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Nonlethal evaluation of the physiological health of unionid mussels: methods for biopsy and glycogen analysis

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Abstract. In long-lived unionid mussels, many short-term measures of growth are of limited value. Changes in physiological condition may be an early indication of stress, because the increased energy demand associated with stress often results in a depletion of glycogen reserves, the principal storage form of carbohydrates in unionid mussels. Our goal was to nonlethally extract tissue from freshwater mussels and then to develop a rapid and dependable method for the analysis of glycogen in the tissue extracts. A biopsy technique was developed to remove between 5 and 10 mg of foot tissue in *Amblema plicata plicata*. The survival rate did not differ between biopsied and non-biopsied mussels during a 581-d observation period, demonstrating that the biopsy technique will allow nonlethal evaluation of the physiological condition of individual mussels through measurement of changes in contaminant, genetic, and biochemical indicators in tissue. We also modified the standard alkaline digestion and phenol-sulfuric acid analysis of glycogen for use on the small samples of biopsied tissue and to reduce analysis time and cost. We present quality control data, including method detection limits and estimates of precision and bias. The modified analytical method is rapid and accurate and has a method detection limit of 0.014 mg glycogen. Glycogen content in the biopsied samples was well above the method detection limit; it ranged from 0.09 to 0.36 mg, indicating that the method should be applicable to native mussels.

Key words: Unionidae, nonlethal sampling, glycogen, foot biopsy, freshwater mussels, *Amblema*, physiological condition.

Species diversity and abundance of North American populations of freshwater mussels have declined substantively in recent years (Williams et al. 1993, Naimo 1995, Wiener et al. 1995); consequently, many conservation efforts are now directed at this faunal group. Traditional methods for obtaining tissue samples for contaminant (Naimo et al. 1992), genetic (Stiven and Alderman 1992), and biochemical (Haag et al. 1993) analyses commonly require sacrificing the animal. Nonlethal techniques for removing tissue samples are needed for temporal studies of threatened, endangered, or otherwise environmentally sensitive species. Berg et al. (1995) have recently shown that mantle biopsies can be used to obtain 35-mg samples without adversely affecting mussel survival for up to 1 y. More studies, using different species and tissue types, are needed to assess long-term effects of tissue sampling techniques on survival of freshwater mussels.

The identification of sublethal indicators of stress in unionid mussels is critical because traditional measures of growth and reproduction are often inappropriate for these long-lived animals. Glycogen is the principal storage form of carbohydrates in many aquatic invertebrates (Stetten and Stetten 1960, DeZwaan and Zandee 1972, Hummel et al. 1989), and it has been used as an indicator of the energetic status of freshwater bivalves (Holopainen 1987, Hemelraad et al. 1990). Glycogen concentrations have been used successfully as an indicator of physiological condition in unionid mussels after exposure to contaminants (Hemelraad et al. 1990) and after infestation by zebra mussels (Haag et al. 1993). Furthermore, changes in glycogen concentrations in long-lived unionid mussels as a result of stress may be observed long before changes in either growth or survival appear. For example, a 3-mo exposure to zebra mussels significantly reduced glycogen concentrations in *Amblema plicata* but did not adversely affect survival (Haag et al. 1993).

Measurement of glycogen in animal tissue is

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a 2-step process: 1) digestion and extraction of glycogen from tissue, and 2) quantification. Three methods have been used to extract glycogen from tissue: alkaline (Good et al. 1933, Montgomery 1957, Van Handel 1965, Lo et al. 1970), acidic (Roe et al. 1961), and enzymatic (Roehrig and Allred 1974). Likewise, glycogen has been quantified with several reagents, e.g., phenol-sulfuric acid (Dubois et al. 1956, Montgomery 1957, Lo et al. 1970) and anthrone (Carroll et al. 1956, Van Handel 1965). Although each method has specific advantages and disadvantages, the alkaline digestion and the phenol-sulfuric acid spectrophotometric methods appear to be the most efficient and cost effective (Montgomery 1957). These methods use stable reagents (Montgomery 1957) and can be performed on relatively small amounts of tissue (35–50 mg, Lo et al. 1970). Few of the published analytical methods for glycogen discuss the validity of the method being evaluated; method detection limits and other evidence of method accuracy are seldom reported.

