Measurements of resistance and reactance in fish with the use of bioelectrical impedance analysis: sources of error

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Abstract—New technologies can be riddled with unforeseen sources of error, jeopardizing the validity and application of their advancement. Bioelectrical impedance analysis (BIA) is a new technology in fisheries research that is capable of estimating proximate composition, condition, and energy content in fish quickly, cheaply, and (after calibration) without the need to sacrifice fish. Before BIA can be widely accepted in fisheries science, it is necessary to identify sources of error and determine a means to minimize potential errors with this analysis. We conducted controlled laboratory experiments to identify sources of errors within BIA measurements. We concluded that electrode needle location, procedure deviations, user experience, time after death, and temperature can affect resistance and reactance measurements. Sensitivity analyses showed that errors in predictive estimates of composition can be large (>50%) when these errors are experienced. Adherence to a strict protocol can help avoid these sources of error and provide BIA estimates that are both accurate and precise in a field or laboratory setting.

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Successful application of promising new technologies is predicated on understanding and controlling sources of errors. The need to identify sources of error with the development of new fisheries technologies is documented in genetic studies (Blanca et al., 2009), in mark and recapture studies (Curtis, 2006), in the tracking of vessels with global position systems (GPS) (Palmer, 2008) and in measuring water clarity with beam transmissometers (Larson et al., 2007). To identify sources of errors for new technologies, measurements are often compared with those from established technologies (Larson et al., 2007), simulated theoretical ones (Palmer, 2008), or known measurements (Curtis, 2006). Regardless of the process, the desired end result is to identify and reduce sources of error to increase the accuracy of measurements, thereby enhancing technology to provide accurate and reliable results within the fields of fisheries research and management.

Bioelectrical impedance analysis (BIA) has the potential for wide application in fisheries as a tool to quickly and accurately perform a number of physiologically important field measurements. The BIA method is capable of estimating proximate composition, fish condition, and energy content in fish quickly, cheaply, and (after calibration) without the need to sacrifice fish (Cox and Hartman, 2005). Bioelectrical impedance analysis has been found to be accurate for measuring compositional mass (i.e., measured in grams), (Cox and Hartman, 2005), but not so accurate for measuring estimates of percentages or energy per wet weight (Pothoven et al., 2008). Bioelectrical impedance analysis involves measuring the impedance, resistance (R), and reactance (X_c) of fish tissues to an electrical current, and relating those measurements to the proximate composition, condition, or energy content of the fish. Linear models relating impedance to compositional components are highly significant ($P<0.001$) with coefficients of determination ($r^2>0.96$ (Cox and Hartman, 2005). Relationships between observed and predicted values have slopes equal to one and intercepts that do not differ from zero. Estimations of body composition, condition, and energy content with BIA may be an asset to a variety of fisheries-related research and management projects by increasing the number of observations taken in the field and providing a means to take repeated measurements on individuals.

Physiological parameters are estimated from measured resistance (R) and reactance (X_c) values. Resistance

of a substance is proportional to the voltage of an applied current as it passes through a substance, or $R = V/C$, where V is applied voltage (volts), and C is current (amps). When the current is low enough, the current does not pass through the cell membrane (owing to the nonconductive lipid bilayer sandwiched between two conductive protein layers). The low current allows R to be reflective of everything extracellular. Reactance is the opposition to alternating current by a capacitor (cell membranes), and can be mathematically expressed by the following equation: $X_c = 1/(2\pi fC)$, where f is frequency in Hertz, and C is capacitance in Farads (Keller et al., 1993). Higher current frequencies will cause cell membranes to become capacitive so that X_c becomes reflective of the total amount of cell membrane material within the current. Both values are thus related to the cross sectional area of the entire fish, conductor length of the organism, and the signal frequency of the current (Lukaski, 1987). The phase angle is the ratio of R to X_c of tissue and has been found to be sensitive to the health and condition of fish (Cox and Heintz, 2009). By using R and X_c, one can estimate the composition and condition of fish.

In order for BIA to be accepted in fisheries science, it is necessary first to identify sources of error, and then to use that knowledge to minimize errors. With the use of BIA in studies of human nutrition and body composition, the identification of error sources was used to establish protocols that minimized errors (Rallison et al., 1993). More specifically, predictions of proximate composition parameters with BIA were found to be accurate with established procedures, but without them, these estimates became inaccurate (Ursula et al., 2004). In previous fish research, BIA protocols were established to minimize any unforeseen sources of error (Cox and Hartman, 2005). Although protocols were established and error may have been minimized, the actual sources of error were not identified. More recently, studies with BIA methods have shown inconsonant results. For example, in a study of cobia (*Rathycentron canadum*), there was a high correlation between BIA and most cobia proximate composition values (Duncan et al., 2007); whereas in another study involving yellow perch (*Perca flavescens*), walleye (*Sander vitreus*), and lake whitefish (*Coregonus clupeaformis*), it was concluded that considerable work needs to be completed before BIA can provide reliable predictions of whole body energy and percent lipid content (Pothoven et al., 2008). Furthermore, it was indicated that there needs to be an understanding of how temperature, locations where the electrode needle is placed (possible sources of error), and lipid distribution within a fish affect BIA measures.

The objective of our study was to identify sources of error in measurements of fish with BIA and errors of R and X_c. The cumulative effects of both significant and nonsignificant errors were examined through sensitivity analysis modeling. We conclude by identifying a protocol that will minimize the sources of error and maximize the potential of BIA in providing measures of body composition and condition in the field and laboratory.

Methods

We conducted laboratory experiments to identify sources of errors within BIA measurements of R and X_c. Specifically, we considered how electrode needle location, procedure deviation, user training, time after death, temperature of the fish, and stomach fullness affected measurements of R and X_c. For a comparison, we used a reference (control) that followed the protocol outlined by Cox and Hartman (2005). For all experiments, a handheld Quantum X impedance analyzer (RJL Systems, Point Heron, MI) was used, except for temperature measurements, for which a desktop Quantum II analyzer was used. In either case, a fixed current at 800 µA, AC, and 50 kHz was used. Electrode needles were either "standard" 12 mm×28 gauge subdermal stainless steel disposable low-profile EEG needle electrodes (Grass Technologies, West Warwick, RI) as used in Cox and Hartman (2005), or "nonstandard" 38 mm×14 gauge standard hypodermic needles with a polypropylene hub. Brook trout (*Salvelinus fontinalis*) used in this study were obtained from the Bowden West Virginia State Fish Hatchery, Bowden, WV, and Chinook (*Oncorhynchus tshawytscha*), pink (*O. gorbuscha*), and coho (*O. kisutch*) salmon were obtained from the Sheldon Jackson College (SJC) salmon hatchery, Sheldon Jackson College, Sitka, AK. Approximately 100 brook trout were maintained in a living stream tank at West Virginia University at 15°C and fed standard hatchery pellets at a rate of 3% body weight per day until used in experiments. Juvenile Chinook, pink, and coho salmon used in this study were taken from the SJC hatchery round pens and adult salmon used in this study were selected from returning adults. Treatment methods are those described below for each particular experiment. Fish that were sacrificed were killed by a blow to the head. In all experiments, sample size was determined by iterative power analysis with a significance of 0.05 and a power of 0.96 (Zar, 1996). In cases where variances of sample sets were not available from previous data, sample data were collected for power analysis before testing.

Linear mixed-effects (LME) models were used to test for the effects of electrode needle location, procedure deviation, user experience, and time on R and X_c measures (Pinheiro and Bates, 2000). The effects of temperature and gut fullness were tested with regression analysis to test for differences in slopes. In each experiment, measured R and X_c values were compared between treatment and controls. Statistical tests on response measures were performed by using program R, vers, 2.4.1 (R Development Core Team, Vienna, Austria). Significance (α) was set at 0.05.

