Research Article

Impact of Two Commercial *In Vivo* Transport Methods on Physiological Condition of the Japanese Oyster (*Crassostrea gigas*)

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The effect of two commercial *in vivo* transport methods (cardboard boxes and ixtle sacks) on the physiological condition of Japanese oyster (*Crassostrea gigas*) was evaluated. Total carbohydrates, glycogen, adenosine $5'$-triphosphate (ATP) and related products, adenylate energy charge (AEC), and pH of transported oysters in simulated conditions were determined. The results showed that the ATP initial concentration was low from the beginning of the experiment, and AEC decreased in both transport methods. With respect to the total carbohydrates and glycogen, the samples maintained in cardboard box and ixtle sack decreased during transport, respectively. Similarly, significant changes in pH were observed for both methods. Our results showed that physiologically the best *in vivo* transporting method for Japanese oyster is in cardboard boxes.

1. Introduction

The Japanese oyster (*Crassostrea gigas* Thunberg, 1793) is a bivalve mollusk cultivated throughout the world. In México, the oyster total production has considerably increased in recent years from 34,762 t in 1998 to 42,945 t in 2013, with aquaculture as the main source of production, providing 33,479 and 38,714 t, respectively, in the same years [1]. Currently, marketing oysters occurs in various presentations, such as without shell, where the oyster flesh is placed in glass bottles and canned smoked oyster flesh. However, the most traditional market for oyster and its consumption is in the shell, which is consumed widely in the region, as well as nationwide and internationally. Because there is a high demand for fresh oysters in the shell, the organism must be transported alive from its capture or culture site to the retail area that demands it, and depending on the distances, transportation can be very long, which may affect their physiological condition [2].

Several studies have reported that when living organisms are placed in an uncommon environment, or when environmental conditions change, for example, from one season to another, they undergo physiological changes in the muscle, allowing them to survive in the new environmental conditions [3]. The main compensatory physiological adjustments are the decrease in total carbohydrates, glycogen, ATP

concentration, and AEC [3, 4]; these can cause a muscle biochemical composition modification and have an impact in the organism and final product intrinsic quality. If the stress conditions generated during collection and transport do not remain within the organism physiological tolerance, then death and deterioration are inevitable [5].

At present, transport of the living organisms is done on water tanks or under moist conditions emersion. The first method is used for short distance shipping, because bacterial growth, decreased dissolved oxygen, and feces and nitrogen compounds such ammonia accumulation can quickly reduce transporting water quality. Also, this method has the disadvantage that the cost for transporting water is expensive. The second method is more economical than the former, but it involves the drawback that has a physiological cost to the animals, which can result in high mortality if the transport conditions are not right [6].

Because of the above, and considering that currently there is a strong demand for live oysters, it is necessary to develop new methods that allow transport of the live product up to any area that has the demands for it, with minimal stress and maximum survival. In this study, the physiological impact of two commercial *in vivo* transport methods, cardboard box and ixtle sacks, was evaluated.

2. Materials and Methods

2.1. Transport and Handling of the Organisms. In this study, adults of *Crassostrea gigas* with a 9.5 ± 0.5 cm height, 4.8 ± 0.5 cm length, 3.3 ± 0.5 cm thickness, and an average weight of 63.7 ± 0.5 g, which were commercially purchased in July 2010, at the Estero Santa Cruz located in Bahia de Kino, Sonora, Mexico, were used (12 kg per treatment). Two different simulated transport methods were applied; the first consisted of placing organisms in cardboard boxes and stored in refrigeration (7°C). In the second method, organisms were placed in ixtle sacks, which were moistened prior to beginning transport and maintained at room temperature (24°C).

2.2. Experimental Design. The two simulated *in vivo* commercial transport methods were studied using a completely randomized factorial design with two factors: (1) transport simulation method and (2) transport time. For each method of transport, ATP, ADP, AMP, AEC, total carbohydrates, glycogen, and pH were determined at 0, 8, 20, 28, and 36 h. For each determination and time, 4 organisms (independent determinations) were immediately frozen in liquid nitrogen and then stored at −80°C prior to analysis. In our case, all parameters in total wet tissues of the organisms were evaluated, since oysters are eaten whole except for the shell.

