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Effect of Ice Storage on Freshness and Biochemical, Physical, Chemical, and Microbiological Quality of Leg Muscle Samples from Bullfrog (*Lithobates catesbeianus*)

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Abstract: The present study evaluated the effect of ice storage on the freshness and quality of bullfrog (*Lithobates catesbeianus*) leg muscle. Biochemical, chemical, physical, and microbiological changes during 24 days of storage were analyzed. A rapid degradation of ATP into its intermediates (AMP, IMP, inosine, and hypoxanthine) was observed, with a significant increase in K-index (6.78% to 79.33%) and hypoxanthine concentration (3.93 ± 0.87 µmol/g), indicating a progressive reduction in freshness. The pH initially decreased due to post-mortem glycolysis but subsequently increased due to microbial activity and protein degradation. Volatile basic nitrogen (TVB-N) content increased significantly, reaching 27.36 mg/100 g, reflecting protein breakdown. A loss of texture was recorded, with a reduction in muscle firmness from 21.93 ± 1.36 Nw to 10.87 ± 1.08 Nw. Microbiological analyses showed an increase in bacterial load, with mesophiles and psychrophiles reaching 6.75 and 6.45 log CFU/g, respectively. These results indicate that the freshness and quality of bullfrog leg under ice storage remain within acceptable limits until day 18, but its quality and freshness decrease significantly toward the end of the study period.

Keywords: frog leg; bullfrog; freshness; biochemical; physical; chemical; microbiological quality; K-value; *Lithobates catesbeanus*; storage; ice

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1. Introduction

Aquaculture is one of the most important economic activities globally and considerably impacts human nutrition. For example, aquaculture accounts for 18% of the total output of fisheries in México [1]. Raniculture is a type of highly productive aquaculture activity that involves the cultivation and/or breeding of frogs in captivity [2] as well as the widespread

export and import of frogs, primarily for their meat. Frog legs are the main edible portion of frogs alongside some other byproducts [3].

According to the current frog production statistics estimated by the Cultured Aquatic Species Information Program of the FAO, ~148 thousand tons of frogs are chiefly produced in countries such as China, Brazil, Ecuador, Guatemala, and México [4].

The bullfrog (*Lithobates catesbeianus*) is considered of great importance in México and is cultivated in the states of Sonora, Sinaloa, Jalisco, Michoacán, Tamaulipas, Veracruz, Tabasco, and Morelos. Taxonomically, *L. catesbeianus* belongs to the subphylum Vertebrata, class Amphibia, order Anura, family Ranidae, genus Lithobates, and species catesbeianus (Shaw, 1802). The nickname bullfrog comes from the sound emitted by the male bullfrog, which resembles that of a bull (laryngeal dimorphism), during the reproductive season. This species is native to North America and is characterized by its large size, as bullfrogs can reach a maximum length and weight of 43 cm and 2.5 kg, respectively. Frog legs are traditionally consumed in several countries worldwide [5,6].

Bullfrog production has several advantages that can be commercially exploited, such as their rapid life cycle and increased demand in international markets as well as the public preference for fresh over frozen products, which consequently results in higher prices and income associated with its sale during certain occasions [7].

Frog meat is considered a healthy food and is beneficial for resolving diet-associated issues such as obesity and malnutrition. Additionally, frog meat can aid in the alleviation of gastrointestinal disorders. Similarly to fish, frog meat is considered white meat and is lean, rich in essential amino acids, therapeutic for high cholesterol and hypertension, and easily digestible [8–10]. Furthermore, it has low carbohydrate and saturated fat content but high protein and water content [11], which is beneficial for consumer health.

Bullfrogs are considered a very important species in aquaculture, and similar to other aquatic products, their freshness and quality reduce with storage time. The loss of freshness and quality of fresh and frozen aquatic foods, as well as their deterioration pattern, is caused by postmortem changes, which occur in four stages: rigor mortis, dissolution of rigor, autolysis (loss of freshness), and bacterial spoilage. The duration of each stage depends on the species, physiological conditions, microbial contamination, and the storage temperature of the product [12,13].

