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Evaluations of the efficacy of Hypersaturated Saline Solutions for the preservation of fish and amphibians for educational purposes

Avaliações da eficácia de Soluções Salinas Hipersaturadas para preservação de peixes e anfíbios para fins educacionais

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Fish and amphibians are traditionally preserved in formalin or ethyl alcohol, but these fluids can compromise their morphological characteristics. Hypersaturated saline solutions, already used in veterinary medicine, have emerged as effective preservation alternatives, maintaining the appearance of specimens similar to that of living organisms. This study evaluated the efficacy of hypersaturated saline solutions (26% and 30%) for the preservation of fish and tadpoles (Anura) as teaching materials, comparing them with conventional techniques, such as 10% formalin and 70% ethyl alcohol, in addition to the use of 20% curing salt. Treatments with 20% curing salt and 26% or 30% saline solutions (T3 and T4) showed good morphological results, preserving colors and malleability similar to those of living animals, but presented bacterial and fungal growth, leading to decomposition after 8 months. Treatments T1 and T2, with prior fixation in 10% formalin and preservation in hypersaturated saline solution, were more effective, maintaining morphological quality for more than five years without microbial growth. Preservation in 30% hypersaturated saline solution, preceded by fixation in formalin, proved to be the most effective and low-cost method for educational purposes, preserving morphological characteristics and with antiseptic properties.
Key words: animal anatomy, vertebrate, anurans.

Peixes e anfíbios são tradicionalmente conservados em formalina ou álcool etílico, mas esses fluidos podem comprometer suas características morfológicas. Soluções salinas hipersaturadas, já utilizadas na medicina veterinária, surgiram como alternativas eficazes de preservação, mantendo a aparência dos espécimes semelhante à de organismos vivos. Este estudo avaliou a eficácia de soluções salinas hipersaturadas (26% e 30%) para a preservação de peixes e girinos (Anura) como materiais didáticos, comparando-as com técnicas convencionais, como formalina 10% e álcool etílico 70%, além do uso de sal de cura 20%. Os tratamentos com sal de cura 20% e soluções salinas 26% ou 30% (T3 e T4) apresentaram bons resultados morfológicos, preservando cores e maleabilidade semelhantes às dos animais vivos, mas apresentaram crescimento bacteriano e fúngico, levando à decomposição após 8 meses. Os tratamentos T1 e T2, com fixação prévia em formalina a 10% e preservação em solução salina hipersaturada, foram mais eficazes, mantendo a qualidade morfológica por mais de cinco anos sem crescimento microbiano. A preservação em solução salina hipersaturada a 30%, precedida pela fixação em formalina, mostrou-se o método mais eficaz e de baixo custo para fins educacionais, preservando as características morfológicas e com propriedades antissépticas.
Palavras-chave: anatomia animal, vertebrados, anuros.

1. INTRODUCTION

The use of properly conserved animal specimens is essential for teaching and for obtaining a deeper understanding of anatomy and physiology, especially in the health and biological sciences

areas as they contribute to the development of the applicative, assimilative, and comprehensive skills of students [1].

Various techniques have been used to preserve and study animals and their tissues. Formaldehyde, discovered in 1868 by the German chemist August Wilhelm von Hofmann, is still widely used in the preservation of cadavers, especially for teaching animal morphology and anatomy [2]. Seventy percent ethyl alcohol is another common preservative used with invertebrates and small vertebrates; it is volatile, however, and requires periodic replacement [3]. Glycerin is widely used to preserve invertebrates such as crustaceans, as it maintains specimen characteristics similar to those of living animals [4].

According to Fox et al. (1985) [5], Mies (1994) [6], and Silva et al. (2008) [2], 10% formalin is the most widely used preservative for animal specimens and anatomical organs. It allows the preservation of tissues and organs for long periods of time and facilitates both their manipulation and dissection without significantly compromising their structures [7]. It can, however, cause dehydration, hardening, and changes in tissue color, and has adverse health effects on those handling them, including headaches and irritation [8].

Researchers have sought to find replacements for formaldehyde as a preservative in order to create more conducive environments for teaching and learning. In this context, Costa et al. (2021) [4] evaluated glycerin for preserving crustaceans. Cury et al. (2013) [1] experimented with four anatomical techniques for preserving dogs, cats, and donated organs: freeze dehydration, glycerination, and latex or vinylite injection. These techniques proved to be effective in meeting students' needs in anatomical studies and allowed the clear visualization and easy handling of their internal and external structures.

Due to the vast diversity of animal groups, it will be necessary to define alternative conservation techniques specific to each type of organism. These techniques must ensure that the specimens' characteristics remain as close as possible to their living states and preserve their *in vivo* morphological and physiological properties. The use of methods appropriate to each group will improve the quality of their conservation, enrich the learning experiences of students, and promote a deeper and more meaningful understanding of animal anatomy and biology.

