

Changes in the Salivary Biomarkers Induced by an Effort Test

Authors

V. N. de Oliveira¹, A. Bessa¹, R. P. M. S. Lamounier¹, M. G. de Santana², M. T. de Mello², F. S. Espindola¹

Affiliations

¹Universidade Federal de Uberlândia, Instituto de Genética e Bioquímica, Uberlândia, Brazil

²Universidade Federal de São Paulo, Departamento de Psicobiologia, São Paulo, Brazil

Key words

- exercise
- lactate
- salivary alpha-amylase
- protein
- anaerobic threshold
- electrophoresis

Abstract

Physical exercise induces biochemical changes in the body that modify analytes in blood and saliva among other body fluids. This study analyzed the effect of an incremental effort test on the salivary protein profile to determine whether any specific protein is altered in response to such stress. We also measured thresholds of salivary alpha amylase, total salivary protein and blood lactate and searched for correlations among them. Twelve male cyclists underwent a progressive test in which blood and saliva samples were collected simultaneously at each stage. The salivary total protein profile revealed that physical exercise primarily affects the polypeptide corresponding to salivary alpha-amylase, the concentration of

which increased markedly during the test. We observed thresholds of salivary alpha-amylase (sAAT), total salivary protein (PAT) and blood lactate (BLT) in 58%, 83% and 100% of our sample, respectively. Pearson's correlation indicates a strong and significant association between sAAT and BLT ($r = 0.84$, $p < 0.05$), sAAT and PAT ($r = 0.83$, $p < 0.05$) and BLT and PAT ($r = 0.90$, $p < 0.05$). The increased expression of the salivary alpha-amylase (sAA) polypeptide suggests that sAA is the main protein responsible for the increase in total protein concentration of whole saliva. Therefore, monitoring total protein concentration is an efficient tool and an alternative noninvasive biochemical method for determining exercise intensity.

accepted after revision
January 25, 2010

Bibliography

DOI <http://dx.doi.org/10.1055/s-0030-1248332>
Published online:
March 18, 2010
Int J Sports Med 2010; 31:
377–381 © Georg Thieme
Verlag KG Stuttgart · New York
ISSN 0172-4622

Correspondence

Dr. Foued Salmen Espindola
Universidade Federal de
Uberlândia
Instituto de Genética e
Bioquímica
Av. Para 1720
38400982 Uberlândia
Brazil
Tel.: +34/32182477
Fax: +34/32182203
fouedespindola@gmail.com

Introduction

Traditional biochemical approaches for studying individual proteins have provided structures and functions of a number of major salivary proteins. However, many salivary proteins and their functions remain uncharacterized [17]. Physical exercise induces biochemical changes in the body, which modify blood and saliva analytes among other body fluids [2, 10, 18]. Based on these observations, analyzing changes in the salivary protein profile may help to identify novel biomarkers for work load, recovery and injury.

Measuring salivary analytes, such as total protein, alpha-amylase, electrolytes, lactate, cortisol and catecholamines may represent a noninvasive method to determine the relationship between intensity of exercise and the blood lactate threshold (BLT) [4, 6, 9]. The BLT is characterized by the transition from a linear to an exponential increase in blood lactate concentration, and its measurement has been of great use in both experimental and routine studies of physical performance

[21, 30]. Previous studies have shown that a salivary threshold exists beyond which a continuous increase in these analytes may serve as a salivary biomarker of exercise intensity [4, 6, 9].

Activity of the sympathetic nervous system increases progressively with intensity of exercise [32], altering some salivary components [3, 10, 38]. Bortolini et al. [4] investigated whether the total protein concentration of whole saliva (TPWS) reflects the anaerobic threshold during an incremental exercise test, and they observed a profound correlation between the total salivary protein threshold (PAT) and the BLT. Analysis of salivary proteins in rat parotid saliva revealed that the protein content in the parotid is markedly influenced by the type of stimulation (sympathetic or parasympathetic) used to induce secretion [1].

