# Biomarkers of stress in rats exercised in swimming at intensities equal and superior to the maximal estable lactate phase

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## ABSTRACT

**Introduction:** The level of stress during acute/chronic exercise is important, since higher levels of stress may impair animal welfare. The adrenocorticotrophic (ACTH) and corticosterone hormone concentrations, as well as cholesterol and ascorbic acid concentrations in adrenal gland, are considered an important stress biomarker. **Purpose:** To analyze the sensitivity of the different biomarkers during acute swimming exercise in different intensities performed by rats. **Methods:** Male Wistar adult rats (n = 18) previously adapted to swimming were submitted to three 25 min. swimming tests with loads of 5.0; 5.5 and 6.0% of their body weight (BW), for maximum lactate steady state (MLSS) determination. After MLSS attainment, the animals were divided into two groups: M (n = 9) sacrificed shortly after a 25 min. session of exercise at the MLSS intensity or S (n = 9) sacrificed after exhaustive exercise at intensity 25% above MLSS. For comparison purposes, a control group C (n = 10) was sacrificed in rest. **Results:** Serum ACTH and corticosterone concentrations were higher after exercise for the two groups (M and S) when compared with control group C ($P < 0.05$). The group S presented higher concentrations for both hormones in relation to the group M ($P < 0.05$). The concentrations of the cholesterol and ascorbic acid in adrenal were lower after exercise for the two groups (M and S) when compared with control group C ($P < 0.05$). No significant differences in adrenal ascorbic acid and cholesterol levels were observed when the two exercise intensities (M and S) were compared ($P < 0.05$). **Conclusion:** All biomarkers of HPA activity pointed alterations in the stress level of the rats submitted to acute swimming exercise. ACTH and corticosterone serum concentrations showed to be more sensitive to small alterations in the effort intensity.


**Keywords:** Continuous exercise. Effort intensity. Hypothalamus-pituitary-adrenal axis.

## INTRODUCTION

Physical exercise is known to be a stressor stimulus both in humans and animals which leads to countless physiological alterations aiming to provide the augmentation of the energetic demand as well as the reach for a new situation of homeostasis[1-3].

It is known that high intensity exercises result in higher increases in the function of stress hormonal biomarkers which respond to effort as ACTH, cortisol and catecholamines. The augmentation in these hormones activity not only influences metabolism but also the cardiovascular, respiratory, digestive and renal systems[4-5]. Concerning prolonged exercises, there is an augmentation of the hormones which sustain the availability of energetic substrates, including cortisol, the growth hormone (GH), glucagon and catecholamines[2,6-7]. These alterations are important to guarantee good exercise performance.

On the other hand, prolonged periods of high activity of glucocorticoids (in men, mainly cortisol, and in most rodents, corticosterone), may trigger undesirable responses to the body, such as: insulin resistance, suppression of the immune system and of the hypothalamic-hypophysis-adrenal axis (HHA)[3,8-10] as well as increase in the incidence of cardiovascular diseases[11-13].

In the skeletal muscle, the corticosterone acts directly promoting protein degradation, especially in the white muscles rich in glycolytic fibers. Such fast mobilization of amino acids from muscle storages makes them available both as energetic source and synthesis of other compounds[3,14-15]. Such fact, although useful in the preservation of homeostasis, implies in harm for the muscle function. Therefore, acute-chronic exercise in animal models may cause high levels of stress. They may even interfere in the expected training results. This situation shows the relevance for the identification of the animal's stress level during exercise.

The issue on how to define the animal's well-being as well as how to evaluate it is still inconclusive. A potent indicator of wellness is the lack of stress, but the standard stress definition is controversial, and no biochemical marker for its measuring has been found in the literature. The activity of the hypothalamus-hypophysis-adrenal axis (HHA) is directly linked with the body's physiological responses to stress. Once known that the HHA axis is formed by the adrenocorticotrophic hormones (ACTH) and corticosterone, the plasma concentrations of these hormones are considered an important stress indicator[9,16]. It is known that the corticosterone synthesis by the adrenal cortex is derived from the cholesterol molecule in an oxi-reduction process, where the ascorbic acid participates. Therefore, another stress indicator which can be used is the depletion of the cholesterol and ascorbic acid concentrations, in the adrenal gland[17-19].

Although the participation of the previously mentioned biomarkers in the stress response has been well elucidated, until the present moment, there is a lack of investigations concerned about whether the verification of ACTH and corticosterone hormonal responses and the depletion of adrenal ascorbic acid and cholesterol would have the same sensibility to detect discreet alterations in the stress level imposed to rats by acute exercise in swimming at known and distinct intensities.

