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Evaluation of an alternative technique for preserving crustaceans in dry conditions with joint mobility: a proposal for didactic purposes

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ABSTRACT. Although crustaceans are traditionally preserved in liquids (formaldehyde and/or ethyl alcohol), those substances tend to alter their morphological aspects. Glycerin, used in human anatomy, is considered a good substitute for formaldehyde, as it preserves animals in states similar to *in vivo* conditions. There are no records in the literature, however, concerning the use of glycerin for conserving invertebrates. The objective of this work was to elaborate and evaluate alternative techniques for conserving the crustacean *Ucides cordatus* (Linnaeus, 1763). Six fixatives (1, 3, 4 and 5% formaldehyde, 70% alcohol, and dietrich solution) and two controls (positive and negative) were tested, as well as the effects of freezing before fixation on the integrity of *U. cordatus* specimens. Our results were evaluated with respect to nine variables. The treatments that demonstrated the best aesthetic results were 4% formaldehyde and 70% ethyl alcohol. The freezing of the animals resulted in brittle organs in all treatments tested. The technique discussed here is extremely promising for the conservation of animals for educational purposes, as it produces preserved specimens that are aesthetically similar to their *in vivo* conditions.

Keywords: preservation; glycerin; *Ucides cordatus*; teaching.

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Introduction

The preservation of the anatomical organs of animals is considered essential to supply the continual demands of educational institutions and zoological collections, and several preparation techniques are commonly used (Zaher & Young, 2003; Silveira, Teixeira, & Oliveira, 2008).

The scientific literature currently indicates the use of fixative substances such as formaldehyde at concentrations of from 10 to 20% (Oliveira, Mindêllo, Martins, & Filho, 2013; Soares, Carreiro, Leitão, & Abrantes, 2017) together with 70% ethyl alcohol (Papavero, 1994; Karam, Cury, Ambrósio, & Mançanares, 2016), although those substances can cause losses of color and joint mobility in specimens, as well as present high risks to the health of those who handle them (Viegas et al., 2010).

The continuous replacement of anatomical specimens used for teaching purposes is a problem faced by educational institutions, as those specimens have limited durability for studies in practical classes, in addition to being expensive and inconvenient to acquire.

Glycerin can be a good substitute for formaldehyde for the preservation of anatomical specimens, both in terms of its atoxic nature and preservation of specimen morphology, flexibility, color, and tissue consistency – thus facilitating the identification of structures difficult to visualize/manipulate in practical studies of internal and external morphology (Silva, Dias, Tavares, Marques, & Furtado, 2008). Its use in human anatomy is widespread, although there are no records in the scientific literature of its use with invertebrates.

It should also be noted that glycerination can reduce the need for the constant sacrifice of animals, as the useful lives of the specimens are extended. Glycerination also allows the animals to be conserved in a dry state, reducing maintenance costs, and those specimens are odorless and atoxic, improving their safety for handling.

We therefore evaluated alternative techniques for preparing zoological material for educational purposes, through glycerination, using the crustacean *Ucides cordatus* (Linnaeus, 1763) to determine: (i) the best

fixative, (ii) the effects of freezing prior to fixation, and (iii) other steps necessary to obtain specimens that maintain the integrity of their morphological characteristics, including joint mobility, with few changes in specimen color.

Material and methods

Were acquired 32 specimens of *U. cordatus* from fishermen (ICMBio collection authorization 59283-1). The animals were euthanized with 7.5% magnesium chloride, following Saldanha (1972), and were cleaned using running water and neutral detergent to remove all sediments. A solution containing 50% of the formaldehyde, 70% ethyl alcohol, and dietrich fixative and 50% of pure bi-distilled glycerin was then applied to the specimens to prevent them from becoming rigid during fixation.

The experiments were divided into two groups of 16 individuals each: (i) animals subjected to previous freezing, and (ii) animals not subjected to freezing (to test its effects on animal integrity); the specimens were then maintained for seven days in a freezer (-4ºC) and subsequently thawed and tested.

Six tests with different fixatives were performed with each group: F1 (1% formaldehyde), F2 (3% formaldehyde), F3 (4% formaldehyde), F4 (5% formaldehyde), A70 (70% ethyl alcohol), and D (dietrich), plus 2 controls (positive - PC, and negative - NC), all with two specimens (a male and a female). The animals used in the experiment varied little in size (Table 1) to avoid size influences on morphology and color evaluations.