It has been proposed that salivary alpha-amylase (sAA) activity is regulated by the sympathetic-adrenal medullary (SAM) system through the action of norepinephrine on the salivary glands [8, 34, 40]. Activity of sAA increases in response

to exercise on a treadmill [12], running [24,33] and cycling [8,37]. Furthermore, sAA assessment has been proposed as a useful tool for evaluating psychological stress [13,25,35,36]. Under exercise-induced stress, sAA activity increases faster than blood cortisol levels, and it declines rapidly after removal of the stress factor [22]. Despite widespread studies of alterations in cortisol levels under different stress conditions, this and recent studies have indicated sAA activity as a marker of exercise-induced stress [8,39].

The goal of this study was to analyze the effect of an incremental effort test on the salivary protein profile and to determine whether any specific protein was altered in response to such stress. We also measured salivary alpha-amylase threshold (sAAT), PAT and BLT and searched for correlations among them.

Methods



Subjects

Twelve national-contending Caucasian male cyclists participated in the study. The average (\pm SD) age, height and mass of the participants was 22.62 ± 3.51 years, 1.78 ± 0.21 m and 71.80 ± 5.76 kg, respectively. Blood biochemical analyses had been previously carried out to obtain a baseline health status of the participants. None of the athletes was using pharmaceuticals, anabolic steroids or tobacco at the time of the study.

The subjects, familiar with the laboratory conditions and testing procedures, were asked to limit their physical activity and abstain from stimulating (coffee, guarana, etc.) or dye-containing substances for 24 h before the test. Participants were asked to drink about two liters of water on the day preceding the test to avoid dehydration during the day of the test. They were also advised to have their last meal at least two hours before the test procedures.

Participants signed a consent form to indicate their awareness of all procedures that would be carried out during and after the test. The experimental protocol began only after it was submitted to and approved by the Ethical Human Research Committee (decision no. 140/04) at the Federal University of Uberlandia, Brazil. The study meets the ethical standards of the IJSM [15].

Procedures

The tests were carried out on a cycle-ergometer (ERGO-FIT 167, Pirmasens, Germany) between 15:00 and 17:00 h at a room temperature maintained between 24 and 26 °C. Participants engaged in stretching exercises and a brief 2 min warm-up on the cycle-ergometer with no load before the test started. Heart rate was measured continually with a cardiac monitor (Polar Electro Oy, Kempele, Finland). The test began with a 50 W load, which was increased 25 W every two minutes up to exhaustion. The pedal rotation frequency was kept between 68 and 75 rpm. Saliva was stimulated by chewing gum and collected by the spit method [23]. Participants were required to perform oral asepsis before the test procedures to avoid contamination of the samples with cellular debris and other materials. Subjects drank 400 mL of water before the test and were not allowed to drink more water during the test. Saliva was collected immediately before the exercise (at rest), at the end of every stage, at which time the exercise load was increased, and in the 5th and 15th minutes after voluntary exercise interruption (upon exhaustion). Saliva samples were placed in pre-cooled (4 °C) mini-tubes and kept on ice until centrifuged at 12 000 g (4 °C). Sediment was

discarded and the supernatant frozen at -20°C until the day of analysis. All analyses were carried out in duplicate. The supernatant was probed for TPWS content by the Coomassie Brilliant Blue-R 250 method [5] and sAA activity was measured by the kinetic method at 405 nm, using as substrate 2-chloro-p-nitrophenyl- α -D-maltotrioxide (CNP-G3) following the manufacturer's protocol (Amylase 405, liquid line, Wiener Lab, Argentina). Unidimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as previously described [20] using minigels with a 5–22% polyacrylamide gradient. The same amount of protein (3 μg) was introduced into each lane of the gel. Identification of the sAA polypeptide was based on its migration within the gel in relation to polypeptides of known molecular mass (46–60 kDa), according to [29]. The sAA polypeptide was also visualized by western blot immunodetection. The relative intensity of sAA polypeptide in each stage of the progressive test was quantified by gel densitometric analysis using the Image Master VDS version 2.0 software, and the results were expressed as the integrated optical density (IOD).