It is already well-known in humans that the exercise intensity modifies, among other aspects, stress conditions, and this condition results in distinct physiological responses in acute or chronic effort. However, research involving animals which is concerned about precisely determining the different effort intensities during swimming and its specific responses to stress is rare.

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Thinking about the existing gap in the literature concerning evaluation protocols in animals, our research group has been developing methods to precisely identify the individual exercise intensity for rats submitted to efforts in distinct ergometers, adapting tests already applied in humans to animal evaluation[20-21]. Such research however, left the specific stress responses concerning different effort intensities unanswered.

The gold standard protocol for the identification of the metabolic aerobic/anaerobic transition during exercise both for humans and animals is the Maximal Lactate Steady State (MLSS)[20-23]. The MLSS represents the highest intensity in which blood lactate stabilization occurs during continuous exercise, due to the balance between lactate production and its removal from the blood stream[20-21,23-24].

Considering the importance in evaluating the stress of animals submitted to exercise as well as the possible effects of the intensity in these responses, the present study had two aims: 1- to analyze the activity of the HHA axis in different intensities; 2- to analyze which biomarker (plasma ACTH and corticosterone concentrations or ascorbic acid cholesterol concentrations in the adrenal glands) more sensitive to more exactly measure the stress level of rats during acute swimming exercise in different effort intensities. The intensities used in the present study were: MLSS, interpreted as the maximal intensity with aerobic predominance and load of 25% higher than it, characteristically considered anaerobic.

## METHODS

### Animals

Male Wistar (*Rattus Norvegicus albinus*) rats (n = 28) with 60 days of age in the beginning of the experiment were used. The animals from the Central Animal Facility of the UNESP – Botucatu, were kept in the Animal Facility of the Biodynamics Laboratory of the Physical Education Department of the UNESP – Rio Claro Campus, in collective cages (five rats per cage), at 25 ± 1°C and clear (beginning at 7:00hs)/dark (beginning at 19:00hs) cycle of a 12/12 hours photoperiod, and with free access to water and food (balanced standard food, Purina). All experiments with animals were performed according to the Brazilian specific resolutions about Bioethics in Experiments with Animals (Law # 6,638, from May 08, 1979 and Resolution # 24,645 from July 10, 1934).

### Outlining and experimental groups

The animals were distributed in three groups, according to their sacrifice condition (acute exercise intensity) in:

- ***Maximal Lactate Steady State in Swimming (M):*** constituted of rats (n = 9) which were randomly selected after being adapted to swimming and performed the individual test of maximal lactate steady state. The animals from this group were sacrificed immediately after 25 minutes of continuous exercise at maximal lactate steady state intensity.
- ***Intensity Higher than the Lactate Steady State in Swimming (S):*** constituted by rats (n = 9) which were randomly selected after being adapted to swimming and having performed the individual test of maximal lactate steady state. The animals from this group were sacrificed immediately after exhaustive exercise at intensity 25% higher than the maximal lactate steady state.
- ***Control (C):*** composed of rats (n = 10) which were adapted to the liquid medium and sacrificed at rest.

### Adaptation to the swimming exercise

The adaptation was performed in a 120 cm deep x 80 cm wide cylindrical tank, with water temperature of 31 ± 1°C. It consisted of daily swimming efforts, five days/week, during three weeks, with overload of 0; 1 and 2% of body weight and duration of 1-5 min. The aim of the adaptation was to familiarize the rat to the liquid medium, to the increase attached to the torso and the exercise itself, with no promotion of physiological alterations concerning the physical training[20].

### Maximal lactate steady state (MLSS) in swimming

After having been adapted to the exercise, each animal (n = 18) was submitted to three exercise tests with intensities equivalent to overloads (plumb weight attached to the animal's torso) of 5.0; 5.5 and 6.0% of the body weight (BW). The tests were performed with 48 hours of interval between them; with the intensities sequence (overloads) being randomly distributed. Each test consisted of 25 continuous minutes of swimming in the intensity established or until exhaustion, being performed by all the eighteen animals. Blood samples were collected through a small section on the extremity of the tail of the animals at rest and at every five minutes of exercise during the test. Blood collection was performed in 30 s, in order to avoid animal's removal from water for prolonged time, causing additional stress, interfering thus in the exercise response. Lactacidemia during the test was considered steady when there was no difference higher than 1 mmol/L from the 10 to 25 minutes of exercise.

