METHODS

A Standardized Protocol for Measuring Bioelectrical Impedance in Green Turtles (*Chelonia mydas*)

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ABSTRACT

Bioelectrical impedance analysis (BIA) is gaining popularity in wildlife studies as a portable technology for immediate and nondestructive predictions of body composition components, such as fat-free and fat masses. Successful application of BIA for field-based research requires the identification and control of potential sources of error, as well as the creation of and adherence to a standardized protocol for measurement. The aim of our study was to determine sources of error and to provide a standardization protocol to improve measurement precision of BIA on juvenile green turtles (*Chelonia mydas*; $n = 35$). We assessed the effects of altered environmental temperature (20°C–30°C), postprandial state (2–72 h), and time out of the water (2 h) on five impedance parameters (resistance at infinite frequency [R_{inf}], resistance at zero frequency [R_0], resistance at 50 kHz [R_{50}], phase angle at 50 kHz [PhA_{50}], and intracellular resistance [R_i]) using a bioimpedance spectroscopy device. Technical reproducibility of measurements and interanimal variability were also assessed. We found an inverse exponential relationship between change in environmental temperature and impedance parameters R_{inf}, R_0, and R_{50}. Postprandial state significantly increased R_{inf} and R_i 72 h after feeding. BIA measurements were reproducible within individual juvenile green turtles at temperatures from 20°C to 30°C. Significant variation in impedance values was found between animals at all temperatures, sampling times, and postprandial states, but the relative differences (%) were small in magnitude. Our study suggests that measurement precision is improved by measuring animals at consistent environmental temperatures close to their preferred thermal range. We propose a standardized protocol of measurement conditions to facilitate laboratory and field use of BIA for body composition assessment studies in turtles.

Keywords: bioelectrical impedance spectroscopy, bioelectrical impedance analysis (BIA), sea turtles, temperature, postprandial state, resistance, reptile.

Introduction

Body composition assessment is a valuable parameter in ecological and clinical studies. The macrocomposition of the body (i.e., fat mass and fat-free mass) may reflect nutritional imbalances and ill health (Ward 2018*b*). Fat mass is critical for survival and reproduction and can be used as an indicator to assess the health condition or fitness of threatened species, such as green turtles (*Chelonia mydas*). Quantifying fat mass may also assist in conservation studies, for example, when examining the nutritional status of green turtles in specific foraging areas. A variety of quantitative and qualitative methods exist to assess body composition, each with different accuracy and precision (Janmahasatian et al. 2005). Impedance-based body composition assessment methods, such as bioelectrical impedance analysis (BIA), are common in nutritional and clinical research in humans. BIA has been used to estimate fat mass and fat-free mass in humans (Grundmann et al. 2015; Ward 2018*a*) and other species, such as fishes (Cox and Hartman 2005; Hartman et al. 2015; Champion et al. 2020), domestic and laboratory animals (Ward et al. 2009; Lindinger 2014; Santarossa et al. 2017; Muller et al. 2021), and wildlife (Sciullo et al. 2016; Teisberg et al. 2016). The portability, minimal invasiveness, and nondestructiveness of BIA make this

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technique potentially suitable for field-based wildlife studies (Cox and Hartman 2005; Barthelmess et al. 2006; Vue et al. 2015).

BIA measures electrical resistance (R) from which body composition can be derived using predictive equations. A tetrapolar electrode arrangement is used in which a pair of electrodes attached to the skin pass a harmless electrical current through the body while another pair of proximally placed electrodes measure impedance. Impedance is the opposition of body tissues to the passage of the alternating current (typically in the frequency range 5–1,000 kHz; Stahn et al. 2012). Impedance comprises two components: R and reactance (X_c). R represents the opposition to current flow through body water, while X_c is the opposition to current flow due to the capacitive nature of cell membranes and tissue interfaces. Both impedance and R are inversely and quantitatively related to conductive volume (Van Marken Lichtenbelt 2001; Ward et al. 2009). The conductive volume formula is as follows:

$$\text{volume} = \rho \frac{L^2}{Z\ (\text{or}\ R)}, \tag{1}$$

where L is the interelectrode length, or surrogate measure (cm); Z is the measured impedance (ohm); R is the measured resistance (ohm); and ρ is the resistivity (ohm) of the conductor. "Volume" refers to the conductive volume of body tissues.

The body's response to an alternating current depends on the frequency of the current applied. At low frequency, ideally zero frequency, current flow does not penetrate the cells, and Z (or R) is related to the volume of the extracellular water. At high frequency, ideally infinite frequency, current flow penetrates the cells, and volume is that of total body water, from which fat-free mass can be derived (fig. 1). Other physical (e.g., body geometry) and chemical (e.g., membrane and tissue composition) factors may affect R and X_c. For a detailed explanation of the physical processes involved in impedance measurements, refer to Van Marken Lichtenbelt (2001) and Ward et al. (2009). Body tissues vary in the R that they present depending on both the amount and the ionic composition of water they contain. Muscle tissue is highly conductive (i.e., lower impedance) because of its higher water content. In contrast, adipose tissue presents a higher R per unit length and area; thus, electrical current flows preferentially through the fat-free mass. Measured R parameters are used to predict the fat-free mass of an individual, with fat mass being derived by difference with body weight (Kyle et al. 2004*a*).

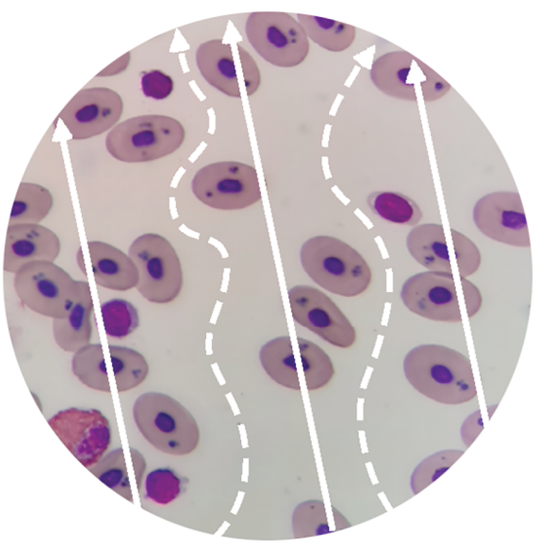

Figure 1. Current flow at low and high frequencies. Low frequencies (dashed lines) do not penetrate the cells, measuring volume of extracellular water. High frequencies (solid lines) penetrate the cells, measuring volume of total body water (intra- and extracellular water).