Hypersaturated saline solutions were introduced into veterinary medicine as alternative techniques for preserving canine anatomical specimens [9]. This method has proven to be highly effective for preserving texture, flexibility, and coloration; it is also non-toxic, environmentally safe, readily available, and has low costs [9, 10]. Additionally, studies have indicated the absence of fungal and bacterial growth, supporting the effectiveness of hypersaturated saline solutions for preservation [11]. A 30% salt solution is prepared by dissolving 1 kg of salt in 2.8 L of water [12].

Although saline solutions have only relatively recently been adopted for preserving anatomical specimens for educational purposes, the use of salt dates back more than 5,000 years to the mummification processes of Ancient Egypt [13]. Salt is also effective for preserving food, as demonstrated by Goulas and Kontominas (2005) [14], who analyzed the effects of salting and smoking on mackerel (*Scomber japonicus*) quality. Their study showed that salt extended the shelf life of the fish, preserved their sensory characteristics, and inhibited the growth of microorganisms.

In addition to the application of conservation techniques for teaching purposes, anatomical museums have conducted studies focusing on the preservation of specimens having great historical value. These studies have included detailed microbiological analyses of the preservation fluids to identify possible factors contributing to specimen deterioration. This approach not only aids specimen conservation but also provides insights into the conditions that could affect specimen integrity over time and allows the implementation of effective preventive measures [15].

Considering the potential advantages of saline solutions when used for specimen preservation, this study evaluated the effectiveness of hypersaturated saline solutions (26% and 30%) for preserving fish and tadpole (Anura) specimens for educational purposes. We compared the hypersaturated saline solution method with conventional techniques such as 10% formalin and 70% ethyl alcohol, as well as the effectiveness of 20% curing salt. Our study also included physicochemical and microbiological evaluations of the preservation fluids to ensure specimen quality.

2. MATERIALS AND METHODS

This study was conducted using 42 specimens of vertebrate animals (24 fish and 18 larval stage anurans [tadpoles]). Among the fish, 18 specimens belonged to the species *Sphoeroides testudineus* (Linnaeus, 1758) and six were *Chloroscombrus chrysurus* (Linnaeus, 1766). All 18 tadpole specimens were of the species *Boana semilineata* (Spix, 1824).

The experiment with fish consisted of two positive controls and four to six treatments, with three specimens distributed in each control and treatment protocol:

- Control 1 (C1): specimens fixed with 10% formalin for one week and subsequently preserved in 10% formalin;
- Control 2 (C2): specimens fixed with 10% formalin for one week and subsequently preserved in 70% ethyl alcohol;
- Treatment (T1): specimens fixed with 10% formalin for one week and subsequently preserved in 26% hypersaturated saline solution;
- Treatment 2 (T2): specimens fixed with 10% formalin for one week and subsequently preserved in 30% hypersaturated saline solution;
- Treatment 3 (T3): specimens fixed with 20% curing salt for one week and subsequently preserved in a 26% hypersaturated saline solution;
- Treatment 4 (T4): specimens fixed with 20% curing salt for one week and subsequently preserved in a 30% hypersaturated saline solution;
- Treatment 5 (T5): specimens fixed with 10% formalin for one week and subsequently preserved in a 26% hypersaturated saline solution for five years;
- Treatment 6 (T6): specimens fixed with 10% formalin for one week and subsequently preserved in a 30% hypersaturated saline solution for five years.

Treatments T5 and T6 were exclusively applied to the species *Chloroscombrus chrysurus*, as these specimens had been preserved for five years.

In terms of the tadpoles, the experiments likewise involved three specimens in each control and treatment protocol, although the controls and treatments were modified in relation to the fish group, with a positive control and four treatments. Transeau Control (CT): specimens fixed in 10% formalin and subsequently preserved in Transeau solution; the first four treatments applied to the fish group (T1 to T4) were likewise applied to the tadpoles. The 26% and 30% hypersaturated saline solutions were prepared using table salt (Cavalinho brand) and distilled water. To prepare the 26% saline solution, 3.2 liters of distilled water were used for every 1 kg of table salt. To prepare the 30% saline solution, 2.8 liters of distilled water were used for every 1 kg of table salt. The 20% curing salt solution was prepared using 8 liters of distilled water for every 1 kg of salt (Adicel).

The experimental specimens were subjected to three types of evaluations: physical-chemical, morphological, and microbiological. The physical-chemical evaluations were performed weekly to characterize the preservative solutions of treatments T1 to T6 (preserved in saline solution). The salinity and pH variables of the solutions were measured using a portable salinometer (model RHS-10ATC) and a microprocessor-based digital pH meter (model DLA) respectively.

The morphological evaluations were performed at two times: at three and six months after the specimens were fixed and preserved. To that end, a quality assessment protocol for educational purposes based on an adaptation of the fish quality index of Amaral and Freitas (2013) [16] was used. Seven parameters were considered in these evaluations: appearance, musculature, coloration, texture, eye opacity, malleability, and odor.