Our goal was to nonlethally extract tissue from freshwater mussels and then to develop a rapid and dependable method for the analysis of glycogen in the tissue extracts. Our objectives were 1) to determine if the biopsy of foot tissue from *Amblema plicata plicata* adversely affected survival; 2) to develop a modified analytical method for glycogen that would be rapid, use lesser quantities of reagents, and be suitable for small masses of tissue with low concentrations of glycogen; and 3) to measure the accuracy (precision and bias, sensu APHA et al. 1995) and detection limits of the method.

Methods

Biopsy technique

Amblema p. plicata was chosen as the test organism because of its commercial value and its numerical abundance in unionid communities in the upper Mississippi River. To biopsy *A. p. plicata*, we gently opened the shell ~5 mm with a pair of reverse pliers and inserted a kidney biopsy needle (23-mm Bard Monopty) into the ventral margin of the foot tissue. Two tissue plugs were removed to obtain an average of 7.7 mg (±0.3 SE, $n = 30$) of wet tissue, a sufficient mass for analysis of glycogen with the modified method. We chose foot tissue because it can be sampled nonlethally, and because glycogen is uniformly distributed in this tissue (T. J. Naimo, unpublished data).

In July 1995, SCUBA divers removed 60 mussels (shell length 80–90 mm) from Navigation Pool 9 of the upper Mississippi River. The 60 mussels were free of zebra mussels. Mussels were transported in coolers containing river water and wet burlap to the laboratory where 30 individuals were randomly allocated into non-biopsied and biopsied groups. All individuals were uniquely marked with a marine shellfish tag. Two tissue plugs were removed from biopsied mussels and were placed into a cryogenic freezer (<−80°C) for later analysis of glycogen. All mussels were then placed into 2 suspended nylon mesh bags in an artificial pond at the Upper Mississippi Science Center in La Crosse, Wisconsin, to measure survival. This 0.04-ha earthen pond was fed by well water (12°C) and had a water retention time of ~1 wk. Water in the pond was aerated and circulated by a 0.5 horsepower Sweetwater® Regenerative Blower (Model S31).

Effect of biopsy on survival

Survival of mussels was checked weekly for the first 7 wk and then checked 11 times over the next 17 mo, for a total of 581 d of observation. We used the Cox proportional hazards regression (Cox 1972) to test the null hypothesis that survival rate did not differ between biopsied and non-biopsied mussels. The hazard function, the conditional probability of dying at time t given survival up to t, is:

$$h(t) = \lim_{\Delta t \to 0} \frac{P(t \le T \le t + \Delta t \mid t \le T)}{\Delta t}$$

where P(x) is the probability of event x, T is the time until death, and Δt is an arbitrary time interval. The hazard function describes the rate of death because its dimension is the number of events per interval of time, where that interval is taken to be vanishingly small. For our data, we chose the model:

$$h(t) = \lambda_0 \exp(\alpha)$$

where λ_0 is the baseline hazard constant and α is the effect of biopsy status (biopsied or non-biopsied). The parameters in proportional hazards models have meaningful interpretations. In our model, $\exp(\alpha)$ is the ratio of the estimated hazard for biopsied to non-biopsied mussels.

Glycogen concentrations in biopsied samples were reported on a wet-weight basis. Foot tissue in *A. p. plicata* is ~88% water, so wet-weight concentrations ×0.12 converts to glycogen on a dry-weight basis.