Several factors may confound impedance measurements when using BIA in field or laboratory settings. Studies on humans and fishes have reported effects of environmental temperature (Caton et al. 1988; Gudivaka et al. 1996), core body temperature (Hartman et al. 2011), hydration status (Cornish et al. 1998; Marini et al. 2015), and postprandial state (Slinde and Rossander-Hulthén 2001). Field-based wildlife research is subject to a highly variable environment, which may result in larger variability of impedance measurements. Reptiles have wider physiological tolerances compared with mammals, as they are poikilothermic, present intermittent breathing, and tolerate longer fasting periods (Munns 2000; Hartzler et al. 2006; Sacchi et al. 2020). These traits may result in higher interanimal variability in comparison to mammals. Current literature from human studies recommends strong adherence to a standardized protocol to minimize the impact of confounding factors on impedance measurements (Khalil et al. 2014; Brantlov et al. 2017). To our knowledge, standardization protocols of BIA for body composition assessment have not been published for any reptile species, although preliminary work has been undertaken in freshwater turtles and crocodiles (Symonds 2003; Peucker and Jack 2006). Without standardization of the BIA technique, variability of the impedance measurements will increase, and prediction of body composition will be unreliable.

The aim of this study was to establish a validated protocol to enable the use of BIA for field-based fat mass (adipose tissue) quantification in sea turtle research and rehabilitation. Our objectives were to determine measurement reproducibility and the impact of likely field variables on impedance measurements on healthy, juvenile green turtles (*C. mydas*).

Material and Methods

Animals

A total of 35 juvenile green turtles (*Chelonia mydas*) were used for experiments. The animals had been collected after hatching from the same clutch from Heron Island (−23°26′18.71″S, 151°54′30.23″E) in southern Queensland, Australia, in February 2017. All procedures were approved by the Department of Environment and Science (government permits SPP18-001167 and PTU18-001419-2) and James Cook University's Animals Ethics Committee (permits A2309, A2525, and A2585). Housing was provided at the Turtle Health Research Facility (James Cook University) in recirculated, UV-sterilized

seawater under controlled environmental conditions (water temperatures of 25°C ± 1°C, salinity of 30–35 ppt). The turtles had a mean (±SD) age of 34 ± 0 mo, length (measured to the nearest millimeter from the nuchal scute to the caudal tip of the supracaudal scute using a measuring tape) of 21.1 ± 1.5 cm, and weight (measured to the nearest milligram using a digital bench scale) of 1.07 ± 0.24 kg. Turtles were fed 5 d wk^{-1}. Diet consisted of blended whole sardines and fish fillets (20% w/w), vegetables (16% w/w), fish pellets (7% w/w), vitamins (Sea Tabs) in gelatin (7% w/w), and water (50% w/w) at a rate of 5% body weight per day. The turtles were under regular veterinary observation and assessed to be clinically healthy. These animals were accustomed to daily handling and monthly blood sampling procedures.

BIA Measurements

A handheld impedance spectroscopy analyzer (SFB7, ImpediMed, Brisbane, Australia) measured R and X_c at 256 logarithmically spaced frequencies in the range of 10–500 kHz. Device calibration was checked daily. Turtles were dried and placed in a prone position on a nonconductive plastic surface, and the head was loosely covered with a nonconductive cohesive bandage or a polyester hood to reduce visual stimuli and minimize stress. Subdermal needle electrodes (27 gauge × 0.5 inch [13 mm], Terumo, Tokyo, Japan) were attached via alligator clips to the electrode leads of the SFB7 impedance analyzer. The skin at the electrode sites was disinfected with 70% ethanol. Ethanol evaporation also removed any remaining moisture on the skin. Needles were inserted subdermally at specific and consistent anatomic locations, in the right front limb and in the right hind limb (fig. 2). The selected anatomical landmarks were the largest peripheral scale on the right front limb and the lateral claw on the right hind limb. Needles were inserted to a maximum depth of 2 mm, with at least 3 cm of distance between electrodes to avoid potential current interference. The signal electrode of each pair was placed laterally (i.e., outer electrodes), and the detector electrode was placed medially (i.e., inner electrodes). Discomfort at the side of needle insertion cannot be ruled out, and some animals involuntarily flinched during the procedure. The animals were used to handling and regular blood sampling procedures, and we assumed that the discomfort related to the needles would not cause any greater levels of stress. Stress was assessed by observing whether the animals presented peripheral vasodilation or jaw clenching. These symptoms were observed in only one turtle, which was excluded from the study.

Impedance measurements were recorded in replicate ($n = 10$), with each measurement taking 800 ms. In the first experiment, two duplicated sets of impedance measurements were obtained immediately and 5 min after taking the turtles out of the water. All repeated impedance measurements were obtained without removing the electrodes or touching the subject.

Blood Sampling

Hydration state was determined from packed cell volume (PCV; %). Blood samples from each animal were collected immediately before the BIA measurement at time point 0 min (PCV 1) and immediately after the BIA measurement at time point 120 min (PCV 2). A 1-mL aliquot of blood (25 gauge × 0.75 inch [19 mm], Terumo) was drawn from the external jugular vein after disinfection of the sample site with 70% ethanol. PCVs were read manually using a hematocrit reader (Livingstone microhematocrit capillary tubes, Livingstone, Mascot, Australia, and Pico 17 microcentrifuge, Thermo Fisher Scientific, Waltham, MA).

Protocol

Before BIA measurements, turtles were fasted for 25 ± 3 h and equilibrated to the test temperature in 10-L saltwater tanks for 2 h. Water and air temperatures were maintained within ±0.5°C of the test temperature (8402-20 Thermistor 237 thermometer, Cole-Palmer Instruments, Vernon Hills, IL). Body temperature was measured using a thermocouple (8402-20 Thermistor 237 thermometer, Cole-Palmer Instruments) and by inserting the probe 5 cm into the cloaca.

A physical examination was conducted before and after each experiment to ensure animal welfare. All procedures were conducted by a veterinarian experienced in reptile medicine and handling (S. Kophamel). Experiments 1 and 2 were performed on each animal once, with a minimum of 3 d between successive experiments. Once measurements were complete, animals were returned to their usual housing and monitored for the rest of the day.

Experiment 1: Temperature, Reproducibility, and Dry-Docking Time

Experiment 1 was conducted at three test environmental temperatures (20°C, 25°C, and 30°C), each on separate days, and simulated 2 h of dry-docking time at each temperature (i.e., turtles were removed from the saltwater tanks and were kept out of the water for 2 h; fig. 3). These parameters were chosen to mimic commonly occurring field conditions: green turtles are captured in waters ranging from 20°C to 30°C and might be left out of the water, after capture, for several hours before processing. Measurements were made in turtles at 25 ± 3 h postprandial, which is the estimated time of peak metabolic response to digestion in reptiles (Secor 2009; Merritt 2021). One animal was excluded from the 30°C and postprandial state experiments because it showed signs of stress (i.e., jaw clenching and peripheral vasodilation). Impedance was measured at 0, 5, and 120 min to determine intra- and interanimal reproducibility. Hydration state was determined via PCV (%; see "Blood Sampling").

