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Proximate composition, lipid utilization and validation of a non-lethal method to determine lipid content in migrating American shad *Alosa sapidissima*

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Lipid content forms the most important energy reserve in anadromous fish and can limit survival, migration and reproductive success. A fat meter was evaluated and compared with a traditional extractive method of measuring available lipid for migrating American shad *Alosa sapidissima* in the Connecticut River, U.S.A. The fat meter gives rapid (<10 s) and non-lethal lipid measurements, whereas traditional methods require lethal sampling that is both time consuming and expensive. The fat-meter readings had a strong relationship to traditional lipid extractions for 60 fish, 30 whole body ($R^2 = 0{\cdot}72$) and 30 fillet only ($R^2 = 0{\cdot}81$). Additional validation showed that fat-meter readings captured the gradual decrease of lipid in individual fish over time, were not affected by removal of gonads or scales and were stable for fish exposed to water or air for 24 h after death. These experiments indicate that the fat meter can be used as a reliable tool for future *A. sapidissima* energetic studies, allowing for larger sample sizes and non-lethal sampling.

Key words: *Alosa sapidissima*; fat meter; lipid; lipid utilization; non-lethal sampling; proximate composition.

INTRODUCTION

Many anadromous fish species rely on lipid reserves to transverse long distances to complete their life cycle (Roff, 1988). Lipid availability and allocation are considered primary drivers of successful spawning for anadromous species and have important implications for traits such as iteroparity (Jonsson *et al.*, 1997; Berg *et al.*, 1998; Doucett *et al.*, 1999). Traditionally, energetic studies have relied on costly and time consuming proximate composition analysis to determine available lipid in fish. These methods require lethal sampling and typically limit sample sizes due to the time and cost of these analyses. Recent technological advances have transitioned these studies to using a rapid, non-lethal technique employing a handheld fat meter that emits microwaves to estimate lipid concentration in tissue.

Several studies have tested the fat-meter technology with a variety of species including: salmonids (*Salmo salar* L. 1758; *Oncorhynchus* spp.) (Hendry & Beall, 2004; Crossin & Hinch, 2005; Quillet *et al.*, 2005), Atlantic herring *Clupea harengus* L.

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1758 (Davidson & Marshall, 2010; McPherson *et al.*, 2010) and European eel *Anguilla anguilla* (L. 1758) (Klefoth *et al.*, 2013). Each of these studies found a strong correlation between the fat meter and proximate composition analysis; additionally, the fat meter was found in many of these studies to have a better estimate of lipid content then other common measurements, including bioelectric impedance, relative condition factor and Fulton's *K*. Before carrying out comprehensive studies, this approach should be validated for each new species against traditional methods to determine the relationship between the fat-meter readings and lipid content. In addition, many aspects of this approach, such as what portion of the body is actually being evaluated for lipid content, have not been examined for any species.

Alosa sapidissima (Wilson 1811) is an anadromous species native to eastern North America that makes spawning migrations in rivers from Florida, U.S.A. to Canada (Greene *et al.,* 2009). *Alosa sapidissima* show clinal variation in their reproductive strategy, being semelparous in their southern range and gradually becoming more iteroparous in northern populations (Glebe & Leggett, 1981*a*). In the Connecticut River, *A. sapidissima* are partially iteroparous and deplete their energy resources by 35–60% during migration (Glebe & Leggett, 1981*a*; Leonard & McCormick, 1999). *Alosa sapidissima* had been thought to stop feeding during migration (Leim, 1924; Nichols, 1959), placing an additional burden on energetic reserves. However, in the York River, VA, USA *A. sapidissima* were shown to feed prior to finishing the remainder of their migration to spawning grounds and then began to feed again during downstream migration once returning to the estuary (Walter & Olney, 2003). Despite possible estuarine feeding before and after spawning, *A. sapidissima* were not able to maintain prior migration lipid levels, probably due to lipid required for migration, spawning and reallocation to gonads (Walter & Olney, 2003).

Available lipid of migrating *A. sapidissima* is thought to directly affect iteroparity (Leonard & McCormick, 1999; Castro-Santos & Letcher, 2010) and be strongly affected by temperature (Glebe & Leggett, 1981*b*). Glebe & Leggett (1981*b*), when comparing semelparous and iteroparous populations of *A. sapidissima* suggested that once a threshold of 60% of energy reserves had been exhausted, *A. sapidissima* would tend to be semelparous; Leonard & McCormick (1999) suggested a threshold of 35–40% for Connecticut River *A. sapidissima*. This suggests that iteroparity rates would decrease if higher amounts of lipid are utilized during migration. Further, Leonard *et al.* (1999) found that temperature had a significant effect on metabolic rate, which would lead to *A. sapidissima* having a less efficient capacity to utilize lipid when at higher temperatures, effectively leading to a higher utilization of lipid and lower iteroparity.

Previous work that described *A. sapidissima* available lipid in the Connecticut River used traditional methods to determine lipid content, which limited their sample sizes (Glebe & Leggett, 1981*a*, 1981*b*; Leonard & McCormick, 1999). *Alosa sapidissima* lipid content remains an important research topic, especially as climate change increases water temperatures (Graham & Harrod, 2009), potentially changing the rate of lipid utilization for *A. sapidissima*. Future studies with higher sample sizes can address remaining unknowns related to lipid use of *A. sapidissima*. Groundwork studies on Connecticut River *A. sapidissima* lipid use (Glebe & Leggett, 1981*a*, 1981*b*) occurred before the implementation of a fish lift at Holyoke Dam (139 km from river mouth) and now *A. sapidissima* have access to spawning grounds up to and beyond Bellows Falls Dam (280 km from river mouth). Leonard & McCormick (1999)

is the only study to investigate *A. sapidissima* lipid use since the spawning grounds have been expanded and this study focused on whole-body energy use for each tissue, strictly limiting the number of fish that could reasonably be tested.

