UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Validation of automated assays for the assessment of stress and welfare in saliva

Validación de ensayos automatizados para la evaluación del estrés y el bienestar en saliva

Dña. María Dolores Contreras Aguilar

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"Validation of automated assays for the assessment of stress and welfare in saliva"

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Memoria presentada por la licenciada en veterinaria

María Dolores Contreras Aguilar

Para optar al grado de Doctor en Ciencias Veterinarias con Mención Internacional

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A mi perrita Panchosa. Siempre te tendré en mi corazón

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DOCTORAL THESIS

AS COMPENDIUM

OF PUBLICATIONS

DOCTORAL THESIS AS COMPENDIUM OF PUBLICATIONS

The recent Ph.D. Thesis, after the authorization of the directors of the Ph.D. Thesis and the Academic Commission responsible for the Veterinary Sciences Ph.D. Program, is presented as a compendium of one book chapter and twelve studies previously published. Therefore, the Ph.D. Thesis is composed of the following **book chapter** and **article** references:

I. **Contreras-Aguilar, M.D.**, & Gómez-García, F. (2020). Salivary Glands' Anatomy and Physiology. In A. Tvarijonaviciute, S. Martínez-Subiela, P. López-Jornet, & E. Lamy (Eds.), *Saliva in Health and Disease: The Present and Future of a Unique Sample for Diagnosis*. Springer International Publishing (1st ed., pp. 3–21).

II. **Contreras-Aguilar, M.D**., Escribano, D., Martínez-Subiela, S., Martínez-Miró, S., Rubio, M., Tvarijonaviciute, A., Tecles, F., Cerón, J.J. (2017). Influence of the way of reporting alpha- Amylase values in saliva in different naturalistic situations: A pilot study. *PlosOne,* 12(6), 1-13.

III. **Contreras-Aguilar, M.D.**, Vialaret, J., Deville de Périère, D., Escribano, D., Lehmann, S., Tecles, F., Cerón, J.J., Hirtz, C. (2019). Variation of human salivary alpha-amylase proteoforms in three stimulation models. *Clinical Oral Investigations*, 24(1), 475-486.

IV. **Contreras-Aguilar, M.D**., Tecles, F., Martínez-Subiela, S., Escribano, D., Bernal, L.J., Cerón, J.J. (2017). Detection and measurement of alpha- amylase in canine saliva and changes after an experimentally induced sympathetic activation. *BMC Veterinary Research,* 13(266), 1-6.

V. **Contreras-Aguilar, M.D**., Escribano, D., Martín-Cuervo, M., Tecles, F., Cerón, J.J. (2018). Salivary alpha-amylase activity and cortisol in horses with acute abdominal disease: a pilot study**.** *BMC Veterinary Research*, 14(156), 1-7.

VI. **Contreras-Aguilar, M.D**., Martínez-Subiela, S., Cerón, J.J., Martín-Cuervo, M., Tecles, F., Escribano, D. (2019). Salivary alpha-amylase activity and concentration in horses with acute abdominal disease: Association with outcome. *Equine Veterinary Journal,* 51(5), 569-574.

VII. **Contreras-Aguilar, M.D**., Escribano, D., Martínez-Subiela, S., Martínez-Miró, S., Cerón, J.J., Tecles, F. (2018). Changes in alpha-amylase activity, concentration and isoforms in pigs after an experimental acute stress model: an exploratory study. *BMC Veterinary Research,* 14(256), 1-8.

VIII. **Contreras-Aguilar, M.D**., Escribano, D., Martínez-Subiela, S., Martín-Cuervo, M., Lamy, E., Tecles, F., Cerón, J.J. (2019). Changes in saliva analytes in equine acute abdominal disease: a sialochemistry approach. *BMC Veterinary Research,* 15(187), 1-9.

IX. **Contreras-Aguilar, M.D**., Henry, S., Coste, C., Tecles, F., Escribano, D., Cerón, J.J., Hausberger, M. (2019). Changes in Saliva Analytes Correlate with Horses' Behavioural Reactions to An Acute Stressor: A Pilot Study**.** *Animals,* 9(11), 1-13.

X. **Contreras-Aguilar, M.D.**, Escribano, D., Martínez-Miró, S., López-Arjona, M., Rubio, C.P., Martínez-Subiela, S., Cerón, J.J., Tecles, F. (2019). Application of a score for evaluation of pain, distress and discomfort in pigs with lameness and prolapses: correlation with saliva biomarkers and severity of the disease. *Research in Veterinary Science,* 126, 155-163.

XI. **Contreras-Aguilar, M.D**., Escribano, D., Quiles, A., López-Arjona, M., Cerón, J.J., Martínez-Subiela, S., Hevia, M.L., Tecles, F. (2019). Evaluation of new biomarkers of stress in saliva of sheep. *Animal,* 13(6), 1278-1286.

XII. **Contreras-Aguilar, M.D**., Monkeviciene, I., Cerón, J.J., Silinskas, I., Vallejo-Mateo, P.J., Tecles, F., Martínez-Subiela, S., Tvarijonaviciute, A., Zelvyte, R. (2019). Biochemical changes in saliva of cows with inflammation: A pilot study. *Research in Veterinary Science,* 124, 383-386.

XIII. **Contreras-Aguilar, M.D**., Hevia, M.L., Escribano, D., Lamy, E., Tecles, F., Cerón, J.J. (2020). Effect of food contamination and collection material in the measurement of biomarkers in saliva of horses. *Research in Veterinary Science,* 129, 90-91.

Additionally, it is considered appropriate to include in the **Annex** of the following Ph.D. Thesis three research results related to the work carried out during the Thesis. In the recent future, those data will be submitted for their possible publications.

I. **Contreras-Aguilar, M.D.**, Mateo, S.V., Tecles, F., Hirtz, C., Escribano, D., Cerón, J.J. Changes occurring on the activity of salivary alpha-amylase proteoforms in two naturalistic situations using a spectrophotometric assay.

II. **Contreras-Aguilar, M.D.**, Cerón, J.J., Muñoz, A., Ayala, I. Changes in saliva biomarkers during a standardized increasing intensity field exercise test in endurance horses.

III. **Contreras-Aguilar, M.D.**, Escribano, D., Hevia, M.L., Lamy, E., Tecles, F., Cerón, J.J. Circadian rhythm in horse's salivary biomarkers related to acute stress and disease.

ABBREVIATIONS

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AAD: acute abdomen disease
ADA: adenosine deaminase
ANS: autonomic nervous system
BChE: butyrylcholinesterase
CI: confidence interval
CK: creatinine kinase
CNP: 2-chloro-4-nitrophenol
$CNPG_3$: 2-chloro-4-nitrophenyl-α-D-maltotrioside
CoA: Concanavalin A
CV: coefficient of variation
EAAPS-1: equine acute abdominal pain scale-version 1
ELISA: enzyme-linked immunosorbent assay
gGT: γ-glutamyl transferase
GsAA: glycosylated sAA
HPA: hypothalamic-pituitary-adrenal
HR: heart rate
HRV: heart rate variability
IgG: immunoglobulin G
IQR: interquartile range
LA: lactate
LC-MS/MS: liquid Chromatography with tandem mass spectrometry
Lip: lipase
LLD: lower limit of detection
LLOQ: lower limit of quantification
NGsAA: non-glycosylated sAA
r: coefficient of correlation
R^2: coefficient of determination
RR: respiratory rate
sAA: salivary alpha-amylase
SAA: serum amyloid A
SAM: sympathetic adrenal medullary
SD: standard deviation
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis, 0.1% (w/v)
SET: standardized exercise test
SIRS: systemic inflammatory response syndrome
TAC: total antioxidant capacity
TEA: total esterase
TP: total protein
TR-IFMA: time-resolved immunofluorometric assay
TsAA: total sAA.
TSST: trier social stress test
V_{200}: velocities at which heart rates of 200 beats/min are reached
V_4: velocities at which blood lactate concentrations of 4 mmol/L are reached
WB: western-Blot
WBC: white blood cell

INTRODUCTION

INTRODUCTION

The stress and low welfare conditions produce inappropriate behaviors and anxiety in animals leading to a decrease in the efficiency of animal production in livestock (Elsa Lamy & Mau, 2012; Martínez-Miró et al., 2016). The leading causes of stress can be psychological, physical such as intense exercise or pain, and inflammation or disease (Murata, 2007; Strahler, Skoluda, Kappert, & Nater, 2017). The accurate assessment of stress in order to detect it and try to avoid its causes and consequences is one of the main challenges in the field of animal welfare research since verbal self-report is not possible (Schiavenato & Craig, 2010).

Stress can interfere with the individual's biological mechanisms (Grandin, 1998), and induces changes at physiological level (Altmann, 1974; Wolf & Goodell, 1981) that can be objectively evaluated. In this context, saliva is recently achieving a growing interest as an alternative to blood as a biological sample for stress assessment. This is because it is easy to collect in a non-invasive way without any need for specialized training, leading to the possibility of repeating the collection of a large number of samples even at short-time intervals (Elsa Lamy & Mau, 2012; Yoshizawa et al., 2013). Besides, some analytes in saliva can be measured by automated assays, with the advantage of being accurate, fast, and with a high sample throughput ability. This allows an advantage for monitoring of animal welfare by a non-stressful and painless methodology.

Traditionally, the cortisol has been used to evaluate physiological changes in stress (Martínez-Miró et al., 2016) since it provides information about the HPA axis (Hyyppä, 2005). However, there are other pathways involved in the stress reaction, such as the activation of the ANS and the SAM axis through catecholamines (Everly & Lating, 2002; Hyyppä, 2005). These pathways of stress reaction can also directly or indirectly interact on different systems and induce changes to immunity level or in the oxidative status (Everly & Lating, 2002; Fazio, Casella, Giannetto, Giudice, & Piccione, 2015; Murata, Shimada, & Yoshioka, 2004). Each of these pathways and interactions can be evaluated by compounds that can be measured in saliva and that could be considered as biomarkers of stress. For example, sAA is a biomarker of the ANS and SAM axis (Strahler et al., 2017), the ADA is a marker of the immune system (Adams & Harkness, 1976), and the TAC is a marker of oxidative status (Rubio et al., 2019).

However, the evaluation of biomarkers of stress other than cortisol in saliva in veterinary species is still in its infancy. In addition, there is a lack of studies in which different analytes are applied in an integrated way using profiles that could evaluate the stress by its different pathways and interactions. To illustrate the importance that the search of new biomarkers in saliva in farm animals is currently having, a European project (Clear-Farm, grant agreement No. 862919) in the framework of the Horizon 2020 Programme was recently awarded to the research group in which the Ph.D. student is included. This European project aims to find possible welfare biomarkers in pigs and cows, and integrate them into a new platform to inform both farmers and consumers to assist their decision-making.

OBJECTIVES

OBJECTIVES

The objectives of this Ph.D. Thesis were to advance in the study of the sAA in different veterinary species and to increase the knowledge in other possible biomarkers of stress and welfare that can be measured in saliva by automated assays. In addition to the study in different animal species, this Thesis also used humans as experimental model for the increase in the knowledge of sAA, since in this species saliva is particularly easy to obtain and has very high sAA concentrations. Therefore, the specific aims were:

- **Objective 1**. To gain knowledge about sAA, mainly by studying the optimal way to report the results, the investigation of possible proteoforms' changes at different stress situations, and the validation of assays in different species such as in dogs, horses, and pigs. This objective led to the published papers (indicated in the section *Articles*) nº 1 - 6, and one experiment included in Annex (Experiment 1).

- **Objective 2**. To validate automated assays in saliva from horse, pig, sheep, and cow for measuring stress and welfare biomarkers different than sAA, and to study how these biomarkers behave in different situations related to psychological, physical, and inflammatory stressors. Namely, the salivary biomarkers studied were TEA, BChE, Lip, ADA, TP, gGT, CK, urea, total bilirubin, phosphorus, and LA. This objective led to the published papers (indicated in the section *Articles*) nº 7 - 11 and by the experiment described in Annex (Experiment 2).

- **Objective 3**. To evaluate possible factors that would affect the interpretation of results in the biomarkers of saliva. This objective was covered by the published paper nº 12 indicated in the section *Articles,* and by one experiment included in Annex (Experiment 3).

EXTENDED SUMMARY

EXTENDED SUMMARY

1. Salivary glands' anatomy and physiology

The knowledge of salivary glands´ anatomy and physiology is important for a proper understanding of how saliva is formed and contains the analytes that can be measured for the evaluation of the stress. A complete update about this subject has been presented by the Ph.D. candidate in the chapter entitled *Salivary glands' anatomy and physiology* of the book *Saliva in Health and Disease: The Present and Future of a Unique Sample for Diagnosis,* recently published by the Springer Editorial. From this chapter, three basic ideas about the salivary glands will be presented here:

1.1 There are anatomical differences between species

Salivary glands are classified as Major, which produce the major volume of saliva, and Minor salivary glands (Edgar, 1990), having differences between species. For example, in horses and swine, as well as in humans, the parotid gland is the largest one. Whereas the submandibular gland in cows is the largest one and brings more saliva secretion, and dogs have the Zygomatic gland that does not exist in other species (Vázquez-Autón et al., 2002). Also, excretory ducts from the Major salivary glands lead the saliva to the oral cavity differently, according to the specie (Contreras-Aguilar & Gómez-García, 2020). These differences could contribute to the changes in salivary composition that exist between species (Carpenter, 2013; Edgar, 1990).

1.2 Glands' innervation affects the saliva composition

Acinar cells and their associated myoepithelial cells are innervated by the sympathetic and parasympathetic branches of the ANS (Emmelin, 1987). Parasympathetic nerve impulses mainly produce high-flow, low-protein saliva, whereas sympathetic impulses produce low-flow, high-protein saliva (Carpenter, 2013; Proctor & Carpenter, 2007). This innervation system's effect on salivary protein and fluid secretion can differ between glands and between the different species (Proctor & Carpenter, 2007).

Acetylcholine and noradrenaline are the principal neurotransmitters released by the parasympathetic and sympathetic neurons that innervate the salivary glands. The salivary glands are also susceptible to being stimulated by the adrenaline released by the adrenal medulla, which is related to the SAM axis (Hyyppä, 2005).

1.3 There are different ways in which analytes can reach saliva

The most important mechanism of protein secretion from salivary glands' cells is the **exocytosis**, where the proteins firstly synthesized and packaging into acinar cells are released into saliva due to neurotransmitters activation, such as occurs with the sAA (Asking & Gjorstrup, 1987; Castle, Jamieson, & Palade, 1975; Nater & Rohleder, 2009; Segawa & Yamashina, 1998).

In addition, there are other mechanisms, such as the **intracellular diffusion**, which is used for some analytes, such as cortisol, to be passively transported into saliva from plasma. In the case of cortisol, this transport is made as nonprotein-bound fractions through the salivary glands' cells due to its solubility (Vining, Mcginley, & Symons, 1983). Regarding water, it is released by the salivary glands by different processes. One is the **ultrafiltration** from plasma to saliva between the acinar cells (paracellular) (Young, Cook, Lennep, & Roberts, 1987). The other is by **aquaporins' transport**, which allows the transepithelial water movement (Gresz et al., 2001; Melvin, Yule, Shuttleworth, & Begenisich, 2005).

It is essential to point out that the whole saliva contains not only secretions of the Major and Minor salivary glands, but also constituents of non-salivary origin, as gingival crevicular fluid (serum exudate and inflammatory cells); desquamated epithelial cells; other fluids as expectorated bronchial and nasal secretions; extrinsic substances as food debris; as well as microbiota and its products, viruses, and fungi (Kaufman & Lamster, 2000). Also, in cases of intraoral bleeding, blood could appear in saliva. Overall, proteomic studies suggest the existence of a different saliva composition among species (de Sousa-Pereira et al., 2015; E Lamy et al., 2009).

2. Materials and Methods

2.1 Ethics considerations

Procedures related to handling in animals or ethics considerations in humans were approved by Bioethical Committee (Comité Ético de Experimentación Animal, CEEA) and Ethics Commission (Comisión Ética de Investigación, CEI) of Murcia University, respectively, under the protocol numbers 171/2015 (studies in the veterinary species), 1349/2016 (humans studies), 288/2017 (dog and horse studies), and 235/2018 (pig studies). The sheep study was approved by the ministry of agriculture, livestock, fisheries, and aquaculture from

Murcia's Region under the protocol number A13180603. Moreover, the study performed in cows was approved by the Lithuanian National Commission of Ethics under the license number G2-60, 2017-02-15. Additionally, this Thesis got a favorable report from the Ethics Commission of Murcia University under the license number 2133/2018. All the previous animal experimental procedures were conducted following the current Spanish and European legislation: 'Real Decreto 53/2013, de 1 de febrero', and Directive 2010/63/EU of the European Parliament and of the Council, of 22 September 2010, on the protection of animals used for scientific purposes.

For the clinical studies performed in horses, informed consent for the animals' inclusion from the owner was given. Also, the participants in human beings' studies were all informed about the procedure, sampling methods, and the objective of the experiment and signed a consent form. Additionally, informed consent was obtained from all individual participants for whom identifying information is included in this article.

2.2 Sampling procedure

The saliva sampling procedure was different depending on the species and the objective to achieve. The sample collections made in the different species are described in Table 1 (Appendix 1):

After collection, in human beings, saliva samples were centrifuged at 4 500 x g for 10 min at 4˚C to remove cells and mucus. In the veterinary species, the collection device was centrifuged at 2 000 – 4 000 ×g for 8-10 min at 4 °C, depending on the case. Finally, the supernatants obtained in all the sampling procedures after centrifugation were transferred in 1.5 mL Eppendorf tubes and stored at –80 ºC until analysis in less than six months, except in the saliva samples obtained in the articles nº 9 and 11 from pigs and sheep, respectively, since they were freshly analyzed once arrived at the laboratory. In all cases, samples after collection were refrigerated or stored on ice until arrival at the laboratory to be processed. In addition, saliva sampling was always performed before any potentially painful procedures or stressful situations, such as venipuncture to obtain blood samples.

