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# Preservation Effects on Common Macroinvertebrates of the Intermountain West

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# PRESERVATION EFFECTS ON COMMON MACROINVERTEBRATES OF THE INTERMOUNTAIN WEST

by

**Megan Paxton**

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**Abstract**

Dry mass/wet mass ratios are essential for estimating energy flow through ecosystems, determining energy budgets, and studying energy allocation in organisms. Preserving specimens by freezing or storing them in ethanol has known effects on the wet mass measurements. These storage methods are used regardless of their effects – altering the wet mass and thereby changing the mass ratio for the organism. We evaluated the effects of ethanol storage and freezing on six different taxa from the Intermountain West: *Hesperoperla, Isoperla, Rhithrogena, Drunella, Arctopsyche, and Rhyacophila*. All the taxa studied except *Hesperoperla* and *Rhyacophila* showed a significant loss in wet mass when treated with ethanol, with organisms retaining only 17.7% - 79.9% of their original wet mass. Freezing had varied effects. Only *Rhithrogena and Drunella* showed significant losses in wet mass after being frozen (retaining 29.8% - 45.9% of their original wet mass). *Hesperoperla*, *Isoperla*, and *Arctopsyche* showed no significant loss or gain in wet mass after treatment. *Rhyacophila* was the only taxa to have a significant mass gain after being frozen, taking on an additional 23% of its original wet mass. Freezing specimens had less of an impact on their wet mass than storing them in ethanol. Dry masses were not significantly affected by either treatment.

**Acknowledgements**

I would like to acknowledge the mentorship of Dr. Scott Miller on this project. I would also like to thank Amber Summers-Graham, whose help and patience made it possible to complete the project.

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## Introduction

Aquatic macroinvertebrate biomass is a commonly studied factor in aquatic food web analyses (Runck 2007), bioenergetics models (Chips and Wahl 2008), predator-prey interactions (Benoit-Bird 2004), and life history analyses (James et al. 2012). The time requirements needed to estimate biomass for large numbers of individuals commonly requires researchers to preserve specimens to prevent tissue decay and mass loss. Common preservation methods include ethanol, formalin, or freezing, which all have the potential to alter the mass of specimens (Johnston and Mathias 1993, Leuven et al. 1985, Treasurer 1990, Howmiller 1972). Such preservation effects could bias the results of biological and environmental models.

Despite the ubiquitous practice of preserving aquatic insects, there is a paucity of published studies examining preservation effects. For the studies conducted to date, they have resulted in equivocal or conflicting results (Leuven et al. 1985). For example, studies comparing the effects of ethanol and formalin show that ethanol results in significantly lower masses (Donald and Paterson 1977, Howmiller 1972), but others claim no difference between ethanol and formalin (Wetzel et al. 2005, Dermott and Paterson 1974). Additionally, the specimens studied are usually fish, mollusks, crustaceans, or worms. Only a small number of studies have examined preservation effects on Ephemeroptera and Plecoptera specimens, which are two very species and common orders of aquatic insects. This greatly limits the amount of available knowledge concerning the effects of freezing and ethanol preservation on these taxa.

The objective of this study was to determine the effects of preservation by freezing and storage in 95% ethanol (two of the most common preservation methods) on the wet and dry mass measurements of six common genera of aquatic macroinvertebrates (*Hesperoperla*, *Isoperla*, *Drunella*, *Rhithrogena*, *Arctopsyche*, and *Rhyacophila*). Data from preserved specimens was

compared to data from a control group of specimens that were not treated. From this comparison, correction factors may be derived to ameliorate preservation effects in any further calculations based on wet and/or dry mass.

**Methods**

To assess preservation effects on wet and dry mass, I focused on common cold-water Ephemeroptera, Plecoptera and Trichoptera genera of the Intermountain West. Specific taxa chosen for the project were *Arctopsyche, Rhyacophila, Drunella, Rhithrogena, Hesperoperla*, and *Isoperla*. These genera were selected to represent a variety of body sizes and degrees of sclerotization to assess differential preservation effects. Approximately 60 individuals of each taxon were collected in November of 2012 or February of 2013 for the ethanol and freeze treatments, respectively.

