Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Scienze Farmacologiche e Tossicologiche dello Sviluppo e del Movimento Umano

XXXII Ciclo

Settore Concorsuale: 05/B1

Settore Scientifico Disciplinare: BIO/08

Evaluation of body fluids changes in sport practice and effects of dehydration during exercise

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Esame finale anno 2020

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ABSTRACT

Body composition is one of the most monitored aspects in athletes, given its relationship to physical performance. High values of fat mass (FM) negatively affect the ability to express force in specific movements (i.e. during jumping). On the other hand, fat-free mass (FFM) is closely related to strength and muscle power, and its increase is one of the goals included in injury prevention programs. Additionally, in sports as well as in daily life, hydration status plays an important role, as hypo-hydration and fluid accumulation may affect physical function, cognitive performance, and health status. Despite the importance of maintaining a well-hydrated state, studies have shown athletes are not adequately replacing fluid during sport practice. Furthermore, although laboratory clinical tests are typically preferred over signs and symptoms for detecting changes in body fluids, the methods are expensive, involving specialized technicians to perform and analyze the required exams. Yet, practitioners, coaches and researchers face the common problem of a lack of valid and practical methods and techniques to monitor body fluids changes under various conditions. Bioelectrical impedance can be applied to assess body composition using prediction equations. However, the dependency on population-specific equations and hydration status is considered the major weakness of conventional bioelectrical-impedance analysis. Alternatively, the analysis can be performed using raw data, namely phase angle (PA), or bioelectrical impedance vectors, i.e. PA and vector length jointly, as in the bioimpedance vector analysis (BIVA) approaches (classic and specific). However, although largely used, reliability studies of PA, classic or specific BIVA in the assessment of body composition, or of hydration through reference techniques are very scarce in the general population and totally lacking in athletes.

For this reason, the purposes of this thesis were: 1. to examine the accuracy of PA, classic and specific BIVA in body composition assessment of athletes, focusing the analysis on absolute values of body mass [(FM), (FFM), total body water (TBW), extracellular water (ECW), intracellular water (ICW)], and on values independent from body dimensions (%FM, ECW/ICW); 2. to explore the

suitability of BIVA in evaluation long-term body fluid changes, over a competitive season in athletes; 3. to determine the time course of bioimpedance values after a period of controlled exercise and when a cold shower with a standardized duration and water temperature was applied; 4. to determine the effects of dehydration on heart rate (HR) response, neuromuscular functionality, and time trial (TT) performance during a cycling exercise in athletes.

According to the aims, the main results of the studies can be summarized as follows:

Specific BIVA turns out to be more accurate for the analysis of %FM in athletes, while it does not correctly evaluate TBW, for which classic BIVA appears to be a suitable approach. PA, and hence both BIVA approaches, can detect ECW/ICW changes.

The vector changes measured with BIVA convincingly mirror fluids loss or gain over a season. In particular, peripheral vectors lying on the left or right side of the minor axis of the BIVA tolerance ellipses, i.e. with higher or lower PA, indicate more or less soft tissue, respectively. In addition, PA is sensitive and inversely related to ECW/ICW ratio.

BIVA might be a valuable tool in identifying dehydration and fluid shifts after physical exercise. A 10-minute cold shower enables the stabilization of bioimpedance analysis measurements within 20 minutes after 30 minutes running exercise, which might facilitate the assessment of hydration change after exercise. Otherwise, without shower the time until reaching stable bioimpedance values is prolonged to 40 min.

After exercise induced dehydration, the bioimpedance vector significantly lengthens along the major axis of the tolerance ellipse, in conformity with body weight loss (-2%), that indicates fluid loss. Dehydration during a one-hour cycling test and subsequent TT, at 65% and 95% of the VO2max, respectively, caused a significant increase in HR during the first trial part (65%VO2max), while it had no effect on the TT performance. Neuromuscular function, assessed by surface electromyography on the thigh muscles, showed a lower muscle activation in TT performance in dehydration conditions, but

no difference when the athletes cycled at 65% of the VO2max, except for the biceps femoris muscles, which showed a major power output also in the first trial part at 65% of the VO2max.

Key words: BIVA, hydration, phase angle, total body water, vector length.

LIST OF PUBLICATIONS

This thesis is mainly based on the following publications, herein referred to by their Roman numerals:

I. Marini E, **Campa F**, Buffa R, Stagi S, Matias CN, Toselli S, Sardinha LB, Silva AM. Phase angle and bioelectrical impedance vector analysis in the evaluation of body composition in athletes. Clin Nutr. 2019. pii: S0261-5614(19)30070-6. doi: 10.1016/j.clnu.2019.02.016.

II. **Campa F**, Matias CN, Marini E, Heymsfield S, Toselli S, Sardinha L, Silva AM. Identifying Athlete Body-Fluid Changes During a Competitive Season With Bioelectrical Impedance Vector Analysis. Int J Sports Physiol Perform. 2019. doi: 10.1123/ijspp.2019-0285.

III. **Campa F**, Gatterer H, Lukaski H, Toselli S. Stabilizing Bioimpedance-Vector-Analysis Measures With a 10-Minute Cold Shower After Running Exercise to Enable Assessment of Body Hydration. Int J Sports Physiol Perform. 2019. doi: 10.1123/ijspp.2018-0676.

In addition, this thesis is supported by the following conferences presentations, here in referred to by their Roman numerals:

I. **Campa F**, Gatterer H, Lukaski H, Toselli S. A Cold Shower Accelerates the Stabilization of Impedance Parameters after Exercise Allowing the Assessment of Body Hydration Status with Bioimpedance Vector Analysis. 11th International Symposium on In Vivo Body Composition Studies Body Composition Analysis (Structural, Functional, Kinetic): Technologies and Models for Biomedical Research and Clinical Application. Columbia University. 2018 June 25 – 27; New York City, USA.

II. **Campa F**, Matias CN, Marini E, Heymsfield S, Toselli S, Sardinha L, Silva AM. Bioelectrical impedance vector analysis identifies body fluid changes over a season in

athletes playing different sports. 3rd Sport Nutrition International Conference. SINSEB. 2018 November 30 – December 2; Bologna, Italy.

GLOSSARY

BC	Body composition
BCM	Body cell mass
BMC	Bone mineral content
BIA	Bioelectrical impedance analysis
BIVA	Bioelectrical impedance vector analysis
BMI	Body mass index
DXA	Dual-energy X-ray absorptiometry
ECW	Extracellular water
ECW/TBW	Extracellular / total body water ratio
EMG	Electromyography
FFM	Fat-free mass
FFB	Fat-free body
FM	Fat mass
H	Body height
HR	Heart rate
Hotelling‘s T^2 test	Test comparing mean two group vectors
ICW	Intracellular water
PA	Phase angle
R/H	Bioelectrical resistance (R/H when adjusted by height)
RXc-graph	R/H vs. Xc/H or Rsp vs. Xcsp probabilistic plot
Rsp	Resistance adjusted by body geometries
TBW	Total body water
Xc/H	Bioelectrical reactance (Xc/H when adjusted by height)
Xcsp	Reactance adjusted by body geometries

Z	Bioelectrical impedance
Z vector	Vector yield by the RXc-graph

GENERAL INTRODUCTION

Body composition

The research in human body composition (BC) has gained relevance given the recognized health impact of several body components. Many contemporary scientists contributed to the field of BC research as it exists today even though interest in the topic extends back several thousand years. One important advance to build an appropriate structure for BC research and hence, to clearly define human BC as a branch of human biology was the proposed five-level model by Wang et al. in 1992 (Figure 1).

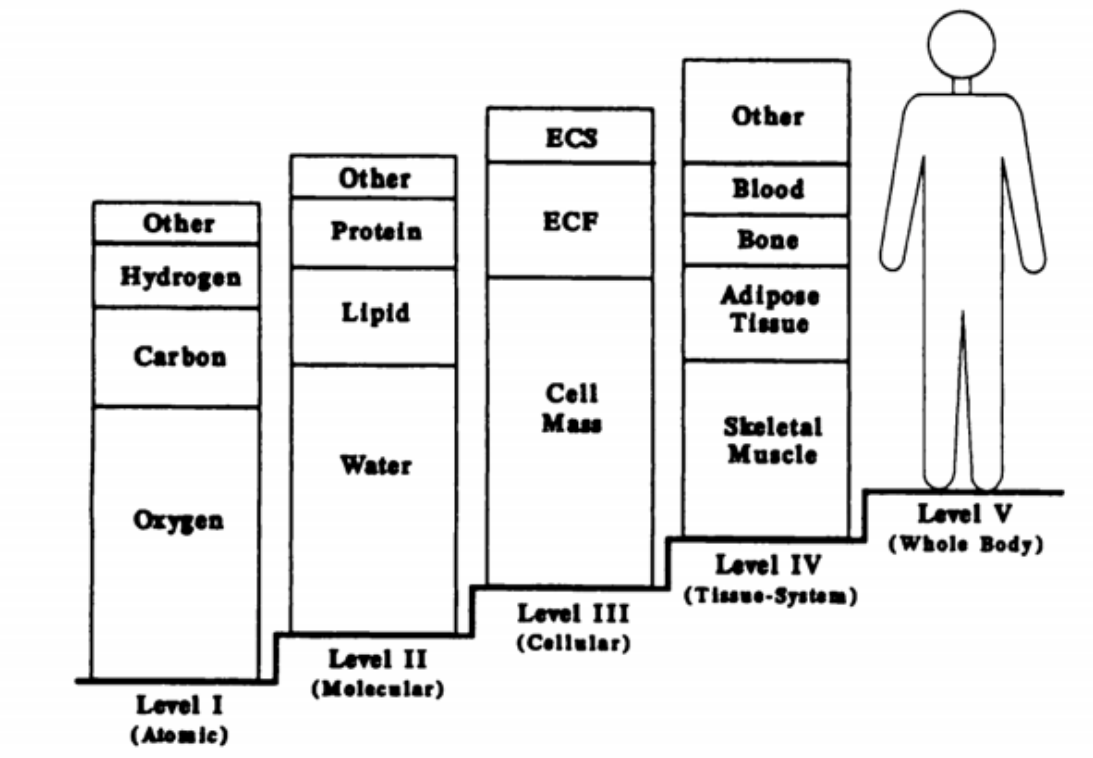

Figure 1. The five body composition levels (Wang et al. 1992).

According to this model, the main body components are organized into five distinct levels of increasing complexity. The five levels are:

1) Atomic: Fifty elements are found in the human body. At this level, 6 elements (oxygen, carbon, hydrogen, nitrogen, calcium, and phosphorus) account about 98% of body weight, and other 44 elements make up the remaining 2%.
2) Molecular: The major components in this approach are water (which comprises 60% of body weight), lipid (simple lipids, compound lipids, steroids, fatty acids and terpenes), protein, mineral and glycogen (Figure 2).

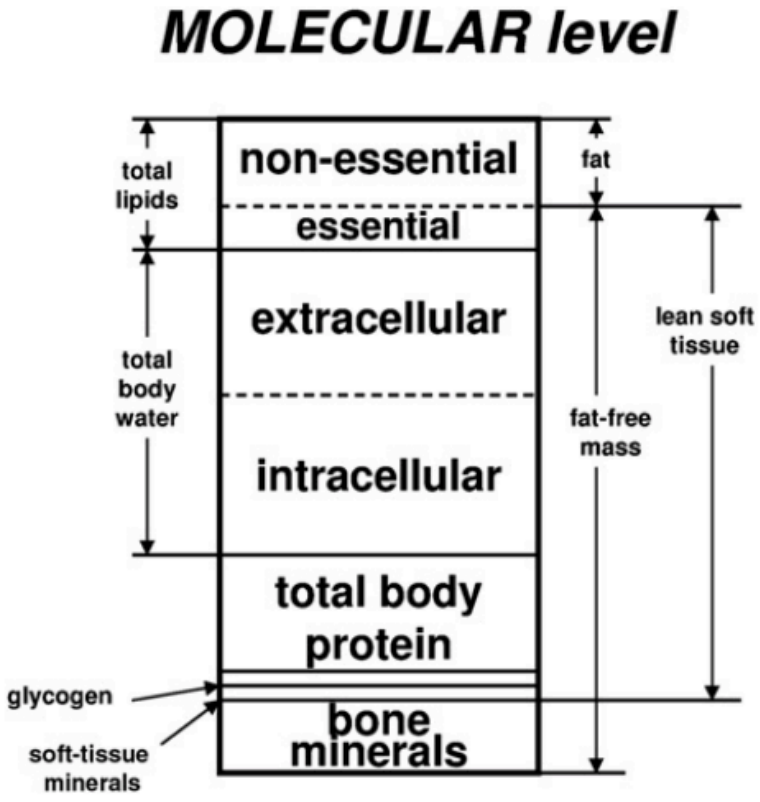

Figure 2. Components of the molecular model (Heymsfield et al. 2005).

This molecular level is the most considered and various level that range from two to four compartments. Behenke et al. (1942) introduced the two-compartment (2C) body composition model which divides body weight into fat mass (FM), with a density of 0.9007 g/cm^3, and fat-free mass (FFM), with a density of 1.1 g/cm^3. Hydrometric methods are also considered 2C models, as body weight is divided in total body water (TBW) and residual. Assuming that TBW represents 73.2% (Wang et al. 1999) of FFM, FM can be simply obtained. At this regard, Pace and Rathbun (1945) were the first to propose that TBW is a constant fraction of FFM on the basis of experiments in pigs. Later, the chemical analysis of 9 human cadavers with an average TBW: FFM of 0.737 provided further support for this assumption (Wang et al. 1999). However, it has been observed that several factors affect the FFM composition and therefore the respective density value, which may be lower or higher than the value of 1.1 g/cm^3 (Durnin and Womersley 1974; Silva et al. 2006). In addition to assume the FM and FFM density as 0.9 and 1.1 g/cm^3, respectively, applying the 2C model requires that 1) the densities of fat and fat-free body (FFB) compartments (water, protein and mineral) are the same for all individuals, 2) the densities of the tissues comprising the FFB are constants within an individual, and their proportional contribution to the lean remains constant, and 3) the individual being measured differs from the reference body only in the amount of fat. Recent technological advances for measuring the water (isotope dilution), minerals, [dual-energy x-ray absorptiometry (DXA)], and proteins (neutron activation analysis) of the FFB have allowed to quantify the FFB composition in vivo using multicomponent models. The assessment of TBW, a major molecular component of FFM, in addition to the estimate of FM obtained by densitometry methods, allows the division of body weight into 3 compartments (3C) composed by FM, TBW and residual mass (protein and mineral) (Siri 1961). Thus, the transition of 2C to a 3C model substantially improves the validity of molecular models in BC assessment, overcoming the limitations of measuring density alone and controlling for inter-individual variation in FFM. However, assuming a constant mineral-to-protein ratio of 0.35 (Withers et al. 1998) it should be used with caution in subjects with depleted body protein or bone mineral content (BMC)

(Ellis 2000; Kuriyan 2018). DXA, the gold standard for BMC measurements, is another widely used 3C method for assessing BC, which considers FFM divided into lean tissue mass and BMC (Heymsfield et al. 1990) (Figure 3). Although reliable in its measurements, concern related to the validity of DXA stems from the assumption of constant hydration of the FFM component. Additionally, FFM can be also considered as a sum of body cell mass (BCM) and extracellular mass (ECM) (Joshi and Bagal 2014) (Figure 3).

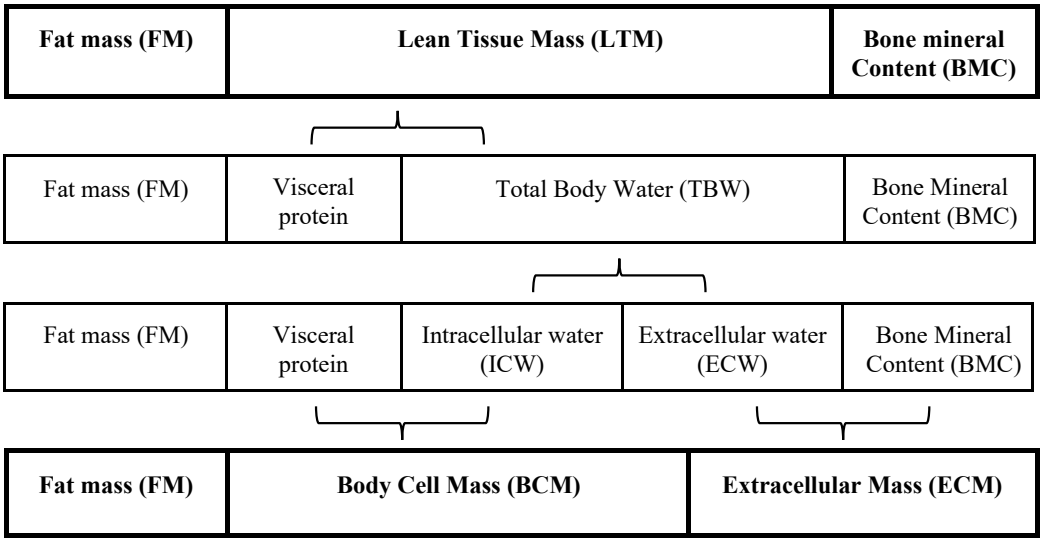

Figure 3. Compartment models of body composition. Adapted from Joshi and Bagal (2014).

In addition, the classic Siri (1961) 3C model can be extended to a four compartment model (4C) by adding BMC (Heymsfield et al. 1990) (Figure 4). Since the 4C model takes into consideration biological variability in both bone mineral and TBW, it is theoretically more valid than the 3C model. Nowadays, the 4C model is considered the state-of-the-art method for body composition measurement as it includes the evaluation of the main FFM component, thus reducing the biological variability.

Lastly, a six component (6C) model exists at the molecular level which considers a sixth component in addition to the five components studied in the classic cadaver analysis (Figure 4). Glycogen is the sixth component which is not considered in the cadaver studies because of its relatively small amount and rapid postmortem autolysis.

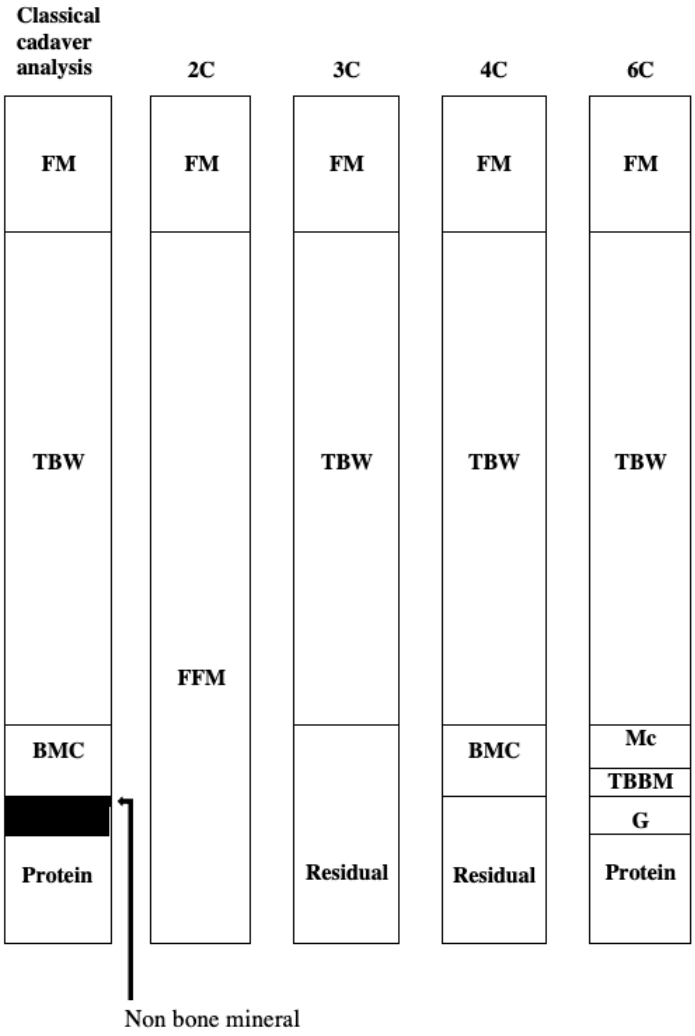

Figure 4. Some of the widely used molecular models and their respective components. BMC, bone mineral content; C, components; FFM, fat free mass; FM, fat mass; G, glycogen; Mc, soft tissue mineral, TBBM, total body bone mineral; TBW, total body water. Adapted from Heymsfield et al. 1990.

3) Cellular: The cellular level includes three components: cells (connective, epithelial, nervous, and muscular), extracellular fluid, and extracellular solids (including three types of fiber: collagen, reticular, and elastic). The cells can be additionally divided into fat and BCM, the latter of which is the actively metabolizing component at the cellular level (Heymsfield et al., 2005; Moore et al., 1963).
4) Tissue-system: The tissue-organ level of body composition consists of major components including adipose tissue, skeletal muscle, visceral organs, and bone. Some tissue-organ-level components are single solid organs such as brain or heart. Others, such skeletal muscle and adipose tissue, are dispersed throughout the body.
5) Whole body: The whole-body level of body composition represents the fifth level and concerns body size, shape, and exterior and physical characteristics. It can be divided into regions such as limbs, trunk, and head. At this level, trunk and limbs are usually described by anthropometric features such as circumferences, skinfolds, and length.

