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**Article Title:** Time to Exhaustion at Maximal Lactate Steady State Continuous and Intermittent During Running Exercise

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## **ABSTRACT**

The purpose of this study was to determine and compare the time to exhaustion (TE) and the physiological responses at continuous and intermittent (ratio 5:1) maximal lactate steady state (MLSS) in well trained runners. Ten athletes ( $32.7 \pm 6.9$  years;  $\text{VO}_2\text{max}$   $61.7 \pm 3.9 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) performed an incremental treadmill test, three to five 30-min constant speed tests to determine the MLSS continuous and MLSS intermittent (5 min of running, interspaced by 1 min of passive rest) and two randomized TE tests at such intensities. Two-way ANOVA with repeated measures was used to compare the changes in physiological variables during the TE tests and between continuous and intermittent exercise. The intermittent MLSS velocity ( $\text{MLSS}_{\text{int}} = 15.26 \pm 0.97 \text{ km}\cdot\text{h}^{-1}$ ) was higher than in the continuous model ( $\text{MLSS}_{\text{con}} = 14.53 \pm 0.93 \text{ km}\cdot\text{h}^{-1}$ ), while the time to exhaustion at MLSS continuous was longer than MLSS intermittent ( $68 \pm 11$  min and  $58 \pm 15$  min,  $p < 0.05$ ). Regarding the cardiorespiratory responses,  $\text{VO}_2$  and R remained stable during both TE tests while HR, VE and RPE presented a significant increase in the last portion of the tests. The results showed a higher tolerance to exercising during continuous MLSS compared to intermittent MLSS, in trained runners. Thus, the training volume of an extensive interval session (ratio 5:1) designed at MLSS intensity should take into consideration this higher speed at MLSS and also the lower TE, when compared with continuous exercise.

**Keywords:** Exercise tolerance; endurance runners; interval training

## INTRODUCTION

Heavy exercise domain is characterized as intensity at which oxygen uptake ( $\text{VO}_2$ ), and blood lactate ([La]) can be maintained at an elevated but steady state level. The maximal lactate steady state (MLSS) has been considered the upper limit of this exercise intensity domain.<sup>1,2</sup> Thus, it can be defined as the highest steady state exercise ( $\sim 80 - 85\% \text{VO}_{2\text{max}}$ ) that can be maintained over time without the continual accumulation of blood lactate<sup>3</sup>.

Since the MLSS is a consistent physiological phenomenon, it can be used as a marker for a submaximal exercise intensity, prediction of endurance performance,<sup>4,5</sup> and also used as an intensity reference to endurance training sessions.<sup>5,6</sup> Commonly, the MLSS intensity has been determined by continuous (MLSS<sub>con</sub>) test protocols, comparing the blood lactate response during the final 20 minutes of a 30 min exercise<sup>7</sup>.

Beneke et al<sup>8</sup> were the first to demonstrate that test interruptions for blood sampling modifies the level of physiological exertion during the tests and may modulate the MLSS workload. This finding led to a novel approach in literature concerning MLSS determination<sup>9-11</sup>. Thus, different studies were conducted with different exercise model combinations (i.e. work/rest ratio; active and passive rest), which is an important aspect to design training sessions. Greco et al<sup>10</sup> comparing the MLSS determination using continuous and intermittent (work/rest ratio of 2:1) protocol, found a difference of 13% (passive rest) and 9% (active rest) higher for intermittent cycling, while Grossl et al<sup>9</sup> found a difference of 6.5% (5:1 with passive rest). Thus, using training loads higher than MLSS<sub>con</sub> during interval training may also determine stability of [La]. Therefore, the protocol used to determine MLSS<sub>con</sub> may have limited applications for extensive interval training prescriptions for endurance athletes.

From a practical point of view, the MLSS training has been named as tempo training which corresponds to the athlete's highest current steady state pace.<sup>12</sup> Highly trained athletes train regularly at these submaximal intensities, at least once per week.<sup>13</sup> Athlete's

performance depends on an adequate distribution between the volume and intensity training during the season. Therefore, the MLSS seems to indicate a borderline intensity with relevance for the prescription of endurance training because above this limit inadequate training load may result quickly with negative consequence leading to symptoms of over-reaching and over-training<sup>8</sup>. In addition, it seems important to measure the time sustained (volume) during exercises performed at (or close) MLSS since it can be used to calibrate the duration of a training session.