### Analysis of blood lactate

The blood samples (25 μL) were collected during the tests and placed in eppendorf tubes (1,5 mL) containing 50 μl of sodium fluoride (1%). They were later stored in a freezer for analysis of the lactate concentration (YSI model 1500 SPORT).

### Animals sacrifice

48 hours after the last MLSS test, the animals were sacrificed by decapitation at rest condition or immediately after a 25 minute-acute or exhaustive exercise session (depending on the group they belong to), with no previous fasting, for blood collection. The serum was separated by centrifugation, aiming immediate dosings of adrenocorticotrophic hormone – ACTH (Coat-A-Count kit by Diagnostic Products Corporation – DPC), corticosterone hormone specific for rats (Coat-A-Count kit by Diagnostic Products Corporation – DPC) and for removal of the adrenal glands, for immediate ascorbic acid (left adrenal)[25] and cholesterol dosings (right adrenal), (Labtest kit).

### Statistical analysis

The statistical procedures included one-way variance analysis (ANOVA) for independent samples, followed by the Newman-Keuls post-hoc test whenever appropriate. The significance level adopted was of $P < 0.05$.

## RESULTS

Figure 1 presents the results of the tests for identification of the maximal lactate steady state (MLSS) during swimming. During

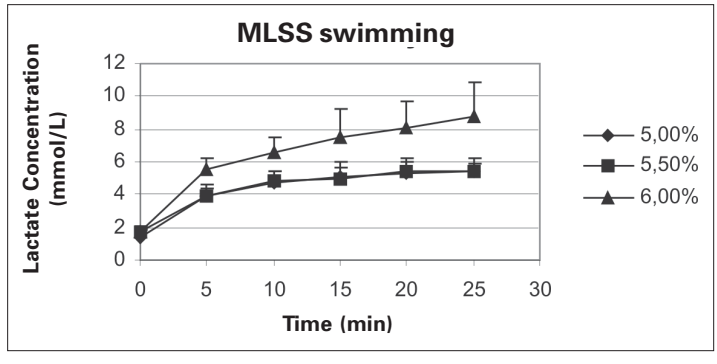


***Figure 1 –*** *Blood lactate concentration (mean ± SD) during identification test of maximal lactate steady state (MLSS) in swimming*

continuous efforts with overloads of 5.0 and 5.5% of body weight (BW), stabilization of the blood lactate concentration from 10 to 25 minutes of exercise, in the mean values of 5.1 ± 0.3 and 5.2 ± 0.3 mmol/L, respectively was observed. In the 6.% of body weight overload there was progressive increase of blood lactate concentration. Thus, the MLSS was obtained in the 5.5% of BW overload.

The results shown in figure 2 are concerning plasma concentrations of the adrenocorticotrophic (ACTH) (A) and corticosterone (B) hormones after animals' sacrifice. The plasma concentration of ACTH found in the groups submitted to swimming (M = 963.37 ± 420.47 and S = 1284.44 ± 361.36 pg/mL) were higher when compared with the concentrations of the animals of the control group (C = 179.32 ± 46.31 pg/mL). When the groups submitted to swimming were compared, the animals from group S had higher plasma ACTH values compared with the values of the animals from group M. Concerning the corticosterone hormone, group S (3845.51 ± 788.8 ng/mL) showed higher concentrations compared with the remaining groups (C = 467.11 ± 262.12 and M = 2661.26 ± 627.89 mg/mL). In addition, group M showed higher values when compared with the values of the control group C.

Data concerning ascorbic acid and cholesterol of adrenal gland of the different groups after sacrifice are found in figures 3 (A) and (B), respectively. The analysis of the results identified higher concentrations of ascorbic acid in the control group (C = 2.54 ± 0.53 µg/mg) when compared with the concentrations of the groups submitted to swimming (M = 1.32 ± 0.27 and S = 1.28 ± 0.46 µg/mg). The results of the cholesterol concentration of the adrenal gland