The specimens were evaluated using a blind protocol (with the evaluators not knowing which control or treatment group they were evaluating) undertaken by two biologists and two veterinarians, all with links to the field of Zoology. The evaluators analyzed the morphologies of the specimens and compared them to positive controls. Each criterion was scored with values from 0 to 3, with specimens that appeared closer to their natural living states receiving the highest scores. The specimens that most deviated from their natural living states received scores closer to 0 (Table 1).

Specimens of *Chloroscombrus chrysurus* preserved in saline solution for five years (T5 and T6) were used only for microbiological and physical-chemical characterizations in light of their morphological differences in relation to the specimens used here in the three- and six-month experiments.

Data analysis was performed using mode scores that identify the central tendency of each data set, allowing the identification of the scores most frequently attributed by the evaluators and, thus, evaluating the effects on the qualities of the specimens. After determining the mode among the results provided by the evaluators, the final result (FR) value was calculated, adding the attributed scores of all of the evaluation criteria. This value was then used to compare the different groups, controls, and treatments, in order to identify the most suitable use protocol. This analysis was performed using the Graphpad Prism 5.0 program.

Microbiological evaluations of the fish were performed three months, six months, and five years after the specimens were fixed and preserved. These evaluations were designed to determine the occurrence of bacteria and/or fungi in the preservative solutions. Microbiological analyses of the tadpole solutions occurred after only 3 months of conservation, however, due to the initiation time of the experiment. Samples of the preservative solution (100 ml) were collected and 5 dilutions were performed (10-1 to 10-5); those dilutions were seeded (in duplicate) in nutrient agar culture medium, using the Pour Plate technique, and incubated at 36º C for 24 h to promote bacterial growth [17].

Samples were also seeded on MacConkey agar and Mannitol agar using a bacteriological loop and incubated at 36º C for 24 hours to observe colony morphologies, count Colony Forming Units (CFU), and to perform possible bacterial identifications based on Gram staining. Samples were also seeded on Sabouraud agar and potato dextrose agar using a sterile swab, and incubated at 30º C for 7 days; during those seven days they were observed daily to evaluate fungal growth and count Colony Forming Units (CFU) [10].

Table 1: Morphological parameters, criteria, and the scores used to evaluate the morphological qualities of the control and treatment groups of Sphoeroides testudineus *and* Boana semilineata*. Adaptation of the fish quality index proposed by Amaral and Freitas (2013) [16].*

Morphological Parameters	Criteria	Scores
Appearance	Opaque	0
	Slightly opaque	1
	Shiny	2
	Very shiny (closest to that of living animals)	3
Musculature	Musculature falling apart when touched	0
	Musculature very stiff	1
	Musculature not very stiff	2
	Musculature close to that of living animals	3
Coloring	Completely whitish	0
	Partially whitish	1
	Coloration near that of live animals, but with perceptible differences	2
	Coloration similar to that of living animals	3
Texture	Disintegrated, soft, or fragile texture	0
	Reasonably preserved, showing signs of alteration	1
	Firm texture, pleasant to the touch	2
	Preserved texture, with firm consistency and pleasant to the touch	3
Eye opacity	Cloudy, opaque eyes, with visible damage	0
	Relatively preserved eyes, without major damage or discoloration	1
	Eyes preserved, with a clear appearance and without visible damage	2
	Eyes preserved, without discoloration, having a crystalline appearance	3
Malleability	Rigid, difficult to move	0
	Flexible, but with limited movements	1
	Flexible, with relatively natural movements	2
	Highly flexible, allowing natural movements	3
Odor	Very strong preservative smell	0
	Strong preservative smell	1
	Bearable preservative smell	2
	No preservative smell	3

This study was conducted with the approval of the Ethics Committee for the Use of Animals (CEUA) of the Federal University of Bahia – Multidisciplinary Institute of Health – Anísio Teixeira Campus (UFBA – IMS – CAT), under registration number 122/2023. Animal collections were authorized by the Brazilian Ministry of the Environment (ICMBio License Nº. 89731-1).

3. RESULTS

Physicochemical evaluations indicated little salinity or pH variations during the study among both the fish preservative solutions and those of the tadpoles. Treatments T1 and T3, exposing fish to the 26% hypersaturated saline solution, evidenced average salinities of 26.42% ± 1.00 and 26.25% ± 0.97 respectively. Treatments T2 and T4, using fish that had been fixed in 20% curing salt and subsequently preserved in the 30% saline solution had average salinities of 30.00% ± 1.13 and 30.33% ± 0.65 respectively (Table 2).

