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Research Article

Effects of Storage Time and Temperature on Subsequent Wet Weight and Percent Dry Weight of *Fundulus heteroclitus*

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Fisheries monitoring and research often requires preservation of fish samples for later analysis in the laboratory. Freezing is a common method, but it can affect subsequent fish size and composition, and the interpretation of metrics based on them. We evaluated the effects of freezing on fish wet weight, dry weight, and the percentage dry weight (a measure often used to estimate energy density). Specifically, we quantified changes in the weight of individual mummichogs (*Fundulus heteroclitus*) frozen at different temperatures (−20°C and −80°C) for 1–3 months. In addition, a temperature change treatment where fish were initially frozen at −80°C for one month before transfer to −20°C for an additional 1–3 months was included to simulate protocols typically used at sea. Individual wet weights were measured before freezing, and after freezing, the wet weights, dry weights, and percentage dry weights were determined. All freezing treatments resulted in minor but significant ($p < 0.05$) water loss. While the change was measurable and directional, it did not have a significant effect on the percentage dry weight except for samples held at −20°C for 3 months. A similar increase in percent dry weight was not evident in the temperature change group that was also stored at −20°C for 3 months. The least amount of weight loss, and therefore bias, was in the −80°C group, followed by the temperature change group, with the −20°C treatments losing the most water. The results indicate that if specimens for percent dry weight estimation have to be frozen, −20°C is okay for a short period of time (1–2 months), but when longer storage times are needed, −80°C is the best, even if only for the initial freezing period.

1. Introduction

The energy content of fish is variable throughout their life, influenced by many factors including migration [1], season [2, 3], and reproductive state [4]. Therefore, monitoring energy content can provide useful insights into these aspects of a species' life history, their role within the trophic system, and energy allocation strategies related to growth and reproduction. The amount of energy (in joules [J]) contained in an organism—the sum of energy from macronutrients (lipids, proteins, and carbohydrates)—is often expressed per body weight to calculate energy density (expressed as $kJ\,g^{-1}$ wet weight). Energy density is often estimated by the percent dry weight of a sample [5], which requires accurate wet and dry weights. The use of energy density values estimated from percent dry weights, which may be biased due to the changes in water weight during freezing and storage, can potentially introduce inaccuracies in energy density estimates and subsequent calculations in bioenergetics models [6, 7].

Freezing has been shown to affect fish morphometrics, with shrinkage in length and loss of weight common [8–11]. The amount of weight loss varies by freezing method and fish size and can be significant depending on the type of analyses performed on thawed specimens. Long-term storage of samples at very low temperatures can also affect fatty acid composition and lipid content [12–14]. Therefore, studies utilizing frozen fish samples should evaluate if freezing protocols, both in terms of temperature and length of

storage, have an effect on the morphometrics and chemical composition determined after freezing.

Sampling programs to assess the energy dynamics of marine ecosystems are necessarily conducted at sea, which presents challenges with sample collection and accuracy of wet weights due to problematic (unstable) weighing environments, lack of precision equipment, and limited space to save and store samples for later analysis. Because of the time (e.g., > 5 days) and precision (e.g., 0.001 g) needed to obtain accurate dry weights, it is impractical to do so at sea and requires land-based laboratory analysis of frozen specimens. However, processing frozen samples in the laboratory on land can cause additional issues, as there is no way to accurately account for any potential water loss that may have occurred during transport and storage. In addition, different freezing methods (in both temperature and duration) may introduce biases in the amount of water lost.

