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[Note]

Sample Preparation Techniques for Determination of Fish Energy Density via Bomb Calorimetry: An Evaluation Using Largemouth Bass

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Abstract.—We evaluated three homogenization and subsampling techniques for preparing fish tissue samples for bomb calorimetry to identify differences in efficiency for estimating fish energy density. We compared (1) drying the whole fish and homogenizing the dried fish tissue, (2) homogenization prior to drying and then drying the subsample of fish tissue, and (3) homogenization after autoclaving to soften the hard structures and then drying a subsample of the homogenized fish tissue. Sample drying time and energy density estimates were compared among techniques across a size range (wet mass = 32–1,080 g) of largemouth bass *Micropterus salmoides*. Both of the subsampling techniques reduced drying time by about 40% relative to drying the whole fish. All three methods provided statistically similar estimates of largemouth bass energy densities. The autoclave process was most efficient, minimizing both sample preparation time and drying time. Variance of energy density estimates was greater for both subsampling methods compared with the traditional whole-fish method. Thus, subsampling can decrease sample preparation time for bomb calorimetry but may reduce power to detect differences among variables of interest (e.g., season). Lastly, estimates of energy density for largemouth bass were a function of body mass, suggesting that using a constant energy density in bioenergetics models is not appropriate.

Bioenergetics models are commonly used for estimating fish consumption and growth (Chipps and Wahl 2008). Predator energy density is an essential parameter required for bioenergetic modeling because it determines the net energy required for growth. Energy density has been found to vary within fish species as a function of body size, geographic location, sex, and reproductive status (e.g., Stewart et al. 1983; Rand et al. 1994; Anthony et al. 2000), which can bias consumption or growth estimates if not considered. However, determining the energy density of fish, particularly large individuals, can be labor intensive and can require expensive equipment to homogenize and subsample an individual fish. Thus, it is common practice to borrow energy density estimates from related species or use indirect measures to determine energy densities (Ney 1993; Hartman and Brandt 1995). Although these practices can prove expedient, it is often necessary to verify the energy density of the fish species in question with direct observation.

Determining the whole-body energy density of a fish traditionally requires the entire fish to be dried and homogenized, and replicate samples are then ignited in a bomb calorimeter (Rand et al. 1994). Caloric density per gram of wet mass can then be calculated by multiplying caloric density per gram of dry mass by the dry mass : wet mass ratio. Grinding dry tissue permits the use of burr or centrifugal grinders that can effectively pulverize hard tissues (such as scales and bone) to facilitate homogenization. Depending on the biomass, drying a whole fish may require a large amount of time, particularly for large-bodied fish. Drying a subsample of the whole-body fish tissue decreases the amount of drying time and allows a greater number of samples to be dried at one time in limited drying oven space. However, calcified structures within fish (e.g., vertebrae) and other tough tissue (e.g., skin) may make manual homogenization of wet fish tissue difficult and can require expensive equipment for blending. The inability to completely homogenize both wet and dry samples makes proper subsampling a challenge and can result in additional variation in analyses within samples.

In this study, we compared three methods of homogenizing and subsampling fish tissue for bomb calorimetry: (1) homogenization after drying the whole fish, (2) drying a subsample of the fish tissue after using a meat grinder to homogenize the whole fish, and (3) drying a subsample of the fish tissue after subjecting the fish to autoclaving and homogenization. An autoclave was used to soften the hard structures of the fish to facilitate homogenization and subsampling. The three methods were evaluated in terms of energy density estimates as a function of percent dry mass and wet mass as well as the amount of time required for drying across a wide size range of largemouth bass *Micropterus salmoides*.

Methods

Fish collection.—Largemouth bass were collected from a pond at Auburn University's E. W. Shell

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Fisheries Research Station, Auburn, Alabama, on 16 September 2008 using a boat-mounted electrofisher (Smith-Root Model 7.5 GPP, 7,500 W). Fifteen largemouth bass were collected across a size range (150–450-mm total length at 20-mm increments) for each sample preparation treatment (i.e., traditional, subsample, and autoclave plus subsample). Total length (nearest mm) and mass (nearest g) were recorded for each largemouth bass, and the stomach contents were removed.