It has been previously demonstrated that exercising at MLSS leads to exhaustion (TE) in about 60 min during a continuous exercise<sup>5,9,14,15</sup>. However, to our knowledge, only one study verified the TE at MLSS during both continuous and intermittent protocol during cycling exercise<sup>9</sup>. The authors found a difference of 24% higher during intermittent cycling (using an exercise: rest ratio of 5:1) compared to continuous. Interestingly, they found a significant higher tolerance during intermittent cycling compared to continuous, even with the workload being higher in intermittent model. This result has an important implication to cycling interval training. However, it is unclear if the same happens for other exercise mode, as running. Considering the physiological differences between cycling and running, such as different contractions evolved (i.e. eccentric vs concentric),<sup>16</sup> delta efficiency,<sup>17</sup> arterial hypoxemia,<sup>18</sup> glycogen depletion<sup>19</sup> and muscle blood flow, it seems important to verify the time to exhaustion in running at both MLSS. Thus, the aim of the present study was to determine and compare the TE and physiological responses at continuous and intermittent (ratio 5:1) MLSS in trained runners.

## **METHODS**

### **Subjects**

Ten well- trained endurance male runners, with at least 5 years of experience in the modality and who were competing in regional to national level volunteered in the present study ( $32.7 \pm 6.9$  years;  $75.3 \pm 5.3$  kg;  $176.8 \pm 5.7$  cm;  $11.6 \pm 4.0$  % body fat). Preceding the period of the study, the athletes had a weekly training volume of about 40km. Prior to any testing all participants was familiarized with the experimental procedures and gave a written informed consent as well as informed of the associated risks and benefits of participation. The procedures were approved by the Federal University of Santa Catarina (protocol 799/2010).

### **Experimental Procedures**

In order to avoid undue fatigue before testing, subjects were instructed to avoid heavy training during the preceding 24 hours. Athletes were advised to maintain a regular diet during the day before testing (i.e. 60%, 25% and 15% of carbohydrates, fat and protein, respectively) and to refrain from smoking and caffeinate drinks during the two hours preceding testing and arrived at the laboratory in a rested and fully hydrated state. Each participant was tested at the same time of day ( $\pm 2$ h) to minimize the effects of biological variation. All running tests were performed on a motorized treadmill (Imbramed Millennium Super, Brazil) with the gradient set at 1%.

Firstly were performed anthropometric measures (body mass, stature, and skinfold measures to estimate percent body fat) followed by an intermittent treadmill test for the assessment of maximal oxygen uptake ( $VO_{2max}$ ), peak velocity (PV), maximal ventilation ( $VE_{max}$ ), maximal heart rate ( $HR_{max}$ ) and onset of blood lactate accumulation (OBLA). Based on the OBLA, on different days, three to five submaximal tests were performed to

determine the velocity at maximal lactate steady state using both a continuous ( $vMLSS_{con}$ ) and an intermittent protocol ( $vMLSS_{int}$ ). Following the determination of the  $vMLSS$  in both models two time to exhaustion tests were performed on different days (randomized). The rate of perceived exertion (RPE) was measured using Borg’s category scale<sup>20</sup>, which consists of 12 statements scored from 0 to 10 (from nothing to maximal).

## **Testing Procedures**

### **Incremental test**

An incremental test was performed in order to measure the maximal oxygen uptake ( $VO_{2max}$ ) and maximal aerobic velocity ( $vVO_{2max}$ ). The initial starting speed was of 10.0  $km.h^{-1}$  and treadmill speed was subsequently increased by 1.0  $km.h^{-1}$  every 3 minutes until subjects achieved volitional exhaustion. Between each stage there was a rest interval of 30 seconds to collect 25 $\mu$ l of capillary blood from the ear lobe to measure blood lactate concentration ([La]). The analysis of lactate was performed using an electrochemical analyzer (YSI 2700 STAT, Yellow Springs, OH, USA) and the OBLA was determined according Berg et al procedures<sup>21</sup>.

Respiratory gases were measured breath by breath (Quark, Cosmed, Rome, Italy) during the incremental test using a pre-calibrated online metabolic system, and the data were reduced to 15s averages. The attainment of  $VO_{2max}$  was defined using the criteria proposed by Howley et al.<sup>22</sup> The  $vVO_{2max}$  was identified as the lowest speed where  $VO_{2max}$  occurred and was maintained for at least one minute. Heart rate (HR) was recorded continuously during the test by a HR monitor incorporated into the gas analyser. The HRmax was the highest 5-sec. average HR value achieved during test.