Specimens were collected from the Logan River, which flows from southeast Idaho through northern Utah and drains into the Bear River (Figure 1). The river typically experiences cold, snowy winters (with air temperatures from -9°C to 0°C and average precipitation of 4.0 cm in January) and hot, dry summers (air temperatures from 15°C to 31°C and average precipitation of 1.6 cm in July). Climatic conditions result in a snowpack hydrologic regime with maximum discharge (16 $m^3/s$) occurring from April – June and base flows (3 $m^3/s$) dominating from August – March (Budy et al. 2008). There are three low-head dams on the lower part of the river which provide water for irrigation canals and local recreation; all samples were collected from reaches above the dams.

In the laboratory, all live specimens were stored in river water and refrigerated at 5.3°C for a maximum of 56 hours. No specimens were processed during the first 24 hours to allow individuals to clear their guts and thus minimize variability in mass estimates.

To quantify the effects of freezing and ethanol preservation, wet weights were measured before and after each treatment, and dry mass was measured after each treatment. Wet weights were obtained by blotting specimens on a paper towel for three minutes prior to being weighed to the nearest ±0.01 mg for larger specimens and ±0.001 mg for smaller specimens. Following wet weight estimates, individuals were assigned to one of three treatment groups: fresh (no preservation), frozen, or 95% ethanol. Individuals within a particular genus were assigned to treatments at random such that the average wet weight and standard deviation of the treatment groups were as close as possible (Table 1).

Frozen specimens were kept in the freezer at a temperature of -15°C for 15 days. In order to thaw the specimens, they were removed from the freezer, 20 specimens at a time, and placed on a counter at room temperature (22°C). Thawing time was approximately 10 minutes. Specimens in the ethanol treatment were left in 1.5 ml vials of 95% ethanol for 75 days. The ethanol treatment was terminated after 75 days because results from past studies suggest that no significant losses or gains in mass are to be expected after that point (Leuven et al. 1985, Shields and Carlson 1996, Wetzel 2005).

Following the treatments, a second set of blotted wet weights was obtained for each specimen. Specimens were then placed in tin weighing boats and dried in an oven at 60°C for 48 hours, after which they were removed and allowed to cool to room temperature in desiccators. Dry weights were taken once specimens had cooled.

To assess preservation effects I relied on both graphical analyses and t-tests. Specifically, paired t-tests were used to compare wet weights before and after preservation for treatment and control groups. In contrast, preservation effects on dry mass were assessed by

testing for significant differences between post-treatment ethanol and freeze means, to the means of the respective control groups. Statistical significance was assessed at the 0.05 alpha level. Post-treatment wet weights were also graphed with the corresponding dry weight for each specimen to examine the dry mass/wet mass relationships.

**Results**

I found that overall, ethanol resulted in greater mass reductions than freezing and that wet weights were more significantly reduced than dry weights (Tables 2 and 3, Figure 2). Treatment responses varied among taxonomic orders, but were consistent within orders with the exception of Trichoptera. Within that order, *Rhyacohpila* showed a significant weight gain after the freeze treatment, and although *Arctopsyche* did gain weight after freezing, it was not significant. The only order to show significant mass changes from both treatments was *Ephemeroptera*. *Rhithrogena* was the most drastically affected by both treatments,retaining only 17.7% of wet mass after the ethanol treatment and 29.8% after being frozen. *Drunella* also had significant mass reductions in response to both treatments,retaining only 41.3% after preservation in ethanol and 45.9% after freezing. *Isoperla* lost a significant portion of their mass after being treated with ethanol, retaining 65.5% of their original wet mass. In the freeze treatment, 77.5% of the original wet mass was retained, which is not a significant mass change. *Rhyacophila* specimens ended the ethanol treatment with 78.8% of their original wet mass, which was not a significant change, and gained an extra 23.0% of their original wet mass after being frozen. This mass gain was significant. After ethanol and freeze treatments, *Hesperoperla* specimens showed no significant mass changes and retained 98.8% and 92.9% of their original wet mass, respectively. *Arctopsyche* did not have a significant change in mass from either treatment and retained 79.9%

of their original wet mass after the ethanol treatment and had 107.0% of their original wet mass after freezing.