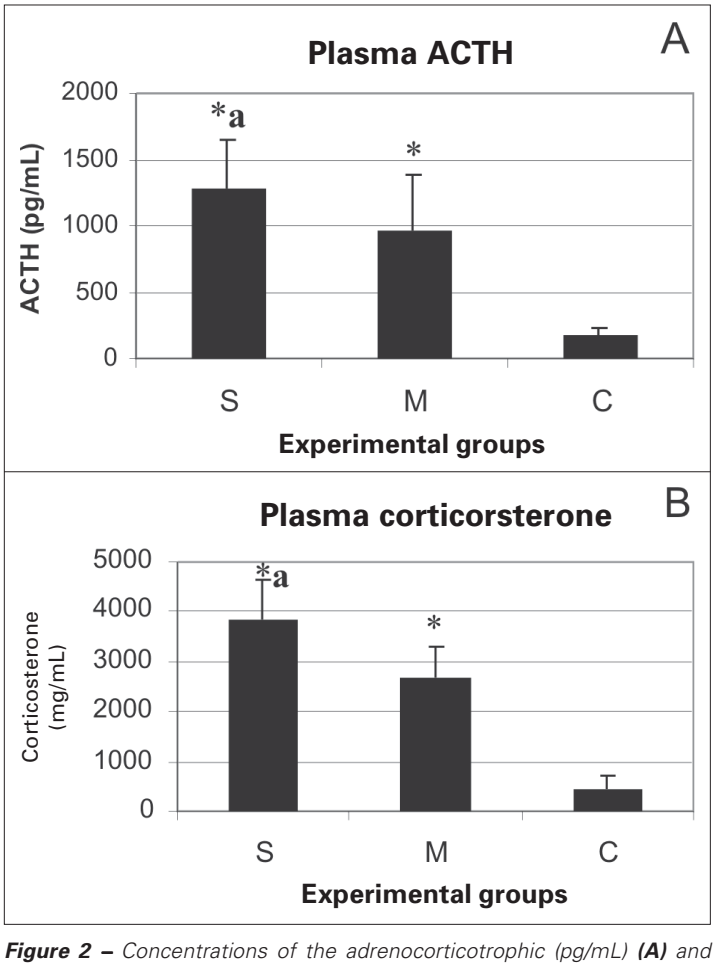


***Figure 2 –*** *Concentrations of the adrenocorticotrophic (pg/mL)* ***(A)*** *and corticosterone hormones (ng/mL)* ***(b)*** *(mean ± SD) of the animals at the end of the experiment at rest and after exercise session at intensity equivalent to the maximal lactate steady state (MLSS) and 25% higher than this in swimming. M = maximal lactate steady state-MLSS; S = 25% higher than the MLSS and C = control. *, P < 0.05 difference in relation to the control group; the P < 0.05 difference in relation to the M group.*

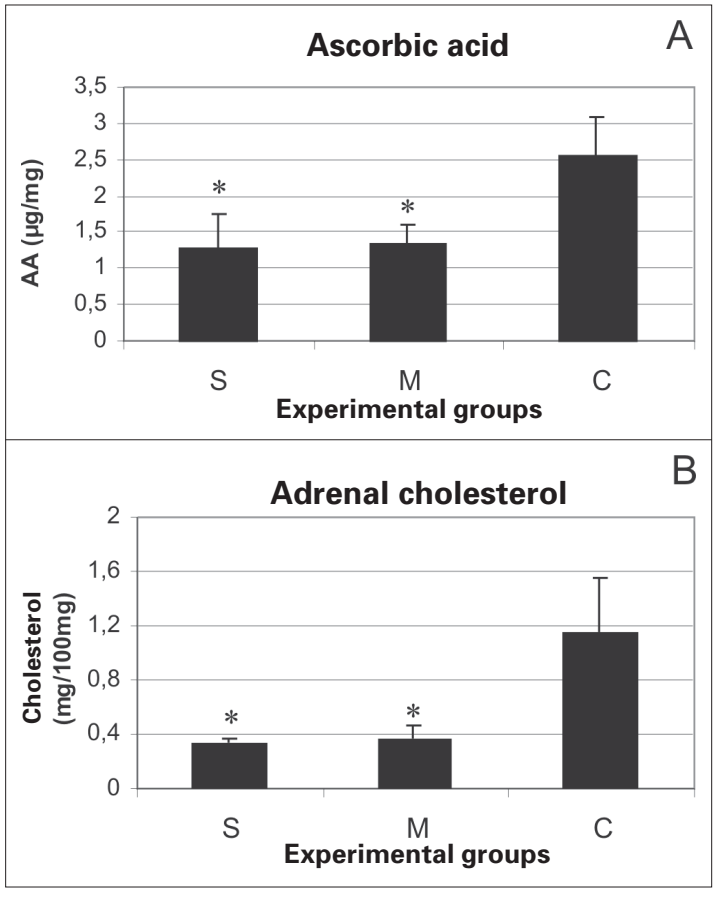


***Figure 3 –*** *Ascorbic acid (µg/mg)* ***(A)*** *and cholesterol of the adrenal gland (mg/100mg)* ***(B)*** *of the animals at the end of experiment at rest and after 25 minutes of exercise at intensity equivalent to the maximal lactate steady state (MLSS) and 25% higher than it in swimming. M = MLSS; S = 25% higher than the MLSS and C = control. *, P < 0.05 significant difference in relation to the control group.*