Table 2: Physicochemical parameters, salinity, and pH of the control groups and treatments with the fish Sphoeroides testudineus *and* C. chrysurus *as well as* Boana semilineata *tadpoles. C1 (fixed in 10% formalin and subsequently preserved in 10% formalin); C2 (fixed with 10% formalin and subsequently preserved in 70% ethyl alcohol); CT (fixed with 10% formalin and subsequently preserved in Transeau solution), T1 (fixed with 10% formalin and subsequently preserved in a 26% hypersaturated saline solution); T2 (fixed with 10% formalin and subsequently preserved in a 30% hypersaturated saline solution); T3 (fixed with 20% curing salt and subsequently preserved in a 26% hypersaturated saline solution); T4 (fixed with 20% curing salt and subsequently preserved in a 30% hypersaturated saline solution); T5 (C. chrysurus fixed with 10% formalin and subsequently preserved in a 26% hypersaturated saline solution for 5 years); and T6 (C. chrysurus fixed with formalin 10% and subsequently preserved in a 30% hypersaturated saline solution for a period of 5 years). Mean ± standard deviation (minimum-maximum), "-" indicates that it was not possible to measure the parameters.*

Groups	Salinity %		pH	
	Fish	Tadpoles	Fish	Tadpoles
C1	-	-	4.86 ± 0.35 (4,61-5,04)	-
C2	-	-	7.66 ± 0.28 (7,46-7,90)	-
CT	-	-	-	3.75 ± 0.09 (3.65-3.89)
T1	26.42 ± 1.00 (25-28)	27.22 ± 1.30 (26-30)	6.52 ± 0.10 (6.35-6.64)	6.01 ± 0.01 (6.00-6.10)
T2	30.00 ± 1.13 (29-32)	29.56 ± 0.53 (29-30)	7.40 ± 0.19 (7.03-7.59)	7.11 ± 0.05 (7.07-7.15)
T3	26.25 ± 0.97 (25-28)	27.11 ± 1.36 (26-30)	6.82 ± 0.25 (6.31-7.05)	6.98 ± 0.04 (6.95-7.08)
T4	30.33 ± 0.65 (30-32)	29.56 ± 0.53 (29-30)	6.82 ± 0.11 (6.23-6.89)	7.15 ± 0.01 (7.06-7.20)
T5	24.00 ± 0.85 (23-25)	-	6.75 ± 0.17 (6.54-6.99)	-
T6	27.00 ± 0.74 (26-28)	-	6.95 ± 0.41 (6.05-7.08)	-

The salinity concentration treatments T1 and T3 with tadpoles kept in 26% saline, on the other hand, presented salinity averages of 27.22% ± 1.30 and 27.11% ± 1.36 respectively – values slightly higher than expected (26%). Treatments T2 and T4 with tadpoles kept in a saline solution with an initial concentration of 30% evidenced salinity averages within the expected range (29.56% ± 0.53 and 29.56% ± 0.53 respectively) (Table 2).

Little pH variation was observed among the different treatments, with averages ranging from slightly acidic to alkaline (from 6.01 to 7.66). However, the control groups C1 for fish (fixed with 10% formalin for one week and subsequently preserved in 10% formalin) and CT for tadpoles (fixed with 10% formalin and subsequently preserved in Transeau solution) had the lowest pH values, with averages of 4.86 ± 0.35 and 3.75 ± 0.09 respectively (Table 2).

The evaluation of the morphological parameters of *S. testudineus* after three months of conservation revealed that the treatments T3; T2, and T4 presented the best results based on their FR values (19, 18, and 17 respectively) in terms of the parameters analyzed (Figure 1; Table 3).

