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# Drying, freezing, and ethanol preservation lower shell strength of two freshwater pulmonate gastropods

## **Cover Page Footnote**

This work was funded by a grant from the University System of Georgia STEM Initiative II Project. We would like to thank Georgia Gwinnett College for lab space and equipment, Peter Sakaris for statistical advice, and Kathryn Zimmermann for discussions about shell chemistry.

## DRYING, FREEZING, AND ETHANOL PRESERVATION LOWER SHELL STRENGTH OF TWO FRESHWATER PULMONATE GASTROPODS

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### ABSTRACT

Many studies have measured gastropod shell strength to investigate abiotic interactions and responses to predation. Shells are often preserved before strength is measured, but preservation may affect shell biomechanics, potentially influencing the outcome of these studies. We hypothesized that commonly used preservation methods (ethanol, freezing, and drying) lower shell strength of two pulmonate snails, *Physella* sp. and *Pseudosuccinea columella*. Compared to controls, all preservation methods significantly lowered strength for both species, except freezing in *Ps. columella*. To date, no studies have addressed the effects of preservation on shell strength in freshwater pulmonates. These results suggest that preservation methods should be considered when using shell strength as a response variable in ecological studies. We also provide one of the few direct measurements of shell strength in freshwater snails.

**Keywords:** snail, strength, biomechanics, preservation, drying, freezing, ethanol

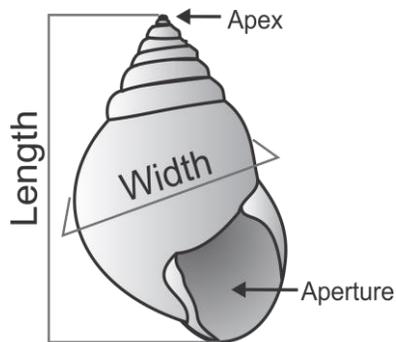
### INTRODUCTION

Many studies have investigated snail shell strength to better understand the interactions of these organisms with biotic and abiotic factors (Vermeij and Currey 1980; LaBarbera and Merz 1992; Brown 1998; Jordaens et al. 2006; Chaves-Campos et al. 2012; Dillon and Jacquemin 2015). However, shells are often preserved before evaluating strength by drying (e.g., Evers et al. 2011), freezing (e.g., Tucker et al. 1997), or storing in chemical preservatives (e.g., Currey and Hughes 1982). Only one study has evaluated the effects of drying on shell strength and found no effect on shell strength except when shells were dried at high temperature (~100 °C) (Currey 1979). We hypothesized that preserving shells lowers their strength. Here, we examine how drying and two additional commonly-used preservation methods affect the shell strength of two freshwater pulmonate snails. Pulmonate snails are typically thin-shelled, but are generally tolerant of many conditions and are found in lakes and ponds, rivers and creeks, and bogs and ephemeral pools (Johnson et al. 2013).

## MATERIALS & METHODS

### Collection and Treatments

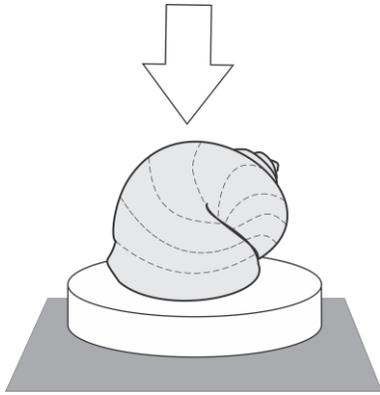
Individuals of *Pseudosuccinea columella* (n = 54) and *Physella* sp. (n = 214) were collected by hand on 27 February 2015 from a small retention pond on the campus of Georgia Gwinnett College, Lawrenceville, Georgia. The taxonomy of *Physella* is currently in flux so we maintain the conservative non-specific epithet *sp.* Voucher specimens are housed at Georgia Gwinnett College. Snails were immediately transported to the laboratory and indiscriminately assigned to four treatments: (1) live treatment (control) - snails were tested for strength the day they were collected; (2) ethanol treatment - snails were preserved in 95% ethanol; (3) dry storage treatment - snails were dried at 70 °C for 28 h then stored at room temperature; and (4) frozen treatment - snails were stored at -4 °C. Temperatures were determined by the limits of the equipment available at the time. All preserved snails were stored for 14 d prior to testing. Before testing strength, we measured shell size as the area of a frontal-section, (i.e., length by width, Figure 1) with an SPI analog caliper (Garden Grove, California). Simple linear measurements have been used to understand shell morphometrics for over a hundred years (Dillon and Jacquemin 2015). Only snails with undamaged shells were tested.