Using the fat meter could increase the sample sizes several fold compared with traditional methods. Multi-year studies could investigate how year-to-year changes in temperature and flow affect lipid levels throughout the *A. sapidissima* migration, potentially leading to a better understanding of iteroparity rates. Higher sample sizes would lead to a better understanding of how lipid is utilized throughout the migration, sections of the migration where lipid availability is less certain (*e.g.* entrance from ocean, post-spawn out-migrating fish) and further elucidate dimorphic differences between females and males. The present study evaluated the performance of the fat meter and validated its measurements to determine the efficacy of this technology to measure the lipid content of migrating *A. sapidissima*.

MATERIALS AND METHODS

FISH COLLECTION

Migrating adult *A. sapidissima* were sampled at four locations constituting the majority of the length of spawning grounds available in the Connecticut River and providing opportunity to measure the full range of lipids that could be observed. Locations included: Old Lyme, CT (1 km upstream), Holyoke Dam, Holyoke, MA (139 km upstream), Cabot Station, Turners Falls, MA (198 km upstream) and Vernon Dam, Vernon, VT (228 km upstream). Fish were captured by gillnet (Old Lyme) or trapped at hydroelectric facilities (Holyoke Dam, Cabot Station, Vernon Dam). Six fish were sampled with the fat meter for comparison to proximate composition analysis at each station over the entire course of the migration, from 30 April to 30 June 2015, covering the extent of the migration; additional fish were collected opportunistically throughout this sampling period for subsequent tests.

FAT METER

A Distell Model 692 Fish Fat Meter (Distell Inc., West Lothian, Scotland, U.K.), chosen for its larger sensor head, sized appropriately to measure larger fish such as *A. sapidissima*. The fat meter is a handheld device that uses microwave technology to determine water content in fish tissue, which is inversely related to lipid content (Craig *et al.*, 1978). The fat meter has a variety of settings made by the manufacturer for particular fish species. There is no manufacturer setting for *A. sapidissima* and no global setting, therefore the most similar species setting available, herring-1, which is based on *C. harengus*, was used. We then used our results from proximate analysis of lipid composition described below to convert readings from the herring-1 setting to values that were calibrated correctly for *A. sapidissima*.

After collection, fish were sacrificed and placed on their side on a measuring board, held down by one person while another person pressed the fat-meter sensor head firmly onto the fish's skin, targeting the white muscle, above the midline just below the dorsal fin (position 1), anterior to position 1 (position 2) and posterior to position 1 (position 3) (Fig. 1). Each fish had two replicate fat-meter readings for each of the three positions and on both sides. All fat-meter readings (at each position and including replicates) took <30 s. After fat-meter readings were taken, whole fish (excluding gonads) were immediately placed in plastic bags, sealed and stored at −20° C for 3 months. Sixty fish were selected for proximate composition analysis, nine for scale and gonad effects, 19 for water and air exposure and 61 for repeated measures at hatchery.

PROXIMATE COMPOSITION ANALYSIS

For each fish selected, proximate composition analysis was performed for either whole body (excluding gonads and scales) or fillet (excluding scales) to determine if the fat meter is a good

FIG. 1. (a) Side-view of an *Alosa sapidissima* indicating positions (1, 2 and 3) where fat-meter readings were taken and (b) fat meter in use at position 2.

indicator of lipid content for the entire fish, or only for the fillet. Specific fish which represented the full range of fat-meter readings were selected for analysis. Whole-body or fillet samples were homogenized with an equal volume of de-ionized water. The resulting homogenates were sampled for percent lipid, moisture (water), protein and ash (minerals) content. Lipid was extracted from the homogenate (1 ml) using 20 volumes of 2:1 chloroform-methanol following the methods of Folch *et al.*, (1957). The homogenate and chloroform-methanol solution was homogenized for 60 s in a 20 ml scintillation vial. The resultant mixture was filtered, rinsed with chloroform-methanol solution and 2 ml of 0·73% NaCl solution was added to a pre-weighed scintillation vial, mixed and let stand at room temperature for 1 h. After 1 h the sample separated into an upper aqueous and lower organic layer. The upper layer was removed and the lower layer was washed with a solution (48% 1·7 mM MgCl2, 49% methanol and 3% chloroform). The bottom layer, which contained the extracted lipid, was placed in an oven at 50° C overnight until a constant mass was achieved. Moisture content was determined by drying 1 ml of homogenate to a constant mass at 50° C (Busacker *et al.*,1990). Protein content was determined by the bicinchoninic acid method (BCA Protein Kit, Pierce; www.thermofisher.com). Ash content was determined (with a muffle furnace; Thermolyne 62 700, Dubuque, IA, USA)) by ashing the previously dried homogenate from the moisture content analysis at 450° C until a constant mass was achieved.

SCALE AND GONAD EFFECTS

It is unknown if fat-meter readings are affected by the presence of scales. *Alosa sapidissima* possess large scales and if scales affect fat-meter accuracy, requiring removal for accurate results, this would offset the convenience of the fat meter's rapid sampling ability, as well as

remove the non-lethal component. Although it is assumed that the fat-meter measures fat content of the muscle and not the abdominal cavity including gonads, to our knowledge this assumption has not been tested. To investigate if scales or gonads affected fat-meter readings, *A. sapidissima* fat-meter readings were taken for individual fish for each of the following treatments: whole fish, whole fish with gonads removed, fillet, fillet with scales removed. These fish were not included in the proximate composition analysis and were specifically selected to represent a wide range of lipid content.