Experiment 2: Postprandial State

The effect of postprandial state on impedance measurements was examined at 25°C 2 h (i.e., fed state) and 72 h (i.e., fasted state) after consumption of a voluntary meal equivalent to 5% of the animal's body weight (meal information detailed in "Animals"). Each animal was weighed before feeding. Impedance measurements were taken using the same protocol used in experiment 1 (fig. 3).

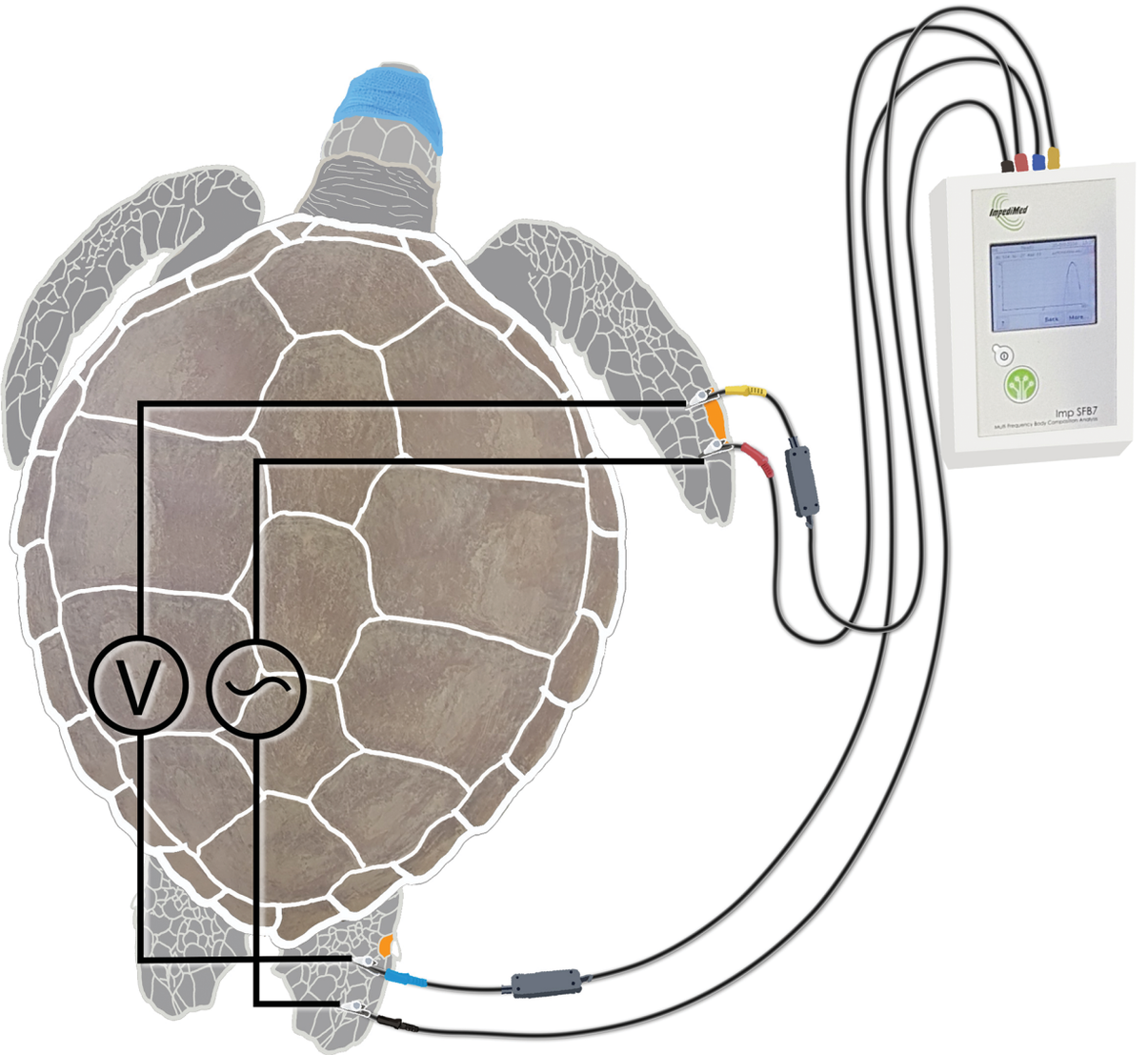

Figure 2. Anatomical locations for placing bioelectrical impedance analysis electrode needles on a juvenile green turtle (*Chelonia mydas*) using a handheld SFB7 multifrequency impedance analyzer. Electrode placement was standardized at consistent anatomic markers. On the right front limb, the inner electrode (yellow) is placed at the medial side of the scale next to the longest scale at the periphery. On the right hind limb, the inner electrode (blue) is placed at the medial side of the scale next to the claw of the right hind limb (highlighted in orange). The outer electrodes (red and black) introduce the current and are placed ≥3 cm apart from the inner electrodes to avoid current inferences. The outer electrodes are placed at the same level as the inner electrodes. The inner electrodes (yellow and blue) record the voltage using a high-input impedance voltmeter. All electrodes were inserted 2 mm subdermally.

Data Analysis

Recorded impedance values were analyzed using BioImp (ver. 5.4.0.3; ImpediMed), which calculates R (ohm), X_c (ohm), and phase angle (arctangent X_c/R; degrees) at each frequency and uses Cole analysis (fig. S1) to obtain estimated resistance at infinite frequency (R_{inf}) and at zero frequency (R_0; Cornish et al. 1993; for further details, refer to the manufacturer's website: https://www.impedimed.com/). The extracted parameters of interest were R_{inf} (predictor of total body water and fat-free mass), R_0 (predictor of extracellular water), intracellular resistance (R_i, an index of intracellular water; Van Marken Lichtenbelt 2001; Ward et al. 2009; eq. [2]), resistance at 50 kHz (R_{50}), X_c at 50 kHz, and phase angle at 50 kHz (PhA_{50}) for comparison with studies using single-frequency (50-kHz) BIA devices. The R_i formula is as follows:

$$R_i = \frac{R_0 \times R_{inf}}{R_0 - R_{inf}}. \quad (2)$$

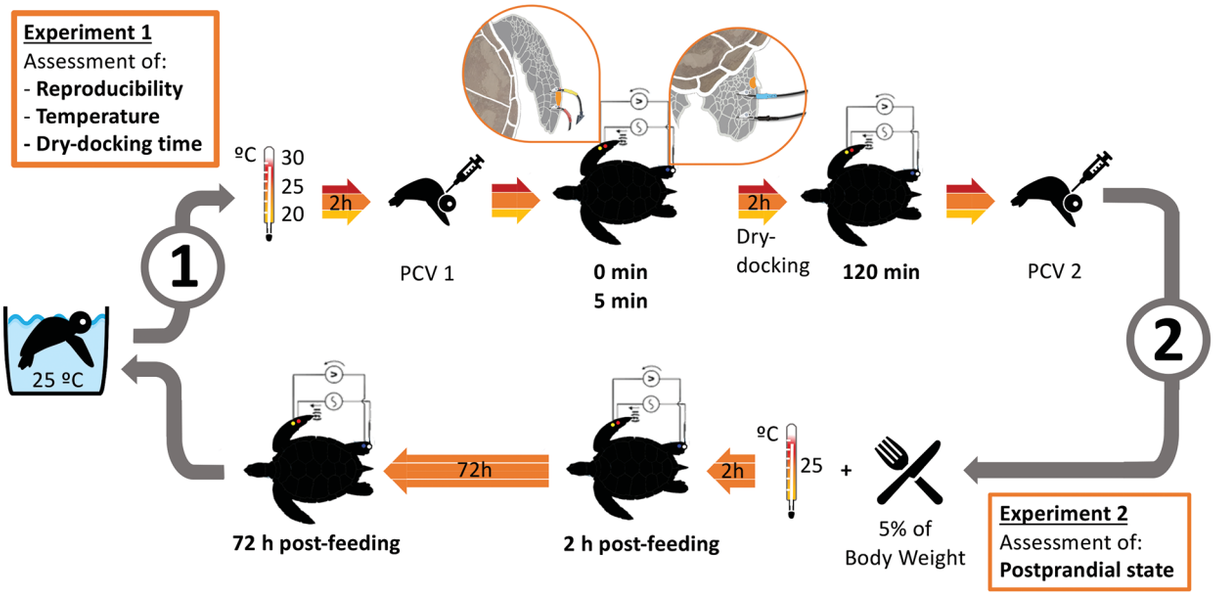

Figure 3. Experimental design for identification of potential sources of error on impedance measurements of juvenile green turtles (*Chelonia mydas*; $n = 34$ or 35). A minimum of 3 d of rest was maintained between successive experiments. PCV = packed cell volume.

The influences of temperature and postprandial state on impedance variables were analyzed using second-order polynomial models and linear mixed effects models fitted by maximum likelihood. Model assumptions (e.g., normality and variance homoscedasticity) were visually and statistically confirmed. In a model selection process based on the lowest Akaike and Bayesian information criteria, the following models best explained the data (table 1).

Tukey post hoc multiple-comparisons tests were used to assess differences between temperatures, dry-docking time, and postprandial time (emmeans package in R, $\alpha = 0.05$; Lenth 2016). Statistical analyses of R_i and PhA_{50} (nonparametric distributions) were conducted using Wilcoxon's signed rank test and Bonferroni correction. Intra-animal variability (i.e., device reproducibility and reproducibility of the BIA measurements) was assessed by conducting replicate measurements ($n = 10$) and by calculating their coefficient of variance (CV) with Tukey post hoc multiple-comparisons tests. Interanimal variability for R_{inf}, R_0, R_{50}, and R_i was determined by the standard error of the mean R values for all animals. Effect sizes were determined using Hedges's g method. Spearman's rank correlation coefficients were also calculated (figs. 4, 5). Sample size and power calculations were conducted using GPOWER (ver. 3.1.9.6 for Macintosh; Erdfelder et al. 1996). Statistical analyses were completed in R (ver. 3.6.1; R Core Team 2019). All data are mean ± SE unless otherwise stated. All data for assessing the validity of our work (.xlsx, .ods., and .csv formats) are available at James Cook University data repository (https://doi.org/10.25903/h5ah-b817).

Results

Technical Reproducibility of Measurements and Intra-animal Variability (Experiments 1 and 2)

Variation (median CV) for all experiments was 2.3% ± 3.8% for R_{inf}, 0.9% ± 3.8% for R_0, 1.3% ± 3.6% for R_{50}, and 7.6% ± 13.8% for R_i ($n = 35$; table 2; for an example of a typical animal, see fig. S1). For experiment 1, turtles at 25°C (24.8 ± 3.0 h postprandial) accounted for the majority of the observed variation (6.9% ± 3.9% for R_{inf}, 8.3% ± 3.4% for R_0, 7.9% ± 3.1% for R_{50}, and 6.8% ± 11.8% for R_i). For experiment 2, significantly smaller CV ($P < 0.05$) was measured at 25°C 72 h postprandially in all impedance parameters

Table 1: Variables of interest, models fitted, and standard error of the estimate of impedance parameters in juvenile green turtles (*Chelonia mydas*; $n = 34$ or 35)

Variable of interest	Type of model	Equation
Temperature	Second-order polynomial	Impedance parameter ~ temperature + weight
Dry-docking time	Linear mixed effects	Impedance parameter ~ time point + temperature + weight + (1|ID)
Postprandial state	Linear mixed effects	Impedance parameter ~ time postprandial + weight + (1|ID)

Note. Temperature is air temperature (°C); weight is total body weight (kg); dry-docking time is 0, 5, or 120 min; postprandial state is the hours after feeding; and 1|ID is animal ID as a random factor.