Total body water and human fluid balance

Water is the most abundant constituent of the body (Heymsfield et al. 2005). The average adult human body is 50-65% water, averaging around 57-60%, but the exact percentage varies based on a number of factors (e.g. age and gender). Adult males have a mean water content of 60%, females about 50%. This can be explained by the sexual difference in body composition: tissues fat cells contain the least amount of water, and females have a larger fat mass.

Water is an important constituent at the molecular, cellular, and tissue levels describing BC (Wang et al. 1992). Unlike the other components of the body at the molecular level, the water compartment consists of a single molecular species, hydrogen oxide. This unique molecular structure simplifies the task of measurement, and thus TBW is a common method for the assessment of body composition at the molecular level.

The extracellular compartment is comprised of the large somewhat ill-defined spaces between cells and the arteries, veins and lymph vessels. Seventy-five percent of extracellular fluid is interstitial and located mainly in lean tissue, whereas the remaining 25% is located in the intravascular space as plasma, which represents about 8% of TBW. Water within the cells accounts for about 50% of TBW. Cells may either expand or shrink as they take up or lose water. The intracellular fluid is isotonic mainly as a result of concentration of potassium ions and is in osmotic equilibrium with the extracellular compartment, where the dominant ion is sodium (Arnaud 1998).

The percentage of water in infants is much higher, typically around 75-78% water, dropping to 65% by one year of age. TBW amounts to 94% of body mass in the first trimester of fetal life, it decreases to 86% by the 24th week of gestation, and to 78% in the mature newborn. By the 24th week of gestation extracellular water (ECW) makes 59% of body mass and is 44% at delivery. During the same time, intracellular water (ICW) would increase from 27 to 34%. Neonatal weight loss mainly arises from a decrease in TBW. In childhood a relative reduction of the ECW compartment has been

observed along with an increase of ICW. By the end of the growth period, about 45% of TBW is ECW and 55% is ICW. The two compartments differ therefore even in adults (Bodzsar and Susanne 2004).

Hydration and water balance are of major importance in human health. Adequate hydration is essential throughout life from the newborn period to elderly stages of life and fluid balance is particularly important in circumstances relating to exercise, sports, and certain illnesses. Homeostasis requires the coordination of sensitive detectors at different sites in body linked by neural pathways with integrative centers in the brain that process this information. Most of the components of fluid balance are controlled by homeostatic mechanisms responding to the state of body water. A water deficit produces an increase in the ionic concentration of the extracellular compartment, which takes water from the intracellular compartment, causing cells to shrink, on the contrary when the body contains an excess of water, the lower ionic concentration of body fluids allows more water to reach the intracellular compartment. For this reason, these fluid spaces are not static volumes, as daily fluctuations in fluid-loss and fluid gain result in dynamic osmotically regulated fluid exchange between compartments (Nicolaidis 1998). Water balance is achieved when water losses are compensated by fluid gain through metabolic water production and from food and fluid intake. The recommendations for adequate daily water intake range from 1.7 to 3.5 L per day, with an average intake of 2.5 L for adult males and 2.0 L for adult females (Armstrong and Johnson 2018; Insitute of Medicine 2004; Nimrouzi et al. 2016).

Table 1. Example of typical fluid balance in a young sedentary man eating 8.8 MJ (2010 kcal) daily. Adapted from Nicolaidis (1998).

	Water (ml/day)
Intake	
Water in solid food	1106
Liquid as drinks	1180
"Metabolic water"	279
Total intake	2565
Losses	
Urine	1294
Faeces	56
Evaporation (sweating, perspiration, breathing)	1215
Total losses	2565
Water balance	0

Body composition assessment

A wide range of methods for assessing BC are available, ranging from the more accurate, historically used as reference methods DXA, densitometry, hydrometry, to the less accurate, but inexpensive, such as anthropometry. These techniques allow for the measurement of body components (e.g. FM, skeletal muscle mass, TBW) know to be related to health risk (Buffa 2017).

The gold standard for BC analysis is cadaver analysis, so no in vivo technique may be considered to meet the highest criteria of accuracy. Multicomponent models are now considered accurate enough to serve as references for the molecular approach to measuring BC (Wells and Fewtrell 2006). Today there is a lack of consensus among investigators on a single accepted reference approach for body composition assessment by the 2C model (FM and FFM). The 2C methods remain dependent on theoretical assumptions, such as constancy of the composition of FFM. Such

assumptions may largely hold true for healthy subjects but are more problematic in patients who may have deranged BC or hydration (Wells and Fewtrell 2006). To overcome this problem, more sophisticated multicomponent models have been designed. For example, the 4C model, considered now as a gold standard, divides body weight into FM, TBW, fat-free tissue protein and BMC. It requires measurements of body weight, TBW by hydrometry, FM by densitometry, and BMC by DXA (Buffa 2017). Consequently, the method to use depends on the aim of the study, accuracy, economic resources, invasiveness, time, and sample size.

On the basis of the underlying theoretical and methodological bases, BC techniques can be classified in three main categories: direct (total body counting, isotope dilution and neutron activation), criterion (densitometry, DXA, computed X-ray tomography, magnetic resonance imaging), and indirect methods [anthropometry, bioelectrical impedance analysis (BIA)]. Direct methods are very accurate, but because of their complexity, costs, invasiveness, are generally applied in research setting. Criterion methods, used as reference techniques, are able to analyse accurately BC at tissue level. Due to their costs and operative complexity, these techniques are generally not suitable for large scale studies. Lastly, indirect methods are generally less accurate, but easy to use and noninvasive (Table 2).

Table 2. Comparative advantages and disadvantages of a selection of methods of body composition assessment. Adapted from Andrews, Beattie, and Johnson (2019); Buffa (2017); Toomey et al. (2015).

	Method	Measure	Advantages	Disadvantages
Direct	Whole body counting	BCM, FFM	High accuracy	Costs, technical difficulties
	Neutron activation analysis	FFM	High accuracy	Costs, technical difficulties, radiation exposure
	Hydrometry (D_2O, NaBr)	TBW, ECW, ICW	Suitable for all age group	Costs, low acceptability, delayed results, inaccuracy if disease affects hydration of FFM
Criterion	Densitometry (underwater weighing, air-displacement plethysmography)	FM, FFM	Relatively fast and non-invasive	Costs, hydration assumptions, effects of disease on lean mass reduce accuracy, distribution of fat unable to be determined.
	DXA	FM, Body cell mass, Bone density, FFM	Reliable and repeatable. Can give regional fat distribution as well as total FM	Small radiation exposure. Can overestimate FM. Limited accuracy regarding regional fat distribution.
	Imaging techniques (magnetic resonance, computed tomography)	FM, BCM, lean tissue mass, adipose visceral tissue	High reproducibility, accurate assessment of adipose tissue volume, assessment of regional adiposity and of intra-	Costs, not suitable for all infants due to need for transfer to scanner and time required for scan acquisition

			abdominal vs subcutaneous adiposity. Relatively non-invasive	
Indirect	Anthropometry	Subcutaneous adipose tissue	Simple measurement of regional fat	Population specific, poor accuracy in individuals and groups, training required
	BIA	FM, FFM, TBW, ICW, ECW	Quick and relatively non-invasive. Cumulative accuracy makes useful for repeated measures	Over-estimation of TBW. Inconsistent individual accuracy. Distribution of fat unable to be determined.

Techniques measuring hydration status and absolute fluid volumes

Numerous hydration markers have been proposed to either measure absolute fluid volumes or to indirectly identify hydration status. The authors of several published review papers (Armstrong 2007; Nissensohn et al. 2015; Popkin, D'Anci, and Rosenberg 2010; Villiger et al. 2018) claim that a single gold standard for hydration assessment is not possible. Current developments suggest that these hydration markers vary greatly in their applicability due to differences in practicality, accuracy and validity. Table 3 presents selected characteristics of 13 hydration assessment techniques that are commonly utilized in physiological, clinical, industrial, military, and athletic settings. These techniques involve either whole-body, hematologic, urinary, or sensory measurements.

Table 3. Selected Characteristics of 13 Hydration Assessment Methods (Adapted from Amstrong 2007).

Hydration Assessment Technique	Body Fluids Involved	Cost of Analysis	Technical Expertise Required
Stable isotope dilution	All (ECW and ICW)	High	High
Neutron activation analysis	All	High	High
Bioelectrical impedance spectroscopy (BIS)	Uncertain	Moderate	Moderate
Body mass change using a floor scale	All	Low	Low
Plasma osmolality	ECW	High	High
% plasma volume change	Blood	Moderate	High
Urine osmolality	Excreted urine	High	High
Urine specific gravity	Excreted urine	Low	Moderate
Urine conductivity	Excreted urine	Moderate	Moderate
Urine color	Excreted urine	Low	Low
24-hour urine volume	Excreted urine	Low	Low
Salivary flow rate, osmolality, total protein	Whole, mixed saliva	High	High
Rating of thirst	Hypothalamus	Low	Low

Armstrong et al. (2007) suggest that certain laboratory hydration assessment techniques can be effective, but only under controlled conditions (i.e., when experimental, postural, activity, dietary, and environmental factors are controlled). In particular, when body fluids are equilibrated, TBW and plasma osmolarity (P_{osm}) provide objective measurements of volume and concentration at a single point in time. On the contrary, when fluid compartments are constantly fluctuating (i.e., during daily activities or exercise), a direct evaluation of a single body fluid could not provide valid information about hydration. Thus, no measurement is valid for all situations. In sports field, the body fluids

changes are usually assessed as the difference in weight during the assessment period expressed as a percentage of the initial body mass. This method is based upon the assumption that 1 kg weight lost is equal to 1 L of TBW lost. TBW can be measured using the dilution principle. Application of the dilution principle in vivo, however, is more complex than in vitro. This complexity arises because the tracers used in the vivo requires careful attention to these deviations from the basic assumptions underlying the dilution principle. Tracers that have been used include antipyrine, ethanol, urea, and isotopically labeled water.

The volume of ECW can be also measured in vivo using the dilution technique. Investigators have proposed a number of tracers for the measurement of ECW, including bromide, chloride, thiocyanate, sulfate, insulin, sucrose and mannitol. Bromide and isotopic chloride dilution come the closest to approximating ECW (Edelman and Leibman 1959) and, with the advent of improved analytical techniques, bromide has become the most commonly used tracer. Nowadays, with a reported sensitivity of 0.8 L dilution techniques are popularly regarded as the gold standard for assessing TBW (Armstrong 2005).

Blood and plasma volume can also be measured by isotope or dye dilutions and have been shown to track TBW losses associated with dehydration (Harrison 1985).

Lastly, absolute TBW, ICW and ECW can be determined by BIA and bioelectrical impedance spectroscopy (BIS).

Bioelectrical Impedance Analysis (BIA)

Historical background

Back in the 1870's Hermann (1871) first described the electrical proprieties of tissues. The pioneering work of relating electrical impedance measurements to biological function was conducted by Nyboer (1950) who studied arterial pulse waveforms and pulsatile blood flow to organs using electrical impedance plethysmography. At the beginning of the 1960's, studies using electrical

impedance measurements as an index of TBW where presented (Thomasset 1962). The four-surface electrode BIA technique were first introduced by Hoffer et al. (1969). From this moment forward, the foundations of BIA were well established, mainly related to the body water content variable, and by the 1990's, a variety of single and multi-frequency BIA analyzers became commercially available and widely sold, since its portability and safeness. By the 2000's, segmental BIA corrected inconsistencies between R and body mass of the trunk region (Kyle et al. 2004). Finally, new variables available from R and Xc were proposed, to assess cell health, such as phase angle (PA) and classic and specific vectors of BIA (Marini et al. 2013; Piccoli et al. 1994).

Principle of body composition measurement by impedance

BIA is a fast, safe and noninvasive method to obtain quantitative estimates of BC. Multifrequency BIA and, specifically, bioimpedance spectroscopy is preferable for fluid volume measurements, though for general BC assessment BIA at 50 kHz is more widely used (Moon 2013). Bioelectrical impedance is composed by resistance (R, ohm) and reactance (Xc, ohm) [$Z = (R^2 + Xc^2)^{0.5}$]. R represents the opposition offered by the body to the flow of an alternating electrical current and is inversely related to the water and electrolyte content of tissues. Xc, which is detectable by phase sensitive devices only, is related to the capacitance properties of the cell membrane and to variations that can occur depending on its integrity, function and composition (Baumgartner, Chumlea, and Roche 1988).

When the electric current passes a cell membrane, a time delay occurs, expressed as PA [PA = arctn Xc/R 180/ π] (Norman et al. 2012) and it may be also interpreted as an indicator of water distribution between the extra- and intracellular spaces. In fact, the higher the PA, the greater the proportion of ICW compared to ECW (Foster and Lukaski 1996).

Measurement protocol

The procedure requires that the surveys are carried out in accordance with precise condition regarding both measurements environment and the subject under study. The measurement environment conditions include a non-conductive examination table, a room temperature of 22°C and the calibration of the analyser at the start of every measurement session. The subject should urinate within 30 minutes prior the test. He should also avoid eating or drinking in the 4 hours prior the test, exercising in the 12 hours prior the test; consuming alcohol in the 48 hours prior to the test and taking diuretics in the seven days prior the test. He should not carry any biomedical device (pacemakers, etc.). Pregnant women, and in general those in premenstrual phase are retaining water, therefore should be excluded.

In the whole body measurement, the subject lies supine on the examination table with the lower limbs spaced by a 45° angle and the upper limbs apart from the body by 30° angle (Figure 5). Measures are taken on the right side of the body, on healthy skin. The analyser leads are connected to the electrodes whit terminals which are black for the detector electrodes and red for the injector ones. Before the electrodes are positioned, the underlying skin must be cleaned with alcohol.

- Hand: the electrode (proximal position; black) is placed on the dorsal surface of the wrist, at level of the styloid process of the ulna, while the injector /distal position; red) is positioned at the distal extremity of the third metacarpal.
- Foot: the detector electrode (proximal position; black) is placed on the dorsal surface of the foot, at the median point of the tibial tarsal joint, and the injector (distal; red) at the base of second and third metatarsal.

The distance between the electrodes should be at least 5 cm in order to avoid the risk of a short-circuit (Piccoli et al. 1999).

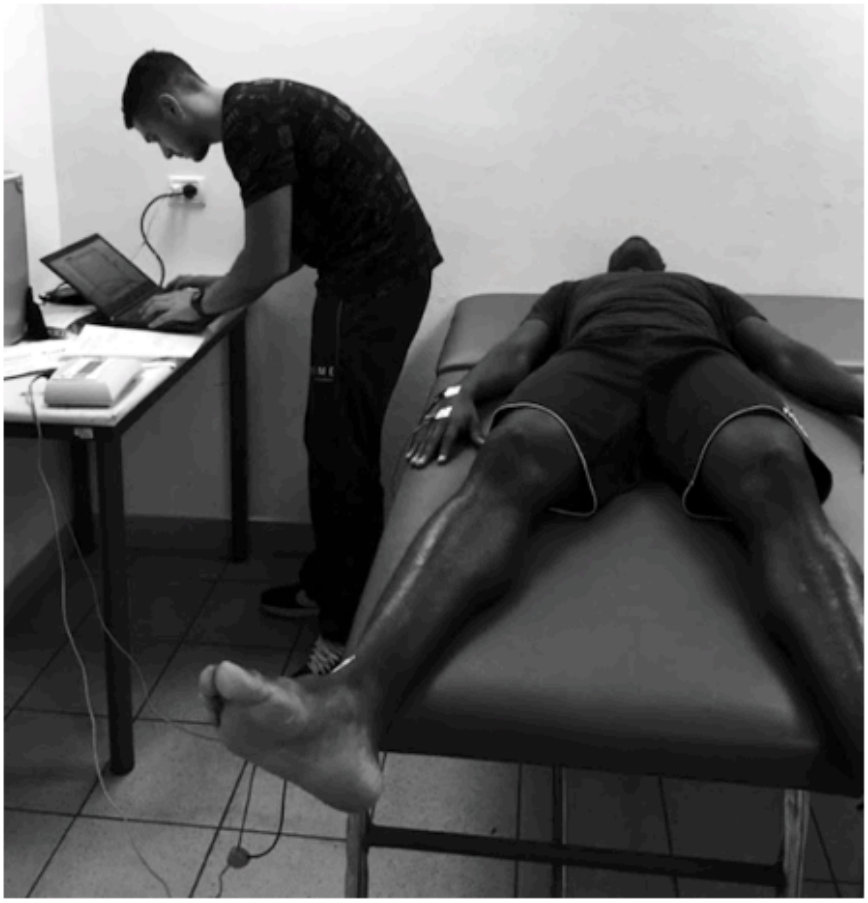

Figure 5. Electrode positioning for whole-body bioimpedance.

Limitation of BIA

BIA measurements must be standardized in order to obtain reproducible results. The use of "general" prediction equations across different age and ethnic groups without prior testing of their validity should be avoided. Choosing a BIA equation that is adapted to the populations studied continues to be a limiting factor of BIA (Kyle et al. 2004). Empirical regression models are population-specific once BIA integrates various body segments with variable physical effects of hydration, fat fraction, geometrical boundary conditions etc. on tissue conductivity. Body water alterations or body geometry abnormalities can alter BIA measurements validity. Also, the ratio ECW/ICW is a factor

known to limit the applicability of predictive equations generated by BIA to populations with varying hydration (Heitmann 1994).

Additionally, one limitation of BIA in some clinical applications and physical activity is moderating physiological factors that affect its validity to monitor hydration (Campa et al. 2019a). These factors include increased cutaneous blood flow, elevated body temperature, accumulation of electrolytes on the skin, and elevated rates of respiration (Buono et al. 2004).

Classic and Specific Bioelectrical Impedance Vector Analysis (BIVA)

BIA data, R and Xc, through BIVA are used to evaluate changes in soft tissues and body fluids in response to a training program (Campa, Silva, and Toselli 2018; Souza et al. 2017) after a sports performance (Carrasco-Marginet et al. 2017) or through a competitive season in athletes (Bonuccelli et al. 2011; Campa et al. 2019b), solely by considering impedance components. BIVA provides a classification (e.g. normal or not normal) and ranking (e.g. better or worse after treatment or intervention) tool; it does not provide estimates of volume or mass. In contrast to other bioimpedance methods this approach does not yield any absolute hydration state, or the electrical model of cell membranes and is unaffected by regression adjustments.

Bioelectrical impedance vector analysis is based on the analysis of impedance vectors (at 50 kHz), projected on a RXc-graph in relation to tolerance ellipses, or for intergroup comparisons (confidence ellipses). This capability allows for various applications in healthy populations and its value is nowadays recognized in different sports (Campa and Toselli 2018; Giorgi et al. 2018; Micheli et al. 2014; Pollastri et al. 2016). The RXc-graph method was proposed for the first time by Piccoli et al. (1994) (Figure 6). The RXc-graph represents elliptical probability regions in the R/H-Xc/H or Rsp-Xcsp, for classic and specific BIVA, respectively plan with curves or surfaces showing the values of a probability function for the joint distribution of R and Xc values. Three tolerance ellipses are

considered in the RXc-graph, namely the median, the third quartile, and the 95th percentile, that are regions including 50%, 75% and 95% of individual point, respectively.

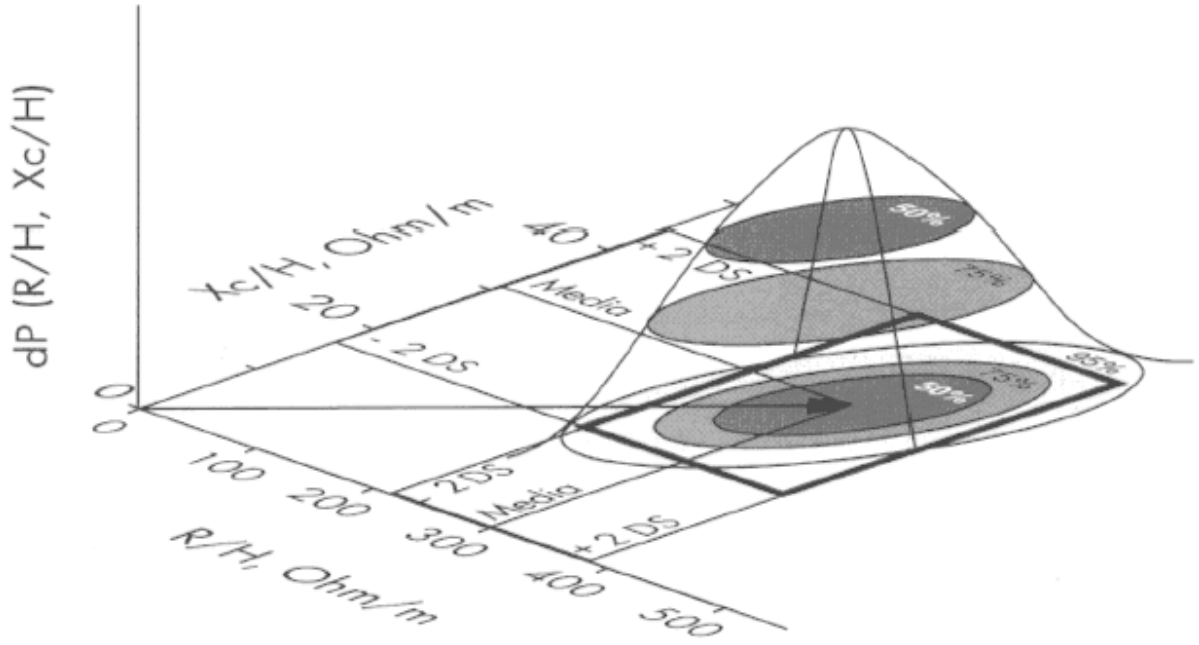

Figure 6. Univariate and bivariate tolerance interval (Adapted from Piccoli 1999).