On average, treating specimens with ethanol resulted in more consistent dry weight reductions than the freeze treatment (Figure 2). Ethanol preservation caused significant dry weight changes in *Rhithrogena*, *Drunella,* and *Arctopsyche*. Freezing specimens only caused a significant dry weight change in *Rhyacophila*, which experienced an increase in mass. Other taxa showed no response to treatments in their dry weight measurements.

## Discussion

Given the pervasive use of preserved specimens for obtaining biomass estimates, I assessed the impacts of two preservation techniques, ethanol and freezing, on common Ephemeroptera, Plecoptera, and Trichoptera taxa of the intermountain west. Overall, freeze treatments had less of an effect on wet and dry weights than ethanol preservation; however, results varied among taxonomic orders with Ephemeroptera being more significantly impacted than Trichoptera or Plecoptera. Furthermore, both ethanol preservation and freezing had a greater impact on wet than dry weights. Although these results span a wide range of responses, it must be noted that the taxa included in the study also vary widely in their body types and composition, leading to the differential responses I observed. In this discussion, the results will be reviewed in two different contexts: the differential responses among taxa and the differential responses between treatments.

### Differential responses among taxa

The responses I observed among taxa were widely different, although differences in preservation-induced weight changes were narrow between specimens of the same taxonomic family. *Ephemeroptera* wet weights were greatly reduced by preservation treatments, but

*Plecoptera* specimen masses were hardly affected at all. Such a broad range of responses is not unheard of, however. Howmiller (1972) and Stanford (1972) both observed large changes in mass due to preservation that had a similar range. Additionally, Maslin and Pattee (1981) saw a 40% decrease in the mass of *Ephemeroptera* specimens as a result of ethanol preservation, while Stanford (1972) and Maslin and Pattee (1981) observed Trichoptera specimens that retained 69 – 75% of their original wet weights after ethanol preservation. The *Plecoptera* specimens of Maslin and Pattee (1981) retained 73 – 85% of their original wet mass after ethanol preservation, which is similar to the response I saw in *Isoperla* specimens. Overall, my results seem to be consistent with those from other studies.

One noteworthy aspect of my results is the morphologically-based pattern of wet mass responses to preservation. The organisms with softer bodies (such as *Rithrogena*, *Drunella*, and *Arctopsyche*) showed the greatest losses in wet mass, while the *Isoperla* and *Hesperoperla*, which have harder exteriors, were less affected by preservation. This could be due to the greater degree of sclerotization present in the Plecoptera specimens but not in the other taxa. Organisms with this hardened outer layer would be less likely to become dehydrated in ethanol preservation or to have tissue damage after being frozen.

In addition to the issue of body type (soft versus hard), specimen size is another critical factor that could explain differential responses among taxa. The range in mean biomass among taxa was extensive, with masses ranging from 0.0016 g to 0.2119 g before treatment. Wetzel et al. (2005) observed that smaller specimens are prone to being more drastically affected by preservation treatments than larger specimens, but no explanation is offered for why that would be. This is consistent with what I saw in my results. *Hesperoperla* and *Arctopsyche*, which were the largest specimens, were the least affected by preservation. *Rhyacophila*, which were still big,

but not as large as *Arctopsyche*, were also not significantly affected by either preservation method. In contrast, *Drunella* and *Rhithrogena* (the smallest specimens) showed the greatest response to preservation. This expansive spectrum of sizes included in the experiment may partially account for some of the minor discrepancies between my data and previously published results, as well as the large range of post-preservation wet mass changes I observed. Several other variables may have impacted the results of this study. Factors such as the body volume of specimens, the ratio of specimen volume to preservative volume, the ambient temperature, seasonal variations in biomass, and gut tube clearance may all influence the results (Leuven et al. 1985, Landahl and Nagell 1978).