For the immunodetection of sAA by western blotting, saliva samples were electrophoretically transferred to a nitrocellulose filter and probed with polyclonal antibody to human sAA (Sigma-Aldrich, St. Louis, USA) [34]. The immunoreactive polypeptide was revealed using the chromogenic NBT/BCIP assay. The broad range protein molecular weight standard consisted of a marker with nine clearly identifiable bands (Promega, Madison, USA). Capillary blood (25 μL) obtained from the earlobe was collected in mini-tubes containing 50 μL of 1% NaCl, stored on ice for < 3 h, and frozen at -20°C until analysis. Blood lactate (BL) was analyzed using an electro-enzymatic method (Lactate Analyzer YSI 1500 Sport, Yellow Springs, USA). A computerized automated method based on a bisegmented regression model was used to determine thresholds [16]. The model allowed us to identify thresholds of all variables examined in this study.

Statistical analysis

Normality among BL, TPWS concentrations and sAA for each parameter were calculated by the Kolmogorov test. All data are expressed as the mean \pm SD. Statistical differences were determined using One-way ANOVA and Tukey's post-hoc test. Correlation analysis was performed by Pearson's method. For all analyses, $P < 0.05$ was considered to be statistically significant.

Results



Physical exercise altered the 1D electrophoresis profile of salivary total protein mainly by increasing the concentration of the polypeptide corresponding to sAA (◉ Fig. 1a). This response was observed in all samples of saliva collected despite subject-to-subject variations present in salivary protein profiles. Immunoblots of total saliva probed with human anti-alpha-amylase identified a polypeptide with a molecular mass of approximately 51 kDa corresponding to sAA (◉ Fig. 1b). The intensity of the stained protein band increased with each increment of exercise load, suggesting increased secretion of the polypeptide. The intensities of the sAA bands observed after each stage of the test were quantified by densitometric analysis (◉ Fig. 1c), revealing differences ($p < 0.05$) between the intensity of alpha-amylase collected immediately before and during the last stage of exercise (exhaustion), as well as five and fifteen minutes after its conclusion.