Glycogen analysis

We developed an analytical method for measurement of low concentrations of glycogen in unionid mussel tissue. We analyzed 10 sets of samples (including tissue plus quality control samples) over 9 mo, and present the results as evidence of the validity of the procedure. All glassware was acid-washed (10% HNO_3 for 24 h) to eliminate cellulose lint, which can interfere with glycogen analyses (Montgomery 1957, Lo et al. 1970).

A glycogen stock solution (5000 mg/L) was prepared by dissolving 250 mg of powdered glycogen (Type VII, *Mytilus edulis*, Sigma Chemical Company) in deionized water and diluting to 50 mL. The stock solution was serially diluted to prepare 4 aqueous calibration standards (i.e., 150, 500, 1000, and 1500 mg/L). The dilution resulted in a final mass of 0.0375–0.3750 mg glycogen in a 250-μL aliquot. We were unable to find a certified reference material, so we created an in-house reference material by compositing homogenized foot tissue from 3 fat pocketbook mussels, *Lampsilis cardium*. This reference material was prepared by adding 2.0 mL of 30% KOH per gram of foot tissue to a 10-mL screw-cap plastic tube and heating for 20 min in a water bath at 100°C. After heating, the contents were vortexed and cooled on crushed ice for 5 min.

We used the method of standard additions (Klein and Hach 1977) to demonstrate the absence of matrix interferences; 3 matrix standards were used to establish the slope of a matrix standard curve for comparison to the slope of the aqueous calibration standard curve. Matrix standards were prepared identically to aqueous calibration standards, except that matrix standards also contained a 10-μL aliquot of the in-house reference material, which contained ~3.8 mg of mussel tissue.

Bias associated with our glycogen determinations was estimated by recovery of known additions using matrix standards. Precision during glycogen analyses was estimated from triplicate analyses (relative standard deviation, RSD) of all matrix standards. We repeatedly measured the in-house reference material as another measure of precision. Method detection limit (MDL) and limit of quantification (LOQ) were estimated according to APHA et al. (1995) for 2 of the analytical sets by replicate analysis of the in-house reference material. The following quality control samples accompanied each set of samples analyzed: 1 procedural blank, at least 2 replicates of the in-house reference material, and 3 replicates of each aqueous calibration standard and matrix standard.

Reagents for the extraction and analysis of glycogen include 30% aqueous KOH (w/v), 95% ethanol (EtOH), 80% aqueous phenol (v/v), and reagent-grade concentrated H_2SO_4. The procedure for the extraction (steps 1–6) and the spectrophotometric analysis (steps 7–12) of glycogen follows:

1) Analyze or freeze (<−80°C) samples rapidly after dissection to minimize enzymatic breakdown of glycogen.
2) Place appropriate amounts of material to be analyzed (tissue, reagent blank, aqueous calibration standard, matrix standard, or a 10-μL aliquot of an in-house reference material) into a 2-mL screw-cap cryovial.
3) Add 30% KOH to each vial at a ratio of 2–10× the volume of material in the vial (e.g., for aqueous calibration standards, we added 250 μL of the standard and 500 μL of 30% KOH; for the biopsy samples, we added 100 μL of 30% KOH). The volume of KOH is not critical as long as the tissue is completely covered to ensure a complete digestion. Heat vials for 20 min in a water bath at 100°C.
4) After heating, vortex vials for 30 s and place on crushed ice for 5 min.
5) Add 95% EtOH to each vial at a ratio of 1.5× the volume of KOH. To prevent the coprecipitation of other polysaccharides, the resulting solution should be ~50% EtOH (Good et al. 1933, Van Handel 1965). Vortex each vial for 5 s and heat for an additional 15 min in a water bath at 100°C.
6) Extraction of glycogen from tissue samples should now be complete; this tissue digestate can be analyzed for glycogen immediately or it can be stored at <−80°C for up to 9 mo prior to analysis. If stored frozen, allow samples to thaw at room temperature prior to analysis.
7) Wash the contents of each vial into a 10-mL