2.3 Human sAA purification and polyclonal antibody production

To achieve the objective 1 of the following Thesis, sAA (a-1,4-a-D-glucan 4-glucanohydrolase; EC 3.2.1.1) was purified from saliva samples, and a rabbit anti-human-sAA polyclonal antibody was developed.

2.3.1 Human sAA purification

Salivary alpha-amylase was purified from humans since sAA is in high amounts in human saliva (Nater & Rohleder, 2009). It was performed according to a modified procedure from Peng *et al*. (2012) based on the enzyme-substrate specific interaction between amylase and glycogen.

This procedure demonstrated to be effective and repeatable when a mini SDS-PAGE was performed in four purified samples at different days stained with Coomassie Brilliant Blue (Coomassie® Brilliant Blue R 250, Bio-Rad Laboratories S. A, CA, USA) (Figure 1).

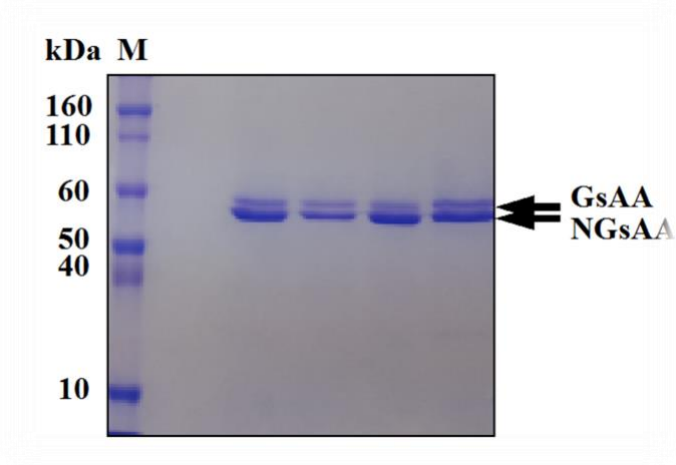


Figure 1. SDS-PAGE gel of four human sAA purified from saliva samples (10µg protein each). Molecular (M) weight markers (Novex Sharp Pre-Stained, Invitrogen, Carlsbad, California). The arrows show the native sAA proteoforms at 59 kDa (GsAA) and 56 kDa (NGsAA).

2.3.2 Rabbit polyclonal antibody against human sAA

Purified human sAA was used as immunogen to produce the rabbit anti-human-sAA polyclonal antibody according to a standard protocol (University of California, 2014): immunization each week with mixed in Complete Freund's Adjuvant for the first injection, and Incomplete Freund's Adjuvant for the second and successive ones. After achieving a high immune

response and excellent cross-reactivity with the purified human sAA and a commercial one (Purified human sAA (ab 77,875, Abcam, Cambridge, UK), evaluated by ELISA screening; the IgG content from the rabbit (NZ, female, 2.5 kg) was purified using a HiTrapTM Protein G HP column, according to the manufacturer's instructions (GE Healthcare Life Sciences, Munich, Germany) using a chromatography system (ÄKTA pure - GE Healthcare Life Sciences). An SDS-PAGE showed an efficient purification of the IgGs produced (Figure 2).

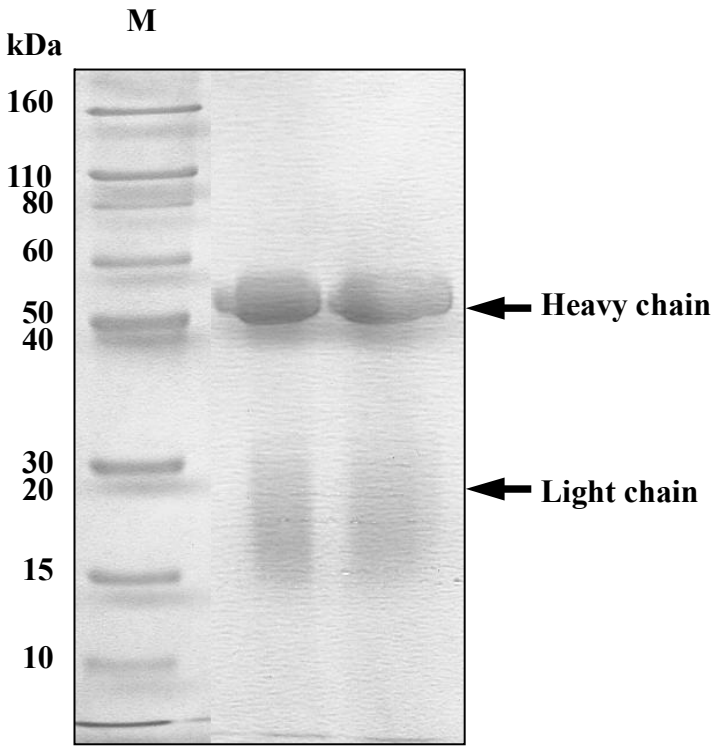


Figure 2. SDS-PAGE gel of two rabbit anti-human sAA polyclonal antibodies purified (5µg protein each). Molecular (M) weight markers (Novex Sharp Pre-Stained, Invitrogen, Carlsbad, California). The arrows show the heavy and light chain.

2.4 Analyte determination in saliva

2.4.1 Salivary stress biomarkers

2.4.1.1 sAA measurements

Enzymatic assay

The sAA activity was measured using a colorimetric commercial kit (Alpha-Amylase, Beckman Coulter Inc., Fullerton, CA, USA) following the International Medicine (IFCC) method

(Rohleder & Nater, 2009; van der Heiden, Bais, Gerhardt, Lorentz, & Rosalki, 1999) in an automatic analyzer for biochemical assay (Olympus Diagnostica GmbH, Beckman Coulter, Ennis, Ireland). It uses $CNPG_3$ as substrate of enzyme, which directly reacts with sAA producing CNP. The resulting absorbance increase per minute measured at 405 nm is directly related to the sAA activity in the sample. Results were expressed as units per milliliter (IU/mL) in humans and per liter (IU/L) in the rest of the veterinary species.

Time-resolved immunofluorometric assay

The sAA concentration was analyzed by a TR-IFMA. This assay was based on the anti-human sAA polyclonal antibody purified, as described in the sub-heading 2.3.2. It consisted of a noncompetitive indirect sandwich method with the anti-human sAA polyclonal antibody firstly biotin-labeled as a capture reagent, and then Eu^{3+}−chelates labeled as a detector. Streptavidin-coated plates (Streptavidin Microtitration Strips, DELFIA, PerkinElmer, Turku, Finland) were used to develop this assay. Results were expressed as µg/mL in humans and ng/mL in horses and pigs.

Proteomics assays

The sAA was also detected by the WB procedure in humans (paper nº 2), dogs (paper nº 3), and pigs (paper nº 6) using an indirect method. Proteins separated in SDS-PAGE were then transferred to nitrocellulose membrane (Bio-Rad Laboratories Inc., Hercules, CA, USA), and an available commercial rabbit anti-human sAA polyclonal antibody (ab 173163, Abcam, Cambridge, UK) in dogs at 1:500 dilution, and the rabbit anti-human sAA polyclonal antibody produced as described in the sub-heading 2.3.2 in humans at 1:6000 and in pigs at 1:8000 dilution; were used as primary antibody. The horseradish peroxidase-conjugated goat polyclonal antibody anti-rabbit (ab 6721, Abcam, Cambridge, UK) at 1:2000 in humans and dogs and 1:12000 in pigs was employed as secondary antibody, being detected using Pierce ECL2 kit (Pierce, Thermo Fisher Scientific, USA), by the Thyphoon 9410 scanner (GE Healthcare, Wilmington, MA, USA) in dogs and by the ImageQuant™ scanner (GE Healthcare, Uppsala, Sweden) in humans and pigs.

2.4.1.2 Cortisol

Salivary cortisol was analyzed using an immunoassay method (Immulite 1000, Siemens Healthcare Diagnostic, Deerfields, Illinois, USA), which uses a solid-phase competitive enzyme-amplified chemiluminescent immunoassay. Results are given in µg/dL.

2.4.2 Other biomarkers that can be measured by automated assays

The assays to measure other possible salivary stress biomarkers different that sAA and cortisol studied in the present Thesis (Lip, TEA, BChE, and ADA) are detailed in Table 2 (Appendix 1). All of them were measured by an automated biochemical analyzer (Olympus AU400 or AU600, Olympus Diagnostica GmbH, Beckman Coulter, Ennis, Ireland).

The rest of the analytes measured in saliva in the following Thesis that we postulated not to be directly associated with stress, but could be potential candidates to evaluate welfare conditions, were TP, gGT, CK, urea, total bilirubin, phosphorus, and LA. These assays were measured by an automated biochemical analyzer (Olympus AU600, Olympus Diagnostica GmbH, Beckman Coulter, Ennis, Irlanda) using commercial kits from Beckman (Beckman Coulter Inc., Fullerton, CA, EE. UU), with the except of the TP measurement where a commercial colorimetric assay to measure urine and proteins from the Low Complexity Region (protein in urine and CSF, Spinreact, España) was used. All of them were adapted according to the manufacturer's instructions regarding reagents, the sample volumes, and the wavelength, except for the CK and lactate methods that were adapted to the saliva of the veterinary species.

2.5 Analytical validation

All the assays used in the present Thesis were analytically validated following the recommendations of the Food and Drug Administration (Food and Drug Administration, 2001), the analytical validation was performed evaluating the following parameters:

2.5.1 Precision

Precision or repeatability was evaluated by the within-run (or intra-assay) precision and by the between-run (or inter-assay) precision. In both, some samples or pooled samples at a different range of activities or concentrations were analyzed five or more times per one, in the same analytical series or once a day within 5 days, respectively. The possible effect of repetitive thawing and freezing in the between-run precision was removed by storing the samples or pool samples in separate vials (aliquots) and using a new one for each measurement. Precision was evaluated by the CV, calculated as the percentage of the SD of the replicates divided by the mean.

2.5.2 Accuracy

It was indirectly evaluated by the linearity under the dilution test, since to the authors' knowledge, no gold standard assays are available to determine the analytes in saliva evaluated in

the present Thesis. Samples or pooled samples at different activities or concentrations were serially diluted with deionized water (spectrophotometric assays) or buffer (TR-IFMA), and observed results were compared with those expected by linear regression analysis. The slope, y-intercept, and R_2 were also calculated.

2.5.3 Sensitivity

This parameter was evaluated by the LLD and LLOQ.

The **LLD** was defined as the lowest concentration of the analyte that could be distinguished from a specimen of zero value, and it was calculated based on data from 13 replicate determinations of the zero standard (deionized water for spectrophotometric assays, or buffer for the TR-IFMA) as mean value plus three standard deviations.

The **LLOQ** was calculated as the lowest activity or concentration that could be measured above the limit of detection with a CV <20%. A saliva sample was serially diluted in deionized water (spectrophotometric assays) or buffer (TR-IFMA), and each dilution was analyzed in five replicates in the same run. CVs for each dilution were estimated as previously described.

3. Experimental design, results and discussion

This data will be presented in the form of the different papers published from the Thesis.

3.1 Objective 1

The objective 1 was achieved by seven studies corresponding to papers nº 1 and nº 2, and the experiment nº 1 (Annex) performed in human beings; paper nº 3 in dogs; papers nº 4 and nº 5 in horses; and paper nº 6 in pigs.

3.1.1 Studies in humans

3.1.1.1 What is the best way to report the sAA results? (paper nº 1).

Aims and experimental design

Two different methods of sAA measurements (by its concentration with the fluorometric assay and by its activity with the spectrophotometric assay), and three different way of expressing sAA results (without any correction, corrected by flow rate, and corrected by protein concentration); were evaluated in three different situations of stress. These stressful situations were a final football match (physical stress), an academic activity consisting of the resolution of clinical cases (apparently of low psychological stress), and another academic activity consisting of pig blood collection (apparently of high psychological stress).

Results and discussion

Comparing the different ways of measuring sAA. The sAA concentration and activity values showed similar behavior in all the evaluated stressful situations, and there was a high correlation between sAA concentration and activity ($r < 0.70$, $P < 0.0001$). However, in general, the changes in concentration were of lower significance than those produced in the enzymatic activity. Therefore, sAA enzymatic activity was more sensitive than concentration to detect the changes induced by stress in the studied situations.

Comparing the different ways of reporting the results of sAA. When values were multiplied by the flow rate or divided by protein concentration, the results obtained in different stress models changed compared to without correction. For example, significant changes after situation 1 were detected only for sAA activity but not for sAA concentration when multiplied by the flow rate, being these changes of lower significance and magnitude that those observed for sAA activity

without any correction. Overall, how sAA is reported and the factors involved in the different ways of expressing sAA should be taken into consideration for an objective interpretation of sAA values.

3.1.1.2 Expression of the different sAA 's proteoforms (paper nº 2).

Aims and experimental design

The possible changes in the sAA proteoforms' expression during different situations that produce increases in sAA activity were evaluated by WB analysis and LC-MS/MS in six healthy women. These situations were an acetic acid stimulation, psychological stress using the standardized TSST, and physical effort using the Cooper treadmill test.

The times just before (Tb) from the acetic acid stimulation and just after (T+0) in each situation for each participant was used for the WB analysis, while for the LC-MS/MS, one gel was prepared mixing equal amount of saliva (100 µL) from all the six participants at each time. Quantification of each sAA's protein band (µg) shown in the WB was estimated by comparing a natural human sAA protein (77875, Abcam, Cambridge, UK) of known quantity and analyzed using ImageQuant™ TL 8.1 (GE Healthcare, Uppsala, Sweden).

Results and discussion

The three models produced an increase in sAA activity. The WB analysis (Figure 3) demonstrated seven common bands at 59 kDa (native GsAA form, band 1), 56 kDa (native NGsAA form, band 2), 48 kDa (band 3), 45 kDa (band 4), 41 kDa (band 5), 36 kDa (band 6), and at 14 kDa (band 7), in which sAA protein was identified after the LC-MS/MS analysis.

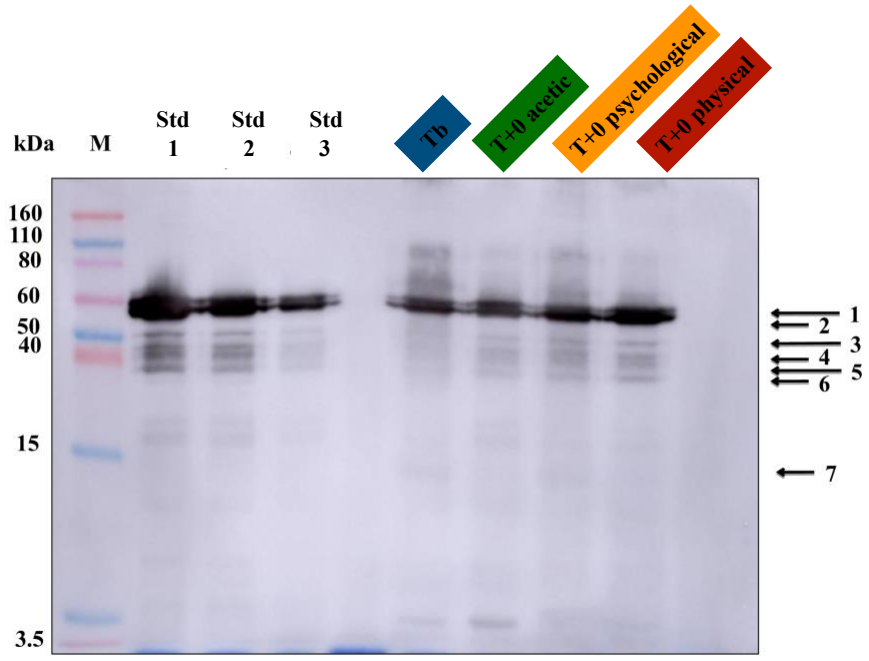


Figure 3. WB analysis for the sAA's proteoforms detection in the acetic acid stimulation at basal time (Tb, blue) and just after (T+0, green), the TSST just after (T+0, orange), and the Cooper treadmill test just after (T+0, red). Saliva pool (3.5 µg of total protein per lane) was used, where M was the Molecular weight markers (Novex Sharp Pre-Stained, Invitrogen, Carlsbad, California); Std was the standards (77875, Abcam, Cambridge, UK) of known quantity; and the numbers on the side (1–7) label the common bands.

Both WB analysis and label-free quantification from the LC/MS-MS analysis indicated different responses from the different proteoforms depending on the type of stimulation. For example, higher increases after the psychological and physical situation compared to Tb in band 2 was observed (Figure 4), which corresponded to the native NGsAA proteoform. Also, this band was the only one correlated with sAA activity ($r = 0.56$, $P = 0.001$). Therefore, the expression of the sAA proteoforms seems to be regulated depending on the stimulation models.