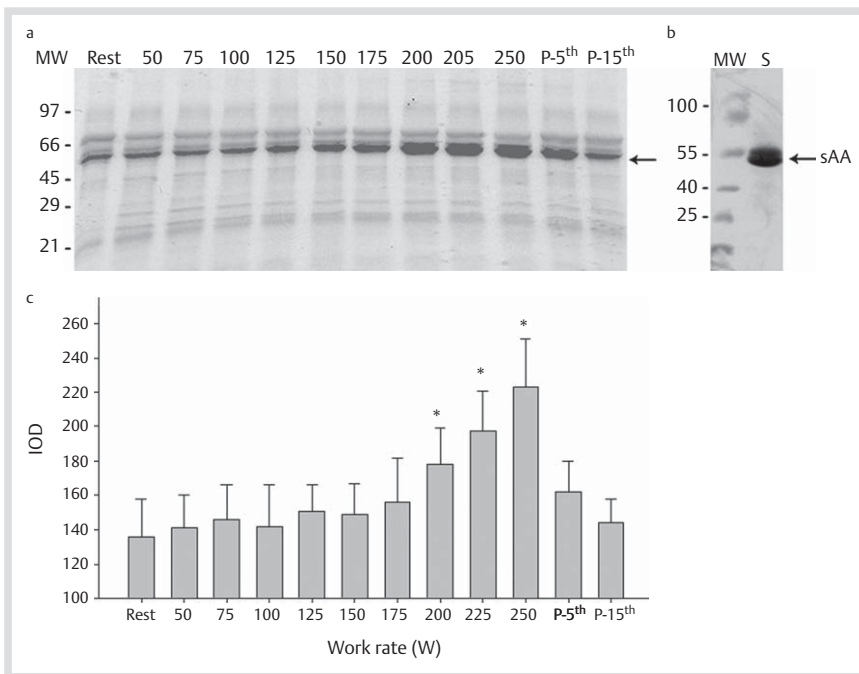


Fig. 1 Electrophoresis pattern of total saliva protein during incremental exercise, immunodetection and densitometric analysis of human salivary alpha-amylase. **(a)** Digitized image of gel stained with Coomassie Blue-R 250 containing 3 μ g of salivary total protein per lane, corresponding to each stage of the exercise in watts (Rest, 50, 75, etc.). P5 and P15 represent samples collected during the 5th and 15th minute post-exercise. Arrow indicates alpha amylase. MW indicates the protein standards used, and their respective relative molecular masses are indicated by the numbers to the left of the gel; **(b)** Immunoblotting of total saliva probed with anti-human salivary alpha-amylase (sAA) antibody; the strongest staining polypeptide in the gel electrophoresis as alpha-amylase; **(c)** Increase in the mean values and standard deviations (\pm SD) ($n = 8$) of the densitometric analysis of salivary alpha-amylase concentration, expressed in watts (W), at each stage of exercise and post-exercise (P5 and P15). (*) indicates difference from rest ($p < 0.05$).

We observed sAAT, PAT and BLT in 58%, 83% and 100% of our sample, respectively. Pearson's correlation indicates a strong and significant association among these variables during the incremental effort test (• Fig. 2). The average duration of the entire test was twenty-four minutes.

Analysis of TPWS and BL concentrations, as well as sAA activity, revealed that all these parameters increased from the resting state to the point of exhaustion and then decreased after the end of the effort test. A one-way ANOVA test revealed significant differences at the moment of exhaustion in relation to resting conditions, exhaustion in relation to post-exercise and post-exercise in relation to resting conditions for TPWS and BL concentrations ($p < 0.01$) and for sAA activity ($p < 0.05$). It should be noted that TPWS, BL and sAA values declined significantly 15 min after the end of the effort test (• Fig. 3).

Discussion

The salivary protein profile changed during the effort test, with a notable increase in the concentration of alpha amylase polypeptide. The PAT, sAAT and BLT were also determined and found to be correlated. After the end of the test, all biochemical parameters returned to resting levels within 15 min, except for blood lactate, which remained at an intermediate level between rest and exhaustion.

The electrophoretic profile of salivary proteins revealed a significant increase in the concentration of alpha-amylase during the incremental effort test. A possible mechanism for such an increase is the release of sAA stored in membrane-bound secretory granules via exercise-induced activation of the beta receptor [7], which can increase sympathetic activity and alter salivary gland secretion [30]. It is unlikely that an increase in *de novo* synthesis of alpha amylase could account for this boost in sAA levels as there would be insufficient time for biosynthesis and secretion of the enzyme. Other authors have reported that increases in salivary protein concentration are associated with dehydration [38] and parasympa-

thetic withdrawal leading to decreased salivary flow [14]. Our data from the electrophoretic profile, which was carried out using samples of similar protein concentrations instead of equivalent volumes, indicates that alterations in TPWS caused by exercise are directly related to alpha-amylase since other salivary proteins were not altered. A previous study showed the relative independence of sAA secretion from changes in saliva flow rate [26], thus eliminating the influence of dehydration, evaporative loss of salivary water and parasympathetic withdrawal in our results.

A possible biological role for this response is that, during exercise, sAA activity may provide a protective effect against infection, since this enzyme has been shown to inhibit bacterial attachment to oral surfaces [27]. This may be a compensatory response to the lowering of immunoglobulin A (IgA) after intense exercise as previously reported [11], although we did not observe alterations in the corresponding molecular weight band to IgA. In agreement with our results, it has been suggested [4,6,10] that alpha-amylase in saliva strongly correlates with lactate threshold. We also observed a correlation between BLT and PAT. These changes in salivary total protein concentration might have been elicited in response to sympathetic activation, and we speculate that they also affect blood lactate concentration, which may explain the correlation between salivary and lactate thresholds [21]. Previous work measured lactate in saliva [28] as an alternative to its measurement in blood [31], but one disadvantage of this technique is that oral mucosa is bacteria-rich, and once exposed to air, bacteria produce lactate because of the inactivation of pyruvate formate-lyase, which catalyzes the first step of pyruvate conversion to formate, acetate and ethanol [19]. Such a scenario could render this method inaccurate for determining exercise-induced lactate production.

