UNIVERSIDAD DE MURCIA

FACULTAD DE VETERINARIA

Advances in the knowledge of Chromogranin A and Other Salivary Biomarkers of Stress in Pigs

Avances en el Conocimiento de la Cromogranina A y Otros Biomarcadores Salivares de Estrés en Cerdos

D. Damián Escribano Tortosa

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"Advances in the knowledge of chromogranin A and other salivary biomarkers of stress in pigs"

"Avances en el conocimiento de la cromogranina A y otros biomarcadores salivares de estrés en cerdos"

Memoria presentada por el licenciado

Damián Escribano Tortosa

Para optar al grado de Doctor en Veterinaria con Mención Internacional

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1. Measurement of chromogranin A in porcine saliva: validation of a time-resolved immunofluorometric assay and evaluation of its application as a marker of acute stress. Escribano, D., Soler, L., Gutiérrez, A.M., Martínez-Subiela, S., Cerón, J.J., 2013. Animal 7, 640-647.

2. Saliva chromogranin A in growing pigs: A study of circadian patters during daytime and stability under different storage conditions. Escribano, D., Gutiérrez, A.M., Fuentes-Rubio, M., Cerón, J.J., 2014. The Veterinary Journal 199, 355-359.

3. Validation of an automated chemiluminescent immunoassay for salivary cortisol measurements in pigs. Escribano, D., Fuentes, M., Cerón, J.J., 2012. Journal of Veterinary Diagnostic Investigation 24, 918-923.

4. Salivary testosterone measurements in growing pigs: validation of an automated chemiluminescent immunoassay and its possible use as an acute stress marker. Escribano, D., Fuentes-Rubio, M., Cerón, J.J., 2014. Research in Veterinary Science, http://dx.doi.org/10.1016/j.rvsc.2014.04.001.

5. Escribano, D., Gutiérrez, A.M., Martínez-Subiela, S., Tecles, F., Cerón, J.J., 2012. Validation of three commercially available immunoassays for quantification of IgA, IgG, and IgM in porcine saliva samples. Research in Veterinary Science 93, 682-687.

6. Effect of repeated administration of lipopolysaccharide on inflammatory and stress markers in saliva of growing pigs. Escribano, D., Campos, P., Gutiérrez, A.M., Le Floc'h, N., Cerón, J.J., Merlot, E., 2014. The Veterinary Journal 200, 393-397.

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Vista la solicitud presentada el día 26 de mayo de 2014, por D. DAMIÁN ESCRIBANO TORTOSA, con D.N.I.: 48520165-D, sobre autorización para presentación de tesis doctoral como compendio de publicaciones con carácter previo a la tramitación de la misma en la Universidad de Murcia, le comunico que la Comisión de General de Doctorado, vistos:

- el informe previo del Presidente de la Comisión Académica del Doctorado en Tecnología de la Reproducción y Medicina Veterinarias , y
- el visto bueno de la Comisión de Ramas de Conocimiento de Ciencias de la Salud ,

resolvió, en su sesión de 11 de julio de 2014, **ACCEDER** a lo solicitado por el interesado pudiendo, por lo tanto, presentar su tesis doctoral en la modalidad de compendio de publicaciones.

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Presidente de la Comisión General de Doctorado

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To whom it may concern:

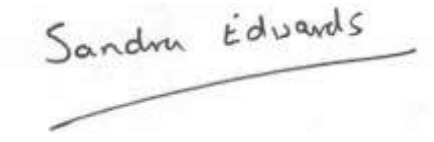
I have studied the material for the doctoral thesis of **Damián Escribano Tortosa** entitled **"Advances in the knowledge of chromogranin A and other salivary biomarkers of stress in pigs"**, comprising a thesis summary, six papers which are published or in press, and a seventh paper which has been submitted for publication.

Together these constitute a significant body of scientific work on the development of laboratory tests for potential biomarkers of stress in pig oral fluid and their practical validation by assessment of samples taken from animals in different stressful situations. This topic is one of growing scientific and practical interest, as we continue to seek better objective measures of animal welfare which can be applied in on-farm circumstances using non-invasive techniques.

The individual publications demonstrate a good knowledge of stress physiology in different species, expertise in the development and validation of novel laboratory assays and in appropriate animal experimentation to assess their practical exploitation. They have all have the candidate as first author and have been accepted by good quality international journals in their field. They are well written with meticulous experimentation, clear presentation of data and good appreciation of context in Introduction and Discussion.

The summary concisely presents an overview of the work, whose objectives are clearly justified by both the scientific rationale and the practical need. The key points of each paper are described in sufficient detail to substantiate the clear conclusions drawn from the work.

I therefore consider that the thesis shows a level of knowledge and scientific output which is in accordance with that expected for a doctoral degree, that the work is of good quality and that it is likely to have significant future application in the field of animal health and welfare. I would have no hesitation to recommend this thesis for a doctoral degree.



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TO WHOM IT MAY CONCERN

Dear Sir / Madam

Ana Cristina Silvestre Ferreira, Assistant Professor, Department of Veterinary Sciences, University de Trás -os- Montes e Alto Douro, concerning the PhD thesis entitled " **Advances in the knowledge of chromogranin A and other salivary biomarkers of stress in pigs**" presented by Mr **Damián Escribano Tortosa** and after good read, I consider that it meets the scientific conditions for public presentation and defense, to obtain a doctoral degree.

The thesis is well presented with a correct organization and an appropriate methodology to fulfill the proposed objectives, finding themselves backed into a vast and current bibliography.
It consists of 4 published articles and 2 being published in scientific peer-reviewed journals with good impact factor, as well as another submitted for publication, demonstrating a very good level research program, that certifies the quality of scientific work by the candidate, as well as its recognition by the scientific community.

The search for new biomarkers in clinical biochemistry for the recognition of the disease state is nowadays a field of work for doctors, veterinarians and researchers. The lack of good biomarkers for the identification of stress in porcine makes the subject of this thesis, apart from very current, a field in new and interesting lines of research.

Sincerely,

Ana Cristina Ferreira, DVM PhD
Professor Auxiliar
Vila Real, May 20th, 2014

Saint-Gilles, 13th of May, 2014-05-13

Damián Escribano Tortosa, born on the 04/10/1985 in Murcia (Spain), PhD student at the Department of Animal medicine and Surgery, University of Murcia, 30100 Espinardo in Murcia, spent 3 months in the PEGASE Laboratory (UMR 1348, INRA, F-35590 Saint-Gilles, France) for a scientific collaboration in the context of his PhD work. He stayed in the laboratory from the 16/09/2012 to the 19/12/2012.

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Esta tesis no hubiera posible llevarla a cabo con éxito sin la ayuda de muchas personas a las que debo mi sentido y sincero agradecimiento.

Quiero agradecer en primer lugar a mi director el **Dr. José Joaquín Cerón** por su increíble dirección y ayuda incalculable antes y durante todo el desarrollo de esta tesis. Por confiar en mí, y seguir haciéndolo, para formar parte de su grupo de investigación. Por su gran capacidad de motivación, tan necesarios y oportunos, en esos momentos de agobio en los que necesitas ánimos. Por tratarme más como compañero y amigo que como jefe y, en definitiva, por ser el principal culpable de contagiarme la pasión por este mundo de la investigación.

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A mi compañera, amiga de fatigas durante todo este largo trayecto y recientemente **Dra. María Fuentes**, porque ella sabe por todo lo que hemos pasado para llegar al final del camino. Porque las largas jornadas de trabajo conjuntas, durante todo este tiempo, nos han hecho convivir todo tipo de situaciones. Sin tu ayuda no lo podría haber hecho y, sobre todo, no hubiera sido igual. Independiente de cómo se separen nuestros caminos, que sepas que gracias a lo que hemos compartido durante este, tienes en mi a un amigo para siempre.

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A mi "grupo" del café del viernes o grupo de técnicos de Lab4; **Isabel** la immulite del grupo, **Susana** la de los módulos de formación, **Josefa** (con mucho cariño) la de la cofradía, **José María** el generoso y la "reciente" incorporación el impromune **Luis**. Por ayudarme siempre que lo he solicitado y, lo más importante y necesario, por los momentos de distracción, conversación y risas, dentro y fuera del laboratorio, que han facilitado mucho la realización de este trabajo.

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Por último quiero reservar las últimas palabras para mi familia. A mis padres **Pedro y Antonia** porque todo lo que soy y consiga ser en esta vida se lo debo a ellos, gracias, sois los mejores padres que un hijo puede tener. A mi hermano **Pedro** y a mí cuñada **Teresa** por los "paseos" y los momentos de risas y distracción tan imprescindibles. A mi abuela **Mercedes** y a mis difuntos abuelos **Pedro, Damián y Juana** porque siempre velan por mí, para que todo me salga bien, y a mi novia **Carmen** por compartir conmigo el mismo camino tanto en lo profesional como, el más importante en la vida, en lo sentimental.

INDEX

ABBREVIATIONS

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APPs: acute phase proteins

CgA: chromogranin A

CRP: C-reactive protein

CV: coefficient of variation

ELISA: enzyme-linked immunosorbent assay

Eu: europium

HP: haptoglobin

HPA: hypothalamic-pituitary-adrenal

HPG: hypothalamus pituitary gonadal

IgA: immunoglobulin A

IgG: immunoglobulin G

IgM: immunoglobulin M

Igs: immunoglobulins

KLH: keyhole limpet hemocyanin

LPS: lipopolysaccharide

r: coefficient of correlation

SAM: sympatho-adrenomedullary

SEM: standard error mean

SD: standard deviation

SPF: specific pathogen free

PMWS: postweaning multisystemic wasting syndrome

PRRS: porcine reproductive and respiratory syndrome

TR-IFMA: time-resolved immunofluorometric assay

INTRODUCTION

INTRODUCTION

Animal welfare is of great importance in animal production systems nowadays and can be evaluated by different ways such as behavioural indicators or biochemical determination of biomarkers reflecting the physiological response of the animal to stress (Smulders et al., 2006; Fuentes et al., 2011). Unfortunately, the methods of observations have some potential for error or misinterpretation so the quantification of physiological responses to a stimulus by biomarkers may offer a more objective and sensitive measurement of stress than behavioural observation in most species (Hart, 2012).

These biomarkers can be measured in blood but its collection is achieved by venepuncture and the lack of superficial blood vessels in pigs usually requires that the extremely invasive method of surgical implantation of cannulas must be used in order to obtain repeated blood sampling (Bushong et al., 2000). In order to overcome this trouble, saliva sampling has been postulated as a non-invasive and stress-free methodology, in comparison with blood sampling which can be a confounding factor in stress models (Merlot et al., 2011). In addition, the repeated saliva sampling over short time intervals can be carried out without greatly influencing the stress response (Heintz et al., 2011) which facilitates ongoing animal monitoring. Other advantage is that saliva sampling can be carried out by individuals with limited training. For all above mentioned, saliva is considered to be an ideal material for evaluating the stress condition in pigs, by using salivary stress biomarkers (Muneta et al., 2010; Fuentes et al., 2011; Soler et al., 2013).

Different stress biomarkers have been postulated to be used for the survey of different axis or systems; HPA, SAM, HPG and immune system; activated during stress condition. For example, salivary cortisol indicates activity of the HPA axis in response to different stressor in pigs (Cook et al., 1996; Schönreiter et al., 1999; Merlot et al., 2011).

Also, in human, measurements of salivary CgA could be used as a sensitive and reliable quantitative tool for monitoring activity of the sympathetic nervous system, which constitutes the initial response to stress (Kanno et al., 1998; Nakane et al., 1998); being a possible alternative to catecholamines, as the latter are considered to be poor markers of acute changes in SAM activity (Schwab et al., 1992; Kennedy et al., 2001). Salivary testosterone, is a product of the HPG axis (Schoofs and Wolf, 2011), and has been related in human studies with the modulation of pain sensation (Choi et al., 2012), depression and anxiety disorders (Giltay et al., 2012). On the other hand, it has been reported that salivary IgA, a major component of adaptive immune mucosal defence, might be a sensitive and specific biomarker of the acute stress during restraint in pigs (Muneta et al., 2010).

At the beginning of this PhD we detected that there were no commercially available assays for CgA measurements in pigs, there were no comprehensive studies of analytical validation of assays for measurements of cortisol, testosterone and immunoglobulins in saliva of pigs; as well as a lack of studies where the biomarkers were globally evaluated in stress models.

OBJECTIVES

OBJECTIVES

This PhD thesis was designed in order to produce advances in the measurement of salivary biomarkers of stress and to develop and validate a panel of salivary biomarkers to evaluate the different physiological systems (SAM, HPA, HPG and immune system) that are taking part in stress mechanism. With this purpose, the specific objectives were:

1. To develop and validate a TR-IFMA for porcine salivary CgA measurements, using a species-specific antibody, and evaluate its behaviour in an acute stress model. In addition, to study if salivary CgA concentrations exhibit any circadian pattern during the daytime, and to evaluate its stability under different storage conditions.

2. To validate commercially available immunoassays for cortisol, testosterone and IgA quantification on the saliva of pigs.

3. To evaluate the response of a panel of several salivary biomarkers to repeated administration of LPS.

4. To investigate the responses of a panel of several salivary biomarkers after applying a psychosocial stressor model in pigs based on isolation and regrouping.

EXTENDED SUMMARY

EXTENDED SUMMARY

1. MATERIALS AND METHODS

1.1 Biomedical ethics

Procedures involving animal handling were approved by the University of Murcia Ethics Committee and followed the recommendations of the European Convention for the Protection of Vertebrate Animals Used for Experimental 139 and Other Scientific Purposes (Council of 140 Europe, ETS Number 123). Moreover, as experimental procedure of article 6 was performed in the experimental unit of the National Institute for Agricultural Research (INRA, France), procedures were conducted according to established guidelines for animal care approved by the regional ethical committee (C2EA-07, agreement R-2012-NLF-02. Approval date: 22 June 2012).

1.2 Sampling procedure

Saliva was collected from all animals using Salivette tubes (Sarstedt, Nümbrecht, Germany) containing a sponge, instead of the commercial cotton rod, as reported before (Gutiérrez et al., 2009). Pigs were allowed to chew the sponge, which was clipped to a flexible thin metal rod, until the sponge was thoroughly moistened (1–3 min). The sponges were then placed in the tubes and centrifuged at 3000*g* for 10 min. Saliva samples were collected and stored at –20°C or –80°C until analysis.

1.3 Analyte determination in saliva

1.3.1 CgA determinations

For the measurement of CgA levels in porcine saliva, a TR-IFMA was developed.