WATER AND AIR EXPOSURE

Often it is not possible to sample fish immediately upon capture from the wild. To assess the effects of prolonged post-mortality exposure to air or water *A. sapidissima* were repeatedly measured with the fat meter at 0, 4, 19 and 24 h to determine if there were any changes to fat-meter readings over time. These fish were not included in prior analyses and were specifically selected to represent a wide range of lipid content. After collection, these fish were sacrificed and exposed to air (21·1° C) or water (20·1° C) for 24 h.

REPEATED MEASURES AT HATCHERY

Non-feeding animals should show a reduction in lipid content over time and the ability of the fat meter to detect this change provides further validation. *Alosa sapidissima* were held at North Attleboro National Fish Hatchery (www.fws.gov/northattleboro) and individuals were sampled three times over the course of their normal migratory period. Fish were trapped at Holyoke Dam on 7 May 2015, transferred to the hatchery and held in a 3·7 m diameter tank with a mixture of city and well water at a flow of 227·1 l min^{-1}. These fish were not fed and tank temperatures ranged from 13·9 to 21·1° C. Fish were individually tagged with PIT tags and fat-meter readings were taken before fish first entered the tank on 7 May 2015 (day 0), on 11 June 2015 (day 35) and 9 July 2015 (day 63).

ANALYSIS

Simple linear regression analysis was conducted to determine the relationship between fat-meter readings and lipid content. Each position and position combination (*e.g.* average of position readings 1 + 2) was evaluated to determine which reading or reading combination provided the most accuracy. Additionally, the relationship between lipid and moisture content was investigated. As *A. sapidissima* migrate and deplete their lipid reserves, moisture content (water) replaces the proportion of composition previously made up of lipid. A simple linear regression was conducted to ensure the expected inverse relationship was observed. Lipid and protein content relationships were also evaluated with a simple linear regression. As both lipid and protein are utilized as energetic reserves in migrating fish, the relationship between how each are used can indicate a change in energetic utilization. Additionally, proximate composition analysis parameters between whole-body and fillet samples were compared with *t*-tests. Statistical analyses were conducted with R statistical software (www.r-project.org).

For each of the following analyses, raw fat-meter readings were adjusted by the regression formula between fat-meter readings and extracted lipid content of the position or positions with the highest R^2 generated from the previous section. This adjusted lipid value represents an accurate lipid measurement for *A. sapidissima*. For the scale and gonad effects analysis, lipid content of whole fish, whole fish with gonads removed, fillet and fillet with scales removed was analyzed with a linear mixed model (LMM) fit by maximum likelihood. The dependent variable was per cent lipid content, fixed effect was treatment (whole fish, whole fish with gonads removed, fillet, fillet with scales removed) and the random effect was fish, the same individual being re-measured at each category. The model was fit using the lmer function in the lme4 package in R (Bates *et al.*, 2013). Differences between each treatment were determined by a type II Wald χ^2-test (ANOVA function in car package) and if significant ($\alpha \leq 0{\cdot}05$) a *post hoc* general linear hypothesis test–Tukey all-pair comparisons was conducted (glht function in the multicomp package in R).

Fish exposed to water and air analyses were analysed with LMMs following the methods of the prior section. The dependent variable was per cent lipid content, fixed effect was hour and the random effect was individual fish.

For the repeated measures at hatchery analysis, lipid content between days elapsed was analysed with a LMM following the same methods from prior sections. The dependent variable was per cent lipid content, fixed effect days elapsed and the random effect was individual fish. Confidence intervals were calculated using bootstrapping from the bootMer function in the lme4 package in R with 200 simulations deriving 95% confidence intervals using the predict function.

RESULTS

FAT METER AND EXTRACTED LIPID RELATIONSHIP

Sixty *A. sapidissima* were sampled, 30 whole body and 30 fillets. Fat meter readings were $\log_e$ transformed to provide a one-to-one relationship between fat-meter readings and extracted lipid content; a natural log transformation has previously been applied to other fat-meter studies (Crossin & Hinch, 2005; Hanson *et al.*, 2010; J. Colt & K. D. Shearer, unpublished data). The relationship between per cent extracted lipid to natural logarithmic transformed fat-meter readings was strong for both whole-body and fillet samples, as well as most position and position combinations (all relationships had a $R^2 > 0{\cdot}63$) (Table I). Positions 2, 1 + 2, 2 + 3 and 1 + 2 + 3 had the highest R^2 values (0·72 for each) for whole-body samples (Fig. 2) and position 1 and position 1 + 2 had the highest R^2 values (0·81 for each) for fillet samples (Fig. 3). Position 3 had the weakest relationship for both sample-types (R^2 of 0·63 and 0·69 for whole-body and fillet, respectively). Position 1 for the fillet samples had the best relationship (highest R^2; Table I) between extracted lipid and the fat-meter readings and the least number of readings, therefore it was selected to adjust raw fat-meter readings for the subsequent analyses (scale and gonad effects, water and air exposure and repeated measures at hatchery), representing an accurate lipid measurement for *A. sapidissima*.