**Differential responses between treatments**

Despite the differential responses among taxa present in this study, all specimens had something in common: there was a more dramatic response in wet weight after chemical preservation than there was after freezing. This could be due to the fact that during ethanol preservation, water is removed from the organisms in order to "fix" or preserve the tissues (Shields and Carlson 1996). This dehydration would cause a significant loss in wet mass for specimens, especially in those with softer bodies such as *Rhithrogena*, *Drunella*, and *Arctopsyche*. Freezing specimens does not entail such a drastic removal of fluids from organism tissue and, therefore, would not induce the same mass loss seen in specimens preserved in ethanol. The dehydration process that ensues during ethanol preservation would also account for the lack of change in specimen dry weight after preservation. The drying process would remove any remnants of moisture from the specimen, but this would not significantly alter the final dry weight from what it would have been without the preservation. Frozen specimens undergo something similar. Specimens frozen in water may lose some mass due to tissue damage during

the freezing process, but they lose about as much water in the drying process as the control specimens. Therefore, the final dry weights are not significantly changed.

My results concerning chemical preservation versus freezing contrast with those of Johnston and Mathias (1993), which asserts that freezing had a more pronounced impact on specimen wet weights than chemical preservation. However, the chemical preservative used in that study was formalin and not ethanol. Some researchers claim the effects of formalin on specimens are widely different from the effects induced by ethanol preservation (Donald and Paterson 1977). More claim that specimens show no difference in their response between ethanol and formalin treatments (Dermott and Paterson 1974, Wetzel et al. 2005). When significant differences are seen between the effects of formalin and ethanol, formalin is the preservative with the lesser impact (Howmiller 1972). Therefore, Johnston and Mathias (1993) may have seen freezing as the preservation method with the larger impact on specimen wet weight only because formalin does affect wet weights to the same extent as ethanol.

Out of all the data that emerged from this project, there is one point that deserves special attention. *Arctopsyche* had a minor increase in wet mass after being frozen that was not statistically significant, but the weight gain that *Rhyacophila* experienced after the frozen treatment was substantial. This is interesting, considering that both *Arctopsyche* and *Rhyacophila* are from the same taxonomic order and share many morphological and compositional characteristics. It is possible that because these specimens have very soft, fleshy bodies that are easily damaged they were somehow maimed while being processed. Even minor tears or penetrations of the outer skin from forceps would allow extra water to seep in and cause additional tissue trauma and fluid accumulation during the freezing process, which may generate error in the post-treatment mass measurements (Gaston et al. 1996). More investigation is

necessary in order to establish a concrete explanation for why these taxa would gain weight after being frozen.

**Implications**

Using any preservation method on specimens will result in some mass change. Alterations to specimen wet mass throws off wet mass/dry mass ratios and subsequently bias the models and estimates using these ratios. This is especially problematic in bioenergetics research. If inaccurate wet mass/dry mass ratios were used in energy density equations, the resulting data would create bioenergetics models predicting incorrect foraging behaviors and energy budgets (James et al. 2012). After investigating the effects of ethanol preservation and freezing on specimen wet weight, it appears that freezing specimens in water is the less detrimental preservation method. Because freezing has the smaller effect on wet mass and it does not alter the final dry mass, it is the better preservation method for aquatic macroinvertebrate specimens.

Previous studies have sought to establish correction factors for preserved specimens to correct for altered specimen wet masses (Leuven et al. 1985, Shields and Carlson 1996). If correction factors were to be calculated for my results, they would be taxon specific. They would also be non-linear because the effects of preservation are greater for smaller specimens and lesser for larger specimens. Therefore, I find it impractical to calculate correction factors for my data. Instead, I recommend specimens be frozen to keep preservation bias in results to a minimum. This will allow wet mass/dry mass ratios to remain as close to their true values as possible and permit researchers to use the ratios without losing accuracy in later calculations.