Production and labelling of polyclonal antibody: A recombinant peptide from porcine CgA protein conjugated to KLH was selected as immunogen to produce polyclonal antibodies. Particularly, the peptide fragment used was the CgA359-379 region and the sequence was obtained from the database UniProt; (http://www.uniprot.org/uniprot/P04404). The recombinant CgA peptide fragment was obtained commercially

(EnoGene Biotech, New York, NY, USA). The specific polyclonal antibodies against porcine CgA were produced in our laboratory according to standard protocol (University of California Berkley Animal Care and Use Committee, 2009). The animal that gave the best response was selected and its IgG content was purified using a HiTrap™ Protein G HP column, according to the manufacturer's instructions (GE Healthcare Life Sciences, Munich, Germany). The purity of the immunoglobulins was assessed by SDS-PAGE using 4% to 12% acrylamide gels and quantified using RC/DC protein assay (Bio-Rad Laboratories, Madrid, Spain).

An aliquot of 1 mg of the polyclonal antibodies produced (rabbit anti-recombinant CgA359-379) was labelled with biotin using a commercial kit (No weigh Sulfo-NHS-biotin, Pierce, Thermo Fisher Scientific, Barrington, IL, USA) and used as a capture antibody in the TR-IFMA assay developed. An additional aliquot of 1 mg of the same polyclonal antibodies was labelled with a Eu chelate (DELFIA Eu-labeling kit, PerkinElmer Life and Analytical Sciences, Turku, Finland), following the manufacturer's instructions and was used as detection antibody. The assay procedure after optimization was as follows:

1.- Add 100 µL/well of biotinylated antibody (100 ng/well).
2.- Incubation with continuous shaking at room temperature (20-25°C) for 60 min and wash 4 times with wash buffer (DELFIA, PerkinElmer Life and Analytical Sciences).
3.- Add 100 µL/well of standards (six standard concentrations were chosen for routine use: 4.68, 9.37, 18.75, 37.5, 75 and 150 ng/well), or saliva sample dilutions at 1:4 with DELFIA assay buffer.
4.- Incubation with continuous shaking at room temperature for 60 min and wash 4 times with DELFIA wash solution.
5.- Add 100 µL/well of Eu-labelled antibody at the optima working dilution (200 ng/well) in DELFIA assay buffer.
6.- Incubation with continuous shaking at room temperature for 60 min and wash 4 times with DELFIA wash solution.
7.- Add 200 µL/well of DELFIA enhancement solution and incubate at room temperature for 5 min with shaking.
8.- The fluorescence was measured in a VICTOR^2 1420 multilabel counter, and concentrations were calculated by the Wallac MultiCalc program.

1.3.2 Cortisol determinations

Cortisol concentration was measured using a solid-phase, competitive chemiluminescent enzyme immunoassay following manufacturer instructions (Immulite/Immulite 1000 cortisol, Siemens Medical Solutions Diagnostics, Los Angeles, CA).

1.3.3 Testosterone determinations

Total testosterone concentration was measured using a solid-phase, competitive chemiluminescent enzyme immunoassay following manufacturer instructions (Immulite/Immulite 1000 Total Testosterone, Siemens Medical Solutions Diagnostics, Los Angeles, CA).

1.3.4 IgA, IgG and IgM determinations

Saliva Igs concentrations were determined by three commercially available sandwich ELISAs (IgA, IgG, and IgM ELISA Quantitation Kit; Bethyl Laboratories, Montgomery, TX, USA). The performance conditions for the three immunoassays were optimized. Different concentrations of coated (25, 50, 100, 200, 300, 400, 500, and 1000 ng/well) and detection antibody (1:10,000–1:80,000) were tested. Optimal sample dilution for each Ig was selected as the dilution which showed the greatest difference between the samples with high and low concentrations. The procedures of the assays after antibody optimization were as follows:

1. Add 100 µL/well of capture antibody at 100 ng/well for IgM and 50 ng/well for IgG and IgA in carbonate/bicarbonate buffer.
2. Incubation at room temperature for 60 min and wash 3 times with wash solution.
3. Add 200 µL/well of blocking solution and incubate for 60 min and wash 3 times.
4. Add 100 µL/well of standards (according to manufacturer instructions) or saliva sample dilutions at 1:200 for IgG and IgM and 1:800 for IgA.
5. Incubation at room temperature for 60 min and wash 3 times with wash solution.
6. Add 100 µL/well of HPR at the optima working dilution (IgA 1:80,000; IgG, 1:70,000, or IgM, 1:70,000) in conjugate diluent.
7. Incubation at room temperature for 60 min and wash 5 times with wash solution.
8. Add 100 µL/well of TMB and incubate in the dark at room temperature for 5 min.
9. Stopped the reaction with 100 µL/well of 2 M sulfuric acid and the absorbance was measured at 450 nm with PowerWave XS, Bio-Tek Instruments Inc.

1.4 Analytical validation

To perform the analytical validation the following parameters were evaluated:

1.4.1 Assessment of intra-assay and inter-assay precision: The intra-assay precision, expressed as the CV, was calculated after analysis of pools of saliva samples containing high and low levels of analyte, respectively, five times in a single analytical run. Each pool was prepared by mixing samples of saliva from animals with similar concentrations of analyte previously quantified by the assay itself. The same pools were used to determinate the inter-assay precision by analysing them on six different days within a 15-day period. The samples were frozen in aliquots, and vials were only thawed as required for each analytical run in order to prevent any possible variation as a result of repeated freeze-thaw cycles (Soler et al., 2011).

1.4.2 Calculation of the analytical limit of detection: The detection limit was defined as the lowest concentration of analyte that could be distinguished from a specimen of zero value. It was calculated for the immunoassay on the basis of data from 10 replicate determinations of the zero standard (assay buffer) as mean value plus two standard deviations (Eckersall et al., 1999).

1.4.3 Estimation of the lower and upper limit of quantification: The lower and upper limit of quantification was calculated based on the lowest and highest analyte concentration, respectively, that could be measured in the linear part of the calibration curve with a CV <20%, and above the detection limit in the case of the lower limit. To estimate the lower limit of quantification, a saliva sample was serially diluted in assay buffer and each dilution was analysed in five replicates in the same run (Park et al., 2010).

1.4.4 Assessment of assay accuracy: As, to the authors knowledge, no gold standard assay is available to quantify analyte in porcine saliva, the accuracy was indirectly investigated by recovery experiment and linearity under dilution as follows.

The recovery experiment was performed as previously reported (Parra et al., 2005). A sample with high analyte concentration was mixed with different amounts of a sample with low analyte concentration in order to achieve different sample dilutions.

Therefore, the high analyte level sample was diluted 2- (50%), 4- (25%), and 10-fold (10%) with the low analyte level saliva sample (50%, 75%, and 90%, respectively). In addition, the low analyte level saliva sample was diluted 4-fold (25%) with the high analyte level saliva sample (75%). Detected and expected analyte levels for each diluted saliva sample were compared, and the percentages of recovery were calculated.

Linearity under dilution was determined by using two porcine saliva samples with high levels of salivary analyte serially diluted (1:2, 1:4, 1:8, 1:16, 1:32, 1:64) with deionized water, and the analyte concentrations was measured by the assay itself. The concentration was measured in duplicate for each dilution. Afterward, curves representing salivary measured analyte concentrations versus expected analyte concentration were constructed (Gutiérrez et al., 2009).

2. EXPERIMENTAL DESIGN, RESULTS AND DISCUSSION

2.1 Objective 1

The objective 1 was covered by 2 experiments corresponding to Article 1 (experiment 1) and Article 2 (experiment 2).

2.1.1 Experiment 1. Measurement of chromogranin A in porcine saliva: validation of a time-resolved immunofluorometric assay and evaluation of its application as a marker of acute stress

Experimental design

To monitor CgA levels under a stress condition, we used an experimental acute stress model that has been proved to increase salivary cortisol concentration in saliva samples (Geverink et al., 2002) and the sympathetic activation (Merlot et al., 2011). For this purpose, 30 animals were sampled for saliva (at 08.00 h in the morning), 15 pigs that were subjected to an immobilization of 3 min with a nose snare (stress group), and another 15 pigs that were not subjected to any stressor stimulus (control group). Saliva samples were collected in both groups of animals, stressed and control pigs, before (Baseline) and after 15 min (T 15 min) and 30 min (T 30 min) of the stressor stimulus,

respectively (Fig. 1). The animals set for stress induction were from different pens in order to avoid the possible psychological stress that the animal could suffer because of the observation of nasal snare application to others. In addition, the animals of the control group were sampled in a different room of the stressed animals to avoid the possible stress due to noise.

Results and discussion

Analytical validation: The intra-assay variation showed CVs that ranged between 6.36% and 4.54% for pool with low and high CgA concentration, respectively, whereas the inter-assay variation provided CVs that ranged between 7.10% for the pool with low level of CgA and 5.77% for the pool with high level of CgA. The analytical limit of detection calculated gave a result of 4.27×10^{-3} µg/ml. The lower limit of quantification was 24.5×10^{-3} µg/ml and the upper limit of quantification was established above 12 µg/ml. The dilution of two porcine saliva samples with high CgA concentrations resulted in linear regression equations, where the coefficients of correlation obtained were of r=0.983 and r=0.967 for samples 1 and 2, respectively. Mean of recovery was 92.35%. The overall results of the analytical validation of immunoassay that were assessed, namely, precision, sensitivity (limit of detection and lower limit of quantification) and accuracy (linearity under dilution and recovery), indicated that the assay developed was able to detect porcine CgA in saliva samples.

Model of experimental acute stress: Statistically significant increases at 15 min were obtained in both salivary cortisol, as reported before (Geverink et al., 2002; Merlot et al., 2011), and CgA concentrations (Fig. 1) in comparison to the baseline. The mean saliva CgA concentration increased from levels of 1.75 µg/ml at baseline to 2.56 µg/ml at 15 min, which means an increase of 31%, similar to the increases described in human acute stress studies after clinical examination in adults (26%; Takatsuji et al., 2008) and venipuncture in children (49%; Lee et al., 2006). These data described herein provide the first evidence of the usefulness of salivary CgA as a biomarker of acute stress in pigs.

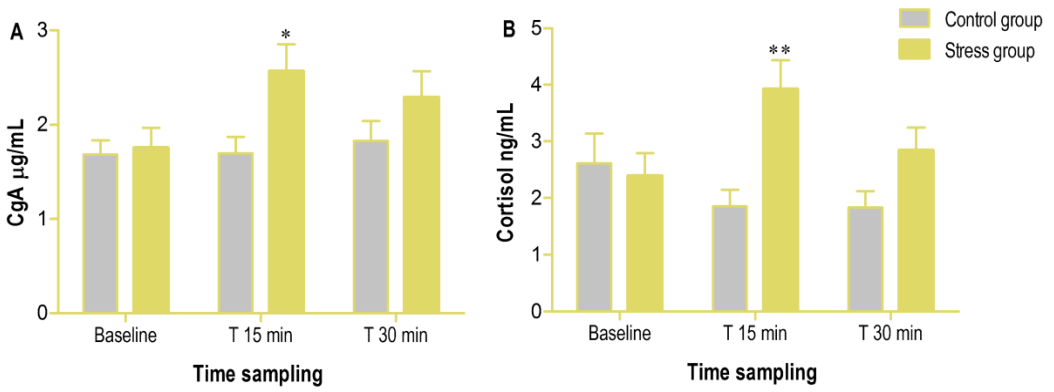


Fig. 1 Salivary chromogranin A (CgA) levels (**A**) and salivary cortisol levels (**B**) in control and stress group (n = 15) before (Baseline) and after 15 min (T 15 min) and 30 min (T 30 min) of the stressor stimulus (immobilization by nose snare). Asterisk indicates statistically significant difference (* P < 0.05; ** P< 0.01) in relation to control group. Values are mean ± standard error.

2.1.2 Experiment 2. Saliva chromogranin A in growing pigs: A study of circadian patterns during daytime and stability under different storage conditions

Experimental design

Evaluation of circadian pattern of salivary CgA secretion in pigs during the daytime: The experiment was conducted on a high sanitary/health-status farm in the southeast of Spain at the end October (the autumn season) and at the end of April (the spring season). A total of 80 animals (40 for each season) were randomly selected for inclusion in the experimental procedure. Pigs were housed in groups of 10: pens 1 and 2 housed 17- and 21-week old non-castrated males, respectively; pens 3 and 4 contained 17- and 21-week old females, respectively. Animals were kept under general commercial housing and had access to a nutritionally-balanced diet and water *ad libitum*. Selected pigs were sampled on two consecutive days, to discriminate between cyclic and random sources of variation, at 07.00, 11.00, 15.00 and 19.00 h. This sampling regime had been previously used in pigs for the study of circadian rhythms of cortisol (Gallagher et al., 2002) and APPs (Gutiérrez et al., 2013). The temperature of the pens at these sampling time-points varied from 18°C in the morning to 30°C at midday in the month of April and from 15°C in the morning to 25°C at midday in the month of October.

Assessment of the stability of salivary CgA: Samples were obtained from 35 randomly-selected pigs of different ages and gender, on the experimental farm of the University of Murcia. To have a sufficient volume of saliva for evaluation of CgA stability, the samples were combined into six pools of saliva, with three different CgA concentrations (two with high, two with intermediate, and two with low concentrations of CgA). Aliquots of 100 µl were prepared from each pool to allow analysis after defined periods of storage at different temperatures. All aliquots were prepared in duplicate, with a protease-inhibitor cocktail (1 µl/ml, P8340; Sigma–Aldrich) added to one of the replicate samples. One aliquot from each pool was immediately analysed to determine a baseline (day 0) CgA concentration. The remaining aliquots were analysed after storage at 4°C, –20°C or –80°C after 2, 5, 15, 30, 90, 180 and 360 days in the presence or absence of the protease inhibitor. Two additional larger aliquots (1 mL) from each of the saliva pools were stored at –20 °C or –80°C for evaluation of the effects of freezing and thawing on the CgA concentration. These aliquots were measured in parallel with the other aliquots, but were subjected to repeated freezing and thawing (seven cycles) at each of the eight analysis time points (Gutiérrez et al., 2011).

Results and discussion

Circadian pattern of CgA in porcine saliva during the daytime in two different seasons: No statistically significant differences were observed between sampling times over the two consecutive days, either in April or in October. Our results are in agreement with previous studies of salivary CgA in dogs (Kanai et al., 2008). The fact that salivary CgA does not seem to be influenced by circadian variation could be considered as an advantage in comparison to other salivary markers of stress in pigs, such as cortisol (Gallagher et al., 2002; Hillmann et al., 2008), IgA (Muneta et al., 2010) or interleukin-18 (Muneta et al., 2011) that vary during the day. However, salivary CgA concentrations were significantly higher in the group of pigs sampled in the autumn compared to those sampled in the spring ($P < 0.0001$). It is possible that the recorded seasonal variation in salivary CgA might be due to temperature differences between seasons, although several other environmental factors are also possible.