PROXIMATE COMPOSITION ANALYSIS

Between whole-body and fillet samples, only per cent protein and ash content differed, with whole-body samples containing about 1% less protein and almost twice the amount of ash content. Lipid, moisture and the total of all components were

TABLE I. Correlation coefficients (R^2) between lipid content and $\log_e$ transformed fat-meter readings of whole body ($n = 30$) and fillet ($n = 30$) samples of *Alosa sapidissima*

Positions		Whole body	Fillet
1	Midline just below the dorsal fin	0·71	0·81
2	Anterior to position 1	0·72	0·79
3	Posterior to position 1	0·63	0·69
1 + 2		0·72	0·81
1 + 3		0·69	0·76
2 + 3		0·72	0·77
1 + 2 + 3		0·72	0·79

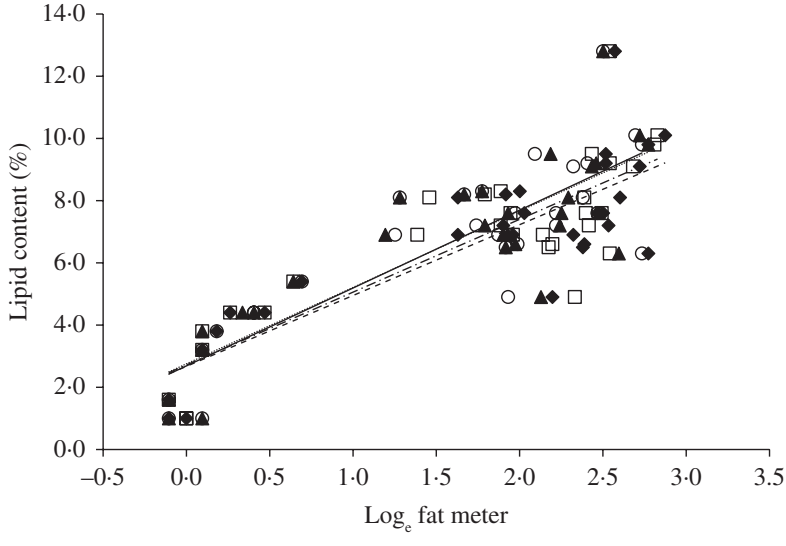

FIG. 2. Relationship between extracted lipid content and fat-meter readings of *Alosa sapidissima* for whole-body samples at position 2 (♦, _ _ _ _, $y = 2{\cdot}277\log_e x + 2{\cdot}673$, $n = 30$, $R^2 = 0{\cdot}72$, $P < 0{\cdot}001$), position 1 + 2 (□, _ . _ . , $y = 2.341\log_e x + 2{\cdot}718$, $n = 30$, $R^2 = 0{\cdot}72$, $P < 0{\cdot}001$), position 2 + 3 (○, ____, $y = 2{\cdot}504\log_e x + 2{\cdot}687$, $n = 30$, $R^2 = 0{\cdot}72$, $P < 0{\cdot}001$) and position 1 + 2 + 3 (▲, dotted line, $y = 2.454\log_e x + 2{\cdot}756$, $n = 30$, $R^2 = 0{\cdot}72$, $P < 0{\cdot}001$).

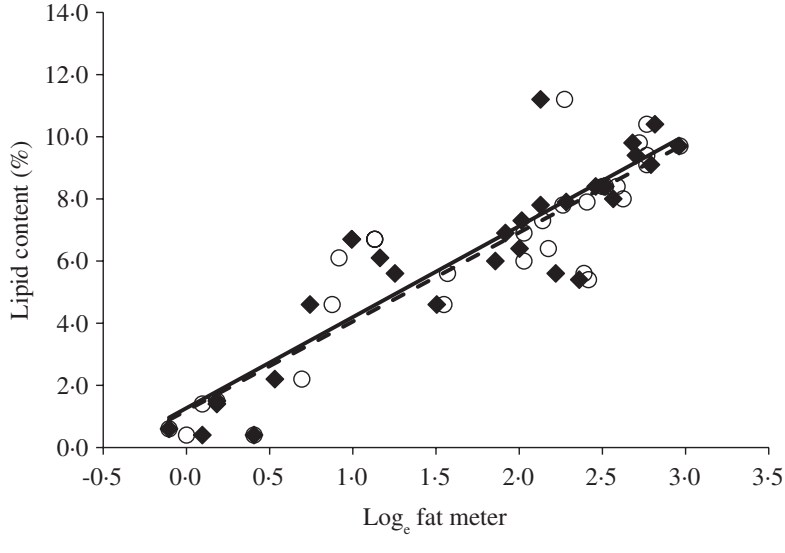

FIG. 3. Relationship between extracted lipid content and fat-meter readings of *Alosa sapidissima* for fillet samples at position 1 (♦, ____, $y = 2.927\ln x + 1{\cdot}269$, $n = 30$, $R^2 = 0{\cdot}81$, $P < 0{\cdot}001$) and position 1 + 2 (○, _ _ _ _, $y = 2.866\ln x + 1{\cdot}189$, $n = 30$, $R^2 = 0{\cdot}81$, $P < 0{\cdot}001$).

TABLE II. Composition (wet mass) of whole-body and fillet samples of *Alosa sapidissima* ($n = 30$, d.f. = 58)

	Composition (%)			
Component	Mean	S.D.	*t*-value	*P*
Moisture				
Whole body	72·1	3·4	0·178	>0·05
Fillet	72·3	4·7		
Protein				
Whole body	15·0	2·2	2·128	<0·05
Fillet	16·4	2·7		
Lipid				
Whole body	6·7	2·7	−0·587	>0·05
Fillet	6·2	3·1		
Ash				
Whole body	2·5	0·5	−10·822	<0·001
Fillet	1·5	0.1		
Total				
Whole body	96·3	2·1	0·165	>0·05
Fillet	96·4	2·1		

similar ($P > 0·05$ in all cases; Table II). Total fish composition was 96·3 and 96·4% for whole-body and fillet samples, respectively (Table II). Glycogen was not measured and has been shown to be *c*. 1% of migrating *A. sapidissima* composition (Leonard & McCormick, 1999).