To address potential biases introduced by how samples are frozen and stored, efforts have been made to evaluate different methods with the goal to minimize and standardize any effects due to freezing. Approaches include immersion in liquid nitrogen before storage, glazing with deionized water or a saline solution before storing in bags and frozen [7], glazing with or frozen in water, vacuum sealing then freezing [6], or freezing initially at −80°C until they are later transferred to −20°C freezers [3]. In some cases, the effects of these different freezing methods were evaluated and showed that the initial (prefreezing) wet weight of specimens differed significantly from the (post-thaw) wet weights after freezing [6, 7]. Crane et al. [6] found that the storage method (glazed with or frozen in water and vacuum sealed) affected the post-thaw wet weights in Emerald Shiner (*Notropis atherinoides*) and Rudd (*Scardinius erythrophthalmus*) and that the impact varied with fish size. Baltasar et al. [7] found that wet weights of thawed mummichogs (*Fundulus heteroclitus*) from three different freezing methods (flash freezing with liquid nitrogen, coating the fish in saline solutions, or coating the fish in water) also differed from the original wet weights. These results indicate the potential for bias in wet weights obtained from frozen samples, and therefore the resulting percentage dry weights, due to the loss of water during the freezing process. Some of the freezing methods mentioned (e.g., using liquid nitrogen, glazing with various solutions) may be feasible for limited studies in close proximity to laboratory facilities, but are not easily scaled to large-scale surveys operating at sea over large areas for many weeks. To explore this further, we designed an experiment to simulate freezing methods available on many research platforms and to determine potential water loss under different protocols and periods of time. Research vessels are often equipped with −20°C freezers and may also have limited volume ultra-low (−80°C) freezer capabilities. We were specifically interested in determining if initial freezing in −80°C provides measurable benefits in terms of accuracy and precision of resulting percent dry weight estimates.

2. Methods

For our experiment, we collected samples of *Fundulus heteroclitus* (total length [TL] = 5.36 cm ± 1.52 [SD], whole weight = 2.985 g ± 2.403 [SD]) and used a range of sizes across 10 different freezing treatments. We selected *F. heteroclitus* as a study species due to the availability near land-based laboratory for immediate processing and their small size, which made it possible to store a large sample size for many months in the limited capacity −80°C freezer. Given the increased surface area to volume of smaller fish compared to larger fish, this also provided a more sensitive test of water loss that should be applicable to other fish species. We chose freezing durations of 1–4 months to mimic common storage times for the treatments that we evaluated [3]. We compared the individual wet weights pre- and posttreatment to determine whether there was significant water loss associated with different freezing treatments described below.

Fundulus heteroclitus samples were collected over a 7-day period in August 2020, from the east branch of the Westport River in Massachusetts. *F. heteroclitus* were selected for this experiment due to local availability and their relatively small size for storage purposes. In total, 300 individuals were collected using minnow traps and brought back alive to the University of Massachusetts Dartmouth. All sampled fish were then pooled and randomly divided into one of 10 freezing treatment groups (Table 1). After the fish were sorted, they were euthanized using 9% solution of Eugenol, blotted dry, and their initial TL (±0.5 cm) and whole weight (±0.001 g) were recorded prior to freezing.

Individuals from the control group were separated out immediately, transferred to preweighed aluminum trays/pans, and placed in a 60°C drying oven until reaching a stable dry weight [15]. Dry weights were considered final when only minimal changes (< 0.01 g) in weight occurred on consecutive weighing dates. The mean difference between the last two dry weights was 0.0060 g, ±0.00750 SD. For all other groups, individuals were double bagged in resealable polyethylene bags and placed in either a −80°C or −20°C freezer. To simulate commonly used sampling procedures on land and on research vessels [3], three different general freezer approaches were employed, and within each approach, three different storage times were evaluated independently. Three groups were stored at −80°C initially for one month, and then, all groups were transported to a −20°C freezer and one group was removed each month for three months (temperature change treatment, two to four total months frozen). Three different groups were stored in a −80°C freezer (−80°C treatment) with one group removed at one, two, and three months to assess the effects of storage time at −80°C freezing. Lastly, three groups were stored in a −20°C freezer (−20°C treatment) and one group was removed at one, two, and three months to assess the storage time freezing water loss.

After each treatment and storage time, the samples were thawed, re-weighed for wet weight, transferred to preweighed aluminum trays/pans, and dried in a 60°C drying oven until a stable dry weight, as described above, was obtained. After all dry weights were obtained, the percent dry weight was calculated as 100 ∗ (dry weight)/(wet weight) for each sample. Since both the pre- and postfreezing treatment wet weights were recorded, we were able to calculate the %DW using each (i.e., a prefreezing %DW and postfreezing %DW).