Effect of gender and age on circadian pattern of CgA in porcine saliva during the daytime: There was no significant effect of gender on salivary CgA concentrations. Mean ± SD salivary CgA concentrations were 0.76 ± 0.05 µg/ml for males vs. 0.81 ± 0.08 µg/ml for females in spring whereas in autumn these were 2.07 ± 0.15 µg/ml for males vs. 1.91 ± 0.09 µg/ml for females. In addition, there was no significant age effect on salivary CgA concentrations. Mean ± SD salivary CgA concentrations were 0.81 ± 0.04 µg/ml for pigs of 17 weeks of age vs. 0.75 ± 0.08 µg/ml for pigs of 21 weeks of age in spring whereas in autumn, values were 1.91 ± 0.10 µg/ml for pigs of 17 weeks of age vs. 2.07 ± 0.14 µg/ml for pigs of 21 weeks of age. Similarly, Kanai et al. (2008) did not find significant differences between male and female in the salivary CgA concentration in dogs.

Effect of storage temperature on salivary CgA concentrations: The samples showed no significant reduction in CgA concentrations at day 2, but by day 5 of refrigeration, there was a reduction in salivary CgA of 24% at intermediate concentrations and 26% at low concentrations. After 30 days of refrigeration, there was a reduction of 10% at high concentrations. Refrigeration had a major impact on salivary CgA measurements after 90 days storage (35%, 47% and 36% for the high, intermediate and low pools, respectively). Salivary CgA was relatively stable when samples were stored at –20°C, with a modest reduction of 5–10% after 360 days of storage, over the range of different concentrations. Furthermore, the values did not differ significantly after seven cycles of repeated freezing and thawing, with a reduction of 10–15%. When saliva was stored frozen at –80°C, similar results were obtained with a reduction of 4–7% after 360 days of storage, over the range of different concentrations. A modest reduction of 6–14% was observed, after seven cycles of repeated freezing and thawing. In the present study, addition of a protease inhibitor did not provide any additional improvement in CgA stability during the experiment, as it has also been described by Gutiérrez et al. (2011) for salivary CRP.

2.2 Objective 2

El objective 2 was covered by 3 experiments corresponding to Article 3 (experiment 1), Article 4 (experiment 2) and Article 5 (experiment 3).

2.2.1 Experiment 1. Validation of an automated chemiluminescent immunoassay for salivary cortisol measurements in pigs

Experimental design

Two models of stress induction, animal transport and immobilization, were used to evaluate the effect of stress on salivary cortisol concentrations using the chemiluminescent assay system described herein. A group of 10 pigs (165 days of age and approximately 100 kg bodyweight) were transported commercially by road from farm to slaughterhouse in the month of December, with a transport time of 30 min. The commercial transporter allowed 1.5 m^2 per pig, with no sawdust, feed, or water provided. The animals were sampled immediately before loading (pre-stress or baseline) and again upon arrival at slaughterhouse pens (post-stress). A second group of 10 pigs was immobilized for 1 min with a nose-snare (stressor stimulus), and saliva samples were collected before (pre-stress or baseline) and 15 min after (post-stress) the stressor stimulus (Fig. 2). The animals designated for stress induction were from different pens in order to avoid the possible psychological stress that the animal could suffer due to the observation of the nasal snare application to others.

Results and discussion

Results of analytical validation are shown below in table 1.

The results showed that both models of stress provided a significant increase in salivary cortisol levels after application of the stressor stimulus compared to those obtained at baseline (Fig. 2). In the first model, the median saliva cortisol concentration increased with a mean magnitude of 78%, higher than those obtained in previous studies after a transport of 25 min (Geverink et al., 1998) (approximately 57%) and 30 min (Schönreiter and Zanella, 2000) (approximately 60%). Moreover, the second model showed similar increases in saliva cortisol concentration (approximately 50%) to those

were obtained by a previous study (Geverink et al., 2002), whereas greater increases, of 72%, were obtained by Merlot et al. (2011). These results demonstrate that the assay system described herein is able to detect increases in salivary cortisol concentrations when pigs are subjected to stressful stimuli.

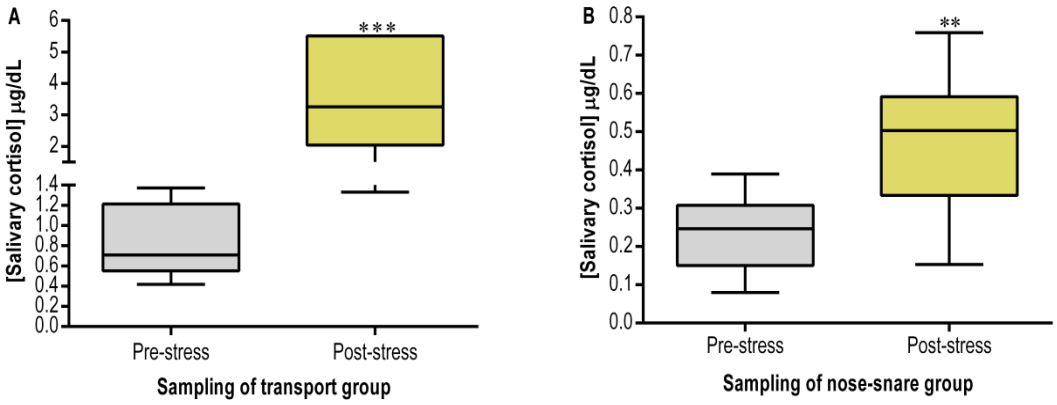


Fig. 2 Evolution of salivary cortisol levels in pigs before (Pre-stress) and after (Post-stress) application of stressor stimuli: **A**, stress of transport group (n = 10); **B**, stress of immobilization with nose-snare group (n = 10). The plot shows median (line within box), 25th and 75th percentiles (box), 5th and 95th percentiles (whiskers). Asterisk indicates statistically significant difference (** P < 0.01; *** P < 0.001) in relation to Pre-stress sampling.

2.2.2 Experiment 2. Salivary testosterone measurements in growing pigs: validation of an automated chemiluminescent immunoassay and its possible use as an acute stress marker

Experimental design

Two models of stress induction, immobilization and transport, were used to evaluate the effect of stress on salivary testosterone concentrations.

A group of eight entire male pigs, 30 weeks old, was immobilized for 1 min with a nose-snare (stressor stimulus), and saliva samples were collected (at 08.00 h) before the immobilization of animals (pre-stress or baseline), and remaining samples were taken at 0 (immediately after restraint with nose-snare), 15 and 30 min after restraint stress (T0, T15 and T30, respectively). A second group of six entire female pigs, 30 weeks old, was transported commercially by road from farm to slaughterhouse in the month of January (between 07.00 h and 11.00 h), with a transport time of approximately 30 min. The

commercial transporter was realized and allowed the welfare recommendations described in Directive 2001/88/EC, 2001 and Directive 2001/93/EC, 2001. Animals were unloaded on arrival at the slaughterhouse in the reception pen, and after the first sampling the pigs were moved to a lairage area (20 °C, stocking density of 150 kg/m^2). During lairage, the studied pigs were mixed with unfamiliar animals and had free access to water. Saliva samples were collected at the farm prior to any handling of the pigs (pre-stress or baseline), after unloading (post stress) at the slaughterhouse in the reception pen (T0), after 30min (T30) and after 60 min (T60) in the lairage area.

Results and discussion

Results of analytical validation are shown below in table 1.

The increment obtained in salivary cortisol and CgA after a nose-snare stress model (Fig. 3A) were similar to those reported before (Escribano et al., 2013). Salivary testosterone increase (2.76-fold increase) was of higher magnitude than those found for cortisol (1.96-fold increase) and CgA (1.64-fold increase) concentrations. The mechanism for the increase in testosterone to acute stress remains unclear. One reason could be the higher sensitivity of the testes to luteinizing hormone as result of the activation of the SAM axis by norepinephrine (Chichinadze and Chichinadze, 2008), which has been shown to stimulate production of luteinizing hormone in both men and women (Chrousos et al., 1998). Significantly higher salivary cortisol concentrations were observed after short road transport model. However, salivary cortisol levels (Fig. 3B2) returned to basal levels as has been reported before (Geverink et al., 1998; Soler et al., 2013), whereas the highest magnitudes of increase in salivary testosterone (Fig. 3B1) (4.48-fold increase) and CgA (Fig. 3B3) (2.69-fold increase) were found at T60. A possible explanation for the evolution of salivary testosterone and CgA levels is that due to factors as uncertainty, social pressure (by mixing with unfamiliar animals) and fear have induced a "fight-flight" response in pigs with the activation of the SAM system (von Borrell, 2001).

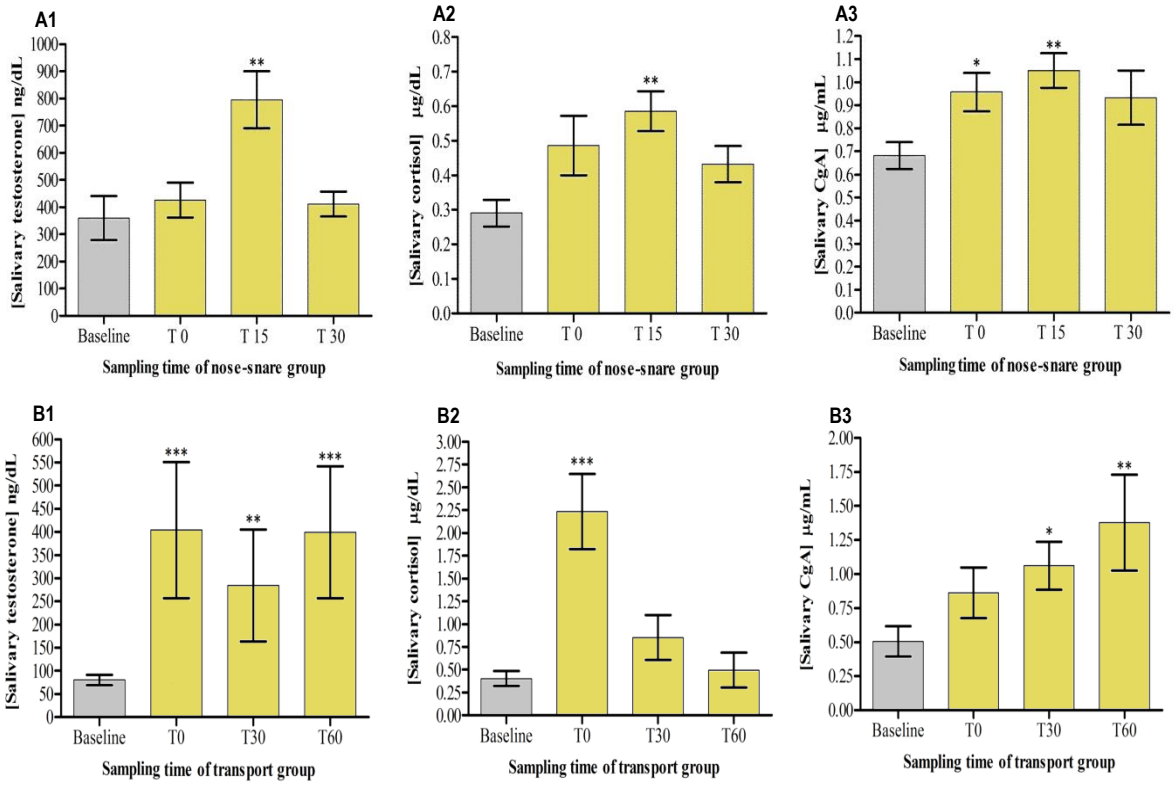


Fig. 3 Evolution of salivary testosterone (1), cortisol (2) and chromogranin A (CgA) levels (3) in two stress models. (**A**) In first stress model, where eight male pigs were sampled prior to any immobilization (baseline), immediately after immobilization (post-stress) over 1 min with nose-snare (T0), after 15 min (T15) and after 30 min (T30) the application of stressor. (**B**) A second stress model, where six female pigs were sampled prior to any handling of the pigs (baseline), immediately after unloading (post-stress) at the slaughterhouse (T0), after 30 min (T30) and after 60 min (T60). Asterisk indicates statistically significant difference (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$) in relation to baseline levels. Values are mean ± standard error.

2.2.3 Experiment 3. Validation of three commercially available immunoassays for quantification of IgA, IgG, and IgM in porcine saliva samples

Experimental design

The ability of these immunoassays to distinguish healthy and diseased pigs was investigated by comparing saliva of fifteen animals from two farms of different sanitary status. Animals were divided in two groups according to the farm of origin. Healthy group: clinically normal pigs from SPF farm. Animals with serology negative to PRRSV, PMWS, Aujeszky's disease and Enzootic pneumonia infection and without the presence of lesions in postmortem examinations. Diseased group: pigs from a farm seropositive to PRRSV and PMWS that exhibited clinical signs compatible with the disease such as: diarrhea, abscesses, wasting, apathy, external injuries or respiratory signs. The post-

mortem examinations showed lesions such as: pulmonary edema, interstitial pneumonia, interstitial hepatitis, lymphoid depletion, gastric ulcer or abscesses.

Results and discussion

Results of analytical validation are shown below in table 1.

Clinical validation: The concentration of total IgA in saliva was statistically significantly higher in the group of diseased animals versus healthy animals ($P < 0.05$; $P = 0.0144$). Median IgA concentrations of 101.4 µg/ml and of 234 µg/mL were found in healthy and diseased animals, respectively. The IgM also showed differences statically significant between healthy and diseased groups of animals ($P < 0.01$; $P = 0.0014$). Median IgM concentration in healthy animals was of 16.33 µg/ml and in diseased animals was of 65.26 µg/ml. The largest differences statically significant between healthy and disease animal groups were obtained in the IgG ($P < 0.0001$). In this case the median IgG concentrations were of 9.09 µg/ml and of 102.6 µg/ml for healthy and diseased animals, respectively. Therefore, the three immunoassays used for Igs determinations were able to detect different Igs concentrations of healthy and diseased animal groups and could be a suitable biomarker to evaluate the humoral immune status of pigs by a non-invasive and no stressful procedure.

Results and discussion of analytical validations of experiments 1, 2 and 3

Table 1 Analytical validation data of different assays to measure cortisol, IgA, IgG, IgM and testosterone in porcine saliva samples.

Analyte	**Units**	**Precision Cv,%**	**Accuracy**			**DL**	**DLL**
			Linearity		**Recovery**		
		Intra Inter	**Equation**	**R**	**%**		
Cortisol	µg/dl	<12 <16	y=0.964x+1.90; y=0.978x-0.35	0.998; 0.996	88.0-95.6	0.016	0.197
IgA	µg/ml	<7 <13	y=1.004x+7.54; y=1.008x+3.62	0.989; 0.994	82.4-99.3	8.25×10^{-6}	4.94×10^{-6}
IgG	µg/ml	<8 <10	y=1.015x+0.79; y=0.978x+0.46	0.992; 0.999	84.7-102	7.74×10^{-6}	2.12×10^{-6}
IgM	µg/ml	<10 <12	y=1.005x-4.18; y=1.007x-5.73	0.989; 0.992	115-119	8.56×10^{-6}	3.80×10^{-6}
Testosterone	ng/dl	<8 <10	y=1.020x-15.48; y=0.981x-31.97	0.999; 0.999	93.4-104	13.60	19.60

Cv: Coefficient of Variation ; DL: Detection Limit; DLL: Detection Lower Limit

The automated chemiluminescent assay system tested in the current study provided good analytical performance and allowed for an adequate discrimination between samples with low and high cortisol and testosterone concentrations. The assay could be a used as a non-invasive and nonradioactive system to analyse salivary cortisol and testosterone in stress research, and its ease of use and high throughput of samples make this assay system an alternative to radioimmunoassay and manual ELISAs techniques. Moreover, the three ELISAs would be suitable for use in porcine saliva samples for Igs determinations, at the dilutions established in our study, since they also provide a good precision, sensitivity and accuracy.