Location of the electrode needle

To determine if different electrode needle locations influence impedance, comparisions of R and X_c measurements were made between different electrode locations within an individual fish (Fig. 1A). A location refers to the simultaneous location of all four (tetrapolar) elec-

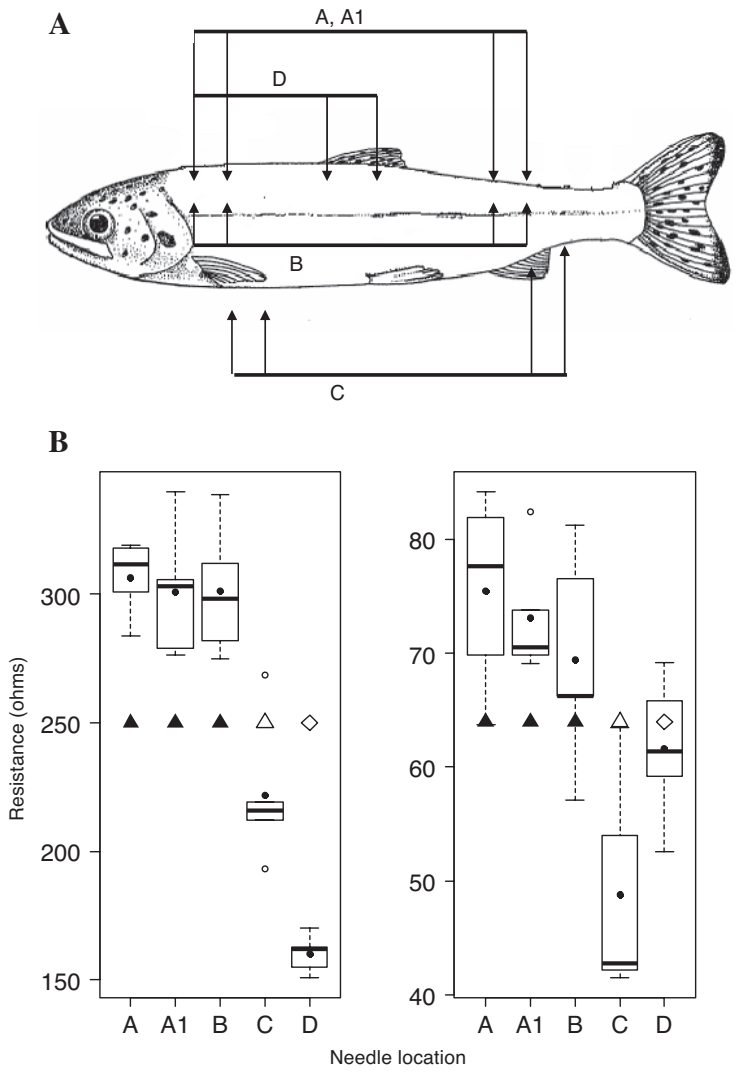

Figure 1

(**A**) Schematic diagram of needle electrode locations where resistance (R) and reactance (X_c) measurements are compared between each set of locations. Location A is the location used in Cox and Hartman (2005) with needles at a depth of 5 mm, A1 is the same location but the needles are inserted only 1 mm deep, and locations B, C, and D are different from A and have needles inserted to 5 mm. (**B**) Boxplots of mean resistance and reactance values taken from five pink salmon (*Oncorhynchus gorbuscha*) from different anatomical locations. Electrode locations are abbreviated as follows: control (A); reinserted (A1, the posterior set of electrodes were moved back to the original holes and the anterior electrodes were removed and reinserted into the exact same holes as in control); below (B, where both sets of electrodes were removed and placed approximately 1 cm below the holes in the control fish (but above the lateral line); ventral (C, where electrodes were removed and placed on the ventral portion of the fish and one set of electrodes was inserted on the anterior region one centimeter above the pelvic fin and the second set of electrodes was placed on the posterior end one centimeter above the anal fin); and half distance (D, where the posterior set of electrodes were moved forward and placed below the dorsal fin at a midpoint from the lateral line). Open circles (○) represent outliers determined by a Grubbs test. Different symbols indicate differences in means. Closed circles (●) represent mean values.

trodes. Spawning pink salmon (n=5, mean fork length=494.6 mm, standard deviation [SD]=8.9 mm) were killed, measured for fork length and weight, and placed on ice for 1 hour. A fish was randomly chosen and placed on a nonconductive board in a left-facing orientation, and measured for R and X_c for each of five electrode locations (Fig. 1A). This procedure was repeated for all five fish that were treated. Electrode locations were the following: 1) control (A, identical to measures found in Cox and Hartman, 2005); 2) reinserted (A1, both sets of electrodes were removed and reinserted into the "control" holes); 3) below (B, where both sets of electrodes were placed approximately 1 cm below the holes in the control (but above the lateral line); 4) ventral (C, where electrodes were placed on the ventral portion of the fish, and one set of electrodes was inserted on the anterior region one cm above the pelvic fin and the second set of electrodes was placed on the posterior end 1 cm above the anal fin); and 5) half distance (D, where the posterior set of electrodes was moved forward and placed below the dorsal fin at a midpoint to the lateral line). Measurements of R and X_c were recorded to the nearest 0.1 ohms (Ω).

Procedure deviations

To determine whether deviations from the procedures in Cox and Hartman (2005) would affect impedance measures, R and X_c measurements from five different treatment were compared to R and X_c measurements from the control group. Specific deviations were as follows 1) switched wires—the signal and detector leads were switched (by unplugging the leads while leaving the needle electrodes in the fish); 2) salt—two cups of seawater (31 practical salinity units) were poured under the fish; 3) conductive board—the fish was placed on a stainless steel conductive board; 4) needle size—the 28 gauge needles were replaced with larger 14 gauge hypodermic needles; and 5) needle depth—BIA electrode needles were placed in the fish in the same orientation as that in the control, except the needles were inserted to a depth of 1 mm rather than 5 mm. Spawning pink salmon (n=5, mean fork length=543.0 mm, SD=20.2) were killed, measured for length and weight, and placed on ice. Each of the five fish was randomly chosen and measured for R and X_c according to control protocols and also for each of the five treatment methods.

User experience

To determine if untrained users produce different and more variable R and X_c measurements compared to an experienced user, R and X_c measurements were compared between a user with training and four users without training. In this experiment, the single trained user had taken over 5000 BIA measurements on fish and the untrained users had no experience or previous training with BIA. Thirty juvenile coho salmon (n=30, mean weight=9.7 g, SD=2.1) were killed and randomly split into five groups of six fish, placed in plastic bags, and covered with ice. Before the experiment, four untrained users were introduced to the concepts of BIA and also to the protocols used by Cox and Hartman (2005). They were allowed to observe the trained user take R and X_c measurements on all six fish in a group. Each person was then randomly assigned a bag of fish. Immediately, each of the four inexperienced persons took R and X_c measures on all six fish within a group. During the time of taking measurements, untrained users were not allowed to ask for assistance. All fish from all groups were measured within 1 hour.

Time

We examined the effect of the time between death of the fish and BIA to determine how long dead fish can be held on ice before R and X_c measurements are compromised. Juvenile coho salmon (n=60, mean fork length=99.2 mm, SD=7.7 mm, and mean weight=10.3 g, SD=2.3 g) were killed and groups of six fish were randomly placed in plastic bags and placed on shaved ice. At 0, 3, 6, 9, 12, 24, 36, 48, 60, 72, and 96 h, a bag of fish was randomly removed and all six fish were measured for length, weight, R and X_c. It was assumed that because of the small size of the fish, the temperatures equilibrated within one hour of placement in ice and remained stable and therefore would minimize the effect of temperature on measurements. Measurements of R and X_c were taken according to the procedures found in Cox and Hartman (2005).