## **Determination of MLSS during continuous and intermittent running**

Subjects performed several constant speed testing, at least 48h apart, in order to determine MLSS. For the determination of MLSS, each constant speed test lasted 30 min and started with a 10-min warm-up phase, being 5 min at 50%  $v\text{VO}_2\text{max}$  and 5 min at 60%  $v\text{VO}_2\text{max}$ . The first 30-min trial was performed at OBLA speed. Blood sampling were collected on the 10<sup>th</sup> and 30<sup>th</sup> min of these tests. The initial speed for determination of  $\text{MLSS}_{\text{int}}$  was 5% above the  $\text{MLSS}_{\text{con}}$ . The identification of  $\text{MLSS}_{\text{int}}$  was similar to the continuous protocol, but with a total duration of 35 min due to the 1-min rest period (passive recovery) after every 5 min of running, characterizing an exercise: rest ratio of 5:1. Blood samples for measurement of [La] were collected on the interval of the second, fourth and last 5 min effort. If during the first constant speed test a stability or a decrease in lactate was observed, further subsequent 30-min tests with 5% higher speed were performed on separate days until [La] steady state could be maintained. On the other hand, if the first constant test resulted in a clearly identifiable increase in the [La] and/or could not be completed due to exhaustion, further tests were conducted with subsequently reduced (5%) speed. The MLSS, in both protocols, was determined as the highest speed that could be maintained with [La] increase lower than  $1 \text{ mmol}\cdot\text{L}^{-1}$  during the last final 20min of appropriate tests<sup>4</sup>.

## **Time to exhaustion**

All subjects were asked to perform a running until exhaustion at the speed corresponding to MLSS previously determined. Physiological ( $\text{VO}_2$ , VE, RER, HR) and perceptual (RPE) parameters were continuously measured during entire test. Nevertheless, because of the different times to exhaustion of the subjects, these parameters were expressed and analyzed as percentage of total time ( $t_{10\%}$ ,  $t_{20\%}$ ,  $t_{40\%}$ ,  $t_{60\%}$ ,  $t_{80\%}$  and  $t_{100\%}$ ). Blood lactate samples were collected at rest, 30th min and at the end of the test. Furthermore, at the 30th



min ~250 mL of water were given to athletes in order to avoid dehydration. The RPE was required every 4 min of exercise.

## **Statistical Analyses**

Analyses were performed using GraphPad Prism software package for Windows (v. 5.0 GraphPad Prism Software Inc, San Diego, CA) and SPSS version 15.0. Data are presented as mean  $\pm$  standard deviation (SD). Normality was assessed by the Shapiro-Wilk test. A mixed model analysis of variance (ANOVA) was used in combination with post hoc testing (Bonferroni), where appropriate, to compare the changes in physiological variables during the tests ( $T_{10\%}$  -  $T_{100\%}$ ) and between continuous and intermittent exercise. The level of significance was  $p < 0.05$  for all statistical analyses. The magnitude of the difference was assessed by the Effects Size (ES) and the scale proposed by Cohen<sup>23</sup> was used for interpretation.

## **RESULTS**

### **Incremental Test**

Mean  $v\dot{V}O_{2\max}$  reached by the subjects was  $17.6 \pm 1.0 \text{ km}\cdot\text{h}^{-1}$  and corresponded to a  $\dot{V}O_{2\max}$  of  $61.7 \pm 3.9 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ . Mean HR, VE and [La] values obtained during the incremental test were  $186 \pm 5.3 \text{ bpm}$ ;  $156.6 \pm 19.0 \text{ L}\cdot\text{min}^{-1}$  and  $11.2 \pm 2.0 \text{ mM}$ , respectively.

### **Maximal Lactate Steady State parameters**

Mean  $MLSS_{\text{con}}$  and  $MLSS_{\text{int}}$  velocities were  $14.5 \pm 1.0 \text{ km}\cdot\text{h}^{-1}$  and  $15.2 \pm 1.0 \text{ km}\cdot\text{h}^{-1}$  corresponding to 82% and 86% of  $v\dot{V}O_{2\max}$ , respectively. The mean velocity,  $\dot{V}O_2$  and [La] obtained in  $MLSS_{\text{con}}$  were significantly lower than  $MLSS_{\text{int}}$  (Table 1).