2.3 Objective 3

The objective 3 was covered by one experiment which corresponds to Article 6.

2.3.1 Experiment. Effect of repeated administration of lipopolysaccharide on inflammatory and stress markers in saliva of growing pigs

Experimental design

The salivary biomarkers used in the present study were selected to allow evaluation of both immune and neuroendocrine systems in response to LPS challenge. Thus, a comprehensive picture of the physiological mechanisms involved in the systemic inflammatory response could be obtained, exploring the interaction between the immune and neuroendocrine systems (Vakharia and Hinson, 2005).

Sixteen crossed Piétrain × (Landrace × Large White) growing pigs of 35 kg bodyweight (77 days old) were used in the experiment. Equal numbers of entire males and females were selected. Animals were fitted with a jugular catheter under general anaesthesia, according to the technique previously described by Melchior et al. (2004), prior to being housed in an experimental room containing 16 individual pens (0.85 × 1.30 m). Every 2 days, the catheters were flushed with 5 mL of sterile saline containing 50 IU/ml heparin to prevent blood clotting. The ambient temperature of the room was maintained at 24 ± 0.2°C and a 12 h light/dark cycle (08.00 to 20.00 h) was

implemented. Pigs had free access to water, and a nutritionally-balanced diet was provided every morning at 08.30 h.

Animals were adapted to experimental conditions for 3 weeks, after which 10 pigs received injections of Escherichia coli LPS (LPS; O55:B5, Sigma–Aldrich) and six control pigs were left untreated. LPS was administered via the catheter on three occasions at 48 h intervals (T1, T2 and T3), at an initial dosage of 30 μg/kg bodyweight, increased by 12% at each subsequent injection in an effort to maintain the inflammatory response (Ziegler-Heitbrock, 1995). LPS was diluted in sterile saline solution (400 μg/ml), and an appropriate volume was administered intravenously to each pig according to its bodyweight (between 3.7 and 6.4 ml). Injections were performed slowly (over 5 min) at 11.00 h. Rectal temperatures were measured at baseline and 3 h after T1 and T3, using a digital thermometer (Microlife Corporation).

Results and discussion

Rectal temperature: After T1, LPS-treated pigs showed evidence of pyrexia, relative to baseline ($P < 0.001$). Exposure to LPS during infection with Gram-negative bacteria elicits release of pro-inflammatory cytokines, as part of the host innate immune response. These cytokines act systemically to induce a febrile response and promote synthesis of APPs from the liver (Eckersall, 2000).

Salivary flow rate: There was no significant difference comparing control and LPS-treated groups at any time point. However, LPS-treated pigs had reduced salivary flow rates, relative to baseline, after T1 ($P < 0.01$), T2 ($P < 0.001$) and T3 ($P < 0.001$). A reduction in secretion of saliva has been reported following LPS treatment in other studies (Lomniczi et al., 2001; Fernandez-Solari et al., 2010).

Analysis of APPs in saliva: Considering the age of the pigs, the concentrations of Hp and CRP were higher than the reference range (Gutiérrez et al., 2009b) after the first two injections of LPS, which confirms that the inflammatory response could be detected in saliva. The increase in salivary Hp (Fig. 4A) and CRP (Fig. 4B) concentrations, relative to baseline, was greater than that reported by Frank et al. (2003) for serum samples after similar LPS administration.

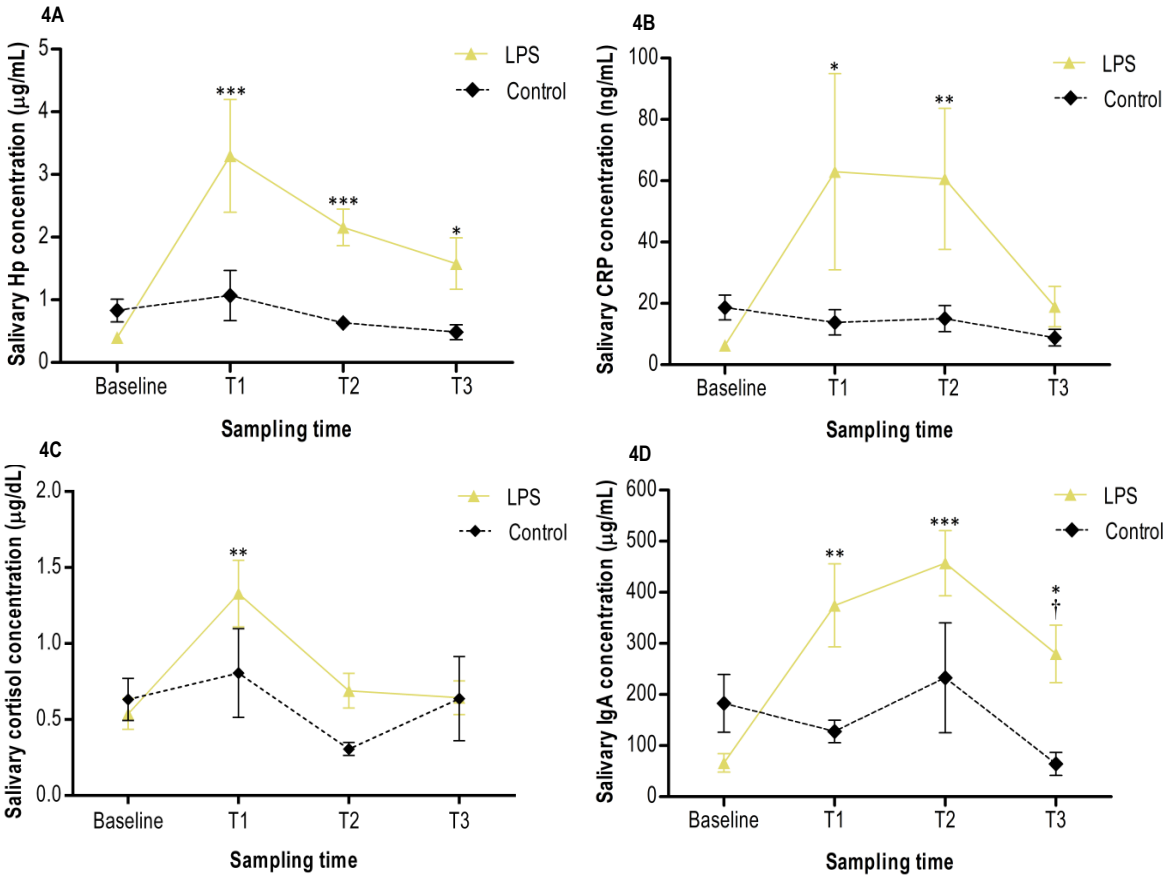


Fig. 4 (**A**) Salivary Haptoglobin (Hp), (**B**) C-reactive protein (CRP), (**C**) Cortisol and (**D**) Immunoglobulin A (IgA) concentrations in control and LPS-treated pigs before (Baseline), after the first (T1), the second (T2) and third (T3) administration of Escherichia coli lipopolysaccharide (LPS). Values shown are means ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to baseline for each group. † $P < 0.05$ comparing LPS-treated and control pigs.

Analysis of stress biomarkers in saliva: The significant increase in cortisol after the first LPS administration (Fig. 4C) is consistent with endotoxaemia inducing activation of the HPA axis in pigs (Williams et al., 2009). The magnitude of the response in salivary cortisol seen at T1 was in a similar range as previously reported in pigs by Lay et al. (2011) and Koopmans et al. (2012). In the present study there was no significant change in salivary CgA after LPS administration. Moreover, we demonstrated that salivary IgA concentrations also increased in response to LPS challenge in pigs (Fig. 4D). Alternatively, salivary secretion of IgA might be linked to activation of the SAM system, as has been proposed in humans (Allgrove et al., 2008). An increase in salivary IgA has been reported in pigs as a response to acute stress during restraint (Muneta et al.,

2010). Thus an increase in salivary IgA is not only associated with activation of the adaptive immune system, can also occur non-specifically in response to stress.

The results of the study demonstrated that repeated administration of LPS can induce significant elevation in salivary biomarkers of the neuroendocrine and immune systems of pigs. Measurement of Hp, CRP, IgA and cortisol in saliva could be used as a practical tool, which is simple to perform and non-invasive, for evaluation of the combined response to endotoxaemia in pigs. In the case of chronic inflammatory states, salivary Hp and IgA might be more sensitive markers than CRP and cortisol.

2.4 Objective 4

The objective 4 was covered by one experiment which corresponds to annex.

2.4.1 Experiment. Changes in saliva biomarkers of stress and immunity in domestic pigs exposed to a psychosocial stressor

Experimental design

The experimental design employed in the present study was adapted from others available in the literature for psychosocial stress assessment, based in social "isolation" (Tuchscherer et al., 2009; Soler et al., 2013) and "regrouping" or mixing (Coutellier et al., 2007; Oster et al., 2014).

Five days before the beginning of the experimental period, all animals were accustomed to human contact and saliva collection methodology for 5 days. The experimental design was divided into three periods: pre-stressor, isolation and regrouping. On each day, during the three periods, saliva samples were collected at 11.00 h in both test and control groups. Two additional saliva samples were collected at 30 min after isolation and at 30 min after regrouping. In the study of isolation the behaviour and lesion scores of each animal were measured each day after sampling based on ethogram of behavioural used by Ruis et al. (2001). After mixing the accumulation of skin lesions as a predictor of individual aggressiveness in pigs were

measured by a previously described method (Turner et al., 2006).

The pre-stressor period lasts the first 5 days (day -1d to -5d; Fig. 5). Test (n = 7) and control (n = 7) pigs remained during this period in the same housing conditions (stocking density of 1.1 m^2 per pig). In the day 6 of this period, baseline sample of isolation (0dI) for test and control groups were obtained. For the isolation procedure, 7 pigs were relocated to a new barn where each one in the same room was isolated in individual pens (7.78 m^2 each), where they had olfactory and auditory but not physical contact with other pigs. From day 0 of isolation (0dI; Fig. 5) till day five of isolation (5dI; Fig. 5) the test pigs were kept in this experimental condition. In the day 6 of this period were taken the baseline samples of regrouping (0dR) for test and control groups. The regrouping period started after the isolation period. Test pigs were regrouped and remaining in these conditions during 3 days (day 0 of regrouping, 0dR, to day 3 of regrouping, 3dR; Fig. 5), returning to their original group with the initial conditions (stocking density of 1.1 m^2 per pig). The overall isolation and regrouping procedure took about 3 min.

The control pigs were remained in the same housing conditions during all the experimental period. The temperature of the pens at these sampling time-points was 21.2 ºC ± 0.8 (mean ± SD) and animals were maintained during experimental period with natural lighting.

Results and discussion

Animal behaviour after isolation and body lesions after regrouping: Isolation caused a significant ($P < 0.05$) increase in exploratory behavioural (sniffing, touching and walking through the pen) and vocalizations in test group compared to control group and also in relation to its baseline values (0dI). In addition, all pigs had a higher number of body lesions after regrouping ($P < 0.05$) compared to its baseline values (0dR), and the test group had a higher number of body lesions ($P < 0.05$) compared to the control group after regrouping. The increase of vocalization that was observed after isolation and the aggression and the significant increase of skin lesions that was found after regrouping would indicate an activation of the stress response in pigs (Ruis et al.,

2001; Turner et al., 2006) after applying our psychosocial stressor model.

Salivary total protein and flow rate: Salivary flow rate and salivary total protein concentration did not change in our stress model. Therefore, it can rule out that the significant changes observed in our study are due to the possible dilution or concentration of markers in saliva because of the lack/excess of saliva or the thickness thereof.

Salivary CgA and IgA: The magnitude and time of increase after isolation and regrouping in salivary CgA response (Fig. 5A) was similar to previously described after applying others models of stress, as restriction with nose-snare (Escribano et al., 2013) or transport and accommodation in slaughterhouse (Escribano et al., 2014). Although more investigations are needed to clarify this topic, the salivary IgA showed a delayed response compared with CgA after isolation (Fig. 5B). A hypothesis for the lack of increase of salivary IgA after regrouping could be the fact of the decrease of the immune response. Since, de Groot et al. (2001) have reported that social stress after mixing could hinder the immune response in pigs and therefore the production of different Igs.

Salivary cortisol and testosterone: In our study, no significant changes on salivary cortisol and testosterone after isolation were observed (Fig. 5C; 5D). These results may indicate a quickly adaptation of the domestic pigs to the housing experimental conditions, as has been reported before for salivary cortisol by Jaskulke and Manteuffel (2011). Relative to regrouping baseline (0dR) and compared to control pigs, cortisol and testosterone concentrations were higher after 30 min in test pigs. The magnitude of the increase in salivary cortisol was in a similar range as reported in previous studies on mixing, where typically are involved unfamiliar pigs (de Groot et al., 2001; Merlot et al., 2004; Coutellier et al., 2007). Also, a similar result for testosterone after regrouping in the slaughterhouse has been found (Escribano et al., 2014).