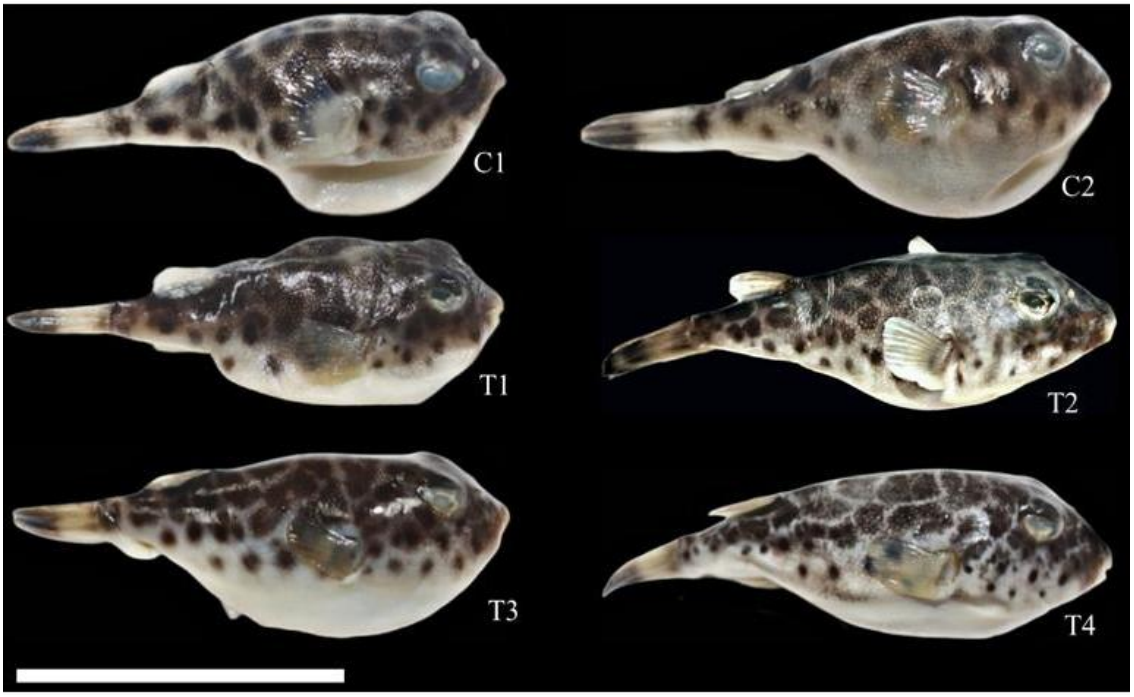

Figure 1: Control and treatment groups of Sphoeroides testudineus *specimens after exposure to C1 (fixed with 10% formalin and subsequently preserved in 10% formalin); C2 (fixed with 10% formalin and subsequently preserved in 70% ethyl alcohol); T1 (fixed with 10% formalin and subsequently preserved in a 26% hypersaturated saline solution); T2 (fixed with 10% formalin and subsequently preserved in a 30% hypersaturated saline solution); T3 (fixed with 20% curing salt and subsequently preserved in a 26% hypersaturated saline solution); T4 (fixed with 20% curing salt and subsequently preserved in a 30% hypersaturated saline solution). White bar = 5 cm scale.*

Table 3: Morphological parameters and scores after evaluations of the control and treatment groups of Sphoeroides testudineus *specimens after three and six months of fixation and subsequent conservation, as well as the morphological parameters and scores after evaluations of the control and treatment groups of* Boana semilineata *after three months of fixation and conservation. C1 (fixed with 10% formalin and subsequently preserved in 10% formalin); C2 (fixed with 10% formalin and subsequently preserved in 70% ethyl alcohol); CT (fixed with 10% formalin and subsequently preserved in Transeau solution); T1 (fixed with 10% formalin and subsequently preserved in a 26% hypersaturated saline solution); T2 (fixed with 10% formalin and subsequently preserved in a 30% hypersaturated saline solution); T3 (fixed with 20% curing salt and subsequently preserved in a 26% hypersaturated saline solution); T4 (fixed with 20% curing salt and subsequently preserved in 30% hypersaturated saline solution). FR= Final result of the sum of all evaluation criteria values. The values used in the table correspond to the mode.*

Morphological parameters	*S. testudineus* (after three months)						*S. testudineus* (after six months)						*B. semilineata* (after three months)				
	C1	C2	T1	T2	T3	T4	C1	C2	T1	T2	T3	T4	CT	T1	T2	T3	T4
Appearance	1	1	2	3	3	3	1	1	2	2	3	3	1	1	2	2	2
Musculature	1	1	2	2	3	3	1	1	2	1	0	2	2	2	2	2	1
Coloration	0	0	2	3	3	3	0	0	1	2	3	3	1	2	3	3	2
Texture	2	2	2	3	3	2	1	1	1	2	3	2	1	0	0	3	1
Opacity of eyes	0	0	1	2	1	1	0	0	1	2	0	0	1	1	1	2	3
Malleability	0	1	1	2	3	2	0	0	1	1	3	2	2	2	3	3	3
Odor	0	0	2	3	3	3	0	0	1	2	3	3	0	2	2	2	3
RF	4	5	12	18	19	17	3	3	9	12	15	15	9	10	13	17	15

The best results for the appearance parameters of *S. testudineus* specimens were observed after three months of conservation in treatments T2, T3, and T4, all having scores of 3 (very shiny,

closest to the appearance of a live animal) (Tables 1 and 3). In all of these treatments, 26% or 30% hypersaturated saline solutions were used as the preservative. The most unfavorable results for the appearances of the specimens, on the other hand, were recorded in the control groups C1 and C2, both with scores of 1 (specimens slightly opaque) (Tables 1 and 3). In these groups, the specimens were fixed with 10% formalin and subsequently preserved in 10% formalin or 70% ethyl alcohol respectively.

The best results for the musculature parameter were observed with treatments T3 and T4, both having scores = 3 (musculature close to that of a living animal) (Tables 1 and 3). The specimens in these treatments were fixed in 20% curing salt and subsequently preserved in 26% or 30% saline solutions respectively. The most unfavorable results, on the other hand, were obtained in the control groups C1 and C2, both with scores = 1 (very rigid musculature) (Tables 1 and 3), when the specimens were fixed with 10% formalin and subsequently preserved in 10% formalin or 70% alcohol respectively (Table 3).