## Time to Exhaustion

As presented in Table 1, the TE at  $MLSS_{con}$  was ~15% longer than at  $MLSS_{int}$  and consequently the distance covered was about 14% higher at  $MLSS_{con}$  ( $p < 0.05$ ). The ES showed a moderate effect when compared the TE in both protocols. Furthermore, [La]  $MLSS$  at the end of the corresponding  $MLSS$  test was lower in continuous running compared to intermittent one. Related to the mean [La] found at the end of the TE test was found  $4.31 \text{ mmol}\cdot\text{L}^{-1}$  for continuous and  $4.76 \text{ mmol}\cdot\text{L}^{-1}$  for intermittent.

## Physiological and perceptual parameters

Mean values and standard deviations of distance covered, RPE and physiological parameters at different percentages of TE performed at  $MLSS_{con}$  and  $MLSS_{int}$  are presented in table 2. As demonstrated by ANOVA mixed model, there were no significant interaction for the physiological and perceptual parameters. Significant main effect by time (Table 2) were evident on VE ( $F_{[5,45]} = 36.76$ ,  $P < 0.001$ ), HR ( $F_{[5,45]} = 79.7$ ,  $P < 0.001$ ), RPE ( $F_{[5,45]} = 141.4$ ,  $P < 0.001$ ), and RER ( $F_{[5,45]} = 4.65$ ,  $P < 0.05$ ) but not on  $VO_2$  ( $F_{[5,89]} = 1.2$ ,  $P = 0.30$ ). Significant main effect by model was observed only for VE ( $F_{[1,9]} = 10.23$ ,  $P = 0.01$ ).

Regarding both exercise models, only the total distance covered presented significant difference between the two protocols.

## DISCUSSION

The main finding of the present study was that the time to exhaustion at  $MLSS_{con}$  was longer than TE at  $MLSS_{int}$  ( $68.8 \pm 11.6$  vs.  $59.8 \pm 12.9$  min;  $p = 0.03$ ;  $ES = 0.73$ ) in trained runners. Although these times were very similar with the previously reported,<sup>5,9,14,15</sup> interestingly the study conducted by Grossl et al,<sup>9</sup> using the same work:rest ratio during cycling exercise, found an opposite result concerning the continuous and intermittent time sustained. Grossl et al<sup>9</sup> found a TE about 67 min for intermittent and 55 min for continuous

cycling, representing a difference of 24%. Conversely, our study also found a 15% higher TE, but with the continuous condition being the longer. It still not clear if these opposite results occurred due to the different exercise modes or it was a coincidental finding. However, considering that during running exercise there are more involvement of muscle mass compared to cycling, leading to a higher energy expenditure,<sup>24</sup> this could support the possible difference between them (i.e. cycling and running).

Despite these controversial results, there are consistent data in the literature supporting the higher MLSS intensity (i.e. speed or power output) during intermittent protocols, compared to continuous one. In agreement, the present research found a speed 5% higher at MLSS<sub>int</sub> than at MLSS<sub>con</sub> ( $15.2 \pm 1.0$  vs.  $14.5 \pm 0.8$  km.h<sup>-1</sup>;  $p < 0.05$ ). Previous studies have reported differences about 3% to 4% in swimming,<sup>12</sup> 6% to 10% in cycling,<sup>8,9</sup> and 6% in running.<sup>25</sup> It is important to highlight that the aforementioned studies have used different work:rest ratio to characterize the intermittent protocol in such sports. Hence, different exercise modes and also work:rest ratio could explain the percentage range found among studies.

The 5 min interval-exercise was chosen in the present study, since it has been suggested, to be near the optimal duration for training the aerobic energy system in long-lasting interval sessions.<sup>26</sup> Also it was supported by traditional long interval sessions frequently used by endurance runners, as repetitions of 1200-1600m (ie. around 5 min), depending on the performance level.<sup>27</sup>

During both TE test (continuous and intermittent) the VO<sub>2</sub> and RER remained stable over time, in accordance with previous studies.<sup>9,14</sup> Thus, it can be observed that the pulmonary VO<sub>2</sub> did not rise at this intensity. Regarding the HR response during TE, it can be noted that there is a similar increase in both protocols. This rise could be explained by several

factors, including the increase in circulating norepinephrine concentrations,<sup>28</sup> hyperthermia, dehydration and associated mechanisms to maintain cardiac output.<sup>29</sup>