Table 1. Measurements of the specimens of *Ucides cordatus* (Crustacea: Decapoda) used in the glycerination technique. CW = carapace width; CL = carapace length; NC: Negative Control (post-mortem specimens without fixation); PC: Positive Control (Fixed in 10% formaldehyde and preserved in 70% alcohol), and the other treatments: F1: 1% formaldehyde; F2: 3% formaldehyde; F3: 4% formaldehyde; F4: 5% formaldehyde; A70: 70% alcohol; and D: dietrich. Values in millimeters (mm).

Groups	Male		Female	
	CW	CL	CW	CL
PC	62.3	39.4	66.1	51.2
NC	66.6	47.3	67.9	52.1
F1	64.3	45.6	65.8	49.8
F2	73.1	48.7	69.7	50.6
F3	69.8	45.5	68.5	52.4
F4	68.3	45.3	68.1	50.3
A70	66.2	45.0	68.7	48.5
D	63.3	41.5	62.2	46.3
Mean	66.7	44.8	67.1	50.2
Standard deviation	2.7	2.2	1.8	1.5

The formaldehyde used in the treatments was buffered with sodium tetraborate to avoid any effects of fixative acidity on the Calcium Carbonate ($CaCO_3$) composing the crab carapace (Martin, 2016).

The positive control (PC) consisted of the traditional method of preparing zoological material recommended by the scientific literature (Wetzer, 2015; Martin, 2016) that is, the specimens were fixed in 10% formaldehyde prepared on the same day as they were euthanized and subsequently preserved in 70% ethyl alcohol until the day of analysis. The negative control (NC) consisted of the newly euthanized animals without any exposure to fixatives.

The organisms were held in containers with lids and were completely immersed in the fixing solution for 7 days.

After the fixation period, the animals were washed in running water to remove excess fixative, and subsequently drained of excess water. When the specimens were partially dry (approximately 2 hours), they were immersed in an impregnation solution of pure (98%) bidistilled glycerin (with 1 g of camphor diluted in 5 ml of absolute ethyl alcohol per liter of glycerin, to avoid fungal proliferation).

After an impregnation period of 60 days, the specimens were removed from the glycerin, drained, and then held in a climatized environment (20ºC) with an air dehumidifier for 30 days. After the drying period, the organisms were evaluated to determine their quality as compared to an *in vivo* model.

The quality evaluations of the specimens for didactic purposes was adapted from the fish quality index (Amaral & Freitas, 2013).

Eight parameters were used to evaluate the specimens: appearance, cephalothorax consistency, level of discoloration, texture, malleability of the appendages, odor, color of the brachiae and abdominal cavity, and dryness by time of exposure (simulating the average time in which the specimens would be used in a laboratory class).

The specimens were evaluated separately by five teachers with links to zoology studies who analyzed and verified the internal and external morphologies of the animals as compared to the negative control. The specimens received numerical grades ranging from 1 to 4 for each parameter, with the specimens having appearances that were closest to their natural state (*in vivo* - NC) receiving the highest grades (Table 2).

The analysis of the data was performed through mode calculations, which evaluate the assigned values of the data set and identifies those that obtained the most consistently high evaluations, using GraphPad Prism version 5 software. The value of the final result (FR) was calculated as the sum of the scores attributed to all items.

The glycerinated animals were subsequently deposited in the Didactic Collection of the Zoology Laboratory of the Federal University of Bahia - CAT/IMS in containers with lids, containing hygienic mats moistened with an antiseptic solution (1 liter of 70% alcohol, 2 ml of 2% iodine, and 10 g of camphor diluted in 10 ml of absolute alcohol) to maintain the asepsis of the materials. Envelopes containing silica gel and camphor were also placed in the containers to prevent fungal growth.

Maintenance every 6 months is suggested to ensure the maximum conservation of the specimens, involving simply wiping with a cloth moistened with the aforementioned antiseptic solution, drying at room temperature, and repackaging with new hygienic mats and envelopes containing silica gel and camphor.

Table 2. Parameters used to assess the quality of crustaceans prepared by the glycerination technique. Adapted from the Amaral & Freitas (2013) fish quality index.

Variables	Assessment	Punctuation
Appearance	Very bright	4
	Bright	3
	Slightly opaque	2
	Opaque	1
Consistency of the cephalothorax	Rigid shell	4
	Slightly fragile carapace	3
	Fragile carapace, showing irregularities	2
	Fragile carapace, breaking when pressing	1
Discoloration	Absent	3
	Detectable	2
	Excessive	1
Texture	Rigid	4
	Uneven surface	3
	Yielding to the touch	2
	Brittle	1
Appendages malleability	Very malleable	3
	Malleable	2
	Hard	1
Odor	Presence of odor characteristic of the species	2
	Total absence of odor characteristic of the species	1
Branchiae coloring	Branchiae with characteristic coloration of the species	2
	Branchiae with blackish and/or whitish coloring	1
Coloring of the abdominal cavity	Original coloring	2
	Discolored	1
Dryness after 2 hours of exposure at room temperature	No change	2
	With alteration and drying of the material	1

Results

The treatments with 4% formaldehyde and 70% alcohol gave satisfactory results considering all of the variables analyzed, receiving maximum values of external and internal quality (Table 3).