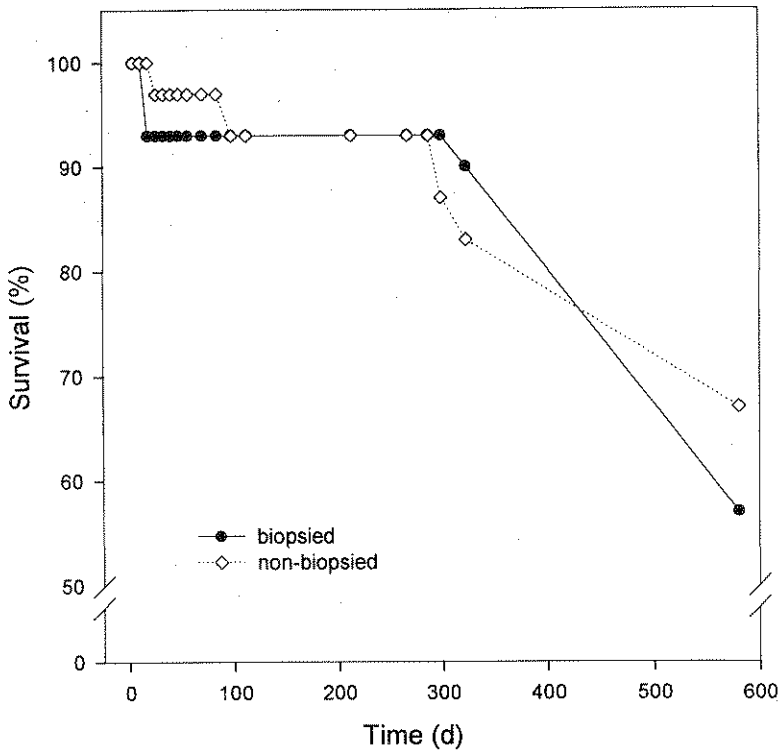

FIG. 1. Percent survival of biopsied and non-biopsied *Amblema plicata plicata* in mussels after a 581-d observation period. $n = 30$ for each group.

graduated test tube with 5 successive rinsings of 1000 μL of deionized water. Dilute all samples to a common volume with deionized water (we used a final volume of 6600 μL). Vortex the contents of each test tube for 10–15 s.

8) Remove a single 2-mL aliquot of the solution and place into a test tube (16-mm dia).
9) Add 100 μL of 80% phenol to each test tube.
10) To the same test tube, rapidly add 5 mL of H_2SO_4, directing the stream of acid to the liquid surface, and vortex the tube for 5–10 s. Repeat steps 8, 9, and 10 on all remaining samples.
11) Allow the samples to remain at room temperature for 30 min to obtain maximum color development.
12) Determine the absorbance of each sample at 490 nm on a spectrophotometer (e.g., Beckman DU-640, 1-cm path length).

The above method simplifies earlier methods in 3 ways. We evaluated each simplification by comparing the slopes and intercepts of aqueous or matrix standard curves with and without the simplification. First, some investigators recommend using Na_2SO_4 with the KOH when low concentrations of glycogen are expected (Van Handel 1965, Lo et al. 1970). However, there were no significant differences in the slopes ($p = 0.06$) or intercepts ($p = 0.38$) of the 2 resulting aqueous curves, 1 with Na_2SO_4 and 1 without Na_2SO_4. Second, some investigators used centrifugation to isolate glycogen from remaining tissues (Good et al. 1933, Lo et al. 1970), whereas others suggested that centrifugation results in lower recoveries of glycogen in small sample sizes, perhaps as a result of decanting a significant fraction of glycogen in the supernatant after centrifugation (Van Handel 1965, Dietz and Stern 1977). We again found that the slopes ($p = 0.18$) and intercepts ($p = 0.25$) of the regression lines from matrix standard curves were not statistically different with and without centrifugation. Third, Good et al. (1933) showed that KOH at a ratio of 2× the tissue mass was necessary to breakdown tissue and liberate glyco-

TABLE 1. Means (ranges in parentheses) for least squares regression parameters of aqueous calibration standard and matrix standard curves from 10 analytical sets for the determination of glycogen. There were no statistical differences ($p > 0.05$) in the slopes of the regression lines between the 2 curve types on 9 of the 10 analysis dates.