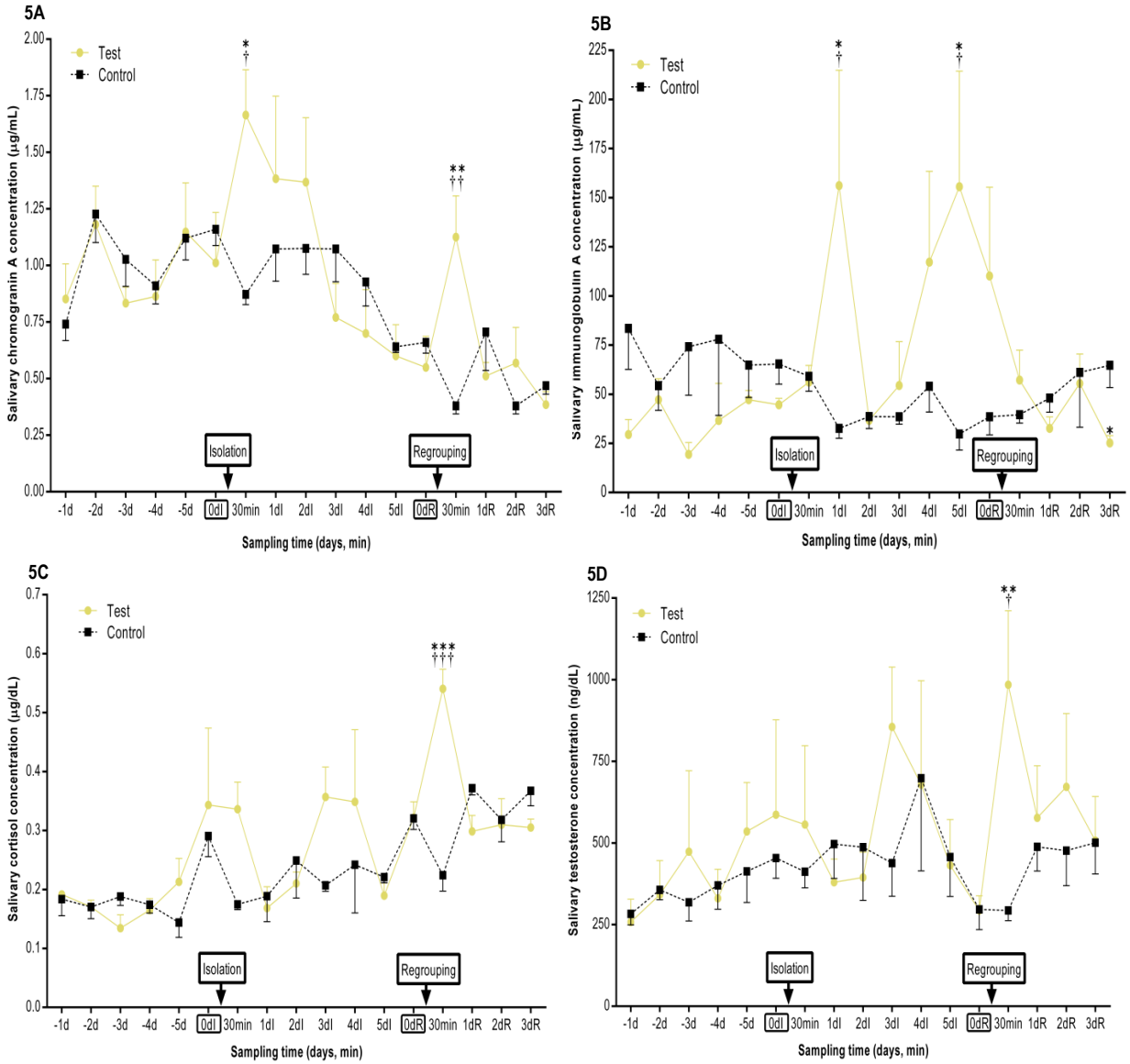


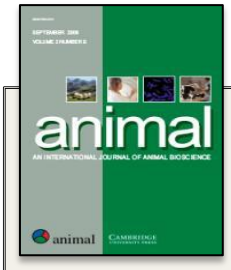
Fig. 5 (**A**) Salivary chromogranin A (CgA), (**B**) immunoglobulin A (IgA), (**C**) cortisol and (**D**) testosterone concentrations in test (n = 7) and control pigs (n = 7); that was divided into three periods: pre-stressor (days -1d to -5d), isolation (0dI to 5dI) and regrouping (0dR to 3dR). Values shown are means ± SEM. * P < 0.05; ** P < 0.01; *** P < 0.001 compared to baseline values (0dI and 0dR) in test or control pigs after isolation and after regrouping for each group. † P < 0.05; †† P < 0.01; ††† P < 0.001 comparing test and control pigs.

Salivary Hp and CRP: Our results indicate that salivary Hp and CRP concentrations did not significantly change in the stress models used in our study. This is in line with a previous report in which no changes in Hp were found after social isolation and short road transport stress (Soler et al., 2013). Then, it should be recognised that, based on our results, these markers do not appears to be indicators of stress. However, salivary Hp and CRP can be used to assess the health status and well-being of pig herds

(Gómez-Laguna et al., 2010). In our case, considering the age of the pigs, the concentrations of Hp and CRP were always lower than the internal reference cut-off values of the laboratory for diseased animals (Gutiérrez et al., 2009b), indicating that animal used in this study were healthy.

The use of several salivary biomarkers could be essential in stress research since our results demonstrated that these appear to react differently to various types of stressors. In the case of isolation, salivary CgA and IgA might be more sensitive markers than cortisol and testosterone, with CgA showing a faster response than IgA. While in the process of regrouped, salivary cortisol, testosterone and CgA might be more sensitive markers than salivary IgA. Moreover, Hp, CRP and total protein concentrations and flow rate in saliva of pigs did not significantly changed after applying this psychosocial stressor as stress model. The Hp and CRP allow to discrimination between healthy and diseased animals while total protein and flow rate can rule out that the significant changes observed are due to alterations in volume or concentration of saliva. Therefore, although further studies are needed, these salivary biomarkers could be used as a practical and non-invasive tool for reflecting the activity of different physiology systems involved in stress response in pigs.

ARTICLES



ARTICLE 1 (Published)

Measurement of chromogranin A in porcine saliva: validation of a time-resolved immunofluorometric assay and evaluation of its application as a marker of acute stress

Article 1 (Animal)

Abstract

The objective of this study was to develop and validate a time-resolved immunofluorometric assay (TR-IFMA) for porcine salivary chromogranin A (CgA) measurements, using a species-specific antibody, and evaluate its behaviour in an acute stress model. Polyclonal antibodies were produced in rabbits immunized with a synthetic porcine fragment of $CgA_{359-379}$ and used to develop a sandwich TR-IFMA. This TR-IFMA was analytically validated and showed intra- and inter-assay coefficients of variation of 6.23% and 5.82%, respectively, an analytical limit of detection of 4.27×10^{-3} μg/mL and a limit of quantification of 24.5×10^{-3} μg/mL. The assay also demonstrated a high level of accuracy, as determined by linearity under dilution (r = 0.975) and recovery tests. When a model of experimental acute stress, in which animals were immobilized for 3 min with a nose-snare (stressor stimulus), was applied, a significant increase ($P < 0.05$) in CgA levels in saliva were detected at 15 min post-stressor stimulus. These results indicate that the assay developed in this study could measure CgA in porcine saliva in a reliable way and that the concentrations of CgA in saliva samples of pigs increase after an acute stress situation.

URL:

http://journals.cambridge.org/action/displayAbstract?fromPage=online&aid=8844045&fileId=S1751731112002005

ARTICLE 2 (Published)

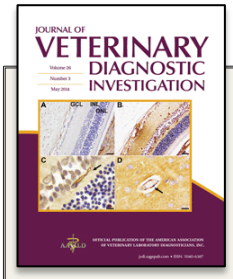
Saliva chromogranin A in growing pigs: A study of circadian patters during daytime and stability under different storage conditions

Article 2 (The Veterinary Journal)

Abstract

Salivary chromogranin A (CgA) is considered to be a biomarker of activation of the sympatho-adrenomedullary system, and has recently been proposed as a useful indicator of the acute stress response in pigs. The aim of the present study was to determinate whether salivary CgA concentrations in healthy growing pigs exhibits any circadian pattern during the daytime, and to evaluate its stability under different storage conditions. A total of 80 pigs (40 in spring and another 40 in autumn) of two different ages and genders were used. To establish the circadian pattern, saliva samples were collected at 07.00, 11.00, 15.00 and 19.00 h on two consecutive days. Pooled samples were used for the stability study and were measured on the day of sampling and periodically for up to 360 days later. Samples were stored at 4 °C, -20 °C or -80 °C and the effect of repeated freezing and thawing was also evaluated. No circadian pattern was detected for salivary CgA in either season and there were no significant effects of gender or age. However, mean salivary CgA concentrations were significantly higher ($P < 0.0001$) in the pigs sampled in autumn, compared to those sampled in the spring. Short term storage at 4 °C is recommended for up to 2 days, whereas frozen samples can be stored for 1 year at -20 °C or -80 °C, without substantial reduction in CgA values. In addition, samples can be frozen and thawed up to seven times without significant loss of the biomarker.

URL: http://www.sciencedirect.com/science/article/pii/S1090023314000148



ARTICLE 3 (Published)

Validation of an automated chemiluminescent immunoassay for salivary cortisol measurements in pigs

Article 3 (Journal of Veterinary Diagnostic Investigation)

Abstract

The aim of the current study was to validate an automated immunoassay for cortisol quantification in the saliva of pigs. The assay had intra- and inter-assay coefficients of variation lower than 16%, in all cases. The limit of detection was 0.016 µg/dL, and the lower quantification limit was 0.197 µg/dL. The assay also demonstrated a high level of accuracy, as determined by linearity under dilution and recovery tests. In addition, this assay was used to quantify cortisol in 2 models of stress: 1 in which animals were immobilized with a nose-snare and 1 in which pigs were transported for a duration of 30 min. In both cases, a significant increase ($P < 0.01$) in salivary cortisol was detected after the stressful situation. Overall, the assay validated in the present study could be used for the evaluation of cortisol changes in stressful situations.

URL: http://vdi.sagepub.com/content/24/5/918.abstract

ARTICLE 4 (Published)

Salivary testosterone measurements in growing pigs: validation of an automated chemiluminescent immunoassay and its possible use as an acute stress marker

Article 4 (Research in Veterinary Science)

Abstract

This study aimed to validate the use of an automated chemiluminescent immunoassay analyser for salivary testosterone measurements in growing pigs and study how circadian pattern during daytime and stress can influence its values. The test method had intra- and inter-assay coefficient of variation lower than 10%. The method showed good linearity and recovery, and detection limits were low enough to detect salivary testosterone levels. No significant differences were observed in testosterone concentrations at different sampling time, and age and gender did not influence circadian pattern. In addition, this assay was used to quantify testosterone in two models of acute stress and, in both cases, significant increases ($P < 0.01$) in salivary testosterone were detected. Therefore, the automated assay system tested for porcine testosterone determinations would be suitable for its use in saliva samples and, furthermore, salivary testosterone levels could be used as a possible marker of acute stress in pigs.

URL: http://www.sciencedirect.com/science/article/pii/S003452881400099X

ARTICLE 5 (Published)

Validation of three commercially available immunoassays for quantification of IgA, IgG, and IgM in porcine saliva samples

Article 5 (Research in Veterinary Science)

Abstract

The objectives of this study were to perform the optimization and validation of three commercially available immunoassays for the measurement of IgA, IgG and IgM (Igs) in porcine saliva samples and to determinate if their concentrations may be used to distinguish healthy from diseased animals. Intra and inter assay coefficients of variation were lower than 15% in all cases. All methods showed good linearity and recovery; and detection limits were low enough to detect Igs levels in healthy and diseased animals. The clinical validation showed an increase statistically significant ($P < 0.05$) in the group of diseased animals versus healthy pigs. Therefore, these assays may be used in porcine saliva samples, in addition, the measurement of Igs in saliva could be a practical tool, simple and minimally invasive, to evaluate the humoral immune status of pigs.

URL: http://www.sciencedirect.com/science/article/pii/S0034528811003778

ARTICLE 6 (Published)

Effect of repeated administration of lipopolysaccharide on inflammatory and stress markers in saliva of growing pigs

Article 6 (The Veterinary Journal)

Abstract

Although saliva could be considered to be an ideal biological sample for evaluation of biomarkers relating to stress and inflammatory responses in pigs, little is known about how these might be influenced by the presence of endotoxaemia. In the present study, the response to repeated administration of lipopolysaccharide (LPS) was investigated, using a panel of salivary stress markers such as chromogranin A (CgA) and cortisol, as well as inflammatory/immune markers such as haptoglobin (Hp), C-reactive protein (CRP) and immunoglobulin A (IgA). Sixteen growing pigs were adapted to experimental conditions for 3 weeks, after which time 10 of the pigs were selected to receive three doses of LPS at 48 h intervals. Saliva samples were taken from all pigs prior to any LPS administration (baseline) and at time points corresponding to 3 h after each injection (T1, T2 and T3). Results showed that repeated administration of LPS induced significant elevation of salivary markers of sympathetic (IgA), hypothalamic-pituitary-adrenal (cortisol) and immune (Hp, CRP and IgA) activity compared to baseline levels ($P < 0.05$). However, rectal temperature, CRP and cortisol data suggested that the amplitude of the inflammatory response decreased with successive LPS administrations. Thus, measurement of salivary biomarkers could be a practical tool for evaluating the inflammatory response to endotoxaemia in pigs. In the case of chronic inflammatory states, salivary Hp and IgA might be more sensitive markers than CRP or cortisol.

URL: http://www.sciencedirect.com/science/article/pii/S1090023314001531

CONCLUSIONS

CONCLUSIONS

1. The assay developed allows the quantification of CgA in porcine saliva samples in a reliable way and its concentrations increase after an acute stress situation. No circadian pattern has been detected for salivary CgA and this biomarker was fairly stable being able to be stored till 1 year at -20ºC or -80ºC.

2. The commercial immunoassays validated for cortisol, testosterone and IgA concentrations can be suitable for its use in saliva samples of pigs with a good precision, sensitivity and accuracy.

3. A single administration of LPS in pigs produces increases of salivary IgA and cortisol. However, repeated administrations only produced increases in IgA whereas cortisol returned to baseline values. No variation in CgA concentrations were observed neither in single nor in repeated LPS administration.

4. The use of a panel of salivary biomarkers would be essential in stress research, since these react differently to various types of stressors. In the case of isolation, CgA and IgA appear to be more sensitive markers than cortisol and testosterone, whereas after the process of regrouped, cortisol, testosterone and CgA seem to be more sensitive markers than IgA.

RESUMEN GENERAL

RESUMEN GENERAL

1. INTRODUCCIÓN

El bienestar animal es de gran importancia en los sistemas de producción animal de hoy en día y puede ser evaluado de diversas maneras, como por ejemplo, con indicadores de comportamiento o con determinaciones bioquímicas de biomarcadores que reflejan la respuesta fisiológica del animal al estrés (Smulders et al., 2006; Fuentes et al., 2011). Desafortunadamente, los métodos de observación tienen algunos potenciales de error o mala interpretación por lo que la cuantificación de las respuestas fisiológicas a un estímulo mediante biomarcadores puede ofrecer una medida más objetiva y sensible de estrés que la observación del comportamiento en la mayoría de las especies (Hart, 2012).

Estos biomarcadores pueden medirse en la sangre, pero su obtención se realiza por punción venosa y la falta de vasos sanguíneos superficiales en los cerdos por lo general requiere la utilización de un método extremadamente invasivo, como la implantación quirúrgica de cánulas, con el fin de obtener muestras de sangre repetidas (Bushong et al., 2000). Para superar este problema, la toma de muestras de saliva se ha postulado como una metodología no invasiva y libre de estrés, en comparación con el muestreo de sangre que puede ser un factor de confusión en los modelos de estrés (Merlot et al., 2011). Además, la toma repetida de muestras de saliva en cortos intervalos de tiempo puede llevarse a cabo sin influir en gran medida la respuesta al estrés (Heintz et al., 2011), lo que facilita el seguimiento de los animales monitorizados. Otra ventaja es que el muestreo de saliva puede ser llevado a cabo por personas con formación limitada. Por todo lo anteriormente mencionado, la saliva es considerada como un material ideal para la evaluación de la condición de estrés en los cerdos, mediante el uso de biomarcadores de estrés salivares (Muneta et al., 2010; Fuentes et al., 2011; Soler et al., 2013).