Temperature

The effect of temperature on R and X_c measurements was examined by taking repeated measurements on individual freshly killed fish over a range of temperatures (~0° to 12.5°C). The length of the experiment was <3 hours (the time it took the fish to freeze) and data in this study indicated that R or X_c do not change significantly within that short period and should nullify confounding effects of time on body condition after death. Regression analysis was used to test whether slopes and intercepts differed from 0 for regressions of R and X_c measurements on temperature. Adult pink salmon (n=4, mean fork length~550 mm) and juvenile (n=1, fork length~100 mm) Chinook salmon were killed and connected to a BIA Quantum-II Desktop by using standard needle electrodes and orientations as described by Cox and Hartman (2005). The Quantum-II was set to record impedance every 5 minutes for 12 hours. An ibutton thermometer (Maxim Integrated Products Inc., Sunnyvale, CA) was placed 1 cm (for juvenile) or 3 cm (for adults) inside the dorsal musculature of the fish and was set to record temperatures every 5 minutes. The juvenile fish was brought from cold (0.5°C) to warm (8.0°C) and the remaining adults were brought from warm (ambient water temperature) to cold (freezing). One adult had a starting temperature of ~12.5°C and the remaining three had a starting temperature of ~8.0°C. The automated Quantum II and the ibutton thermometers were synchronized to start recording at the same time. Each transferred fish was placed on a 4-in stand in the empty freezer compartment of a standard refrigerator. After 12 h, the fish was removed from the freezer, and R and X_c measurements and temperature logs were downloaded onto a computer. The one juvenile fish was removed from an outside cold tank, killed, and placed on a standard laboratory bench at room temperature. Initial temperature for the juvenile fish was ~0.5°C. For regression analysis, only impedance measurements taken when the fish temperature was >0°C were used. Significance tests were performed on each fish to test for slopes=0 by using a standardized major axis (SMA) test. The Bartlett-corrected likelihood ratio test (L) was used to test for differences between slopes of regression lines.

Stomach fullness and electrode location on live fish

To determine if R and X_c measurements in fish are affected by stomach fullness and electrode location, R and X_c values were taken and compared from locations on whole-body lengths and half-body lengths and with both full and empty stomachs. Measurements were taken across the length of the whole body (A1) and half its length (D) (Fig. 1A). Half- body length refers to the electrode orientation; one set of electrodes was placed toward the head region and the second was placed around midpoint of the fish (see D in Fig. 1A). Whole-body length refers to the second orientation that followed methods in Cox and Hartman (2005), where one set of electrodes was placed towards the head region and the second set was placed towards the tail region (see A in Fig. 1A). A two factor analysis of variance (ANOVA) was used to simultaneously test for differences in half-body and whole-body R and X_c measurements for brook trout with full and empty stomachs. If significance was found, a Tukey multiple comparison test was used to identify like values of R and X_c. Brook trout (n=20, length range=110–130 mm) were randomly split into four groups: A) half-body length full-stomach; B) half-body length empty-stomach; C) whole-body length full-stomach; and D) whole-body length empty-stomach (i.e., five replicates for each of the four combinations). All sets of fish were starved for three days before the experiment to ensure that the stomach was empty. Within 2 hours of the start of the experiment, the full-stomach group was fed fly larvae (*Sarcophaga bullata*) (Grubco Inc., Hamilton, OH), to satiation while those with the empty-stomach designation remained unfed. Before R and X_c measurements were taken, fish

Table 1

List of all possible bioelectrical equations that could be correlated with specific fisheries parameters (TBW=total body water, TBP=total body protein, FFM=fat-free mass, TBA=total body ash, DW=dry weight, and TBF=total body fat) or overall condition of the fish. Fisheries parameters listed here and their correlated equations are the ones presented by the authors in this article. Resistance (R) and reactance (X_c) are measured in biological tissue and the values are then inserted into each equation. In some equations, the distance between electrodes (L_d) is needed.

Obtained	Name	Equation symbol	Electrical equation	Volume symbol	Electrical volume equation	Fisheries parameter
Measured	Resistance in series	R	R	R_{sv}	L_d^2/R	TBW, TBP, FFM
Measured	Reactance in series	X_c	X_c	X_c	L_d^2/X_c	—
Derived	Resistance in parallel	R_p	$R+(X_c^2/R)$	R_{pv}	L_d^2/R_p	TBA
Derived	Reactance in parallel	X_c	$X_c+(R^2/X_c)$	$X_{c\,v}$	L_d^2/X_c	DW, TBF
Derived	Capacitance (farad)	C_{pf}	$(1{\cdot}10^{-12})/(314000{\cdot}X_c)$	C_{pfv}	L_d^2/C_{pf}	—
Derived	Impedance series	Z_s	$\sqrt{(R^2+X_c^2)}$	Z_{sv}	L_d^2/Z_s	—
Derived	Impedance parallel	Z_p	$(X_c{\cdot}R)/\sqrt{(X_c^2+R^2)}$	Z_{pv}	$L_d^2 \cdot Z_p$	—
Derived	Phase angle	phase angle	Arctan/(X_c/R)	—	—	Condition

were anesthetized in a tricane methanesulfonate (MS-222) solution of 1 g/9 L water.

Sensitivity analysis

To evaluate how sensitive models were to errors, six significant and nonsignificant errors along with six errors in distance between electrodes were incorporated into predictive models of total body water, dry weight, and phase angle (Table 1). Equations used in models were derivatives of R, X_c, and combinations of these two values which can be representative of biological tissue. Significant and nonsignificant differences (e.g., errors) from the previous experiments were converted into a percent difference from controls and used in the sensitivity analysis. The brook trout data set used in this analysis were from Cox and Hartman (2005) and the trout ranged in size from 10 to 227 g. For the predictive models used to determine total body water, dry weight, and total body fat estimates, equations including R in series (for total body fat), X_c in parallel (for dry weight and total body fat), and the electrical equation phase angle (for condition) (Table 1) were used. The significant R errors (in percentages) ranged from –58% (conductive board) to 10% (decreased needle depth) (Table 2). The significant X_c errors (in percentages) ranged from –45% (high temperature) to 47% (decreased needle depth) (Table 2). All length errors ranged from 0% to 5%. To consider how nonsignificant errors affect parameter estimates, a range of the nonsignificant R and X_c errors was inserted into each equation. The range of nonsignificant R errors (in percentages) that was inserted was –3% (full-stomach) to 3% (time=3 h). The nonsignificant X_c errors (in percentages) ranged from 1% (A1) to 9% (full-stomach) (Table 2).

For total body water, a single 6×6 matrix consisting of 36 combinations was formed from the six R and length errors. For dry weight estimates, two matrices were formed, one for R in parallel (used in the predictive model which had both R and X_c terms in it), and a second for the actual predictive model that estimated dry mass (Table 1). The data were plotted in three-dimensional matrix plots, with the x and y axes describing the range of values for either the length between detectors, R, X_c, or R in parallel, and with the z axis depicting the difference (in percentage) between predicted estimates with and without errors. In phase angle models, length between detectors is not a variable, and therefore R and X_c values were the only variables modeled. During analysis with significant errors added, phase angle values seemed to offset one another. To clarify this relationship, range of error values of –10% to 10% were added to both the R and X_c values. Three-dimensional matrices were plotted with the x and y axes representing R and X_c values and with the errors and the z axis depicting the difference between phase angle estimates with and without errors.

Results

Anatomical location of the electrode needle

The insertion of electrode needles in different locations within the fish resulted in different R and X_c mean values (Fig. 1A). Specifically, mean R and X_c values at locations C and D were significantly different from A for both R (LME $t_{16,\,25}>8$, $P<0.001$), and X_c (LME $t_{16,\,25}>3$, $P<0.001$). The difference in mean R values between location A (mean=306.62 Ω) and location C was –84.82 Ω (–28%), and D was –146.44 Ω (–47%). The difference in X_c means between location A (mean=75.44 Ω) and location C was –26.64 Ω (–35%), and between location A and D the difference was –13.80 Ω (–18%). There was not enough evidence to indicate that locations A1 and B were significantly different from A for either R (LME $t_{16,\,25}<2$, $P>0.12$)

Table 2

Resistance (R) and reactance (X_c) mean values, significance levels, and percent difference for experiments to determine effects of needle location, covariates, different users, time needed for measurements, and stomach fullness on R and X_c measures. Each category has adjustments that may be a source of error when compared to a standard protocol (control) found in Cox and Hartman (2005).