presented similar pattern to the one found for the ascorbic acid concentration: the control group (C = 1.15 ± 0.4 mg/100mg) presented higher values compared with both groups submitted to swimming (M = 0.37 ± 0.09 and S = 0.33 ± 0.04 mg/100mg). No difference was found in the ascorbic acid and cholesterol of the adrenal gland concentrations when the two exercise intensities were compared. (M and S).

## DISCUSSION

Exercising causes increase or reduction in blood concentrations of some hormones in relation to rest concentrations, so that the body is able to provide greater energetic demand as well as to keep homeostasis[7,26]. Very frequently, the increases or reductions directly reflect in adjustments in the hormonal secretion rhythm on behalf of an endocrinal gland[4,27].

A large number of studies report that submaximal exercises result in augmentation in stress hormones which belong to the HHA-ACTH-corticosterone axis, being the latter secreted by the adrenal gland[2,4]. During the corticosterone synthesis by the adrenal cortex, biochemical alterations, such as depletion of the ascorbic acid and cholesterol concentrations, occur in these glands[18,28-29]. This is an essential factor for the successful development of research involving animals and physical exercise is their wellness. Moreover, the lack of stress is a pre-requisite for this situation. Therefore, the present study had the aim to analyze the HHA axis activity and to verify which biomarker (hormonal plasma concentrations – ACTH and corticosterone – or biochemical concentrations of the adrenal glands – ascorbic acid and cholesterol) is more sensitive in

order to more accurately measure the stress level of rats during acute swimming exercise in distinct and known effort intensities: MLSS and 25% higher than it.

The gold standard procedure for the identification of the aerobic/anaerobic metabolic transition during exercising is the Maximal Lactate Steady State (MLSS), which represents the higher exercise intensity in which a balance between the release and removal of lactate from the blood stream occurs[24]. In previous studies our group demonstrated to be possible to determine the MLSS in rats[20-21].

The MLSS was found in the 5.2 ± 0.3 mmol/L concentration of blood lactate, at the intensity of 5.5% of body weight (BW); these results are similar to the ones obtained by Gobatto *et al.*[20] (2001), who have found the MLSS of sedentary rats in the mean concentration of 5.5 mmol/L of blood lactate at the intensity of 5-6% of BW. Nonetheless, these values are different from the ones found by Manchado *et al.*[21] (2005), who used the same principle of exercise with continuous loads in running exercise on treadmill. In running, the MLSS in the 20 m/min velocity in lactate concentration was of 3.9 ± 0.3 mmol/L. The difference in the blood concentrations of lactate observed between the present study and the research by Manchado *et al.*[21] (2005) may be attributed to the kind of exercise applied, highlighting the ergometer dependence of the maximal lactate steady state test applied to rats, as well as the one observed in humans.

After the MLSS, the animals were submitted to a 25-minute exercise session in the MLSS intensity or exhaustive exercise session in the intensity 25% higher than the MLSS, in the trial to explicit that subtle alterations in the load attached to the rats' torso may imply in distinct stress-related physiological responses.

Although it has been well-established for decades in the literature that the increase in the HHA axis activity of humans during exercising is proportional to its intensity[30-33], studies with animal models which are concerned about accurate identification of the effort intensity used in acute/chronic exercise as well as its consequent response in the HHA axis are scarce. Moreover, until the present date, there is a lack of works in the literature which associate the sensibility of different stress biomarkers during acute swimming exercise for rats in distinct intensities.

The performance of swimming exercise in both intensities (MLSS and 25% higher) implied in increases of ACTH and corticosterone concentrations compared with animals at rest. These results were similar to the ones reported by Oliveira *et al.*[34] (2004) in studies about the acute effect of swimming exercise in rats, in the HHA axis activity. In research conducted with rats exercised on treadmill, Kawashima *et al.*[35] (2004) found similar results, such as increase in the concentrations of the corticosterone and ACTH hormones. It is worth mentioning that in both studies there was no concern about the accurate identification of the effort intensity of the animals.