Notably, quality indices based on nucleotide degradation have garnered special attention over the past few decades for freshness surveillance of aquatic products during manipulation and conversion. The concentration of the principal adenine nucleotides and their associated compounds in the postmortem muscle exhibits good correlation with the deterioration of the quality of a large variety of aquatic products. In this regard, the total molar concentration (TMC) of adenosine 5′ triphosphate (ATP) and its related metabolites in muscle tissue, along with the rate and pattern of its degradation at varying concentrations during storage, is influenced by both the species and the specific type of muscle tissue. Additionally, irrespective of species or muscle type, ATP undergoes rapid degradation within the first 24 h postmortem [14]. In aquatic and fishery organisms, ATP catabolism follows a sequential metabolic pathway: ATP → adenosine 5′-diphosphate (ADP) → adenosine 5′-monophosphate (AMP) → inosine 5′-monophosphate (IMP) → inosine (HxR) → hypoxanthine (Hx) [12,15]. The K-value, an established freshness indicator for aquatic and fishery products, is derived from the concentrations of ATP and its degradation products and serves as a key metric for assessing the rate of nucleotide degradation. This index is expressed as the percentage ratio of the sum of HxR and Hx concentrations to the total concentration of ATP and all its related catabolic intermediates [14,16]. Furthermore, the K-value has been shown to correlate strongly with storage duration, making it a reliable indicator of postmortem quality deterioration [14].

Previous studies have revealed that the postmortem changes that strongly affect the freshness and quality of aquatic products include the protein and ATP degradation, pH decrease, lipid oxidation, as well as the production of undesirable compounds such as trimethylamine (TMA-N) and total volatile bases of low molecular weight (TVB-N) as a result of bacterial action, which directly impacts the texture, water-holding capacity (WHC), and color of the muscle tissues [17–19]. Various methods that are currently used to evaluate the freshness and quality of the products of different aquaculture species are based on assessing changes in sensory, biochemical, chemical, and physical parameters as well as in microbiological composition [20]. Like other aquatic products, bullfrog meat is highly perishable due to microbial activity and handling conditions, highlighting the presence of psychrotrophic bacteria such as *Pseudomonas* spp., *Shewanella* spp., and *Aeromonas* spp. [21], which are responsible for spoilage. In addition, they may contain foodborne pathogenic microorganisms such as *Salmonella* spp. [22] and *Escherichia coli* O157:H7 [23]. While specific studies on the microbiota of bullfrog meat are limited, previous research has identified spoilage and pathogenic microorganisms in the skin, mouth, and intestine of different frog species, where the presence of *Lactobacillus* spp., *Pediococcus* spp., *Micrococcus* spp., *Enterococcus faecalis*, *Enterococcus faecium*, and members of the Enterobacteriaceae family, as well as *Escherichia coli*, *Salmonella typhi*, *Vibrio cholerae*, and *Staphylococcus aureus* have been reported [21,24]. This diversity in frog meat depends on environmental factors, habitat, management practices, etc.

The most important factors affecting the freshness and quality of bullfrog legs during ice storage are the same as those that apply to aquatic products in general. In fact, the freshness and quality of bullfrog leg muscle during ice storage are influenced by several factors, including physical, biochemical, microbiological, and sensory factors. However, these factors depend primarily on temperature control, in this case, on the ice-chilling process. To maintain constant cold conditions, frequent ice changes are essential, as this reduces the rate of microbial growth and slows down the chemical and enzymatic reactions responsible for product spoilage [13]. A key aspect for efficient temperature control is the constant replacement of ice because, over time, ice in contact with the muscle melts, reducing the cooling area and causing a slight increase in temperature. This can accelerate deterioration processes, favoring the appearance of undesirable changes in texture, color, odor, and microbial growth. Another determining factor in the quality and freshness of frog legs is storage time, since, as storage time increases, chemical, enzymatic, and microbiological reactions advance, affecting the stability of the product. Hence, the importance of precise temperature control [12]. In addition, proper handling during harvest and storage, together with good hygiene and packaging practices, can minimize initial contamination and prolong the shelf life of the product.