The two BIVA approaches differ each other in that classic BIVA analyses bioelectrical values standardized for subject's height (which represents the conductor's length), whereas in specific BIVA R and Xc values are corrected also for cross-sectional areas, in order to reduce the effect of body dimensions. According to classic BIVA, variations of bioelectrical vectors along the major axis of tolerance ellipses indicate changes in total body water (dehydration towards the upper pole, fluid overload towards the lower pole). The minor axis refers to variations of absolute amount of body cell mass, FM, and FFM (left side: more mass; right side: less mass) and to variations of extracellular/intracellular water ratio (ECW/ICW) (low values in the left side). Within classic tolerance ellipses, the left upper side would correspond to athletic individuals, whereas the left lower side to obese ones. In specific BIVA, the major axis relates to %FM variation (higher values towards the upper

pole), while the minor axis gives the same information as in classic BIVA (more mass and lower ECW/ICW ratio on the left side). In fact, the minor axis is mainly related to variations of phase angle, which is unaffected by the correction.

PA allows the interpretation of TBW and BCM (Gonzalez et al. 2016; Norman et al. 2012). However, the analysis of PA only, without considering the information furnished by vector length (Z), can lead to interpretation errors. In fact, groups of individuals characterized by quite identical PA, but different Z, may show different body fluids or %FM (Lukaski et al. 2017; Marini et al. 2019; Mereu et al. 2016). The vectorial approach appears to be more efficient, as it considers both influential variables, PA and Z.

Statistical inference with the RXc-graph method

The two-sample Hotelling's T^2 test (Hotelling 1931) is a multivariate extension of the Student's t test for unpaired data in comparison of mean vectors, i.e. two or more variables, from two groups. It is more sensitive than the Student's t test performed on each variable and entails a smaller risk of erroneously rejecting the null hypothesis. The results of this test can be represented on the main graph (Piccoli and Pastori 2002). In general, with unbalanced groups, if the 95% confidence intervals of group means do not overlap, the group means are significantly ($p < 0.05$) different (Figure 7).

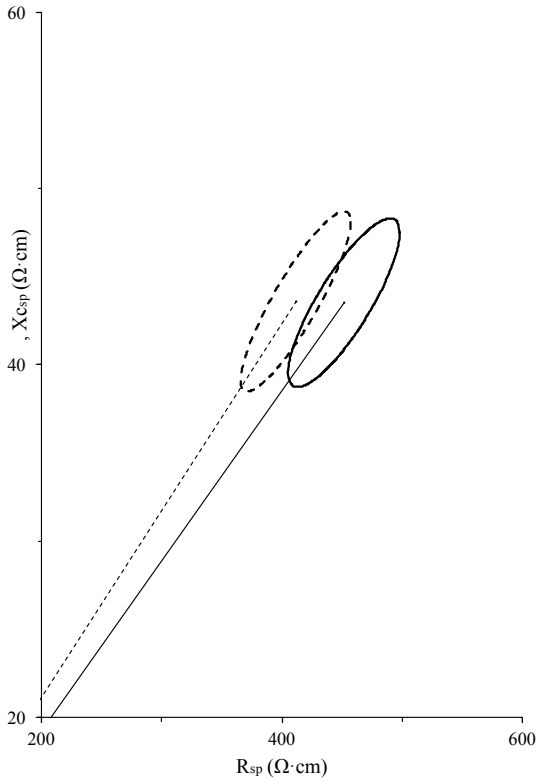

Figure 7. Main graph and 95% confidence impedance ellipses showing a show a significant difference two groups (Adapted from Campa, Silva, and Toselli 2018).

The paired one-sample Hotelling's T^2 test is just a multivariate extension of the Student's t test for paired data in comparison of mean different vectors, i.e. the difference of two or more variables considered two times in the same subjects. The results of this test can be represented on the paired graph. In this graph, if the 95% confidence ellipse of mean vector difference does not cover the zero point, then the statistical test is significant at $p < 0.05$. If it covers the zero point, the statistical test is not significant (Figure 8).

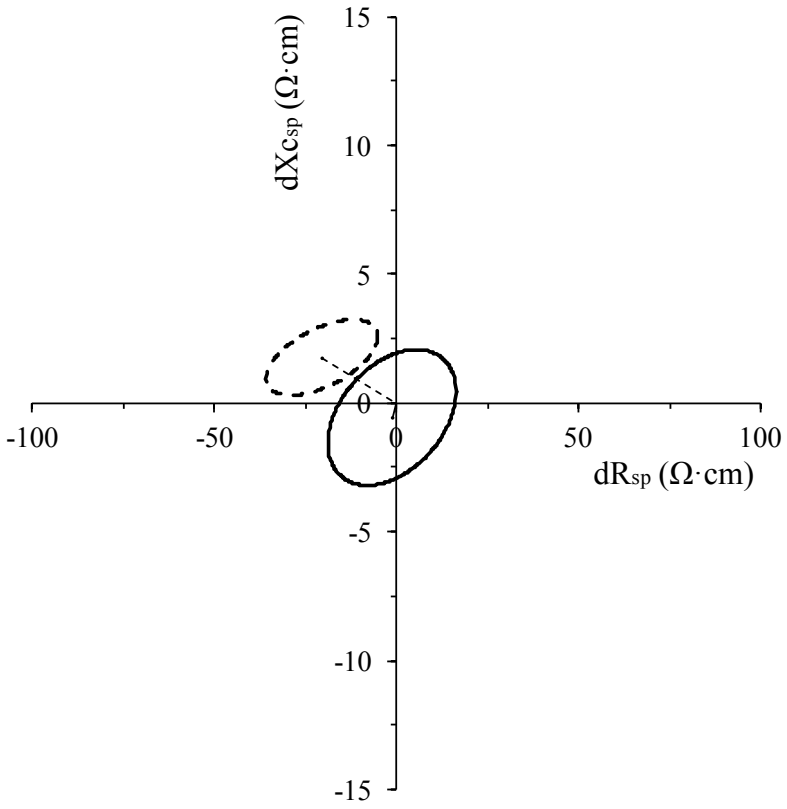

Figure 8. Paired Graph and impedance vector displacements with 95% confidence ellipses from a baseline to a post-intervention measurement. Dotted and continuous lines for the groups which showed, and which did not show a significant change, respectively. (Adapted from Campa, Silva, and Toselli 2018).

Dehydration and sport practice

Water and electrolyte balance are critical for the function of all organs and a person's health. Different techniques are used by physiologists to measure the state of hydration (e.g. plasma osmolality, urine-specific gravity, or body weight). However, this variety of methods makes it difficult to agree on the exact definition of the term dehydration. Generally, this concept refers to the reduction of TBW below the average basal value. (Armstrong 2007). Today's well-accepted notion that water loss ≥ 2% bodyweight impairs physiological functioning.

The type of dehydration is determined by the composition of fluid and electrolytes lost. Fundamentally, a deficit of TBW is characterized by the tonicity of fluid remaining in the body and whether there is a depletion of intracellular and / or extracellular fluid spaces. When the concentration of fluid-loss is hypo-osmotic with respect to plasma, the dehydration is called intracellular dehydration, on the contrary, when the concentration of fluid-loss is iso-osmotic with respect to plasma due to a greater loss of electrolytes with water this type of dehydration is called extracellular dehydration (Nadel, Pedersen, and Maddock 1941). The extracellular dehydration can be caused by illness (e.g. secretory diarrhea or vomiting), natriuresis and diuresis from cold or altitude exposure, whereas intracellular dehydration can occur with hypotonic sweat-loss compounded by thermal stress, exercise, physical activity or prolonged periods of inadequate fluid intake or fluid restriction.

Person performing exercise in the heat will often incur a body water deficit. If a dehydrated person exercises in the heat, they will incur significant adverse effects (Sawka 1998). Generally, the subject dehydrates during exercise because of fluids nonviability or a mismatch between thirst and body water requirements. In these instances, the person starts to exercise as euhydrated, but incurs an exercise-heat mediated dehydration over a prolonged period. This scenario is common for many athletic and occupational settings.

Athletes performing high intensity exercise commonly have sweating rates of 1.0 to 2.0 L/h while in the heat. These high sweating rates, however, are not maintained continuously and are dependent upon the person need to dissipate body heat. As little as 1–2% BM loss from sweating has been shown to compromise physiological functioning during exercise. Evidence indicating a detrimental effect of dehydration on cognitive, heart and gastrointestinal function, and hemodynamic response (Popkin et al. 2010). Exercise in hot conditions with inadequate fluid replacement is associated with hyperthermia, reduced stroke volume and cardiac output, decreases in blood pressure, and reduced blood flow to muscle. During exercise in the heat, a principal problem is to avoid hypohydration by matching fluid consumption to sweat loss. This is a difficult problem because thirst

does not provide a good index of body water requirements (Sawka 1998). Thirst is probably not perceived until an individual has incurred a water deficit of about 2% body weight loss. In addition, thirst is a poor index of body water requirements and ad libitum water intake during exercise in the heat results in an incomplete replacement of body water loss. As a result, it is likely that unless forced hydration is stressed, some dehydration will occur during exercise.

JUSTIFICATION AND AIMS

The analysis and monitoring of BC are fundamental in sport as well as in daily life, because of its relevance for health and sports performance. Such analysis can be performed in different contexts and with different approaches, e.g. in cross-sectional studies aimed to characterize sporting group samples, in longitudinal researches finalized to define short-term or long-term changes, or in applications aimed to detect and monitor muscle injuries (Castizo-Olier et al. 2018). Variations of BC can interest diversely athletes practicing different sport, because of their different exercise type and requirements for body physique and composition. Analyzing and monitoring BC is a very important topic in sport, because its variation in time can affect the athletes' performances. The excess FM seems to be counterproductive in the fast movements reducing performance and increasing energy demands during the execution of a particular action. Conversely, lean mass is closely linked to speed, strength and power, and injury prevention. Furthermore, while overhydration is quite uncommon in athletes, physiological dehydration processes can be induced by physical activity, leading to hypotonic, isotonic, or hypertonic dehydration (Oppliger and Bartok 2002). A fluid loss of 2% body mass decreases cardiovascular and cognitive performance in warm/hot conditions, and a fluid loss of 3% body mass can degrade the same performance factors under cold stress (Muth et al. 2019). Thus, an accurate assessment of their nutritional status and monitoring of their BC are essential to maintain athletes' health and to optimize their performance.

Despite the availability of gold standard methods in the assessment of BC, such as dilution techniques and DXA, the bioelectrical impedance method appears as a valid, easy to use, more epidemiological and relatively low-cost alternative to asses BC. However, validity of BIA is influenced by sex, age, ethnicity, level of fatness and health status. To overcome predictive errors associated to equations, alternative methods, such as BIVA and its variant specific BIVA have been conceived.

BIVA, described in detail by Piccoli et al. (1994), Lukaski and Piccoli (2012), and Buffa et al. (2014), considers the impedance components (R and Xc) independently of regression predictions of

fluid volumes or assumptions about the constant chemical composition of the fat-free body. Although specific BIVA was found to be more accurate than classic BIVA in the evaluation of %FM in older adults, there are no evidence on athletes. In addition, to the best of our knowledge, no studies have compared classic and specific BIVA in the body fluids assessment and evaluated the association between ECW/ICW, obtained by reference methods, and PA.

Additionally, one limitation of BIA in some clinical applications and physical activity is moderating physiological factors that affect its validity to monitor hydration (Gatterer et al. 2104). These factors include increased cutaneous blood flow, elevated body temperature, accumulation of electrolytes on the skin, and elevated rates of respiration.

Given the practicality of BIA and BIVA in assessing BC, increased knowledge and improvement linked to methodological aspects concerning impedance analysis can help the work of researchers, clinicians and coaches. In fact, modest levels of dehydration (2-3% body mass loss) are typically experienced by athletes in daily life as result of training and competition. From previous investigations it is unclear if modest dehydration negatively affects people's capacity to complete exercise.

For these reasons, the present doctoral thesis is composed by four studies: a cross-sectional study (Study I), an observational study (Study II) and two experimental studies (Studies III and IV). The main objectives of Study I were to utilize the two BIVA approaches (classic and specific), evaluating their accuracy in the BC assessment in athletes. Study II was aimed to compare body fluid assessment obtained with dilution techniques and BIVA in athletes throughout a competitive season, whereas the purpose of study III was to determine the time course of BIA values when a cold shower with a standardize duration (10 min) and water temperature (22°C) was applied after a period of controlled exercise. Lastly, study IV was aimed to deeply investigate the effects of dehydration on heart rate (HR) and oxygen uptake (VO2) responses, neuromuscular functionality, and time trial (TT)

performance during a cycling exercise in athletes, evaluating also the variation of the exanimated parameters when the athletes hydrate during the trial.

STUDY I

Introduction

PA, classic and specific BIVA have been applied in different groups, particularly obese, athletic subjects, and in the elderly, and in the clinical setting (Buffa et al. 2014; Kyle et al. 2004; Marini et al. 2013; Norman et al. 2012). A growing body of literature on BIVA in sport and exercise research and practice is also noticeable (Campa et al. 2018; Campa and Toselli 2018; Carrasco-Marginet et al. 2017) and specific BIVA has been proposed as a promising approach in this field (Castizo-Olier et al. 2018). Although largely used, reliability studies of PA, classic or specific BIVA in the assessment of BC (Buffa et al. 2013; Gonzalez et al. 2016) or of hydration (Gonzalez et al. 2016; Lukaski and Piccoli 2012) through reference techniques are very scarce in the general population and totally lacking in athletes (Castizo-Olier et al. 2018).

Therefore, the aim of this research was to evaluate the accuracy of PA, classic and specific BIVA in BC assessment of athletes, focusing the analysis on quantitative estimates of BC parameters (FM, FFM, TBW, ECW, ICW), and on values independent from body dimensions (%FM, ECW/ICW). At this purpose, DXA was used as a reference technique for the estimate of FM, FFM and %FM, and dilution techniques for TBW and ECW.

Materials and methods

Participants

This was a cross-sectional, observational study on 202 athletes (139 men and 63 women) over 16 years of age (men: 21.5 ± 5.0; women: 20.7 ± 5.1). The sample included athletes involved in a total of 11 sports (Athletics, Basketball, Handball, Judo, Karate, Pentathlon, Rugby, Soccer, Swimming, Triathlon, Volleyball). The results of a medical screening indicated that all subjects were in good health.

The following inclusion criteria were used: 1) 10 or more hours of training per week, 2) negative test outcomes for performance-enhancing drugs, and 3) not taking any medications. All subjects and their parents or guardians were informed about the possible risks of the investigation before giving written informed consent to participate. All procedures were approved by the ethics committee of the Faculty of Human Kinetics, Technical University of Lisbon, and were conducted in accordance with the declaration of Helsinki for human studies of the World Medical Association.

Procedures

On each subject, all the measurements were obtained in the same morning. Subjects came to the laboratory after an overnight fast (12 h fast), refraining from vigorous exercise at least 15 h, no caffeine and alcohol during the preceding 24 h, and consuming a normal evening meal the night before (Figure 9).

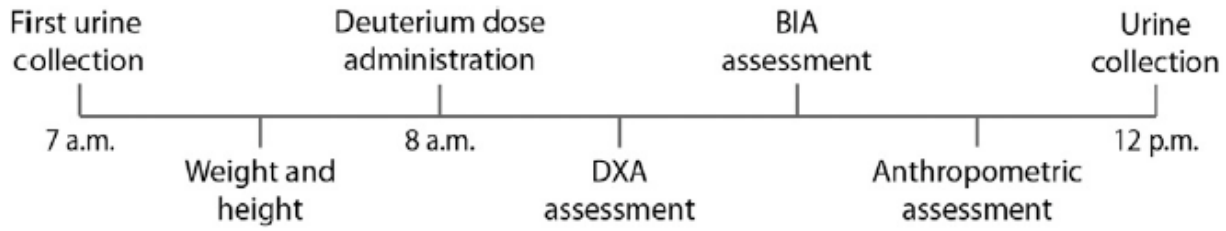

Figure 9. Timeline of stations performed by the athletes involved in the study.

Measurements

The impedance measurements were performed with BIA (BIA 101 Anniversary, Akern, Florence, Italy) using an electric current at a frequency of 50 kHz. Measurements were made on an isolated cot from electrical conductors, the subjects were in the supine position with a leg opening of 45° compared to the median line of the body and the upper limbs, distant 30° from the trunk (Lukaski

and Piccoli 2012). After cleaning the skin with alcohol, two electrodes (Biatrodes Akern Srl, Florence, Italy) were placed according to a standardized protocol (Lukaski and Piccoli 2012). BIVA was carried out using the classic and specific BIVA methods, i.e. normalizing R and Xc for height (H) in meters [classic BIVA; (Piccoli et al. 1994)], or multiplying R and Xc by a correction factor (A/ L), where A is the estimated cross-sectional area (or 0.45 x arm area + 0.10 x waist area + 0.45 x calf area) and L the length of the 'conductor' (1.1 x height) [specific BIVA (Marini et al. 2013)]. The length of the vector (Z) was calculated as the hypotenuses of individual impedance normalized values. Bioelectrical PA was calculated as the arctangent of Xc/R × 180°/π. Prior to each test the analyzer was calibrated with the calibration deemed successful if R value is 383 Ω. and Xc equal to 46 Ω. The test-retest CV in 10 participants in our laboratory for R and Xc is 0.3% and 0.9%, respectively. Italo Spanish bioelectrical specific values (Ibanez et al. 2015) were used as a reference. Italo Spanish bioelectrical classic values (unpublished data) were: R/H (men: 284.9 ± 33.6; women: 391.2 ± 41.1); Xc/H (men: 38.0 ± 5.0; women: 44.0 ± 5.8).

Subjects came to the laboratory refraining from exercise and alcohol or stimulant beverages and fasting for at least 3 hours. All anthropometric data were collected by a physician specifically trained according to a standardized protocol (Lohman, Roche, and Martorell 1988). Body weight was measured with a scale without shoes and wearing minimal clothes, to the nearest 0.01 kg and height was measured to the nearest 0.1 cm with a stadiometer (Seca, Hamburg, Germany). Body Mass Index (BMI) was calculated as the ratio of body mass to height squared (kg/m^2). Girths were measured by using an anthropometric tape (Lufkin W606PM; Apex Tool Group, Sparks, MD, USA). Skinfold thicknesses were measured by use of a Slim Guide caliper (Creative Health Products, Ann Arbor, MI, USA).

The subjects came to the laboratory after an overnight fast (12 h fast), refraining from vigorous exercise at least 15 h, no caffeine and alcohol during the preceding 24 h, and consuming a normal evening meal the night before. Athletes underwent a whole-body DXA scan according to the

procedures recommended by the manufacturer on a Hologic Explorer-W fan-beam densitometer (Hologic, Waltham, MA, USA). The equipment measures the attenuation of X-rays pulsed between 70 and 140 kV synchronously with the line frequency for each pixel of the scanned image. According to the protocol described by the manufacturer, a step phantom with six fields of acrylic and aluminum of varying thicknesses and known absorptive properties was scanned to serve as an external standard for the analysis of different tissue components. For athletes who were taller than the scan area, we used a validated procedure that consisted of the sum of a head and a trunk plus limbs scans (Santos et al. 2013). The same technician positioned the participants, performed the scan, and executed the analysis (QDR for Windows software version 12.4; Hologic, Waltham, MA, USA) according to the operator's manual by using the standard analysis protocol. The DXA measurements included whole-body measurements of absolute FM (kg), percentage of FM (%FM) and FFM (kg).