	Curve type	
Parameter	Aqueous calibration standard	Matrix standard
Mean slope	3.317 (2.565–4.095)	3.377 (2.667–4.244)
Mean intercept	−0.008 (−0.053–0.034)	0.111 (0.064–0.151)
Mean adjusted R^2	0.991 (0.969–0.998)	0.986 (0.957–0.998)
Mean % difference between slopes[a]	1.8	

[a] Mean % difference between slopes was calculated as the absolute difference between the mean aqueous calibration standard curve slope and the mean matrix standard curve slope divided by the grand mean of the 2 slopes. The quotient was multiplied by 100 to obtain the relative % difference.

gen. When the mass of tissue to be digested varies among samples, this method would require varied amounts of KOH to be added to each sample and, hence, varied amounts of all other reagents to maintain a similar composition of the digestion solution. The method would become tedious and error-prone when analyzing hundreds of tissue samples of varying masses. To determine if excess KOH (more than 2× the standard volume) would affect the quantification step of the method, we compared 2 aqueous standard curves, 1 with varied and 1 with constant volumes of KOH. This comparison revealed that the slopes ($p = 0.15$) and intercepts ($p = 0.82$) of the regression lines were not statistically different. Based on these results, the proposed glycogen method does not use Na_2SO_4 with the KOH, does not involve centrifugation, and uses a constant volume of KOH.

Glycogen concentrations were normally distributed and had homoscedastic variances, so all subsequent analyses were performed on untransformed data. Aqueous calibration standard and matrix standard curves were generated with linear regression models (SAS Institute 1988). To evaluate possible matrix interferences via the standard additions procedure, we compared the slopes of the aqueous calibration standard and matrix standard curves for each date with analysis of covariance (Sokal and Rohlf 1981). A Type I error (α) of 0.05 was used to judge the significance of all statistical tests.

Results

We recovered 100% of both biopsied and non-biopsied mussels during all observation periods. We did not detect significant differences in survival between biopsied and non-biopsied mussels ($p = 0.20$) during the 581-d observation period. The ratio of hazards of biopsied to non-biopsied mussels [exp(α)] was 0.56 with a 1-tailed 95% confidence upper bound of 1.18. Therefore, the excess hazard to mussels subjected to the biopsy technique was at most 18%. Survival of *A. p. plicata* exceeded 83% in both biopsied and non-biopsied mussels throughout the 1st y after the biopsies were performed (Fig. 1).

The wet weights of the biopsied tissues ranged from 4.4 to 11.4 mg per mussel. They had a mean (±1 SE) glycogen concentration of 22.3 ± 1.4 mg/g (range 11.6–37.4 mg/g). Glycogen content of the biopsy samples ranged from 0.09 to 0.36 mg, well above both the MDL (0.01–0.02 mg) and the LOQ (0.03–0.04 mg) determined for this analytical method (results presented below).

Aqueous standard and matrix standard curves were linear from 0.0375 to 0.3750 mg of glycogen in our method. R^2 values always exceeded 0.96. Slopes of the aqueous calibration standard and matrix standard regression lines were not significantly different ($p > 0.05$) in 9 of the 10 analytical sets (Table 1), thus demonstrating the absence of matrix interferences and validating the use of the aqueous calibration standard curve to predict the mass of glycogen. Across all analytical sets, the mean slope was 3.32 for the aqueous calibration standard curve and 3.38 for the matrix standard curve, a difference of 1.8% (Table 1).