		Mean		Significance		% Difference	
Category	Error source	R (Ω)	X_c (Ω)	R (Ω)	X_c (Ω)	R	X_c
Needle location	A (control)	306.62	75.44	—	—	—	—
	A1	300.80	73.12	0.58	0.54	2	1
	B	301.12	69.44	0.60	0.13	2	2
	C	221.80	48.80	<0.01	<0.01	–28	–35
	D	160.18	61.64	<0.01	<0.01	–47	–18
Covariates	Control	261.48	70.34	—	—	—	—
	Switched wires	262.38	71.02	0.87	0.81	<1	<1
	Salt	226.04	64.24	<0.01	<0.01	–14	–9
	Conductive board	108.96	56.30	<0.01	<0.01	–58	–20
	Needle size	236.38	64.04	<0.01	<0.01	–10	–9
	Needle depth	288.24	103.62	<0.01	<0.01	10	47
Different users	Control	915.35	155.73	—	—	—	—
	1	969.00	156.33	0.09	0.93	6	<1
	2	929.03	173.52	0.66	0.01	1	11
	3	857.13	142.20	0.07	0.04	–6	–8
	4	795.27	140.62	<0.01	0.02	–13	–10
Time (h)	0	896.17	166.77	—	—	—	—
	3	924.45	173.92	0.34	0.38	3	4
	6	900.85	174.62	0.87	0.34	<1	5
	9	909.87	178.03	0.64	0.17	2	7
	12	919.90	206.20	0.42	<0.01	3	24
Stomach fullness	Half-empty	321.00	89.00	0.95	0.99	–3	1
	Half-full	311.60	89.60				
	Completely empty	830.40	204.60	0.49	0.14	3	9
	Completely full	805.20	185.20				

or X_c (LME $t_{16,\ 25}$<1, P >0.55) (Fig. 1B). Although differences were not statistically significant, differences in mean R values between location A (mean=306.62 Ω) and locations A1 and B were –5.82 Ω (–2%) and –5.50 Ω (2%), respectively. Similarly, nonsignificant differences in X_c means between location A (mean=75.44 Ω) and locations A1 and B were –2.32 Ω (–1%) and –6.00 Ω (–2%), respectively. The anatomical locations of C and D differed the most from A, whereas locations A1 and B had the greatest similarity (Fig. 1A). Location C represented the entire length of the ventral region and D represented the forward half of the dorsal region of the fish. The location of A1 represented the same area of the fish as in A, except there was a second puncture, and location B represented an area slightly below A, but was still within the dorsal musculature (Fig. 1A).

Procedure deviations

Some procedural deviations from those outlined in Cox and Hartman (2005) significantly affected R and X_c measures. Specifically, changes in needle depth and size, and placing the fish on a conductive surface or on salt water caused significant changes in R and X_c, (LME $t_{20,30}$>3, P<0.001) (Table 2, Fig. 2). Ranked differences in R means and percentages from highest to lowest between the control and deviations were as follows: 1) –152.52 Ω, –58% for fish placed on a conductive board; 2) –35.44 Ω, –14% for fish placed on salt water; 3) 26.76 Ω, 10% for shallow needle depth; and 4) –25.10 Ω, –10% for the larger needle size (Table 2, Fig. 2). Ranked differences in X_c means from highest to lowest and percent differences between the control and assorted covariates were as follows: 1) 33.28 Ω, 47% for shallow needle depth; 2) –14.04 Ω, –20% for fish placed on a conductive board; 3) –6.30 Ω, –9% for the larger needle size; and 4) –6.10 Ω, –9% for fish placed on salt water (Fig. 2). Differences were not significant between the control means of R and X_c and switched wires for either R (mean R=262.38Ω, LME $t_{20,30}$<0.3, P>0.80), or X_c (mean X_c=71.02 Ω, LME $t_{20,30}$<0.5, P>0.70) (Fig. 2).

User experience

Untrained users produced different and more variable R and X_c measurements than the experienced user. Pairwise tests indicated that one untrained user obtained a significantly different mean for R (LME $t_{20,30}>3$, $P<0.009$) than the trained user (mean=915.35 Ω) with means differing by –120.08 Ω (–13%) (Fig. 3). Although differences were not significant, two of the untrained users had mean differences greater than 5% from the control. Significance was not found because of the high variance obtained from untrained users (mean deviation=46.75) and when compared to the trained user, standard deviations from untrained users were 4.6 times larger. Reactance values were significantly different for the trained user compared to those for the three untrained users (LME $t_{20,30}>2$, $P<0.04$) with differences ranging from –15.12 to 17.78 Ω (–10% to 11%). Variability of X_c standard deviations was greater (1.3×) in three of the untrained users when compared to variability in the control, but was not as great as the R variability (1.3× vs. 4.6×) (Table 2, Fig. 3).

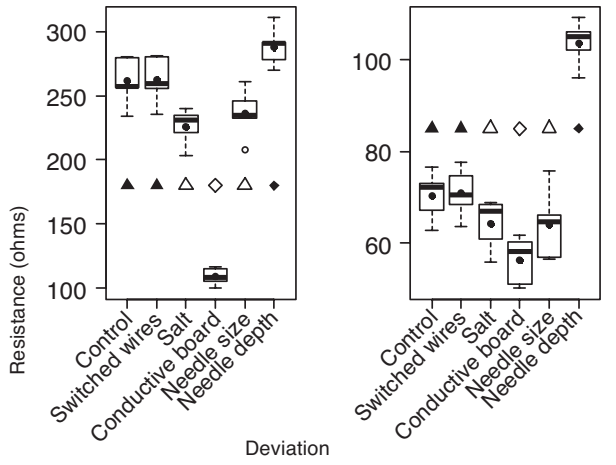

Figure 2

Boxplots of repeated resistance and reactance measurements taken from five pink salmon (*Oncorhynchus gorbuscha*) (control) and when an additional variable was added to the procedure (deviation). Specific deviations were 1) switched wires: the signal and detector leads were switched; 2) salt: two cups of seawater (31 practical salinity units) were poured under the fish; 3) conductive board: the fish was placed on a stainless steel conductive board; 4) needle size: the 28 gauge needles were replaced with larger 14 gauge hypodermic needles; and 5) needle depth: BIA electrode needles were placed in the fish in the same orientation as that used in the control, except needles were inserted to a depth of 1 mm. Open circles (○) represent outliers determined by a Grubbs test. Closed circles (●) represent mean values. Different symbols indicate differences determined by the statistical tests applied.

Time

The time period between death and BIA measurements did not affect R or X_c measures on iced fish from 0 to 72 h and from 0 to 9 h, respectively (Fig. 4, A and B). In this study, R differences between 0 and 72 h were not significant, LME ($t_{55,\ 66}<2$, $P>0.16$) (Table 2, Fig. 4A). Although during the first 72 h, significant differences were not detected, non-\significant differences in mean R between 0 h (mean R=896.16 Ω) and subsequent times (3–60 h) ranged from to 28.28 Ω (3%) at 3 h to –41.48 Ω (–5%) at 60 h (Fig. 4A). The mean deviation of all grouped R values was 47.81. Mean values of X_c were not significantly different between 0 and 9 h (LME $t_{55,66}<2$, $P>0.17$) (Table 2, Fig. 4B). Although differences were not detected, nonsignificant differences in X_c means between 0 h and subsequent times (3, 6, and 9 h) were 7.15 Ω (4%), 7.85 Ω (5%), and 11.26 Ω (7%), respectively. Starting at 12 h, mean values of X_c were significantly different from 0 h LME ($t_{55,66}>4$, $P>0.001$) and increased from 166.76 Ω (mean at 0 h) to 206.19 Ω (24%) (mean at 12 h). The mean deviation for all grouped X_c values was 12.81.