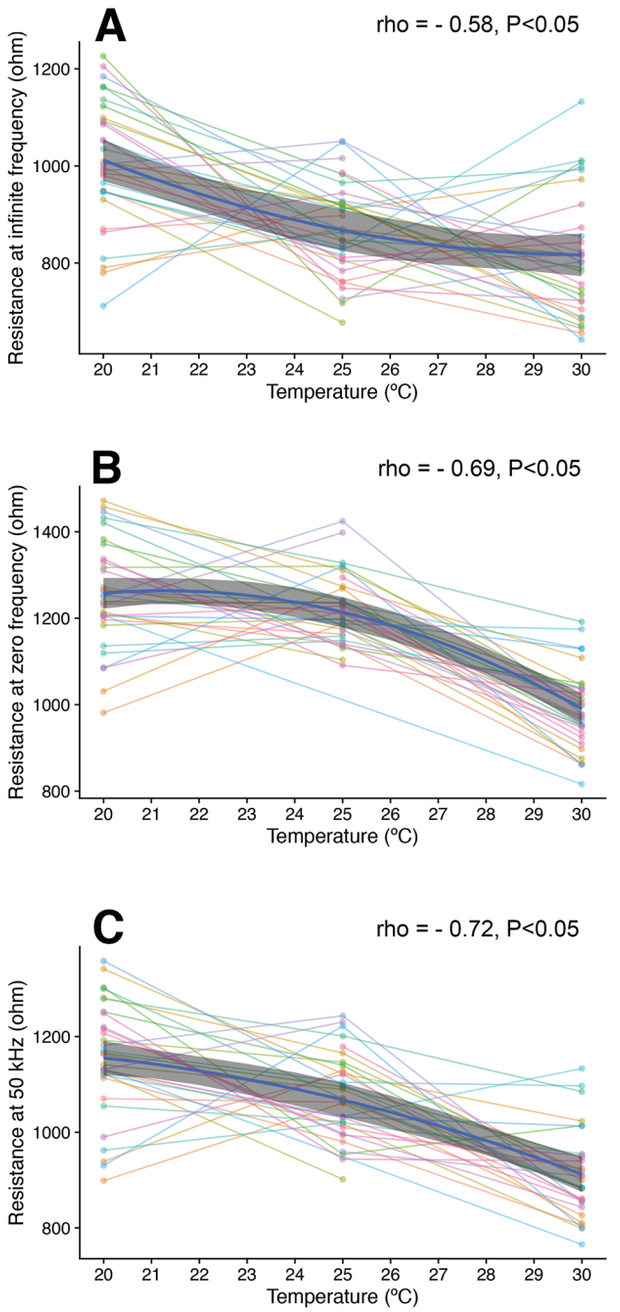

Figure 4. Second-order polynomial models, with 95% confidence intervals, of predicted impedance measurements in juvenile green turtles (*Chelonia mydas*) from 20°C to 30°C measured after a 4-h acclimatization period to the target temperature (2 h of acclimatization and 2 h of measurements; $n = 34$ or 35). *A*, Resistance at infinite frequency (ohm): $R^2 = 0.38$, Spearman's correlation coefficient $\rho = -0.58$, $P < 0.05$. *B*, Resistance at zero frequency (ohm): $R^2 = 0.63$, $\rho = -0.69$, $P < 0.05$. *C*, Resistance at 50 kHz (ohm): $R^2 = 0.56$, $\rho = -0.72$, $P < 0.05$. Superimposed line plots represent each individual's response (raw data).

(1.2% ± 1.5% for R_{inf}, 0.5% ± 0.5% for R_0, 0.7% ± 0.9% for R_{50}, and 5.6% ± 10.0% for R_i). The overall variation for all experiments except experiment 1 at 25°C was 0.6% ± 0.9% for R_0, 7.0% ± 14.5% for R_i, 1.4% ± 2.5% for R_{inf}, and 0.8% ± 1.4% for R_{50}. No other significant variation differences between time points and temperatures were found ($P > 0.05$).

Interanimal Variability (Experiments 1 and 2)

Significant variation in impedance measurements was measured at each experimental temperature and at each sampling time. Interanimal variability in R_{inf}, R_0, and R_{50} was highest at 20°C (R_{inf}: 15.4%; R_0: 11.8%; R_{50}: 12.1%) and decreased to a nadir at 25°C (R_{inf}: 12.6%; R_0: 6.4%; R_{50}: 8.6%) before increasing at 30°C (R_{inf}: 15.5%; R_0: 10.4%; R_{50}: 11.2%). Standard deviation was largest at 20°C (R_{inf}: 1,062.9 ± 163.5 ohm; R_0: 1,330.5 ± 157.2 ohm; R_{50}: 1,205.8 ± 146.0 ohm), was decreased at 30°C (R_{inf}: 861.5 ± 134.0 ohm; R_0: 1,048.4 ± 109.1 ohm; R_{50}: 964.2 ± 107.9 ohm), and was smallest at 25°C (R_{inf}: 872.7 ± 109.9 ohm; R_0: 1,213.0 ± 77.9 ohm; R_{50}: 1,070.3 ± 92.0 ohm) experiments. R_i demonstrated the greatest interanimal variability compared with R_{inf}, R_0, and R_{50}. Interanimal variability of R_i ranged from 30.8% to 94.4%, with mean values ± SD ranging from 3,328.2 ± 1,025.7 to 7,203.3 ± 3,510.4 ohm. Interanimal variability did not follow a specific pattern as dry-docking time increased (table S5). Tables S4 and S5 give the interanimal variability results for all impedance variables at each temperature, hours postprandial, and time points.

Effect of Temperature and Dry-Docking Time (Experiment 1)

In general, *R* declined with an increase in environmental temperature but not consistently between animals or impedance parameters (fig. 4). Data were well fitted by the models but did not account for the significant variation in responses between individuals. Overall, significant differences were found for R_{inf} between temperatures 20°C–25°C and 25°C–30°C ($P < 0.05$, df = 91, Hedges's $g = 1.37$ [for 20°C–25°C] and 0.09 [for 25°C–30°C], $t = 5.2$ [for 20°C–25°C] and 2.2 [for 25°C–30°C]), for R_0 between temperatures 20°C–30°C and 25°C–30°C ($P < 0.05$, df = 92, Hedges's $g = 2.08$ [for 20°C–30°C] and 1.74 [for 25°C–30°C], $t = 11.2$ [for 20°C–30°C] and 9.5 [for 25°C–30°C]), and for R_{50} between temperatures 20°C–25°C, 20°C–30°C, and 25°C–30°C ($P < 0.05$, df = 92, Hedges's $g = 1.11$ [for 20°C–25°C], 1.88 [for 20°C–30°C], and 1.06 [for 25°C–30°C], $t = 3.7$ [for 20°C–25°C], 10.1 [for 20°C–30°C], and 6.5 [for 25°C–30°C]).

Impedance parameters decreased as dry-docking time increased. Significant differences were found between time points 0–120 min and 5–120 min across all temperatures (R_{inf}: $P < 0.05$, df = 272, $t = 3.5$ [0–120 min]; R_0: $P < 0.05$, df = 272, $t = 5.4$ [0–120 min], $t = -3.2$ [5–120 min]; R_{50}: $P < 0.05$, df = 272, $t = 5.4$ [0–120 min], $t = -3.2$ [5–120 min]; table S5). No significant differences ($P > 0.05$) were found between time points 0 and 5 min for R_{inf}, R_0, and R_{50} and between time points 5 and 120 min for R_{inf}. No significant differences in PhA_{50} were found between temperatures and between impedance measurements at different time points ($P > 0.05$). We found no significant changes in PCV (%; data