2.3. ATP, ADP, AMP, and AEC. The extraction, identification, and quantification of the ATP, ADP, and AMP in the oyster flesh were conducted through high resolution liquid chromatography. The methodology described by Ryder [7] was used; it consisted of injecting 20 μL of the diluted extract into a Varian Prostar chromatograph using a 4.6 × 150 mm C18 reversed phase column (Varian Inc., Lake Forest, CA).

The mobile phase used was a phosphate buffer made up of 0.04 M KH_2PO_4 and 0.06 M K_2HPO_4. The flow rate utilized was 1 mL min^{-1}, and detection was carried out at 254 nm in a UV-Vis Varian Prostar 325 detector. The AEC was calculated in accordance with Maguire et al. [8], using the following equation:

$$AEC = \frac{[(ATP) + 1/2\,(ADP)]}{[(ATP) + (ADP) + (AMP)]}. \tag{1}$$

2.4. Total Carbohydrates and Glycogen. The total carbohydrate and glycogen content were determined in accordance with the method of Racotta et al. [9]. One gram of oyster flesh was homogenized with cold 10% trichloroacetic acid (TCA). The homogenate was centrifuged at 3000 ×g at −5°C for 5 min. For glycogen, 0.1 mL of the supernatant was mixed with 1 mL of 95% ethanol and centrifuged under the same conditions. The precipitate (glycogen) obtained was resuspended in 0.1 mL distilled water. Then, 1 mL of anthrone reagent (0.1% dissolved in 76% sulphuric acid) was added to tubes that were incubated at 90°C for 5 min. Absorbance was read at 620 nm. For total carbohydrates, 0.1 mL of TCA supernatant was mixed with 1 mL of anthrone reagent, was incubated, and was read under the same conditions to quantify the glycogen.

2.5. pH. The measurement of pH was carried out by introducing in the oyster flesh a Hanna HI 90140 penetration pH meter (Hanna Instruments, Inc.). Equipment was calibrated daily with commercial standard solutions.

2.6. Statistical Analysis. Data analyses were performed with the NCSS Version 2000 [10] statistical program. An ANOVA analysis was achieved in a completely randomized factorial design with two factors (simulated transport method and transport time). A Tukey's multiple mean comparison test was applied when significant differences were found. A sample size $n = 4$ for each factor combination and 5% significance level were used in all statistics.

3. Results and Discussion

3.1. ATP, ADP, and AMP. The dry bases ATP, ADP, and AMP concentration is shown in Table 1. The ATP initial concentration found in this study was 0.06 μmol/g of dry muscle. This value is similar to the 0.07 μmol/g of dry muscle reported by Ocaño-Higuera et al. [11] found in *A. ventricosus*. The low initial ATP concentration found in this study indicates evidence of stress in the organisms even in the cultivation system. Such stress can be due to the high temperatures at the culture site in the month of July (ambient temperature ≈ 45°C), which promotes the consumption of this metabolite as organisms respond to the environmental conditions. In regard to the type of simulated transport, no significant differences ($P > 0.05$) were found for the ATP concentration over time, neither for comparing them at each sampling point, except for the cardboard box samples at the end of the experiment (36 h), which showed a significant decrease ($P < 0.05$) in the levels of this metabolite.

TABLE 1: Nucleotides, nucleosides (μmol/g dry weight), and AEC values of oyster *Crassostrea gigas* during *in vivo* transport in cardboard box (CB) and ixtle sacks (IS).