Temperature

Temperature affected R and X_c measurements. As temperature increased, R and X_c decreased and slopes were not equal to zero (Fig. 5, A and B). Individual regressions of X_c and R with temperature were significant and all individual regressions had $r^2>0.92$. Individual slopes from each of the five R regressions were negative (–12.19, –11.64, –11.61, –10.95, and –12.01) and the mean slope was –11.65 (Fig. 5A). In the R regressions, there was no evidence of differences between slopes (L=3.82, P=0.43). In the X_c regressions, significant differences were found between slopes (L=93.65, $P<0.05$) (Fig. 5B). Slopes from the each of the five X_c regressions were negative (–4.86, –2.77, –2.87, –2.53, and –1.59). In X_c regressions, the maximum and minimum slopes were possible outliers and represented the only juvenile fish (closed circle symbol in Fig. 5B, slope=–4.86) whose temperature went in the opposite direction to that of the rest of the fish (i.e., cold to warm rather than warm to cold) and an adult fish (circle symbol in Fig. 5B, slope=–1.59) with an initial temperature 3°C higher than the others. When these two fish were removed from the regression analysis, the three remaining X_c slopes were not found to be different from each other (L=3.97 and P=0.14) (Table 2, Fig. 5B).

Stomach fullness

Stomach fullness did not affect R or X_c measures in either half- or whole-body measures (Fig. 6, A and B). Differences in R measures were not significant between fish with full or empty stomachs for half-body measurements (Tukey HSD, P=0.95) or full-body measurements (Tukey HSD, P=0.49) (Fig. 6A). Mean

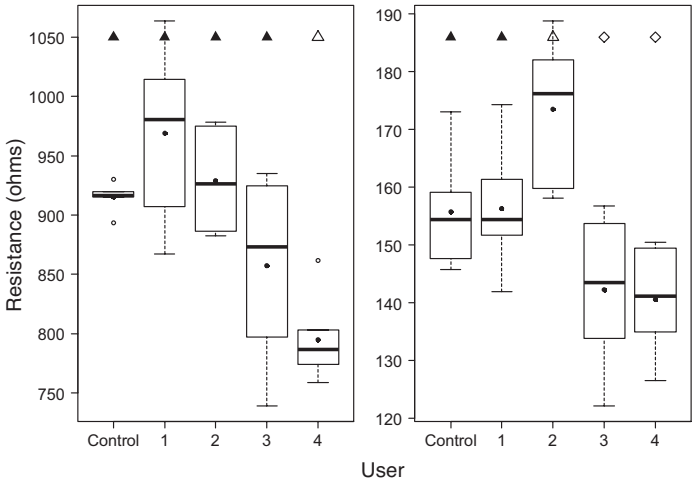

Figure 3

Boxplots of resistance and reactance measurements from five different people in a comparison of a trained user (control) with users without any training (1–4). Five coho salmon (*Oncorhynchus kisutch*) were measured by each person. Open circles (○) represent outliers determined by a Grubbs test. Closed circles (●) represent mean values. Different symbols indicate differences as determined by the statistical tests applied.

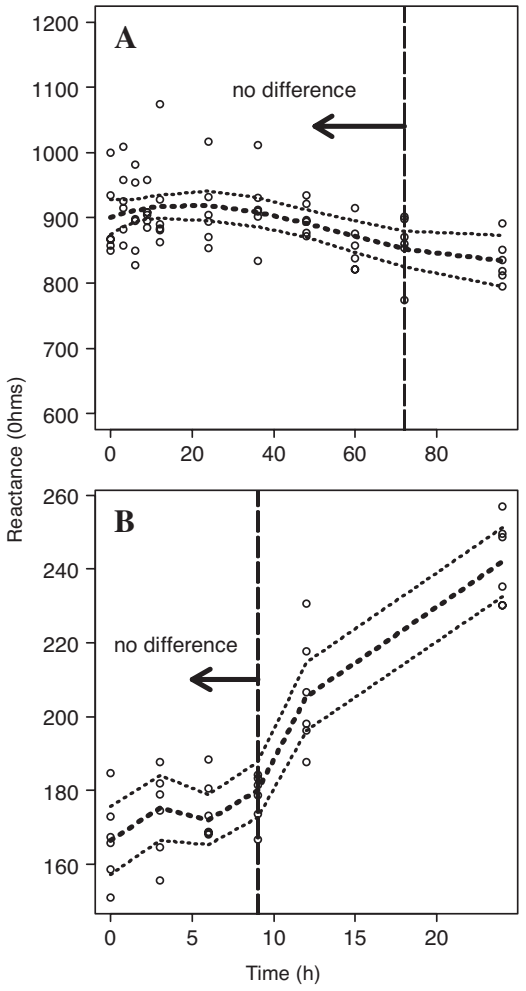

Figure 4

Resistance (**A**) and reactance (**B**) measurements taken over time on six groups of six coho salmon (*Oncorhynchus kisutch*) (n=36) that were killed and placed on ice. Open circles (○) represent outliers determined by a Grubbs test. The heavy dashed line indicates mean values and the light dashed line indicates 95% confidence intervals. The vertical dashed line and the arrow from it represent the times in which there was no difference in resistance or reactance measurements.

values of R for combinations of fish with full or empty stomachs with both half- and full-body measurements were 321, 312, 830, and 805 Ω, respectively (Fig. 6A). Although there was not a significant difference between mean values of R, nonsignificant differences in R means between full and empty stomachs for both half- and full-body measurements were –9.4 Ω (–3%) and –25.2 Ω (–3%) (Table 2). Mean X_c values were not significantly different between fish with full and empty stomachs for mid-body measurements (Tukey HSD, P=0.99) or full-body measurements (Tukey HSD, P=0.14) (Table 2, Fig. 6B). Mean values for R for fish with full or empty stomachs for both half-, and full-body measurements were 89, 89, 204, and 185 Ω, respectively (Table 2, Fig. 6B). Although there was not a significant difference between mean values of X_c, nonsignificant differences in means between fish with full or empty stomachs for both half- and full-body measurements were 0.6 Ω (<1%) and –19.4 Ω (9%). Variation of the estimations was also greater in the half-body measures in both R and X_c measures (Fig. 6, A and B).

Sensitivity analysis

Predictive models for estimating total body water were highly inaccurate when significant errors were inserted into the models and considerably more accurate when nonsignificant errors were inserted into the models (Fig. 7, A and B). Inserted significant errors (–58% to 10%) were inversely correlated with parameter estimation errors. The maximum significant negative error (–58%, conductive board) resulted in an overestimation >120% and a 10% error (decreased needle depth) resulted in an underestimation >–10% (Fig. 7A). The addition of length errors (0% to 5%) produced results that were positively correlated with parameter estimation errors and compounded the overall parameter estimation error (Fig. 7A). Inserted nonsignificant errors (–3% to 3%) were also inversely correlated to parameter estimates (Fig. 7B). The maximum nonsignificant negative error (–3%, full-stomach) resulted in an underestimation of 2.7%, and the maximum nonsignificant positive error (3%, R at 3 h) resulted in an overestimation of 2.6% (Fig. 7B). Length error alone caused overestimations to range from