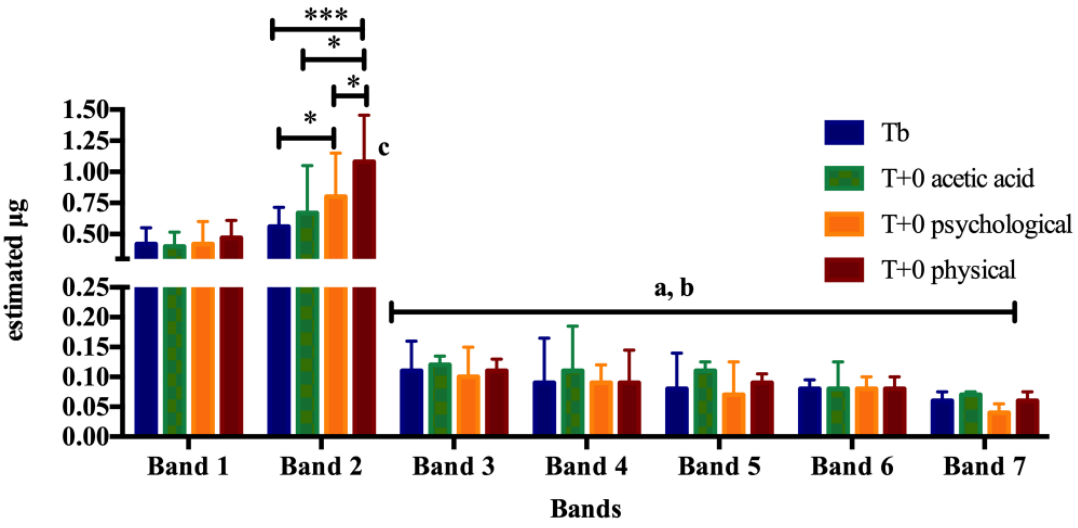


Figure 4. Estimated µg of each common band marked in the individual WB images from the six participants. Bars show the median values with the interquartile range; asterisks indicate significant post hoc differences between the sAA stimulations in each band; the letters the significant post hoc differences between the bands in each sAA stimulation (a with respect the band 1 at Tb and T+0 in all sAA stimulations; b with respect the band 2 at Tb and T+0 in all sAA stimulations; c with respect the band 1 at T+0 in the acetic acid stimulation, the psychological stress model, and the physical stress model).

3.1.1.3 Activity estimation from the two native sAA (GsAA and NGsAA) by a simple and fast to set up method (Experiment 1, Annex).

Aims and experimental design

A fast and simple method for the measurement of both NGsAA and GsAA was developed and validated. This method consisted of applying of a de-glycosylation procedure with CoA, and the measurement of the activity before (TsAA activity) and after (NGsAA activity) the de-glycosylation procedure. The difference between them was considered as GsAA activity.

Once validated, this method was applied to two stress models. One was a physical effort consisting of CrossFitWODs, where samples were obtained five min before the exercise (Tb), between rounds (T+01), after complete the exercise (T+02) and 10 min after (T+10). The second was a psychological challenge, the standardized TSST, where samples were collected 5 min before the interview (Tb), just after the arithmetic task (T+0), and 15 min later (T+15).

Results and discussion

De-glycosylation procedure validation. The imprecision of the de-glycosylation procedure was lower than 10%. Linear regression analysis showed R^2 higher than 0.995 with slopes not significantly different from one and intercepts not significantly different from zero. Overall, the de-glycosylation procedure demonstrated to reduce statistically the glycosylated band by an easy, repeatable, and accurate way.

Results of activity in the stress models. After the de-glycosylation procedure, the GsAA significantly decreased (Figure 5), and sAA activity in the stress models was reduced of average a 74.6 ± 11.63%. TsAA, GsAA, or NGsAA showed changes of different magnitude and significance in both stress models. Therefore, the measurement of the different sAA proteoforms activity by the present de-glycosylation method can provide additional information in situations of physical and psychological stress by a simple and fast way.

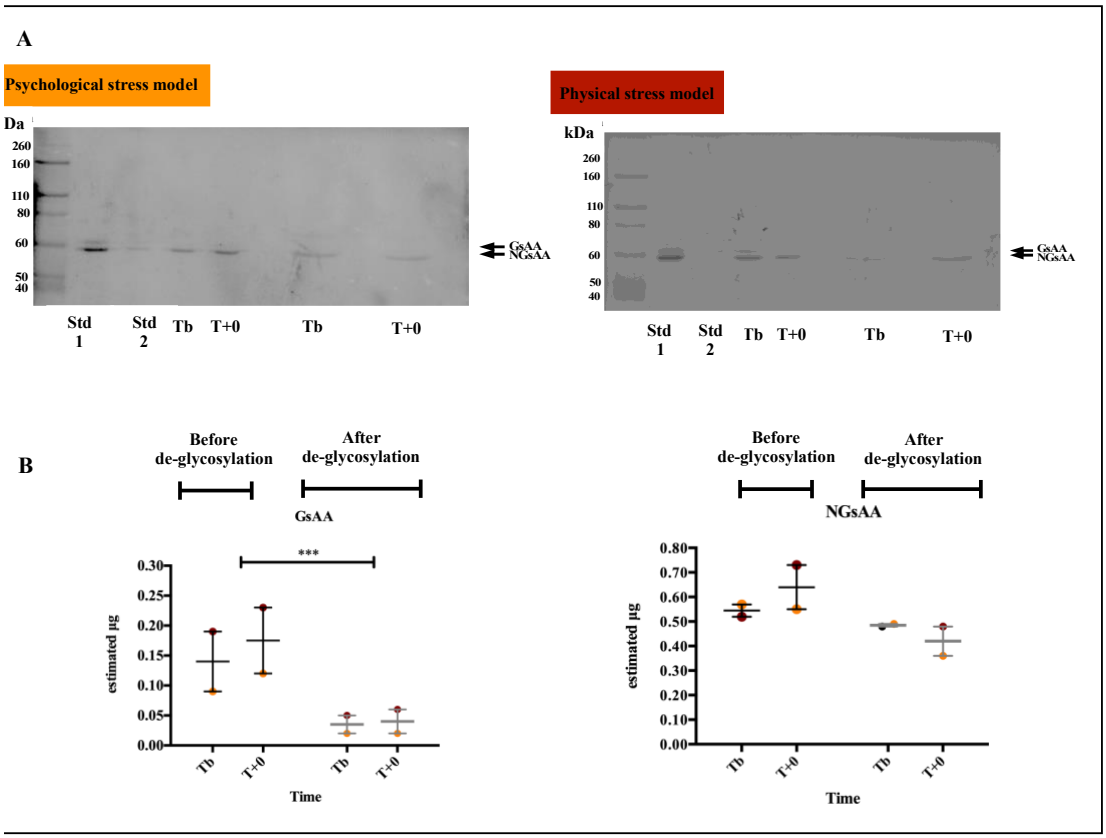


Figure 5. SDS-PAGE (**A**) and statistic study (**B**) from two participants during the TSST and the CrossFitWODs stress models, before and after the de-glycosylation procedure. Saliva samples were added at 1.5 µg per lane. The Standards (Std) were a natural commercial purified human sAA protein (77875, Abcam, Cambridge, UK) of known quantity. Molecular weight markers (Novex Sharp Pre-Stained, Invitrogen, Carlsbad, California). The arrows show the sAA proteoforms at 59 kDa (up) and 56 kDa (down). Asterisks indicate significant post hoc difference with respect the previous time.

3.1.2 Study in dogs

3.1.2.1 Is the sAA present in dog's saliva? (paper nº 3).

Aims and experimental design

This experiment aimed to demonstrate the presence of sAA in the saliva of dogs. For this purpose, a WB was performed with the antibody produced as described in point 2.3.2. In addition, a spectrophotometric assay for the measurement of sAA activity was

analytically validated in dog saliva and was applied to an experimentally induced sympathetic activation (Kutzler, 2005) to evaluate if the sAA activity could change in this situation.

The samples used for the WB were a pool from four canine saliva specimen obtained after the sympathetic activation at 25 µg of total protein, and 10 µg of total protein from a concentrated saliva specimen from a healthy dog. The sympathetic activation model consisted on semen collection in 10 healthy male beagles, where saliva samples were obtained at least 30 min before the ejaculation at basal time (TB), just starting the ejaculation (T-0), just after ejaculation (T + 0), and 30 min later (T + 30).

Results and discussion

Detection of sAA in the WB. A band with a low intensity was identified in both the concentrated and non-concentrated dog saliva between 60 kDa and 50 kDa, where the human saliva specimen and the purified human sAA (ab 77,875, Abcam) was also detected. This confirms the existence of sAA in dog saliva. Compared to human saliva, the lower intensity band observed in dogs was probably due to the lower sAA concentration that dogs have compared to humans, by following per under the SDS-PAGE findings and enzymatic activity results obtained in our study (see below).

Analytical validation. The intra- and inter-assay imprecision was lower than 15%. It showed a good linearity in serially diluted saliva pools, with mean R_2 of 0.998 and slopes not significantly different from one, and the intercepts not from zero. The LLD and LLOQ were 1.6 IU/L, lower than the minimum recorded value in the experimental model (15.2 U/L). These results demonstrated that the spectrophotometric assay for the measurement of sAA activity was precise, accurate, and sensitive.

Results in the experimentally induced sympathetic activation. The sAA median activity significantly increased just after ejaculation compared with just before the ejaculation, indicating that sAA could increase after sympathetic activation in this species. The median values observed in the dogs of our study (89.5 IU/L) were much lower than those obtained in humans after psychological stress or intense exercise (Koibuchi & Suzuki, 2014; Nater & Rohleder, 2009; Rohleder & Nater, 2009).

3.1.3 Studies in horses

3.1.3.1 Relation between sAA activity and pain in horses with AAD (paper nº 4).

Aims and experimental design

To evaluate sAA as a possible pain-induced stress biomarker in horses, a prospective observational study was performed in a group of privately owned horses with AAD, and compared with a group of healthy control horses, by an unpaired Student's t-test. Also, sAA activity was compared with its EAAPS-1 scale (pain scale), HR and RR values, plasma LA concentration, SIRS score, and SAA concentration by a Spearman correlation test.

Results and discussion

In 19 horses with AAD due to a variety of causes resolved by medical treatment, sAA activity (24.5 median-fold, $P < 0.001$) and cortisol (1.7 median-fold, $P < 0.01$) were significantly higher than in 11 healthy horses. The highest values of sAA (706.7 U/L and 680.9 IU/L) were presented in the two no-survivor horses. Also, a significant correlation between sAA activity and the pain scale ($r = 0.78$, $P < 0.001$), and the SIRS score ($r = 0.49$, $P < 0.05$), was observed (Table 3). It was not observed with cortisol.

Overall, based on our results, sAA activity, but not cortisol, is related to the pain level in horses with AAD than with the inflammation state in that disease.

Table 3. Coefficients of correlation between sAA activity and the EAAPS-1, HR and RR, plasma LA, SIRS score, and SAA, in 19 horses with AAD.

	sAA (IU/L)	*P* values
EAAPS-1 score	0.78 (0.38, 0.89)	**< 0.001**
HR (beats/min)	0.22 (−0.27, 0.62)	0.362
RR (breaths/min)	0.32 (−0.17, 0.68)	0.184
SAA (µg/mL)	0.29 (−0.21, 0.67)	0.235
Plasma LA (mmol/L)	0.44 (−0.05, 0.76)	0.069
SIRS score	0.49 (0.03, 0.78)	**0.032**

Results are expressed as Spearman r-value (95% CI). SIRS score (4 point-score) is based on the number of abnormal SIRS criteria among the following: HR > 52 beats/min, RR > 20 breath/min, WBC above or below 5.0-12.5 × 10^9/L, and temperature below or above 37.0-38.5 ºC.

3.1.3.2 Use of sAA to objectively evaluate critical illness and prognosis for survival in horses with AAD (paper nº 5).

Aims and experimental design

A prospective study was performed in healthy horses and horses with AAD referred to the Veterinary Teaching Hospital of the University of Extremadura (Spain), where sAA activity, using the colorimetric commercial kit, and concentration, using the TR-IFMA, were measured. Previously, the TR-IFMA was analytically validated in horse saliva. Then, associations between survival to discharge and sAA activity and concentration, and other clinical parameters were examined using univariable logistic regression and Spearman correlation.

Results and discussion

Analytical validation of TR-IFMA for sAA. The intra-assay precision was 11.9 ± 8.2%, and the linearity under dilution test yielded a mean R^2 of 0.947 ± 0.010. The LLD was 4.5 ng/mL. These results demonstrated that the TR-IFMA used to measure sAA concentration was precise, accurate, and sensitive.

sAA activity and concentration values. The 33 horses with AAD treated medically or by surgery included in this study showed higher sAA activity and concentration ($P < 0.001$) than the 25 healthy horses. Eight horses did not survive, having sAA activity, but not concentration, significantly higher compared to survivors. The optimal cut-off point for predicting non-survival was a sAA activity of ≥151.2 IU/L with a resulting sensitivity of 75% and specificity of 84%, with higher sensitivity than for sAA concentration.

Association with the outcome and other clinical variables. sAA activity and concentration correlated moderately ($P < 0.001$). Both correlated moderately with the HR ($P < 0.001$) and plasma LA concentration ($P < 0.001$). However, only sAA activity correlated with the SIRS score ($r = 0.43$, $P = 0.01$) and was associated with survival (Table 6). These results suggest that sAA activity is preferred to sAA concentration on evaluating the prognosis of horses with AAD.

Table 6. Univariate analysis of associations between non-survival and sAA activity and concentration, HR, RR, plasma LA concentration, and SIRS score in 33 horses with AAD.

Variable	Crude odds ratio[a] (95% CI)	P value
sAA activity (IU/L)	2.30 (1.18-4.42)	**0.01**
sAA concentration (ng/mL)	0.91 (0.60-1.37)	0.7
HR (beats/min)	5.41 (1.52-20.00)	**0.009**
RR (breath/min)	2.42 (1.21-4.84)	**0.01**
Plasma LA (mmol/L)	6.76 (1.48-30.94)	**0.01**
SIRS score (0-4)	2.82 (1.15-6.94)	**0.02**

[a]The Crude odds ratio is the increase in odds of non-survival for every one-unit increase in the variable with non-survival being the referent.

3.1.4 Study in pigs

3.1.4.1 How can the sAA activity, concentration and isoforms change in pigs after a stressful situation? (paper nº 6).

Aims and experimental design

Employing the standardized nasal snare immobilization commonly used in veterinary practice, which has been demonstrated to produce high stress to pigs (Escribano, Soler, Gutiérrez, Martínez-Subiela, & Cerón, 2013; Martínez-Miró et al., 2016), sAA was evaluated by the spectrophotometric assay (activity), the TR-IFMA (concentration), and by WB (proteoforms). The TR-IFMA was previously validated with pig saliva.

Six pigs were subjected to the temporary restraining for at least 1 min with a nasal snare or loop (Escribano et al., 2013). Saliva was obtained the day before the experiment took place (basal level, Tb), 5 min before restrain (T-5), for the first 30 s while the nasal snare immobilization was performed (T + 0A), for the second 30 s during immobilization (T + 0B), just after immobilization (T + 0C) and 15 min later (T + 15).

Results and discussion

Analytical validation of TR-IFMA for sAA. The intra- and inter-assay precision was < 10%. The linearity under dilution test yielded a mean R_2 of 0.978 ± 0.006. The LLD and the LLOQ calculated were 1.4 ng/mL and 18.14 ng/mL, respectively. These results demonstrated that TR-IFMA used to measure sAA concentration was precise, accurate, and sensitive.

Changes in sAA measurements. sAA activity showed significant changes along the times ($P < 0.05$), but the multiple comparisons test did not show any significant increase at any time. However, sAA concentration did not show significant changes along the time. In addition, both did not correlate ($P = 0.338$). This divergence between activity and concentration could be due to different activity of the sAA proteoforms, as observed in the WB analysis performed in saliva from three pigs (Figure 6). The bands with different intensity according to the sampling time and sAA activity levels were observed at 59-60 kDa, but also at 65-66 kDa in some of them. This could open a new line for the evaluation of possible selected proteoforms of sAA as potential biomarkers of stress in pigs, for example, the proteoform with a molecular weight of 65–66 kDa since could be related to major response in sAA activity after stress.

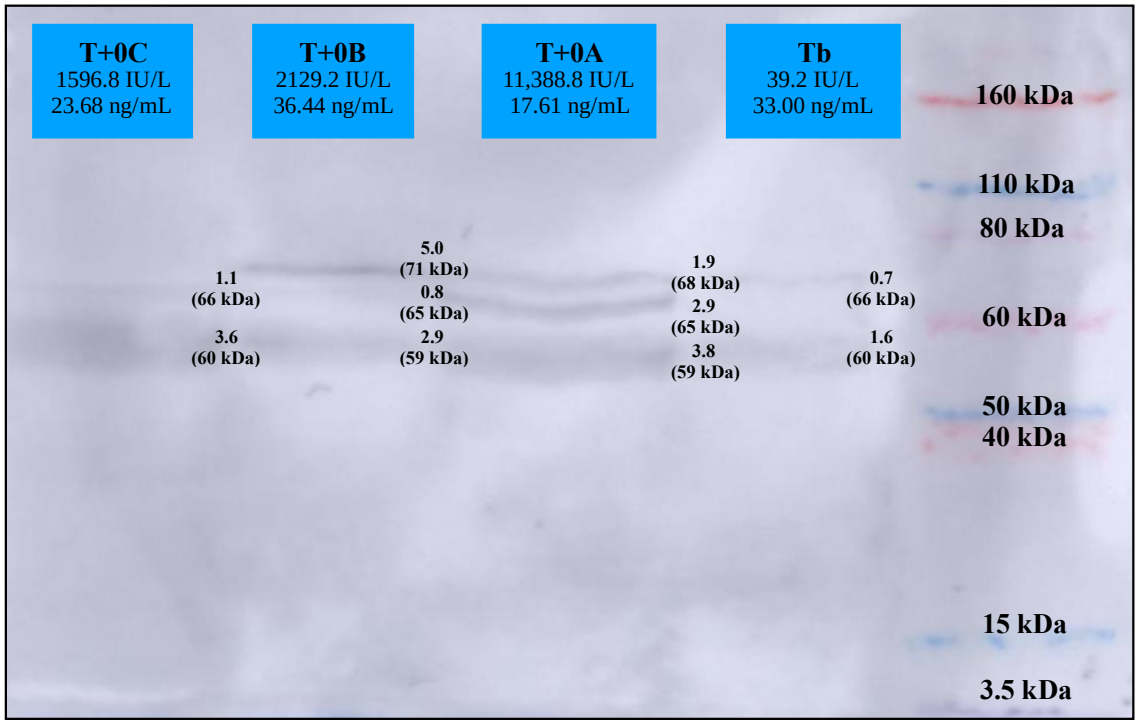


Figure 6. WB from the saliva of pigs 1 in the experimental nasal snare immobilization. The saliva samples (1 ng of sAA per well) was added at Tb, T + 0A, T + 0B, and T + 0C. Molecular weight markers (Novex Sharp Pre-Stained, Invitrogen, Carlsbad, California). Numbers next to the positive bands in pig's saliva indicate the average intensity ($\times 10^3$).