Se han postulado diferentes biomarcadores de estrés que pueden ser utilizados para el estudio de los diferentes ejes o sistemas; HPA, SAM, HPG y sistema inmune;

activados durante condiciones de estrés. Por ejemplo, el cortisol salivar indica la actividad del eje HPA en respuesta a diferentes factores de estrés en los cerdos (Cook et al., 1996; Schönreiter et al., 1999; Merlot et al., 2011). Además, en humanos, las mediciones de CgA salivar podrían ser utilizadas como una herramienta sensible y fiable para monitorizar la actividad del sistema nervioso simpático, lo que constituye la respuesta inicial al estrés (Kanno et al., 1998; Nakane et al., 1998); siendo una posible alternativa a las catecolaminas, las cuales son consideras pobres marcadores de los cambios agudos en la actividad SAM (Schwab et al., 1992; Kennedy et al., 2001). La testosterona salivar, un producto del eje HPG (Schoofs and Wolf, 2011), ha sido relacionada en estudio humanos con la modulación de la sensación de dolor (Choi et al., 2012), la depresión y los trastornos de ansiedad (Giltay et al., 2012). Por otra parte, se ha sugerido que la IgA salivar, un componente importante de la defensa adaptativa inmune de mucosas, podría ser un biomarcador sensible y específico del estrés agudo durante la restricción en cerdos (Muneta et al., 2010).

Al comienzo de esta tesis doctoral detectamos que no había ensayos comerciales disponibles para la medición de CgA en cerdos, tampoco había estudios exhaustivos de validación analítica de ensayos para las mediciones de cortisol, testosterona, e inmunoglobulinas en la saliva de los cerdos; así como una falta de estudios donde los biomarcadores fueran evaluados globalmente en modelos de estrés.

2. OBJETIVOS

Esta tesis doctoral ha sido diseñada con el fin de producir avances en la medición de biomarcadores de estrés y desarrollar y validar un panel de biomarcadores salivares para evaluar los diferentes sistemas fisiológicos (SAM, HPA, HPG y sistema inmune) que participan en el mecanismo de estrés. Con este propósito, los objetivos específicos fueron:

1. Desarrollar y validar un TR-IFMA para las mediciones de CgA salivar porcina, utilizando un anticuerpo específico de la especie, y evaluar su comportamiento en un modelo de estrés agudo. Además, estudiar si las concentraciones de CgA salivar exhiben un patrón circadiano durante el día, y evaluar su estabilidad bajo diferentes condiciones de almacenamiento.

2. Validar inmunoensayos disponibles comercialmente para la cuantificación de cortisol, testosterona e IgA en la saliva de los cerdos.

3. Evaluar la respuesta de un panel de varios biomarcadores salivares a la administración repetida de LPS.

4. Investigar la respuesta de un grupo de varios biomarcadores salivares después de aplicar un modelo de estrés psicosocial en cerdos basado en el aislamiento y el reagrupamiento.

3. MATERIAL Y MÉTODOS

3.1 Ética biomédica

Los procedimientos que implican la manipulación de animales fueron aprobados por el Comité de Ética de la Universidad de Murcia y siguieron las recomendaciones de la Convención Europea 139 para la Protección de los Animales Vertebrados Utilizados para la Experimentación y Otros Fines Científicos (Consejo de Europa 140, ETS Número 123). Por otra parte, como el procedimiento experimental del artículo 6 fue realizado en la unidad experimental del Instituto Nacional de Investigación Agronómica (INRA, Francia), los procedimientos se llevaron a cabo de acuerdo con las pautas establecidas para el cuidado de animales aprobados por el comité de ética regional (C2EA-07, acuerdo R-2012-NLF-02. Fecha de aprobación: 22 Junio 2012).

3.2 Procedimiento de muestreo

La saliva de todos los animales fue recogida utilizando tubos Salivette (Sarstedt, Nümbrecht, Alemania), conteniendo una esponja en lugar de la barra de algodón comercial, según estudios anteriormente publicados (Gutiérrez et al., 2009). A los cerdos se les permitió masticar la esponja, que se sujeta a una barra de metal delgada y flexible, hasta que la esponja se humedeció a fondo (1-3 min). Posteriormente, las esponjas fueron colocadas en los tubos y se centrifugaron a 3000 *g* durante 10 min. Las muestras de saliva fueron recogidas y almacenadas a -20°C o -80°C hasta su análisis.

3.3 Determinación del analito en saliva

3.3.1 Determinación de CgA

Para la medición de los niveles de CgA en la saliva porcina se desarrolló un TR-IFMA.

Producción y marcado de anticuerpo policlonal: Se seleccionó un péptido recombinante de proteína CgA porcina conjugado con KLH como inmunógeno para producir anticuerpos policlonales. En particular, el fragmento de péptido utilizado fue la región CgA359-379 y la secuencia se obtuvo de la base de datos UniProt; (http://www.uniprot.org/ uniprot/P04404). El fragmento peptídico de CgA recombinante se obtuvo comercialmente (EnoGene Biotech, Nueva York, NY, USA). Los anticuerpos policlonales específicos contra la CgA porcina se produjeron en nuestro laboratorio de acuerdo con el protocolo estándar (Universidad de California Berkley Animal Care and Use Committee, 2009). El animal que dio la mejor respuesta fue seleccionado y su contenido de IgG se purificó utilizando una columna de afinidad HiTrap™ columna G HP, de acuerdo con las instrucciones del fabricante (GE Healthcare Life Sciences, Munich, Alemania). La pureza de las Igs se evaluó por SDS-PAGE, usando geles de acrilamida del 4% al 12%, y fueron cuantificadas mediante el ensayo de proteínas RC/DC (Bio-Rad Laboratorios, Madrid, España).

Una alícuota de 1 mg de los anticuerpos policlonales producidos (conejo anti-recombinante CgA359-379) se marcó con biotina utilizando un kit comercial (No weigh Sulfo-NHS-biotina, Pierce, Thermo Fisher Scientific, Barrington, IL, USA) y se utilizó como un anticuerpo de captura en el ensayo TR-IFMA desarrollado. Una alícuota

adicional de 1 mg, de los mismos anticuerpos policlonales, se marcó con un quelato de Eu (kit de DELFIA Eu-labelling, PerkinElmer Life y Analytical Sciences, Turku, Finlandia), siguiendo las instrucciones del fabricante y se usó como anticuerpo de detección. El procedimiento de ensayo después de la optimización es el siguiente:

1.- Añadir 100 µL/pocillo de anticuerpo biotinado (100 ng/pocillo).
2.- Incubación, con agitación continua, a temperatura ambiente (20-25°C) durante 60 min y lavar 4 veces con tampón de lavado (DELFIA, PerkinElmer).
3.- Añadir 100 µL/pocillo de los estándares (seis concentraciones de estándar fueron escogidas para el uso habitual: 4,68; 9,37; 18,75; 37,5; 75 y 150 ng/pocillo), o de las muestras de saliva diluidas a 1/4 con DELFIA tampón de ensayo.
4.- Incubación, con agitación continua, a temperatura ambiente durante 60 min y lavar 4 veces con DELFIA solución de lavado.
5.- Añadir 100 µL/pocillo de anticuerpo marcado con Eu a la dilución óptima de trabajo (200 ng/pocillo) en DELFIA tampón de ensayo.
6.- Incubación, con agitación continua, a temperatura ambiente durante 60 min y lavar 4 veces con DELFIA solución de lavado.
7.- Añadir 200 µL/pocillo de solución DELFIA intensificadora e incubar a temperatura ambiente durante 5 min con agitación.
8.- La fluorescencia se midió con VICTOR^2 1420 multilabel counter, y las concentraciones fueron calculadas por el programa Wallac MultiCalc.

3.3.2 Determinaciones de Cortisol

La concentración de cortisol fue medida usando un inmunoensayo enzimático quimioluminiscente competitivo en fase sólida siguiendo las instrucciones del fabricante (Immulite/Immulite 1000 cortisol, Siemens Medical Solutions Diagnostics, Los Angeles, CA).

3.3.3 Determinaciones de testosterona

La concentración total de testosterona fue medida usando un inmunoensayo enzimático quimioluminiscente competitivo en fase sólida siguiendo las instrucciones del fabricante (Immulite/Immulite 1000 Total Testosterone, Siemens Medical Solutions Diagnostics, Los Angeles, CA).

3.3.4 Determinaciones de IgA, IgG e IgM

Las concentraciones de Igs en saliva se determinaron mediante tres ensayos ELISA disponibles en el mercado (IgA, IgG e IgM ELISA cuantificación Kit; Bethyl laboratorios, Montgomery, TX, USA). Diferentes concentraciones de anticuerpo de captura (25, 50, 100, 200, 300, 400, 500, y 1000 ng/pocillo) y de detección (1/10,000-1/80,000) fueron testadas y se optimizaron las condiciones de funcionamiento de los tres inmunoensayos. La dilución óptima de la muestra para cada Ig fue seleccionada como aquella dilución que mostró la mayor diferencia entre las muestras con concentraciones altas y bajas. Los procedimientos de los ensayos, después de la optimización de anticuerpos, fueron los siguientes:

1. Añadir 100 µL/pocillo de anticuerpo de captura a 100 ng/pocillo para IgM y 50 ng/pocillo para IgG e IgA de concentración en tampón carbonato/bicarbonato.
2. Incubación a temperatura ambiente durante 60 min y lavar 3 veces con solución de lavado.
3. Añadir 200 µL/pocillo de solución de bloqueo.
4. Bloquear a temperatura ambiente durante 60 min y lavar 3 veces con solución de lavado.
5. Añadir 100 µL/pocillo de estándares (de acuerdo a las instrucciones del fabricante) o las muestras de saliva diluidas a 1/200 para IgG e IgM y 1/800 para IgA.
6. Incubación a temperatura ambiente durante 60 min y lavar 3 veces con solución de lavado.
7. Añadir 100 µL/pocillo de HPR a la dilución óptima de trabajo (IgA 1/80.000; IgG, 1/70.000 or IgM, 1/70.000) en el diluyente del conjugado.
8. Incubación a temperatura ambiente durante 60 min y lavar 5 veces con solución de lavado.
9. Añadir 100 µL/pocillo de TMB e incubar en oscuridad a temperatura ambiente durante 5 min.
10. Parada de la reacción con 100 µL/pocillo de ácido sulfúrico 2 M y la absorbancia fue medida a 450 nm con PowerWave XS, Bio-Tek Instruments Inc.

3.4 Validación analítica

Para realizar la validación analítica se evaluaron los siguientes parámetros:

3.4.1 Evaluación de la precisión intra-ensayo y la inter-ensayo: La precisión intra-ensayo, expresada como CV, se calculó después de analizar las mezclas o "pools" de muestras de saliva, que contienen niveles altos y bajos de analito respectivamente, cinco veces en una sola prueba analítica. Cada pool se preparó usando muestras de saliva de los animales con concentraciones similares de analito previamente cuantificados por el propio ensayo. Estos mismos pools se utilizaron para determinar la precisión inter-ensayo mediante su análisis en seis días diferentes dentro de un período de 15 días. Las muestras se congelaron en alícuotas y sólo se descongelaron los viales requeridos para cada serie de análisis, con el fin de evitar cualquier posible variación como resultado de los ciclos repetidos de congelación-descongelación (Soler et al., 2011).

3.4.2 Cálculo del límite analítico de detección: El límite de detección fue definido como la concentración más baja de analito que podía distinguirse de una muestra de valor cero. Este fue calculado para el inmunoensayo a partir de 10 determinaciones repetidas del estándar cero (tampón de ensayo) como el valor medio más dos SD (Eckersall et al., 1999).

3.4.3 Estimación del límite inferior y superior de cuantificación: El límite inferior y el superior de cuantificación fue determinado en base a la concentración de analito más baja y más alta, respectivamente, que puede ser medida en la parte lineal de la curva de calibración con un CV <20%, y por encima del límite de detección en el caso del límite inferior. Para estimar el límite inferior de cuantificación, una muestra de saliva se diluyó en serie en tampón de ensayo y se analizaron cinco repeticiones de cada dilución en la misma prueba analítica (Park et al., 2010).

3.4.4 Evaluación de la exactitud del ensayo: Como, en conocimiento de los autores, no hay un estándar de referencia disponible para cuantificar el analito en la saliva porcina, la exactitud se investigó indirectamente mediante el experimento de recuperación y la linealidad bajo dilución.

El experimento de recuperación fue realizado siguiendo procedimientos descritos anteriormente (Parra et al., 2005). Una muestra con alta concentración de analito se mezcló con diferentes cantidades de una muestra con una baja concentración de

analito con el fin de conseguir diferentes diluciones de la muestra. Por lo tanto, la muestra con alto nivel de analito se diluyó 2 - (50%), 4 - (25%), y 10 veces (10%) con la muestra de saliva de nivel bajo (50%, 75%, y 90%, respectivamente). Además, la muestra de saliva con bajo nivel de analito se diluyó 4 veces (25%) con la muestra de saliva de alto nivel de analito (75%). Los niveles de analito detectados y esperados para cada muestra de saliva diluida fueron comparados, y se calcularon los porcentajes de recuperación.

La linealidad bajo dilución se determinó mediante el uso de dos muestras de saliva de porcino con altos niveles de analito diluidas en serie (1/2, 1/4, 1/8, 1/16, 1/32, 1/64) con agua desionizada. Después, se construyeron las curvas representando las concentraciones de analito medidas frente a la concentraciones del analito esperadas (Gutiérrez et al., 2009). Las concentraciones del analito fueron medidas por el propio ensayo y se midieron por duplicado para cada dilución.

4. DISEÑO EXPERIMENTAL, RESULTADOS Y DISCUSIÓN

4.1 Objetivo 1

El objetivo 1 fue cubierto por 2 experimentos correspondientes al artículo 1 (experimento 1) y al artículo 2 (experimento 2).