**Figure 1.** Length was measured from the apex to the base of the aperture. Width was measured as the diameter of the greatest whorl. These measurements were combined into a frontal sectional area for analysis.

### Measuring strength

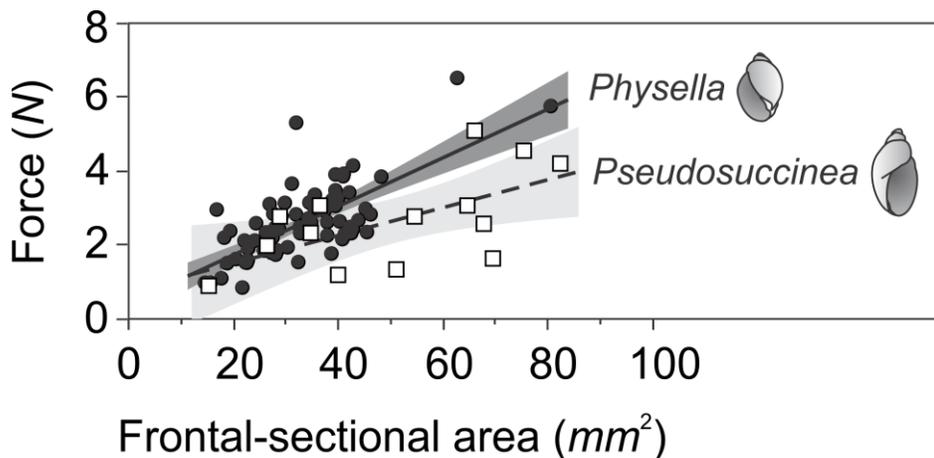
A PASCO Economy Force Sensor, model CI-6746 (Roseville, California), was used to measure the maximum force in Newtons required to crush the shell's body whorl. Force was measured by applying pressure dorsally on the body whorl until failure (Figure 2). This method simulates the crushing action of many durophagous predators, and provides a comparable indication of shell sturdiness across treatments (Currey and Hughes 1982; DeWitt et al. 2000; Jordaens et al. 2006).



**Figure 2.** Force (arrow) was measured dorsoventrally on the body whorl until failure.

### Analysis

Maximum force at failure was recorded with DataStudio (Roseville, California) and analyzed using JMP 11 (Cary, North Carolina). Force measurements for *Physella* did not fit assumptions of normality and were log transformed before analysis. Since strength is influenced by size, we used ANCOVA with Dunnett's post hoc test on the least squares means (LSM) of shell strength to compare the strength of each preservation treatment to live snails while controlling the confounding effects of size (Parris 2011). Because preservation is not likely to increase shell strength, we performed one-tailed tests to determine if shells are significantly weaker after preservation.



**Figure 3.** Comparison of snail shell strength between species (control groups only) with frontal-sectional area (width x length) as a covariate, showing that *Pseudosuccinea* (open squares, dotted line) are larger on average, but relatively weaker than *Physella* (solid circles, solid line) (ANCOVA,  $F = 8.75$ ,  $P < 0.005$ ). 95% confidence intervals are shown.

## RESULTS

We found a significant correlation between shell size and strength for both species (*Ps. columella*:  $r^2 = 0.52$ ,  $P < 0.0001$ , *Physella*:  $r^2 = 0.32$ ,  $P < 0.001$ ) (Figure 3), supporting the use of size as a covariate. Although *Ps. columella* was significantly larger than *Physella* (frontal-sectional area: *Ps. columella* =  $51 \pm 28$  mm<sup>2</sup>, *Physella* =  $33 \pm 11$  mm<sup>2</sup>,  $t = 4.66$ ,  $df = 57$ ,  $P < 0.0001$ ), shells of live *Physella* were significantly stronger than *Ps. columella* when correcting for size (ANCOVA using log N for both species,  $F = 8.75$ ,  $P < 0.005$ ) (Figure 3). Compared to controls, all preservation methods significantly lowered strength for both species, except freezing in *Ps. columella* (Tables I and II).

**Table I.** ANCOVA table of *Pseudosuccinea columella* shell strengths with frontal-sectional area as a covariate. Comparisons of each preservation method to the live controls are analyzed using one-tailed Dunnett's post hoc test on the least squares means which are adjusted for frontal-sectional area. Bold font represents values that were significantly different from control.