It is clear that physical exercise induces acute alterations in sAA activity, TPWS and BL concentrations. These parameters increased considerably from the resting state to the last stage of exercise then returned rapidly to pre-test levels 15 min after conclusion of the test.

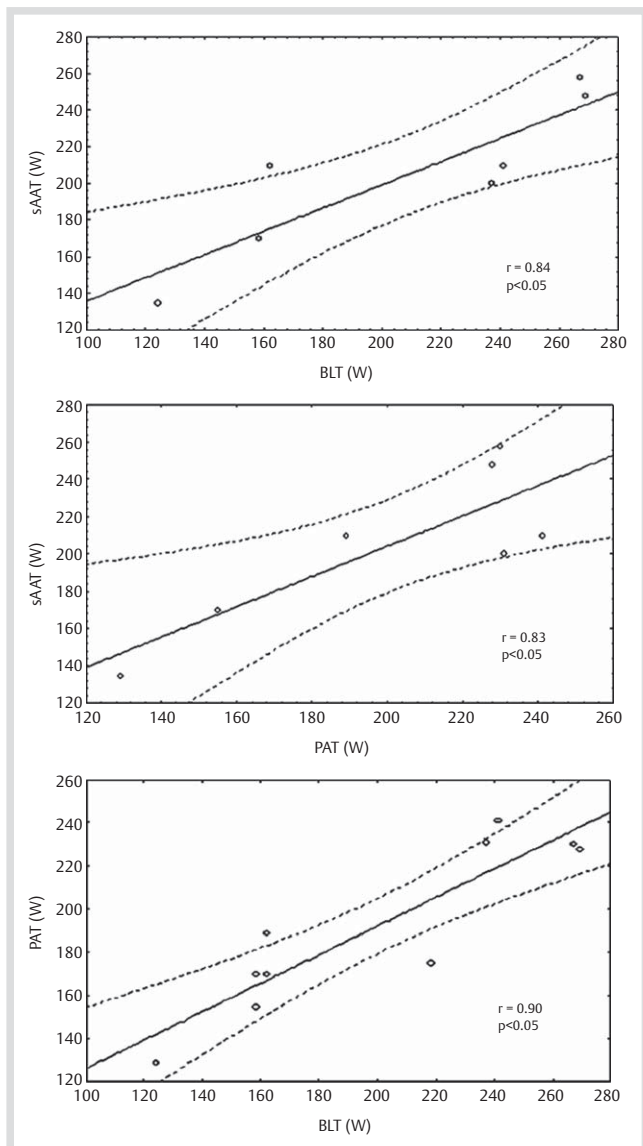


Fig. 2 Correlation between salivary and lactate thresholds. Relationship of the blood lactate (BLT), total salivary protein (PAT) and amylase (sAAT) thresholds expressed in watts. Upper and lower lines represent the confidence level (95%) for the values predicted by the regression equation (central line), while (r) represents the coefficient of linear correlation between the analyzed variables.

Conclusion

Our findings suggest that BL, TPWS and sAA were altered due to exercise in a manner directly correlated to exercise load. The saliva as a potential source of exercise noninvasive biomarkers offers less of a biohazard to both donor and collector than blood. Increased expression of sAA polypeptide during the incremental effort test suggests that sAA is the main protein responsible for the increase in TPWS concentration. Due to its relative simplicity, monitoring TPWS is an efficient method for determining exercise intensity when compared to measuring sAA activity. Further research is needed to evaluate whether such salivary biomarkers would have a shift in their curve in chronic response to physical exercise.