Table 1: Sample groups and length of time stored in −80 °C and −20 °C freezers for freezing method experiment.

Treatment	Time in −80°C freezer	Time in −20°C freezer	Total time in freezer	Total length (cm) ± S.D.
Control (0 months)	—	—	—	5.48 ± 1.53
Temperature change 2 months	1 month	1 month	2 months	5.30 ± 1.29
Temperature change 3 months	1 month	2 months	3 months	5.30 ± 1.49
Temperature change 4 months	1 month	3 months	4 months	5.10 ± 1.55
−80°C 1 month	1 month	—	1 month	5.66 ± 1.72
−80°C 2 months	2 months	—	2 months	5.43 ± 1.62
−80°C 3 months	3 months	—	3 months	5.56 ± 1.59
−20°C 1 month	—	1 month	1 month	5.43 ± 1.42
−20°C 2 months	—	2 months	2 months	5.18 ± 1.69
−20°C 3 months	—	3 months	3 months	5.17 ± 1.43

Note: After each freezer treatment, the final dry weight was obtained for all samples.

The size distribution (initial wet weights and final dry weights) of fish in each treatment was compared using ANOVA. Because the levels for total time in freezer were not equivalent across freezer temperatures and we were interested in evaluating thespecific protocols independently, we collapsed the freezer temperature and duration levels into a single treatment factor with 10 levels including the control. Freezing treatment effects were evaluated in several ways to evaluate differences between freezing protocols on water loss and determine whether freezing treatment affected our estimates of the %DW. First, to test whether significant water loss occurred during freezing, we evaluated whether significant water change occurred using an ANOVA across all treatments with pre- and posttreatment weights (i.e., not including the control). The difference between the initial (pretreatment) wet weight and after freezing (post-thaw) wet weight (i.e., change in water) of *F. heteroclitus* was used as the response variable and freezing treatment, and TL as the independent variables. Follow-up tests (Tukey's HSD) were used to determine which treatments were different. Similarly, to determine how changes in wet weights during freezing might affect the calculated %DW, we evaluated the difference in %DW of individuals using wet weights before and after freezing. In addition, to provide context on how changes in %DW of individuals due to freezing might affect estimates of the mean %DW for a sample of fish, we compared the mean %DW across all treatments. An ANOVA was also conducted to compare percent dry weight across all treatments and the control as a function of freezer treatment. Significance was determined at an alpha level of 0.05 for all analyses, and follow-up tests (Tukey's HSD) were used to determine which treatments were different from the control and other treatments. All tests were performed in r [16]. Effect sizes (ESs) were evaluated using Omega^2, calculated with the r package "effectsize" [17], and interpreted as very small, $ES < 0.02$; small, $0.02 \leq ES < 0.13$; medium, $0.13 \leq ES < 0.26$; large, $ES \geq 0.26$ [18].

3. Results

Out of the initial 300 individuals across the 10 freezing treatments, four individuals were removed from further analysis due to transcription errors, resulting in a final sample size of 296. ANOVA revealed that there was not a statistically significant difference between the control or any freezer treatments in mean initial wet weights ($F_{(9,286)} = 0.4853$, $p = 0.8841$) or final dry weights ($F_{(9,286)} = 0.338$, $p = 0.9617$).

There was a range in %DW across all freezer treatments, and individuals in all treatments lost some amount of water during freezing and storage as illustrated by the comparison of posttreatment to pretreatment (true) %DW (Figure 1) and the measured loss of weight in grams postfreezing within each treatment (Figure 2). ANOVA indicated that there were significant differences among the nine (noncontrol) freezing treatments on water loss (Table 2). All nine treatment groups resulted in a significant loss of water, and freezing treatment had a significant and large effect on water loss, but length did not. Post hoc tests on a reduced model that did not include length were used to determine which treatments were different from each other (Figure 1). Samples stored for 3 months at −20°C had significantly more water loss than all other groups. Water loss in the temperature change groups stored for three and four months was less than the −20°C 3-month treatment, but significantly higher than most of the remaining treatments.