3.2 Objective 2

The objective 2 was achieved by six studies corresponding to paper nº 7 and nº 8, and experiment nº 2 (Annex) performed in horses; article nº 9 in pigs; article nº 10 in sheep; and nº 11 in cows.

3.2.1 Studies in horses

3.2.1.1 Equine AAD: a sialochemistry approach (paper nº 7)

Aims and experimental design

A panel of 23 analytes was analytically validated in saliva of horses, and the possible changes in these analytes in a pilot study with six healthy horses and six horses with AAD were evaluated. Then, the analytes with significant changes were evaluated in a larger population of healthy and diseased horses.

Diseased horses were diagnosed in the Veterinary Teaching Hospital of the University of Extremadura (Spain) as having acute gastrointestinal disease due to a variety of causes. In contrast, healthy ones were horses admitted for castration or routine health check in the Veterinary Teaching Hospital of the University of Extremadura and of privately owned horses from a stable in the province of Almería (Spain).

Results and discussion

In the pilot study, the analytes that showed a significant increase in horses with the AAD compared to healthy horses were gGT, CK, urea, total bilirubin, TP, phosphorus, and sAA, which confirm its increase in the diseased horses when they were evaluated in the broader population (Figure 7).

Elevations in serum of gGT and total bilirubin have been associated with several causes of AAD in horses (Gardner, Nydam, Mohammed, Ducharme, & Divers, 2005). Increases in CK in saliva in situations of muscle damage have been described in dogs (Tvarijonaviciute et al., 2017) and humans (Barranco et al., 2018), as it has been described as occurring in the AAD by ischemic injuries (Krueger, Ruple-Czerniak, & Hackett, 2014). Increases in serum urea with expected creatinine values are associated with reduced hydration status (Morgan, Carver, & Payne, 1977) or bleeding in the gastrointestinal tract (Ernst, Haynes, Nick, & Weiss, 1999). A higher concentration of TP observed in saliva in horses with AAD could also indicate reduced hydration

status, as previously described in serum (Muñoz, Riber, Trigo, Castejón-Riber, & Castejón, 2010). In this study, phosphorus in saliva was correlated with the SIRS score but not with phosphorus in plasma; therefore, further studies should be performed to clarify the mechanisms of the presence of phosphorus in saliva. Finally, the increases in sAA activity on horses with the AAD agreed with the previous studies performed in horses.

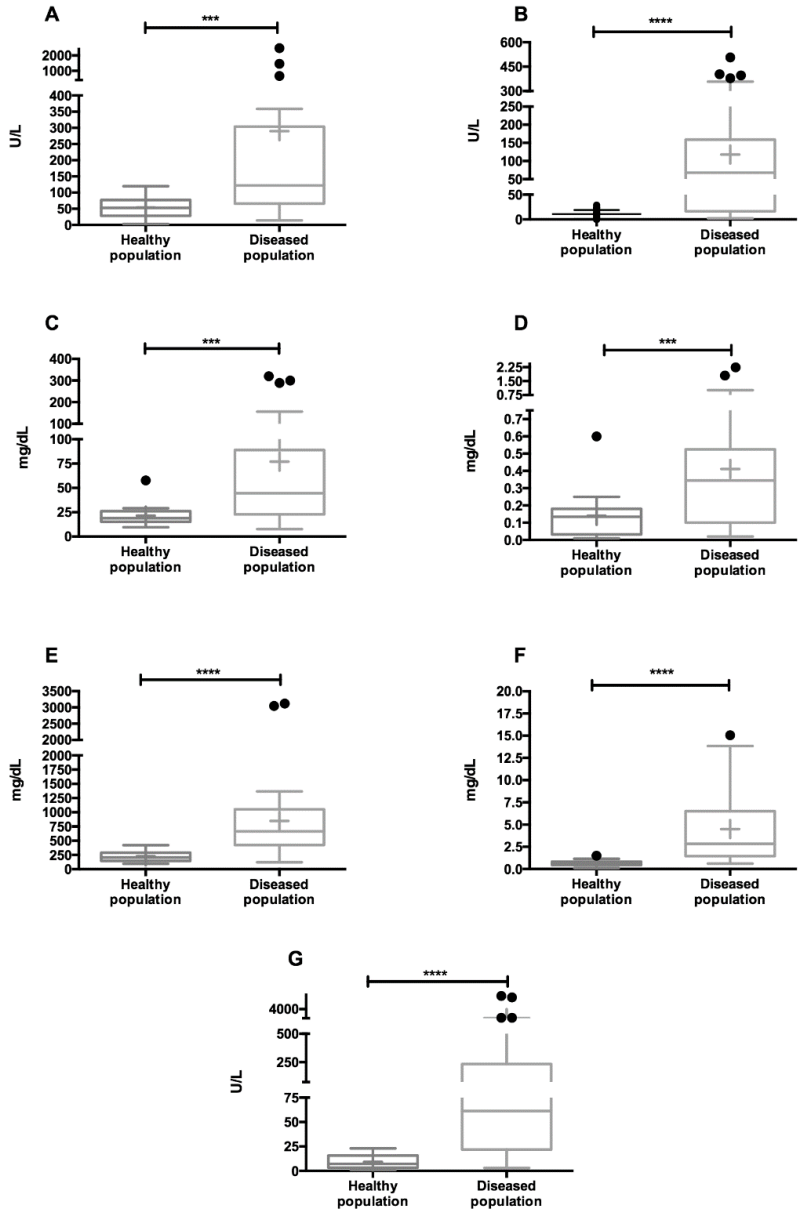


Figure 7. Results of gGT (**A**), CK (**B**), urea (**C**), total bilirubin (**D**), TP (**E**), phosphorus (**F**), and sAA (**G**) in saliva of the healthy horses population (n = 20) and the diseased horses population (n = 37) with AAD. The plot shows median (line within box), 25th–75th percentiles (box), 5th and 95th percentiles (whiskers) and outliers (•). The cross inside the box shows the mean. Asterisk indicates statistically significant differences between groups.

3.2.1.2 Association between the salivary stress biomarkers and the behavioral reactions to an acute stressor in horses (paper nº 8)

Aims and experimental design

This pilot study was performed in the Station Biologique of Paimpont (France) with the help of the `Laboratoire Ethologie et humaine´ of the `Université de Rennes´ (UMR CNRS 6552).

It consisted in evaluating the changes of sAA activity, Lip, TEA, BChE, ADA, and cortisol, and their potential link with horses' emotionality expressed by behavior after a fearfulness test based on suddenness: opening an umbrella in front of riding horses just after releasing them in an indoor familiar covered arena (Figure 8, **A**). The horses' behavior was video-recorded at T3, evaluating behavioral patterns related to the fearfulness (Hausberger, Bruderer, Scolan, & Pierre, 2004; Lesimple, Fureix, LeScolan, Richard-Yris, & Hausberger, 2011; Stomp et al., 2018) to calculate their frequencies of occurrence and two indexes previously validated (emotionality and laterality) (D'Ingeo et al., 2019; Hausberger et al., 2004; LeScolan, Hausberger, & Wolff, 1997) at each horse.

Results and discussion

Changes in the salivary stress biomarkers. Salivary alpha-amylase (P = 0.050), Lip (P = 0.002), TEA (P = 0.009) and BChE (P = 0.004) showed significant changes between the sampling times as happened with the cortisol (Figure 8, **B**).

Association with emotionality behavioral patterns. BChE was correlated positively with the index of emotionality (r = 0.68, P = 0.049) and with the alarm acoustic signals (snoring/blowing) (r = 0.69, p = 0.046); and sAA negatively with "quiet behaviors" such as "sniffing at the ground" (r = −0.65, P = 0.043).

Therefore, this preliminary research opens the possibility of wider use of selected biomarkers in saliva for evaluating acute stress in horses, such as BChE, for being the most reliable predictor of behavioral responses or sAA of quieter behaviors.

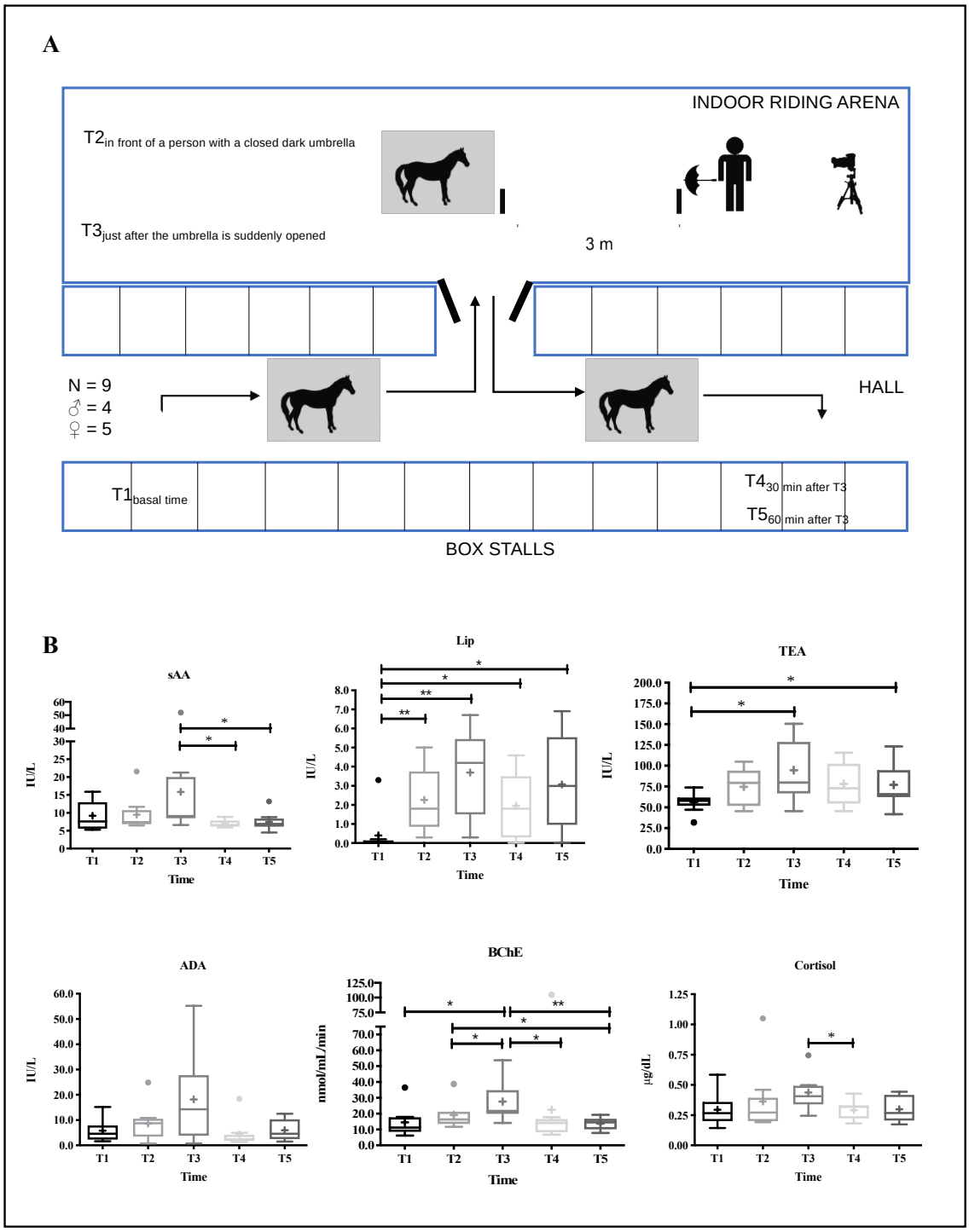


Figure 8. Scheme of the sudden stress experimental design (**A**), and results (**B**) of sAA, Lip, TEA, BChE, ADA, and cortisol. The plots show median (line within box), 25th and 75th percentiles (box), 5th and 95th percentiles (whiskers), and outliers (•). The cross inside the box shows the mean. Asterisks indicate a statistically significant difference between times.

3.2.1.3 Changes in salivary biomarkers in endurance horses after an increasing intensity exercise (Experiment 2, Annex)

Aims and experimental design

Salivary alpha-amylase activity, cortisol, LA, and CK in saliva were evaluated in 10 horses during a SET, where samples were obtained at the basal time (TB), after the seven bouts of velocity increased by 3 km/h (T+01 to T+07), and 5, 15, 30, and 45 min later (T+5, T+15, T+30, and T+45).

Besides, possible association with HRV parameters related to sympathetic and parasympathetic tone, fitness level (V_4 and V_{200}), and skeletal muscle damage by measuring CK in plasma, was also evaluated.

Results and discussion

Changes of the selected salivary biomarkers during the SET. Increases in sAA ($P = 0.01$) and CK ($P = 0.002$) levels were only observed in horses' saliva due to the SET. Specifically, the increases compared to TB were observed at T+04 ($P = 0.03$), T+06 ($P = 0.01$), and T+07 ($P = 0.009$) in CK, and at T+30 ($P = 0.03$) and T+45 ($P = 0.03$) in sAA. Further studies should be performed to clarify the sAA delayed-release after exercises of different intensities and durations when in human sAA activity increases immediately after an intense physical effort (Koibuchi & Suzuki, 2014; Strahler et al., 2017).

Correlation results. sAA and CK in saliva were positively correlated (r values = 0.53-0.72) with the sympathetic tone and negatively (r values = –0.53 - –0.70) with the parasympathetic tone, not with the fitness levels parameters. Only a low positive correlation ($P < 0.001$) was observed between CK in saliva and plasma, and no changes in plasma CK were showed during the SET. This divergences between saliva and plasma CK response would indicate that salivary glands could directly synthesize CK.

These results would indicate that sAA and CK in saliva could be more related to the perception of intensity in exercise, and therefore the exercise-related stress, than to the horses' fitness level.

3.2.2 Study in pigs

3.2.2.1 Association between selected salivary biomarkers and the grade of pain, distress and discomfort in pigs with lameness and prolapses (paper nº 9)

Aims and experimental design

A panel of stress (cortisol, sAA activity, TEA, BChE, and Lip) and immunity (ADA, isoenzyme 1 -ADA1- and 2 -ADA2-) biomarkers, among others, was evaluated in 22 healthy pigs and 44 pigs with lameness and rectal prolapse subdivided in those without (score < 4) and with (score ≥ 5) evident pain, distress and discomfort according to a new score system by modifying a protocol previously published (Morton & Griffiths, 1985) (Table 7, Appendix 1). Then, the salivary biomarkers were correlated with their distress status.

Results and discussion

Changes in saliva analytes: Lame pigs and prolapsed pigs with pain showed higher salivary levels of cortisol ($P < 0.05$ and $P > 0.001$), sAA ($P < 0.01$ and $P > 0.01$), TEA ($P < 0.05$ and $P > 0.001$), BChE ($P < 0.05$ and $P > 0.01$), ADA1 ($P < 0.01$ and $P > 0.001$), and ADA2 ($P < 0.05$ and $P > 0.01$), respectively, compared with the healthy pigs.

Association with the distress status: Salivary cortisol, TEA, BChE, ADA1, and ADA2 correlated moderately with the pain score. Therefore, these salivary biomarkers could be used as tools for pain assessment in lame and prolapsed pigs.

3.2.3 Study in sheep

3.2.3.1 Salivary biomarkers for the acute stress assessment in sheep (paper nº 10)

Aims and experimental design

In this experiment, Lip, BChE, TEA, ADA in sheep's saliva were analytically validated and evaluated during two acute stress episodes: (1) being faced with a dog, and (2) being sheared. Then, they were compared with results of cortisol and sAA activity obtained in saliva, which have previously proven to be stress biomarkers in sheep (Fuentes-Rubio, Fuentes, Otal, Quiles, & Luisa Hevia, 2016), as well with the HR results. The results obtained in both stress episodes were compared with those collected from a control group sampled without any stress conditions.

Results and discussion

Analytical validation. Lipase, BChE, TEA, and ADA assays showed within-run CVs lower than 5% and a R^2 higher than 0.990 when linearity under dilution was assessed. Therefore, all these assays in sheep's saliva were precise and accurate.

Changes in the acute stress episodes. Depending on the intensity or the duration of the stressful stimulus, salivary biomarkers changed differently along the time. The Lip was the only analyte that showed significant changes between the stress and the control group in both experiments (Table 8, Appendix 1), with less overlap between groups than sAA activity and cortisol. Overall, it can be concluded that the panel of analytes measured in this study in sheep's saliva can be used for evaluating acute stress episodes, with a particular interest in the Lip.

3.2.4 Study in cows

3.2.4.1 Biochemical changes in saliva due to inflammation states in cows (bovine mastitis) (paper nº 11)

Aims and experimental design

In this study, a panel of salivary biomarkers related to stress, inflammation and immune system, and oxidative stress, in which sAA activity, cortisol, ADA, BChE, and LA were included; were measured in 18 cows with active clinical mastitis and 18 clinically healthy cows to evaluate possible changes that in saliva can occur in inflammatory states in cattle. Previously, all these analytes in saliva were validated.

Results and discussion

Significant increases in sAA ($P = 0.028$) and cortisol ($P = 0.038$) were observed in the the mastitis group, indicating possible activation of the SNS and the HPA axis. Higher LA levels in saliva were observed in mastitis group ($P = 0.015$), correlating with some parameters of oxidative stress in saliva and serum. Finally, a significant decrease in BChE activity in saliva was detected in cows with mastitis.

These results indicate that cows with active mastitis show changes in salivary biomarkers that can reflect the presence of stress, inflammation, and oxidative stress in the animals.