4.1.1 Experimento 1. Medición de la cromogranina A en saliva porcina: validación de un ensayo inmunofluorométrico a tiempo retardado y evaluación de su aplicación como un marcador de estrés agudo

Diseño experimental

Para monitorizar los niveles de CgA bajo una situación de estrés se utilizó un modelo experimental de estrés agudo que ha demostrado aumentar la concentración de cortisol en muestras de saliva (Geverink et al., 2002) y la activación simpática (Merlot et al., 2011). Para este propósito, 30 animales fueron muestreados (a las 08:00 h de la mañana), 15 cerdos fueron sometidos a una inmovilización de 3 min con un acial (grupo de estrés), y otros 15 cerdos no fueron sometidos a ningún estímulo de estrés

(grupo control). Las muestras de saliva se recolectaron en ambos grupos de animales, cerdos estresados y controles, antes (Basal) y después de 15 min (T 15 min) y 30 min (T 30 min) del estímulo estresante, respectivamente (Fig. 1). Los animales elegidos para la inducción de estrés eran de diferentes parques con el fin de evitar el posible estrés psicológico que pudieran sufrir debido a la observación de la aplicación del acial en otros animales. Además, los animales del grupo de control se muestrearon en una habitación diferente de los animales estresados para evitar el posible estrés debido al ruido.

Resultados y discusión

Validación analítica: La variación intra-ensayo mostró CVs que oscilaron entre 6,36% y 4,54% para el pool con baja y alta concentración de CgA, respectivamente, mientras que la variación inter-ensayo proporcionó CVs que oscilaron entre 7,10% para el pool con bajo nivel de CgA y 5,77% para el pool con un elevado contenido de CgA. El límite analítico de detección calculado dio un resultado de $4{,}27\times10^{-3}$ µg/ml. El límite inferior de cuantificación fue de $24{,}5\times10^{-3}$ µg/ml y el límite superior de cuantificación se estableció por encima de 12 µg/ml. La dilución de dos muestras de saliva de porcino con altas concentraciones de CgA dio lugar a ecuaciones de regresión lineal, donde los coeficientes de correlación obtenidos fueron de r = 0,983 y r = 0,967 para las muestras 1 y 2, respectivamente. La media de recuperación fue de 92,35%. Los resultados generales de la validación analítica del inmunoensayo que fueron evaluados, a saber, la precisión, la sensibilidad (límite de detección y límite inferior de cuantificación) y la exactitud (linealidad bajo dilución y recuperación), indicaron que el ensayo desarrollado fue capaz de detectar CgA porcina en muestras de saliva.

Modelo experimental de estrés agudo: Se obtuvieron incrementos estadísticamente significativos de las concentraciones a los 15 min respecto a los niveles basales, tanto en el cortisol salivar como en la CgA (Fig. 1), lo que coincide con resultados anteriormente publicados (Geverink et al., 2002; Merlot et al., 2011). La concentración media de CgA aumentó de niveles de 1,75 µg/ml al inicio a 2,56 µg/ml a los 15 min, con un media de incremento del 31%, similar a los incrementos descritos en estudios humanos sobre el estrés agudo debido al efecto de un examen (26%; Takatsuji

et al., 2008) y después de la venopunción en niños (49%; Lee et al., 2006). Estos datos descritos en el presente estudio proporcionan la primera evidencia de la utilidad de la CgA salivar como un biomarcador de estrés agudo en cerdos.

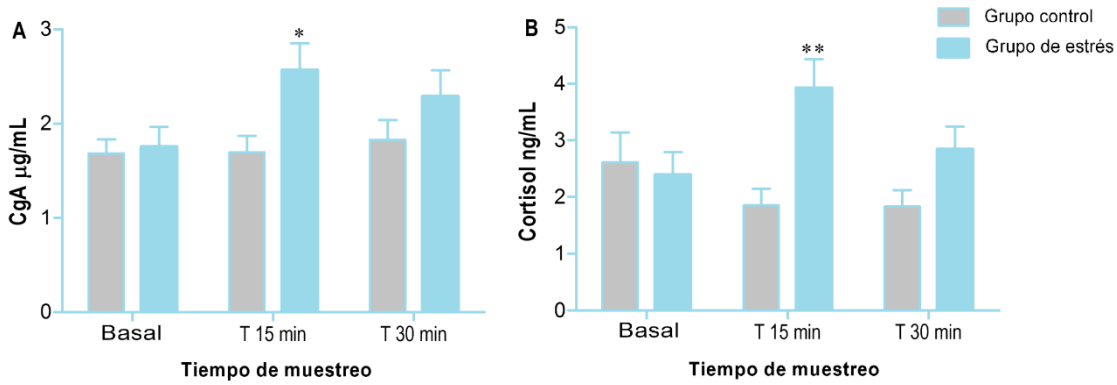


Fig. 1 Niveles de cromogranina A (CgA) (**A**) y de cortisol salivar (**B**) en el grupo control y de estrés (n = 15) antes (basal) y después de 15 min (T 15 min) y 30 min (T 30 min) del estímulo estresante (inmovilización con acial). El asterisco indica diferencia estadísticamente significativa (* P < 0,05; ** P < 0,01) en relación con el grupo control. Los valores son la media ± error estándar.

4.1.2 Experimento 2. Cromogranina A salivar en cerdos en crecimiento: un estudio de los patrones circadianos durante el día y la estabilidad bajo diferentes condiciones de almacenamiento

Diseño experimental

Evaluación del patrón circadiano de la secreción salivar de CgA en los cerdos durante el día: El experimento fue realizado en una granja con alto estatus sanitario, en el sureste de España, a finales de octubre (la estación de otoño) y al final del mes de abril (estación de primavera). Se seleccionaron un total de 80 animales (40 para cada estación) al azar para la inclusión en el procedimiento experimental. Los cerdos fueron alojados en grupos de 10: corrales 1 y 2 contenían machos no castrados de 17 - y 21-semanas de edad, respectivamente; corrales 3 y 4 contenían hembras de 17 - y 21-semanas de edad, respectivamente. Los animales fueron alojados bajo condiciones comerciales generales y tuvieron acceso a una alimentación nutricionalmente balanceada y agua *ad libitum*. Los cerdos seleccionados fueron muestreados en dos días consecutivos, para discriminar entre las variaciones cíclicas y aleatorias de variación, a las 07:00, 11:00, 15:00 y 19:00 h. Este régimen de muestreo ha sido utilizado

previamente en cerdos para el estudio de los ritmos circadianos de cortisol (Gallagher et al., 2002) y APPs (Gutiérrez et al., 2013). La temperatura de los corrales en estos tiempos de muestreo varió de 18°C en la mañana a 30°C al mediodía en el mes de abril y de 15°C en la mañana a 25°C al mediodía en el mes de octubre.

Evaluación de la estabilidad de la CgA salivar: Se obtuvieron muestras, en la granja experimental de la Universidad de Murcia, de 35 cerdos seleccionados al azar de diferentes edades y sexo. Para tener un volumen suficiente de saliva, para la evaluación de la estabilidad de la CgA, las muestras se combinaron en seis pools de saliva con tres concentraciones diferentes de CgA (dos con alta, con dos intermedia, y dos con bajas concentraciones de CgA). A partir de cada pool se prepararon alícuotas de 100 µl para permitir el análisis después de determinados períodos de almacenamiento a diferentes temperaturas. Todas las alícuotas se prepararon por duplicado, con un cóctel inhibidor de la proteasa (1 µl/ml, P8340; Sigma-Aldrich) añadidos a una de las muestras replicadas. Una alícuota de cada pool se analizó inmediatamente para determinar la concentración basal de CgA (día 0). El resto de alícuotas fueron analizadas después del almacenamiento a 4°C, -20°C o -80°C después de 2, 5, 15, 30, 90, 180 y 360 días con la presencia o ausencia del inhibidor de la proteasa. Dos alícuotas adicionales de mayor tamaño (1 ml) de cada uno de los pools de saliva se almacenaron a -20°C o -80°C para la evaluación de los efectos de la congelación y descongelación sobre la concentración de la CgA. Estas alícuotas se midieron en paralelo con las otras alícuotas, pero fueron sometidas a los ciclos de congelación y descongelación (siete ciclos) en cada uno de los ocho puntos de tiempo de análisis (Gutiérrez et al., 2011).

Resultados y discusión

Patrón circadiano de la CgA en la saliva porcina durante el día en dos estaciones diferentes: No se observaron diferencias estadísticamente significativas entre los tiempos de muestreo en los dos días consecutivos, ya sea en abril o en octubre. Nuestros resultados están en concordancia con estudios previos de CgA salivar en perros (Kanai et al., 2008). El hecho de que la CgA salivar no parece estar influenciada por la variación circadiana podría ser considerada como una ventaja en comparación a otros marcadores salivares de estrés en los cerdos, tales como el cortisol (Gallagher et

al, 2002; Hillmann et al., 2008), IgA (Muneta et al., 2010) o interleucina-18 (Muneta et al., 2011) que varían durante el día. Sin embargo, las concentraciones de CgA salivar fueron significativamente mayores en el grupo de cerdos muestreados en el otoño en comparación con los muestreados en la primavera ($P < 0,0001$). Es posible que la variación estacional registrada en la CgA salivar pudiera ser debida a diferencias de temperatura entre estaciones, aunque otros diversos factores ambientales también son posibles.

Efecto del género y la edad en el patrón circadiano de la CgA en la saliva de porcino durante el día: No hubo un efecto significativo del sexo sobre las concentraciones de CgA salivar. Las concentraciones de CgA medias ±SD en la primavera fueron 0,76 ± 0,05 µg/ml para los machos frente a 0,81 ± 0,08 µg/ml en las hembras, mientras que en el otoño fueron de 2,07 ± 0,15 µg/ml para los machos frente a 1,91 ± 0,09 µg/ml para las hembras. Además, no hubo un efecto significativo de la edad sobre las concentraciones de CgA salivar. Las concentraciones de CgA medias ±SD en la primavera fueron 0,81 ± 0,04 µg/ml para los cerdos de 17 semanas de edad frente a 0,75 ± 0,08 µg/ml para los cerdos de 21 semanas de edad, mientras que en el otoño los valores fueron 1,91 ± 0,10 µg/ml para cerdos de 17 semanas de edad frente a 2,07 ± 0,14 µg/ml para los cerdos de 21 semanas de edad. Del mismo modo, Kanai et al. (2008) no encontraron diferencias significativas entre machos y hembras en la concentración de CgA salivar en los perros.

Efecto de la temperatura de almacenamiento sobre las concentraciones de CgA salivar: Las muestras no mostraron ninguna reducción significativa en las concentraciones de CgA en el día 2, pero el día 5 de la refrigeración hubo una reducción de la CgA salivar del 24% en concentraciones intermedias y del 26% en concentraciones bajas. Después de 30 días de refrigeración, hubo una reducción del 10% en concentraciones altas. La refrigeración tuvo un impacto importante en las mediciones de CgA salivar después de 90 días de almacenamiento (35%, 47% y 36% para los pools de alta, intermedia y baja concentración, respectivamente). La CgA salivar fue relativamente estable cuando las muestras se almacenaron a -20°C con una modesta reducción del 5-10% después de 360 días de almacenamiento, a lo largo del rango de diferentes concentraciones. Por otra parte, los valores no fueron diferentes

significativamente después de siete ciclos de congelación y descongelación repetidos, con una reducción del 10-15%. Cuando la saliva se almacenó congelada a -80°C se obtuvieron resultados similares, con una reducción del 4-7% después de 360 días de almacenamiento, a lo largo del rango de diferentes concentraciones. Se observó una ligera reducción del 6-14% después de siete ciclos de congelación-descongelación. En el presente estudio, la adición de un inhibidor de la proteasa no proporcionó ninguna mejora adicional en la estabilidad de CgA durante el experimento, como también ha sido descrito por Gutiérrez et al. (2011) para la CRP.

4.2 Objetivo 2

El objetivo 2 fue cubierto por 3 experimentos correspondientes al artículo 3 (Experimento 1), el artículo 4 (experimento 2) y el artículo 5 (experimento 3).

4.2.1 Experimento 1. Validación de un inmunoensayo quimioluminiscente automatizado para las mediciones de cortisol salivar en cerdos

Diseño experimental

Se utilizaron dos modelos de inducción de estrés, el transporte de animales y la inmovilización, para evaluar el efecto del estrés sobre las concentraciones de cortisol salivar utilizando el sistema de ensayo quimioluminiscente descrito con anterioridad. Un grupo de 10 cerdos (de 165 días de edad y aproximadamente 100 kg de peso corporal) fueron transportados por carretera de la granja al matadero, en el mes de diciembre, con un tiempo de transporte de 30 min. El transporte comercial fue realizado con 1,5 m^2 por cerdo, sin serrín y sin suministrar comida o agua. Los animales fueron muestreados inmediatamente antes de cargar (pre-estrés o basal) y de nuevo a su llegada a los parques del matadero (post-estrés). Un segundo grupo de 10 cerdos se inmovilizó durante 1 min con un acial (estímulo estresante), y las muestras de saliva se recogieron antes (pre-estrés o basal) y 15 min después (post-estrés) del estímulo estresante (Fig. 2). Los animales designados para la inducción de estrés fueron de diferentes parques con el fin de evitar el posible estrés psicológico que el animal pudiera sufrir debido a la observación de la aplicación del acial sobre otros animales.

Resultados y discusión

Los resultados de la validación analítica se muestran posteriormente en la tabla 1.

Los resultados mostraron que ambos modelos de estrés provocan un aumento significativo en los niveles de cortisol salivar después de la aplicación del estímulo estresante en comparación con los obtenidos a nivel basal (Fig. 2). En el primer modelo, la concentración de cortisol en la saliva aumentó con una magnitud media de incremento del 78%, mayor que las obtenidas en estudios descritos con anterioridad después de un transporte durante 25 min (Geverink et al., 1998) (aproximadamente 57%) y 30 min (Schönreiter y Zanella, 2000) (aproximadamente el 60%). Por otra parte, el segundo modelo mostró aumentos similares en la concentración de cortisol salivar (aproximadamente 50%) a los mostrados en un estudio previo (Geverink et al., 2002), mientras que mayores incrementos, del 72%, fueron obtenidos por Merlot et al. (2011). Estos resultados demuestran que el sistema de ensayo descrito en el presente estudio es capaz de detectar aumentos en las concentraciones de cortisol salivar cuando los cerdos son sometidos a estímulos estresantes.