The highest scores for *S. testudineus* coloration coincided with the results obtained for the appearances of the specimens, with treatments T2, T3, and T4 presenting scores = 3 (coloration similar to that of living animals) (Tables 1 and 3). In contrast, the most negative results likewise followed the patterns observed for appearance and musculature, with controls C1 and C2 standing out negatively; similarly these controls presented the lowest scores in terms of the parameter coloration (equal to 0; specimen completely whitish).

Treatments T2 and T3 presented the best results in terms of the textures of the specimens with scores = 3 (texture preserved, with a firm consistency and pleasant to the touch) (Tables 1 and 3). The other controls and treatments received scores = 2 (texture firm and pleasant to the touch), indicating little variation in this parameter among the different protocols analyzed.

No group achieved a maximum score in terms of the parameter eye opacity. Only treatment T2 (fixed with 10% formalin and subsequently preserved in a 30% saline solution) obtained a score = 2 (eyes preserved, with a clear appearance and without visible damage) (Tables 1 and 3). The other control groups attained only the lowest scores, equal to 0 (cloudy, eyes opaque with visible damage) (Tables 1 and 3).

The best result for the malleability variable was observed in treatment T3, with a score = 3 (highly flexible, allowing natural movements); it had been fixed in 20% curing salt and subsequently preserved in a 26% saline solution (Tables 1 and 3). The lowest score was recorded in control C1, with score = 0 (rigid, with difficulty for movement) (Tables 1 and 3).

The results for the odor parameter were similar to those of the color parameter: treatments T2, T3, and T4 were assigned scores of 3 (without preservative odor) (Tables 1 and 3), while the control groups C1 and C2 were assigned the lowest scores (0 = very strong preservative odor).

The morphological analyses of the specimens of *S. testudineus* after six months of conservation indicated that treatments T2, T3, and T4 continued to present the best results when compared with evaluations carried out after only three months. The classifications of the treatments did change, with T3 and T4 being assigned the same FR of 15, although T2 was assigned a FR of 12 (Table 3). The analyses of the morphological parameters of *S. testudineus,* after six months of conservation, revealed similar results to those obtained after three months, especially with regard to specimen appearance. The only exception was treatment T2, whose score dropped from 3 to 2 (shiny) (Tables 1 and 3).

In terms of the musculature parameter, all of the treatments evidenced variations in their scores when comparing the three and six month time periods. The best scores (2 = muscles not very rigid) were observed in treatments T1 and T4, while treatment T3 presented the lowest score (0 = muscles falling apart when touched), which had not been assigned in any of the three-month evaluations (Tables 1 and 3).

In terms of the parameter coloration, only treatments T1 and T2 evidenced variations in their scores when compared to the evaluations carried out at three months. Treatment T1, which received a score of 2 (coloration similar to that of live animals) became altered to score = 1 (partially whitish). Treatment T2 varied from score 3 (coloration similar to that of live animals) to score 2 (coloration near that of live animals, but with perceptible differences) (Tables 1 and 3).

In terms of the parameter texture, only treatments T3 and T4 maintained their score values, (3 and 2 respectively) when compared to the evaluations carried out at three months. All of the other groups showed score variations (Table 3).

In terms of eye opacity, treatments T3 and T4 exhibited changes from one evaluation to the next, altering from score 1 (relatively preserved eyes, without major damage or discoloration) to score 0 (cloudy, opaque eyes and visible damage) (Tables 1 and 3).

In terms of the malleability parameter, only the control group C2 and treatment T2 evidenced changes when compared to evaluations performed after three months. The specimens of control C2 decreased from score 1 (with flexibility, but with limited movements) to score 0 (rigid and with difficulty for movement) in the second evaluation. Malleability varied from score 2 in treatment T2 (flexible, with relatively natural movements) to score 1 (with flexibility, but with limited movements) (Tables 1 and 3).

In terms of the parameter odor, treatments T1 and T2 evidenced changes between the two evaluation periods. Treatment T1 was assigned score 2 (tolerable preservative odor) in the first evaluation, but score 1 in the second evaluation (strong preservative odor). Treatment T2, on the other hand, varied from score 3 (without preservative odor) to score 2 (with a tolerable preservative odor) in the second evaluation (Tables 1 and 3).

The morphological analysis of *Boana semilineata* specimens after three months of conservation revealed that the best treatment results for T2, T3, and T4 were the same as those identified for *S. testudineus* after three and six months, although their classification orders were different: T3 was assigned FR=17, followed by T4 with FR=15, and T2 with FR=13. The lowest scores were recorded in the CT control group and in the T1 treatment (Figure 2; Table 3).