Following the collection of a baseline urine sample, each participant was given an oral dose of 0.1 g of 99.9% 2 H_2O per kg of body weight (SigmaeAldrich; St. Louis, MO) for the determination of TBW by deuterium dilution using a Hydra stable isotope ratio mass spectrometer (PDZ, Europa Scientific, UK). Subjects were encouraged to void their bladder prior to the 4-h equilibration period and subsequent sample collection, due to inadequate mixing of pre-existing urine in the bladder. Urine samples were prepared for 1 $H/^2H$ analyses using the equilibration technique by Prosser and Scrimgeour (1995). ECW was assessed from a baseline saliva sample using the sodium bromide (NaBr) dilution method after the subject consumed 0.030 g of 99.0% NaBr (SigmaeAldrich; St. Louis, MO) per kg of body weight, diluted in 50 mL of distilled-deionized water. ICW was calculated as the difference between TBW and ECW.

Statistical analysis

Descriptive statistics including means ± standard deviations were calculated for all outcome variables. Normality was evaluated using Shapiro-Wilk test. Since the data showed a normal

distribution, the association between bioelectrical impedance and BC values was investigated using Pearson's correlation analysis. Multiple regression analyses were performed to understand the associations between FM, %FM, FFM, TBW, ICW, and ECW and bioelectrical values. Model adjustments included age and sport practiced. If more than one variable was a predictor in the model, a variance inflation factor (VIF) for each independent variable was calculated to evaluate multicollinearity, and values below 5 were considered not to have multicollinearity issues. Data were analyzed with IBM SPSS Statistics version 24.0 (IBM, Chicago, IL). Bioelectric variables were calculated using the specific BIVA software (www.specificbiva.unica.it). For all tests, statistical significance was set at $p < 0.05$.

Results

Athletes of both sexes showed a condition of normal weight, with low mean values of BMI and low average values of %FM, as expected in a sample of young sportive subjects (Table 4).

Table 4. Participants' characteristics, including the correlation between bioelectrical variables and the comparison between sexes.

	Men (n=139)	Women (n=63)	t-Student	*p*
Variable	Mean ± SD	Mean ± SD		
Age (y)	21.5 ± 5.0	20.7 ± 5.1	1.0	0.296
Height (cm)	183.3 ± 9.1	171.1 ± 8.2	9.2	0.000
Weight (kg)	77.2 ± 11.4	63.7 ± 8.9	8.3	0.000
Upper arm crf (cm)	32.3 ± 3.2	28.6 ± 2.6	8.3	0.000
Waist crf (cm)	81.3 ± 6.4	76.5 ± 5.7	5.1	0.000
Calf crf (cm)	37.6 ± 2.4	36.1 ± 2.8	3.6	0.000
BMI (kg/m^2)	22.9 ± 2.6	21.8 ± 2.1	3.1	0.002
R (Ω)	467.9 ± 51.4	566.1 ± 67.4	-11.4	0.000

Xc (Ω)	63.1 ± 8.0	67.6 ± 10.5	-3.4	0.001
PA (°)	7.7 ± 0.8	6.8 ± 0.8	7.1	0.000
Rsp (Ω*cm)	324.3 ± 31.2	368.3 ± 46.1	-8.0	0.000
Xcsp (Ω*cm)	43.9 ± 6.2	44.0 ± 7.1	-0.1	0.000
Zsp (Ω*cm)	327.3 ± 31.5	370.9 ± 45.9	-8.2	0.924
R/H (Ω/m)	255.8 ± 30.6	331.5 ± 41.2	-14.6	0.000
Xc/H (Ω/m)	34.6 ± 5.1	39.6 ± 6.4	-6.1	0.000
Z/H (Ω/m)	258.2 ± 30.8	334.3 ± 41.3	-14.5	0.000
FM (kg)	10.8 ± 4.3	15.4 ± 4.4	-6.9	0.000
FM (%)	13.9 ± 3.9	24.1 ± 4.8	-16-0	0.000
FFM (kg)	65.7 ± 8.6	47.9 ± 6.2	14.7	0.000
TBW (kg)	49.5 ± 7.5	35.8 ± 5.3	12.1	0.000
ECW (kg)	19.2 ± 3.1	14.6 ± 1.9	10.2	0.000
ICW (kg)	30.4 ± 5.7	21.2 ± 3.8	10.5	0.000
ECW/ICW (kg)	0.6 ± 0.1	0.7 ± 0.1	-3.4	0.001
r R-Xc	0.577	0.687		
r R/H-Xc/H	0.669	0.729		
r Rsp-Xcsp	0.636	0.716		

Note: r R-Xc, correlation between R and Xc; r R/H-Xc/H, correlation between R/H and Xc/H; r Rsp-Xcsp, correlation between Rsp and Xcsp.

Anthropometric and BC measurements showed significant differences between sexes. Consistently with the known pattern of sexual dimorphism in adults, men showed higher values of all anthropometric measurements, FFM, TBW, ECW, ICW, while women showed higher bioelectrical values (with the only exception of phase angle and specific reactance), and higher FM, %FM, and ECW/ICW (Table 4).

Both men and women showed significantly higher stature ($p<0.001$), larger circumferences ($p<0.001$ for waist and upper arm circumference; $p<0.05$ for calf circumference, only in men), but a

similar BMI with respect to the Italo-Spanish reference population. Classic bioelectrical values (R/H and Xc/H) were significantly lower in Portuguese athletes of both sexes than in the reference population ($p<0.001$), whereas specific values were not significantly different in the two populations, with the exception of Rsp which was higher in the Italo-Spanish group ($p<0.05$). Phase angle was similar in men and significantly higher in Portuguese females ($p<0.001$).

Table 5 shows the correlation matrix between bioelectrical impedance parameters and BC variables. Following adjustment for covariates, including age and sport practiced, bioelectrical values remained significantly associated with BC variables. In fact, in the multicollinearity diagnosis we found no VIF above 5, which is the rule of thumb used in regression models to assess if β is affected.

In classic BIVA, as regards hydration, the correlation between TBW, ECW, ICW and R/H, Xc/H, Z/H was highly and negatively significant in both sexes (Table 5, Fig. 10a). As regards BC parameters, the association between FFM or FM and R/H, Xc/H, Z/H was negative in both sexes (Table 5), while the correlation with %FM was inconsistent in the two sexes (Z/H negatively correlated in men and positively in women) and reached the significance level only in men (Table 5, Fig. 10c).

In specific BIVA, the correlation between FM or %FM and bioelectrical values (Rsp, Xcsp, Zsp) was positive and highly significant in both sexes (Table 5, Fig. 10d), while the association with FFM rarely reached the significance level. The mean vectors of groups with different percentages of body fat (below Q1 vs. above Q3 of the %FM) were significantly separated (Fig. 11d,l). The mean vectors of opposite quartiles were located within the 50% tolerance ellipses and the group with higher %FM (above $Q3_{\%FM}$) towards the pole of higher %FM, as expected. The association of specific bioelectrical values with TBW, ICW or ECW, instead, was not significant, with the only exception of the positive correlation between Xcsp and ICW and TBW in men (Table 5, Fig. 10b, Fig. 11b,h).

Table 5. Correlation between bioimpedance and body composition variables.

	Men									
	R	Xc	R/H	Xc/H	Rsp	Xcsp	PA	Zsp	Z/H	Z
FM	-0.312§	-0.356§	-0.406§	-0.398§	0.602§	0.340§	-0.085	0.588**	-0.443§	-0.316§
%FM	-0.144	-0.228**	-0.160	-0.215*	0.589§	0.313§	-0.105	0.569**	-0.214*	-0.147
FFM	-0.539§	-0.462§	-0.781§	-0.625§	0.173*	0.127	0.010	0.204*	-0.778§	-0.542§
TBW	-0.731§	-0.484§	-0.883§	-0.586§	0.068	0.186*	0.184*	0.099	-0.880§	-0.732§
ECW	-0.484§	-0.565§	-0.701§	-0.694§	-0.028	-0.156	-0.165	-0.019	-0.702§	-0.490§
ICW	-0.705§	-0.339§	-0.792§	-0.405§	0.104	0.326§	0.327§	0.140	-0.783§	-0.703§
ECW/ICW	0.295*	-0.170	0.207*	-0.204*	-0.122	-0.472§	-0.493§	-0.153	0.187*	0.288**
	Women									
	R	Xc	R/H	Xc/H	Rsp	Xcsp	PA	Zsp	Z/H	Z
FM	0.059	-0.128	-0.126	-0.256*	0.734§	0.414§	-0.232	0.737**	-0.127	0.055
%FM	0.281*	0.001	0.222	-0.033	0.774§	0.407§	-0.295*	0.773**	0.218	0.277*
FFM	-0.475§	-0.333§	-0.734§	-0.525§	0.029	0.055	0.052	0.026	-0.731§	-0.475§
TBW	-0.598§	-0.368§	-0.829§	-0.549§	-0.171	-0.018	0.146	-0.156	-0.829§	-0.597§
ECW	-0.543§	-0.489§	-0.781§	-0.667§	-0.033	0.086	0.083	-0.043	-0.788§	-0.545§
ICW	-0.547§	-0.258	-0.746§	-0.419§	-0.219	0.018	0.243	-0.193	-0.743§	-0.545§
ECW/ICW	0.214	0.127	0.244	0.085	0.256	-0.117	-0.408§	0.215	0.229	0.209

Note: r values are reported in the table; *Significant at $p < 0.05$, **$p < 0.01$, § $p < 0.001$.

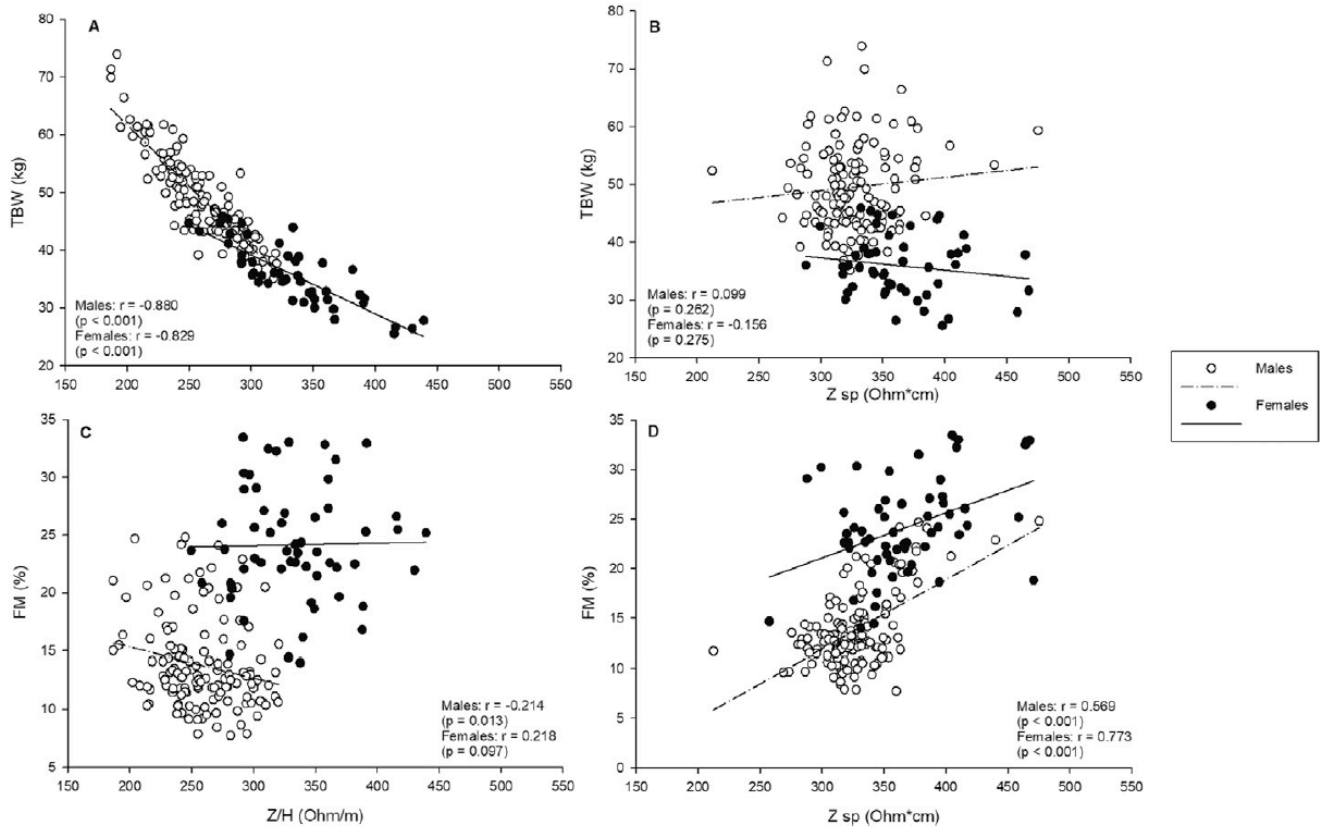

Figure 10. Correlation between classic or specific impedance vectors with total body water and fat-mass%. a: Z/H vs. TBW; b: Zsp vs. TBW; c: Z/H vs. %FM; d: Zsp vs %FM.

PA, and hence both classic and specific BIVA, detected ECW/ICW differences in both sexes, with lower PA values in subjects with higher ECW/ICW ratio (Table 5, Fig. 11e,f,m,n, Fig. 12). It was also positively associated with ICW and TBW in men and negatively associated with %FM in women (Table 5).

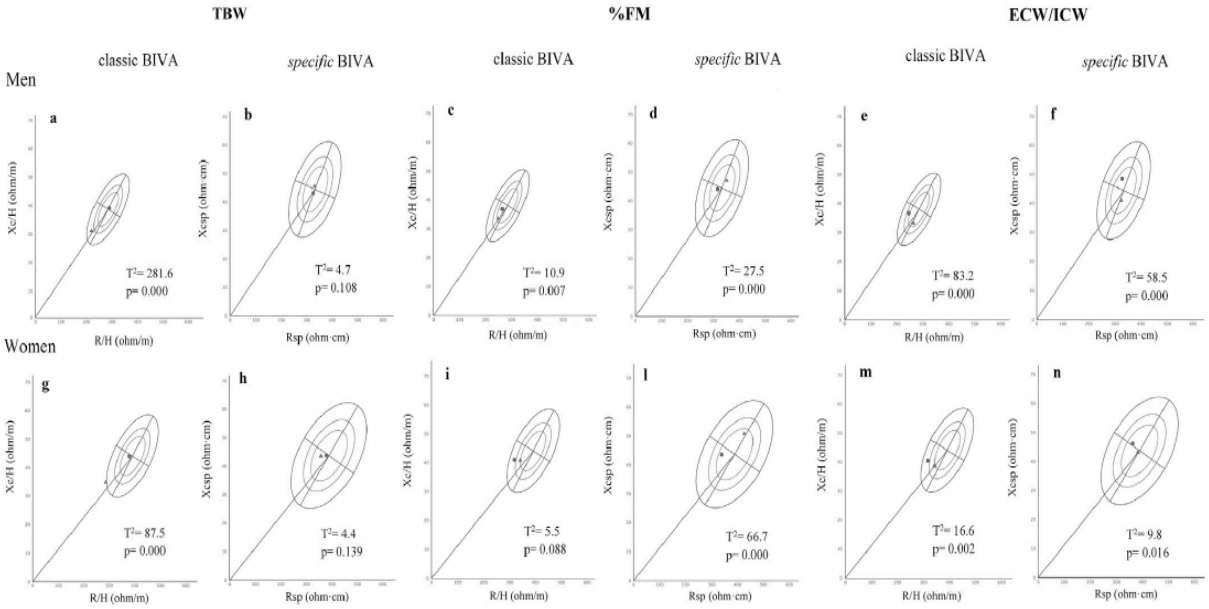

Figure 11. Classic and specific mean vectors of quartiles (below Q1 vs. above Q3) with different total body water, fat-mass%, and extracellular/intracellular water ratio in men. Circles: below Q1; triangles: above Q3; a: classic BIVA and TBW(men); b: specific BIVA and TBW (men); c: classic BIVA and %FM (men); d: specific BIVA and %FM (men); e: classic BIVA and ECW/ICW (men); f: specific BIVA and ECW/ICW (men); g: classic BIVA and TBW(women); h: specific BIVA and TBW(women); i: classic BIVA and %FM (women); l: specific BIVA and %FM (women); m: classic BIVA and ECW/ICW (women); n: specific BIVA and ECW/ICW (women).

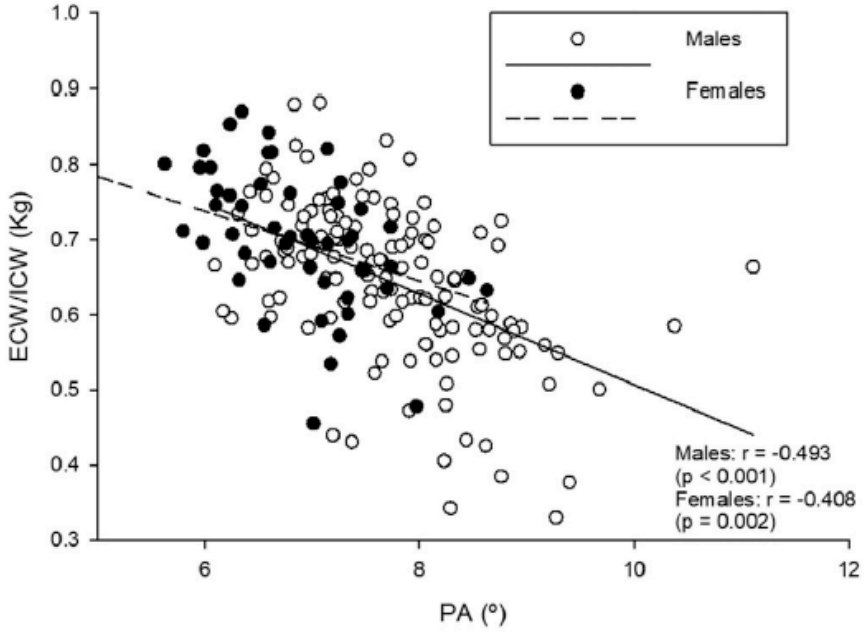

Figure 12. Correlation between phase angle and extracellular/intracellular water ratio.

Discussion

The present study, for the first time, analysed the association of PA, classic and specific BIVA with DXA and dilution techniques, for BC assessment in athletes. Data showed that classic BIVA correctly detect differences of TBW, but was weak in the assessment of %FM. On the contrary, specific BIVA detected changes of %FM, but not those of TBW. The relation between bioelectrical values with FM and FFM was different in classic and specific BIVA: classic bioelectrical values were negatively related to body compartments (particularly to FFM), while specific bioelectrical values showed a positive correlation (particularly with FM). In addition, the relation with water compartments was different: R/H and Xc/H were negatively related to ICW and ECW, while in specific BIVA only Xcsp was positively related to ICW and only in men. PA, which is the same in classic and specific BIVA, was sensitive to ECW/ICW ratio and ICW. These results were unaffected by age and sports practiced. Although the sexual dimorphism, the association between bioelectrical and BC variables was quite similar in the two sexes. The only exception was the stronger relation between classic values with %FM

or FM in men (with an opposite direction in the two sexes in the case of %FM), and the stronger relation between specific values with FM observed in women.

Previous reliability studies on BC assessment in the general population, using DXA as a reference, have shown quite similar results. Indeed, specific BIVA has demonstrated to evaluate FM, FFM, and %FM accurately in US adults (Buffa et al. 2013) and in Italian elderly (Marini et al. 2013) . Further, both specific vector length and phase angle have shown to be able to detect skeletal muscle mass differences (Buffa et al. 2014). The same studies have also shown that classic BIVA can recognize different quantities of absolute mass but does not perform accurately in evaluating %FM and in the recognition of the obesity and athletic regions within the RXc-graph, as in the present research. Furthermore, Wells et al. (2019) recently tested classic BIVA in a sample of healthy children against the 4-component model criterion and recognized inconsistencies in BC outcomes, particularly for FFM. Accordingly, the recent review on the applications of BIVA in sport sciences (Castizo-Olier et al. 2018) has shown that the majority of the studies using classic BIVA did not observe bioelectrical vectors falling in the region of the tolerance ellipses expected for athletes. As suggested by Castizo-Olier et al. (2018), this could indicate the need of reference values for each population or sport. However, as discussed with more detail elsewhere (Buffa et al. 2014), these unexpected results of classic BIVA could be due to the solely effect of body geometry and cross-sectional areas in particular on bioelectrical parameters. In fact, according to the Ohm's law, resistance is directly proportional to the conductor's length and inversely proportional to its cross-section. Indeed, our sample of athletes, characterized by shorter classic vectors (significantly lower values of R/H and Xc/H) with respect to the reference sample of Italo-Spanish young adults (Ibanez et al. 2015) is also characterized by significantly higher circumferences. The correction for cross-sections applied in specific BIVA reduces the differences related to body size and shape, increasing the sensitivity of bioelectrical values to tissues' properties and BC, such as %FM. In fact, the vectors of Portuguese athletes are located towards the obesity region of classic tolerance ellipses of the Italian-Spanish young adults (Ibanez et

al. 2015), while they are centrally located within the specific tolerance ellipses of same reference population.