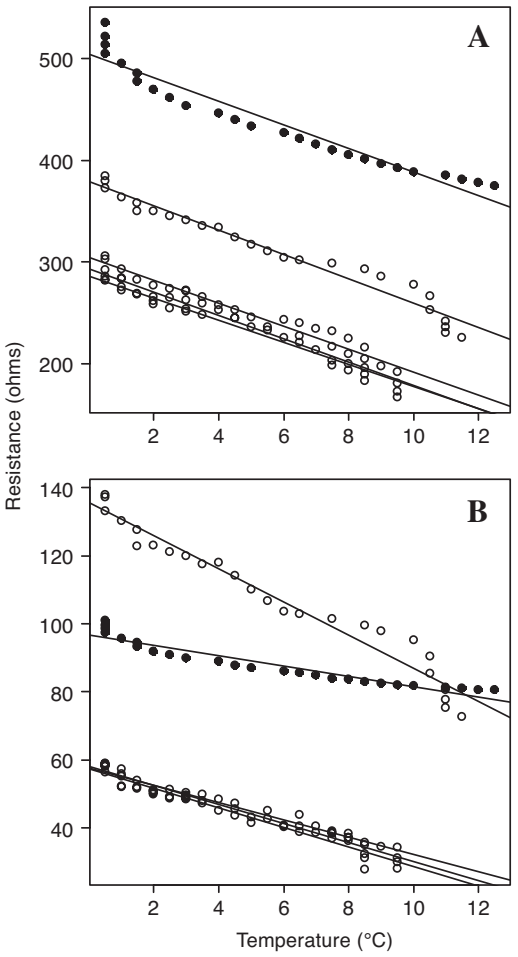

Figure 5

Influence of temperature on repeated measurements of resistance (**A**) and reactance (**B**) on dead Chinook (*Oncorhynchus tshawytscha*) (n=2) and coho salmon (*O. kisutch*) (n=3). All fish except the juvenile fish were measured from warm to cold temperatures. Solid circle=juvenile fish that was measured from cold to warm, and open circle=adult salmon that were measured from warm to cold.

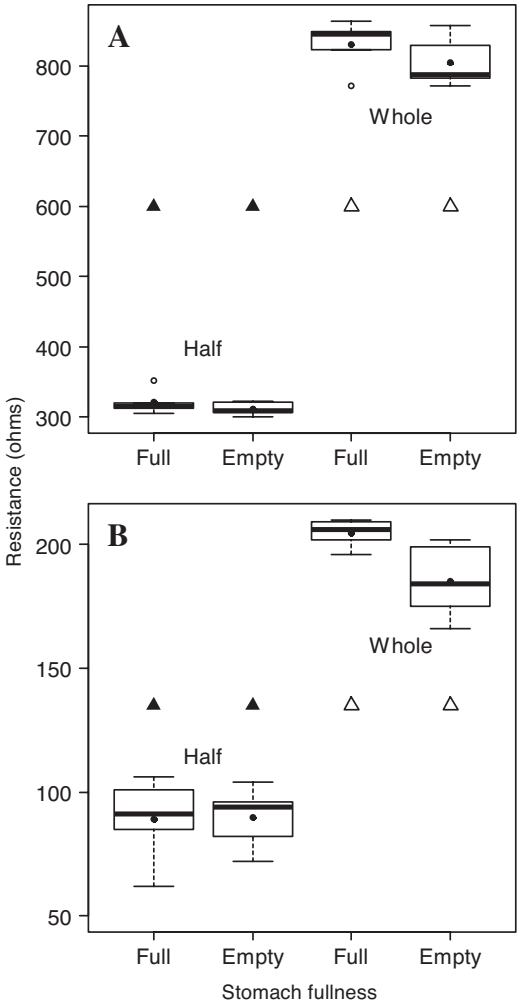

Figure 6

Boxplots describing resistance (**A**) and reactance (**B**) measurements in four groups of live brook trout (*Salvelinus fontinalis*) for different electrode locations and stomach fullness (n=5 per group), where full=full stomach, empty=empty stomach. "Whole" refers to a whole-body length (the entire length of the fish as described in Cox and Hartman [2005]). "Half "refers to a half-body length (one set of electrodes was placed towards the head region and the second being placed around mid-point [under the dorsal fin]). Open circles (○) represent outliers determined by a Grubbs test. Closed circles (●) represent mean values. Other symbols indicate differences as determined by the applied statistical tests.

0% to 9%. Compounding both maximum R (–3%) and length (5%) errors resulted in an overestimation >12%.

Both significant and nonsignificant errors inserted into the derived electrical volume equation X_c in parallel caused inaccuracies in subsequent parameter estimations of dry mass (Fig. 8, A and B). The insertion of significant errors impacted parameter estimations more substantially than nonsignificant ones. The addition of significant R errors (–58% to 10%) and X_c errors (–35% to 47%) into X_c in parallel (nonvolumetric) resulted in errors ranging from –58% to 173%. The subsequent addition of this range of errors into predictive models of DW caused estimations to be inaccurate by –45% to 349%, and length errors compounded the error (Fig. 8A). The addition of nonsignificant R errors (–3% to 3%) and X_c errors (0% to 9%) into X_c in parallel (nonvolumetric) resulted in X_c in parallel errors ranging from –11% to 4%. The subsequent addition of these errors plus the length errors (0% to 5%) into the volumetric equation to predict dry weight (DW) resulted in parameter estimations of DW that were inaccurate by

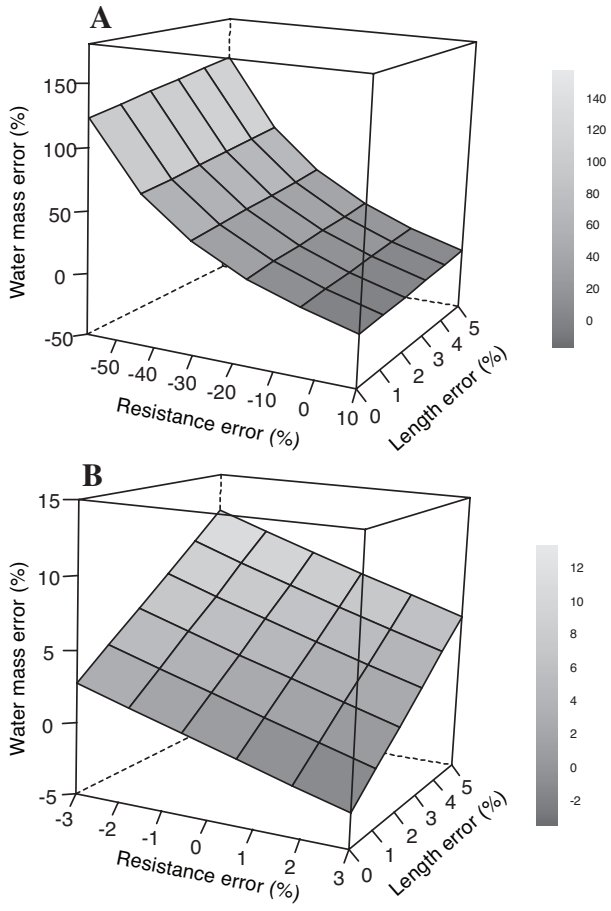

Figure 7

Comparisons of estimates of water mass parameters with resistance errors ranging from –58% to 10% (**A**) and with resistance errors ranging from –3% to 3% (**B**). The data were plotted in three-dimensional matrix plots with the x and y axes describing the range of values for either the length between detectors, R, X_c, or R in parallel, and with the z axis depicting the difference (as a percentage) between predicted estimates, with and without errors.

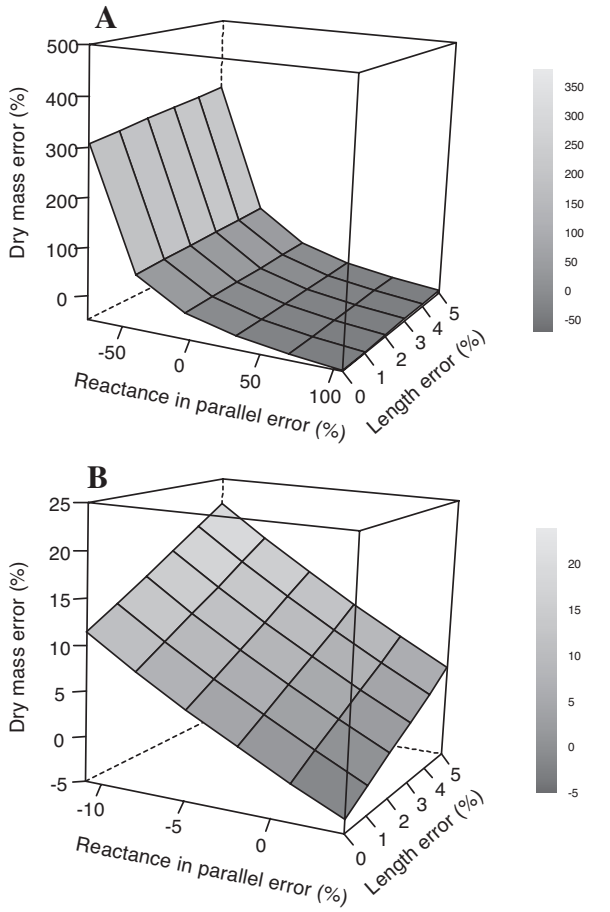

Figure 8

Comparisons of estimates of dry mass parameters with reactance in parallel [$X_c+(R^2/X_c)$] errors ranging from –58% to 173% (**A**) and –11% to 4% (**B**), and length errors in both ranging from 0% to 5%. The data were plotted in three-dimensional matrix plots with the x and y axes describing the range of values for reactance in parallel and the length between detectors, and z axis depicts the difference (as a percentage) between predicted estimates of dry mass, with and without errors.