Parameter	Storage (h)				
	0	8	20	28	36
ATP					
CB	0.06 ± 0.01^{ax}	0.05 ± 0.02^{ax}	0.11 ± 0.07^{ax}	0.05 ± 0.01^{ax}	0.02 ± 0.00^{bx}
IS	0.06 ± 0.01^{ax}	0.06 ± 0.02^{ax}	0.11 ± 0.04^{ax}	0.07 ± 0.02^{ax}	0.04 ± 0.03^{ax}
ADP					
CB	0.42 ± 0.23^{ax}	0.29 ± 0.14^{ax}	1.29 ± 0.54^{bx}	0.43 ± 0.08^{ax}	0.46 ± 0.23^{ax}
IS	0.42 ± 0.23^{ax}	0.53 ± 0.22^{ax}	0.88 ± 0.31^{ax}	0.47 ± 0.09^{ax}	0.43 ± 0.23^{ax}
AMP					
CB	0.55 ± 0.13^{ax}	0.52 ± 0.10^{ax}	2.38 ± 1.10^{bx}	0.60 ± 0.09^{ax}	0.88 ± 0.22^{ax}
IS	0.55 ± 0.13^{ax}	1.09 ± 0.79^{ax}	2.54 ± 1.35^{bx}	0.96 ± 0.32^{ax}	0.78 ± 0.33^{ax}
AEC					
CB	0.26 ± 0.01^{ax}	0.23 ± 0.02^{ax}	0.22 ± 0.03^{ax}	0.24 ± 0.01^{ax}	0.22 ± 0.02^{ax}
IS	0.26 ± 0.01^{ax}	0.21 ± 0.05^{abx}	0.17 ± 0.05^{bx}	0.20 ± 0.03^{by}	0.16 ± 0.03^{by}

Values are mean and standard deviation of $n = 4$.
Means in the same row (a-b) or column (x-y) with different superscript letters for each analysis are statistically different ($P < 0.05$).

As for concentrations of ADP and AMP found during simulated transport time, only in some cases there were significant variations ($P < 0.05$), such as the ADP concentration increase observed at 20 h of storage for cardboard box samples, although these values decrease to the initial levels within hours ($P < 0.05$). This same behavior in the AMP concentrations was observed, for both of the simulated transport methods. When comparing between simulated methods, no significant differences ($P > 0.05$) at the sampling times were observed.

3.2. AEC. Results of AEC for specimens transported in cardboard box and ixtle sacks are shown in Table 1. This parameter is a widely utilized indicator in aquatic organisms, as it gives information regarding the energetic condition. This can be used for evaluating the effect of short term stress at emersion or transport on the energetic status of the studied organisms.

In this study, an AEC initial value of 0.26 was found, which corresponds to organisms with a severe physiological stress; this is according to what the literature has reported, where an AEC value of 0.8–1.0 is for healthy organisms, 0.5–0.7 for moderately stressed organisms, and <0.5 for severely stressed organisms. Organisms with moderate stress will be hard to grow and reproduce, but they can recover, while specimens with severe stress cannot grow and reproduce, as well as compromising its viability; even these were transferred to normal conditions without stress factors [8].

In a study realized with *C. gigas*, a seasonal effect on the AEC of organisms maintained in emersion (short term stress due to the desiccation) was observed. The most affected organisms were caught in May and July and it was due to desiccation, while the ones caught in January remained in an optimum energetic condition [12]. This can be associated with the results obtained in this study, since the organisms caught in July probably experienced a severe thermal stress with physiological consequences, when the culture site presents the annual maximum values of temperature. Furthermore,
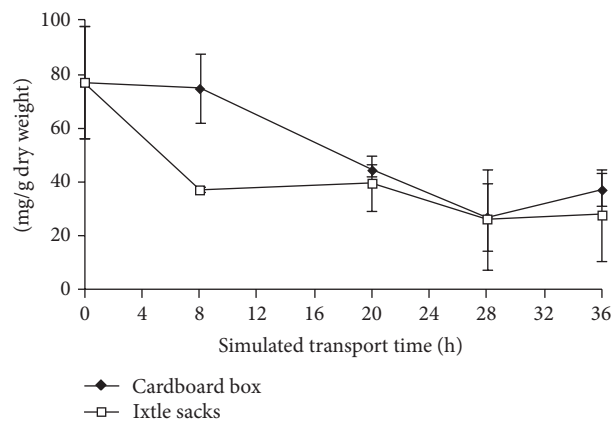

FIGURE 1: Total carbohydrates concentration (mg/g dry weight) of *Crassostrea gigas* oyster during *in vivo* transport in cardboard box and ixtle sacks. Data shown are mean of $n = 4$. Bars represent deviation standard.

when the cardboard box transport was used, the time did not show a significant effect ($P > 0.05$) on AEC and remained in its initial values without changes until the ending of the simulated method. However, when ixtle sacks transport were used, a decrease from 20 h (with respect to the initial value) was observed, remaining so until the end of the experiment. When comparing between both cardboard box and ixtle sacks during transport, higher AEC values can be observed from 28 h until the end of the experiment. These methods differences may be because ixtle sacks samples were kept at room temperature. This is important, if considering that, up to certain levels, higher temperatures increase the reaction velocities [13], specifically the ones related to the conversion of ATP to ADP and AMP, consequently changing the AEC values.