Classic BIVA is commonly used to monitor hydration changes, with fluid overload indicated by shorter vectors, i.e. falling towards the lower pole of the classic tolerance ellipses. The technique has been clinically validated for the evaluation of TBW (Bronhara, Piccoli, and Pereira 2012; Lukaski and Piccoli 2012; Piccoli 2014), and used for detecting body fluids changes in athletes (Gatterer et al. 2014). Further, Wells et al. (2018) showed that BIVA outcomes behaved as expected on the basis of theoretical assumptions in the case of FFM hydration, using the 4-component model as a reference. The vector migration has also shown to be consistent with fluid loss determined using dilution techniques (Heavens et al. 2016; Lukaski and Piccoli 2012). However, Heavens et al. (2016) noticed that the area of normal hydration on the tolerance ellipses is wider than expected on the basis of dilution techniques.

The classic vector length, mainly determined by R/H values, can be also considered indicative of extracellular water (negative relation), being ECW strongly correlated with TBW (Segal et al. 1991), while Xc/H, which is related to body cell mass, should be positively associated with ICW (Piccoli et al. 1994). Instead, in the present study, a negative relation between Xc/H and ICW was observed. However, it should be noted that ICW, as well as ECW, is also positively correlated with TBW. Further, nor R/H or Xc/H are expected to give information on fluid distribution between compartments and tissue hydration, especially if considered separately. Fluid distribution is more related to the ECW/ICW ratio, which is not dependent on body dimensions (and hence on absolute values of ICW, ECW, TBW), and mainly detected by PA. In fact, PA has demonstrated to be related to water distribution between the extra- and intra-cellular spaces using dilution as reference technique: the higher PA, the greater proportion of ICW compared to ECW, i.e. the lower ECW/ICW ratio (or ECW/TBW) (Chertow et al. 1995; Gonzalez et al. 2016). PA is identical in classic and specific BIVA

and, accordingly, the two techniques have demonstrated a similar accuracy in detecting ECW/ICW in US adults, based on the comparison with bioelectrical impedance spectroscopy (Buffa et al. 2013).

BC and body fluids monitoring is a relevant topic in sports. In fact, an elevated body fat mass can negatively affect the quality of movement and performance in athletes (Toselli and Campa 2018), while hypo-hydration and fluid accumulation may compromise physical and cognitive performance, and eventually health (Maughan and Shirreffs 2010a), especially in certain sports (Maughan and Shirreffs 2010b). Furthermore, ICW variations are related to changes in performance (Silva et al. 2010). However, it should be stressed that different physiological adaptations and dehydration processes, diversely affecting the extra cellular and intracellular spaces, can be induced by physical exercise and their relations with bioelectrical changes should be better explored (Cheuvront et al. 2013). Moreover, as also suggested by Wells et al. (2018), further work is needed to improve the understanding of PA meaning at the physiological level.

This research has the main point of strength of being the first study performed in athletes analysing the association of PA, classic and specific BIVA with DXA and dilution techniques in the assessment of BC and body fluids.

Despite the encouraging results obtained in this study, some limitations are present and should be considered. In fact, our results are applicable to BIA equipment using the 50 kHz frequency and to a similar population. Indeed, even if multifrequency devices are widely used with acceptable accuracy at the group level to assess and track FFM (Matias et al. 2012), BIVA was originally developed and proposed using single-frequency devices. Moreover, a recently published research (Silva et al. 2018) showed that BIA values at 50 kHz are not directly comparable to those obtained by single-frequency devices. Thus, further analysis using multifrequency devices are required and could give useful information. Additional studies should focus on health and disease populations, different age groups, ethnicity, and body regions to better define the suitability of BIVA approaches for BC assessment.

Conclusions

The present study shows that specific BIVA is more accurate than classic BIVA in the %FM assessment in athletes, whereas the classic method is able to analyze body fluids with a higher accuracy. PA (and hence both classic and specific BIVA) was sensitive to ECW/ICW ratio. Physicians and sports coaches should consider using both BIVA approaches (classic and specific) to obtain reliable BC evaluations in athletes. More research is needed to analyse the sensitivity of BIVA to each type of dehydration and to body water compartments. Further, validation studies are also necessary with regard to the variations of BC and hydration that occur during the competitive season and in pre-to post-exercise.

STUDY II

Introduction

Classic BIVA has been applied in different sports disciplines and practices. In particular, it has shown to be able to identify changes of body fluids after an exercise session, compared to plasma osmolarity (a hydration biomarker), stable isotope dilution and body weight changes (Gatterer et al. 2014).

However, to the best of our knowledge, no studies have explored the accuracy of BIVA in assessing long-term body fluid changes, through the comparison with dilution techniques, the gold standard method for determining total body water compartments. Therefore, the aim of this investigation was to compare body fluid assessment obtained with dilution techniques and BIVA in athletes throughout a competitive season. Our hypothesis was that vector displacements could reflect changes in body fluid over the season.

Materials and Methods

Participants

This was a longitudinal investigation of 58 athletes engaged in five sports (basketball, swimming, volleyball, handball and triathlon) (men: age 18.7 ± 4.0 years; women: age 19.2 ± 6.0 years). The following inclusion criteria were considered: 1) 10 or more hours of training per week, 2) negative test outcomes for performance-enhancing drugs and 3) not taking any medications. The results of a medical screening indicated that all subjects were in good health. All subjects and their parents or guardians were informed about the possible risks of the investigation before giving written informed consent to participate. All procedures were approved by the ethics committee of the Faculty of Human Kinetics, Technical University of Lisbon, and were conducted in accordance with the declaration of Helsinki for human studies of the World Medical Association.

Procedures

Subjects were evaluated at the beginning (PRE) and after 6 months (POST), during the competitive season. The subjects came to the laboratory after an overnight fast (12 h fast), refraining from vigorous exercise at least 15 h, no caffeine and alcohol during the preceding 24 h, and consuming a normal evening meal the night before. Body weight was measured with a scale without shoes and wearing minimal clothes, to the nearest 0.01 kg and stature was measured to the nearest 0.1 cm with a stadiometer (Seca, Hamburg, Germany). The intra-observer technical error of measurement (TEM) and the coefficient of variation (CV) were calculated in a subsample of ten subjects (height: TEM = 0.06 cm, CV = 0.04; weight: TEM = 0.04 kg, CV = 0.07). Body mass index (BMI) was calculated as the ratio of body mass to height squared (kg/m^2).

Total body water

Following the collection of a baseline urine sample, each participant was given an oral dose of 0.1 g of 99.9%. H_2O per kg of body weight (Sigma - Aldrich; St. Louis, MO) for the determination of TBW by deuterium dilution using a Hydra stable isotope ratio mass spectrometer (PDZ, Europa Scientific, UK). Subjects were encouraged to void their bladder prior to the 4-h equilibration period and subsequent sample collection, due to inadequate mixing of pre-existing urine in the bladder. Urine samples were prepared for 1 $H/^2H$ analyses using the equilibration technique by Prosser and Scrimgeour (1995). The laboratory has reported a coefficient of variation (CV) in ten subjects for TBW of 0.3% (Matias et al. 2013). After the POST assessment, athletes were empirically divided into four groups according to sex and TBW change: those who increased and those who decreased TBW from PRE to POST.

Extracellular water

Extracellular water (ECW) was assessed from the sodium bromide (NaBr) dilution method after the subject consumed 0.030 g of 99.0% NaBr (Sigma - Aldrich; St. Louis, MO) per kg of body weight, diluted in 50 mL of distilled-deionized water. Baseline samples of saliva were collected before sodium bromide oral dose administration, and enriched samples were collected 3 h post-dose administration. ICW was calculated as the difference between TBW and ECW. The test-retest CV in 7 participants for the ECW using high performance liquid chromatography in our laboratory is 0.4% (Matias et al. 2013).

Dual-energy X-ray absorptiometry

Athletes underwent a whole-body DXA scan according to the procedures recommended by the manufacturer on a Hologic Explorer-W fan-beam densitometer (Hologic, Waltham, MA, USA). The equipment measures the attenuation of X-rays pulsed between 70 and 140 kV synchronously with the line frequency for each pixel of the scanned image. For athletes who were taller than the scan area, we used a validated procedure that consisted of the sum of a head and a trunk plus limbs scans. (Matias et al. 2012) The same technician positioned the participants, performed the scan, and executed the analysis (QDR for Windows software version 12.4; Hologic, Waltham, MA, USA) according to the operator's manual by using the standard analysis protocol. The DXA measurements included whole-body measurements of FM (kg) and FFM (kg). In our laboratory, in ten healthy adults, the test-retest CV for both FM and FFM is 0.8% and 1.7%, respectively (Matias et al. 2013).

Bioelectrical impedance analysis

The impedance measurements were performed with BIA (BIA 101 Anniversary, Akern, Florence, Italy) using an electric current at a frequency of 50 kHz. Measurements were made on an isolated cot from electrical conductors, the subjects were in the supine position with a leg opening of 45 ° compared to the median line of the body and the upper limbs, distant 30° from the trunk. After

cleansing the skin with alcohol, two electrodes (Biatrodes Akern Srl, Florence, Italy) were placed according to a standardized protocol (Lukaski and Piccoli 2012). BIVA was carried out using the classic BIVA method, normalizing R and Xc parameters for height (H) in meters (Piccoli et al. 1994). The length of the vector was calculated as the hypotenuses of individual impedance normalized values. Bioelectrical PA was calculated as the arctangent of $Xc/R \times 180°/\pi$. Prior to each test the analyzer was calibrated with the calibration deemed successful if R value is 383 Ω and Xc equal to 46 Ω. The test-retest CV in 10 participants in our laboratory for R and Xc was 0.3% and 0.9%, respectively.

Statistical Analysis

Descriptive statistics including means ± SD were calculated for all outcome variables. Once the data were tested for normality (Shapiro-Wilks test), differences in BC and bioelectrical variables between PRE and POST were analyzed by paired sample t-test. The paired one-sample Hotelling's T^2-test was performed to determine if the changes in the mean group vectors (measured at the first and second time points) were significantly different from zero (null vector). A 95% confidence ellipse excluding the null vector indicated a significant vector displacement. Single and multiple regression analyses were performed to understand the associations between changes in TBW, ICW and ECW/ICW ratio with vector length and PA. Model adjustments included age, stature and sex. Data were analyzed with IBM SPSS Statistics version 24.0 (IBM, Chicago, IL). For all tests, statistical significance was set at $p < 0.05$.

Results

General characteristics of the athletes are shown in Table 6. The majority of athletes (28 males and 11 females) significantly increased TBW from PRE to POST, while 11 men and 8 women showed a decrement.

Table 6. Participants' characteristics.

Variable	Men (n=39)	Women (n=19)
	Mean ± SD	Mean ± SD
Age (y)	18.7 ± 4.0	19.2 ± 6.0
Stature (cm)	79.58 ± 10.23	62.54 ± 8.52
Weight (kg)	188.52 ± 8.19	170.79 ± 4.87
BMI (kg/m^2)	22.36 ± 2.20	21.39 ± 2.26

Table 7 shows the changes in the BC and bioelectric variables from the first to the second measurement. In male and female athletes who significantly increased their fluids during the season, an increase in ICW, FFM and PA, and a reduction in R, R/H, and Z/H were detected. Otherwise, in athletes who reduced TBW from PRE to POST, a decrease in ICW and an increase in all bioelectrical values (R, Xc, R/H, Xc/H, Z/H, and PA) were measured among men, and an increase of Xc and Xc/H among women.

The vector displacements plotted on the RXc graph, from PRE to POST, and the results of the paired one-sample Hotelling's T^2-test were significant in men and women (Figure 13 and 14).

Table 7. Two-way analysis of covariance (ANCOVA) for the comparison at baseline (PRE) and during (POST) the competitive season after adjusting for athletes who increased (H) and decreased (L) body fluids as covariate.

	H				L						
	Men (n = 28)		Women (n = 11)		Men (n = 11)		Women (n = 8)		Gender	Time	Gender x Time
Variable	PRE	POST	PRE	POST	PRE	POST	PRE	POST	Effect	Effect	interaction
									P-value	*P*-value	*P*-value
TBW (kg)	49.2 ± 6.1	51.6 ± 6.0*	31.8 ± 3.1	33.3 ± 3.2*	52.1 ± 6.6	49.8 ± 6.3*	36.7 ± 4.6	35.2 ± 4.5*	<0.001	<0.001	0.403
ECW (kg)	20.3 ± 2.5	21.0 ± 2.5*	14.1 ± 1.8	14.3 ± 1.5	20.4 ± 2.5	19.7 ± 2.0	15.1 ± 1.7	14.7 ± 1.5	<0.001	0.005	0.521
ICW (kg)	28.9 ± 4.1	30.6 ± 4.3*	17.7 ± 1.7	19.0 ± 2.0*	31.7 ± 4.6	30.2 ± 4.9*	21.6 ± 3.2	20.5 ± 3.2*	<0.001	0.001	0.240
ECW/ICW	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.003	0.824	0.279
FM (kg)	11.8 ± 4.7	11.5 ± 4.3	16.3 ± 3.8	16.3 ± 4.2	12.2 ± 3.2	11.8 ± 2.6	15.7 ± 4.8	15.0 ± 4.6	0.034	0.728	0.876
FFM (kg)	67.6 ± 7.4	70.0 ± 7.9*	44.1 ± 4.8	45.8 ± 4.4*	65.5 ± 8.5	67.2 ± 8.2	48.8 ± 6.0	48.8 ± 5.4	<0.001	<0.001	0.712
R (Ω)	491.0 ± 49.9	463.1 ± 45.6*	617.0 ± 51.4	591.4 ± 54.0*	447.9 ± 34.0	461.1 ± 37.1*	557.5 ± 77.2	576.4 ± 84.0	<0.001	<0.001	0.687
Xc (Ω)	60.3 ± 6.3	60.1 ± 6.0	71.0 ± 8.2	71.0 ± 8.3	59.5 ± 4.9	62.9 ± 4.9*	68.1 ± 11.4	72.0 ± 12.5*	<0.001	0.004	0.869
R/H (Ω /m)	258.4 ± 27.0	243.1 ± 24.0*	363.4 ± 36.1	347.7 ± 37.3*	243.6 ± 22.0	250.4 ± 23.0*	324.7 ± 44.4	334.7 ± 46.6	<0.001	<0.001	0.878
Xc/H (Ω /m)	31.8 ± 3.9	31.6 ± 3.8	41.8 ± 5.3	41.7 ± 5.2	32.3 ± 2.9	34.1 ± 2.8*	39.7 ± 6.7	41.8 ± 7.0*	<0.001	0.002	0.879
PA (°)	7.1 ± 0.7	7.5 ± 0.8*	6.6 ± 0.3	6.9 ± 0.4*	7.6 ± 0.7	7.9 ± 0.7*	7.0 ± 0.6	7.2 ± 0.6	0.005	0.034	0.345
Z/H (Ω /m)	260.4 ± 27.1	245.1 ± 24.1*	365.8 ± 36.5	350.2 ± 37.6*	245.8 ± 22.0	252.7 ± 23.0*	327.1 ± 44.8	337.4 ± 47.0	<0.001	<0.001	0.884

Note: Data are expressed as mean and standard deviation; * p< 0.05 vs. PRE.

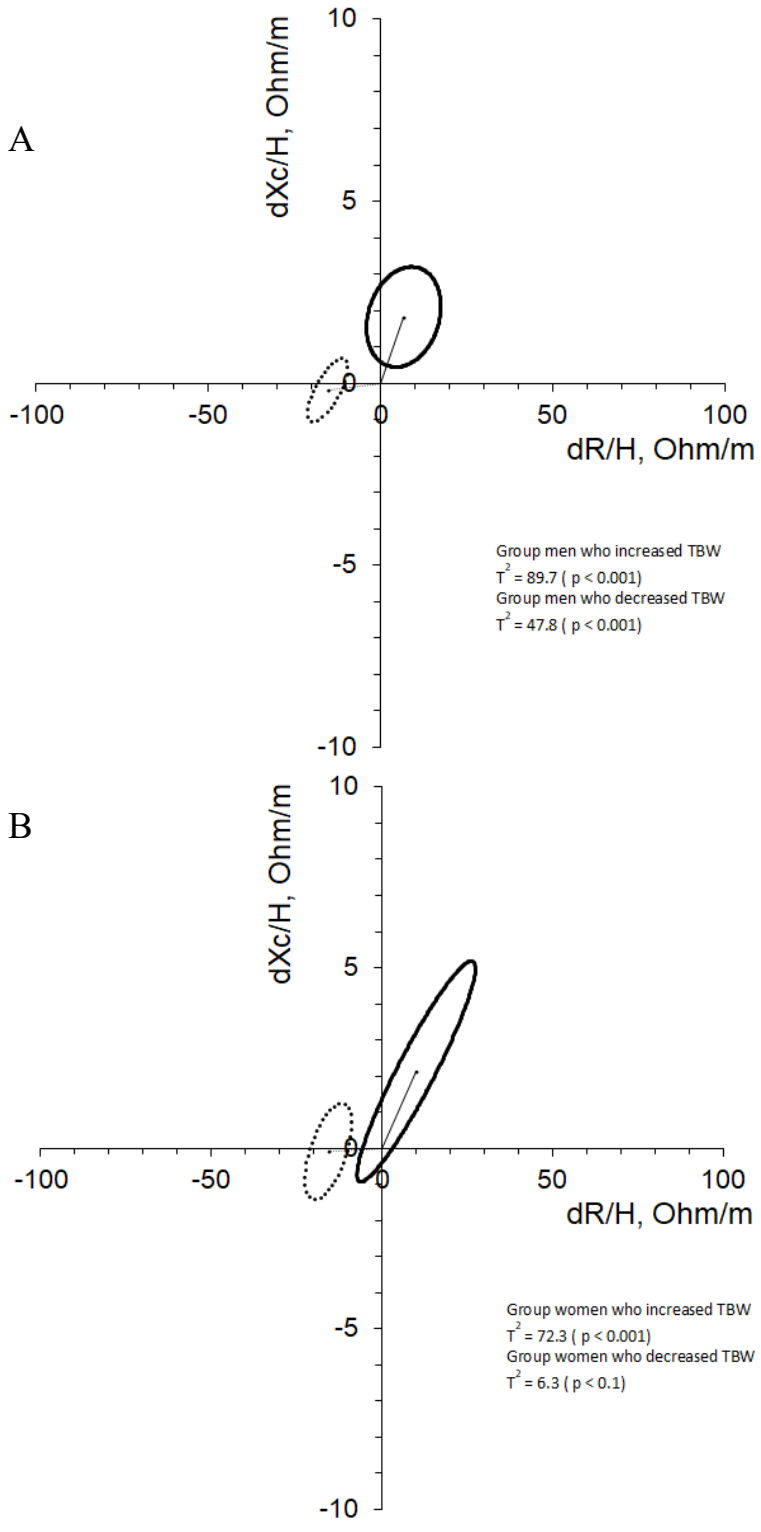

Figure 13. Paired graph and vector displacements with 95% confidence ellipses who increased (dashed line) or decreased (solid line) total-body water in males (panel A) or females (panel B). T^2, Hotelling's T^2 test; p-value.

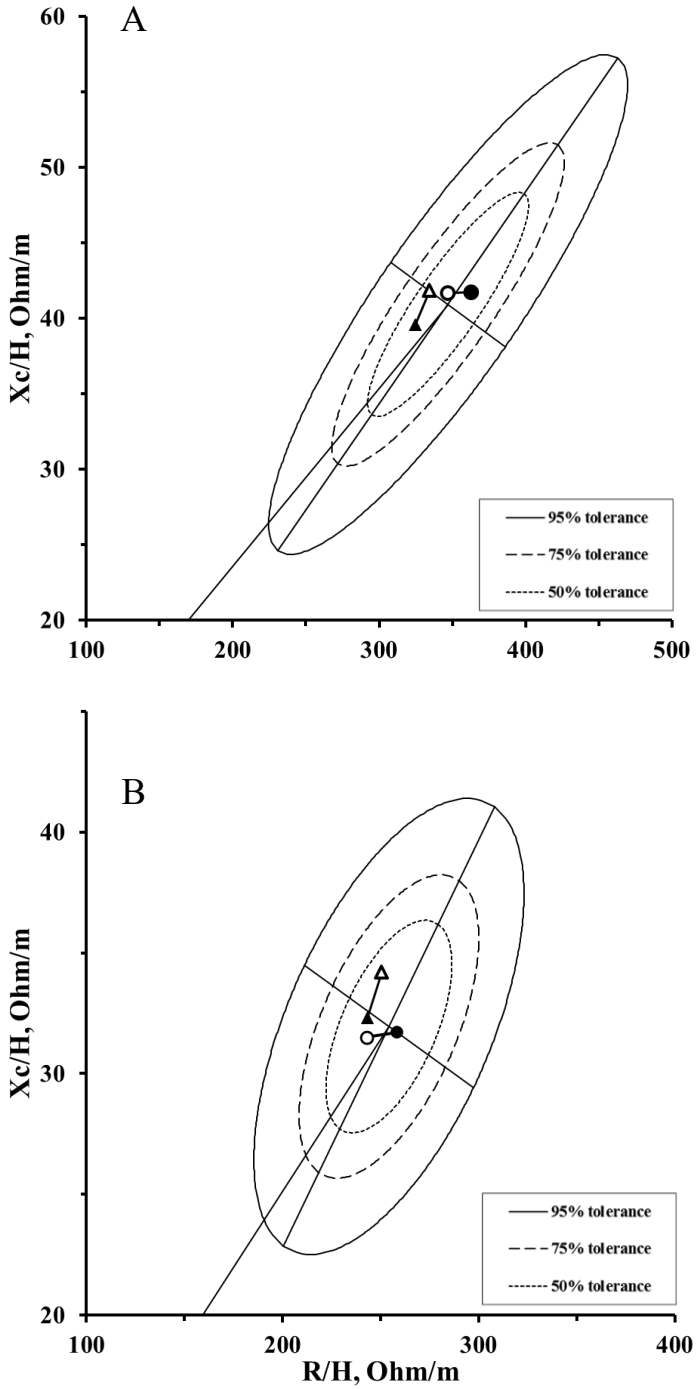

Figure 14. RXc-graph and mean impedance vectors plotted on the tolerance ellipses created from bioimpedance values measured at PRE in women (panel A) and men (panel B). Where circles and triangles represent the clusters that increase or decrease fluids from PRE (black clusters) to POST (white clusters), respectively.