0% to 21% (Fig. 8B). If length errors were not included, parameter estimations of DW were inaccurate by –3% to 11%. The addition of length errors compounded the parameter estimation errors.

Individual errors of R and X_c errors affected phase angle measures, but combined errors tended to offset one another (Fig. 9, A and B). The introduction of significant errors of R and X_c (–58% to 10%, and –37% to 45%, respectively) caused phase angle measurements to vary from –60% to 129%. When inserted errors were in the same direction (i.e., both errors are either negative or non-negative numbers), they offset one another and resulting phase angle errors were closer to 0% (Fig. 9A). When inserted errors were opposite of one another (i.e., when one error was a positive number and the other negative), phase angle errors increased and decreased to their maximum values and did not offset one another, but rather increased errors. Identical errors for both R and X_c showed symmetry in that phase angle errors equaled 0% when inserted R and X_c errors were the same (Fig. 9B). Reactance errors by themselves were inversely correlated with phase angle errors and R errors were positively correlated with phase angle values.

Discussion

The ability to accurately estimate physiological parameters including proximate composition, condition, and energy content with BIA will permit increased preci-

sion in energy flow and proximate composition studies on spatial and temporal scales that were previously impractical. At the individual level, BIA will permit repeated measures on the same individual during the course of investigation, yielding better tracking of energetics components and improved precision in bioenergetic models. At the population level, BIA will permit assessment of the condition of cohorts over time and permit detailed comparisons across cohorts, and temporal and spatial scales. At the community level, BIA will permit the evaluation of growth and energy-flow dynamics across species that may elucidate community dynamics that were previously unknown, or permit correlation of condition with outbreaks of disease. This approach also has potential for the nonlethal study of threatened or endangered species by the use of models developed for closely related species. In order for the application of BIA to reach its potential, sources of error that affect R and X_c measurements need to be continually identified and analyzed.

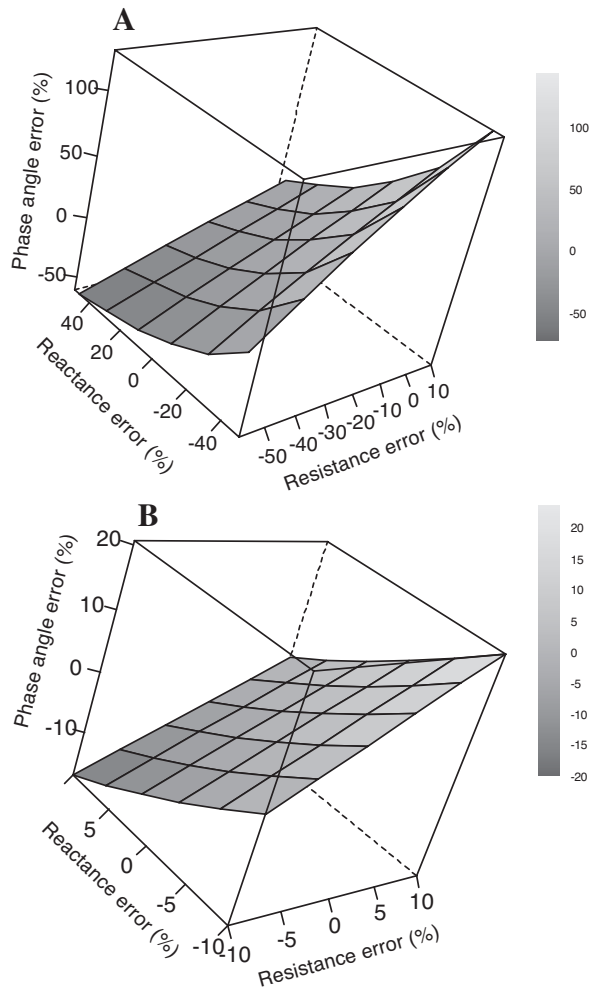

Figure 9

Comparisons of large (**A**) and small (**B**) resistance (R) and reactance (X_c) errors affecting phase angle [arctan/(X_c/R)]. Errors in resistance and reactance values are greater in A than in B. The data were plotted in three-dimensional matrix plots with the x and y axes describing the range of values for reactance and resistance, and the z axis depicting the difference (as a percentage) between phase angle, with and without errors.

Sources of error include different electrode needle locations, procedure, user experience, time periods between death and impedance measurements, and temperature. Measuring impedance in the same anatomical location of the fish is critical to obtaining accurate and reproducible impedance measurements. When electrodes are not placed in the same anatomical location of fish, incomparable and inaccurate results are obtained, but which location is best is still a question. Variability in R and X_c measurements increased with the distance of electrode placement from the control. Impedance values can change for two reasons: 1) the distance between electrodes is directly proportional to the electrical volume (e.g., R in Table 1) and consequently, halving the distance between electrodes leads to reduced values of R and X_c; and 2) when electrodes are placed in different locations on the fish, different tissue types are represented, and moving electrodes from the dorsal side of the fish to the ventral side will not only change the distance between electrodes, but it will also reflect different tissue types. The dorsal side is mainly muscle and the ventral consists of peritoneal tissue and organs. Changing the distance will change the R and X_c values, and changing the electrode location will change the tissue types that are being measured. Sensitivity to tissue types is consistent with Geddes and Baker (1967) who reported different impedance values with different tissue types (i.e., skeletal muscle, liver, and kidney tissues). Therefore, electrodes can be re-inserted into the same holes or moved slightly dorsally or ventrally as long as the same tissue type is measured and the distance stays relatively similar. Impedance measurements are dependent on the anatomical location of the needle electrodes.

Sources of error caused by procedural deviation can also be avoided by standardizing protocols. Measures of R and X_c are affected by covariates such as needle depth, needle size, and conductive surfaces where the measurements are taken. Minimizing these errors can be accomplished by inserting needles to a uniform depth, blot drying the fish before measurements are taken, taking measurements on a nonconductive board, and by using the same gauge of needle electrodes. If procedures are not standardized, R and X_c change as electrical currents are altered by procedural changes. For example, changing the needle depth or size will change the needle surface area that is in contact with the tissue. Because smaller surface areas present more resistance to the electrical current than larger ones, R and X_c values will change. Similarly, taking impedance measurements on a conductive board offers the electrical current a less resistant route. Ohm's law states that when electrical currents are offered a less

resistant route, they tend to take them, and offering the current a path through seawater or a conductive board would allow the current to take a pathway that is the least resistant and that would possibly not even include the fish. This would result in a drop in R and X_c values (which was seen in this study) that may not be representative of values for a fish. A drop in impedance values was also seen in a study by Mirtaher et al. (2005), when an electrical current was measured through increasing concentrations of NaCl. Because impedance values are used to measure the composition and condition of fish tissue, the majority of the electrical pathway needs to be within the fish. Switching the signal and detecting wire leads will not have any effect on R or X_c values, as long as the impedance analyzer unit (e.g., RJL Systems Quantum II) is internally modified to correct for this switch.