In terms of the appearance variable, the best results were obtained by the fixatives: 3, 4 and 5%, formaldehyde, 70% alcohol, and dietrich solution, those being very bright; followed by the most negative results: 10% formaldehyde and 70% ethyl alcohol (PC), with the specimens showing opacity (Figures 1 to 3).

The two variables of consistency of the cephalothorax and texture of the specimens showed good results for all the fixatives analyzed, proving that the glycerination process does not negatively affect the surfaces and stiffnesses of the specimens; that result did not apply to the positive control, however.

Table 3. Evaluation parameters and final result for specimens of *Ucides cordatus* (Crustacea: Decapoda) exposed to the glycerination technique. NC: Negative Control (post-mortem specimens without fixation), PC: Positive Control (Fixed in 10% formaldehyde and preserved in 70% alcohol), F1: 1% formaldehyde, F2: 3% formaldehyde, F3: 4% formaldehyde, F4: 5% formaldehyde, A70: 70% alcohol, and D: dietrich. FR: Final Result – the sum of all values of the evaluation criteria. The values used in the table correspond to the mode.

Variables	Controls		Treatments					
	NC	PC	F1	F3	F4	F5	A70	D
Appearance	4	1	2	4	4	4	4	4
Consistency of the cephalothorax	4	4	4	4	4	4	4	4
Discoloration	3	1	3	3	3	3	3	3
Texture	4	4	4	4	4	4	4	4
Appendages malleability	3	1	3	2	3	2	3	2
Odor	2	1	2	2	2	2	2	2
Branchiae coloring	2	1	2	2	2	1	2	2
Coloring of the abdominal cavity	2	1	2	2	2	2	2	2
Dryness after 2 hours of exposure at room temperature	2	1	2	2	2	2	2	2
FR	26	15	24	25	26	24	26	25

Figure 1. Specimens of *Ucides cordatus* exposed to four different treatments. 1) positive control; 2) negative control; 3) 70% alcohol; 4) Dietrich. Black bar: 5 cm scale.

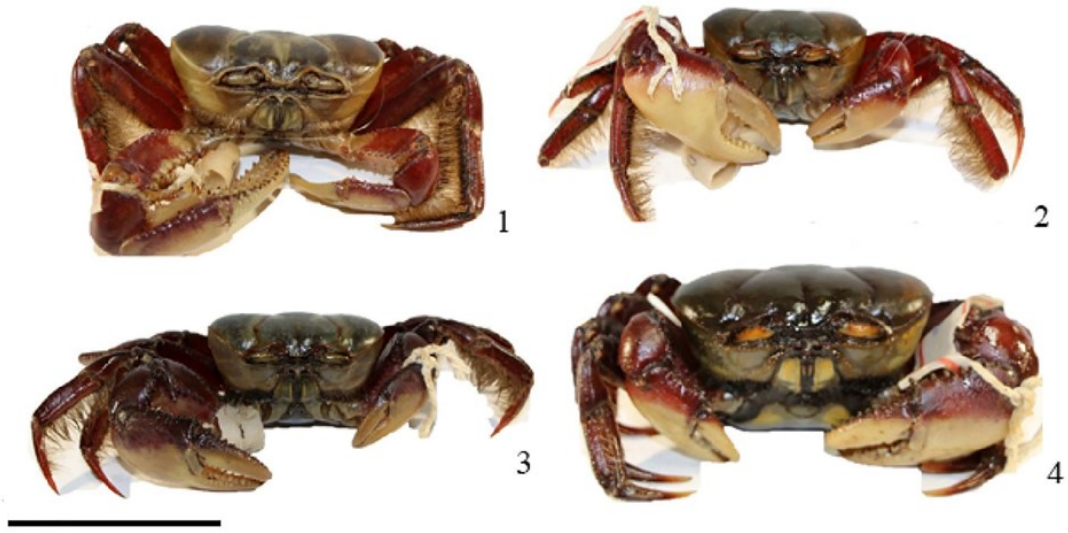

Figure 2. Specimens of *Ucides cordatus* exposed to four different treatments. 1) 1% formaldehyde (F1); 2) 3% formaldehyde (F2); 3) 4% formaldehyde (F3); 4) 5% formaldehyde (F4). Black bar: 5 cm scale.