In Table 8 and 9 results from single and multiple regression analysis are displayed. Vector length was negatively correlated to with TBW and ICW and positively associated with the ECW/ICW ratio, even when adjusted for sex, age and stature. Phase angle was positively associated with TBW and ICW and negatively associated with the ECW/ICW ratio, independently of sex, age, and stature.

Table 8. Regression analyses for body fluids with vector length

	Model	Model [a]
	β (CI 95%)	β (CI 95%)
Δ TBW		
Δ ZL	-0.718 (-0.142; -0.080) **	-0.672 (-0.137; -0.071) **
Δ ICW		
Δ ZL	-0.630 (-0.134; -0.064) **	-0.531 (-0.119; -0.047) **
Δ ECW/ICW		
Δ ZL	0.344 (0.000; 0.004) *	0.217 (0.000; 0.003)

Note: β, standardized beta coefficient; CI, confident interval; Δ, changes;

* Significant at $p < 0.05$

** Significant at $p < 0.01$

[a] Adjusted for sex, age and stature.

Table 9. Regression analyses for body fluids with phase angle

	Model	Model [a]
	β (CI 95%)	β (CI 95%)
Δ TBW		
Δ PA	0.458 (1.228; 4.324) **	0.396 (0.780; 4.024) *
Δ ICW		
Δ PA	0.564 (2.013; 4.929) **	0.455 (1.307; 4.293) **
Δ ECW/ICW		
Δ PA	-0.436 (-0.166; -0.042) **	-0.433 (-0.171; -0.007) *

Note: β, standardized beta coefficient; CI, confident interval; Δ, changes;

* Significant at $p < 0.05$

** Significant at $p < 0.01$

[a] Adjusted for sex, age and stature.

Discussion

The main finding of the present investigation is that changes in body fluids throughout a competitive season are associated with changes in bioelectrical vectors in athletes. In particular, decreases in TBW detected by deuterium dilution were accompanied by increases in vector length and decreases in PA, and viceversa. In all groups there was a significant increase in PA, except for the females whose TBW decreased, where a positive but not significant trend was observed. Additionally, groups showing higher PA values also showed higher values of FFM, significantly among those whose TBW increased. These results support evidence provided in previous studies that highlighted that peripheral vectors lying on the left or right side of the minor axis of the tolerance ellipses, i.e. with higher or lower phase angles, indicate more or less soft tissue, respectively (Buffa et al. 2014; Lukaski 2013; Marini et al. 2013; Piccoli et al. 1994).

Carrasco-Marginet et al. (Carrasco-Marginet et al. 2017) showed that following a loss of fluids PA tends to increase. Actually, higher PA values reflect higher cellularity, cell membrane integrity and better cell function (Norman et al. 2012), and are associated with improved power output in elite road cyclists (Pollastri et al. 2016).

In our investigation, increases in PA were also associated with ECW/ICW ratio decrements and this is in line with the findings of Gonzalez et al. (2016), who suggested that PA is inversely related to ECW/ICW ratio, and with our previous researches on athletes (Marini et al. 2019). In addition, a positive correlation between ECW/ICW ratio and vector length was found. Significant ICW reductions occurred in athletes who decreased TBW. Although it was not our goal to investigate the causes of TBW changes in the athletes, our hypothesis is that the reductions of TBW and ICW can be due to the nutritional habits or the different demands of exercise and the respective recovery process.

The use of BIVA has become a very common practice in sports, to evaluate changes in body fluids in athletes during the competitive season or following an exercise program or a training session. Mascherini et al. (2015) showed that vector movements can occur during a competitive season, highlighting that increases in fluids occur at the end of the pre-season phase and at the end of the season, while fluid leaks can occur during the competitive period. The bioelectrical vector and PA changes have also been associated with increases in strength and decrease in FM after exercise training programs in adults (Campa et al. 2018). In addition, several studies have proposed new BIVA references for sports such as soccer (Micheli et al. 2014) and volleyball (Campa and Toselli 2018), highlighting that BIVA can identify significant differences based on the competitive level, due to different characteristics in athletes of several sports. Although the classic BIVA approach has shown to be weak in the distinction of the relative contribution of fat mass and fat free mass (Buffa et al. 2014), the studies that validated BIVA with accurate laboratory tests for the evaluation of short-term fluid changes (as after a physical exercise) have concluded that BIVA was accurate to assess body fluid changes (Gatterer et al. 2014; Heavens et al. 2016). To our knowledge, this is the first

investigation to examine vector changes over a competitive season in athletes, comparing the results obtained by BIVA with TBW and water compartments from dilution techniques.

Despite the encouraging results obtained in this investigation, some limitations should be addressed. First, our results are applicable to the actual BIA equipment using the 50 kHz frequency. In fact, 50 kHz single frequency devices are among the most used equipment, yet similar studies should be conducted to test other frequencies resulting from multifrequency equipment as predictors of TBW and its compartments. Secondly, it is important to underscore that since athletes were tested at the beginning and at the main stage of the competitive period, but it is unknown if these two measurements represent what happened during the entire season. Lastly, as only five sports were included in this investigation, generalizability of these findings to other sports is limited.

Conclusions

This investigation has shown that vector changes convincingly mirror fluids loss or gain over a season. In particular, peripheral vectors lying on the left or right side of the minor axis of the tolerance ellipses, i.e. with higher or lower phase angles, indicate more or less soft tissue, respectively. In addition, PA is sensitive and inversely related to ECW/ICW ratio. BIVA shifts might be used by nutritionist and coaches as a practical method to monitor body fluid changes and to adapt training and nutrition in athletes.

STUDY III

Introduction

BIA is a fast, safe and non-invasive method to obtain quantitative estimates of BC values and their use is very common in sports. Recently Gatterer et al. (2014) suggested that elimination of some confounding factors which occur after exercise (increased cutaneous blood flow, elevated body temperature, accumulation of electrolytes on the skin, and elevated rates of respiration) by cold shower application would facilitate the use of BIVA to assess post-exercise fluid changes. One limitation of this study, however, was the use of an uncontrolled exposure of the cold application (i.e., duration of the shower and temperature of water) and, thus, the time course required to achieve stable BIA values post shower could not been determined.

The present study aimed to determine the time course of BIA values when a cold shower with a standardize duration (10 min) and water temperature (22°C) was applied after a period of controlled exercise. We hypothesized that 60 min was the time duration needed to stabilize BIA values and that a cold shower considerably speeds up the time course.

Materials and Methods

Participants

Ten healthy, well-trained male adults (age 26.2 ± 4.1 years, BMI 23.9 ± 1.7 kg/m^2 with an average weekly training 9.4 ± 2.9 hours) volunteered to participate in the study. Participants were informed about the objectives and the procedures of the research and signed the informed consent form. The study was approved by the Bioethics Committee of the University of Bologna.

Procedures

The athletes performed two running test sessions separated by one week. During each session, athletes ran on a treadmill for 30 min at an intensity corresponding to a score of 15 (heavy work) on the BORG scale (Borg 1982). They were randomly assigned to have a cold shower or none during the first treadmill running session. Participants who had no shower during the first session had one during the second session. To induce a state of normal hydration, participants were instructed to drink 3 L of fluids over the 24 hr prior to the treadmill run sessions. On each test day, the athletes came to the laboratory of the University of Bologna at 9.00 am. After emptying the bladder, the pre-tests (T0) were performed. Tests included the BIA analysis, body temperature, hand and foot skin temperature, body weight and height measurements. Body height was measured once. Body weight was measured before and after exercise. The remaining parameters were repeatedly measured immediately after exercising (T1) and after 20 (T2), 40 (T3) and 60 min (T4) of recovery. During the shower session the shower was performed after T1. The temperature of the water was held constant at 22°C for 10 min, with the water temperature controlled with a digital thermometer (Checktemp, CL, Hanna instruments). During the recovery period, athletes were seated and were not allowed to drink or eat. All tests and measurements were performed in an environmental chamber set at 23°C and 50% relative humidity.

Measurements

Height was recorded to the nearest 0.1 cm with a stadiometer and weight was measured to the nearest 0.1 kg with a high-precision mechanical scale. The impedance measurements were performed with a bioimpedance analyzer (BIA 101 Anniversary, Akern, Florence, Italy) using a phase-sensitive device with alternating current at a frequency of 50 kHz. The accuracy of the bioimpedance instrument was validated before each test session following the manufacturer's instructions. Measurements were made on a cot isolated from electrical conductors. The subjects were in the supine position with legs (45° compared to the median line of the body) and arms (30 ° from the trunk) abducted. After cleansing the skin with alcohol, two electrodes (Biatrodes Akern Srl, Florence, Italy) were placed according to a

standardized protocol (Lukaski and Piccoli 2012). Bioimpedance values were analyzed according to the BIVA method. R and Xc parameters were divided by standing body height (H) in meters. The body temperature was measured using an infrared thermometer (Mini Flash, TFA Dostmann GmbH & Co, Wertheim, Germany) on the forehead.

Statistical analysis

The Shapiro-Wilk test was used to check the normal distribution of data. The paired, one-sample Hotelling's T^2 test was performed to determine if the changes in the mean group vectors (measured between T0 and T1, T1 vs T2, T2 vs T3, and T3 vs T4) were significantly different from zero (null vector). A 95% confidence ellipse excluding the null vector indicated a significant vector displacement. A 5 × 2 repeated measures analysis of variance was performed to analyze all investigated variables. Time (T0, T1, T2, T3, and T4) was the within-subjects factor, and condition (shower; no shower) was the between-subjects factor. To examine changes between recovery phases (T0, T1, T2, T3, and T4) and baseline values on each condition, a paired sample t-test with Bonferroni correction was used. Data were analyzed with SPSS v20.0 (SPSS, Chicago, IL, USA) or with BIVA software. Data are presented as mean ± SD and the significance level was set at $p < 0.05$. For ANOVA outcomes, effect size was assessed by using partial eta squared ($\eta^2 p$).

Results

Table 10 shows all measured variables in the course of the trials. Pre-exercise physical characteristics were similar among the two groups. Body weight decreased during both running sessions (-0.40 % in the shower tests and -0.41 % in the control test $p<0.001$).

Table 10. Measured parameters before the exercise and during the recovery phase in both trials.

		T0	T1	T2	T3	T4	ANOVA	
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Time effect	Time x condition
R/h (Ω/m)	T	274.6 ± 48.0	264.8 ± 48.4*	274.4 ± 50.5	274.5 ± 50.2	274.4 ± 50.5	F=51.5; p<0.001; η^2_p=0.74	F=4.5; p=0.003; η^2_p=0.20
	CT	274.1 ± 48.1	264.6 ± 47.8*	268.9 ± 47.2*	274.7 ±48.2	275.2 ± 48.3		
Xc/h (Ω/m)	T	37.9 ± 7.1	35.4 ± 6.7*	37.5 ± 6.8	37.5 ± 6.7	37.5 ± 6.9	F=72.8; p<0.001; η^2_p=0.80	F=1.3; p=0.276 η^2_p=0.06
	CT	38.1 ± 6.9	35.6 ± 7.0*	37.0 ± 6.8*	37.4 ± 6.9*	37.3 ± 6.7*		
Vector length (Ω/m)	T	277.3 ±48.4	267.2 ± 48.7*	276.9 ± 50.8	277.1 ± 50.6	276.9 ± 50.8	F=54.1; p<0.001; η^2_p=0.75	F=4.5; p=0.003; η^2_p=0.20
	CT	276.3 ± 48.2	267.0 ± 48.1*	271.5 ± 47.6*	277.2 ± 48.5	277.8 ±48.6		
Phase angle (°)	T	7.9 ± 0.9	7.7 ± 0.9*	7.8 ± 0.8	7.8 ± 0.8	7.8 ± 0.8	F=9.4; p<0.001; η^2_p=0.34	F=0.51; p=0.72; η^2_p=0.02
	CT	7.9 ± 0.8	7.7 ± 0.9*	7.8 ± 0.8*	7.8 ± 0.9*	7.8 ± 0.8		
Body weight (kg)	T	72.5 ± 8.1	72.2 ± 8.1*	-	-	-	-	-
	CT	72.5 ± 8.0	72.2 ± 7.8*	-	-	-		
Forehead temperature (°C)	T	36.0 ± 0.1	37.0 ± 0.1*	36.1 ± 0.2	36.1 ± 0.2	36.1 ± 0.2	F=324.6; p<0.001; η^2_p=0.94	F=29.1; p<0.001; η^2_p=0.61
	CT	36.0 ± 0.1	37.0 ± 0.2*	36.6 ± 0.1*	36.4 ± 0.1*	36.2 ± 0.1*		

Note: T= Shower trial; CT= Control trial; *= $p < 0.05$ vs. T0.

Exercise significantly affected vector position on the paired graph (Fig. 15), R/H, Xc/H, PA and vector length (Table 10, T0 to T1, p<0.05). After the cold shower, vector length, PA, R/H and Xc/H returned to baseline at T2 (Table 10), whereas without shower baseline values were achieved at T3 for R/H and vector length and at T4 for PA (Table 10). In the shower test, Xc/H does not show any significant difference between T0, T2 and T3 (p=0.161 and p=0.173, respectively). From T0 to T4 a trend towards significance was found (p=0.074). In the test without shower, Xc/H remained significantly different from T0 also in T2, T3 and T4 (p<0.001).

In both conditions a higher body temperature was recorded at T1 compared to before exercise (Table 10, p<0.05). Only in the test session without shower body temperature remained increased in the course of the measurements [T0 vs. T2 (p<0.001), T3 (p<0.001) and T4 (p<0.002), respectively; Table 10].

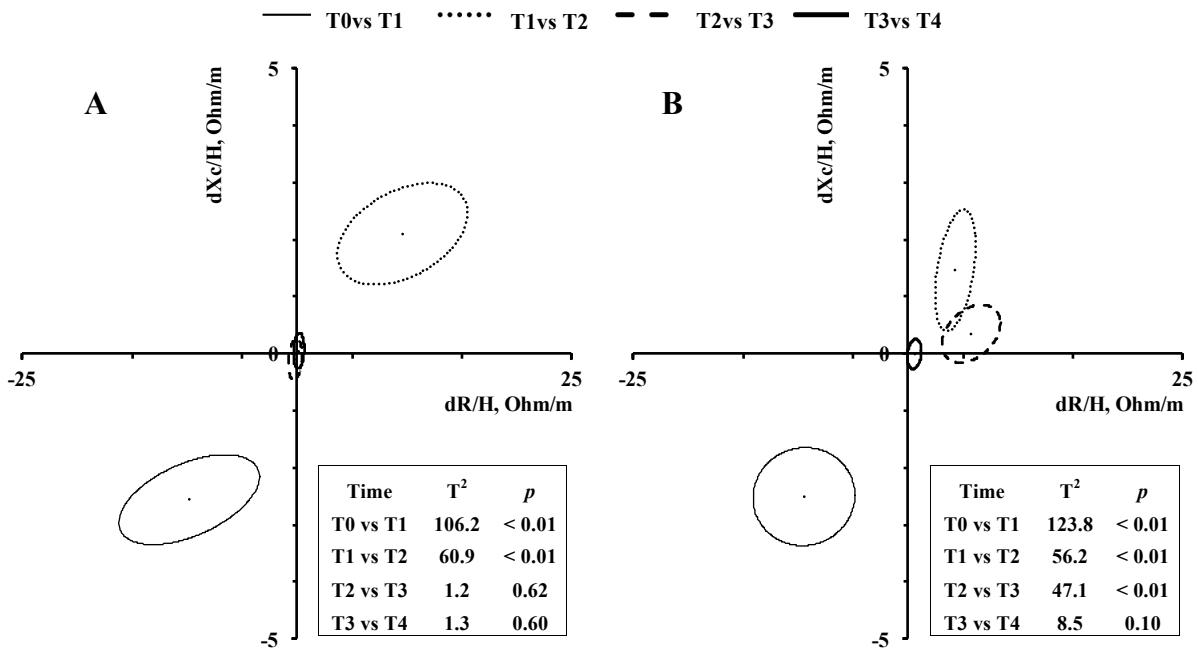

Figure 15. Paired Graph and impedance vector displacements with 95% confidence ellipses for T0 vs T1, T1 vs T2, T2 vs T3 and T3 vs T4 in the shower (A) and control (B) trials.

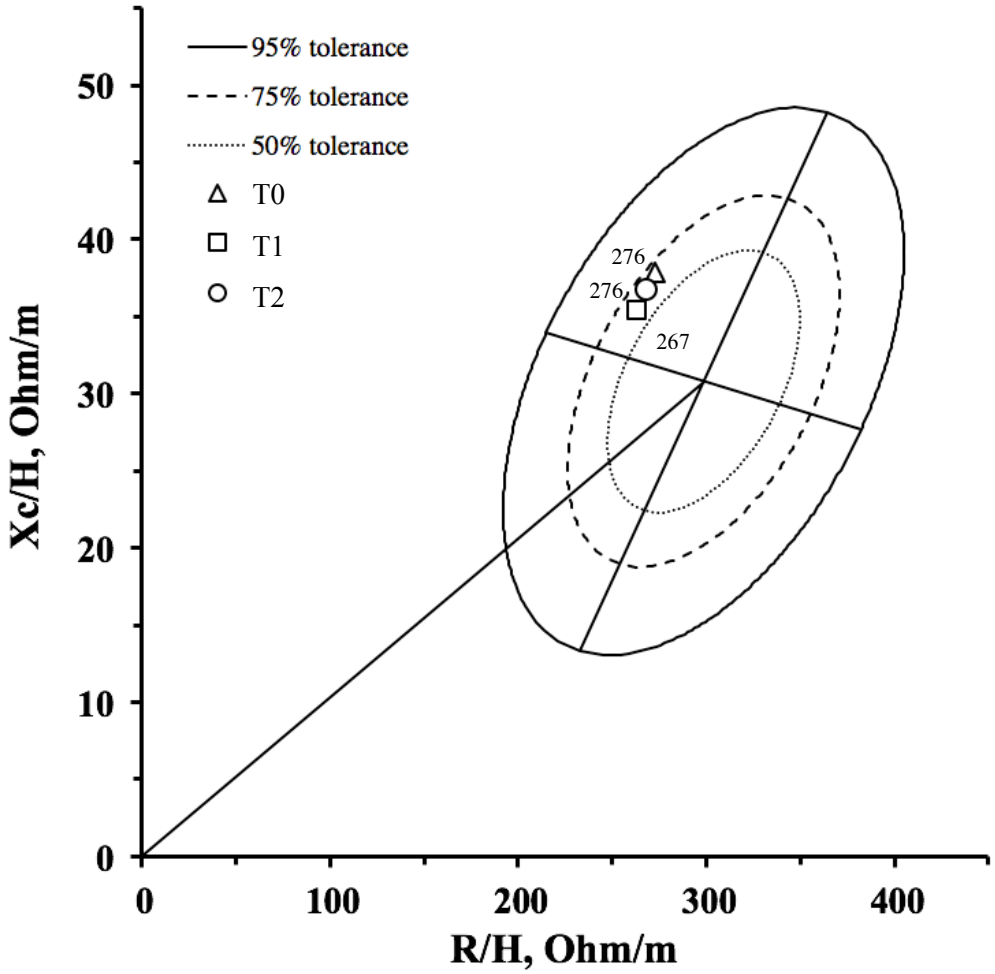

Figure 16. The vector displacement in the first three times (T0, T1 and T3) for the shower trial using the mean R/H and the mean Xc/H plotted on the reference population tolerance ellipses

Discussion

The main findings of the present study are that after a cold shower with a water temperature of 22°C and a duration of 10 min is applied after exercising, BIA values stabilize after approximately 20 min. Without shower the time until reaching stable values was prolonged to 40 min. In addition, the recorded changes in Xc/H and PA could indicate that some fluid shifts between body compartments occurred with unaltered hydration (vector position within the 50th percentile of the tolerance ellipse).