The change in wet weights affected estimates of percent dry weight across the freezer treatments. ANOVA indicated that there were significant differences among the nine (noncontrol) freezing treatments on the difference in percent dry weights estimated before and after freezing, and the effect due to treatments was considered large based on ES (Table 3). The post hoc tests indicated the −20°C treatment at 3 months had significantly higher percent dry weight than all other treatments (Figure 3). The change in %DW was < 0.5 for all other treatments except the temperature change treatment at 4 months.

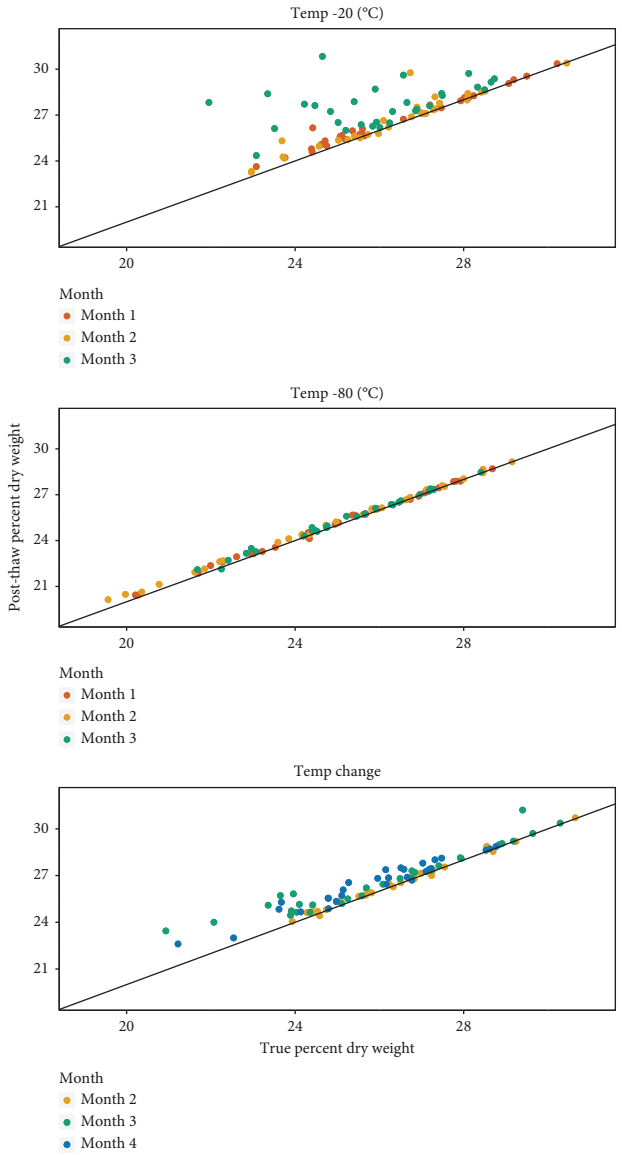

FIGURE 1: The relation between the true percent dry weight and the post-thaw percent dry weight for samples stored for 1–4 months. True percent dry weight is calculated with the wet weight obtained prior to freezing, and the post-thaw percent dry weight was calculated with the wet weight obtained after the freezing treatment. Points that fall above the 1:1 line indicate water loss and overestimated percent dry weights compared to the true percent dry weight, which is especially evident for the −20°C and temperature change groups.

Additionally, an ANOVA was conducted to determine whether the loss in water resulted in a significant change in the estimated percent dry weight for each treatment, including the control. This analysis indicated the significant treatment-wide estimates of %DW; however, the ES indicated the freezer treatments had a small effect on the mean %DW of each treatment (Table 4). Post hoc tests indicated the −20°C freezer treatments at 1 and 2 months were higher than all other treatments and the control. The range in %DW within each treatment (Figure 4) was relatively larger than the change in %DW of individual fish (Figure 3).