When animals or humans are exposed to potentially harmful stimuli (exercise, cold, sleep deprivation, immobilization, among others) an increase of the HHA axis activity occurs, with ACTH secretion and corresponding increase of the circulating concentration of glucocorticoids[4,36-37]. These increases are considered the frontline of the endocrine mechanisms for defending the body against stress conditions, especially increasing the demand of energetic substrates[9]. The glucocorticoids are able to stimulate the glucogenesis by the liver, directly acting in the phosphoenolpyruvate carboxykinase enzyme (PEPCK) or indirectly through the augmented sensibility of the hormones responsible for the hepatic glycolysis, adrenaline and glucagon[38]. However, excessive HHA axis activation and consequent increases in the plasma concentrations of corticosterone, may lead to homeostasis rupture and undesirable effects to the body[3,34].

Glucocorticoids are hormones of antagonist action to insulin, hampering thus, the anabolic action of this hormone. The insulin-resistance caused by the excess of plasmatic glucocorticoids cannot only trigger diabetes mellitus type 2 but also promote the protein catabolism of muscular fibers[14-15].

When the hormonal concentrations are compared in the two selected intensities (MLSS x 25% higher than it), more severe increases were observed in the concentrations of ACTH and corticosterone in intensity 25% higher than MLSS. According to Soya[34] (2001), the activity of the HHA axis during acute exercise depends on its intensity, producing greater activity in high intensities. In exercises performed by humans with a maximal consumption of $O_2$ higher than 60% of the $\dot{V}O_{2\,max}$, the ACTH and cortisol secretion is proportional to the exercise intensity[40]. Considering this aspect, the ACTH and corticosterone results show that the HHA axis activity in acute swimming exercise in rats is similar to the one in humans, with higher concentrations in higher intensities.

The cholesterol and ascorbic acid concentrations in the animals' adrenal after the acute swimming exercise performance were also evaluated. Ascorbic acid values, as well as cholesterol in the adrenal gland, were lower in the animals submitted to swimming compared with the ones at rest. These outcomes were similar to the ones found by Kelliher *et al.*[41] (2000) in a study with animals exercised by swimming. No difference was found in the two intensities (MLSS x 25% higher than it), when the cholesterol and ascorbic acid concentrations in the adrenal were compared.

All human steroids as well as the ones from rats are synthesized from the cholesterol molecule. The cholesterol used in the steroids synthesis may be synthesized by cortex cells through the acetate; however, 80% are derived from the low density lipoproteins (LDL) of the plasma[42]. Therefore, it is possible that the higher synthesis of corticosterone found in the group 25% higher than the MLSS had been supported by the plasma cholesterol (LDL).

It is clear that ascorbic acid is considered a bold physiological reducer and is highly concentrated in the adrenal glands. In the adrenal cortex, the augmentation of the corticosterone synthesis after stress or exogenous administration of ACTH, is usually associated with a decrease in the ascorbate concentration[18]. Nevertheless, its participation in the steroidogenesis is not totally elucidated. In studies conducted with mutant rats unable to synthesize ascorbic acid, Mitani *et al.*[43,44] (2004) and (2005), found alterations in the plasma aldosterone concentrations, with no changes in the corticosterone concentration when the demand of ascorbic acid was reduced. On the other hand, Bornstein *et al.*[45] (2003) and Patak *et al.*[46] (2004), found reductions in the plasma concentration of corticosterone in research conducted with mutant rats with absence of ascorbic acid in the membrane transporter (SVCT2).

Thus, the ascorbic acid and cholesterol concentrations in the adrenal gland presented decreases after acute swimming exercise. Nevertheless, they were not sensitive to alterations concerning intensity.

In sum, the biomarkers analyzed (ACTH and corticosterone plasma concentrations and ascorbic acid and cholesterol concentrations in the adrenal gland) showed changes in the stress level of rats submitted to acute swimming exercise at intensity of MLSS and 25% higher. The plasma hormonal concentrations were more sensitive to little changes in the exercise intensity. Therefore, these biomarkers seem to be more suitable to more accurately interfere in the stress levels of rats exercised in swimming. Yet, further studies are needed in order to relate the role of ascorbic acid from the adrenal in the steroidogenesis.

Once the activity of the HHA axis of rats showed a straight relationship with exercise intensity, our results showed the need for caution in the prescription of physical exercise for rats, since little changes in effort intensity, especially above the aerobic/anaerobic metabolic transition, lead to modifications in the hormonal stress biomarkers, which as known, may influence in the physiological responses specific to exercise. In order to accurately determine the effort load according to the expected aims, we suggest the

use of a maximal lactate steady state protocol, which is considered the gold standard for such determination.


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