Moisture content had a strong and expected inverse linear relationship with extracted lipid content, $R^2 = 0·91$ and 0·81 for whole-body and fillet samples, respectively (Fig. 4). For whole-body samples, lipid and protein content decreased linearly ($R^2 = 0·36$), *i.e*. lipid and protein content decreased at approximately the same proportional rate. For fillet samples lipid and protein content decreased exponentially ($R^2 = 0·46$), as lipid content decreased below approximately 2% an increased amount of protein was being utilized (Fig. 5).

SCALE AND GONAD EFFECTS

Predicted mean lipid content for whole body was 6·3%, whole body with gonads removed 6·6%, fillet 6·5% and fillet with scales removed 6·3% (Table III and Fig. 6). Wald χ^2-test was significant ($\chi^2 = 8·9$, d.f. = 3, $P < 0·05$) and one *post hoc* all-pair comparison was slightly below the $P < 0·05$ (whole body *versus* whole body with gonads removed, $P < 0·05$); all other comparisons were above ($P > 0·05$; Table III).

WATER AND AIR EXPOSURE

Nineteen *A. sapidissima* ($n = 10$ for water; $n = 9$ for air) were sampled and predicted mean lipid content for fish exposed to water was 4·9% at 0 h, 5·2% at 4 h, 4·7% at 19 h and 4·6% at 24 h (Table IV). Wald χ^2-test was significant ($\chi^2 = 29·8$,

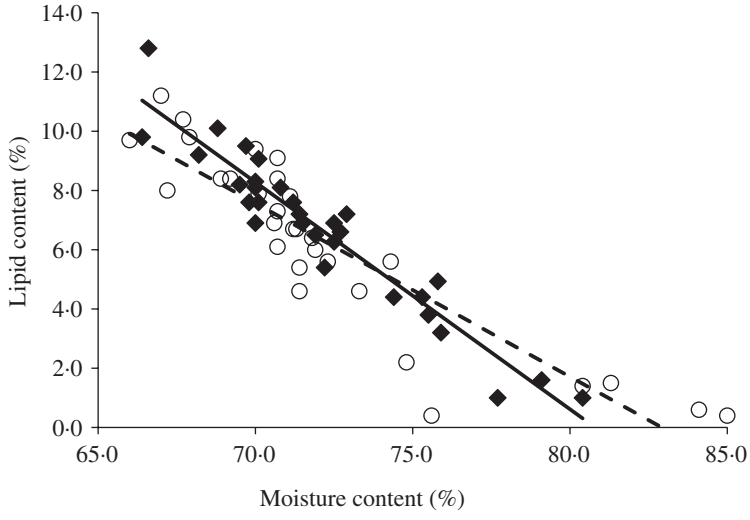

FIG. 4. Relationship between extracted lipid and moisture content of *Alosa sapidissima* for whole-body (♦, solid line, $y = -0{\cdot}767x + 61{\cdot}933$, $n = 30$, $R^2 = 0{\cdot}91$, $P < 0{\cdot}001$) and fillet (○, dashed line, $y = -0{\cdot}586x + 48{\cdot}574$, $n = 30$, $R^2 = 0{\cdot}81$, $P < 0{\cdot}001$) samples.

d.f. = 3, $P < 0{\cdot}001$) and three *post hoc* all-pair comparisons were significantly different ($P < 0{\cdot}001$; Table IV). Predicted mean lipid content for fish exposed to air was 4·9% at 0 h, 5·2% at 4 h, 4·9% at 19 h and 5·0% at 24 h (Table V). Wald χ^2-test was significant ($\chi^2 = 15{\cdot}921$, d.f. = 3, $P < 0{\cdot}001$) and two *post hoc* all-pair comparisons were significantly different ($P < 0{\cdot}001$) (Table V).

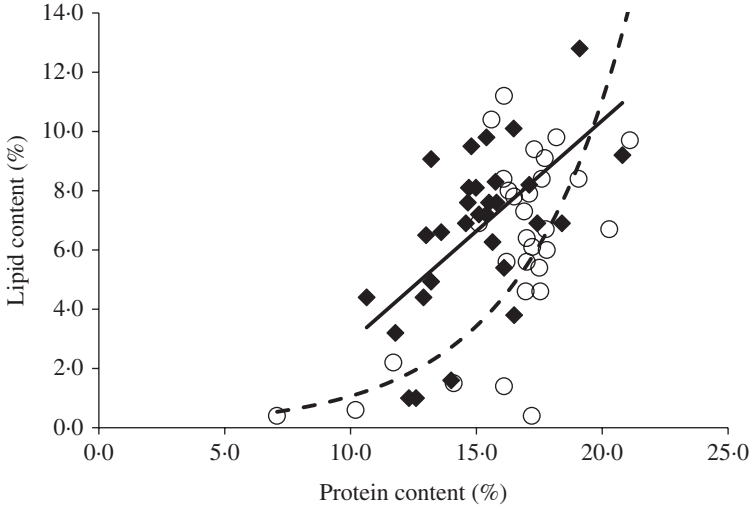

FIG. 5. Relationship between extracted lipid and protein content of *Alosa sapidissima* for whole body (♦, ——, $y = 0.746X - 4{\cdot}558$, $n = 30$, $R^2 = 0{\cdot}36$, $P < 0{\cdot}001$) and fillet (○, – – – –, $y = 0.103e^{0.234X,}$ $n = 30$, $R^2 = 0{\cdot}48$, $P < 0{\cdot}001$) samples.