The RSD for triplicate analyses of matrix standards averaged 9.2% and ranged from 4 to 14% across 3 concentration levels on 10 analysis dates (Fig. 2a, Table 2). The mean % recovery of glycogen added to the in-house reference material (matrix standards) was 103% and varied from 82 to 132% (Fig. 2b), indicating relatively low bias especially at this small mass of glyco-

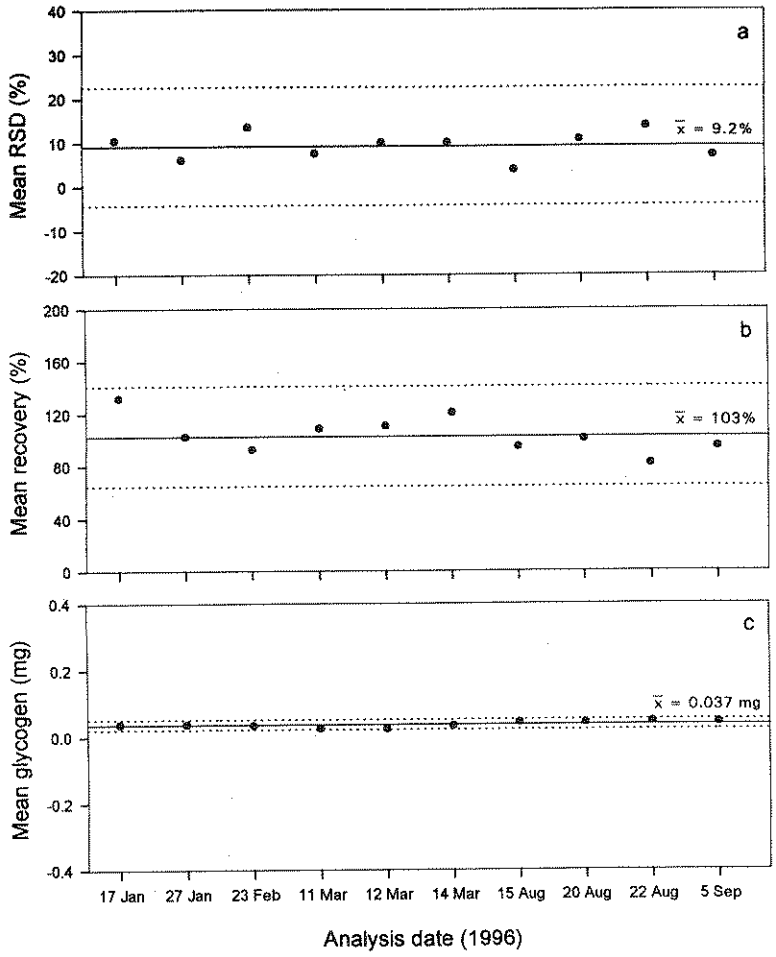

FIG. 2. Variations in (a) the relative standard deviation (RSD) among triplicate analyses of matrix standards, (b) mean % recoveries in matrix standards, and (c) mean estimated mass of glycogen in an in-house reference material on 10 separate analysis dates. Dashed lines represent ±2 SD.

gen in the in-house reference material. Slight differences in the recovery of glycogen between Fig. 2b and Table 2 were the result of averaging recovery of glycogen across analysis dates (Table 2) or across concentration levels (Fig. 2b). Thus, the proposed method consistently recovers close to 100% of the glycogen added to solutions containing between 0.024 mg (mass of glycogen in the 10-µL aliquot of the in-house reference material) and 0.308 mg (mass of glycogen in the 0.250 mg matrix standard) of glycogen.

The mean glycogen content in the in-house reference material has been consistent over the past 9 mo, ranging from 0.024 to 0.045 mg, with a mean of 0.037 mg (Fig. 2c), indicating that the homogenate can be stored for an extended period in a cryogenic (<−80°C) freezer. The RSD in triplicate analyses of the in-house reference material averaged 10.6%, indicating relatively high precision. Estimates of the method detection limit and the limit of quantification were 0.010 and 0.017 mg and 0.025 and 0.043 mg, respectively.