## Tables and Figures

**Table 1:** Means and standard deviations for each randomly assigned treatment and control group. Note that separate specimens were used for the freeze treatment and

| Organism ID | Ethanol | Control | Freeze | Control |
|---|---|---|---|---|
| **Hesperoperla** | 0.2032 (0.08) | 0.2119 (0.08) | 0.14 (0.14) | 0.1476 (0.1) |
| **Isoperla** | 0.0029 (<0.01) | 0.003 (<0.01) | 0.012 (<0.01) | 0.0113 (0.01) |
| **Rhithrogena** | 0.0022 (<0.01) | 0.0024 (<0.01) | 0.0057 (<0.01) | 0.0058 (<0.01) |
| **Drunella** | 0.0016 (<0.01) | 0.0016 (<0.01) | 0.0053 (<0.01) | 0.0057 (<0.01) |
| **Arctopsyche** | 0.0737 (0.05) | 0.0739 (0.05) | 0.057 (0.05) | 0.051 (0.04) |
| **Rhyacophila** | 0.0118 (0.01) | 0.0123 (<0.01) | 0.0129 (<0.01) | 0.0123 (<0.01) |

**Table 2:** Average wet weights among taxa compared before and after the ethanol (top) and freeze (bottom) treatments. Also included are the average post-treatment mass retention and the p-values for the comparison of wet weights before and after treatment.

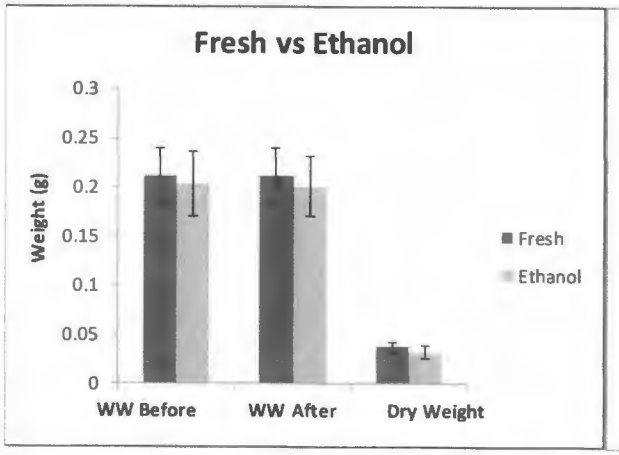
| **Ethanol** | | | | |
|---|---|---|---|---|
| **Organism ID** | **Avg. Wet Weight Before Treatment (g)** | **Avg. Wet Weight After Treatment (g)** | **Avg. % Mass Retention** | **p-value** |
| Hesperoperla | 0.2032 | 0.2009 | 100.0% | 0.628 |
| Isoperla | 0.0029 | 0.002 | 65.5% | 0.022 |
| Rhithrogena | 0.0022 | 0.0004 | 17.7% | <0.001 |
| Drunella | 0.0016 | 0.0006 | 41.3% | <0.001 |
| Arctopsyche | 0.0737 | 0.059 | 79.9% | 0.347 |
| Rhyacophila | 0.0118 | 0.0093 | 78.8% | 0.089 |
| **Frozen** | | | | |
| **Organism ID** | **Avg. Wet Weight Before Treatment (g)** | **Avg. Wet Weight After Treatment (g)** | **Avg. % Mass Retention** | **p-value** |
| Hesperoperla | 0.14 | 0.1301 | 92.9% | 0.769 |
| Isoperla | 0.012 | 0.0093 | 77.5% | 0.365 |
| Rhithrogena | 0.0057 | 0.0017 | 29.8% | <0.001 |
| Drunella | 0.0053 | 0.0024 | 45.9% | 0.006 |
| Arctopsyche | 0.057 | 0.0612 | 107.0% | 0.617 |
| Rhyacophila | 0.0129 | 0.016 | 123.0% | 0.033 |