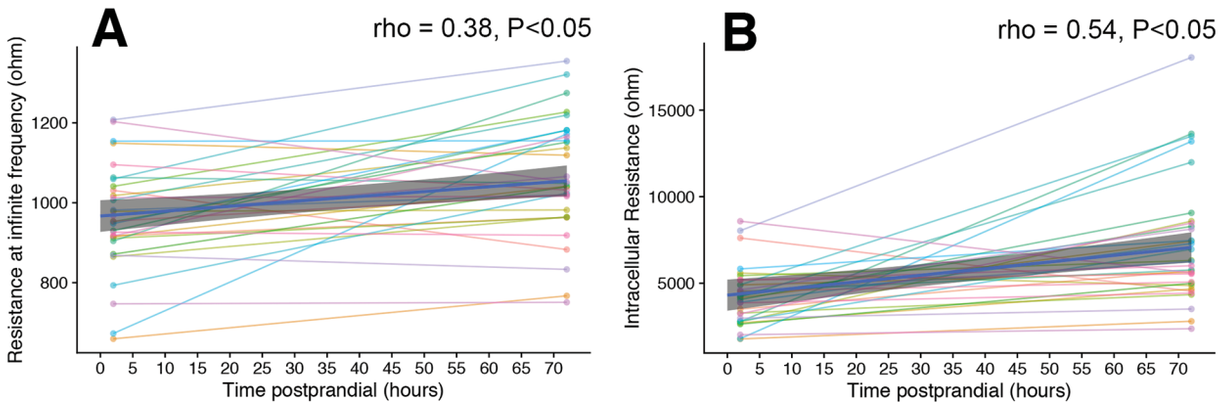

Figure 5. Impedance variables measured in juvenile green turtles (*Chelonia mydas*) at 2–72 h postprandial at 25°C; $n = 34$. *A*, Predicted resistance values at infinite frequency (ohm): $R^2 = 0.51$, Spearman's correlation coefficient $\rho = 0.38$, $P < 0.05$. *B*, Intracellular resistance (ohm): $R^2 = 0.38$, $\rho = 0.54$, $P < 0.05$. Superimposed line plots represent each individual's response (raw data).

normally distributed) after dry-docking the turtles for 2 h at any test temperature (20°C, 25°C, and 30°C; $P > 0.05$). Mean PCV before time point 0 min (PCV 1) was 31.0% ± 3.2% (all temperatures). Mean PCV at time point 120 min (PCV 2) was 30.3% ± 3.3% (all temperatures).

Sample size and power calculations revealed that a sample size of ≥18 subjects (repeated measurements) would be sufficient to achieve a statistical power of 0.8 ($\alpha = 0.05$). Our statistical power (β), calculated for $n = 34$ animals, was $\beta = 1$ ($F = 3.2$, $\alpha = 0.05$, effect size $f = 6.1$). Tables S2 and S3 provide results of the predicted values and of the post hoc tests for pairwise comparisons, including the estimated values, standard errors, degrees of freedom, effect sizes (Hedges's g method), confidence limits, and P values for all impedance parameters.

Effect of Postprandial State (Experiment 2)

R_{inf} and R_i significantly increased 72 h after feeding relative to 2 h after feeding ($P < 0.05$, Hedges's $g = 0.75$ and 1.07, respectively; fig. 5). Postprandial state did not significantly affect R_0, R_{50}, and PhA_{50} ($P > 0.05$). Impedance parameters did not consistently change between animals as postprandial time increased (fig. 5).

Discussion

In this study, we confirmed reproducibility of BIA measurements in juvenile green turtles (table 2), and our data highlight the importance of controlling for environmental temperature to standardize measurement of impedance parameters (general guidelines provided in fig. 6). In the present study, model fitting

Table 2: Intra-animal variability, assessed by the median coefficient of variance (percent ± SD), of 10 replicate impedance measurements

Experiment, temperature	R_{inf}	R_0	R_{50}	R_i
Experiment 1 (25 ± 3 h postprandial):				
20°C[a]	1.8 ± 2.5	.7 ± 1.2	.8 ± 1.7	9.1 ± 14.0
25°C[a]	6.9 ± 3.9	8.3 ± 3.4	7.9 ± 3.1	6.8 ± 11.8
30°C[b]	1.4 ± 3.0	.6 ± .6	.8 ± 1.3	8.5 ± 17.1
Experiment 2:				
25°C, 2 h postprandial[b]	1.1 ± 1.3	.4 ± .6	.6 ± .8	3.5 ± 3.8
25°C, 72 h postprandial[b]	1.4 ± 1.8	.6 ± .3	.7 ± 1.0	10.5 ± 12.1
Median (all experiments except experiment 1 at 25°C)	1.4 ± 2.2	.6 ± .7	.7 ± 1.2	7.9 ± 11.8
Median (all experiments)	2.5 ± 2.5	2.1 ± 1.2	2.2 ± 1.6	7.7 ± 11.8

Note. R_0 = resistance at zero frequency; R_{50} = resistance at 50 kHz; R_{inf} = resistance at infinite frequency; R_i = intracellular resistance.
[a] $n = 35$.
[b] $n = 34$.