3.3. Total Carbohydrates. Figure 1 shows the data for total carbohydrates values in dry basis corresponding to the
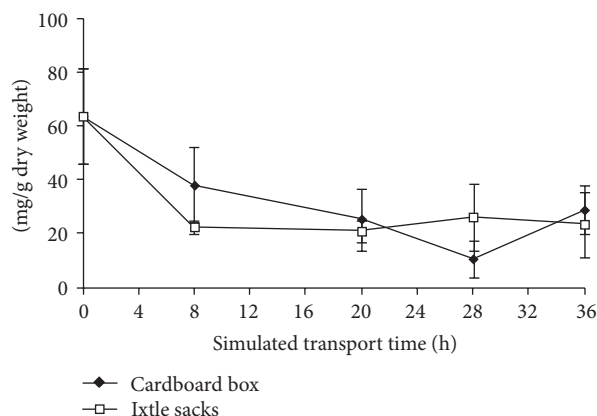
FIGURE 2: Glycogen concentration (mg/g dry weight) of *Crassostrea gigas* oyster during *in vivo* transport in cardboard box and ixtle sacks. Data shown are mean of $n = 4$. Bars represent deviation standard.
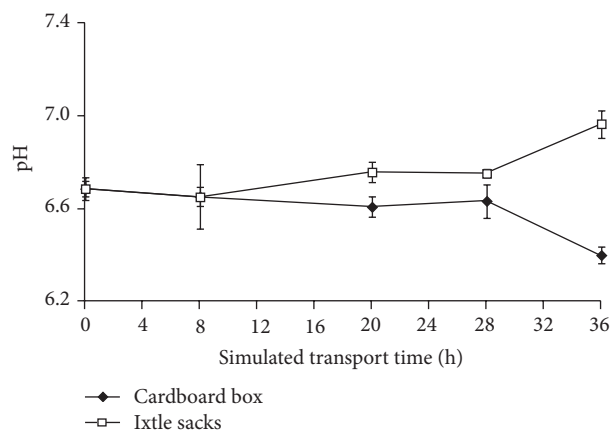

FIGURE 3: pH values of *Crassostrea gigas* oyster during *in vivo* transport in cardboard box and ixtle sacks. Data shown are mean of $n = 4$. Bars represent deviation standard.

simulated transport methods evaluated. The initial value was 77.16 mg/g, which is greater than 4 mg/g reported by Patrick et al. [14], also in adults of *C. gigas* species harvested in July, in a culture system located at the French coast. Furthermore, the value is less than 100 mg/g reported by Delaporte et al. [15] for this species caught in the same month and region but for juvenile organisms. Similarly, the initial values found in this study are less than the ones reported by Ocaño-Higuera et al. and Pacheco-Aguilar et al. [11, 16] who found 302 and 228 mg/g in the *A. ventricosus* and *N. subnodosus* adductor muscle, respectively. The differences observed may be due mainly to the type and size of studied species, the analyzed tissues, and environmental factors of the culture or capture site [17].

Regarding the transport methods, significant differences were observed over storage time. For the cardboard box organisms, initial values decreased until 20 h of storage ($P < 0.05$), remaining without significant variation ($P > 0.05$) until ending the experiment. In contrast, the carbohydrates reserve of the organisms transported in ixtle sacks decreased from 8 h of the simulated transport ($P < 0.05$), remaining at those low values to the storage end ($P > 0.05$). The difference observed in the total carbohydrates of both transport methods may be associated with the thermal stress induced on the organisms placed in ixtle sacks. On the other hand, the results obtained for cardboard box organisms are probably due to the temperature drop during transport simulation, in turn changing its metabolism.