Figure 3. Specimens of *Ucides cordatus* exposed to two different treatments after previous freezing. A) 70% alcohol (F1); B) dietrich (D). Black bar: 5 cm scale.

No discoloration of the specimens was observed in any of the treatments with fixatives or the negative control; there was excessive discoloration, however, with the positive control.

The malleability of the crabs showed positive results using 3 fixatives (1 and 4% formaldehyde, and 70% alcohol) with high malleability; the fixatives 3% formaldehyde, 5% formaldehyde, and Dietrich were judged to be only malleable. The 10% formaldehyde (PC) fixative was the only one that resulted in stiffness. The same pattern was repeated for the variables: odor, color of the abdominal cavity, color of the branchiae (Figures 4 and 5), and dryness after exposure, with the PC receiving a grade of 1, indicating the highest level of discharacterization of the specimen; the other treatments received satisfactory evaluations for the variables in question.

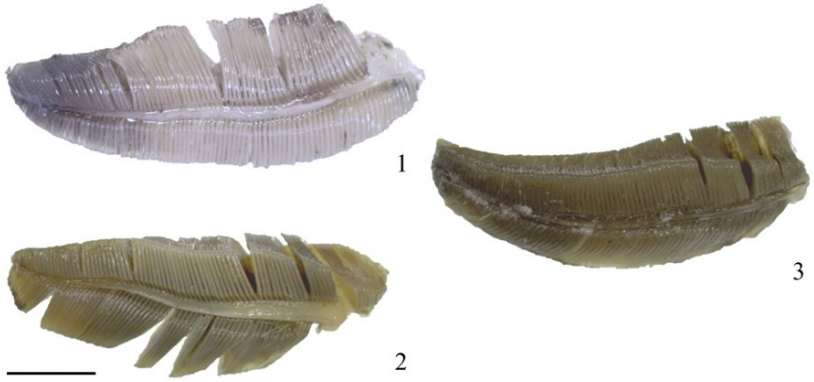

Figure 4. Branchiae of *Ucides cordatus* exposed to three different treatments. 1) positive control; 2) 70% alcohol; 3) Dietrich. Black bar: 5 mm scale.

Figure 5. Branchiae of *Ucides cordatus* exposed to four different treatments. 1) 1% formaldehyde (F1); 2) 3% formaldehyde (F2); 3) 4% formaldehyde (F3); 4) 5% formaldehyde (F4). Black bar: 5 mm scale.

In summary, the 4% formaldehyde and 70% ethyl alcohol fixatives presented the most positive results in relation to the variables analyzed (FR value of 26), followed by the fixatives dietrich and 3% formaldehyde (FR values of 25) as their malleability was affected. The 1% and 5% formaldehyde fixatives were scored as 24, the 1% formaldehyde treatment showed its general appearance affected; the 5% formaldehyde affected

malleability and branchiae color. The positive control received a much lower FR value (15), being considered inappropriate as a preservative protocol.

Discussion

Analyzing the benefits of the glycerination and formaldehyde techniques, it was possible to verify that the quality of the glycerinated specimens was superior to samples submitted to the traditional formaldehyde technique (positive control).

The traditional technique of using high concentrations of formaldehyde produces stiff and brittle specimens with different morphologies than *in vivo* animals – which results in didactic losses and requires constant animal collections (and sacrificing more of them) to replace specimens damaged during classroom handling.

The glycerination proposed here still requires fixation, however, and does not completely exclude the use of formaldehyde. Previous studies of the use of glycerin as a preservative of biological materials found it to be a promising reagent, with antiseptic action (Chirife, Scarmato, & Herszahe, 1982), with high resistance to fungal and bacterial attacks (Brun et al., 2004) and capable of preserving specimens for periods longer than 10 years (Brun et al., 2002).

The concentrations of formaldehyde tested in the present work (4 and 5%), when combined with glycerin, provided satisfactory results and were capable of preventing specimen deterioration and maintain them in less friable and easier to handle conditions.

An important factor in the crustacean evaluations was there malleability, because when fixed in 10% formaldehyde (PC) the specimen becomes more rigid, making it more difficult to understand the articulations of those animals, especially in academic environments. Fixatives such as dietrich and 3% formaldehyde resulted in the loss of malleability of *U. cordatus*, as those fixatives act on muscle fibers, causing them to become stiff. Guastalli et al. (2012) evaluated the effects of formaldehyde (3.8%) on the shear of chicken muscle fibers, and found that sheer value to be seven times greater as compared to *in vivo* muscles. Thus, small amounts of softening agents such glycerol can maintain the flexibility of appendages and help retard their desiccation (Martin, 2016).