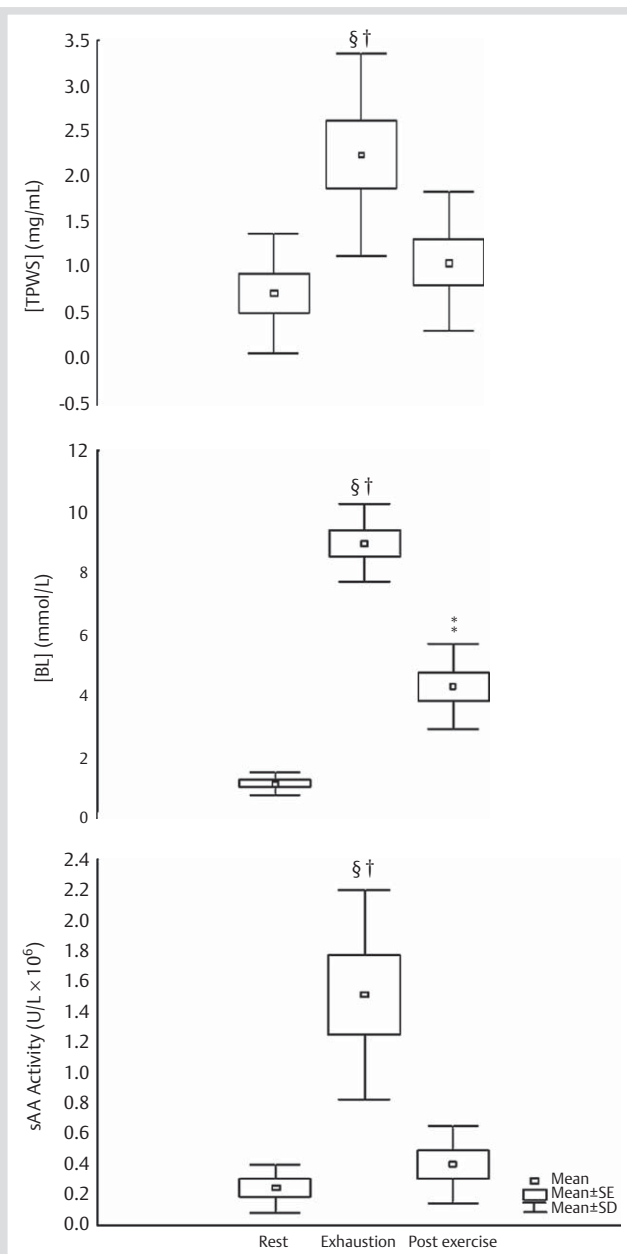


Fig. 3 Exercise intensity markers before the test, during exhaustion and after the test. Alterations in blood lactate (BL) concentrations, total protein concentration of whole saliva (TPWS) and salivary alpha-amylase (sAA) activity during rest, exhaustion and after the test. Plots display absolute values of the mean (\pm standard deviation) of blood lactate (BL) and salivary total protein (TPWS) concentrations as well as salivary alpha-amylase activity (sAA) at the moments of rest, exhaustion and 15 min after the last stage (post-exercise). (\dagger) Indicates $p < 0.05$ vs. exhaustion and rest. (\ddagger) Indicates $p < 0.05$ vs. 15' post-exercise and rest. (\S) Indicates $p < 0.05$ vs. exhaustion and post-exercise. SD = Standard Deviation and SE = Standard Error.