Dead fish can be held on ice for up to 9 h without compromising R or X_c. If measures of R are the only measured impedance value being used, fish may be iced up to 72 h before measurments start to change, but if R and X_c are to be used, fish need to be iced and measured within 9 h of capture. Icing fish delays postmortem rigor mortis and subsequent tissue breakdown. These processes first affect X_c, then R. This sequence is due to X_c reflecting cell membrane integrity, whereas R reflects more extracellular material. After 12 h, X_c starts to increase due to rigor mortis (muscle contraction), and upon resolution, cell membrane integrity is compromised until the cell eventually ruptures. The rupturing of cells in turn releases intracellular fluid into extracellular spaces causing decreases in R. Increasing X_c (due to muscle contractions) followed by decreasing R (due to edema) was observed in two studies. The first, a study of human health showed increases in X_c due to muscle contractions (Kashuri et al., 2007), and a second fish study showed postmortem haddock (*Melanogrammus aeglefinus*) R levels decreasing because of changes in edema (Martinsen et al., 2000). The use of ice to slow these postmortem processes is not a new technique (Orr, 1920), but it is still an important technique that can be applied to extend the time of measuring impedance in killed or dead fish,

Personnel should be trained in taking impedance measurements to increase accuracy and decrease variability of R and X_c measurements. How much training is needed cannot be answered with these data. The large variability in R and X_c values measured by untrained personnel is due to their unfamiliarity with procedures that would increase accuracy and decrease variability. Without the training of personnel, inserted electrode needles may shift during measurements, causing changes in the contact area between tissue and the needles. As the contact pressure of the needle changes, current flow is also altered and results in changes in R and X_c. Also, untrained users take more time to take measurements than do trained users and the additional time allows excess fluid buildup around the needle electrode sites that can affect current flows. Because both fluid buildup and pressure changes can cause fluctuations in impedance values, a standard procedure should be developed to minimize errors. Needles should be placed perpendicular to the fish, inserted to the appropriate depth, and held stable during measurements. Body and hand position of the user must allow the user to view the needles and measurements should be taken in a timely manner (<30 s). Likewise, procedural training can increase the proficiency in obtaining impedance measurements by decreasing variability of hand movements and increasing accuracy of the position of needle insertion. This was observed by Liddell et al. (2002) who demonstrated that formalized training for needle control and position for medical students can have lasting efficiencies on procedures involving needles. Increasing training and experience before taking BIA measures will decrease variability and increase accuracy of impedance measurements.

Temperature affects impedance measurements, but can be standardized by correcting to a set temperature. The inverse relationship between temperature and impedance is widely described in literature concerning conductive metals (Grimnes and Martinsen, 2007). Because the relationship of metals and impedance is known to be linear over a broad range of temperatures (0–1200 K), a similar relationship should exist between biological tissue and temperature. This relationship would also be more constant in cold-blooded species where temperature changes are systemic and not prone to localized temperature changes (e.g., at extremities or skin) as with warm-blooded organisms (Caton et al., 1988). In another human study, Gudivaka et al. (1996) provided a correction factor for changing skin temperatures to normalize impedance measurements by using the inverse linear relationships between impedance measurements and temperature. Because a linear relationship is shown for R in our study, it is possible to determine an empirical approximation for R at a standardized temperature which is shown to be

$$R_m - R_0 = \alpha(T_m - T_0), \quad (1)$$

where R_m = resistance measured at T_m;
R_0 = calculated resistance at T_0;
α = –6.02;
T_m = measured temperature when measured resistance was taken; and
T_0 = 0°C.

The authors would like to point out that this equation is based on five data points and the usage here is intended to show the possibility of correcting for temperature. The set point of 0°C was chosen because fish could be put either on ice or adjusted down to 0°C by using Equation 1. By icing or standardizing measurements to a set point, accuracy will increase in impedance measurements. Reactance measurements could also be standardized to a 0°C temperature by using an empirical approximation similar to R. In the X_c data presented here (with the aforementioned outliers removed), slopes between the remaining three fish are not different and

an empirical approximation for X_c at a standardized temperature (0°C) is identical to that in Equation 1, except that α=–2.8.

Stomach fullness had no effect on response measurements within the half- or whole-body groups. The stomach and alimentary canal are encased by less conductive layers of muscle than the surrounding organs located in the peritoneal cavity (Pethig, 1979). Much like an insulated wire, these less conductive muscle layers will insulate the stomach contents even if the stomach contents are more conductive than the surrounding tissue. The insulation provided by the muscle layer reduces the chance that the current pathway will include stomach contents. Decreased R values (Ω/cm) are seen in various animals in the peritoneal spleen, liver, and kidney, and relatively higher R values in nearby muscle tissue (Pethig, 1979). These insulating muscles, coupled with less resistant alternative pathways (i.e., organs), indicate that stomach fullness does not need to be accounted for in BIA measurements.

Sensitivity analyses show that significant deviations from the procedures found in Cox and Hartman (2005) can lead to unacceptable errors in predictive estimates of R and X_c, but nonsignificant deviations are more acceptable. The average of all significant errors in this study is 26% and would cause parameter estimates to be off by about 25% to 30%, which is too large for most biological studies. The nonsignificant error averages of <3% will cause parameter estimation errors to be around 2% to 4% (or about a 1:1 ratio), which may be acceptable in some studies. It should be noted that if several nonsignificant errors are encountered at the same time, they can be cumulative and result in an estimation error that is significant.

In all electrical volume equations, length between detectors (L_d) is a squared term in the numerator, making predictive estimates extremely sensitive to changes in L_d while also diluting the error effects on the denominator. Likewise, in parallel equations, the term R is either in the numerator (as in X_c in parallel, see Table 1) or in the denominator (as in R in parallel, see Table 1) and is typically a much larger number than X_c, and therefore increases the influence of errors on parallel equations, especially when R is in the numerator as in reactance in parallel (X_c). When the subsequent volume equations are used, predictive estimates are more sensitive to L_d changes. The nonsignificant errors seen and described in this study are still deviations from the standard protocol found in Cox and Hartman (2005); therefore with a standard protocol, these "nonsignificant" errors will not be reflected and any errors that are, would be from other factors not measured here (e.g., anatomy, thickness of skin and scales, condition, or biochemical composition).

In summary, sources of error have been identified and found to significantly affect parameter estimates, but small errors that are not significant may be acceptable. In particular, electrode locations with respect to anatomy can significantly affect parameter estimates, and if electrodes needles are placed in the same anatomical location on each fish, impedance measurements will reflect the same relative volumetric areas within and between fish samples. Measurements need to be taken on a nonconductive surface that is clear of salt water, on blot-dried fish, and standardized with specific needle gauges and depths. New users need to be trained and taught stable body and hand positions and positions that allow a view of the needle to ensure accurate and precise measurements. Because temperature affects R and X_c measurements, internal temperature needs to be measured to allow adjustments of R and X_c values to 0°C or fish need to be stored on ice. Time is critical in taking impedance measurements, but icing fish can add 9 h between fish death and the time of BIA measurements. Stomach fullness of fish does not affect half- or whole-body impedance measurements, and therefore does not have to be accounted for. Sensitivity analysis in our study showed that significant deviations from the procedures of Cox and Hartman (2005) can lead to unacceptable errors in predictive estimates of BIA measurements but nonsignificant deviations are more acceptable. Although adherence to these protocols can provide consistent measurements of impedance, comparability between researchers will depend on the development of training procedures, improved understanding of temperature effects, development of improved electrodes, continuous calibration with actual laboratory measurements, and unified standard protocols. It should also be noted that multifrequency impedance analyzers are available and currents at different frequencies could possibly have different measurements than those with a single frequency. The identification of sources of error illustrated here and subsequent adherence to a standardized protocol will offset the sources of error that may be present in bioelectrical impedance research and allow the technology to advance.

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