4. Discussion

The freezing treatments were designed to simulate commonly used methods for freezing and storing samples before processing for percent dry weight and energy density analysis. The freezing experiment had two parts: first to evaluate differences between freezing protocols on water loss and second to determine whether freezing treatment affected our estimates of the %DW. The first objective was evaluated using repeated measures of wet weight on the same individuals (initial and post-treatment). To evaluate the second objective, we compared % DW of a representative subsample (control) to the %DW of subsamples subjected to each treatment. The temperature change freezing treatment simulated a commonly used freezing protocol; samples are first frozen at sea in a −80°C freezer immediately after collection and later transported to a −20°C freezer for long-term storage until processing. All treatments showed significant water loss during freezing, but the percent dry weights were statistically the same as the control for all but the 1- and 2-month treatments at −20°C. The slightly higher %DW for the 1- and 2-month treatments at −20°C was likely because these treatments had a smaller range in %DW and lacked individual fish with low true %DW (e.g., < 23; Figure 1). Although there were no significant differences in initial wet or dry weights of fish across treatments, it appears there was a difference in the mean %DW of fish assigned to each treatment. We interpret the difference in these two treatments from the control to be due to the lack of low % DW individuals within those treatments and not due to the freezing treatment given the small ES. This indicates that while some water loss occurs under the tested freezing treatments, the change in individual percent dry weights was small compared to the variation across individuals within a treatment.

The magnitude of weight change (water loss) was very low (0.008% of the total wet weight), and this bias was directional and detectable, but the effects on %DW estimates were minimal and only statistically significant for the −20°C 3-month treatment. In nearly all treatments, the magnitude of change in %DW was < 0.5%, which represents a small fraction (5%; 0.5/10) of the range in %DW observed (20%–30%). A bias of 0.5% DW equates to a 1.9%–2.9% change in estimated ED, depending on species-specific models used [3]. Ideally, fresh wet weights should be determined accurately soon after sampling and before storage as recommended by Crane et al. [6] and Baltasar et al. [7], but this may be impractical when sampling large numbers of fish. Initial freezing and storage at −80°C resulted in the least change in wet weight, even after 3 months, but it may be impractical to store large volumes of samples at this ultra-low temperature. Initial freezing and storage at −20°C resulted in acceptable levels of water loss up the third month in storage, when weight changes were significant and would bias percent dry weight measures. This water loss after 3 months at −20°C was significantly greater than in the temperature change treatment at 4 months, demonstrating the benefits