Sample preparation.—Largemouth bass from each 20-mm size-class were randomly assigned to the three treatments. For the traditional method, whole fish were cut into 2.5-cm cubes and were oven-dried at 70°C until a constant mass was achieved for two consecutive days (±0.01 g between days); the final dry mass was then recorded. For the subsample method, the 2.5-cm cubes were pulverized in a meat grinder (LEM number-10 stainless-steel manual meat grinder) prior to drying, and the tissue was homogenized by hand. A 40–60-g subsample was weighed and oven-dried to a constant mass as above. For the autoclave plus subsample method, whole fish were placed into oven bags that were loosely sealed, and bags were placed in a steam-generated autoclave (Barnstead laboratory sterilizer, Model C-1761) at 120°C and 1.4 kg/cm^2 for 1 h. Mass was recorded before and after the autoclave process to document any change in moisture content. The fish was then homogenized using a hand mixer, and a 40–60-g subsample of the homogenate was removed and oven-dried in a manner similar to that described above.

Energy content determination.—Dried samples were blended to a homogeneous mixture using a standard coffee grinder and then were redried to a constant mass (±0.01 g for two consecutive days) to remove any moisture that had accumulated during homogenization. At least two 0.1–0.2-g pellets were formed and ignited in a semi-micro bomb calorimeter (Parr Instrument Co. Model 1425 or 6725) to measure caloric content. A third pellet was analyzed if the caloric values of the first two were not within 2% of each other. Caloric values for all pellets were averaged to estimate caloric density (cal/g dry mass) of the sample. The caloric density per wet mass of the sample was then determined by multiplying the energy density of the dry sample by the proportion of final dry mass to original wet mass.

Statistical analyses.—Analysis of covariance (ANCOVA) was used to determine whether estimates of caloric density were affected by sample preparation method (GLM procedure; SAS Institute 2008). The percent dry mass was used as the covariate to determine the overall effect of treatments on caloric density. We used percent dry mass in lieu of total length or mass to reduce the variation in caloric density in relation to sex, maturity state, and condition. Sample preparation method was used as a class variable to determine whether elevation differences existed among treatments in the regression of caloric density on percent dry mass; percent dry mass was used to determine the effect on caloric density among all treatments; and the interaction between percent dry mass and sample preparation method was used to evaluate whether slope differences existed among treatments in the caloric density–percent dry mass regression. Residuals were assessed for normality and homogeneity of variance as a function of percent dry mass, sample preparation method, and predicted values to ensure that assumptions of ANCOVA were met. Analysis of variance (ANOVA) was used to determine whether drying time differed among the three treatments (GLM procedure; SAS Institute 2008). Normality and homogeneity of variance of the residuals were assessed similar to above to ensure that the assumptions of ANOVA were met.

Piecewise regression was used to determine the relationship between largemouth bass energy density and total wet mass using the program Joinpoint (National Cancer Institute 2008). We fit piecewise regression models containing zero to three knot values and used the Bayesian information criterion (BIC; Tiwari et al. 2005) to determine the best-fit piecewise regression model. The residuals from the best model were compared among methods to examine the effect of sample preparation method while controlling for the effect of body size.

Results

Sample preparation method had a significant effect on drying time (ANOVA: $F_{2,42} = 17.73$, $P < 0.001$). Compared with drying whole fish (i.e., traditional method), both subsampling methods reduced the amount of time (d) required for drying by approximately 40% ($t_{43} > 4.65$, Bonferroni-adjusted $P < 0.001$; Figure 1). Wet mass energy density (cal/g) increased with percent dry mass of the sample (ANCOVA: $F_{1,39} = 142.51$, $P < 0.001$; Figure 2). The sample preparation method did not affect the slope (ANCOVA: $F_{2,39} = 1.98$, $P = 0.15$) or elevation (ANCOVA: $F_{2,39} = 1.96$, $P = 0.15$) of this relationship, indicating that neither the subsampling nor the autoclave process affected energy density estimates. The regression model for all methods pooled was

$$\text{energy density (cal/g wet mass)} = -376.56 + 60.41 \times \text{dry mass (\%)}$$

and explained 83% of the variation in energy density ($F_{1,43} = 204.53$, $P < 0.001$). Additionally, the mean