3.3 Objective 3

The objective 3 was addressed by the paper nº 12, and the experiment nº 3 described in Annex 1, performed both in horses.

3.3.1 Studies in horses

3.3.1.1 Effect of contamination with food and the collection material in the measurement of biomarkers in the saliva of horses (paper nº 12).

Aims and experimental design

Different types of food (oats, grass, and hay) were incubated with horse saliva after cleaning their mouth in an *in vitro* experiment. This was made to evaluate the influence that the presence of food can have on the results of the salivary biomarkers previously evaluated in horses in the present thesis (TP, sAA activity, cortisol, Lip, TEA, BChE, ADA, phosphorus, gGT, Urea, total bilirubin, and CK).

In addition, the influence that different materials for saliva collection (cotton or synthetic polypropylene sponge) can have in the results was assessed by an *in vivo* experiment where saliva samples were obtained with both materials in clean (after washing the mouth) and dirty (just after a feed) saliva.

Results and discussion

Presence of food in the saliva (in vitro experiment). Significant increases were observed in TP ($P = 0.050$) and phosphorus ($P = 0.008$) when saliva was incubated with oats; in TP ($P = 0.037$), sAA activity ($P = 0.018$), TEA ($P = 0.018$), BChE ($P = 0.037$), ADA, $P = 0.037$), and total bilirubin ($P = 0.018$) with grass; and in sAA activity ($P = 0.018$), phosphorus ($P = 0.037$), gGT ($P = 0.004$), and CK ($P = 0.016$) with hay.

Influence of the different materials for saliva collection (in vivo experiment). Lower values were obtained in clean saliva collected with cotton role compared to sponge for sAA activity ($P = 0.030$), TEA ($P = 0.034$), BChE ($P = 0.003$), gGT ($P = 0.002$) and cortisol ($P < 0.001$).

Overall, this study proves that, when biomarkers are going to be measured in saliva of horses, ideally clean saliva and the same absorbent-based collection method during all the experiments must be used to have consistent results. In the case of being not possible, researchers

should be aware that these are factors that can affect salivary analyte measurements, and comparison of results from different studies should be considered.

3.3.1.2 Effect of the circadian rhythm in the analytes in the horse's saliva (Experiment 3, Annex).

Aims and experimental design

The possible effect of the circadian rhythm in the basal levels of cortisol, sAA activity, Lip, TEA, BChE, ADA, gGT, CK, urea, total bilirubin, TP, and phosphorus in horses' saliva was assessed in a population of five clinically healthy mares by collecting saliva from 06.30 to 20.30 each 2 h during two consecutive days on two different photoperiod seasons: winter (sunrise 08.02 and sunset 17.45) and spring (sunrise 06.45 and sunset 21.19).

Results and discussion

Changes along the hours of the day. Although temperature and relative humidity did not change along the hours of the day in each sampled season, changes along the hours of the day were observed in cortisol, sAA activity, TEA, BChE, ADA, and CK (Figure 9). Therefore, the sampling time must be considered in the interpretation of these analytes.

Changes between seasons. Although significant changes were observed between season in temperature and relative humidity, no thermal discomfort was observed. However, the values obtained in sAA activity, Lip, and BChE were significantly different between seasons (Figure 9).

In conclusion, circadian rhythm can modify the basal levels of cortisol, sAA activity, TEA, BChE, ADA, and CK in horses' saliva along the hours of the day and between season. Therefore, the time of the day and season influence saliva analytes in horses and should be taken into consideration for their interpretation.

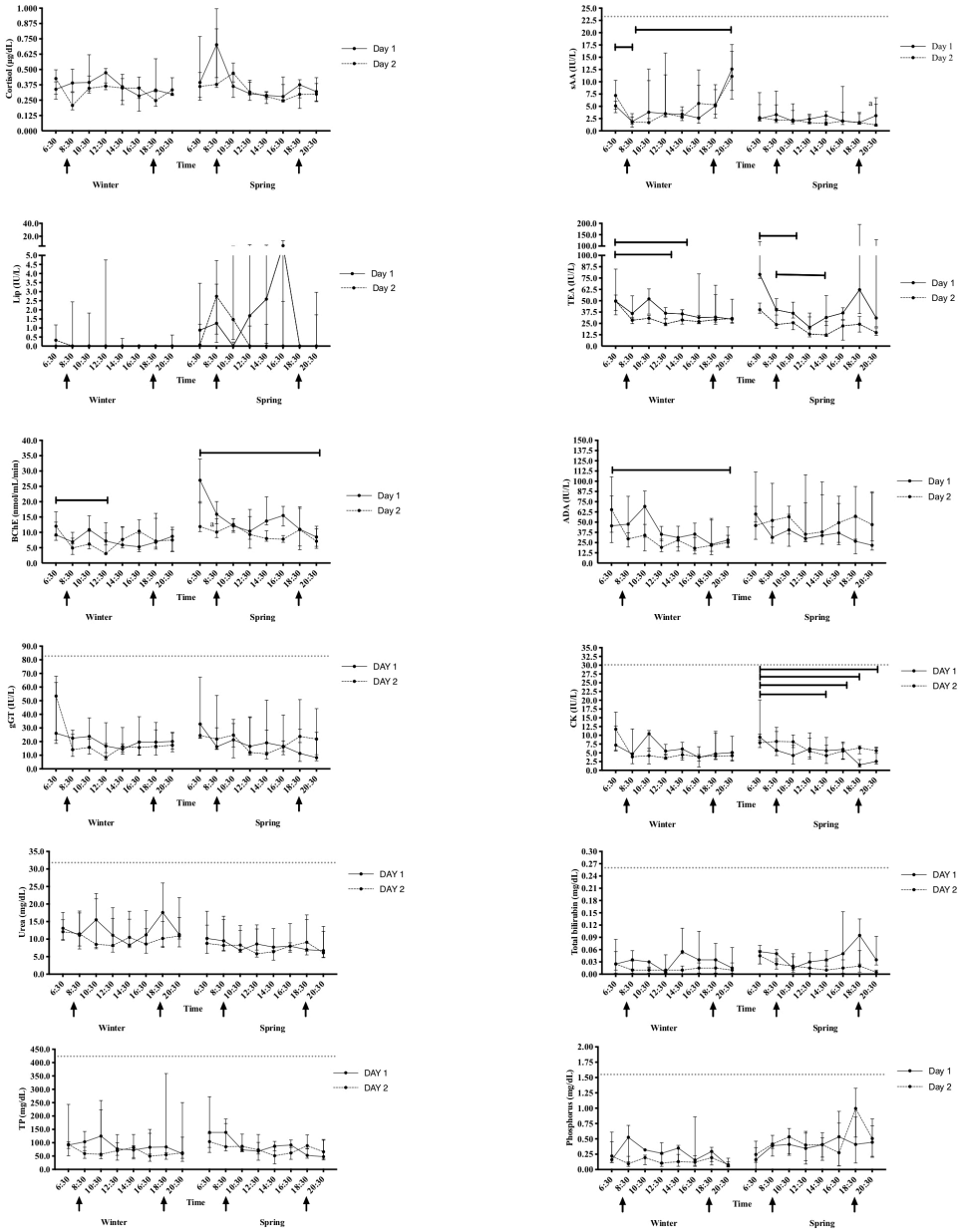


Figure 9. Median and IQR results of cortisol, sAA, Lip, TEA, BChE, ADA, gGT, CK, urea, total bilirubin, TP, and phosphorus in the saliva of five mare horses measured along different hours of the day on two different seasons (winter – December vs. spring – May). Solid lines and letters above results show the significant changes (Fisher's LSD test) between the hours of the day and between seasons, respectively, when occurring for both days. Dot lines show the cut-off points in the salivary analytes for predicting disease due to AAD in horses established in the paper nº 7. Arrows under the X-axis reflect the times of feeding.

BOOK CHAPTER

BOOK CHAPTER 1 (Published)

Salivary Glands' Anatomy and Physiology

Editorial: Springer

Abstract: This contribution aims to show the anatomical and physiological characteristics of the salivary glands as entity to produce saliva, and to present the composition of the "saliva" fluid as a protective medium for the mouth, the start of digestion and as diagnostic medium. All this from a comparative point of view between humans and animals.

URL: https://www.springer.com/gp/book/9783030376802

ARTICLES

OBJECTIVE 1

To gain knowledge about sAA, especially by the study of the optimal way of report the results, the investigation of possible proteoforms' changes at different stress situations, and the validation of assays in different species such as in dogs, horses, and pigs.

IN HUMANS

ARTICLE 1 (Published)

Influence of the way of reporting alpha-Amylase values in saliva in different naturalistic situations: A pilot study

Journal: PLos One

Abstract: The objective of this pilot study was to compare the different ways of measuring salivary alpha-amylase (sAA, enzymatic vs. concentration) and to evaluate the influence that the different ways of reporting the results can have in sAA interpretation. For this purpose, sAA was measured by direct quantification and also by an enzymatic assay in three different naturalistic situations, a physical stressor (situation 1) and two mental stressors of different intensity (situations 2 and 3). The results were expressed in three different ways (without correction, multiplied by flow rate and divided by protein concentration). sAA concentration and activity increased just after situations 1 and 3. When values were multiplied by the flow rate, significant changes after situation 1 were detected only for sAA activity but not for sAA concentration, being these changes of lower significance and magnitude that those observed for sAA activity without any correction. In addition, a significant increase in sAA activity was found at T+15 in situation 2. In situation 3 the significant decrease in sAA at T+15 disappeared. When values were divided by protein concentration, there were no significant changes in situations 1 or 3, but a decrease in situation 2 at T+0 and an increase at T+15. sAA activity and concentration showed a significant correlation in all situations. This pilot study points out that the way of expressing sAA can influence the results obtained in different stress models and also their interpretation. Therefore, how sAA is reported and the factors involved in the different ways of expressing sAA, should be taken into consideration for an objective interpretation of sAA values.

URL: https://www.springer.com/gp/book/9783030376802

IN HUMANS

ARTICLE **2** (Published)

Variation of human salivary alpha-amylase proteoforms in three stimulation models.

Journal: Clinical Oral Investigations

Abstract: To evaluate the sAA proteoforms' expression during different stimulation situations. This study evaluated the salivary alpha-amylase (sAA) proteoforms' behavior by western blot (WB) analysis and high-resolution mass spectrometry (LC-MS/MS) in different situations that produce increases in sAA activity. For this purpose, six healthy women with a similar body mass index,age, and fit, underwent different sAA stimulation tests, such as acetic acid stimulation, psychological stress using the standardized Trier social stress test, and physical effort using the Cooper treadmill test. The three models showed an increase in sAA activity. The WB demonstrated seven common bands observed in the six women (band one at 59 kDa, two at 56 kDa, three at 48 kDa, four at 45 kDa, five at 41 kDa, six at 36 kDa, and seven at 14 kDa), in which sAA protein was identified. The individual WB analysis showed that band two, which corresponded to the native non- glycosylated sAA proteoform, had a higher increase after the three sAA stimulation inducers, and this band was also the only proteoform correlated with sAA activity ($r = 0.56$, $P= 0.001$). In addition, when the label-free quantification analysis was performed, the different proteoforms showed different responses depending on the type of stimulation. This preliminary study showed that the diverse sAA proteoforms' expression depends on the different stimulation models. This study opens new perspectives and challenges for the use of the different alpha-amylase proteoforms as possible biomarkers in addition to the sAA activity.

URL: http://doi.org/10.1007/s00784-019-03021-9

IN DOGS

ARTICLE **3** (Published)

Detection and measurement of alpha-amylase in canine saliva and changes after an experimentally induced sympathetic activation.

Journal: BMC Veterinary Research

Abstract: Salivary alpha-amylase (sAA) is considered a biomarker of sympathetic activation in humans, but there is controversy regarding the existence of sAA in dogs. The hypothesis of this study was that sAA exists in dogs and it could change in situations of sympathetic stimulation. Therefore, the aims of this study were: 1) to demonstrate the presence of alpha-amylase in saliva of dogs by Western-Blot, 2) to validate an spectrophotometric method for the measurement of sAA activity and 3) to evaluate the possible changes in sAA activity after the induction of an ejaculation in dogs which is known to produce a sympathetic activation. Western-Blot demonstrated a band in dog saliva specimens between 60 kDa and 50 kDa, similar to purified sAA. The spectrophotometric assay validated showed an adequate inter- and intra-assay precision, and a high correlation coefficient (r = 0.999) in the linearity under dilution study. sAA median activity significantly increased just after ejaculation compared with just before the ejaculation (2.06-fold, P =0.005). This study demonstrated the existence of alpha-amylase in saliva of dogs and that this enzyme can be measured by a spectrophotometric assay. In addition, results showed that sAA increase after a sympathetic activation and could be potentially used as non-invasive biomarker of sympathetic activity in this species.

URL: http://doi.org/10.1186/s12917-017-1191-4

IN HORSES

ARTICLE 4 (Published)

Salivary alpha-amylase activity and cortisol in horses with acute abdominal disease: a pilot study.

Journal: BMC Veterinary Research

Abstract: The aim of this study was to evaluate salivary alpha-amylase (sAA), considered a non-invasive biomarker for sympathetic nervous system (SNS) activity, and salivary cortisol as possible pain-induced stress biomarker, in horses with acute abdominal disease. Therefore, a prospective observational study was performed in which both biomarkers were analyzed in a group of horses with acute abdomen syndrome, and compared with a group of healthy control horses by an unpaired Student's t-test. In addition, the possible relationship between both biomarkers, the score in Equine Acute Abdominal Pain scales version 1 (EAAPS-1 scale), Heart Rate (HR) and Respiratory Rate (RR), plasma lactate, the systemic inflammatory response syndrome (SIRS) score and serum amyloid A (SAA) concentration was assessed by a Spearman correlation test. A total of 30 horses were included in the study, 19 with acute abdominal disease diagnosed as large colon displacements, simple impactions of the pelvic flexure, spasmodic colics and enteritis and 11 healthy ones. sAA activity (24.5 median-fold, $P < 0.0001$) and salivary cortisol (1.7 median-fold, $P < 0.01$) were significantly higher in horses with acute abdomen than in healthy horses. sAA activity was significantly correlated with EAAPS-1 scale ($r = 0.78$, 95% confidence interval [CI] 0.38–0.89, $P < 0.001$) and SIRS score ($r = 0.49$, 95% CI 0.03–0.78, $P < 0.05$). Neither sAA nor salivary cortisol correlated with HR, RR, plasma lactate and SAA. Although this study should be considered as preliminary one, alpha-amylase measurements in saliva could be a biomarker of pain-induced stress in horses with acute abdominal disease.

URL: http://doi.org/10.1186/s12917-018-1482-4.

IN HORSES

ARTICLE **5** (Published)

Salivary alpha-amylase activity and concentration in horses with acute abdominal disease: Association with outcome.

Journal: Equine Veterinary Journal

Abstract: Salivary biomarkers could be useful to objectively evaluate critical illness and prognosis for survival in horses with acute abdominal disease. To compare salivary alpha-amylase (sAA) activity and concentration in healthy horses and horses with acute abdominal disease, and evaluate the association between sAA activity and concentration with disease severity and outcome. sAA activity, measured using a colorimetric commercial kit, and concentration, measured using a Time-resolved immunofluorometric assay, in 25 healthy horses and in 33 horses with acute abdominal disease was compared using an ANOVA. Associations between survival to discharge and sAA activity and concentration and other clinical parameters were examined using univariable logistic regression and Spearman correlation. sAA activity and concentration were different between healthy (median = 4.3 [2.6–11.2] IU/L and 58.4 [53.4–80.6] ng/mL, respectively) and diseased (median = 29.8 [14.2–168.9] IU/L and 388.3 [189.1–675.8] ng/mL, respectively) (P<0.001). The sAA activity was higher in non-survivors (median = 479.0 [78.7–2064.0] IU/L, n = 8) compared to survivors (median = 19.3 [12.1–103.7] IU/L, n = 25, P<0.001) and sAA activity and concentration correlated (P<0.001) moderately with HR (r = 0.66 and r = 0.61, respectively). sAA activity correlated weakly with salivary cortisol (r = 0.45, P<0.001) and systemic inflammatory response syndrome score (r = 0.43, P<0.05), while activity and concentration correlated (P<0.001) moderately with plasma lactate concentration (r = 0.57 and r = 0.60, respectively). The sAA activity was significantly (P = 0.01) associated with increased risk of nonsurvival. In conclusion, the sAA activity, but not concentration, shows potential as a biomarker of prognosis for survival in horses with acute abdominal disease.

URL: http://doi.org/10.1186/s12917-018-1482-4.

IN PIGS

ARTICLE **6** (Published)

Changes in alpha-amylase activity, concentration and isoforms in pigs after an experimental acute stress model: an exploratory study.

Journal: BMC Veterinary Research

Abstract: Salivary alpha-amylase (sAA) is considered a non-invasive biomarker of acute stress that can be evaluated by changes in activity and concentration, and also by changes in its isoforms, although this last way of evaluation has never been used in veterinary medicine. This research evaluated the changes of sAA by three different ways in which sAA can be evaluated in an experimental acute stress model in six pigs based in a technique of temporarily restraining. These ways of evaluation were 1) activity by a spectrophotometric assay, 2) concentration by a fluorometric assay, and 3) isoforms of the enzyme by a Western blot. Although salivary cortisol significantly increased due to the stimulus of stress and all the pigs manifested signs of stress by high-pitched vocalization, sAA activity showed an increase of different degree in the six pigs after the stress stimulus, while sAA concentration showed decreases in four of the six pigs. sAA activity did not correlate with sAA concentration or salivary cortisol, and a low correlation was observed between sAA concentration and salivary cortisol ($r = 0.48$, $p = 0.003$). The inter-individual variability was higher in sAA activity than in sAA concentration and salivary cortisol. Finally, three possible isoforms of sAA at 154–160 kDa, 65–66 kDa and 59–60 kDa were observed that showed different dynamics after the stress induction. Although this pilot study's results should be taken with caution due to the low sample size, it reveals a different behavior between sAA activity and concentration in pig after an acute stressful stimulus leading to evident external signs of stress by high-pitched vocalization, and opens a new field for the evaluation of possible selected isoforms of sAA as potential biomarkers of stress.