References

- 1 Asking B. Sympathetic stimulation of amylase secretion during a parasympathetic background activity in the rat parotid gland. *Acta Physiol Scand* 1985; 124: 535-542
- 2 Bautmans I, Njemini R, Vasseur S, Chabert H, Moens L, Demanet C, Mets T. Biochemical changes in response to intensive resistance exercise training in the elderly. *Gerontology* 2005; 51: 253-265
- 3 Bishop NC, Blannin AK, Armstrong E, Rickman M, Gleeson M. Carbohydrate and fluid intake affect the saliva flow rate and IgA response to cycling. *Med Sci Sports Exerc* 2000; 32: 2046-2051

- 4 Bortolini MJS, De Agostini GG, Reis IT, Lamounier RPMS, Blumberg JB, Espindola FS. Total protein of whole saliva as a biomarker of anaerobic threshold. *Res Q Exerc Sport* 2009; 80: 604–610
- 5 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72: 248–254
- 6 Calvo F, Chicharro JL, Bandres F, Lucia A, Perez M, Alvarez J, Mojares LL, Vaquero AF, Legido JC. Anaerobic threshold determination with analysis of salivary amylase. *Can J Appl Physiol* 1997; 22: 553–561
- 7 Castle D, Castle A. Intracellular transport and secretion of salivary proteins. *Crit Rev Oral Biol Med* 1998; 9: 4–22
- 8 Chatterton RT Jr, Vogelsohn KM, Lu YC, Ellman AB, Hudgens GA. Salivary alpha-amylase as a measure of endogenous adrenergic activity. *Clin Physiol* 1996; 16: 433–448
- 9 Chicharro JL, Legido JC, Alvarez J, Serratos L, Bandres F, Gamella C. Saliva electrolytes as a useful tool for anaerobic threshold determination. *Eur J Appl Physiol* 1994; 68: 214–218
- 10 Chicharro JL, Lucia A, Perez M, Vaquero AF, Urena R. Saliva composition and exercise. *Sports Med* 1998; 26: 17–27
- 11 Davison G, Allgrove J, Gleeson M. Salivary antimicrobial peptides (LL-37 and alpha-defensins HNP1-3), antimicrobial and IgA responses to prolonged exercise. *Eur J Appl Physiol* 2009; 106: 277–284
- 12 Gilman S, Thornton R, Miller D, Biersner R. Effects of exercise stress on parotid gland secretion. *Horm Metab Res* 1979; 11: 454
- 13 Gordis EB, Granger DA, Susman EJ, Trickett PK. Asymmetry between salivary cortisol and alpha-amylase reactivity to stress: relation to aggressive behavior in adolescents. *Psychoneuroendocrinology* 2006; 31: 976–987
- 14 Hanna SJ, Brelen ME, Edwards AV. Effects of reducing submandibular blood flow on secretory responses to parasympathetic stimulation in anesthetized cats. *Exp Physiol* 1999; 84: 677–687
- 15 Harriss DJ, Atkinson G. International Journal of Sports Medicine – Ethical Standards in Sport and Exercise Science Research. *Int J Sports Med* 2009; 30: 701–702
- 16 Hinkley DV. Inference about the intersection in two-phase regression. *Biometrika* 1969; 56: 495–504
- 17 Hu S, Xie Y, Ramachandran P, Ogorzalek Loo RR, Li Y, Loo JA, Wong DT. Large-scale identification of proteins in human salivary proteome by liquid chromatography/mass spectrometry and two-dimensional gel electrophoresis-mass spectrometry. *Proteomics* 2005; 5: 1714–1728
- 18 Impellizzeri FM, Rampinini E, Marcora SM. Physiological assessment of aerobic training in soccer. *J Sports Sci* 2005; 23: 583–592
- 19 Iwami Y, Takahashi-Abbe S, Takahashi N, Abbe K, Yamada T. Rate-limiting steps of glucose and sorbitol metabolism in *Streptococcus mutans* cells exposed to air. *Oral Microbiol Immunol* 2000; 15: 325–328
- 20 Laemmli UK, Favre M. Maturation of the head of bacteriophage T4. I. DNA packaging events. *J Mol Biol* 1973; 80: 575–599
- 21 Lehmann M, Schmid P, Keul J. Plasma catecholamine and blood lactate cumulation during incremental exhaustive exercise. *Int J Sports Med* 1985; 6: 78–81