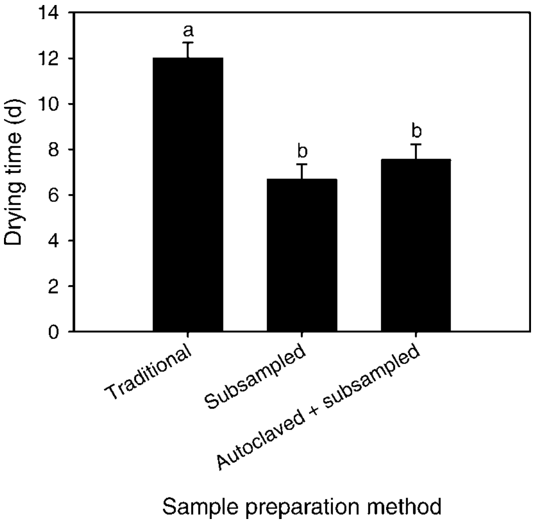

FIGURE 1.—Mean (+SE) drying time (d) of largemouth bass samples prepared for bomb calorimetry via three preparation methods. Different letters above the bars indicate significant differences ($\alpha = 0.05$).

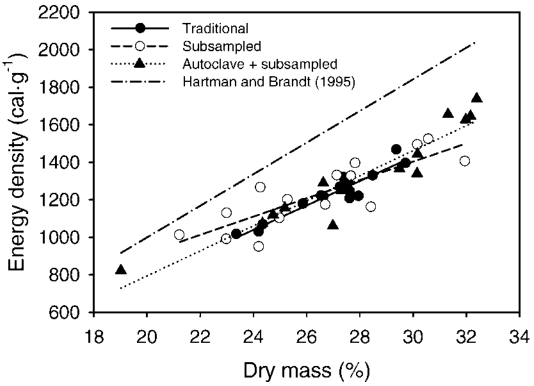

FIGURE 2.—Energy density (cal/g; wet-mass basis) plotted against percent dry mass (with associated regression lines) of largemouth bass samples prepared for bomb calorimetry via three preparation methods. The estimated energy density based on percent dry mass from the relationship proposed by Hartman and Brandt (1995) is also included.

coefficient of variation (CV = 100 × SD/mean) within each fish (i.e., measurement precision) for the traditional, subsampling, and autoclave plus subsampling methods was 2.3, 2.3, and 1.1%, respectively. However, within-fish variance was similar among methods (Levene's test: $F_{2,42} = 0.61$, $P = 0.55$), indicating that sample homogenization was equal among the three techniques.

The relationship between energy density of largemouth bass and wet mass was best explained by a two-piece regression model with a single knot value (Table 1; Figure 3). The mass (*M*) at which the trend in energy density changed (i.e., the knot value) for all methods pooled was 174 g, resulting in the following model:

$$\text{energy density (cal/g wet mass)} = \begin{cases} 816.54 + 3.55M & \text{for } M \leq 174\,\text{g} \\ 1{,}489.44 - 0.31M & \text{for } M > 174\,\text{g} \end{cases}$$

Residuals from the two-piece regression model had unequal variances among the sample preparation methods (Brown and Forsythe's test: $F_{2,42} = 5.23$, $P = 0.009$). Equality of variance tests on residuals indicated that the variance of caloric estimates for the traditional method was approximately seven times smaller than that observed for both subsampling methods (folded $F_{14,14} > 7.69$, $P < 0.001$), but the variance was similar between subsampling methods (folded $F_{14,14} = 1.11$, $P = 0.85$). Controlling for heterogeneous variances, residuals from the two-piece regression model were similar among all methods (Welch's ANOVA: $F_{2,22.46} = 1.70$, $P = 0.21$), indicating that caloric density values were equal among methods when accounting for the effect of body size.

Discussion

We found that the relationship between caloric density (cal/g wet mass) and percent dry mass was similar among all sample preparation methods in terms of elevation and slope, suggesting that preparation method did not affect caloric estimates. Residuals from the two-piece regression model were similar among all methods, also indicating that caloric density values were not statistically different among methods when accounting for the effect of body size. In addition, both subsampling techniques took 40% less drying time compared with the traditional method, increasing the efficiency of the sample preparation process. However, variance of residuals was smaller using the traditional method compared with both subsampling methods but was similar between subsampling methods. These results suggest that subsampling can decrease the time required for determination of energy density but will reduce the power to detect differences among a variable of interest (e.g., season). Despite the higher variability in energy density estimates, subsampling techniques may increase the number of samples that can be processed, which would offset the loss in power due to increases in degrees of freedom. Additionally, it is possible that a larger subsample may decrease the variability in energy density estimates, but this was not tested in our study. In future studies, it may be advantageous to determine an optimal subsampled proportion of fish tissue that minimizes the variance of

TABLE 1.—Piecewise regression model selection results displaying the number of knots, sample size (N), number of parameters (k), sum of squared errors (SSE), and Bayesian information criterion (BIC) for each tested model of largemouth bass energy density versus wet mass.