Treatment	Sample size	Snail frontal-sectional size (mm x mm) $\pm$ SD	Mean strength (N) $\pm$ SD	Least squares means $\pm$ se	<i>P</i> (compared to control)
<i>Pseudosuccinea</i>					
Live (Control)	14	51.0 $\pm$ 21	2.66 $\pm$ 1.3	2.68 $\pm$ 0.2	
Ethanol	12	52.9 $\pm$ 26	2.10 $\pm$ 1.0	2.06 $\pm$ 0.2	<b>0.05</b>
Dried	14	58.0 $\pm$ 40	2.11 $\pm$ 1.0	1.92 $\pm$ 0.2	<b>0.02</b>
Frozen	14	44.3 $\pm$ 22	2.03 $\pm$ 1.1	2.23 $\pm$ 0.2	0.14

**Table II.** ANCOVA table of *Physella* shell strengths with frontal-sectional area as a covariate. Comparisons of each preservation method to the live controls are analyzed using one-tailed Dunnett's post hoc test on the least squares means which are adjusted for frontal-sectional area. Bold font represents values that were significantly different from control.

Treatment	Sample size	Snail frontal-sectional size (mm x mm) $\pm$ SD	Mean strength (N) $\pm$ SD	Normalized mean strength (log N) $\pm$ SD	Least squares means $\pm$ se	<i>P</i> (compared to control)
<i>Physella</i>						
Live (Control)	76	33.3 $\pm$ 11	2.61 $\pm$ 1.0	0.89 $\pm$ 0.4	0.89 $\pm$ 0.1	
Ethanol	48	34.1 $\pm$ 11	2.29 $\pm$ 1.1	0.76 $\pm$ 0.4	0.73 $\pm$ 0.1	<b>0.04</b>
Dried	49	31.5 $\pm$ 10	2.27 $\pm$ 1.3	0.67 $\pm$ 0.6	0.72 $\pm$ 0.1	<b>0.04</b>
Frozen	41	35.2 $\pm$ 12	1.28 $\pm$ 0.9	0.02 $\pm$ 0.7	-0.03 $\pm$ 0.1	<b>&lt;0.0001</b>

## DISCUSSION

We predicted that drying, of all treatments, would cause the greatest reduction in strength, because heating may alter the shell matrix, but this was only true for *Ps. columella*. Indeed, dried *Ps. columella* was 24% weaker than live snails on average. Currey (1979) tested the effects of drying on shell strength of the dog whelk, *Nucella lapillus*. We found an effect of drying at a lower temperature on freshwater pond snails (~70 °C compared to 110 °C) and for less time (28 h compared to 5 d), than he did in whelks. Currey suggested that drying may have caused distortion in the shell's lamellar structure, and variation in response to temperature between genera might be due to differences in shell thickness (Vermeij and Covich 1978; DeWitt et al. 2000).

To our knowledge, this is the first study to test the effects of ethanol and freezing, two common methods by which snails are preserved, on shell strength. Freezing *Physella* induced the greatest reduction in shell strength. Compared to live controls, freezing lowered strength by 51% (Table II). Freezing is the only treatment we tested that could have fitness consequences for snails in the wild (DeWitt et al. 2000), since snails are unlikely to encounter ethanol in their environment, and drying is typically fatal regardless of shell strength. Calcium carbonate, the main structural component of gastropod shells, is more soluble at lower temperatures (Langmuir 1997), which could lead to a reduction in shell strength during fall and winter. However, Génio et al. (2015) found no effect of freezing or ethanol storage on shell trace element concentration in a deep-sea mussel.

Our data caution against preserving snails when they are to be used for studies of shell biomechanics because these methods may significantly alter shell strength. Future studies should investigate these effects on snail species with disparate shell sizes, shapes, and strengths as well as examine if different preservative methods affect shell dynamics differently. For instance, ethanol and drying cause the soft tissues to shrink, thus potentially removing some support, whereas using 10% buffered formalin as a preservative does not distort or desiccate the animals. In addition, further studies could include removing the animals from the shells and filling shells with wax following the methods of Huryn and Denny (1997) to control for the effects of the resistance of the animal on the shell itself. Indeed, the structural causes of decreased shell strength in *Ps. columella* and *Physella* remain unknown. Other studies could test whether or not seasonal changes in environmental temperature affect shell strength and thus susceptibility to predation.

## ACKNOWLEDGMENTS

This work was funded by a grant from the University System of Georgia STEM Initiative II Project. We would like to thank Georgia Gwinnett College for lab space and equipment, Peter Sakaris for statistical advice, and Kathryn Zimmermann for discussions about shell chemistry.

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