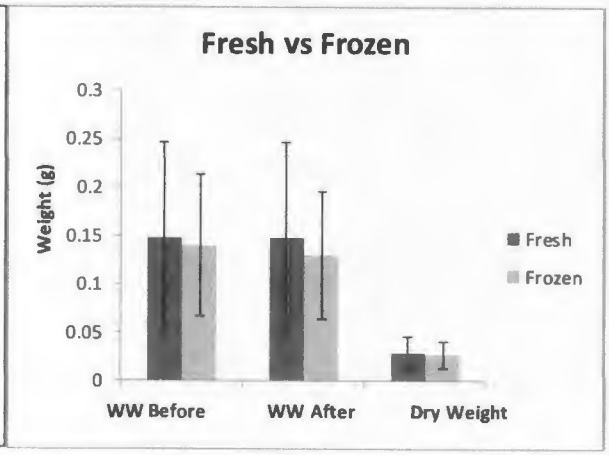
**Table 3:** Average dry weights among taxa compared before and after the ethanol (top) and freeze (bottom) treatments. Also included are the wet weight/dry weight ratios using pre- and post-treatment wet weights, the differences between the ratios, the p-values for the comparison of wet weights before treatment and dry

| Ethanol | | | | | |
|---|---|---|---|---|---|
| **Organism ID** | **Avg. Dry Weight (g)** | **p-value** | **WW (Before):DW** | **WW (After):DW** | **Difference** |
| Hesperoperla | 0.03205 | 0.204 | 6.34 | 6.27 | 0.07 |
| Isoperla | 0.00041 | 0.905 | 7.07 | 4.88 | 2.20 |
| Rhithrogena | 0.00007 | 2.21E-06 | 31.43 | 5.71 | 25.71 |
| Drunella | 0.00014 | 0.031 | 11.43 | 4.29 | 7.14 |
| Arctopsyche | 0.0087 | 0.028 | 8.47 | 6.78 | 1.69 |
| Rhyacophila | 0.00118 | 0.233 | 10.00 | 7.88 | 2.12 |
| **Frozen** | | | | | |
| **Organism ID** | **Avg. Dry Weight (g)** | **p-value** | **WW (Before):DW** | **WW (After):DW** | **Difference** |
| Hesperoperla | 0.02651 | 0.857 | 5.28 | 4.91 | 0.37 |
| Isoperla | 0.00148 | 0.696 | 8.11 | 6.28 | 1.82 |
| Rhithrogena | 0.00034 | 0.509 | 16.76 | 5.00 | 11.76 |
| Drunella | 0.00045 | 0.998 | 11.78 | 5.33 | 6.44 |
| Arctopsyche | 0.01026 | 0.75 | 5.56 | 5.96 | -0.41 |
| Rhyacophila | 0.00282 | 0.066 | 4.57 | 5.67 | -1.10 |