- 22 Li TL, Gleeson M. The effect of single and repeated bouts of prolonged cycling and circadian variation on saliva flow rate, immunoglobulin A and alpha-amylase responses. *J Sports Sci* 2004; 22: 1015–1024
- 23 Navazesh M. Methods for collecting saliva. *Ann N Y Acad Sci* 1993; 694: 72–77
- 24 Nexo E, Hansen MR, Konradsen L. Human salivary epidermal growth factor, haptocorrin and amylase before and after prolonged exercise. *Scand J Clin Lab Invest* 1988; 48: 269–273
- 25 Nierop A, Bratsikas A, Klinkenberg A, Nater UM, Zimmermann R, Ehlert U. Prolonged salivary cortisol recovery in second-trimester pregnant women and attenuated salivary alpha-amylase responses to psychosocial stress in human pregnancy. *J Clin Endocrinol Metab* 2006; 91: 1329–1335
- 26 Rohleder N, Wolf JM, Maldonado EF, Kirschbaum C. The psychosocial stress-induced increase in salivary alpha-amylase is independent of saliva flow rate. *Psychophysiology* 2006; 43: 645–652
- 27 Scannapieco FA, Solomon L, Wadenya RO. Emergence in human dental plaque and host distribution of amylase-binding streptococci. *J Dent Res* 1994; 73: 1627–1635
- 28 Schabmueller CG, Loppow D, Piechotta G, Schutze B, Albers J, Hintsche R. Micromachined sensor for lactate monitoring in saliva. *Biosens Bioelectron* 2006; 21: 1770–1776
- 29 Schenkels LC, Veerman EC, Nieuw Amerongen AV. Biochemical composition of human saliva in relation to other mucosal fluids. *Crit Rev Oral Biol Med* 1995; 6: 161–175
- 30 Schneider DA, McLellan TM, Gass GC. Plasma catecholamine and blood lactate responses to incremental arm and leg exercise. *Med Sci Sports Exerc* 2000; 32: 608–613
- 31 Segura R, Javierre C, Ventura JL, Lizarraga MA, Campos B, Garrido E. A new approach to the assessment of anaerobic metabolism: measurement of lactate in saliva. *Br J Sports Med* 1996; 30: 305–309
- 32 Stainsby WN, Brooks GA. Control of lactic acid metabolism in contracting muscles and during exercise. *Exerc Sport Sci Rev* 1990; 18: 29–63
- 33 Steerenberg PA, van Asperen IA, van Nieuw Amerongen A, Biewenga A, Mol D, Medema GJ. Salivary levels of immunoglobulin A in triathletes. *Eur J Oral Sci* 1997; 105: 305–309
- 34 Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 1979; 76: 4350–4354
- 35 van Stegeren A, Rohleder N, Everaerd W, Wolf OT. Salivary alpha amylase as marker for adrenergic activity during stress: effect of beta-blockade. *Psychoneuroendocrinology* 2006; 31: 137–141
- 36 van Veen JF, van Vliet IM, Derijk RH, van Pelt J, Mertens B, Zitman FG. Elevated alpha-amylase but not cortisol in generalized social anxiety disorder. *Psychoneuroendocrinology* 2008; 33: 1313–1321
- 37 Walsh NP, Blannin AK, Clark AM, Cook L, Robson PJ, Gleeson M. The effects of high-intensity intermittent exercise on saliva IgA, total protein and alpha-amylase. *J Sports Sci* 1999; 17: 129–134
- 38 Walsh NP, Montague JC, Callow N, Rowlands AV. Saliva flow rate, total protein concentration and osmolality as potential markers of whole body hydration status during progressive acute dehydration in humans. *Arch Oral Biol* 2004; 49: 149–154
- 39 Yamaguchi M, Deguchi M, Miyazaki Y. The effects of exercise in forest and urban environments on sympathetic nervous activity of normal young adults. *J Int Med Res* 2006; 34: 152–159
- 40 Yamaguchi M, Kanemori T, Kanemaru M, Kanemaru M, Takai N, Mizuno Y, Yoshida H. Performance evaluation of salivary amylase activity monitor. *Biosens Bioelectron* 2004; 20: 491–497