URL: http://doi.org/10.1186/s12917-018-1581-2.

OBJECTIVE 2

To validate automated assays in saliva from horse, pig, sheep, and cow for the measurement of stress and welfare biomarkers different that sAA, and to study how these biomarkers behave at different situations related to psychological, physical, and inflammatory stressors.

IN HORSES

ARTICLE 7 (Published)

Changes in saliva analytes in equine acute abdominal disease: a sialochemistry approach.

Journal: BMC Veterinary Research

Abstract: The biochemical components of saliva can change in certain pathologies in horses, for example in acute abdominal disease. The aim of this study was (1) to evaluate if a panel of biochemical analytes usually used in serum can be measured in saliva of horses and (2) to study the possible changes of these biochemical analytes in saliva of horses affected by acute abdominal disease. A panel of 23 analytes was analytically validated in saliva of horses and possible changes in these analytes in a pilot study with six healthy horses and six horses with acute abdominal disease were evaluated. The analytes with significant changes were then evaluated in a larger population of 20 healthy and 37 diseased horses. Seven analytes showed significant increases in the pilot study which were confirmed in the larger population. The analytes which showed significant changes, and their median fold increase and significance shown in the larger population were salivary γ-glutamyl transferase (gGT, 2.3 fold, $P = 0.001$), creatine kinase (CK, 6.2 fold, $P < 0.001$), urea (2.3 fold, $P = 0.001$), total bilirubin (2.6 fold, $P < 0.001$), total proteins (3.2 fold, $P < 0.001$), phosphorus (P, 4.5 fold, $P < 0.001$) and alpha-amylase (sAA, 8.5 fold, $P < 0.001$). Total proteins, P and sAA showed sensitivities higher than 70% at their optimal cut-off points and a specificity of 100% in differentiating between healthy horses and those with acute abdominal disease. A panel of 23 biochemical analytes can be measured in saliva of horses, where gGT, CK, urea, total bilirubin, total protein, P and sAA levels are raised in horses with acute abdominal disease.

URL: http://doi.org/10.1186/s12917-019-1933-6.

IN HORSES

ARTICLE **8** (Published)

Changes in Saliva Analytes Correlate with Horses' Behavioural Reactions to An Acute Stressor: A Pilot Study.

Journal: Animals

Abstract: Acute stress induces an array of behavioural reactions in horses that vary between individuals. Attempts to relate behavioural patterns and physiological responses have not always given clear-cut results. Here, we measured the changes in a panel of salivary components: salivary alpha-amylase (sAA), lipase, total esterase (TEA), butyrylcholinesterase (BChE), adenosine deaminase (ADA), and cortisol, and their potential link with horses' behaviours after acute stress. Saliva samples were collected in nine riding horses subjected to a test consisting of opening an umbrella. Saliva sampling was obtained at a basal time point in the stall (T1), in the test indoor arena (T2), at a time of stress (T3), and 30 min (T4) and 60 min (T5) later. The horses' behaviour was recorded at T3 for 1 min. sAA, lipase, TEA, and BChE showed significant changes along time, increasing at T3 for BChE, and decreasing at T4 for sAA and BChE. Butyrylcholinesterase appeared to be the most reliable predictor of behavioural responses, as it correlated with the index of emotionality, of laterality, and the occurrence of alarm signals, while sAA decreased when horses expressed quieter behaviours. These first results bring promising lines for novel, more precise physiological markers of acute stress in horses that can bridge the gap between behaviour and physiology.

URL: https://doi.org/10.3390/ani9110993

IN PIGS

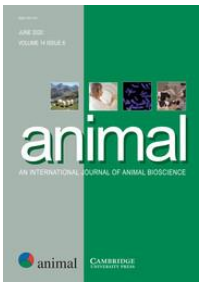
ARTICLE 9 (Published)

Application of a score for evaluation of pain, distress and discomfort in pigs with lameness and prolapses: correlation with saliva biomarkers and severity of the disease.

Journal: Research in Veterinary Science

Abstract: A score system was used to evaluate pain, distress and discomfort in healthy pigs and pigs with two different diseases: lameness and rectal prolapse. In addition, correlations between the results of this score and a panel of salivary biomarkers and severity of disease were studied. This panel included biomarkers of stress (cortisol, salivary alpha-amylase (sAA), total esterase activity (TEA), butyrylcholinesterase (BChE) and lipase (Lip)), immunity (adenosine deaminase isozymes 1 (ADA1) and 2 (ADA2)) and oxidative status (uric acid (UA), Trolox equivalent antioxidant capacity (TEAC), cupric reducing antioxidant capacity (CUPRAC), ferric reducing ability of saliva (FRAS), advanced oxidation protein products (AOPP) and hydrogen peroxide (H2O2)). Based on their score, diseased animals were subdivided in those without and with evident pain. Lame pigs and prolapsed pigs with pain showed higher salivary levels of cortisol, sAA, TEA, BChE, ADA1 and ADA2 compared with the healthy pigs. In addition, the prolapsed pigs with pain showed higher levels of FRAS, AOPP and H2O2 compared with the healthy animals. Salivary cortisol, TEA, BChE, ADA isozymes 1 and 2, FRAS and AOPP correlated with the pain score. This five-point pain score system can be easily applied to lame and prolapsed pigs, and salivary bio- markers could be used as an additional tool for pain assessment in those pigs.

URL: http://doi.org/10.1016/j.rvsc.2019.08.004.



IN SHEEP

ARTICLE **10** (Published)

Evaluation of new biomarkers of stress in saliva of sheep.

Journal: Animal

Abstract: Some routine handling procedures can produce stress in farm animals, and an adequate control of these stressors is important to avoid the negative effects on animal health and production. The measurement of biomarkers in saliva can be a suitable tool for the evaluation and control of stress. In this report, lipase, butyrylcholinesterase (BChE), total esterase (TEA) and adenosine deaminase (ADA) activities in the saliva of sheep were evaluated as biomarkers of stress. For this purpose, they were measured after inducing stress by facing a dog (experiment 1) and shearing (experiment2), and comparing them to other stress salivary biomarkers such as α-amylase (sAA) and cortisol, as well as heart rate (HR). Each analyte was measured at the basal time, and during and just after the end of the stressful stimulus, and at various times for the first hour after the period of stress induction. Values were compared with those obtained from a control group. Lipase was the only analyte that showed significant changes between the stress and the control group in both experiments. Although TEA and ADA increased after stress, no significant differences were seen compared with the control group. Lipase was correlated highly with sAA and HR, in experiment 1; and correlated moderately with cortisol and HR in experiment 2. Lipase showed the greatest percentage increase after the stressful stimuli and less overlap with the control group in the two experiments. From the results of this study it can be concluded that lipase, TEA, BChE and ADA are enzymes present in the saliva of sheep and that they can be measured by using simple and fast colorimetric methods. Further studies should be undertaken with regard to the possible application of lipase as a biomarker of stress in sheep.

URL: http://doi.org/10.1017/S1751731118002707.

IN COWS

ARTICLE **11** (Published)

Biochemical changes in saliva of cows with inflammation: A pilot study.

Journal: Research in Veterinary Science

Abstract: Saliva contains a variety of compounds that can change in local and systemic pathologies including inflammation. Although changes in acute phase proteins and markers of oxidative stress in saliva during inflammation in humans and different animal species have been described, no data exist about possible changes during inflammation in analytes in saliva of cows. The aim of the present study was to evaluate changes in selected salivary biomarkers of stress, inflammation and immune system, and oxidative stress in cows with inflammation. For this purpose, bovine mastitis was used as model. Saliva and serum from 18 clinically healthy cows and 18 cows with clinical mastitis were used in the study. A panel of analytes integrated by alpha-amylase, cortisol, haptoglobin, adenosine deaminase, cholinesterase, total antioxidant capacity, lactate, and uric acid was measured in all samples and differences between the two groups of animals were evaluated. Significant increases in cortisol, alpha-amylase, uric acid, lactate and significant decreases in cholinesterase were detected in saliva of cows with mastitis. These results indicate that that cows with mastitis show changes in salivary biomarkers that reflect presence of stress, inflammation and oxidative stress in the animals.

URL: http://doi.org/10.1016/j.rvsc.2019.04.019.

OBJECTIVE 3

To evaluate possible factors that would affect the interpretation of results in the biomarkers of saliva.

IN HORSES

ARTICLE **12** (Published)

Effect of food contamination and collection material in the measurement of biomarkers in saliva of horses.

Journal: Research in Veterinary Science

Abstract: This study aims to evaluate the effect of the presence of food and the material used in a panel of biomarkers in saliva of horses. For the food effect study, clean saliva was incubated with a known amount of food consisting of oats, hay or grass. Significant changes were observed when saliva was incubated with oats for total protein (P = .050) and phosphorus (P = 0.008), with grass for total protein (P = 0.037), salivary alpha-amylase (sAA, P = 0.018), total esterase (TEA, P = 0.018), butyrilcholinesterase (BChE, P = 0.037), adenosine deaminase (ADA, P = .037), and total bilirubin (P= .018), and with hay for sAA (P = 0.018), phosphorus (P= 0.037), γ-glutamyl transferase (gGT, P = 0.004), and creatine kinase (CK, P = 0.016). For the material-based collection study, saliva using a sponge and a cotton role at the same time were collected and compared. Lower values were obtained in clean saliva collected with cotton role compared to sponge for sAA (P = 0.030), TEA (P = 0.034), BChE (P = 0.003), gGT (P = 0.002) and cortisol (P < .001). In conclusion, the presence of food and the material used for its collection, can influence the results obtained when analytes are measured in saliva of horses.

URL: http://doi.org/10.1016/j.rvsc.2020.01.006.

CONCLUSIONS

CONCLUSIONS

1. The measurement of the activity of sAA is more sensitive than the quantification of its concentration to detect changes in this enzyme induced by stress or pain in humans, horses, and pigs.

2. sAA exists in the dog's saliva, and its activity can increase when an experimental stimulation of the sympathetic nervous system is carried out.

3. The sAA has different proteoforms that show different changes depending on the stress models in humans and pigs. Additionally, the activity of the primary native proteoforms, which are the GsAA (59kDa) and NGsAA (56kDa), can be measured by a spectrophotometric assay developed in our study in humans in a fast and straightforward way.

4. A panel of salivary biomarkers including Lip, TEA, BChE, ADA, TP, gGT, CK, urea, total bilirubin, phosphorus, and LA; can be measured by automated assays in a fast and reliable way and can be associated with stress. Therefore, they can show changes after acute stress in horses and sheep, as well as in different diseases (AAD in horses, lameness and rectal prolapse in pigs, and acute mastitis in cows).

5. The degree of food contamination in saliva, the collection method, and circadian rhythm can modify the values of some of the analytes assessed in the present Thesis. Therefore, ideally, these factors must be considered for their interpretation when those analytes are measured.

RESUMEN

RESUMEN

Las situaciones de estrés o que producen un bajo grado de bienestar dan lugar a comportamientos y estados fisiológicos inapropiados en los animales, que en las especies ganaderas se traduce en un descenso en la producción (Elsa Lamy & Mau, 2012; Martínez-Miró et al., 2016). Las principales causas de estrés pueden ser de origen psicológico, físico como ejercicio intenso o dolor, e inflamatorio o por enfermedad (Murata, 2007; Strahler et al., 2017). Pero la correcta evaluación del estrés para intentar evitar sus causas y consecuencias es uno de los principales retos en el ámbito del estudio del bienestar animal debido a la imposibilidad de los animales de expresarlo verbalmente (Schiavenato & Craig, 2010).

El estrés puede interferir en los mecanismos biológicos del individuo (Grandin, 1998), e inducen cambios a nivel psicológico (Altmann, 1974; Wolf & Goodell, 1981) que pueden evaluarse objetivamente. En este contexto, la saliva está adquiriendo recientemente interés para su uso como muestra biológica para la evaluación de estrés como alternativa a la sangre. Esto ocurre porque la saliva es fácil de obtener y no invasiva, no produce prácticamente estrés en el animal, no necesita una alta capacitación del personal que realiza los muestreos y posibilita la obtención de gran cantidad de muestras de forma repetida (Elsa Lamy & Mau, 2012; Yoshizawa et al., 2013). Esto hace que sea una metodología de muestreo ideal para la monitorización del bienestar. Además, algunos analitos en saliva pueden medirse de forma precisa y rápida mediante ensayos automatizados.

Tradicionalmente el cortisol se ha usado para evaluar los cambios psicológicos que ocurren por estrés (Martínez-Miró et al., 2016), ya que da información sobre la activación del eje HPA (Hyyppä, 2005). Sin embargo, hay otros mecanismos fisiológicos envueltos en la reacción de estrés, como la activación del ANS y del eje SAM mediados por catecolaminas (Everly & Lating, 2002; Hyyppä, 2005). Estos mecanismos de inducción de estrés también pueden directa o indirectamente interactuar e inducir cambios, por ejemplo, en el sistema inmune o en el estado oxidativo (Everly & Lating, 2002; Fazio et al., 2015; Murata et al., 2004). Cada una de estas vías e interacciones pueden evaluarse mediante componentes que pueden medirse en saliva y que podrían considerarse como biomarcadores de estrés. Por ejemplo, la sAA es considerada un marcador del ANS y del eje SAM (Strahler et al., 2017); el ADA un marcador del sistema inmune (Adams & Harkness, 1976); y el TAC un marcador del estado oxidativo (Rubio et al., 2019).

Sin embargo, hay muy pocos estudios que hayan evaluado marcadores de estrés diferentes al cortisol en saliva en las diferentes especies veterinarias. Además, no hay trabajos en los que se aplique un perfil analítico en saliva que, desde un punto de vista integrador, pudiera evaluar el estrés mediante sus diferentes vías de activación e interacción. Para ilustrar la importancia que existe actualmente en buscar nuevos biomarcadores salivales en animales de granja, el grupo de investigación al que la estudiante de doctorado está adscrita ha conseguido un proyecto europeo ('Clear-Farm') dentro del Programa Horizonte 2020 en el marco de trabajo en ganadería. Este proyecto europeo tiene como objetivo buscar posibles biomarcadores de bienestar en el porcino y vacuno, e integrarlos dentro de una nueva plataforma que informe tanto a los granjeros como a los consumidores a la hora de la toma de decisiones.

Los **objetivos** de la actual Tesis doctoral son avanzar en el estudio de la sAA en las diferentes especies domésticas y mejorar el conocimiento sobre otros posibles biomarcadores de estrés y bienestar que puedan determinarse en saliva mediante técnicas de medición automatizadas. Además del estudio en diferentes especies animales, en esta tesis se usó el ser humano como modelo experimental para incrementar el conocimiento de la enzima sAA, ya que en esta especie la saliva es particularmente más fácil de obtener y tiene altas concentraciones de esta enzima. De forma concreta, los objetivos fueron:

1. Aumentar el conocimiento sobre la sAA, especialmente mediante la evaluación de la mejor forma de expresar sus resultados, la investigación de los posibles cambios de sus proteoformas en diferentes situaciones estresantes, y la validación de ensayos en las especies canina, equina y porcina. Este objetivo se alcanzó mediante los artículos publicados (indicados en la sección '*Articles*') nº 1-6 y el experimento nº 1 incluido en la sección '*Annex'*.

2. Validar ensayos automatizados en saliva de la especie equina, porcina, ovina y bovina para la medición de biomarcadores de estrés y bienestar diferentes a la sAA, y estudiar cómo estos biomarcadores se comportan en las diferentes situaciones relacionadas con estresores psicológicos, físicos e inflamatorios. Los biomarcadores estudiados fueron el TEA, BChE, Lip, ADA, TP, gGT, CK, urea, bilirrubina total, fósforo y LA. El desarrollo de este objetivo condujo a los artículos publicados nº 7-11 y al experimento nº 2 ('*Annex'*).

3. Evaluar los posibles factores que podrían afectar a la interpretación de los resultados de los biomarcadores salivales estudiados en la presente Tesis. Este objetivo fue cubierto

gracias al artículo publicado nº 12 y al experimento nº 3 descrito en la sección '*Annex*', ambos desarrollados en la especie equina.

Además de los trabajos publicados, en la presente Tesis se describe brevemente los resultados obtenidos por la doctoranda en un **capítulo del libro** titulado '*Salivary glands' anatomy and physiology* of the book *Saliva in Health and Disease: The Present and Future of a Unique Sample for Diagnosis*', recientemente publicado en la editorial 'Springer'. En éste se profundiza sobre el conocimiento de la anatomía y fisiología de las glándulas salivales para un apropiado conocimiento sobre cómo se forma la saliva y cómo ésta contiene los analitos que pueden ser medidos para la evaluación de estrés.

Con respecto a los estudios experimentales desarrollados en la presente Tesis, todos recibieron aprobación por parte del **Comité Ético** de Experimentación Animal (CEEA), de la **Comisión Ética** de Investigación (CEI) de la Universidad de Murcia, del Ministerio de Agricultura, Ganadería, Pesca y Acuicultura de la Región de Murcia, o de la Comisión Ética Nacional de Lituania. Además, esta Tesis adquirió el informe favorable por parte de la Comisión Ética de la Universidad de Murcia bajo el número de licencia 2133/2018. En todos los estudios clínicos desarrollados en caballos o en seres humanos se informó a los dueños o a los participantes, respectivamente, de todos los procedimientos llevados a cabo, los métodos de muestreos y los objetivos del experimento. Tras aceptarlo, se les ofreció un documento de consentimiento informado que firmaron.