3.4. Glycogen. The dry basis glycogen concentration of the organisms for both simulated transport methods is shown in Figure 2. The glycogen initial value was 63.60 mg/g, which is less than 286 mg/g reported by Beltrán-Lugo et al. [4] for *N. subnodosus*, whose organisms were collected also in summer (September). The difference found may be due mainly to the studied species but also factors such as reproductive state, as well as the environmental conditions of the culture or capture site. It has been observed that the tissues of some oyster species may bear up to 1–8% of glycogen in wet

basis [18]. However, there are reports of glycogen content in some studies conducted with *Crassostrea gigas* as this one realized by Lannig et al. [19], who found levels of ≈0.018, 0.108 and 0.01% (wet basis) in the adductor muscle, mantle, and gills, respectively. The glycogen initial value (wet basis) in this study is 0.56%; however, this value corresponds to the total wet tissues of the organisms, which include the portion that normally is eaten. Therefore, the difference in these values when comparing between studies may be due to the discrepancy in the portions or tissues evaluated.

With respect to the glycogen values over the simulated transport time, in Table 1 is shown a similar pattern to that one found for total carbohydrates content where differences between the utilized methods can be observed. In the organisms placed in cardboard box, the glycogen initial value decreased from 20 h of simulated transport, remaining without significant change ($P > 0.05$) until the end of the experiment. However, according to the results for this metabolite, the ixtle sacks organisms experienced higher energetic spending and, therefore, a higher physiological cost. This is based on what initial glycogen values (as in the total carbohydrates) decreased from 8 h of storage, keeping those concentration levels without variation to the end of the simulated transport ($P > 0.05$). This difference can be explained as in the case of total carbohydrates, where previously it was mentioned that a possible thermal stress was experienced by the organisms when ixtle sacks were maintained at room temperature.

3.5. pH. The pH values of the samples corresponding to the simulated transport in cardboard box and ixtle sacks are shown in Figure 3. A pH initial value of 6.68 can be observed; this initial value is very close to the range reported by Beltrán-Lugo et al. [4], who found 6.59 and 6.78 in the *N. subnodosus* adductor muscle collected in summer and autumn, respectively. However, Pacheco-Aguilar et al. [16] reported for the same species (*N. subnodosus*) an initial value of 6.3, which is slightly lower than the ones found in the aforementioned studies. The difference found when

comparing all studies may be due to the lactic acid production under *postmortem* anaerobic conditions, which decreases the muscle pH. In this regard, it is important to mention that in the study by [16] Pacheco-Aguilar et al. the reported values correspond to day 1 of the experiment, where organisms remained in considerable *postmortem* time and experienced an initial anaerobic glycolysis with concomitant lactic acid production.

Moreover, during the simulated transport for the two evaluated methods the pH values remained without significant changes ($P > 0.05$) up to 28 h. However, for the cardboard box organisms a significant decrease (with respect to the initial values) up to the simulated transport ending (36 h) was observed, coinciding with a decrease in ATP levels. In this regard, it is important to emphasize that along with the lactic acid formation there is another factor influencing the pH decrease, which is associated with inorganic phosphates released during ATP to ADP conversion [20]. As for the cardboard box organisms, for the ones kept in ixtle sacks, a variation in pH was observed since the value of this parameter increased up at the ending transport. This increase may be due to the amino compounds formation from protein degradation [20], because the possible death of the organisms was attributed to the physiological damage caused by this transport method. On the other hand, there was a significant effect when comparing between both methods, since the pH values were different from 20 h until the end of transport. This behavior may be due to the differences in ATP degradation, acid lactic, and amino compounds production in the transport methods evaluated.

4. Conclusions

According to previous studies and the results of this work, very low ATP values were obtained, which in turn affect AEC levels. There was a severe stress in the organisms from the beginning of the experiment. When comparing results between two simulated transport methods, less physiological damage was noticed for the cardboard box organisms. This is important, since organisms with a better energetic or physiological condition allow obtaining a long shelf life product. In addition, the indicators most suitable for evaluating the physiological and biochemical condition of the organisms in this study were total carbohydrates, glycogen, and pH, considering that these ones showed more variation during the simulated transport.

Disclosure

This work is part of the thesis to obtain the B.S. degree of María Elena Duarte-Figueroa, which was developed in the Laboratorio de Investigación en Alimentos of the Departamento de Ciencias Químico Biológicas of the Universidad de Sonora.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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