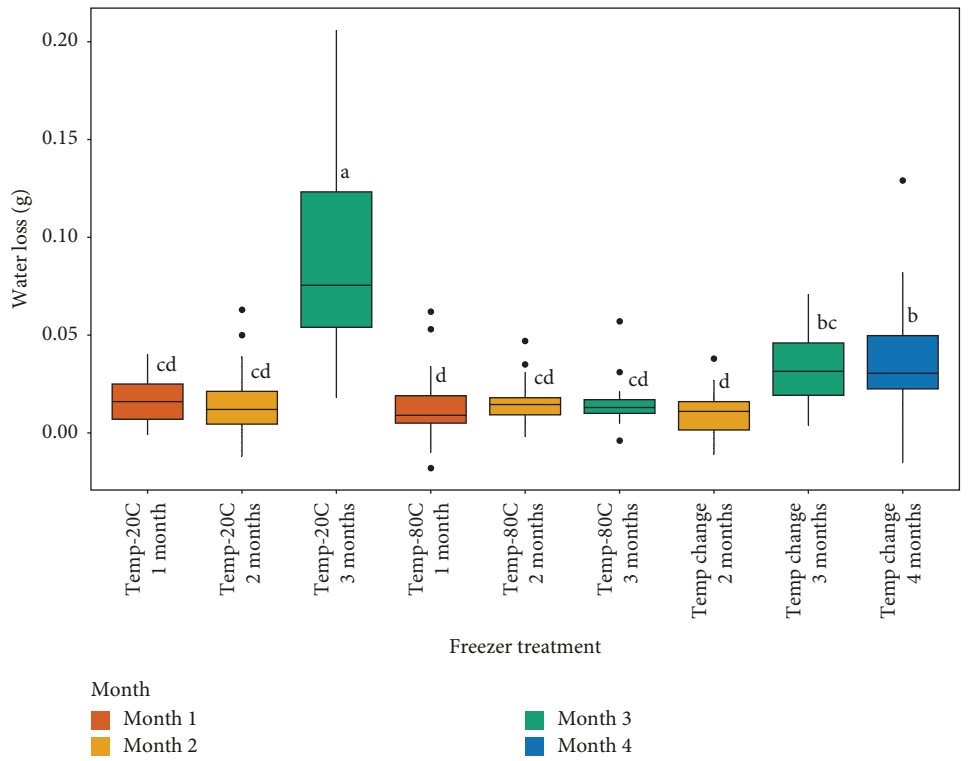

FIGURE 2: Mean water loss of *F. heteroclitus* compared to freezer type and freezing time by month. Treatments with the same letters are not significantly different from each other (Tukey's HSD, $p < 0.05$).

TABLE 2: Summary of the ANOVA (Type II) for the effects of freezing treatment and initial size (length) on water loss (the comparison of differences in pre- and post-thaw wet weights).

	Df	Sum Sq	Mean Sq	*F* value	Pr(> *F*)	Omega^2 (95% C.I.)	Interpretation
Freezer treatment	8	0.16080	0.020100	35.209	< 0.001	0.51 (0.43, 1.00)	Large
Length	1	0.00029	0.000290	0.507	0.477	0.00 (0.00, 1.00)	Very small
Residuals	256	0.14614	0.000571				

Note: Effect size was estimated using Omega^2 and interpreted as described in the methods.

of initial freezing at −80°C (Figure 3). Initial freezing at ultra-low temperatures (−80°C) produces small ice crystals which can reduce cell and tissue leakage when subsequently thawed [7, 19]. The larger ice crystals that form when freezing takes longer likely penetrate beyond the outer surface layers, making it more permeable. This is crucial, as the loss of water and alteration of the wet weight during the thawing process are the main issues when it comes to getting a precise wet weight from previously frozen samples. Samples with intact outer layers can be drained or blotted dry without drawing liquids from below the surface, but damage to the outer layer will increase the release of liquid from the sample. Therefore, the temperature change treatment represents a practical approach to prevent significant decreases in wet weight over several months of storage for large volumes of samples.

As in other studies, we show that freezing and extended storage can affect subsequent wet weight, but in our case, this did not affect the %DW estimate for a sample (compared to a control of similar-sized fish collected at the same time). Baltasar et al. [7] also found that the wet weights of *F. heteroclitus* changed after freezing, and the percent dry weights calculated for individuals from pre- and post-freezing also differed and affected energy density calculation, but they only evaluated samples stored for a period of 6 months in the freezer. The present study evaluated the effect of time in storage, but over a shorter period. We demonstrated a significant change in water loss for fish stored at −20°C after three months, with reduced losses for samples initially frozen at −80°C but frozen for a total of four months. Together, these results highlight the importance of freezing methods and protocols in studies of fish percent dry weight and energy density and underscore the need to evaluate and understand potential biases. The results presented here are based on small-sized fish (< 10 cm TL) compared to the samples routinely processed at the Northeast Fisheries Science Center (8–35 cm [3]). Given

Table 3: Summary of the ANOVA for the effects of freezing treatment on the difference in percent dry weight.

	Df	Sum Sq	Mean Sq	*F* value	Pr(> *F*)	Omega^2 (95% C.I.)	Interpretation
Freezer treatment	8	61.47	7.683	16.48	< 0.001	0.32 (0.23, 1.00)	Large
Residuals	257	119.81	0.466				

Note: Effect size was estimated using Omega^2 and interpreted as described in the methods.