Figure 2: Results for the specimens of Boana semilineata*. CT (fixed with 10% formalin and subsequently preserved with Transeau solution); T1 (fixed with 10% formalin and subsequently preserved in a 26% hypersaturated saline solution); T2 (fixed with 10% formalin and subsequently preserved in a 30% hypersaturated saline solution); T3 (fixed with 20% curing salt and subsequently preserved in a 26% hypersaturated saline solution); T4 (fixed with 20% curing salt and subsequently preserved in a 30% hypersaturated saline solution). White bar = 5 cm.*

In terms of the parameter appearance, the best results were obtained in treatments T2, T3, and T4, all of which were preserved in 26% or 30% hypersaturated saline solutions and were assigned

score 2 (shiny). The lowest scores (1 = slightly opaque) were observed in the CT control group and in treatment T1 (Tables 1 and 3).

In terms of the parameter musculature, most groups were assigned score 2 (musculature slightly rigid), with the exception of treatment T4 (score 1, very rigid musculature) (Tables 1 and 3).

After three months of conservation, the *Boana semilineata* specimens evidenced the best coloration scores (3 = coloration similar to living animals) in treatments T2 and T3. The lowest score (1 = partially whitish) was observed in the CT control group (Tables 1 and 3).

In terms of the parameter texture, treatment T3 was assigned the highest score (3 = texture preserved, with firm consistency and pleasant to the touch); the lowest score (1 = reasonably preserved, showing signs of alteration) was recorded in CT and T4 (Tables 1 and 3).

In terms of the parameter eye opacity, treatment T4 stood out with the highest score (3 = eyes preserved, without discoloration, and with a crystalline appearance). Groups CT, T1, and T2 obtained the lowest scores (1 = eyes relatively preserved, without major damage or discoloration) (Tables 1 and 3).

When considering all of the parameters analyzed, specimen malleability presented the best results, with treatments T2, T3, and T4 having score 3 (highly flexible, allowing natural movements). Groups CT and T1 had score 2 (flexible, with relatively natural movements) (Tables 1 and 3). In terms of the parameter odor, treatment T4 was assigned the highest score (3 = without preservative odor), while CT had the lowest score (0 = very strong preservative odor) (Tables 1 and 3).

It is notable that although the specimens fixed with 20% curing salt and subsequently preserved in 26% or 30% hypersaturated saline solutions (T3 and T4) evidenced good morphological results for both *S. testudineus* and *Boana semilineata* and preserved their colors and malleability similar to those of living animals after 8 months, they did show signs of decomposition. Therefore, when analyzing the alternative conservation fluids examined in this study, it was found that the hypersaturated saline solutions presented superior results as compared to 10% formalin or 70% ethyl alcohol as conservation fluids, and that the use of 10% formalin or 70% ethyl alcohol resulted in gradual losses of the morphological characteristics of the specimens (which became less similar to those of living animals after a certain period of exposure).

Microbiological observations and the quantification of microorganisms present in the preservative liquids holding fish and tadpoles showed satisfactory results, with low incidences of bacterial and fungal growth. Only one Gram-positive bacillus was identified in the analyses of the preservative liquids of the fish group, with 1 CFU in treatment T3 after 180 days of conservation. None of the other groups, including controls and treatments, evidenced bacterial growth in samples analyzed at 90 days, 180 days, or 5 years.

As for fungi, yeast growth was observed (>500 CFU in treatment T3, and 2 CFU in T4) in the 180-day analysis; none of the other groups, controls, or treatments evidenced fungal growth at 90 or 180 days, or after five years (Table 4).

No bacterial growth was recorded in tadpole preservative solutions after 90 days of conservation. Fungi, however, were identified in treatments T3 and T4, with 6 CFU of yeasts in T3 and 1 CFU of filamentous fungi (*Aspergillus*) in T4 (Table 4).

Table 4: Microbiological identification and quantification of the control and treatment groups of the fish Sphoeroides testudineus *and* Chloroscombrus chrysurus *preserved in 26% or 30% hypersaturated saline solutions for 90 and 180 days, and 5 years, as well as the tadpoles* Boana semilineata *preserved in 26% or 30% hypersaturated saline solutions for 90 days. C1 (fixed with 10% formalin and subsequently preserved in 10% formalin); C2 (fixed with 10% formalin and subsequently preserved in 70% ethyl alcohol); CT (fixed with 10% formalin and subsequently preserved in Transeau solution); T1 (fixed with 10% formalin and subsequently preserved in a 26% hypersaturated saline solution); T2 (fixed with 10% formalin and subsequently preserved in a 30% hypersaturated saline solution); T3 (fixed with 20% curing salt and subsequently preserved in a 26% hypersaturated saline solution); T4 (fixed with 20% curing salt and subsequently preserved in a 30% hypersaturated saline solution); T5 (C. chrysurus fixed with 10% formalin and subsequently preserved in a 26% hypersaturated saline solution for 5 years); T6 (C. chrysurus fixed with 10% formalin and subsequently preserved in a 30% hypersaturated saline solution for 5 years). Negative = no microbiological growth, "-" = no analysis was performed during this period, CFU = Colony Forming Units.*