TABLE III. Linear mixed model (LMM) parameters and differences between treatments (Comparisons) for lipid content (%) of *Alosa sapidissima* ($n = 9$) measured as whole fish (Whole), whole fish with gonads removed (No gonads), fillet, fillet with scales removed (No scales)

Treatment	Estimate	S.E.	*t*-value	*P*
LMM				
Whole	6·3	1·026	6·095	<0·001
No gonads	6·6	0·138	2·651	<0·01
Fillet	6·5	0·138	1·847	>0·05
No scales	6·3	0·138	0·482	>0·05
Comparisons			*z*−value	
Whole *v*. no gonads	−0·4	0·138	−2·651	<0·05
Whole *v*. fillet	−0·3	0·138	−1·847	>0·05
Whole *v*. no scales	−0·1	0·138	−0·482	>0·05
No gonads *v*. fillet	0·1	0·138	0·803	>0·05
No scales *v*. no gonads	−0·3	0·138	−2·169	>0·05
No scales *v*. fillet	−0·9	0·138	−1·365	>0·05

REPEATED MEASURES AT HATCHERY

Sixty-one *A. sapidissima* were tagged and analysed with the fat meter and only the 35 that survived for all 63 days were included in the analysis. Predicted mean lipid content for day 0 was 6·1%, day 35 2·5% and day 63 1·5% (Table VI and Fig. 7). Wald χ^2-test was significant ($\chi^2 = 374{\cdot}9$, d.f. $= 2$, $P < 0{\cdot}001$) and each *post hoc* all-pair comparison was significant ($P < 0{\cdot}001$) (Table VI).

DISCUSSION

The fat meter was demonstrated to provide accurate lipid measurements when compared with a traditional lipid extraction method. Additionally, *A. sapidissima* composition was proportionally accurate: lipid, moisture, protein and ash totalled *c*. 96% and glycogen (not measured) is expected to be *c*. 1%. The proportional accuracy of total fish composition indicates that lipid content was being measured accurately and provides further confidence in the relationship between lipid content and fat-meter readings. The readings were not affected by the presence of scales or gonads and are stable in water and air for 24 h after death, indicating they will be robust in the face of a variety of biological and experimental conditions. Further, repeated fat-meter readings over 63 days revealed reductions in lipid content similar to what *A. sapidissima* experience during the course of their migration.

Both whole-body and fillet samples were compared to determine if the fat meter was a good indicator of available lipid for the entire fish, or only for lipid reserves within the muscle. *Alosa sapidissima* have a subdermal fat layer that makes up a significant proportion (27%) of lipid utilized during migration and much of the energy contribution comes from muscle tissue found in the fillet (collectively 92%) (Leonard & McCormick, 1999). The fat-meter readings indicated a strong relationship to whole-body samples ($R^2 = 0{\cdot}72$ at positions 2, 1 + 2, 2 + 3 and 1 + 2 + 3), but the fillet readings had a slightly better relationship ($R^2 = 0{\cdot}81$ at position 1 and position 1 + 2),

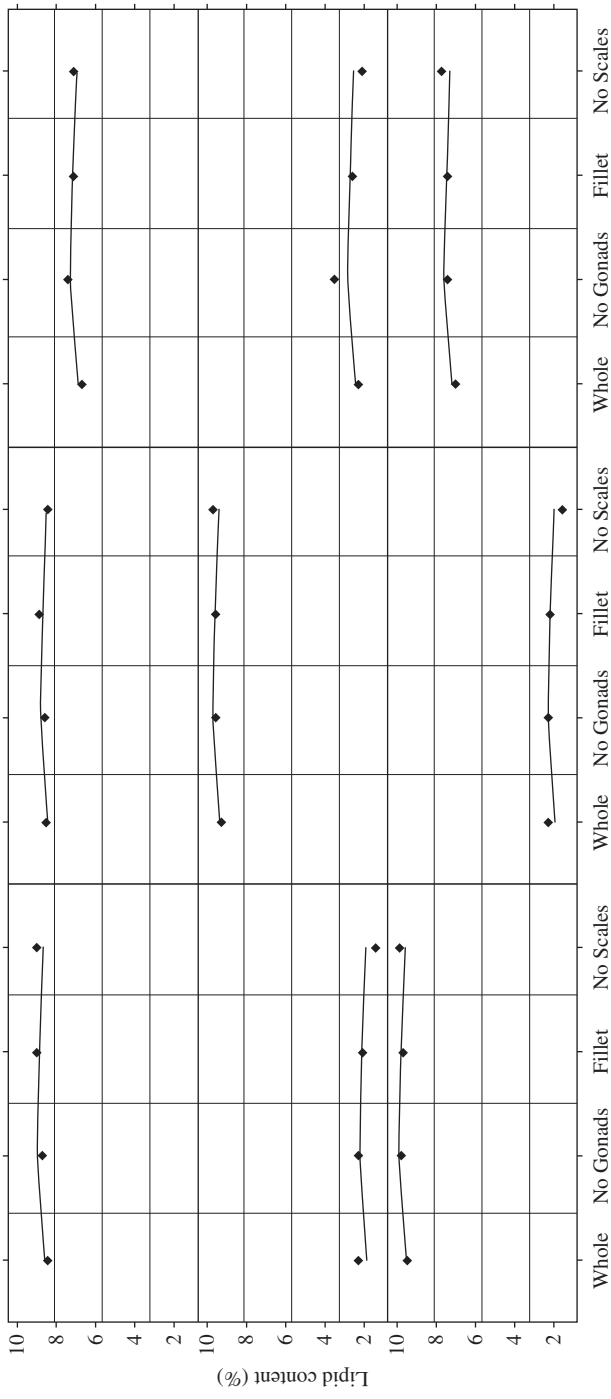

FIG. 6. Predicted curves of mean lipid content from linear mixed models for nine separate *Alosa sapidissima* measured with a fat meter as whole fish, whole fish with gonads removed, fillet, fillet with scales removed.