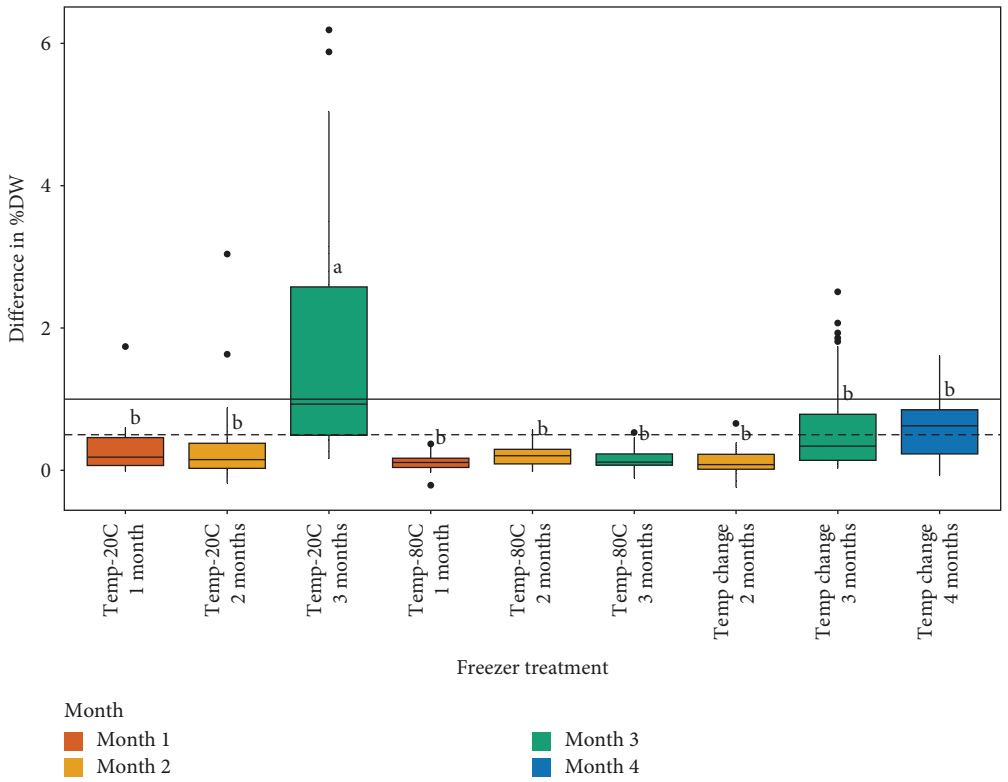

Figure 3: Mean difference in percent dry weight (%DW) of *F. heteroclitus* across the nine freezer treatments. Treatments with the same letters are not significantly different from each other (Tukey's HSD, $p < 0.05$). Horizontal lines are shown at 0.5 (dashed) and 1 (solid) for reference. All groups exhibited a significant amount of water loss.

Table 4: Summary of the ANOVA for the effects of freezing treatment on percent dry weight.

	Df	Sum Sq	Mean Sq	*F* value	Pr(> *F*)	Omega^2 (95% C.I.)	Interpretation
Freezer treatment	9	129.3	14.364	3.624	< 0.001	0.07 (0.01, 1.00)	Small
Residuals	286	1133.7	3.964				

Note: Effect size was estimated using Omega^2 and interpreted as described in the methods.

that the surface area-to-volume ratio impacts how much water is lost, larger fish with a lower surface area-to-volume ratio should presumably be proportionately lower. In a study on eels, Simon [10] showed greater shrinkage in length and weight for smaller size classes. A rigorous evaluation of freezing protocols as presented here provides necessary information to standardize methods used in long-term monitoring efforts like those reported in the annual State of the Ecosystem Report for the Northeast US Shelf [20].

Fish samples are frequently brought to the laboratory for more detailed analyses than can be performed at sea or at remote locations, which requires some form of preservation. Freezing is a practical and convenient method that does not require chemicals or solutions to preserve fish samples for later analysis. The temperature and duration of freezing can have significant effects on subsequent fish size and weight [6, 10]. Researchers often assume freezing to have minimal effects on fish samples and, therefore, negligible effects on subsequent analyses. However, this