El **muestreo de saliva** varió dependiendo de la especie y del objetivo del estudio llevado a cabo. En el caso de la especie humana, la obtención de saliva se realizó mediante flujo pasivo a través de una pajita, recolectando la saliva en un tubo de 5 mL. Sin embargo, en el caso de las diferentes especies animales, ésta se obtuvo de forma activa tras hacerles masticar un material, principalmente una esponja de polipropileno, aunque en algunos experimentos realizados en caballos también mediante un pequeño rollo de algodón o una fibra de gasa; durante un tiempo determinado o cuando éstos se humedecían. Para ello se usaba una varilla de metal flexible que se les introducía en la boca del animal, o en un experimento en concreto en caballos mediante un tubo de plástico con agujeros enganchado a la cabezada. Luego, el material con la saliva se introducía en un tubo de la marca Salivette (Salivette, Sarstedt, Aktiengesellschaft & Co, Nümbrecht, Germany). En cualquier caso, las muestras de saliva tanto en humana como en las especies domésticas evaluadas se centrifugaron, y el sobrenadante obtenido se transfirió a tubos Eppendorf y fueron almacenadas a –80 ºC para su posterior análisis en menos de 6 meses,

excepto en los experimentos descritos en los artículos nº 9 y 10 en los que se analizaron las muestras en fresco.

Para alcanzar el objetivo 1 de la presente Tesis, previamente se **purificó sAA** en muestras de saliva **humana** y se desarrolló un **anticuerpo policlonal contra sAA humana**. Se usó la saliva de esta especie por su alto contenido en sAA (Nater & Rohleder, 2009). La purificación de la sAA se llevó a cabo según el procedimiento modificado de Peng *et al.* (2012) basado en la interacción de especificidad de sustrato entre amilasa y glucógeno. Por otro lado, la purificación del anticuerpo policlonal se obtuvo usando como inmunógeno la sAA purificada de la forma anteriormente descrita, y acorde a protocolos estandarizados (University of California, 2014) usando una columna HiTrapTM Protein G HP (GE Healthcare Life Sciences, Munich, Germany) y un sistema de cromatografía (ÄKTA pure - GE Healthcare Life Sciences). En ambos casos se obtuvieron resultados de purificación satisfactorios.

Con respecto a la **determinación de los analitos** en saliva, se emplearon diferentes kits colorimétricos comerciales adaptados a un analizador automático (Olympus Diagnostica GmbH, Beckman Coulter, Ennis, Ireland) para la medición de la actividad sAA, Lip, TEA, BChE, ADA, TP, gGT, CK, urea, bilirrubina total, fósforo y LA. En el caso del cortisol, éste se midió mediante un método de inmunoquimioluminiscencia (Immulite 1000, Siemens Healthcare Diagnostic, Deerfields, Illinois, USA). Además, la sAA se determinó mediante dos procedimientos: un método inmunofluorométrico llamado TR-IFMA para la medición de su concentración, que ha sido desarrollado por la doctoranda para esta Tesis; y mediante diferentes técnicas proteómicas (WB y SDS-PAGE) para la evaluación de sus proteoformas. En ambos casos se empleó como anticuerpo el policlonal desarrollado para la presente Tesis.

Todos los ensayos descritos anteriormente fueron **validados analíticamente** para cada especie mediante los parámetros de precisión (precisón inter- e intra-ensayo), exactitud (indirectamente mediante el test de linealidad bajo dilución) y sensibilidad (evaluando el LLD y el LLOQ), acorde a recomendaciones previamente publicadas (Food and Drug Administration, 2001). En todos los casos, los métodos mostraron ser precisos, exactos y sensibles para cada especie.

Para poder alcanzar los objetivos previamente descritos, esta Tesis por compendio de artículos se compone 12 **publicaciones científicas**, publicadas en revistas científicas de alto impacto y reconocidas internacionalmente; y de tres experimentos llevados a cabo dentro del

periodo de desarrollo de la tesis, y que en breve los resultados serán suministrados para sus posibles publicaciones. Así pues, quedan resumidas a continuación:

- Objetivo nº 1:

  - *Artículo nº 1*: Se midió la actividad y la concentración de la sAA en saliva humana expresando sus resultados de tres formas (sin corrección, corregidos por el flujo salival y corregidos por proteínas) ante tres situaciones con diferente grado de estrés con el fin de determinar cuál sería la mejor forma para evaluarla y expresarla. Finalmente, la actividad mostró mejores resultados para detectar cambios debido al estrés, y según su forma de expresar los resultados, éstos cambiaron la significancia e intensidad estadística. Por tanto, se debe tener en cuenta la forma de expresar los resultados de sAA para una correcta interpretación de los mismos.

  - *Artículo nº 2*: Se analizaron los posibles cambios de las diferentes proteoformas de la sAA mediante análisis WB y LC-MS/MS en la saliva humana de seis participantes del sexo femenino durante diferentes modelos de estimulación de la sAA (estimulación por ácido acético, psicológica por estrés social y física mediante ejercicio). En todas las muestras se observaron siete bandas comunes en el análisis WB, en las que tras el análisis LC-MS/MS se identificó la proteína sAA. Además, ambos análisis mostraron que dichas bandas tenían diferente respuesta según el tipo de estimulación. Por tanto, la expresión de las diferentes proteoformas de la sAA parece estar regulada dependiendo del modelo de estimulación.

  - *Experimento nº 1*: Se desarrolló un método simple y rápido para la estimación de la actividad de ambas proteoformas nativas de la sAA humana (la GsAA y la NGsAA) tras un proceso de deglicosilación. Éste mostró reducir significativamente la banda glicosilada en repetidas ocasiones y de manera eficiente. Posteriormente, el método se aplicó a un grupo de participantes que se sometieron a una situación de estrés psicológico o físico, donde los cambios en la actividad fueron de diferente magnitud en ambos modelos de estrés según si se medía la actividad total o para cada proteoforma. Esto indicaría que la medición de la actividad de las diferentes proteoformas nativas de la sAA proporcionarían información adicional para la evaluación de diferentes modelos de estrés en humana.

- *Artículo nº 3*: El principal objetivo de este estudio fue demostrar la existencia de la sAA en la saliva de la especie canina. Para ello diferentes salivas procedentes de perros Beagle fueron analizadas mediante WB. Además, se validó el método espectrofotométrico de medición de actividad sAA y se aplicó a muestras de saliva obtenidas tras un modelo experimental de activación simpática (eyaculación). Finalmente se demostró la existencia de la enzima en la saliva de la especie canina, y que su actividad puede incrementar tras una estimulación simpática.

- *Artículo nº 4*. La actividad sAA se midió en 19 caballos diagnosticados con diferentes causas de AAD, en los que se apreció que esta enzima incrementa su actividad en los caballos con este síndrome en comparación con caballos sanos, y estaría relacionada con el dolor percibido.

- *Artículo nº 5*. En este estudio, la enzima sAA se determinó mediante su actividad y concentración en otro grupo de caballos sanos y otro diagnosticados con AAD con el fin de evaluar su validez como evaluador de la supervivencia. Se observó que la actividad sAA fue más sensible en predecir la supervivencia en los caballos diagnosticados con esta enfermedad, estando también asociada con la HR, el LA plasmático y el SIRS.

- *Artículo nº 6.* Se evaluó la enzima sAA ante un modelo estandarizado de estrés agudo en cerdos mediante tres métodos: espectrofotometría obteniendo su actividad, TR-IFMA midiendo su concentración, y WB evaluando las posibles proteoformas en la saliva de cerdo. La actividad sAA, pero no su concentración, mostró cambios significativos durante el modelo de estrés. Además, se observaron diferentes bandas en el WB con diferente intensidad según el tiempo de muestreo. Esto abriría una nueva línea de investigación en esta especie para la evaluación de posibles proteoformas seleccionadas de sAA como potenciales biomarcadores de estrés en cerdos.

- Objetivo nº 2:

- *Artículo nº 7*. En un estudio piloto realizado en seis caballos sanos y seis caballos diagnosticados con AAD debido a diferentes causas, se evaluaron en saliva 23 analitos previamente validados con el fin de elegir los que significativamente variaron, para posteriormente aplicarlo a una población mayor. De todos ellos, los niveles de gGT, CK urea, bilirrubina total, TP, fósforo y la actividad sAA mostraron ser mayores en los caballos enfermos con respecto a los sanos en ambas poblaciones.

- *Artículo nº 8*. En una población de nueve caballos sometidos a un modelo experimental de estrés agudo basado en el miedo, se observaron incrementos significativos en los niveles de la actividad sAA, Lip, TEA y BChE, junto con el cortisol salival. Además, la BChE fue la que correlacionó con el índice de emocionalidad compuesto de una serie de comportamientos y con las señales de alarma que los caballos expresaron en el tiempo de estrés. Por otro lado, niveles bajos de la actividad sAA correlacionaron con los comportamientos que expresan tranquilidad en esta especie.

- *Experimento nº 2*. En un grupo de 10 caballos de la disciplina raid sometidos a un test incremental de ejercicio sub-máximo (test de esfuerzo), se evaluaron en saliva los niveles de actividad sAA, cortisol, LA y CK, y se compararon con los resultados obtenidos de HRV, nivel de entrenamiento y daño muscular. Como resultado, solo se observaron incrementos de la sAA y la CK en saliva durante el test de esfuerzo, correlacionando ambos con el tono simpático (positivamente) y parasimpático (negativamente), indicando que ambos podrían estar más relacionados con el estrés sufrido como consecuencia de la intensidad de ejercicio que con propiamente el nivel de entrenamiento.

- *Artículo nº 9.* Un panel de biomarcadores asociados con el estrés (cortisol, actividad sAA, TEA, BChE y Lip) y con la inmunidad (ADA), entre otros, se evaluaron en la saliva de un grupo de cerdos con diferentes grados de cojeras y prolapsos rectales. Sus resultados se compararon con una escala desarrollada por la candidata a doctora a partir de la modificación de otra previamente publicada; que evalúa dolor, situaciones

de angustia y malestar. Los niveles más altos de cortisol, sAA, TEA, BChE y ADA se observaron en aquellos cerdos con cojera o prolapso rectal que mostraban más grado de dolor y malestar. Además, el cortisol en saliva, TEA, BChE y ADA correlacionaron moderadamente con dicha escala.

- *Artículo nº 10.* En dos grupos de ovejas, sometidos a dos modelos de estrés agudo, se evaluaron en saliva los niveles de Lip, BChE, TEA y ADA para compararlos con los resultados obtenidos de actividad sAA y de cortisol. Dependiendo de la intensidad o de la duración de los estímulos estresantes, los biomarcadores salivares cambiaron de forma diferente a lo largo del tiempo. Pero fue la Lip la que mostró incrementos significativos en el grupo de estrés con respecto a un grupo control en ambos modelos, con menor solapamiento en los niveles entre ambos.

- *Artículo nº 11.* En un grupo de 18 vacas diagnosticadas con mamitis clínica aguda, se evaluaron en saliva un panel de biomarcadores relacionados con el estrés, inflamación y sistema inmune, para compararlos con los obtenidos en la saliva en otro grupo de 18 vacas sanas. Se observaron incrementos en la actividad sAA y concentración de cortisol. Además, los niveles de LA en saliva fueron mayores en el grupo de mamitis, mientras que fueron menores para la actividad BChE.

- Objetivo nº 3:

- *Artículo nº 12*. Para averiguar si la presencia de comida en las muestras de saliva o el tipo de material usado para su obtención pudieran afectar a los analitos evaluados en la presente Tesis en la saliva de caballos, se realizó un experimento *in vitro* con diferentes tipos de comida (avena, hierba y paja) y otro *in vivo* usando una esponja de polipropileno y un rollo de algodón para obtener la saliva en un grupo de caballos. Se observó que, en diferente grado, la presencia de comida puede variar los valores de los analitos medidos. Además, el algodón produce disminuciones en los valores de la actividad sAA, TEA, BChE, gGT y cortisol en saliva.
- *Experimento nº 3*. Se evaluó si el ritmo circadiano podría afectar a los niveles basales en la saliva de caballos de cortisol, actividad sAA, Lip, TEA, BChE, ADA, gGT, CK, urea, bilirrubina total, TP y fósforo. A lo largo del día, se observaron

cambios significativos en los niveles de sAA, TEA, BChE, ADA y CK. Además, los valores de sAA, Lip y BChE fueron diferentes según la estación (Primavera vs. Invierno).

Acorde a los resultados obtenidos, las **conclusiones** de la presente Tesis doctoral fueron las siguientes:

1. La actividad sAA es más sensible que la medida de su concentración para detectar cambios inducidos por estrés o dolor en las especies humana, equina y porcina.

2. La sAA existe en la saliva de la especie canina, y su actividad puede incrementar tras una estimulación simpática.

3. La enzima sAA tiene diferentes proteoformas que muestran diferentes cambios según el modelo de estrés aplicado en las especies humana y porcina. Además, la actividad de las principales proteoformas nativas de esta enzima, que son la GsAA (59kDa) y la NGsAA (56kDa), pueden medirse mediante un ensayo espectrofotométrico desarrollado en la especie humana de forma rápida y sencilla.

4. La Lip, TEA, BChE, ADA, TP, gGT, CK, urea, bilirrubina total, fósforo y LA pueden medirse en saliva a través de ensayos automatizados de una forma rápida y fiable, y pueden ser biomarcadores de estrés. Así pues, sus niveles se modifican ante situaciones de estrés agudo en caballos y ovejas, o ante diferentes enfermedades en caballos (AAD), cerdos (cojeras y prolapsos rectales) y vacas (mamitis aguda).

5. El grado de contaminación de la saliva con comida, el método de recolección de la misma y el ritmo circadiano pueden modificar los valores de algunos de los analitos evaluados en la presente Tesis. Por lo tanto, idealmente estos factores deben tenerse en cuenta a la hora de interpretar los resultados cuando se emplean dichos analitos.

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ANNEX

EXPERIMENT 1

Planned to be submitted for possible publication.

Changes occurring on the activity of salivary alpha-amylase proteoforms in two naturalistic situations using a spectrophotometric assay

Abstract: The research objetive was to evaluate the changes in the activity of both non-glycosylated and glycosylated salivary alpha-amylase proteoforms (NGsAA and GsAA, respectively) in a physical and psychological stress model, measured by a simple and easy to set up method. NGsAA and the GsAA activity were measured after a physical effort and a psychological challenge. The method was precise, and when it was applied on the stress models, significant increases at the time just after the stress in the physical (T+01) and psychological (T+0) model were observed in TsAA, NGsAA and, GsAA activities. GsAA was the only proteoform that showed a significant increase after complete the physical effort (T +02). Also, the NGsAA activity showed the highest magnitude of increase at the stress moment and the highest correlation with the State-Trait Anxiety Inventory Scale in the TSST trial. This innovative method can be applied to saliva samples to estimate the two main sAA proteoforms in a fast and easy way, and it can provide additional information to the TsAA activity in situations of physical or psychological stress.

EXPERIMENT 2

Planned to be submitted for possible publication.

Changes in saliva biomarkers during a standardized increasing intensity field exercise test in endurance horses

Abstract: This study aimed to evaluate if salivary alpha-amylase (sAA), cortisol, lactate (sLA) and creatine kinase (sCK) in saliva of horses can show changes during a standardized exercise test (SET), and if they are related to heart rate variability (HRV) parameters related to sympathetic and parasympathetic tone, fitness level (FL) or skeletal muscle damage. For this purpose, ten endurance horses were submitted to a SET in field condition. Saliva and blood were obtained at basal time (TB), after the seven bouts of velocity (T+01 to T+07), and 5, 15, 30, and 45 min later (T+5, T+15, T+30, and T+45). HRV parameters, FL, and plasma CK (pCK) were also evaluated. Salivary-alpha amylase increased at T+30 and T+45, and sCK increased at T+07 after the SET. They showed a positive correlation with the sympathetic tone and a negative correlation with the parasympathetic tone, respectively. In conclusion, sAA and sCK, but not salivary cortisol and sLA, increased in ten endurance horses after an increasing intensity velocity exercise. Changes in sAA and sCK were associated with HRV and could be used to assess the exercise-related stress after a physical effort.

EXPERIMENT 3

Planned to be submitted for possible publication.

Circadian rhythm in horse's salivary biomarkers related to acute stress and disease

Abstract: The possible effect of the circadian rhythm in the basal levels of cortisol, sAA activity, Lip, TEA, BChE, ADA, gGT, CK, urea, total bilirubin, TP, and phosphorus in horses' saliva was assessed in a population of five clinically healthy mares by collecting saliva at from 06.30 to 20.30 each 2 h during two consecutive days on two different photoperiod seasons: winter and spring. Although temperature and relative humidity did not change along the hours of the day in each sampled season, changes along the hours of the day were observed in cortisol, sAA activity, TEA, BChE, ADA, and CK. Therefore, the sampling time must be considered in the interpretation of these analytes. Significant changes were observed between season in temperature and relative humidity, although no thermal discomfort was observed. However, the values obtained in sAA activity, Lip, and BChE were significantly different between seasons. In conclusion, circadian rhythmic can modify the basal levels of cortisol, sAA activity, TEA, BChE, ADA, and CK in horses' saliva along the hours of the day and between season. Therefore, the time of the day and season influence saliva analytes in horses and should be taken into consideration for their interpretation.

APPENDIX

APPENDIX 1. Tables

Table 1. Summary table about the saliva collection at the different species in study performed in the present Thesis.

Specie	Method	Way to obtain	Collection tube	Collection time	Paper or Experiment nº from this Thesis
HUMANS	- PASSIVE FLOW (without tongue movements under supervision)	THROUGH A STRAW	POLYSTYRENE TUBE (5 mL)	CONTROLLED (1 min)	Paper nº 1, 2 Experiment nº 1
DOGS	- CHEWING MATERIAL Small polypropylene sponge[1] (around the mouth)	WITH FORCEPS	SALIVETTE TUBE[2]	UNCONTROLLED (until thoroughly moist)	Paper nº 3

1 Esponja Marina, La Griega E. Koronis, Madrid, Spain.
2 Salivette, Sarstedt, Aktiengesellschaft & Co, Nümbrecht, Germany.