Model	Number of knots	N	k	df	SSE	BIC
1	0	45	2	43	1,715,498.66	10.72
2	1	45	4	41	932,871.72	10.28
3	2	45	6	39	816,646.72	10.31
4	3	45	8	37	781,087.50	10.44

energy density estimates while maintaining the benefits of increased efficiency.

Caloric density as a function of body mass (g) was best explained by a two-piece regression model. Energetic density increased rapidly up to 174 g, at which point the energy density declined gradually and may represent the rapid accumulation of lipids up to a given threshold as largemouth bass increase in size (Ludsin and DeVries 1997; Garvey et al. 1998). This suggests that the common use of a single fixed value for largemouth bass energy density (i.e., 1,000 cal/g or 4.184 kJ/g) is not appropriate (e.g., Rice et al. 1983). Although our estimates of energy density for largemouth bass represent an improvement over using a fixed value, it is only a point estimate in time; thus, caution should be used in incorporating our function across annual bioenergetics simulations. We also found that predicted estimates of energy density from percent dry mass using an empirically derived relationship pooled across 22 species (Hartman and Brandt 1995) produced energy density estimates that were 5–45% higher than those derived in the laboratory (Figure 2). The resulting slope of the relation between energy density and percent dry mass was significantly different from our bomb calorimetry-derived estimates ($F_{3,86} = 610.68$, $P < 0.001$). This suggests that caution should be used in estimating energy density from the relationship provided by Hartman and Brandt (1995) and that energy density predictions should be verified with direct observation.

There are a number of techniques that can be used for estimating whole-body energetic density of fish, such as component analyses (see review by Paine 1971), but bomb calorimetry remains the most direct and widely used technique. In this study, we identified that some time-saving subsampling techniques can be used to prepare samples for bomb calorimetry when estimating energy density of large-bodied fish. Despite providing largemouth bass energy density estimates similar to those given by other methods, the meat grinder method was the most labor-intensive and required the greatest amount of time in terms of the sample preparation prior to drying. Although the amount of labor required for this method could be reduced by using an electric meat grinder, the whole fish would still need to be cut into pieces small enough to feed through the grinder. While we did not quantify the consistency of the final dried and homogenized tissue among sample preparation techniques, the autoclave process resulted in a very fine powder that facilitated the formation of pellets prior to bomb calorimetry. Scales from the fish still remained in the other samples, which made it more difficult to form pellets. Although the within-fish variation was statistically similar among methods, the lower CV observed using the autoclave method meant that analysis of a third pellet was required for fewer fish (i.e., a third pellet was bombed when the first two deviated more than 2% from each other). Only 20% of the fish from the autoclave method required a third pellet to be bombed in comparison with 47% and 60% of fish from the traditional and subsampling methods, respectively. While the high temperature of an autoclave (i.e., 120°C) may affect the fatty acid profile of fish, our results clearly indicate that the gross energy was not significantly affected by the process. Therefore, the autoclave process offered the most efficient alternative among the three examined methods by minimizing both sample preparation time and drying time while not affecting caloric density estimates.

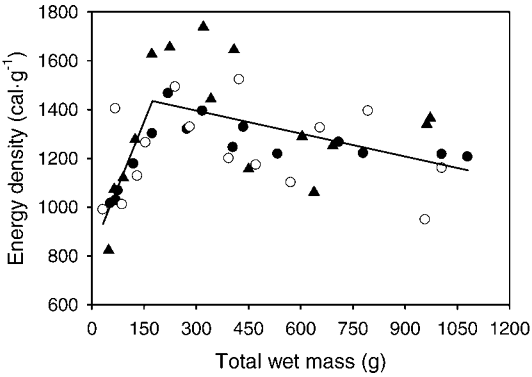

FIGURE 3.—Energy density (cal/g; wet-mass basis) plotted against total wet mass (g) of largemouth bass samples prepared for bomb calorimetry via three preparation methods (filled circles = traditional whole-fish method; open circles = subsampling method; triangles = autoclaving plus subsampling method). The solid line is the best-fit piecewise regression model for all methods combined.

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