Discussion

The removal of 4–11 mg of foot tissue from *A. p. plicata* did not adversely affect survival for up to 19 mo after the biopsy was performed. A

TABLE 2. Mean % recovery of glycogen (±1 SD) in matrix standards and mean relative standard deviation (RSD ±1 SD) among triplicate analyses of matrix standards. Recoveries and RSD are based on 3 replicate samples on each of 10 analysis dates except for the 0.25-mg matrix standards that are based on 3 replicate samples on each of 5 analysis dates. Each matrix standard contained 10 µL of the in-house reference material and 250 µL of a standard.

Amount of glycogen added to in-house reference material (mg)	Mean recovery[a] (%)	Mean RSD[b] (%)
0.0375	103 ± 30	14.0 ± 7.3
0.1250	101 ± 7	4.2 ± 2.9
0.2500	101 ± 4	9.6 ± 3.4

[a] [(measured concentration in spiked sample) − (measured concentration in unspiked sample)/(concentration of known addition)] × 100

[b] [(SD of triplicate matrix standard)/(mean concentration in triplicate matrix standard)] × 100

similar conclusion was reached by Berg et al. (1995), who performed mantle biopsies on *Quadrula quadrula* and *Actinonaias ligamentina* and found no significant differences in survival in either species up to 13 mo after the biopsies. Furthermore, 1-y survival estimates for *A. p. plicata* for this study (83–90%) were similar to 13-mo survival estimates in *Q. quadrula* (84–87%, Berg et al. 1995). This mass of tissue (4–11 mg) is more than adequate for the quantification of glycogen with the modified analytical method.

Although our study showed a substantial decline in survival of *A. p. plicata* after 1 y (Fig. 1), survival still exceeded that commonly reported in the literature for relocated mussels. Cope and Waller (1995), for example, reviewed 33 mussel relocation projects and found a mean survival of 51% with most mortality occurring during the 1st y. Because freshwater mussels can survive for extended periods of time on stored energy reserves, the delayed decline in survival suggests that inadequate nutritional resources in the pond may have contributed to the reduced survival.

The modified method for the analysis of glycogen in small tissue samples (10 mg wet weight) has relatively high precision and low bias, and can detect and quantify relatively small amounts of glycogen extracted from tissue. Compared to other methods (Good et al. 1933, Lo et al. 1970), the modified procedure allows 30% more samples to be analyzed on a given day (because centrifugation is not required) and reduces reagent use and cost several fold. Reliable method detection limits and limits of quantification, reproducible analyses of the matrix standards and the in-house reference material, and acceptable recoveries of glycogen all suggest that the modified method is accurate and linear for the analysis of glycogen in the 35- to 350-µg range. Lower glycogen values have been reported (Van Handel 1965, Lo et al. 1970); however, method detection limits and limits of quantification were not reported in these studies.

Development of methods to biopsy freshwater mussel tissue without adversely affecting survival will allow researchers to measure changes in a number of tissue-related indicators of animal health. These techniques may be particularly beneficial with threatened and endangered species for which sacrificing the organism may jeopardize the population. The measurement of glycogen in these long-lived (up to 70 y) animals is critical because traditional measures of condition, such as shell length or weight, are often inappropriate during short-term observations. Furthermore, the measurement of glycogen is an early indicator of physiological stress in organisms that precedes measurable changes in survival (Haag et al. 1993).

This study focused on quantifying glycogen in samples of foot tissue in unionid mussels, but the procedure can likely be applied to other tissues (mantle, gill) and other organisms. The method has been successfully applied to the measurement of whole-body glycogen concentrations in fingernail clams (*Musculium*) and burrowing mayflies (*Hexagenia*) (T. J. Naimo, unpublished data). Further research is focused on evaluating differences in glycogen concentrations between tissue types in unionid mussels.

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