Table 1. (Continued).

Specie	Method	Way to obtain	Collection tube	Collection time	Paper or Experiment nº from this Thesis
HORSES	- CHEWING MATERIAL Polypropylene sponge[1] (5 × 2 × 2 cm) (toward the side of the cheeks)	FLEXIBLE THIN METAL ROD	SALIVETTE TUBE[2]	UNCONTROLLED (until thoroughly moist)	Paper nº 4, 5, 7 Experiment nº 2
	- CHEWING MATERIAL Polypropylene sponge[1] (5 × 2 × 2 cm) and/or Cotton role (horses' vestibule across the third or fourth maxillary premolar)	FLEXIBLE THIN METAL ROD	SALIVETTE TUBE[2]	CONTROLLED (1 min)	Paper nº 12 Experiment nº 3
	- CHEWING MATERIAL Natural fiber gauze (suspender clips connected the device to the halter)	PLASTIC TUBE WITH A HOLE LONGITUDINALLY	SALIVETTE TUBE[2]	CONTROLLED (1 min)	Paper nº 8

1 Esponja Marina, La Griega E. Koronis, Madrid, Spain.
2 Salivette, Sarstedt, Aktiengesellschaft & Co, Nümbrecht, Germany.

Table 1. (Continued).

Specie	Method	Way to obtain	Collection tube	Collection time	Paper or Experiment nº from this Thesis
PIGS	- CHEWING MATERIAL Polypropylene sponge[1] (5 × 2 × 2 cm) (toward the side of the cheeks)	FLEXIBLE THIN METAL D	SALIVETTE TUBE[2]	CONTROLLED	Paper nº 6
				UNCONTROLLED (until thoroughly moist)	Paper nº 9
SHEEP	- CHEWING MATERIAL Small polypropylene sponge[1] (toward the side of the cheeks)	WITH FORCEPS	SALIVETTE TUBE[2]	CONTROLLED (1 min)	Paper nº 10
COWS	- CHEWING MATERIAL Polypropylene sponge[1] (5 × 2 × 2 cm) (toward the side of the cheeks)	FLEXIBLE THIN METAL ROD	SALIVETTE TUBE[2]	CONTROLLED (1 min)	Paper nº 11

1 Esponja Marina, La Griega E. Koronis, Madrid, Spain.
2 Salivette, Sarstedt, Aktiengesellschaft & Co, Nümbrecht, Germany.

Table 2. Summary table of the analytical methods used for measuring Lip, TEA, BChE, and ADA in saliva from the veterinary species studied in the present Thesis.

Biomarker	Methodology technique	Reagents	Reaction	Measurement units	References
Lip	Spectrophotometric assay	Lipase, Beckman Coulter Inc., Fullerton, CA, USA[1]	Ability of lipase to hydrolyze the heavy- chain esterified fatty acid 1,2-diglyceride to 2-monoglyceride.	IU/L	(Graca, Messick, McCullough, Barger, & Hoffmann, 2005)
TEA	Spectrophotometric assay	4-NA, Sigma-Aldrich Co., St Louis, Mo, USA.	Capacity of TEA to hydrolyze the 4-NA measured at 405 nm and at pH 7.4.	IU/L	(Fernando Tecles et al., 2017)
BChE	Spectrophotometric assay	BTCI, Sigma-Aldrich Co., St Louis, Mo, USA. DTNB, Sigma-Aldrich Co., St Louis, Mo, USA.	BChE activity was based on the absorptivity of the reaction product hydrolyzed between DTNB and thiocholine detected at 405 nm.	nmol/mL/min	(F. Tecles & Cerón, 2001) (Fernando Tecles et al., 2016)
ADA	Spectrophotometric assay	Adenosine Deaminase Assay Kit, Diazyme Laboratories, Poway, CA, USA[1]	Enzymatic deamination of adenosine to inosine, which is specific for this enzyme.	IU/L	(F. Tecles et al., 2018)

[1]Commercially available method.

Table 7. Perception of pain, distress, and discomfort to the pigs scoring system, based on the score system proposed by (Morton & Griffiths, 1985). If a score of 3 is recorded more than once, then all scores of 3 are given one extra mark.

Independent Variables	Scores	Definitions
Unprovoked behavior	0	− Actively moves and is in alert. − In relation to its mates − It approaches the feeder or water dispenser
	1	**Minor changes:** − Less active and less alert (both).
	2	− **Evident reduction** in both **activity** and **alert state.** − **On its own, away from its mates**. May push it head in a corner. − May eat it bedding or injures itself.
	3	− Very restless or **does not move at all.** − **Although is provoked by other pigs remains on its own.** − May have **expiratory grunts.** − May **grate its teeth**.
Behavioral responses to external stimuli	0	− With whiskers **twitching and sniffing** when the experimenter **approaches the box**. − Attempts to escape if the experimenter frightened it.
	1	− Approaches the evaluator when she/he is outside the box, **but without decision: hesitates.**
	2	− **Does not approach the evaluator** when is outside the box. − Inside the box, it avoids the experimenter, but **finally, it gets curious**.
	3	− **Avoids the evaluator all the time.** − **Reacts violently** (in panic) **or** may be very weak as in a **pre-comatose state.**

Table 7. (Continued).

Independent Variables	Scores	Definitions
Appearance	0	− Coat is smooth, lies flat and often has a sheen. − Eyes are clear and bright.
	1	Lack of grooming: − **Stains on the coat, but no other marked changes**
	2	Visible lack of grooming: − Coat starey. − **Eyes and nose may have discharges**. − **External orifices some ungroomed.**
	3	Remarkable lack of grooming: − Coat very starey. − **Ungroomed external orifices.** − **Eyes and nose with visible discharges. Eyes look pale, pupils enlarged and partially closed eyelids.** − **Abnormal posture,** eg, may look hunched up or legs in abnormal positions (i.e. front legs apart with chest pain).
Bodyweight	0	BCS 3: Ideal hips. Hip and spine are easily **palpable with firm pressure**. Ribs **not appreciable** but palpated with firm pressure with the palm of the hand. BCS 4: Fat hips. Hip, spine and ribs **cannot be palpated with firm pressure** with the palm of the hand.
	1	BCS 2: Thin hips. Hip, spine, and ribs **easily palpable without firm pressure**
	2	BCS 2: but with **tired behavior.**
	3	BCS 1: Emaciated hip. Hip, spine, and ribs are **prominent to the eye.**
Clinical signs	0	− Without evident changes in temperature by palpation. − Soaks easily the sponge with less than 1 min of sampling. − Respiratory rate and mucous membranes within the physiological norms.
	1	− **Soaks the sponge** only **after 1 min** of sampling.
	2	− Score 1 + **high temperature to palpation.** − **Respiratory rate may be increased** (>50 breath/min).
	3	− I**ncreased temperature or cold limbs al palpation.** − **No soaks the sponge after 1 min of sampling.** − Shallow respiration or respiratory rate increased (>50 breath/min). − **Abnormal mucous membrane**s (injected, purple or white)

Table 8. Results of cortisol, sAA activity, Lip, BChE, TEA, ADA, and HR in sheep's saliva obtained during the experiment 1 (by facing a dog, n = 14) and 2 (by shearing, n = 14), and in a control group (n = 5, respectively). Sampling was taken before (Tb), during (T + 0A), just after (T + 0B), and after 15 min (T + 15), 30 min (T + 30) and 60 min (T + 60) of the stressor stimulus.

		Experiment 1						
	Group	**Tb**	**T+0A**	**T+0B**	**T+15**	**T+30**	**T+60**	**P value**
Salivary cortisol (μg/dL)	Stress	0.60 (0.495-0.712)	0.88 (0.731-1.019)	0.86(0.694-1.036)	0.95 (0.833-1.058)	1.00 (0.769-1.225)	0.60 (0.481-0.709)	0.38
	Control	0.78 (0.604-0.959)	0.81 (0.652-0.961)	0.89 (0.721-1.067)	0.83 (0.615-1.052)	0.74 (0.478-1.009)	0.62 (0.452-0.785)	
sAA (IU/L)	Stress	7.7 [11.03-23.93]	**17.2a [11.03-23.93]**	**18.6a [11.05-23.95]**	11.2 [8.45-31.68]	11.3 [8.38-16.95]	**6.5b,c,d [4.38-11.48]**	**< 0.01**
	Control	3.2 [3.20-11.10]	3.7 [3.30-6.90]	4.3 [3.75-6.70]	5.2 [3.95-8.30]	3.0 [2.85-4.60]	4.5 [3.75-5.40]	
Lip (IU/L)	Stress	3.9 [3.40-4.40]	**10.0a [6.65-15.15]**	**7.8a [4.45-12.00]**	**4.4b,c [3.20-6.45]**	**4.2b [2.50-6.80]**	**2.7b,c [2.20-3.45]**	**< 0.01**
	Control	1.1 [0.95-1.15]	1.3 [0.92-1.29]	1.3 [1.06-1.35]	1.0 [0.90-1.15]	0.9 [0.83-0.88]	1.0 [0.87-1.18]	
BChE (ng/mL/min)	Stress	22.9 [20.50-25.30]	25.5 [19.70-35.90]	31.0 [25.05-42.95]	23.4 [19.30-37.15]	24.3 [20.80-34.45]	24.3 [19.75-30.50]	0.39
	Control	20.8 [19.35-35.30]	24.1 [21.55-29.80]	39.1 [26.40-39.25]	35.1 [25.35-36.00]	25.6 [23.05-36.85]	38.0 [29.80-42.10]	
TEA (IU/L)	Stress	165.8 [147.6-210.9]	**213.6a [168.9-344.4]**	218.6 [139.7-326.0]	183.2 [125.6-237.9]	160.0[142.8-224.0]	**190.2b [135.9-218.8]**	0.80
	Control	147.2 [126.8-250.0]	185.6 [143.2-216.0]	260.8 [164.8-312.8]	154.4 [160.4-291.2]	184.0 [144.8-251.2]	268.0 [182.4-290.8]	
ADA (IU/L)	Stress	143.9 (100.9-186.9)	**304.0a (155.9-452.2)**	**266.4a(200.8-332.1)**	217.2 (109.0-325.5)	**175.9b (120.4-231.5)**	**148.8b (104.4-193.3)**	0.30
	Control	126.1 (52.6-199.5)	153.2 (25.2-281.1)	214.8 (72.9-356.8)	166.7 (65.8-267.6)	134.7 (37.3-232.1)	157.7 (62.1-253.4)	
HR (beats/min)	Stress	98 (89.8-106.5)	**144a (137.2-151.3)**	**122a,b (111.8-132.5)**	**111a,b(105.0-117.6)**	**97b,c,d(91.1-102.9)**	**97b,c,d(89.8-103.3)**	**< 0.01**
	Control	85 (70.7-98.9)	69 (40.1-98.3)	73 (69.5-72.3)	73 (58.2-87.7)	73 (59.2-87.7)	70 (52.1-87.5)	

Results are in Mean (95% confidence interval); or Median [interquartile range, 25th–75th percentiles]. Subscripts letters mean statistical differences with respect to Tb (a), T+0A (b), T+0B (c), and T+15 (d).

Table 8. (Continued).

		Experiment 2					
	Group	**Tb**	**T+0A**	**T+0B**	**T+30**	**T+60**	**P value**
Salivary cortisol (µg/dL)	Stress	0.73 (0.618-0.840)	**1.17$_{a}$ (0.965-1.376)**	**1.11$_{a}$ (0.905-1.305)**	**1.15$_{a}$ (0.902-1.393)**	**0.95$_{a}$ (0.758-1.145)**	**< 0.01**
	Control	0.72 (0.587-0.844)	0.73 (0.638-0.823)	0.81 (0.706-0.910)	0.67 (0.546-0.787)	0.61 (0.531-0.680)	
sAA (IU/L)	Stress	2.8 [2.50-3.30]	**6.8$_{a}$ [4.90-19.60]**	**9.3$_{a}$ [7.70-14.00]**	4.0 [3.20-4.40]	4.7 [3.80-5.70]	0.12
	Control	5.2 [3.20-10.90]	5.7 [3.65-8.93]	5.8 [4.28-10.43]	4.6 [2.98-10.55]	5.4 [4.10-9.23]	
Lip (IU/L)	Stress	2.7 [1.60-4.00]	**28.9$_{a}$ [11.30-52.50]**	**42.5$_{a}$ [35.60-94.00]**	**11.1$_{a,b,c}$ [5.90-18.40]**	**9.8$_{a,b,c}$ [7.90-16.10]**	**< 0.01**
	Control	2.0 [1.45-2.55]	2.5 [1.50-3.40]	2.4 [1.60-2.93]	1.4 [1.30-2.15]	1.8 [1.50-2.38]	
BChE (ng/mL/min)	Stress	14.8 [12.35-25.48]	**23.5$_{a}$ [18.65-35.20]**	**23.9$_{a}$ [19.40-29.80]**	19.5 [14.95-27.03]	24.8 [13.55-28.78]	0.15
	Control	19.4 [15.25-28.13]	21.6 [16.18-25.20]	26.3 [14.33-39.10]	22.6 [17.93-27.80]	27.8 [15.93-38.70]	
TEA (IU/L)	Stress	72.4 [147.6-210.9]	**172.8$_{a}$ [97.6-284.6]**	**183.2$_{a}$ [111.8-248.0]**	**119.6$_{b,c}$ [78.8-148.4]**	148.8 [76.4-184.6]	0.35
	Control	150.8 [132.8-246.2]	167.6 [143.6-210.8]	166.0 [146.4-280.2]	186.0 [135.8-218.8]	203.6 [162.2-288.2]	
ADA (IU/L)	Stress	124.6 (82.1-167.2)	**344.0$_{a}$ (213.5-474.5)**	**247.2$_{a}$ (160.6-333.7)**	**149.5$_{b}$ (104.0-195.1)**	**183.0$_{b}$ (85.4-280.6)**	0.09
	Control	123.9 (93.6-154.1)	147.0 (93.1-201.0)	183.5 (114.4-252.6)	130.0 (85.4-174.7)	142.1 (94.7-189.5)	
HR (beats/min)	Stress	78 (70.7-84.4)	no measured	**129$_{a}$ (109.9-148.4)**	**94$_{c}$ (79.8-107.8)**	**90$_{c}$ (80.8-98.9)**	**< 0.01**
	Control	71 (46.6-96.2)	no measured	66 (44.6-86.6)	73 (67.1-78.1)	68 (47.4-88.7)	

Results are in Mean (95% confidence interval); or Median [interquartile range, 25th–75th percentiles]. Subscripts letters mean statistical differences with respect to Tb (a), T+0A (b), and T+0B (c).

APPENDIX 2. EDITORIAL AND JOURNAL IMPACT FACTORS

SPI Scholarly Publishers Indicators
In Humanities and Social Sciences

Editoriales extranjeras

Orden	Editorial	ICEE
1	Oxford University Press	1705.000
2	Cambridge University Press	1681.000
3	Routledge (Francis & Taylor Group)	1153.000
4	Springer	670.000
5	Peter Lang Publishing Group	642.000
6	Brill	526.000
7	De Gruyter	386.000
8	Sage Publications	343.000
9	Harvard University press	326.000

	Title20	ISO_ABBREV	ISSN	CATEGORY_DESCRIPTION
1	ANIMAL	Animal	1751-7311	VETERINARY SCIENCES
2	ANIMAL	Animal	1751-7311	AGRICULTURE, DAIRY & ANIMAL SCIENCE
3	ANIMALS	Animals	2076-2615	AGRICULTURE, DAIRY & ANIMAL SCIENCE
4	ANIMALS	Animals	2076-2615	VETERINARY SCIENCES
5	BMC VET RES	BMC Vet. Res.	1746-6148	VETERINARY SCIENCES
6	CLIN ORAL INVEST	Clin. Oral Investig.	1432-6981	DENTISTRY, ORAL SURGERY & MEDICINE
7	EQUINE VET J	Equine Vet. J.	0425-1644	VETERINARY SCIENCES
8	PLOS ONE	PLoS One	1932-6203	MULTIDISCIPLINARY SCIENCES
9	RES VET SCI	Res. Vet. Sci.	0034-5288	VETERINARY SCIENCES

FECYT FUNDACIÓN ESPAÑOLA PARA LA CIENCIA Y LA TECNOLOGÍA

Web of Science Group

	Title20	Publisher	TOT_CITES	IMPACT_FACTOR	ARTL_INFLUENCE
1	ANIMAL	Animal	7550	2400	593
2	ANIMAL	Animal	7550	2400	593
3	ANIMALS	Animals	2181	2323	0
4	ANIMALS	Animals	2181	2323	0
5	BMC VET RES	BMC Veterinary Research	5617	1835	566
6	CLIN ORAL INVEST	Clinical Oral Investigations	6849	2812	643
7	EQUINE VET J	EQUINE VETERINARY JOURNAL	7200	2477	612
8	PLOS ONE	PLoS One	688763	2740	928
9	RES VET SCI	RESEARCH IN VETERINARY SCIENCE	6467	1892	432

FECYT FUNDACIÓN ESPAÑOLA PARA LA CIENCIA Y LA TECNOLOGÍA

Web of Science Group

	Title20	IMMEDIACY_INDEX	CITED_HALF_LIFE	EIGENFACTOR	QUARTILE_RANK
1	ANIMAL	1000	6	0,01	11/142
2	ANIMAL	1000	6	0,01	9/63
3	ANIMALS	398	2,5	0,00343	10/63
4	ANIMALS	398	2,5	0,00343	14/142
5	BMC VET RES	327	4,6	0,01227	32/142
6	CLIN ORAL INVEST	991	4,6	0,01147	15/91
7	EQUINE VET J	1367	13	0,00453	9/142
8	PLOS ONE	499	5,6	1,38886	27/71
9	RES VET SCI	496	8,3	0,00582	31/142