Bioimpedance measurements are known to be influenced by body and skin temperature and blood flow (Gatterer et al. 2014; Ring et al. 2016). In the present study, the body temperature of the

athletes increased significantly after 30 minutes of running at a heavy intensity. Concomitantly to the increased body temperature immediately after running, the BIA vector showed a downward shift along the major axis of the tolerance ellipses (Fig. 16). As the vector length is inversely related to total body water, this should indicate fluid gain. Obviously, this immediate shift is caused by the mentioned physiological adaptations during exercise, as the slightly reduced body weight indicates body fluid loss due to sweating. Accordingly, after the shower, the body temperature returned to baseline 20 min after exercise, and BIA vector returned to baseline as well. On the contrary, without the cold shower, body temperature remained slightly above baseline even though vector length and position returned to baseline. This might indicate that a slightly increased body temperature of approximately 0.2°C with <0.5% loss of body weight has minimal effect on whole-body BIA measurements.

Kyle et al. (2004) reported that BIVA is effective at monitoring hydration changes after exercise. In the present study, the vector returned to baseline and showed no prolongation out of the upper pole of the 50th percentile of the tolerance ellipse. These vector positions (i.e., inside the 50th percentile of the tolerance ellipse) indicate that after the exercise session all participants were normal hydrated (Piccoli et al. 1994) even though the athletes lost about 0.4% or 0.3 kg of their body weight in both tests. Obviously, this study was not designed to induce graded levels of dehydration or to investigate hydration but to establish the time course of bioimpedance parameters after exercise followed by cold shower application.

The ratio between the intra- and extracellular fluid volumes is identified by the PA. In our tests, when the impedance measurement was performed immediately after exercise, the phase angle showed a significant decrease, with a slow recovery thereafter to nearly baseline values. Additionally, Xc/H, reflecting intracellular fluid content 6 remained altered in the non-shower setting and showed some differences between T0 and T4 in the shower setting. These changes possible could indicate that some fluid shifts between body compartments occurred. One speculation is that fluid moved transiently from the intracellular to interstitial space. Such a change would directly impact capacitance because the

distance between cells would be reduced. Findings of the present study are not adequate to address this issue in detail.

Some limitations have to be acknowledged. Lack of data describing the effects of the shower after a more strenuous physical test that would result in greater fluid loss can be considered a limitation of the study. Therefore, the present results are only valid for the condition of exercise-induced modest hypohydration. Additionally, besides BIVA and body weight change no further hydration marker was assessed in the present study. Thus, future studies are needed that focus on identifying and monitoring the physiological parameters involved in altering impedance measurements following exercise.

Conclusions

A cold-water shower lasting for 10 min resulted in an early stabilization of impedance parameters compared to passive recovery. BIVA might be a valuable tool in identifying dehydration and fluid shifts. On that basis, the small fluid losses established by the changed body weight seem not to indicate a state of dehydration.

STUDY IV

Introduction

The search for optimal physical and mental condition in athletes and recovery of physiological parameters after performance sports have always been topics of study for trainers, coaches and high-level athletes. For decades, the relationship between hydration status and performance has been closely evaluated, with hydration status being directly linked with sport performance. Many studies have reported the consequences of dehydration on physical and mental levels, highlighting humoral changes and cognitive deficits, which not only compromise the normal daily actions but can negatively affect sports performance (Masento et al. 2014; Pethick et al. 2019; Zhang et al. 2019). More specifically, it has been shown that the inadequate restoration of fluids during exercise compromises neuromuscular function, increases fatigue perception, technical skills, also affecting metabolic rate and heart rate variability after exercise (Barley et al. 2018; Castro-Sepulveda et al. 2014; Georgescu et al. 2017; Nuccio et al. 2017).

Studies show that only a loss of 1-2% of BM from sweating is sufficient to compromise physiological functioning and sport performance during exercise. On the contrary, maintenance of body water during exercise is thought to provide protection from thermal injury, reduce physiological strain and maintain or even improve sport performance (Convertino et al. 1996).

Dehydration is the process of losing body water. The National Athletic Trainers' Association (NATA) recommends using a combination of methods to assess hydration status, including body mass change, urine color or urine specific gravity (USG) after first morning void, and thirst level to track hydration status (McDermott et al. 2017).

Recently, it has been shown that the BIVA method is able to measure changes in body fluids and assess the state of hydration in the short and long term, even after exercise (Campa et al. 2019a; Campa et al. 2019b; Gatterer et al. 2014).

There is still an incomplete picture regarding the loss of fluids which occurs during exercise and the relationship that it has on physical performance variables. Despite the importance of maintaining a euhydrated state, studies have shown individuals are not adequately replacing fluid during exercise (Muth et al. 2019).

Therefore, the purpose of this study was to thoroughly investigate the effects of dehydration on heart rate (HR), oxygen uptake (VO_2) responses, neuromuscular functionality, and time trial (TT) performance during a cycling exercise in athletes, while also evaluating the variation of the examined parameters when the athletes hydrated during the trial.

Materials and methods

Participants

We selected ten trained male subjects (age 23.4 ± 2.7 years) who volunteered to participate in this study. Subjects were instructed not to practice intense physical activity in the 24 hours prior to the tests, to refrain from consuming alcohol and caffeine for at least 48 hours and avoid using diuretics during the 7 days prior to each evaluation. After being informed on the objectives and the research procedures, participants signed the consent document. The study was approved by the Bioethics Committee of the University of Bologna.

Procedures

The athletes visited the laboratory three times. All tests were performed at the same time of the day (9:00 AM), in a quiet room with stable temperature (21°C; 52% of humidity). On the first visit, subjects performed an incremental cycling test to exhaustion on an electronically braked cycle ergometer (LODE Excalibur, Quinton Instrument, Groningen, the Netherlands) to determine the VO_2peak. The expired gas analysis was performed with the Quark CPET device (Cosmed, Italy). The maximal exercise test lasted until VO_2 plateau was obtained or at least one of the two additional criteria:

(i) a plateau of heart rate despite an increased velocity, or (ii) exercise cessation due to substantial fatigue. VO_2 plateau was defined as an increase in $VO_2 \leq 50$ ml min-1 during the last 30 s despite increased velocity (Yoon, Kravitz, and Robergs 2007). The highest VO_2 values reached during the exercise phase of the incremental test were considered the VO_{2max}. Heart rate (HR) was collected using a Polar RS400 downloadable HR monitor (Polar Electro., Lachine, QC) and oxygen uptake (VO_2) was assessed during the sessions.

On two subsequent occasions, subjects returned to the laboratory for practice trials and cycled at 65% VO_2max for 90 min followed by a time to trial (TT) at 95% VO_2max, in different conditions: dehydration (DEH) and hydration (HYD). For the dehydration practice trial, subjects completed the trial without ingesting fluids (Holland et al. 2017; Logan-Sprenger et al. 2015). On the contrary, in the hydration trial the athletes drank 1 l of water in 4 intervals every 15 minutes. In the shortest time possible, they removed the metabolimeter mask and consumed 0.225 ml of water previously prepared in 4 0.5 L bottles (Backes and Fitzgerald 2016).

Subjects woke, emptied the bladder, and took their body mass (SECA model 874 with precision to 0.01 kg) in shorts prior to each experimental trial. Two hours prior to the practice rides, subjects ingested a meal provided for them (790 kcal; 144 g carbohydrate, 35 g fat, 19 g protein) and 250 mL of fluid. Additionally, subjects drank as they normally would the night before and drank 300 mL of water 90 and 45 min before the trial to ensure they were well hydrated before cycling (Logan-Sprenger et al. 2015). After the TT, subjects removed their shoes and shirt, towel dried and were weighed to determine the body mass loss over the trial. In addition, to assess fluid loss, bioelectric impedance was measured at baseline (T0), immediately after the trials (T1) and after a 10-minute shower (T2) according to the procedures reported by Campa et al. (2019a). The impedance measurements were performed with a bioimpedance analyzer (BIA 101 Anniversary, Akern, Florence, Italy) using a n phase-sensitive device with alternating current at a frequency of 50 kHz. The accuracy of the bioimpedance instrument was validated before each test session following the manufacturer's

instructions. Measurements were made on a cot isolated from electrical conductors. The subjects were in the supine position with legs (45° compared to the median line of the body) and arms (30° from the trunk) abducted. After cleansing the skin with alcohol, two electrodes (Biatrodes Akern Srl, Florence, Italy) were placed on the right hand and two on the right foot. Bioimpedance values were analyzed according to the BIVA method. R and Xc parameters were divided by standing body height (H) in meters.

Surface electromyography (sEMG) was recorded using a sEMG machine (EMG FREE1000, BTS Bioengineering, Italy). In this study, we recorded the activity of the vastus medialis and biceps femoris muscles. Before placement of electrode, the skin was shaved, abraded with sandpaper, and cleansed with ethanol to avoid impedance mismatch and movement artifact. The utmost care was taken when placing electrodes on the midline of the muscle belly, between the myo-tendinous junction and the nearest innervations zone. Detection surface was oriented perpendicularly to the length of the muscle fibers. Ag/AgCl electrodes were adhered to the skin along the direction of muscle fiber orientation. After placing the electrodes, we acquired the maximum voluntary contraction (MVC) in which each subject had to perform, for 5 s, an isometric contraction against a maximum load using isotonic machines (Technogym, Italy).

Data was recorded at a sample rate of 1000 Hz and stored for analysis (Balasubramanian and Jayaraman 2009). All raw EMG signals were band pass filtered (20-450 Hz), resampled at 500 Hz and positively rectified. The EMG signals were normalized to the peak of the MVC. The normalized root mean square (RMS) values were calculated in 100 ms bin from EMG signals using Matlab.

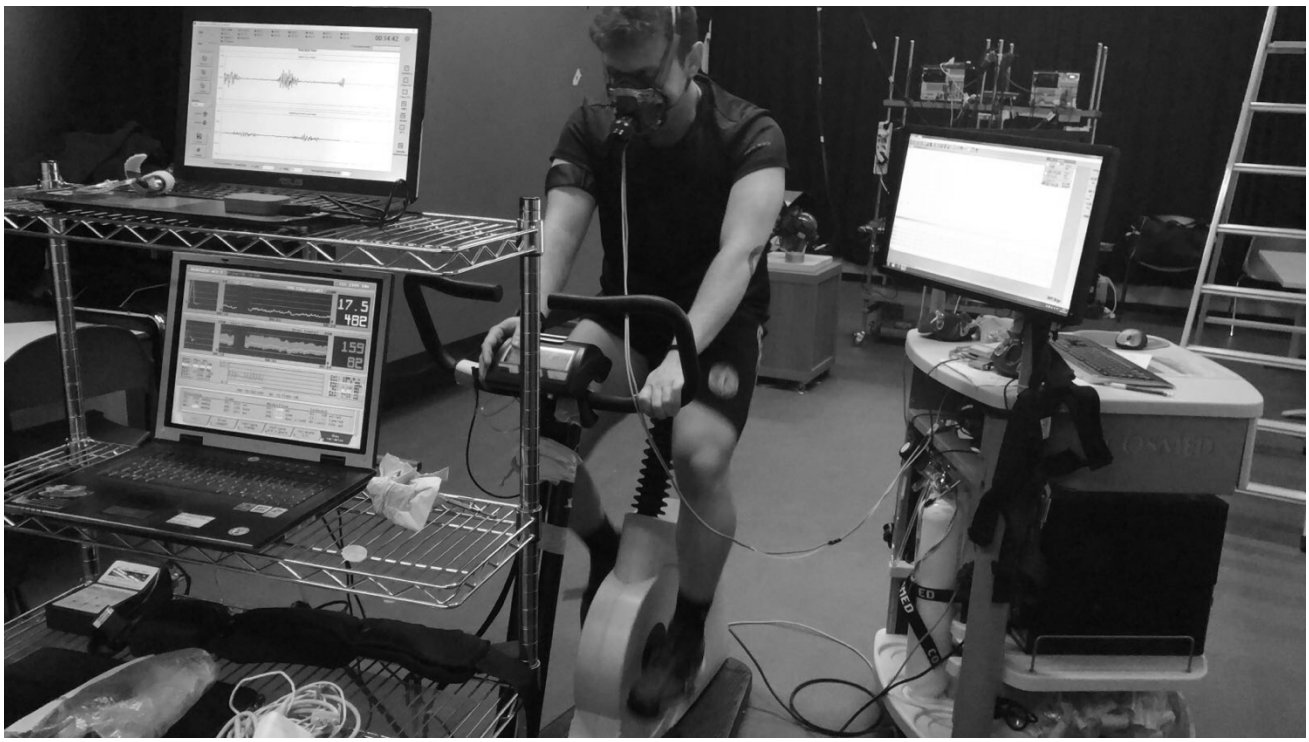

Figure 17. An athlete performing a cycling trial.

Statistical Analysis

Shapiro-Wilk tests were used to check the normal distribution of data. Descriptive statistics including means ± SD and data distribution were calculated for all outcome variables. A 3 × 2 ANOVA for repeated measures was performed to analyze the changes in the bioimpedance variables. Effect sizes were calculated using partial eta squared (ηp^2). To compare changes in sEMG activity, HR, VO_2, and body weight a paired student t-test was performed. The paired one-sample Hotelling's T^2-test was performed to determine if the changes in the mean group vectors (measured between T0 vs T1 and T0 vs T2) were significantly different from zero (null vector). A 95% confidence ellipse excluding the null vector indicated a significant vector displacement. Linear regression analyses were performed to understand the associations between sEMG activity, VO_2 and time during the trials. Data was analyzed with IBM SPSS Statistics version 24.0 (IBM, Chicago, IL) and BIVA software (Piccoli and Pastori, 2002). For all tests, statistical significance was set at $p < 0.05$

Results

Table 11 shows the changes in the bioelectric values. R/H, Xc/H and vector length significantly changed ($p<0.05$) in T1 and T2 compared to T0 in the DEH condition. On the contrary, when the athletes performed the trial in the HYD condition, the same values showed a significant change only in T1. The PA showed a significant reduction in both trials in T1 compared to T0.

Table 11. Bioimpedance parameters before and after the exercise and the shower in both tests.

		T0	T1	T2	ANOVA	
		Mean ± SD	Mean ± SD	Mean ± SD	Time effect	Time x condition
R/H (Ω/m)	H	259.9 ± 36.4	248.7 ± 34.4*	260.4 ± 36.9	F= 146.5; p= <0.001;	F= 19.5; p= <0.001;
	D	255.6 ± 35.8	246.2 ± 34.6*	267.4 ± 36.1*	η^2_p= 0.89	η^2_p=0.52
Xc/H (Ω/m)	H	35.2 ± 5.4	32.6 ± 5.1*	35.3 ± 5.4	F= 172.1; p= <0.001;	F= 18.5; p= <0.001
	D	34.7 ± 6.9	32.1 ± 4.9*	36.9 ± 4.9*	η^2_p= 0.90	η^2_p= 0.50
Vector length (Ω/m)	H	262.3 ± 36.7	250.8 ± 34.8*	262.8 ± 37.2	F= 154.5; p= <0.001;	F= 28.5; p= <0.001;
	D	257.9 ± 36.1	248.3 ± 34.9*	269.9 ± 36.4*	η^2_p = 0.89	η^2_p=0.53
Phase angle (°)	H	7.7 ± 0.5	7.4 ± 0.5*	7.8 ± 0.4	F= 553.4; p= <0.001;	F= 1.34; p= 0.274; η^2_p
	D	7.7 ± 0.4	7.4 ± 0.4*	7.9 ± 0.4	η^2_p = 0.96	= 0.06

Note: H= Hydration condition; D= Dehydration condition; *= $p < 0.05$ vs. T0.

The average body weight of athletes in T0 were 74.75 ± 10.49 and 74.91 ± 10.37 in HYD and DHE conditions, respectively. After both trials body weight showed a significant decrease by measuring 74.52 ± 10.56 in HYD ($p=0.005$) and 73.58 ± 10.39 in DEH ($p<0.001$). When the athletes performed the DEH trial, their body weight decreased by 1.76% ± 0.39%, while when they performed the HYD trial their body weight decreased by 0.3% ± 0.27%.

Figure 18 shows the vector displacements (on the left side) and the Hotelling's T^2 test results (on the right side) for DEH and HYD conditions, panel A and B, respectively.

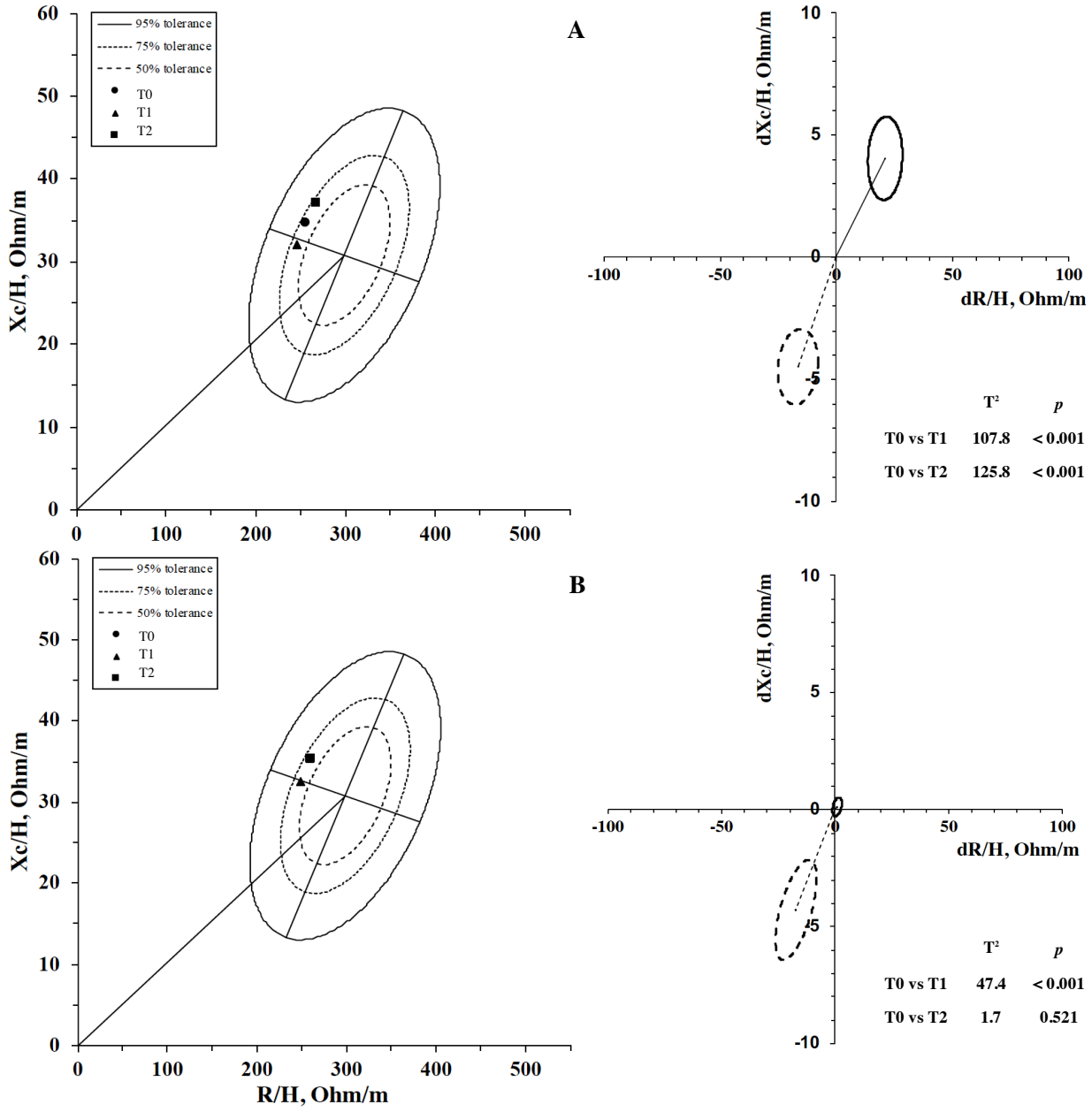

Figure 18. On the left side mean impedance vectors, plotted on the 50%, 75%, and 95% tolerance ellipses of the corresponding healthy male reference population (Piccoli et al. 1994) are displayed both for DEH and HYD trial (A and B, respectively). On the right side, mean vector displacements and results of the Hotelling's T^2 test; continue lines: T0 vs. T2, dotted lines: T0 vs. T1.

Table 12 shows the comparisons between the average HR measured in the two trials. Significant differences between HYD and DEH conditions were found during exercising at 65% of the VO2max.

Table 12. Comparison of the heart rate (HR) values between the two trials.