Fish						
Groups	**Aerobic bacteria (CFU ml-¹)**			**Fungi (CFU ml-¹)**		
	90 days	180 days	5 years	90 days	180 days	5 years
C1	Negative	Negative	-	Negative	Negative	-
C2	Negative	Negative	-	Negative	Negative	-
T1	Negative	Negative	-	Negative	Negative	-
T2	Negative	Negative	-	Negative	-	-
T3	Negative	Gram Positive Bacillus 1CFU	-	Negative	Leveduriforme >500 CFU	-
T4	Negative	Negative	-	Negative	Leveduriforme 2 CFU	-
T5	-	-	Negative	-	-	Negative
T6	-	-	Negative	-	-	Negative
Tadpoles						
Grupos	**Aerobic bacteria (CFU ml-¹)**			**Fungi (CFU ml-¹)**		
	90 days	180 days	5 years	90 days	180 days	5 years
CT	Negative	-	-	Negative	-	-
T1	Negative	-	-	Negative	-	-
T2	Negative	-	-	Negative	-	-
T3	Negative	-	-	Leveduriforme 6 CFU	-	-
T4	Negative	-	-	Filamentous (*Aspergillus*) 1 CFU	-	-

4. DISCUSSION

The minimal variations observed in terms of both the salinity and pH of the preservation fluids can be attributed to alterations of their osmotic pressures. According to Cath et al. (2006) [18], osmotic pressure leads to the movement of water through a semipermeable membrane from an area of lower solute concentration to an area of higher concentration. This phenomenon is essential in both biological and chemical contexts, and affects cellular dynamics, the balance of body fluids, as well as the stability of preservation system solutions. Monthly analyses are therefore essential for monitoring and adjusting the salinity and pH levels of specimen preservation fluids to ensure their stability and prevent the deterioration of preserved material.

The analyses of the morphologies of specimens preserved in hypersaturated saline solutions indicated that this technique resulted in superior preservation quality as compared to traditional preservative fluids, such as formalin and ethyl alcohol. Preservation in 10% formalin increases muscle tissue rigidity, making them 4.4 to 5 times more rigid than observed in live animals. Additionally, formalin contains toxic compounds such as methanol and heavy metals.

Nunes et al. (2011) [19] presented data showing that the use of 70% alcohol as a preservative fluid can result in aesthetic changes in preserved specimens and reduce their softness, leaving them approximately five times more rigid than live animals.

Guaraná et al. (2021) [12] morphologically evaluated swine viscera preserved in 30% hypersaturated saline solution, and analyzed criteria such as color, appearance, texture, flexibility, and odor, and reported results superior to those of the control groups. Similarly, Friker et al. (2007) [20] compared the efficacy of a 30% hypersaturated saline solution to that of formaldehyde for the fixation and preservation of goat viscera, and concluded that the saline solution gave better results in terms of all of the criteria evaluated and therefore represented a promising alternative to traditional methods.

In the morphological analyses performed here, the specimens fixed with 20% curing salts showed good results, maintaining specimen malleable with textural characteristics close to those of living animals. Microbiological analyses of those specimens, however, revealed the presence of bacteria and fungi, suggesting that that treatment alone may not have sufficient antimicrobial properties to maintain specimen integrity over time. Additionally, 8 months after the beginning of the experiment, the specimens of these treatments (T3 and T4) showed signs of decomposition and bacterial and fungal growth. Similarly, Domański et al. (2023) [15] identified the presence of Gram-positive bacteria and Leveduriforme fungi in museum specimens treated similarly. Friker et al. (2007) [20], however, were successful in using curing salts as a fixative when supplemented with 14.5 mg of hexacyanoferrate per kilogram of salt and an antioxidant. The addition of antioxidants may therefore represent a promising strategy for avoiding microbial contamination.

The results of the present study corroborate the experiments of Oliveira (2014) [10] and Brun et al. (2002) [21], with 26% and 30% hypersaturated saline solutions demonstrating significant efficacy in preserving fish and amphibian specimens previously fixed in 10% formaldehyde. These conservation techniques were effective in maintaining the morphological integrity of the specimens and preserving their texture, color, and flexibility close to that of living animals, even after prolonged periods of exposure. The specimens also evidenced good resistance to microbiological contamination, indicating the use of hypersaturated saline solutions as a viable alternative to traditional preservation methods (such as the use of formalin and alcohol) – demonstrating the potential of this technique to meet educational needs while offering safer and less toxic learning environments.

5. CONCLUSION

The results obtained here indicate that fish and amphibian specimens previously fixed with 10% formalin and preserved in 26% or 30% hypersaturated saline solutions are satisfactorily preserved and morphologically surpassed the quality of specimens in control groups. Although slight alterations of some morphological characteristics were observed, the combination of formalin as a fixative and the use of a 26% or 30% hypersaturated saline solution as a preservative gave viable results, surpassing traditional approaches in terms of all of the parameters evaluated. The 30% hypersaturated saline solution can therefore be considered the most effective method for fish and amphibian conservation for educational purposes.

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