TABLE IV. Linear mixed model (LMM) parameters and differences between times (Comparisons) for lipid content (%) of *Alosa sapidissima* ($n = 10$) exposed to water at 0, 4, 19, and 24 h after death

Hour	Estimate	S.E.	*t*-value	*P*
LMM				
0	4·9	0·678	7·298	<0·001
4	5·2	0·125	1·871	>0·05
19	4·7	0·125	−2·138	<0·05
24	4·6	0·125	−3·118	<0·01
Comparisons			*z*−value	
0 *v*. 4	0·233	0·125	1·871	>0·05
0 *v*. 19	−0·267	0·125	−2·138	>0·05
0 *v*. 24	−0·389	0·125	−3·118	<0·01
4 *v*. 19	−0·500	0·125	−4·009	<0·001
4 *v*. 24	−0·622	0·125	−4·989	<0·001
19 *v*. 24	−0·122	0·125	−0·980	>0·05

which may be due to lipid allocated from the viscera and liver, which contributed 6 and 2% of energy allocation as reported by Leonard & McCormick (1999) and perhaps is not represented well by the fat-meter readings, which probably only measure the muscle.

Many studies have determined the relationship between fat-meter readings and extracted lipids (validated) for a variety of fish species. Readings from one to multiple positions are customarily tested if the fish is large enough. Typically, lipid is extracted from the whole animal, fillet, or muscle sample, which may or may not include gonads, skin or red muscle. These decisions can be by design (to include lipid not present in the fillet) or for convenience (fillets homogenize more easily than the whole body).

TABLE V. Linear mixed model (LMM) parameters and differences between times (Comparisons) for lipid content (%) of *Alosa sapidissima* ($n = 9$) exposed to air at 0, 4, 19, and 24 h after death

Hour	Estimate	S.E.	*t*-value	*P*
LMM				
0	4·9	0·710	6·831	<0·001
4	5·2	0·098	3·682	<0·001
19	4·9	0·098	0·511	<0·001
24	5·0	0·098	1·330	<0·001
Comparisons			*z*−value	
0 *v*. 4	0·360	0·098	3·682	<0·001
0 *v*. 19	0·050	0·098	0·511	>0·05
0 *v*. 24	0·130	0·098	1·330	>0·05
4 *v*. 19	−0·310	0·098	−3·171	<0·01
4 *v*. 24	−0·230	0·098	−2·352	>0·05
19 *v*. 24	0·080	0·098	0·818	>0·05

TABLE VI. Linear mixed model (LMM) parameters and differences between days (Comparisons) for lipid content (%) of tagged *Alosa sapidissima* ($n = 35$) measured at day 0, 35, and 63 post tagging

Day	Estimate	S.E.	*t*-value	*P*
LMM				
0	6·1	0·248	24·480	<0·001
35	2·5	0·248	−14·530	<0·001
63	1·5	0·248	−18·350	<0·001
Comparisons			*z*−value	
0 *v*. 35	−3·6	0·248	−14·528	<0·001
0 *v*. 63	−4·5	0·248	−18·347	<0·001
35 *v*. 63	−0·9	0·248	−3·819	<0·001

The present study compared the fat meter's relationship to both whole-body and fillet samples, indicating a better relationship between the fillet and the fat meter, as opposed to the whole body, further suggesting that the fat meter is probably measuring the lipid content of muscle tissue only.

The relationship between protein and lipid content differed between whole-body and fillet samples. In whole-body samples the relationship between protein and lipid content was linear (both approximately decreasing at the same proportional rate), however for fillet samples protein and lipid had an exponential relationship. For fillets with extremely low lipid values (*c*. 2% or less), protein values were relatively much lower, as low as 7·1%, demonstrating that protein reserves had begun to be utilized at an increased rate after lipid reserves had been mostly depleted. The difference in the protein-lipid relationship between whole-body and fillet samples is probably due to

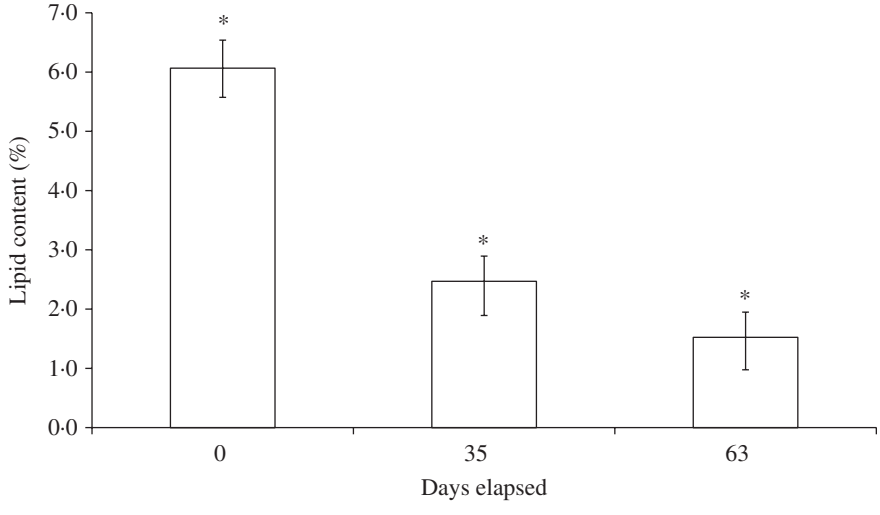

FIG. 7. Predicted mean lipid content from a linear mixed model of *Alosa sapidissima* ($n = 35$) at day 0, 35, 63. Error bars represent 95% confidence intervals determined from bootstrapping. *Significant difference between sample ($P < 0·05$).