Related to the perceived exertion, our results showed that RPE increased reaching maximal values at exhaustion, showing that it is sensitive to the exercise duration, as found in previous studies and increases as a linear function of exercise duration.<sup>14,15,30</sup> Thus, the brain, in response to afferent feedback from multiple organic systems, recognizes that exercise is becoming progressively more demanding, even though the workload remains constant.<sup>31</sup>

The MLSS intensity has been used to control training intensity in longitudinal approaches.<sup>5,6</sup> Such studies demonstrated improvement in both submaximal and maximal aerobic (i.e. VO<sub>2</sub>max) parameters. However, there is a lack of information concerning the prescription of continuous and intermittent stimulus at this particular intensity, and consequently the acute and chronic physiological responses. Although the physiological parameters analyzed in the present study did not show differences between protocols, the intermittent trial trended to induce higher metabolic demand, supported by shorter TE found (-20%). It could be important to plan the overload of an interval training sessions at MLSS.

Additionally, the number of repetitions performed during the MLSS<sub>int</sub>, can be a useful tool to determine the volume of endurance interval training sessions. Thus, the present results suggest that about eleven repetitions of 5-min were sustained until exhaustion. Considering that this volume is linked to exhaustion, it is overly long to apply during interval training sessions. Hence, at 60-80% of time to exhaustion seems to be a valuable volume to apply during endurance interval sessions, and the results point out values of 7 to 9 repetitions of 5-min with 1-min of rest. Moreover, it should be noted that this result is limited to training session pattern studied during current study. Then different work:rest ratio could promote different values than the presented here and more studies are needed to address this topic.

Finally the individualization of intensity and duration of the submaximal exercise (both continuous and intermittent) is likely to be a broad and successful procedure to plan a session of endurance training.

### **Practical Applications**

- The present study is the first to show a higher tolerance to exercising during continuous MLSS compared to intermittent (ratio 5:1), thus caution must be taken to prescribe interval training using the continuous MLSS velocity, to avoid a possible underestimation of training load and volume;
- Time to exhaustion and distance covered in both models (continuous and intermittent) can be used as volume reference to establish endurance training sessions;
- The total volume of 14 km (~ 60min) could be considered as the upper limit to prescribe interval training sessions at MLSS (~ 86%  $v\text{VO}_2\text{max}$ ) using a similar 5:1 ratio, at least when applied to well-trained endurance runners;
- Interval training at intermittent maximal lactate steady state might be more efficient to induce a higher metabolic stress, due to greater absolute running velocity (i.e. 15.2  $\text{km}\cdot\text{h}^{-1}$  vs. 14.5  $\text{km}\cdot\text{h}^{-1}$ ).

### **Conclusion**

The results showed that the TE and distance covered at intermittent MLSS is longer than continuous MLSS running, suggesting references to determine the prescription of endurance interval training sessions. Further, the conventional continuous model of MLSS determination should be applied carefully to interval sessions, since the prior underestimate the appropriated MLSS speed to such sessions.

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**Table 1** – Mean  $\pm$  SD values of speed, time to exhaustion, distance covered and blood lactate during both continuous and intermittent maximal lactate steady state.

<b>Variable</b>	<b>Continuous</b>	<b>Intermittent</b>	<b>p</b>	<b>ES</b>
Speed (km.h <sup>-1</sup> )	14.5 $\pm$ 1.0*	15.2 $\pm$ 1.0	p< 0.0001	1.0
TE (min)	68.8 $\pm$ 11.6*	59.8 $\pm$ 12.9	p< 0.0322	0.7
Distance (km)	16.5 $\pm$ 2.8*	14.3 $\pm$ 2.7	P<0.0280	0.8
[La] <sub>MLSS</sub> (mmol·L <sup>-1</sup> )	3.7 $\pm$ 0.9*	4.4 $\pm$ 1.0	P<0.0238	0.8

Note: TE= Time to Exhaustion; [La]<sub>MLSS</sub>= Blood lactate concentration at MLSS; ES= effect size.

\*p<0.05 compared to the intermittent model.

**Table 2** – Distance covered, cardiorespiratory and perceptual responses during percentages of time to exhaustion trials.