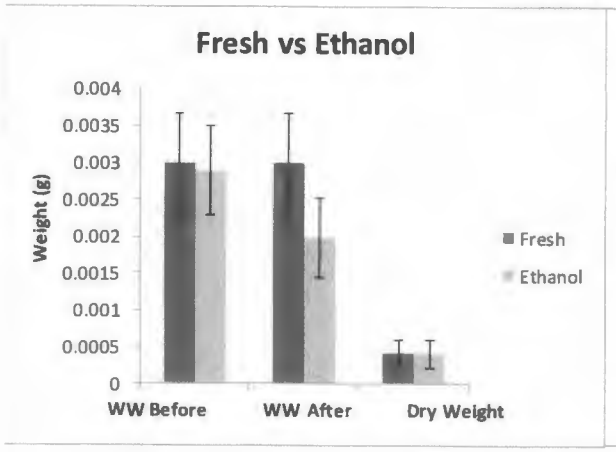

**Figure 1:** Location of sample collection points on the Logan River, UT. Collection sites are marked in red.

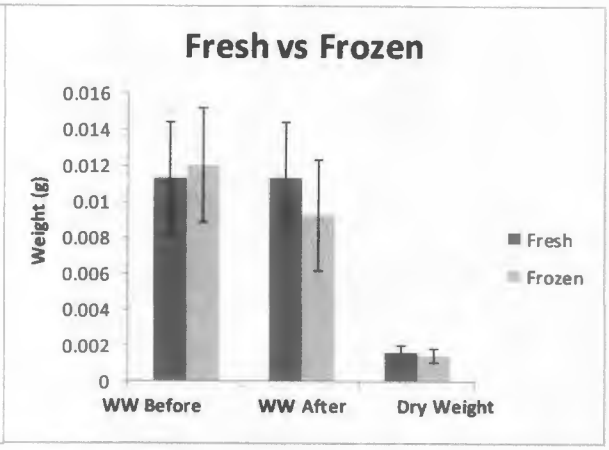
## Hesperoperla



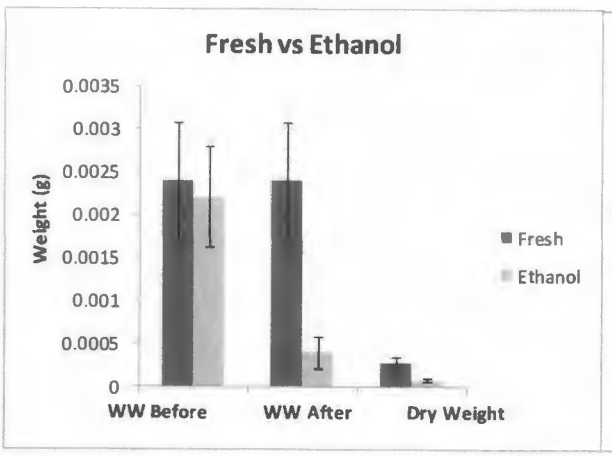



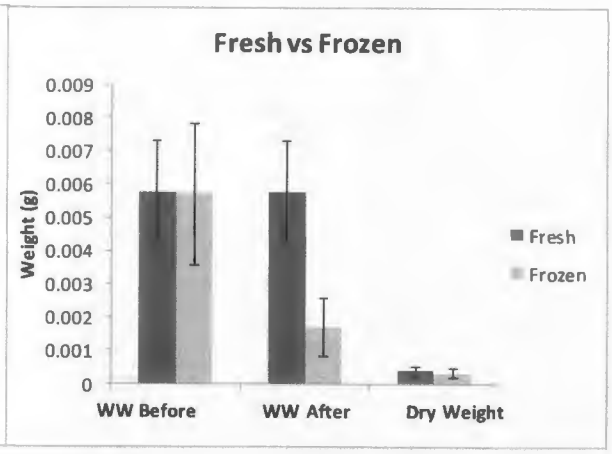

## Isoperla




## Rhithrogena



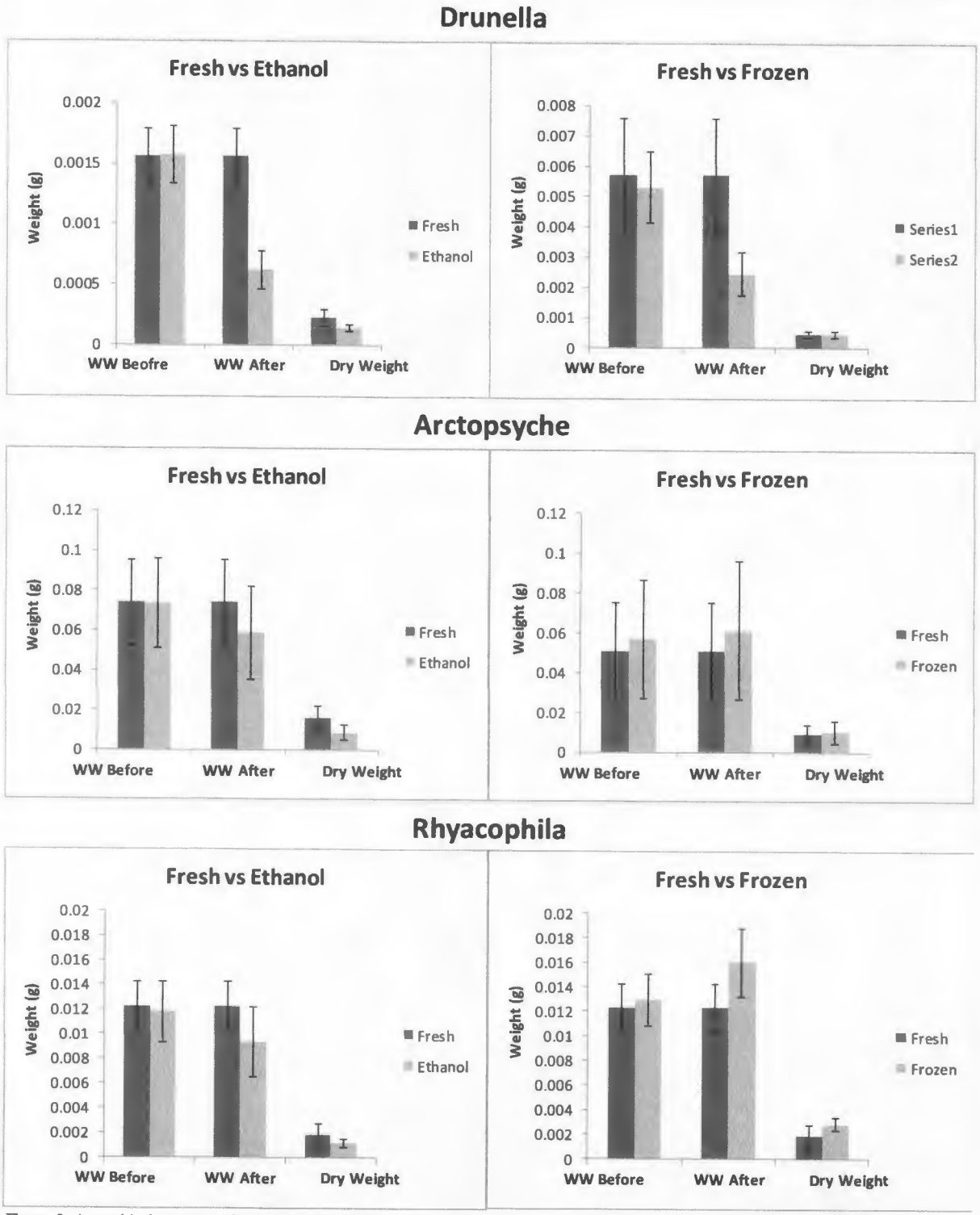



**Figure 2:** A graphical summary of my results comparing the pre- and post-treatment wet weights and dry weights of the control group versus the treatment group. Error bars are shown, indicating the 95% confidence interval used.

**Author's Biography**

Megan Paxton was born in Provo, Utah, in 1991 but was raised in the Midwest, mostly in Iowa. She graduated from Ames High School in May of 2009 and returned to her native Utah as a Presidential Scholar at Utah State University. Although passionate about music, Megan decided on biology as her major. She also earned a minor in chemistry as part of the cellular/molecular emphasis she chose with her degree and supplemented her science-heavy schedule with music courses to complete a music minor. During her time at Utah State, Megan participated in research in the National Aquatic Monitoring Center under Dr. Scott Miller and was also an active officer in the Honors Student Council, a member of the Circle K service organization, a member of the Medical Unity club, and a singer in multiple university and community choirs. She will graduate Magna Cum Laude in May of 2013 with University and Departmental Honors and a Bachelor's of Science in biology.

After graduating, Megan plans to return to her beloved Europe and travel before re-enslaving herself to her education and obtaining a Master of Public Health and pursuing a career as a dietitian.

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