	HYD		DEH		
Condition	Mean	SD	Mean	SD	p-value
Baseline					
HR (bpm)	81.61	7.75	87.62	8.90	0.133
65% VO2max					
HR (bpm)	136.10	8.33	139.95	7.64	0.019
95% VO2max					
HR (bpm)	162.97	14.53	169.09	8.67	0.281

No significant differences between the two trials were found only for VO_2 during cycling at 65% (HYD: 2152.01 ± 315.06 ml/min, DEH: 2191.25 ± 341.66 ml/min, p=0.299) and 95% VO2max (HYD: 2815.39 ± 426.92 ml/min, DEH: 2762.41 ± 539.09 ml/min, p=0.806). Figure 19 shows the VO_2 kinetics and the results of the linear regressions for VO_2 and time in both conditions.

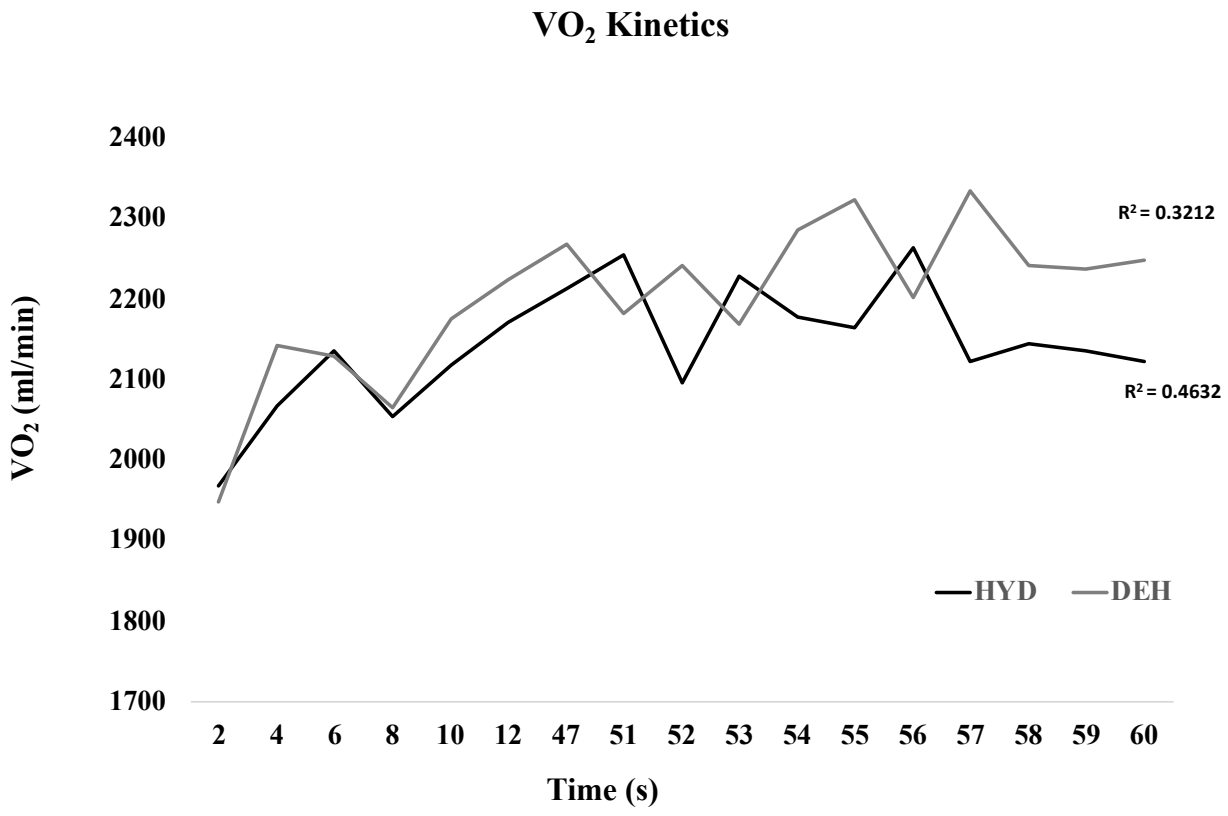

Figure 19. VO_2 kinetics and results of linear regressions for VO_2 and time in both conditions.

Table 13 and 14 show the comparison of the normalized RMS values between the two trials for RVM and RBF muscles, respectively. During cycling at 65% VO2max, significant differences between the two trials were found only for the RBF muscle. RVM and RBF showed higher sEMG activity during the HYD condition in the TT performance.

Scatterplot showing the results of the linear regressions for normalized RMS values during the TT performance are shown in Figure 20 and 21 for RBF and RVM muscle, respectively. In the DEH condition there was a stronger negative correlation between the power expressed by the RVM and RBF muscles and TT time compared to the HYD condition.

Table 13. Comparison of the RVM values between the two trials.

	HYD		DEH		
Condition	Mean	SD	Mean	SD	p-value
Baseline					
NormRMS (% MCV mV)	5.46	3.49	5.48	3.20	0.891
65% VO2max					
NormRMS (% MCV mV)	15.47	5.46	15.46	5.62	0.851
95% VO2max					
NormRMS (% MCV mV)	17.79	8.26	13.47	10.39	<0.001

Table 14. Comparison of the RBF values between the two trials.

	HYD		DEH		
Condition	Mean	SD	Mean	SD	p-value
Baseline					
NormRMS (% MCV mV)	2.40	1.22	2.41	1.40	0.890
65% VO2max					
NormRMS (% MCV mV)	5.33	2.59	4.67	51.37	<0.001
95% VO2max					
NormRMS (% MCV mV)	7.66	2.55	5.58	3.23	<0.001

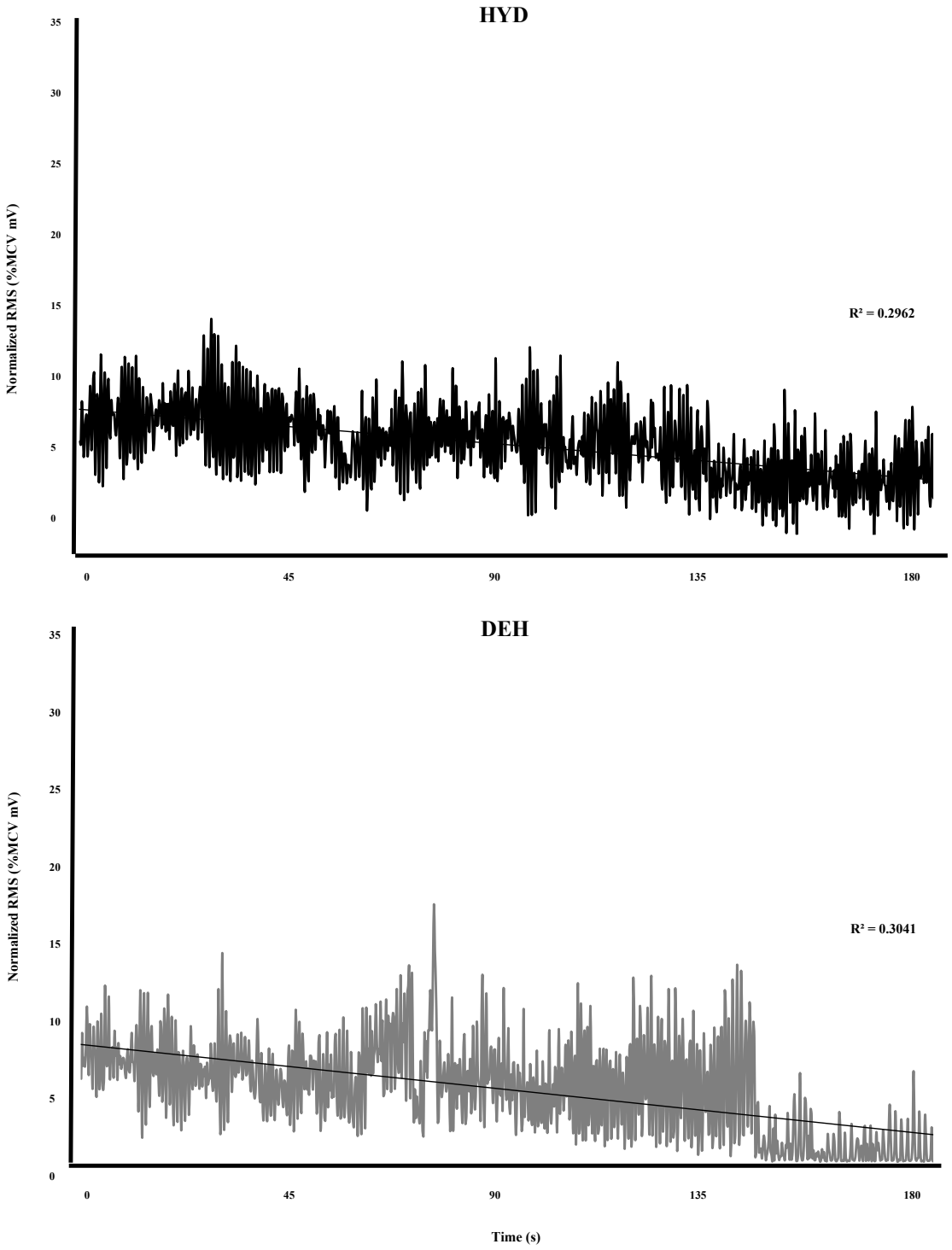

Figure 20. Scatterplot shows the relationship between the normalized RMS and time during the TT performance for the RBF muscle.

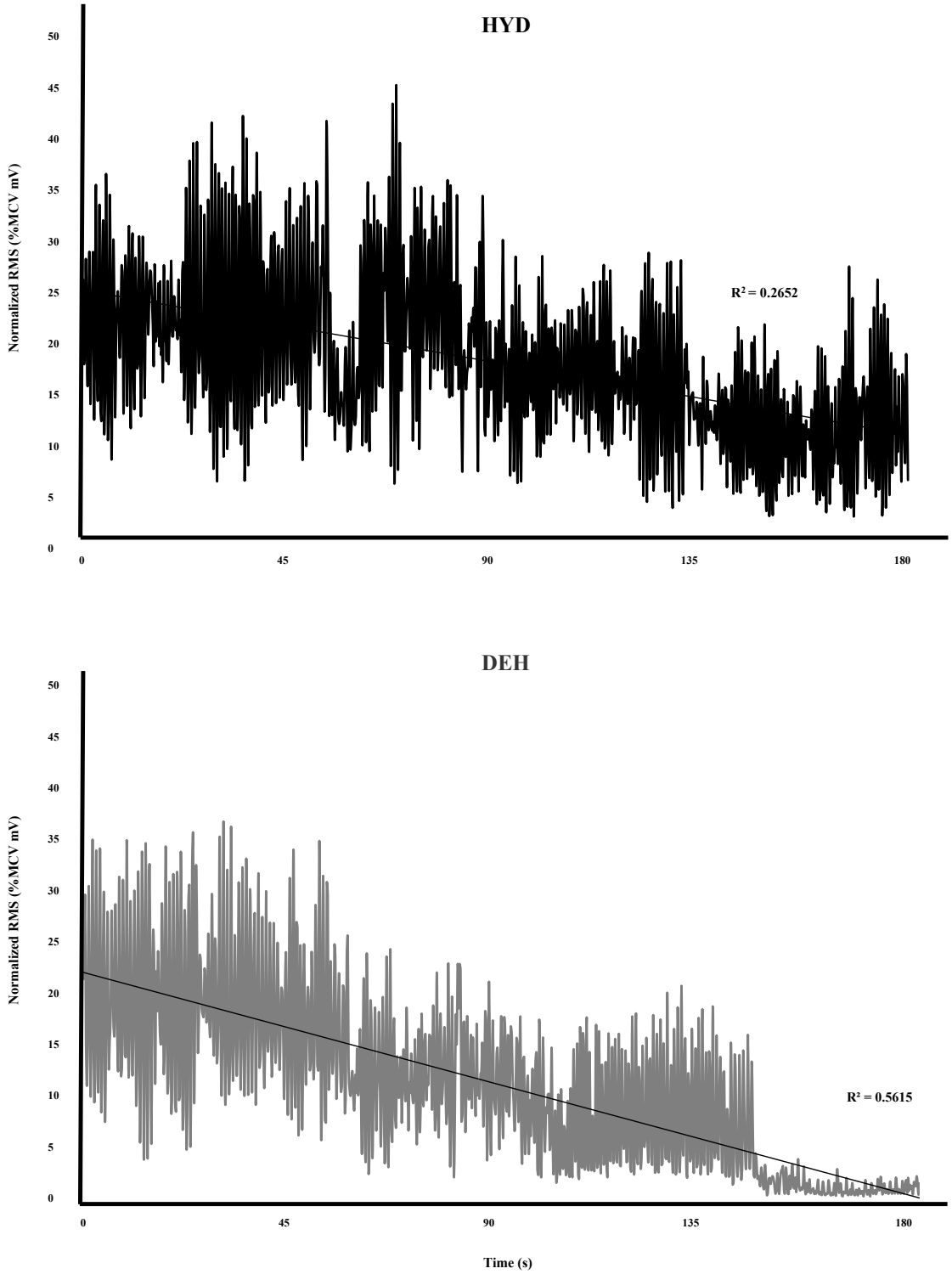

Figure 21. Scatterplot shows the relationship between the normalized RMS and time during the TT performance for the RVM muscle.

Discussion

The aim of the present study was to investigate the effects of dehydration on HR, VO_2 responses, and neuromuscular functionality during a cycling performance in athletes.

Fluid loss and therefore dehydration during the DEH session was evaluated by BIVA, which identified a significant vector displacement along the major axis of the ellipses; on the contrary, when the athletes restored the fluids lost during the HYD trial, no vector displacement was detected. This evaluation was also supported by the body weight change measured after the DEH trial.

The HR variation observed during the cycling performance at 65%VO2max was significantly different between the two conditions; in fact, a higher HR during DEH sessions was observed. Our hypothesis is that HR increases during dehydration because of the blood flow redistribution, which causes a high body temperature and a consequent decrease in the stroke and blood volume, and consequently compensatory increase in HR. In line with these results, previous studies have documented the resultant tachycardia and diminished stroke volume during dehydration. Our results are similar to those obtained by Logan-Sprenger et al. (2015) in an experiment on 9 subjects who completed two cycling trials lasting 60 minutes at 65% VO2max followed by a TT; as in our study, the subjects showed an increase in HR in dehydration condition compared to when the subjects were hydrated, but only in the first part of the test.

No significant difference was measured for VO_2 between the two trials in both phases of the cycling exercise. However, a stronger negative association was found between VO_2 and trial time during the DEH session. Furthermore, the VO_2 kinetics showed tendentially higher values during DEH, although not statistically significant. Probably, a greater percentage of fluid loss is necessary to have significant effects on VO_2 during a cycling exercise carried out at 65%VO2max.

We observed that during the first part of the test, when the athletes were pedaling at 65% VO2max, no significant difference was measured on the power output by the RVM among the trials, while the RBF activity was significantly lower during the DEH session; this could have been due to

the athletes' pedaling technique. When athletes cycled at 95%VO2max, both the RVM and RBF muscle activity was significantly reduced during the DEH test, suggesting that fluid loss compromises the muscle power expression during dehydration. In line with this, our results showed a significant negative association between muscle power and trial time during the TT, and this association was stronger in the DEH session.

The effects of dehydration on muscle performance have been studied using different protocols and measurement techniques. Studies vary in the percentage of lost fluids achieved from 1.7% to 5.8% of body mass reduction (Bowtell et al. 2013; Minshull and James 2013; Pallares et al. 2016; Schoffstall et al. 2001). After a literature review, Judelson et al. (2007) concluded that dehydration consistently attenuates muscle power by approximately 3%. The origin of these reductions has been speculated to reside on alterations in cardiovascular, metabolic or buffering functions. In fact, heat stress, with or without dehydration, compromises blood flow to active muscles and skin during strenuous exercise as the systemic circulation becomes compromised (Crandall and Gonzalez-Alonso 2010).

In a meta-analysis conducted by Goulet (2013) it was showed that levels of exercise induced dehydration, similar to those in the present study, did not reduce cycling TT performance. Additionally, in response to a similar hydration status (progressive loss to 2% body weight loss), no significant differences were reported when trained men completed a 40 km cycling TT performance as measured by power output and mean finish time (Berkulo et al. 2016). These records of data suggest the influence of dehydration may, in part, be protocol specific. In our study, athletes cycled for 60 min at 65% VO2max and performed a 95% VO2max TT in two different tests. During the HYD session, in order to restore the fluids lost during exercise, the athletes ingested 0.5 ml of water every 15 min to maintain a body weight similar to that recorded at the baseline. However, to the best of our knowledge, this is the first experiment to use this experimental protocol to ensure a well-hydration status of the athletes during the exercise and therefore it is not possible to compare our results with other researches. One of the most common potential limitations is the inherent difficulty in blinding subjects to the fact that

they are dehydrating versus rehydrating during a given trial. Another possible limitation in the present study is the participant sample, which may compromise detectable differences among the two hydration conditions in the examined parameters. Nonetheless, other studies evaluating exercise performance and dehydration/rehydration have used samples ranging from n = 6 to 11 (Buono and Wall 2000; Laitano et al. 2012; Minshull and James 2013).

From the discussion above it is clear that more research is needed to address several remaining questions regarding the potential impact of dehydration on sport performance. Valid and reliable protocols should be developed and used in future studies to ensure that tests are able to detect the effects of fluid loss on central and peripheral parameters.

Conclusions

This study demonstrated that many physiological parameters along with neuromuscular function and HR were altered during a moderate intensity exercise when subjects dehydrated versus maintaining a well-hydration status through drinking. After exercise induced dehydration, the bioimpedance vector significantly lengthens along the major axis of the tolerance ellipse, in conformity with body weight loss (-2%), that indicates fluid loss. Dehydration during a one-hour cycling test and subsequent TT, at 65% and 95% of the VO2max, respectively, caused a significant increase in HR during the first trial part (65%VO2max), while it had no effect on the TT performance. On the contrary, neuromuscular function, assessed by surface electromyography on the thigh muscles, showed a lower muscle activation in TT performance in dehydration conditions, but no difference when the athletes cycled at 65% of the VO2max, except for the biceps femoris muscle. Finally, although the VO_2 kinetics showed a greater tendency to consume O2 during dehydration, no statistically significant effect was found. The practical application of this study demonstrated that athletes exercising in a dehydrated state significantly decreased performance; therefore, attention needs to be paid to strategies to maintain

a well-hydration status during exercise. In addition, BIVA has been shown to be a suitable tool to assess body fluid changes and hydration status even after exercise.

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OVERALL CONCLUSIONS

From the present doctoral thesis, the following conclusions were derived:

1. BIVA is a suitable method to assess body composition in athletes. However, it must be considered that specific BIVA is more accurate for the analysis of %FM in athletes, while it does not correctly evaluate TBW, for which classic BIVA appears to be a suitable approach. PA, and hence both BIVA approaches, can detect ECW/ICW changes.
2. Changes in body fluid throughout a competitive season are associated with changes in classic bioelectrical vectors in athletes. In particular, decreases in TBW are accompanied by increases in vector length and decreases in PA, and vice versa. Vector changes convincingly mirror fluid loss or gain over a season in athletes.
3. Peripheral vectors lying on the left or right side of the minor axis of the tolerance ellipses on the RXc-graph, i.e. with higher or lower PA, indicate more or less soft tissue, respectively.
4. When a cold shower with a water temperature of 22°C and a duration of 10 min is applied after a 30 min run, bioimpedance values stabilize after approximately 20 min. During the same conditions, without showering the time until stable values are reached is prolonged to 40 min.
5. Classic BIVA might be a valuable tool in identifying dehydration and fluid shifts after exercise.
6. Dehydration during a one-hour cycling test and subsequent TT, at 65% and 95% of the VO2max, respectively, caused a significant increase in HR during the first trial part (65%VO2max), while it had no effect on the TT performance. Neuromuscular function, assessed by surface electromyography on the thigh muscles, showed a lower muscle activation in TT performance in the dehydration condition, but no difference when the athletes cycled at 65% of the VO2max, except for the RBF which showed a major power output also in the first trial part at 65% of the VO2max .

ACKNOWLEDGMENTS

Firstly, I would like to express my special appreciation and thanks to my supervisor Professor Stefania Toselli for encouraging my research and for allowing me to grow as a research scientist, for her motivation, and immense knowledge. Thanks must also go to Professor Analiza Monica Silva and Professor Luis Sardinha for giving me the opportunity to be part of some exciting clinical and laboratory studies at the Lisbon University. Special thanks must also go to Professor Elisabetta Marini for her invaluable contribution to study design, data analysis, and manuscript preparation for studies I and II. I would also like to thank Professor Milena Raffi and Dr Alessandro Piras for their contribution towards the development of study IV.

Gratitude is also expressed to all my Italian and Portuguese colleagues and thanks to all subjects that took part in this research.

I would especially like to thank my loving wife Vanessa for being the best. Lastly, words can't express the gratitude I owe to my father, my mother, my uncles, Abbondanza, and my cousins. Thank you again for being my team, I love you.

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