Time to exhaustion continuous						
Variable	T <sub>10%</sub>	T <sub>20%</sub>	T <sub>40%</sub>	T <sub>60%</sub>	T <sub>80%</sub>	T <sub>100%</sub>
Distance (km)	1.6 ± 0.3	3.3 ± 0.5	6.6 ± 1.0	9.9 ± 1.6	13.2 ± 2.2	16.5 ± 2.8
VO <sub>2</sub> (mL·kg <sup>-1</sup> ·min <sup>-1</sup> )	51.6 ± 3.8	51.6 ± 4.9	52.2 ± 4.2	52.1 ± 4.4	52.6 ± 4.7	52.5 ± 3.9
VE (L·min <sup>-1</sup> )	108.5±26.0	108.6±10.0	<sup>e</sup> 114.0±9.7	<sup>d</sup> 115.3±9.8	<sup>c</sup> 120.8±13.2	<sup>b</sup> 127.7±17.7
HR (bpm)	159 ± 10.7	<sup>e</sup> 163 ± 9.7	<sup>d</sup> 168 ± 8.9	<sup>c</sup> 172 ± 9.2	<sup>c</sup> 175 ± 9.9	<sup>b</sup> 178 ± 10.6
RER	0.91 ± 0.0	0.93 ± 0.0	0.94 ± 0.0	0.93 ± 0.0	0.93 ± 0.0	<sup>e</sup> 0.95 ± 0.0
RPE	2.6 ± 1.0	<sup>e</sup> 3.3 ± 1.0	<sup>d</sup> 4.6 ± 0.8	<sup>c</sup> 6.1 ± 1.0	<sup>b</sup> 8.1 ± 1.0	<sup>a</sup> 10.5 ± 0.5
Time to exhaustion intermittent						
Variable	T <sub>10%</sub>	T <sub>20%</sub>	T <sub>40%</sub>	T <sub>60%</sub>	T <sub>80%</sub>	T <sub>100%</sub>
Distance (km)	1.4 ± 0.3	2.9 ± 0.5	5.7 ± 1.1	8.6 ± 1.6	11.4 ± 2.2	14.3 ± 2.7
N° of bouts	1.2 ± 0.3	2.4 ± 0.5	4.8 ± 1.0	7.2 ± 1.5	9.6 ± 2.1	12.0 ± 2.6
VO <sub>2</sub> (mL·kg <sup>-1</sup> ·min <sup>-1</sup> )	52.2 ± 5.8	52.9 ± 6.3	53.3 ± 5.7	53.3 ± 4.9	53.6 ± 5.1	53.6 ± 5.5
VE (L·min <sup>-1</sup> )	111.2±13.2	115.7±14.8	<sup>e</sup> 120.4±15.2	<sup>d</sup> 124.0±15.4	<sup>c</sup> 127.2±17.3	<sup>b</sup> 131.5±18.4
HR (bpm)	160 ± 8.9	<sup>e</sup> 165 ± 9.1	<sup>d</sup> 169 ± 8.3	<sup>c</sup> 172 ± 8.5	<sup>c</sup> 175 ± 8.5	<sup>b</sup> 177 ± 9.7
RER	0.91 ± 0.0	0.92 ± 0.0	0.92 ± 0.0	0.92 ± 0.0	0.92 ± 0.0	<sup>e</sup> 0.93 ± 0.0
RPE	3.2 ± 1.4	<sup>e</sup> 3.7 ± 1.5	<sup>d</sup> 4.6 ± 1.6	<sup>c</sup> 6.4 ± 1.4	<sup>b</sup> 7.9 ± 1.3	<sup>a</sup> 10.3 ± 0.7

Note: VO<sub>2</sub>= oxygen uptake; VE= ventilation; HR = heart rate; RER = respiratory exchange ratio, RPE = rate of perceived exertion.

<sup>a</sup>p<0.05 related to t<sub>10%</sub>, t<sub>20%</sub>, t<sub>40%</sub> e t<sub>60%</sub>; t<sub>80%</sub> <sup>b</sup>p<0.05 related to t<sub>10%</sub>, t<sub>20%</sub>, t<sub>40%</sub> e t<sub>60%</sub>; <sup>c</sup>p<0.05 related to t<sub>10%</sub>, t<sub>20%</sub>, t<sub>40%</sub>, <sup>d</sup>p<0.05 related to t<sub>10%</sub>, t<sub>20%</sub>, <sup>e</sup>p<0.05 related to t<sub>10%</sub>.