After only 7 days of exposure to 10% formaldehyde, the treated specimens showed more degraded internal and external morphologies than the other treatments, as formaldehyde contains considerable percentages of methanol, phenolic and urea resins, among other compounds (including heavy metals such as lead and cadmium) that can cause alterations in the tissues being fixed (in addition to being toxic), as noted by Soares et al. (2017).

Glycerin is free of toxic compounds, and does not present risks to the preparer or others who will have contact with the material. Additionally, the specimens are odorless, unlike those treated with formaldehyde, which causes irritation to the mucous membranes (Karam, 2016).

It was possible to observe that the loss of malleability among positive control specimens of *U. cordatus* was due to muscle firmness changes promoted by 10% formaldehyde, becoming 4.4 to 5.0 times more rigid when compared to the *in vivo* state (Guastalli et al. 2012). 10% formaldehyde is still widely used, however, due to its low cost and high tissue penetration (Oliveira et al., 2013).

The use of dietrich is recommended for the fixation of arthropods, as it ensures the maintenance of specimen color (Alejandro & Scaglia, 2018). Its use with decapods, however, was not aesthetically positive, as the specimens showed discoloration and losses of brightness. It is pertinent to note that the dietrich solution is a reagent composed of several fixatives (absolute alcohol, formaldehyde, and acetic acid) at high concentrations, which can result in excessive discoloration (Alejandro & Scaglia, 2018).

The use of 70% alcohol demonstrated promising results, receiving maximum quality evaluations with *U. cordatus* specimens, although it can promote aesthetic differentiations in anatomical pieces after an exposure period of 180 days (Nunes et al. 2011). Therefore, the positive evaluation in the present study may reflect the shorter time of exposure (only seven days) (Black & Dodson, 2003; Martin, 2016). In addition to aesthetic considerations, Nunes et al. (2011) analyzed the textures of muscle tissue subjected to fixation and preservation in alcohol, and found that it promoted reductions of softness, making the specimens approximately five times more rigid than *in vivo* samples. Black & Dodson (2003) pointed out that the best method for preserving cladocerans is to fix them in 95% ethyl alcohol, followed by storage in 70% ethyl alcohol.

Black & Dodson (2003) analyzed the use of ethanol for the preservation of zooplanktonic crustaceans (*Daphnia*), and recommended fixing them in 95% ethanol and storing them in 70% ethanol. Martin (2016) recommended the use of 70% ethanol to preserve crustaceans, especially decapods, citing anecdotal reports that high concentrations of ethanol can make specimens brittle and less useful for didactic manipulation.

It is worth mentioning that the use of alcohol as a fixative allows posterior genetic studies, which are not possible with formalin-treated materials (Sambrook, Fritsch, & Maniatis, 1989). Formalin denatures DNA, making it difficult (often impossible) to recover DNA for molecular studies (Martin, 2016). That was not a concern in the present study, however, as the specimens considered here were for didactic purposes.

Combinations of other substances, such as formalin, ethanol, and glutaraldehyde have been used in the fixation of crustaceans (Felgenhauer, 1987).

Martin (2016) recommended freezing crustaceans before the fixation process, which can avoid autotomy (crustaceans often autotomize their appendages when alarmed). Crustaceans that were frozen in the present study demonstrated negative results in the malleability assessments, as their appendages were easily lost (detected in all treatments and the positive control), making additional analyses unfeasible. Colla and Prentice-Hernández (2003) reported that large ice crystals (and in smaller quantities) are formed under conditions of slow freezing, which causes cell rupturing and injury through osmotic pressure. Therefore, we do not recommend using frozen specimens with this technique.

Another point that merits attention is the influence of ontogenetic variations in animal color. To avoid that factor in the experiments, we chose to use specimens with little size variation. We did not observe differences in the color of the samples of the tested crabs regarding their sex.

Conclusion

We recommend the glycerination protocol after fixation in 4% formaldehyde or 70% ethyl alcohol for preserving *U. cordatus* specimens for didactic purposes. Employing our results, institutions that would otherwise need to frequently replace teaching materials will be able to prepare specimens and use them sequentially, fulfilling teaching needs with specimens of excellent quality that will allow positive educational experiences through direct contact with specimens closer to their natural *in vivo* states of color and malleability, and under dry conditions without toxic residues. Thus, it will be possible to substitute formaldehyde-treated specimens (which present health risks) and reduce the expenses of periodic replacement of reagents such as 70% alcohol.

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