; Suzuki, T. Changes in mortality rate and glycogen content of the Manila clam *Ruditapes philippinarum* during the development of oxygen-deficient waters. *Fisheries Sci.* **2003**, *69*, 936–943.
22. Albentosa, M.; Fernandez-Reiriz, M.J.; Labarta, U.; Perez-Camacho, A. Response of two species of clams, *Ruditapes decussatus* and *Venerupis pullastra*, to starvation: physiological and biochemical parameters. *Comp Biochem Physiol B Biochem Mol*

Biol. **2007**, *146*, 241-249.

23. Patrick, S.; Faury, N.; Goulletquer, P. Seasonal changes in carbohydrate metabolism and its relationship with summer mortality of Pacific oyster *Crassostrea gigas* (Thunberg) in Marennes–Oléron bay (France). *Aquaculture* **2006**, *252*, 328-338.
24. Giacomin, M.; Jorge, M.B.; Bianchini, A. Effects of copper exposure on the energy metabolism in juveniles of the marine clam Mesodesma mactroides. *Aquat Toxicol.* **2014**, *152*, 30-37.
25. Strahl, J.; Dringen, R.; Schmidt, M.M.; Hardenberg, S.; Abele, D. Metabolic and physiological responses in tissues of the long-lived bivalve *Arctica islandica* to oxygen deficiency. *Comp Biochem Physiol A Mol Integr Physiol.* **2011**, *158*, 513-519.
26. Liu, Y.; Zhang, C.; Chen, S. Comparison of active non-volatile taste components in the viscera and adductor muscles of oyster (*Ostrea rivularis gould*). *Food Sci Technol Res.* **2013**, *19*, 417-424.
27. Quinta, R.M.R.; Nunes, M.L. Changes in baseline levels of nucleotides during ice storage of fish and crustaceans from the Portuguese coast. *Eur Food Res Technol. 212*, 141-146.
28. Hong, H.; Regenstein, J.M.; Luo, Y. The importance of ATP-related compounds for the freshness and flavor of post-mortem fish and shellfish muscle: A review. *Crit Rev Food Sci Nutr.* **2017**, *57*, 1787-1798.
29. Lawal, A.T.; Adeloju, S.B. Mediated xanthine oxidase potentiometric biosensors for hypoxanthine based on ferrocene carboxylic acid modified electrode. *Food Chem.* **2012**, *135*, 2982-2987.
30. Da Ros, L.; Nesto, N.; Nasci, C.; Moschino, V.; Pampanin, D.; Marin, M.G. Biochemical and behavioural effects of hydraulic dredging on the target species *Chamelea gallina*. *Fish Res.* **2003**, *64*, 71-78.
31. Houcke, J.; Medina, I.; Maehre, H.K.; Cornet, J.; Cardinal, M.; Linssen, J.; Luten, J. The effect of algae diets (*Skeletonema costatum* and *Rhodomonas baltica*) on the biochemical composition and sensory characteristics of Pacific cupped oysters (*Crassostrea gigas*) during land-based refinement. *Food Res Int.* **2017**, *100*, 151-160.
32. Wang, Q.; Xue, C.; Li, Z.; Fu, X.; Xu, J.; Xue, Y. Changes in the contents of ATP and its related breakdown compounds in various tissues of oyster during frozen storage. *J. Ocean U China.* **2007**, *6*, 407-412.
33. Wu, Y.; Kunihiko, K.; Chen, S. Changes of ATP and ATP-related compounds contents and K value in Sebaste sthompsoni in different storage temperatures. In M. Xu & G. Wang (Eds.), *ASET.* **2015**, *12*, 2145–2150.
34. Thebault, M.T.; Raffin, J.P.; Picado, A.M.; Mendonca, E.; Skorkowski, E.F.; Le Gal, Y. Coordinated changes of adenylate energy charge and ATP/ADP: use in ecotoxicological studies. *Ecotoxicol Environ Saf.* **2000**, *46*, 23-28.
35. Mørkøre, T.; Rødbotten, M.; Vogt, G.; Fjæra, S.O.; Kristiansen, I.Ø.; Manseth, E. Relevance of season and nucleotide catabolism on changes in fillet quality during chilled storage of raw Atlantic salmon (*Salmo salar L.*). *Food Chem.* **2010**, *119*, 1417-1425.
36. Hosoi, M.; Hayashi, I.; Kubota, S.; Toyohara, M.; Toyohara, H. Effect of salinity change on free amino acid content in Pacific oyster. *Fish Sci.* **2003**, *69*, 395-400.
37. Potts, W.T. Excretion in the molluscs. *Biol Rev.* **2010**, *42*, 1-41.
38. Cochet, M.; Brown, M.; Kube, P.; Elliott, N.; Delahunty, C. Understanding the impact of growing conditions on oysters: a study of their sensory and biochemical characteristics. *Aqua Res.* **2015**, *46*, 637-646.
39. Yuasa, M.; Kawabeta, K.; Eguchi, A.; Abe, H.; Yamashita, E.; Koba, K.; Tominaga, M. Characterization of taste and micronutrient content of rock oysters (*Crassostrea nippona*) and Pacific oysters (*Crassostrea gigas*) in Japan. *Int J. Gastron Food S.* **2018**, *13*, 52-57.
40. Melis, M.; Tomassini Barbarossa, I. Taste perception of sweet, sour, salty, bitter, and umami and changes due to l-arginine supplementation, as a function of genetic ability to taste 6-n-propylthiouracil. *Nutrients* **2017**, *9*.
41. Hayashi, T.; Yamaguchi, K.; Konosu, S. Sensory analysis of taste-active components in the extract of boiled snow crab meat. J. Food Sci, **2010**, 46, 479-483.