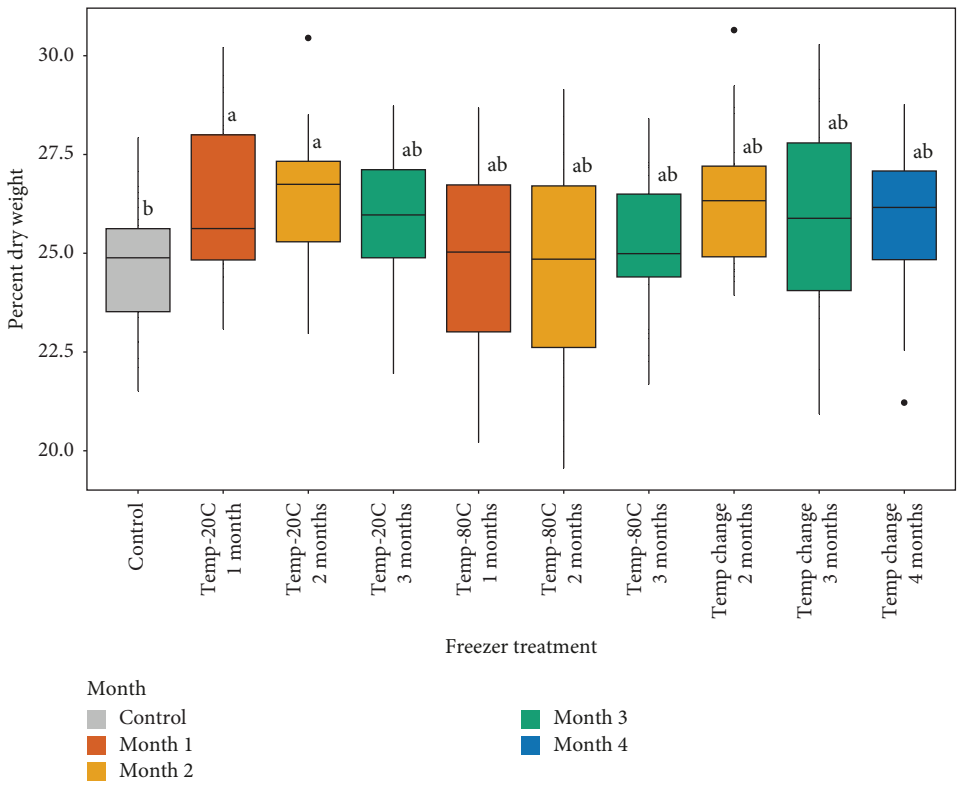

Figure 4: Mean percent dry weights of *F. heteroclitus* compared to freezer treatment. Treatments with the same letters are not significantly different from each other (Tukey's HSD, $p < 0.05$).

assumption should be directly tested depending on specific research objectives (e.g., for fish condition [8]; percentage dry weight [6, 7]; morphology [9, 11]; or chemical composition [12, 14, 21]).

5. Conclusion

In this work, we conducted a freezing experiment using samples of *F. heteroclitus*. We subjected samples to different freezing treatments, with different temperatures (−20°C and −80°C) and times of storage (0–4 months, Table 1), as well as a treatment that was initially frozen at −80°C and then transferred to −20°C. All freezing treatments led to minor but significant water loss. However, the impact of this small measurable loss on the estimated percent dry weight was very low for all groups except the −20°C 3-month treatment. This significant change in weight at 3 months in the −20°C treatment was not evident in the temperature change group which was initially frozen at −80°C before spending a similar 3 months at −20°C. The least amount of weight loss, and therefore bias, was in the −80°C group, followed by the temperature change group, with the −20°C treatments having the greatest weight loss. From a practical standpoint, the results indicate that if specimens have to be frozen, −20°C is okay for a short period of time (1–2 months). If longer storage times are needed, −80°C is the best, but if this is not feasible, then the temperature change protocol will minimize water loss.

Our results further highlight the effects of freezing methods and temperatures, which can affect post-thaw wet weights over time. This is important to consider when designing sampling programs or comparing percent dry weight data across studies using different freezing methods. Future studies of fish energetics that use frozen samples should clearly document their freezing procedures and how those procedures affect percent dry weight values, as done here, to improve confidence in cross-study comparisons.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author.

Conflicts of Interest

The authors declare no conflicts of interest.

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