protein being utilized from the fillet before, or to a larger extent, than from other parts of the fish such as the viscera or the liver. This result echoes the findings of Leonard & McCormick (1999) who reported that although *A. sapidissima* conserve red muscle protein for the duration of the spawning migration, white muscle protein is utilized throughout migration and increasingly at the later stages of migration when lipid levels are low; additionally they found no difference in viscera protein in later stage migration and only small differences in liver protein.

Mean lipid, moisture and total composition were not significantly different between whole-body and fillet samples, indicating that each set of samples had a similar mean composition for these variables. Conversely, protein was significantly higher for fillet samples relative to whole body. This result was expected considering that fillets consist mostly of muscle tissue, have fewer bones and do not include the viscera or liver, which contain much less protein than muscle (Leonard & McCormick, 1999). Also, ash was significantly larger for whole-body samples indicating a larger amount of ash (minerals) is found outside the fillet, which is expected with the inclusion of more bones in the whole-body samples. Regardless, ash has a small overall contribution to *A. sapidissima* composition for both sample-types.

Whole fish are sampled with the fat meter in the field to take advantage of its rapid measuring capacity and non-lethal sampling. The presented study removed scales since they do not homogenize well and do not contain lipid and the gonads were removed to concentrate lipid relationships between tissues that are readily available for lipid utilization: skin, subdermal fat layer and muscle for fillet samples and additionally viscera and liver in the whole-body samples. Results comparing each treatment: whole body, gonads removed, fillet and no scales showed little variation in lipid content between treatments for individual fish and the predicted means (0·3% range). However, analysis indicted a slightly significant difference between the whole-body and whole body with gonad removed treatments ($P < 0{\cdot}05$). While statistically significant, this difference is by a small amount (0·3%) making it not biologically relevant and reducing the concern of this statistical difference as showing a true change in lipid content between these treatments. Additionally, there was no difference between the first treatment (whole body) and the last (no scales) which represent the best measured relationship between the fat meter and the lipid extraction (the highest R^2 was for the relationship between position 1 for the fillet, fat-meter readings were taken for the whole body and the lipid extraction was from a scale-less fillet), further indicating that the difference between these two treatments is not a concern.

The fat meter relies on the measurement of water with microwaves to determine lipid content by using the inverse relationship of water and lipid in tissue. If fish were lethally sampled, but measured with the fat meter at a later time (*e.g.* stored on ice), it was not known how accurate the fat-meter readings would remain. This study's results showed that *A. sapidissima* exposed to water or air for 24 h maintained similar measurements (water 0·6% range; air 0·3% range). *Post hoc* analysis showed multiple significant differences for both exposure to water and air, but with such slight differences in predicted means and no clear trend in change of lipid content, it appears that the fat-meter readings can remain stable in these conditions.

Only one other repeated measures test with a fat meter has been performed on fish (Hendry & Beall, 2004). Being able to follow the decline of lipid content for individual fish both aids in validating the efficacy of the fat meter as a device to measure the lipid content of migrating fish, as well as aids the understanding of the rate at

which *A. sapidissima* utilize lipid over time. *Alosa sapidissima* held at the hatchery utilized 59·0% of their lipid in the first 35 days and 75·4% of the original amount after 63 days, demonstrating similar lipid reductions as other *A. sapidissima* studies (Glebe & Leggett, 1981*b*; Leonard & McCormick, 1999); however fish in our experiment were held in conditions that differ from the Connecticut River, where *A. sapidissima* probably spend less time in the river during migration, have a different temperature regime and will experience the cumulative effects of traversing the river and fish-ways (as opposed to being held in tanks).

The fat meter provided rapid lipid measurements in <30 s when considering reads at every position, including replicates. For both fillet and whole-body samples, multiple positions provided the best relationship between lipid content and fat-meter readings (positions 2, 1 + 2, 2 + 3 and 1 + 2 + 3 for whole body and positions 1 and 1 + 2 for fillet). To reduce sampling time to <10 s and probably reduce handling stress to sampled fish, sampling at one position (*e.g.* at position 1 and not position 1 + 2) should be used in future fat-meter studies with *A. sapidissima*. Additionally between the two sample-types, fillet sample relationships had higher R^2 values and the fillet regression formula should be used when taking fat-meter readings on whole bodies. The fillet regression formula can be applied to whole, live fish because our results indicate that the fat meter is actually measuring the lipid content of the fillet, missing the lipid content of the liver and viscera, providing the more accurate value. Knowing the accuracy and consistency of the fat meter, *A. sapidissima* energetic studies can take advantage of this technology and increase the sample size of studies and not have to sacrifice sampled fish.

In summary, validating the fat meter is a crucial first step before broader analysis. It is important to know the meter's performance under the scenarios in which research will be performed to ensure that accurate lipid measurements are taken and are appropriate for the particular species. Often, few validation procedures beyond a lipid extraction are considered before larger scale studies are started. In this study, the series of validation methods employed ensures that the fat meter provides accurate lipid measurements for the fish species of interest.

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