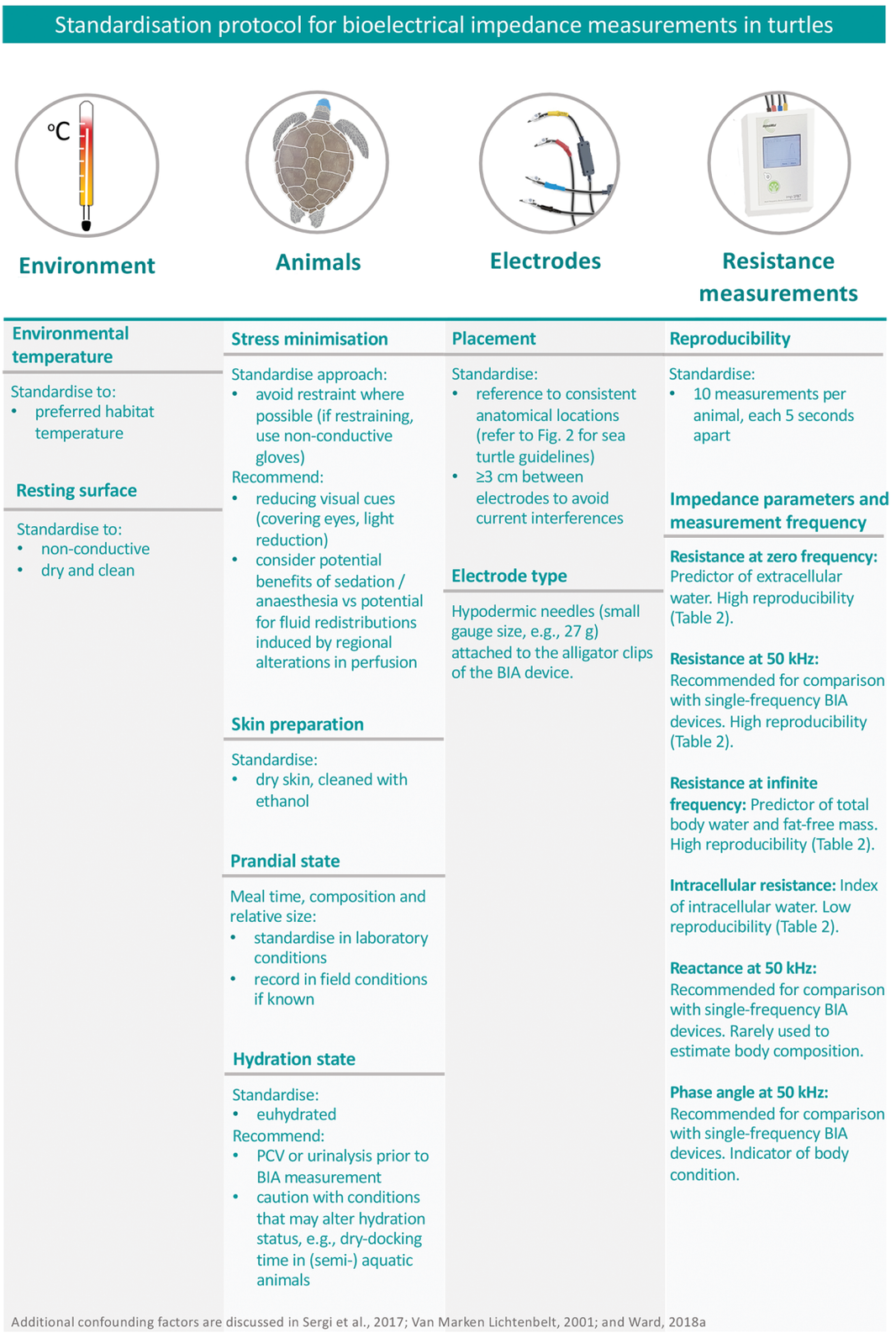

Figure 6. Standardization protocol for bioelectrical impedance measurements in turtles. BIA = bioelectrical impedance analysis; PCV = packed cell volume.

was assessed as standard error (table S2). Standard errors were less than 1.5% for R_{inf}, R_0, and R_{50}, which are comparable with published data in human-based studies (Ward et al. 1997).

Technical Reproducibility of Measurements and Intra-animal Variability

Reproducibility of impedance measurements (i.e., intra-animal variability, assessed by the CV) was assessed as a proxy for precision and was consistent with CV values reported in humans for R_{inf}, R_0, and R_{50} (i.e., 0.7%–2% for single-frequency and multifrequency BIA devices; Van Loan and Mayclin 1987; Steijaert et al. 1994; Ward et al. 1997). Intra-animal variability was low enough to suggest that BIA is appropriate for use in sea turtles. Intra-animal variability was greater for impedance parameters R_{inf} and R_i than for R_0 and R_{50} (table 2). Greater imprecision in measurement of R_{inf} is not surprising, since R_{inf} is calculated by extrapolation of data when fitting the Cole model. Consequently, precision is highly dependent on the goodness of fit of measured data to each model. CV for R_i was also comparatively large (7.0%–7.6%). The large variation for R_i is also not surprising, since R_i is derived from R_0 and R_{inf} (eq. [2]). Both R_i and R_{inf} are estimated by extrapolation and may be subject to propagation of error. An error propagation analysis showed that, on average, 84% $\pm$ 3.6% of the error measured for R_i was determined by variance in R_{inf}, whereas only 16% $\pm$ 3.6% was attributable to R_0 (table S6). The errors in R_i make this impedance parameter less reliable for body composition assessment (Ward et al. 1997; Ward 2009; Sanchez et al. 2013).

An unexpected observation was the fivefold difference in magnitude between experiments conducted at the same temperature (25°C); for example, median R_{inf} CV was 6.9% in experiment 1 (25 $\pm$ 3 h postprandial) but 1.1% (2 h postprandial) and 1.4% (72 h postprandial) in experiment 2 (table 2). The time to peak increase in postprandial metabolic rate varies across reptiles depending on meal size, frequency, and composition and temperature and occurs on average at approximately 25 h postprandial (Secor 2009). Few studies have measured metabolic responses to digestion in marine turtles; however, postprandial metabolic rate peaks at 21 h and remains elevated for a total of 30 h in hawksbill turtles (*Eretmochelys imbricata*) fed a meal equivalent to 5% of the animal's body weight at 30°C (Merritt 2021). The greater intra-animal variability at 25 h postprandial could be related to changes in relative fluid distribution induced by digestion (Secor 2009; Merritt 2021). However, this is not supported by the intra-animal variability at 25 $\pm$ 3 h postprandial at 20°C and 30°C (experiment 1), which was not larger than the intra-animal variability observed in experiment 2. Although the turtles were used to handling and to blood sampling, BIA was a novel procedure that animals had been exposed to only once before experiment 1. Experiment 2, however, was conducted last after an interval of 3–4 d, during which the animals had possibly become familiarized with the experimental procedures. We hypothesize that changes in cardiovascular function (i.e., skeletal muscle activity) might have triggered the greater intra-animal variability at 25 $\pm$ 3 h postprandial at 25°C (experiment 1). Whole-body impedance varies according to the distribution of water between extracellular and intracellular compartments (fig. 1) and redistribution of total body water between body segments via blood flow, which is regulated by the cardiovascular system (Liang et al. 2000). Cardiovascular function, which may be increased by handling stress, leads to redistribution of body fluids between compartments and body regions, such as the limbs. Changes in regional blood flow, and the resulting redistribution of body fluids, have been shown to alter impedance values in humans (Liang et al. 2000). Redistribution of relatively small volumes of blood to the periphery will disproportionately affect whole-body impedance measurements, potentially contributing to increasing intra- and interanimal variability (Liang et al. 2000). Therefore, changes in electrode placement or posture can result in an over- or underestimation of body impedance and, consequently, of fat mass or fat-free mass (Gudivaka et al. 1999; Hafs and Hartman 2011). Electrode placement should be standardized for each species. We recommend minimizing handling stress in field settings (e.g., covering the eyes of the turtles or possibly using sedation in other species), as well as habituating the animals before the BIA procedure in laboratory settings. Nevertheless, intra-animal variability with <10% CV might still be deemed satisfactory depending on the research question. Further research assessing stress hormone levels in sea turtles and the impact of alterations in cardiovascular function and blood flow redistribution on impedance is warranted. This assessment should be carried out at different environmental temperatures (20°C–30°C), in fed (2 h postprandial) and fasted (72 h postprandial) states, and during peak digestive metabolic rate (25 $\pm$ 3 h postprandial).