This study aims to evaluate the effect of ice storage on the freshness, biochemical, physical, chemical, and microbiological quality of leg muscle samples from the bullfrog (*Lithobates catesbeianus*). This is relevant particularly because of little to no previous research on these parameters, and the findings of this study will allow for the more efficient utilization of the species as well as improve its processing and marketing strategies for national and international consumption.

2. Materials and Methods

2.1. Collection of Bullfrogs

The bullfrogs (*L. catesbeianus* (Shaw 1802); n = 55) employed in the present study were obtained from the NutriFrog company (Mazatlán, Sinaloa, México) and included 28 females and 27 males with average body weights of 233.78 ± 22.43 and 260.62 ± 39.07 g, respectively, and an average age of 13 months. The bullfrogs were transported (for a

duration of 4 h) in a container allowing air circulation to Caseros, S. de R. L. M. I (Navojoa, Sonora, México), where they were maintained with adequate food supply and allowed to rest for a period of 4 days to eliminate the stress of transport before their sacrifice.

The bullfrogs were sacrificed via desensitization via a dry blow to the anterior dorsal area, followed by evisceration, skin removal, and procurement of their legs [25]. The bullfrog legs so obtained were washed with potable water and individually placed in high-density polyethylene bags arranged in alternating beds of ground ice and bullfrog legs in a hermetic cooler for transport to the Food Research Laboratory of the University of Sonora (México). The time interval between the sacrifice of the bullfrogs and the receipt of the sample at the laboratory was no longer than 12 h.

2.2. Analysis of Samples During Storage on Ice

Following receipt at the laboratory, the bullfrog leg samples were repacked in polyethylene bags and again arranged in alternating beds of ground ice and bullfrog legs in a hermetic cooler. The frog legs subjected to storage on ice were analyzed over a duration of 24 days, with samples withdrawn on days 0, 3, 6, 9, 12, 15, 18, 21, and 24 to evaluate the biochemical, physical, and chemical changes in them, including ATP and degradation products, K-value, pH, total volatile bases (TVB-N), WHC, texture, and color (L*, a*, and b*). The total bacterial counts (mesophilic and psychrophilic counts) were determined on days 0, 6, 12, 18, and 24 of ice storage. Frog legs from four individual organisms for each day of sampling were employed for biochemical, physical, and chemical analyses, while frog legs from two individual organisms per day were employed for microbiological analyses. Finally, crushed ice was replaced as and when necessary during the storage of the bullfrog legs.

2.3. Analytical Determinations

2.3.1. ATP and Degradation Products

ATP, ADP, AMP, IMP, HxR, and Hx were quantified using high-performance liquid chromatography (HPLC), as detailed previously [26]. An extract was prepared by homogenizing bullfrog leg muscle (3 g) in 0.6 M perchloric acid (15 mL) at 0 °C using an Ultra-Turrax T18 Basic homogenizer (IKA Works Inc., Wilmington, NC, USA) at 18,000 rpm for 1 min. The homogenate was subsequently centrifuged at 5500× *g* for 10 min at 0 °C in a refrigerated Thermo Electron Model IEC-MULTI RF centrifuge (Thermo Fisher Scientific, Asheville, NC 28804, USA). Subsequently, a 7 mL aliquot of the supernatant was neutralized with 1 M KOH to attain a pH of 6.5–6.8 and allowed to rest for 30 min on ice. Potassium perchlorate was then removed via filtration using Whatman No. 4 paper. The supernatant was diluted to 15 mL with distilled water, and the samples were stored at −80 °C until further use.