Temperature, Dry-Docking, and Interanimal Variability

The relationship between increasing temperature and impedance values varied between turtles (fig. 4). The current study indicates an inverse exponential relationship between environmental temperature and mean R values (fig. 4). In addition, interanimal variability (i.e., median CV and standard deviation) was highest at a temperature of 20°C followed by 30°C and 25°C and was lowest at 120 min dry-docking time.

Body temperature of poikilothermic animals, such as sea turtles, is largely determined by environmental temperature. Green turtles are mainly found in waters above 20°C in the Pacific, Atlantic, and Indian Oceans. Reptiles can control their rate of heat transfer to the environment by regulating cardiovascular function (Smith et al. 1986; Sato et al. 1994; Galli et al. 2004). Previous sea turtle studies suggested that blood flow plays an essential thermoregulatory role. The extent to which individuals control heart rate and/or peripheral vascular R for thermoregulatory purposes at a certain temperature might be different across individuals (Heath and McGinnis 1980; Smith et al. 1986; Penick et al. 1996; Hochscheid et al. 2002). High variability in physiological responses is a common finding in reptile studies and is not necessarily related to the experimental methodology (Munns 2000). Our study was conducted under carefully controlled laboratory conditions using a highly

standardized protocol, acclimatizing animals to specific temperature regimens for 4 h. It is therefore unlikely that our experimental methodology caused the heterogeneity in individual responses. We hypothesize that at temperatures lower than the preferred thermal range (25°C–30°C; Bluvias 2010; Stacy and Innis 2017), thermoregulatory mechanisms, including reductions in peripheral blood flow, were induced to slow the rate of cooling. The likely individual variation in the degree of peripheral vasoconstriction, especially to the limbs; the set point at which thermoregulatory mechanisms were employed; and the resulting relative changes in fluid redistribution could all independently or in combination result in the variable impedance responses measured between animals (fig. 4). Cloacal body temperature is likely not an accurate measure of core body temperature, as it is influenced by several factors, such as ambient temperature, regional blood flow, heart rate, or surface-to-volume ratio. In field settings, additionally, core temperature measurements are not practical because of logistical and ethical constraints. The impact of peripheral vasoconstriction on impedance measurements might be confirmed by using longer acclimatization periods and by using stomach or intramuscular temperature loggers to measure core body temperature in a laboratory setting.

R was more sensitive to changes in temperature than PhA_{50}, a body condition indicator used in human studies and to a lesser extent in animals (Cornish et al. 1992; Kyle et al. 2004*b*; Hartman et al. 2015; Ward 2018*a*). This observation suggests that R may be the preferred indicator for studies of temperature effects, whereas the relative stability of PhA_{50} indicates that it may be a useful indicator of body condition. Finally, we did not find any significant differences in PCV, suggesting that 120 min of dry-docking at 20°C, 25°C, and 30°C did not cause dehydration in the animals. In field conditions, animals are often left dry-docking for several hours before processing, with the potential risk of dehydration. Alterations in hydration status are important to consider, as intra- and extracellular fluid distributions define the R values (Lindinger 2014). Urinalysis might have provided a more comprehensive assessment of hydration status compared with PCV. Because of logistical constraints and handling challenges (i.e., difficulties for emptying the bladder in sea turtles) and to minimize stress, PCV was prioritized over urinalysis in this study.

Postprandial State Effects

R_{inf} and R_i were decreased in the fed state (2 h postprandial) in comparison to the fasted state (72 h postprandial) at 25°C. The changes in R_{inf} were small in magnitude, representing $0.1\% \pm 0.7\%$ h^{-1} after feeding. To our knowledge, no other studies have assessed the effect of postprandial state on impedance measurements in animals. In reptiles, at 2 h postprandial, physiological changes related to digestion might not have occurred yet (Secor 2009). Peak metabolic rate in response to digestion occurs at approximately 21–25 h after feeding (Secor 2009; Merritt 2021). The 72-h postprandial state represents the fasted state. The effect of feeding has been studied in humans with equivocal results depending on the body region being measured, posture of the participant, and feeding regimen (Dixon et al. 2013). In the present study, R_0 and R_{50} were more stable 72 h postprandial (fasted state) than R_{inf} or R_i. R_0 is reflective of extracellular water, whereas R_i and R_{inf} are reflective of intracellular and total body water, respectively. In vertebrates, digestion results in increased bicarbonate concentrations (HCO_3^-), with an accompanied increase in blood pH. This so-called alkaline tide is especially marked in reptiles (Hartzler et al. 2006; Sherwood et al. 2012) and has also been reported in green turtles (March et al. 2019). Electrolyte shifts in the fed state, relative to the fasted state, may have led to the increased intra-animal variability observed for R_i and R_{inf} 72 h postprandial. In field-based studies, it is likely that postprandial state will not be known. Hence, the small but significant effect of postprandial state needs to be included in reliability and confidence assessments of impedance measurements.

Study Limitations

The transformation of impedance values to the predicted body composition parameters of fat-free mass and fat mass requires the BIA device to be calibrated against a reference method, such as computed tomography. Calibration studies to develop the required predictive equations have recently been completed by our research team. The predictive equations to estimate fat-free mass and fat mass in green turtles are detailed in Kophamel et al. (2023). The transformation of impedance parameters to a body composition parameter requires additional measurements, such as interelectrode length, and several assumptions. These assumptions include a constant known as hydration fraction, resistivity coefficients (or their implicit values in regression-based predictor algorithms), and body proportion parameters (Ward et al. 1998). Errors in these parameters, not accounted for in the present study and logistically complex to account for in field studies, may be of proportionally smaller or larger magnitude than the errors reported in this study. Minimization of overall error therefore requires attention to all of these factors.

Conclusions

BIA demonstrated strong validity and reliability in juvenile green turtles (*Chelonia mydas*), and we propose a standardization protocol to enable field-based use in other turtle species. Impedance measurements were highly reproducible across experimental temperatures and sampling times and resulted in low interanimal variability at the species' preferred thermal range. We identified environmental temperature and postprandial state as confounding factors on the measurements. Animals' responses to handling (cardiovascular and skeletal muscle activity) may increase impedance variability, and future study protocols should aim to minimize these impacts by minimizing or standardizing potential stressors. The heterogeneity in the individuals' responses across temperatures and postprandial time suggests that correction algorithms are inefficient for BIA use in the field. We therefore emphasize the need to control for environmental temperature in laboratory and field settings where possible and provide general guidelines to standardize the R measurements in turtles (fig. 6).

Precision, accuracy, and reproducibility of measurement will depend on operator training, an understanding of the basic principles of impedance measurement, the effects of confounding factors on *R* values, and the responses of each individual. We highlight the potential for BIA, with careful application and standardization, to become a very valuable tool for sea turtle body composition assessment in field-based studies.

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