The separation and quantification of the compounds via HPLC was performed by injecting the neutralized and diluted supernatant (20 µL) onto a Varian Prostar 240 chromatograph (Varian, Inc., Walnut Creek, CA, USA) that employs a Varian C18 Ultrasphere ODS reverse phase column (Beckman Instruments, Inc., Fullerton, CA, USA) with an inner diameter of 4.6 mm and a length of 250 mm. The mobile phase comprises phosphate buffer (0.04 M KH_2PO_4 and 0.06 M K_2HPO_4), at a flow rate of 1 mL/min. Nucleotides, nucleosides, and nitrogenous bases were detected at 254 nm using a UV-visible detector (Varian, Inc., Walnut Creek, CA, USA).

2.3.2. Calculation of K-Value

It is important to highlight that high-performance liquid chromatography (HPLC) is highly efficient for quantifying ATP and its degradation products due to its ability to separate molecules according to their polarity and partition coefficient. In reverse phase

C18 columns, more polar compounds elute faster than less polar compounds, allowing accurate separation. In addition, UV detection at 254 nm ensures high sensitivity and specificity, since nucleotides have aromatic conjugated structures that absorb at this wavelength [26]. Compared to other methods such as UV-Vis spectrophotometry and enzymatic methods, to mention a few, HPLC allows quantifying each metabolite individually [27]. In addition, it combines sensitivity, reproducibility, and ease of application, being ideal for freshness and quality studies in biological and food products [14].

The concentrations of ATP, ADP, AMP, IMP, HxR, and Hx were employed for calculating the K-value using the equation described in a previous study [28], as follows:

$$\text{K-value (\%)} = [(\text{HxR} + \text{Hx})/(\text{ATP} + \text{ADP} + \text{AMP} + \text{IMP} + \text{HxR} + \text{Hx})] \times 100.$$

2.3.3. Determination of the pH of Frog Leg Muscle

The pH of the bullfrog leg muscle was determined according to a previously reported method [29]. For this purpose, bullfrog leg muscle (2 g) was homogenized in distilled water (18 mL) using the Ultra-Turrax T18 Basic homogenizer (IKA Works Inc., Wilmington, NC, USA) at 18,000 rpm for 1 min. The pH of the homogenate was determined by introducing the tip of the electrode of a potentiometer into it (Orion 420 A, Thermo Electron Corporation, Waltham, MA, USA). The equipment was calibrated daily with the standard pH buffer solutions.

2.3.4. Color

The color of bullfrog leg muscle was assessed using tristimulus colorimetry within the CIE Lab color space, employing a MiniScan HunterLab colorimeter (Reston, Virginia, VA, USA). The instrument operated in reflectance mode with a 0.5 cm reading port opening, capturing the L (lightness), a (red–green axis), and b (yellow–blue axis) values. Color measurements were conducted on both surfaces of the muscle tissue. Prior to data collection, the colorimeter was calibrated according to the manufacturer's guidelines.

2.3.5. Texture

The texture of bullfrog leg muscle was evaluated using an EZ-S Shimadzu 346-54909-33 texture analyzer (Shimadzu Corp., Model EZ-S, Kyoto, Japan) equipped with a Warner–Bratzler shear cell. The maximum shear force (N) required to cut samples measuring $10 \times 10 \times 20$ mm^3 was recorded. A 50 kg compression cell was used, applying force at a head speed of 20 cm/min. The force was exerted perpendicular to the muscle fibers, and the required shear force was measured following the International System of Units (SI) standards.

2.3.6. Water Retention Capacity (WHC)

The water-holding capacity (WHC) of bullfrog leg muscle was determined according to a previously established method [30]. Two grams of muscle tissue were weighed and placed into a 50 mL centrifuge tube, followed by centrifugation in a refrigerated Thermo IEC-MULTI RF centrifuge (Thermo Fisher Scientific, Asheville, NC, USA) at 19,600$\times$ g for 60 min at 4 °C. The WHC was calculated as the percentage of water loss relative to the initial moisture content using the following formula:

$$\text{WHC (\%)} = 100 - ([\text{Wi} - \text{Wf}]/\text{Wi} \times 100),$$

where Wi and Wf correspond to the initial weight of fresh bullfrog meat and the final weight after centrifugation and surface drying, respectively.