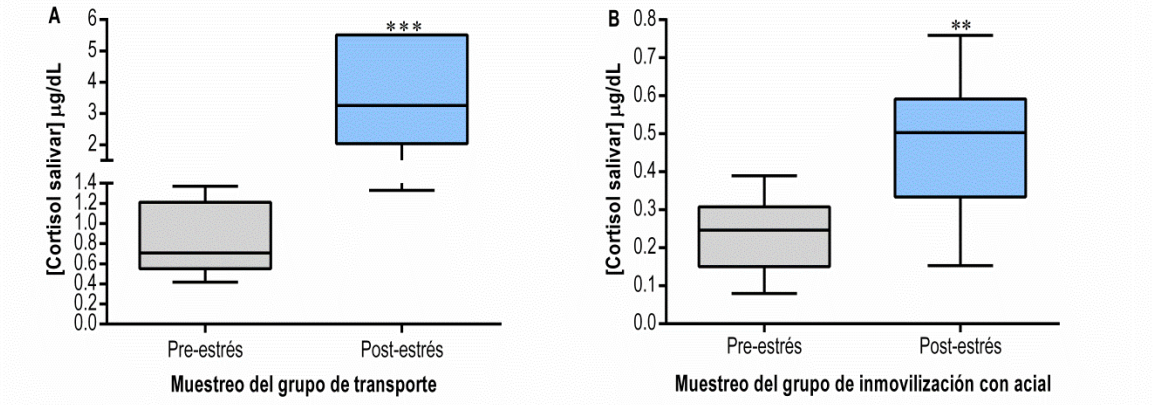


Fig. 2 Evolución de los niveles de cortisol salivar en cerdos antes (Pre-estrés) y después (Post-estrés) de la aplicación de los estímulos estresantes: **A**, estrés del grupo de transporte (n = 10); **B**, estrés del grupo de inmovilización con acial (n = 10). La gráfica muestra la mediana (línea dentro de la caja), percentiles 25th y 75th (caja), percentiles 5th y 95th (barras). El asterisco indica una diferencia estadísticamente significativa (** P < 0,01; *** P < 0,001) en relación con el muestreo Pre-estrés.

4.2.2 Experimento 2. Mediciones de testosterona salivar en cerdos en crecimiento: validación de un inmunoensayo quimioluminiscente automatizado y su posible uso como marcador de estrés agudo

Diseño experimental

Se utilizaron dos modelos de inducción de estrés, la inmovilización y el transporte, para evaluar el efecto del estrés sobre las concentraciones de testosterona salivar.

Un grupo de ocho cerdos enteros, de 30 semanas de edad, fueron inmovilizados durante 1 min con un acial (estímulo estresor), y las muestras de saliva se recogieron (a las 08:00 h) antes de la inmovilización de los animales (pre-estrés o Basal), y las restantes se tomaron a las 0 (inmediatamente después de la restricción con el acial), 15 y 30 min después del estrés por restricción (T0, T15 y T30, respectivamente). Un segundo grupo de seis cerdas enteras, de 30 semanas de edad, fueron transportadas comercialmente por carretera desde la granja al matadero en el mes de enero (entre la 07:00 h y las 11:00 h), con un tiempo de transporte de aproximadamente 30 min. El transporte comercial fue realizado siguiendo las recomendaciones de bienestar descritos en la Directiva 2001/88/CE, 2001 y la Directiva 2001/93/CE, 2001. Los animales se descargaron a su llegada al matadero, en el parque de recepción, y después de la primera toma de muestras los cerdos fueron trasladados a un área de estabulación (20°C, la densidad de carga de 150 kg/m^2). Durante la estabulación, los cerdos estudiados fueron mezclados con animales desconocidos y tuvieron acceso libre al agua. Las muestras de saliva fueron recolectadas en la granja antes de cualquier manipulación de los cerdos (pre-estrés o basal), después de la descarga (post-estrés) en el corral de recepción del matadero (T0), después de 30 min (T30) y después de 60 min en el área de estabulación (T60).

Resultados y discusión

Los resultados de la validación analítica se muestran posteriormente en la tabla 1.

Los aumentos de cortisol y CgA salivar obtenidos con el modelo de estrés del acial (Fig. 3A) fueron similares a los descritos previamente (Escribano et al., 2013). El aumento de la testosterona salivar fue de mayor magnitud (aumento de 2,76 veces) que los encontrados para las concentraciones de cortisol (aumento de 1,96 veces) y CgA (aumento de 1,64 veces). El mecanismo por el cual aumenta la testosterona en el estrés agudo sigue siendo incierto. Una razón podría ser la mayor sensibilidad de los

testículos a la hormona luteinizante como resultado de la activación del eje SAM por la norepinefrina (Chichinadze y Chichinadze, 2008), que se ha demostrado que estimula la producción de la hormona luteinizante en hombres y mujeres (Chrousos et al., 1998). Concentraciones significativamente más elevadas de cortisol salivar fueron observadas después del modelo de transporte corto por carretera. Sin embargo, los niveles de cortisol salivar (Fig. 3B2) volvieron a los niveles basales como se ha descrito anteriormente (Geverink et al., 1998; Soler et al., 2013), mientras que las mayores magnitudes de incremento de la testosterona (Fig. 3B1) (aumento de 4,48 veces) y la CgA salivar (Fig. 3B3) (aumento de 2,69 veces) fueron encontradas en el T60. Una posible explicación de la evolución de los niveles de testosterona y CgA salivar es que,

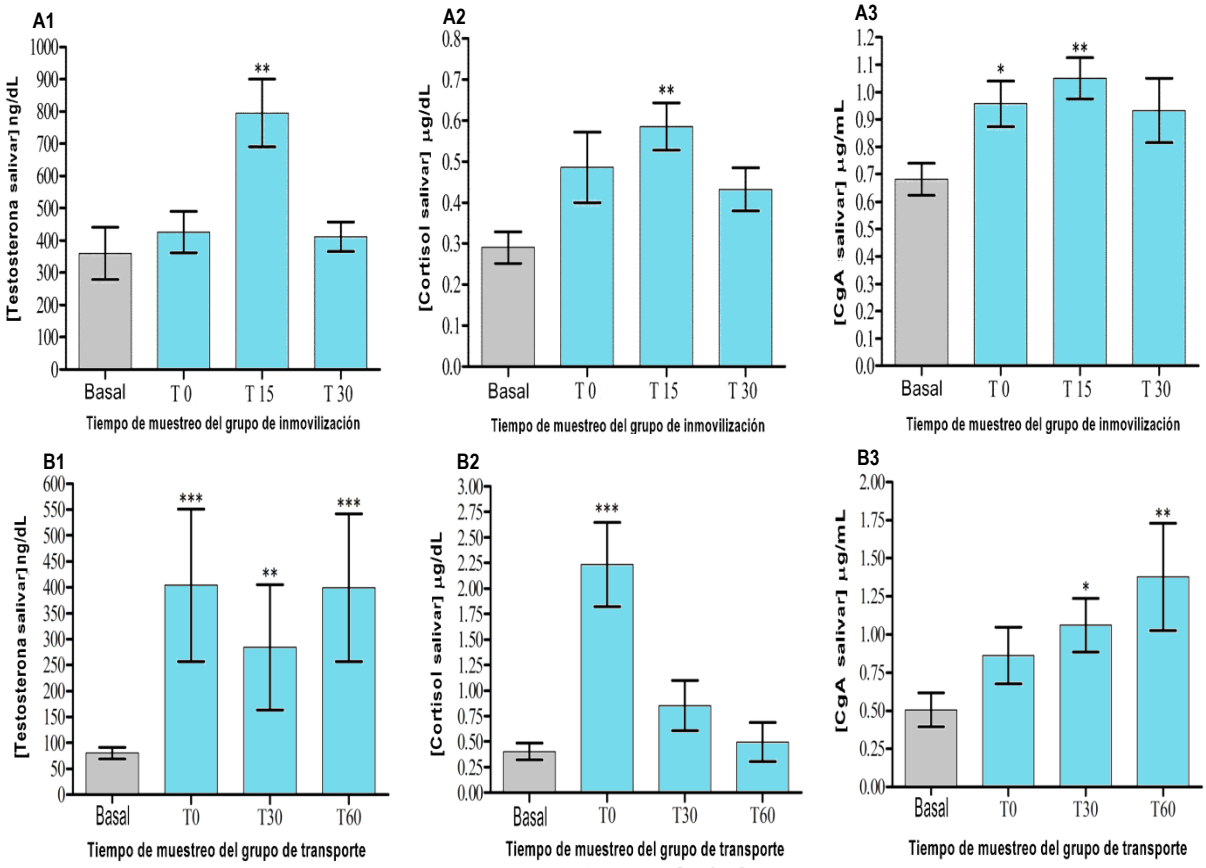


Fig. 3 Evolución de los niveles de testosterona (1), cortisol (2) y cromogranina A (CgA) salivar (3) en dos modelos de estrés. (**A**) Un primer modelo, donde ocho cerdos fueron muestreados antes de la inmovilización (basal), inmediatamente después de la inmovilización (post-estrés) durante 1 min con el acial (T0), después de 15 min (T15) y después de 30 min (T30) de la aplicación del estresor. (**B**) Un segundo modelo de estrés, donde seis cerdas fueron muestreadas antes de cualquier manipulación (basal), inmediatamente después de descargarlas (post-stress) en el matadero (T0), después de 30 min (T30) y después de 60 min (T60). El asterisco indica una diferencia estadísticamente significativa (* P < 0,05; ** P < 0,01; *** P < 0,0001) en relación con el muestreo basal. Valores son medias ± error estándar.

debido a factores como la incertidumbre, la presión social (por la mezcla con animales desconocidos) y el miedo, se haya inducido una respuesta de "lucha-huida" en los cerdos con la activación del sistema SAM (von Borrell, 2001).

4.2.3 Experimento 3. Validación de tres inmunoensayos disponibles comercialmente para la cuantificación de IgA, IgG, e IgM en muestras de saliva porcina

Diseño experimental

La capacidad de estos inmunoensayos para distinguir entre cerdos sanos y enfermos fue investigada comparando la saliva de quince animales de dos granjas de distinto estatus sanitario. Los animales fueron divididos en dos grupos en función de la explotación de origen. Grupo saludable: cerdos clínicamente normales de una granja SPF. Animales con serología negativa para PRRS, circovirosis porcina, la enfermedad de Aujeszky y la infección por neumonía enzoótica y sin la presencia de lesiones en los exámenes *post mortem*. Grupo de enfermos: cerdos de una granja seropositiva a PRRS y PMWS que mostraron signos clínicos compatibles con la enfermedad, tales como: diarrea, abscesos, pérdida de masa, apatía, lesiones externas o signos respiratorios. Las necropsias mostraron lesiones tales como: edema pulmonar, neumonía intersticial, hepatitis intersticial, depleción linfoide, úlcera gástrica o abscesos.

Resultados y discusión

Los resultados de la validación analítica se muestran posteriormente en la tabla 1.

Validación Clínica: La concentración de IgA total en saliva fue significativamente mayor en el grupo de animales enfermos en comparación con los animales sanos ($P < 0,05$; $p = 0,0144$). La mediana de la concentración de IgA fue de 101,4 µg/ml y de 234 µg/ml en animales sanos y enfermos, respectivamente. La IgM también mostró diferencias estadísticamente significativas entre los grupos de animales sanos y enfermos ($P < 0,01$, $p = 0,0014$). La mediana de la concentración de IgM en animales sanos fue de 16,33 µg/ml y en los animales enfermos fue de 65,26 µg/ml. Las mayores diferencias estadísticamente significativas entre los grupos de animales sanos y enfermos se obtuvieron en la IgG ($P < 0,0001$). En este caso las medianas de IgG fueron

de 9,09 µg/ml y de 102,6 µg/ml para los animales sanos y enfermos, respectivamente. Por lo tanto, los tres inmunoensayos utilizados para las determinaciones de Igs fueron capaces de detectar diferentes concentraciones de Igs entre los grupos de animales sanos y enfermos y podrían ser biomarcadores adecuados para evaluar el estado inmune humoral de los cerdos mediante un procedimiento no invasivo ni estresante.

Resultados y discusión de las validaciones analíticas de los experimentos 1, 2 y 3

Tabla 1 Datos de la validación analítica de los diferentes ensayos para medir cortisol, IgA, IgG, IgM y testosterona en muestras de saliva porcina.

Analito	**Unidades**	**Precisión Cv,%**	**Exactitud**			**LD**	**LMD**
			Linealidad		**Recuperación**		
		Intra Inter	**Ecuación**	**R**	**%**		
Cortisol	µg/dL	<12 <16	y=0.964x+1.90; y=0.978x-0.35	0.998; 0.996	88.0-95.6	0.016	0.197
IgA	µg/mL	<7 <13	y=1.004x+7.54; y=1.008x+3.62	0.989; 0.994	82.4-99.3	8.25×10^{-6}	4.94×10^{-6}
IgG	µg/mL	<8 <10	y=1.015x+0.79; y=0.978x+0.46	0.992; 0.999	84.7-102	7.74×10^{-6}	2.12×10^{-6}
IgM	µg/mL	<10 <12	y=1.005x-4.18; y=1.007x-5.73	0.989; 0.992	115-119	8.56×10^{-6}	3.80×10^{-6}
Testosterona	ng/dL	<8 <10	y=1.020x-15.48; y=0.981x-31.97	0.999; 0.999	93.4-104	13.60	19.60

Cv: Coeficiente de Variación ; LD: Limite de Detección; LMD: Límite Menor de Detección

El sistema de ensayo de quimioluminiscencia automatizado probado en el actual estudio proporciona un buen rendimiento analítico y permite una adecuada discriminación entre las muestras con bajas y altas concentraciones de cortisol y testosterona. El ensayo puede ser utilizado como un sistema no invasivo y no radioactivo para analizar cortisol y testosterona salivar en la investigación del estrés, y su facilidad de uso y el alto rendimiento de las muestras hacen de este sistema de ensayo una alternativa al radioinmunoensayo y las técnicas ELISAs manuales. Por otra parte, las tres pruebas ELISAs podrían ser adecuadas para su uso en muestras de saliva de la especie porcina para la determinación de Igs, a las diluciones establecidas en nuestro estudio, ya que también proporcionan una buena precisión, sensibilidad y exactitud.

4.3 Objetivo 3

El objetivo 3 fue cubierto por 1 experimento que corresponde al artículo 6.

4.3.1 Experimento. Efecto de la administración repetida de lipopolisacárido sobre marcadores inflamatorios y de estrés en la saliva de cerdos en crecimiento

Diseño experimental

Los biomarcadores salivares que se utilizaron en este estudio fueron seleccionados para permitir una evaluación tanto del sistema inmune como del sistema neuroendrocrino en respuesta al LPS. Puesto que, una visión global de los mecanismos fisiológicos que intervienen en la respuesta inflamatoria sistémica se podría obtener explorando la interacción entre los sistemas inmune y neuroendocrino (Vakharia and Hinson, 2005).

Dieciséis cerdos cruzados Piétrain × (Landrace × Large White) en crecimiento de 35 kg de peso corporal (77 días de edad) fueron utilizados en el experimento. Se seleccionaron el mismo número de machos y hembras enteras. Los animales fueron equipados con un catéter yugular bajo anestesia general, de acuerdo con la técnica descrita previamente por Melchior et al. (2004), antes de ser alojados en un cuarto de experimentación que contenía 16 corrales individuales (0,85 × 1,30 m). Cada 2 días, los catéteres fueron lavados con 5 ml de solución salina estéril conteniendo 50 UI/ml de heparina para prevenir la coagulación de la sangre. La temperatura ambiente de la sala se mantuvo a 24 ± 0,2°C y se puso en práctica un ciclo de 12 h luz/oscuridad (de 08:00 a 20:00 h). Los cerdos tuvieron libre acceso al agua y se proporcionó una dieta nutricionalmente equilibrada todos los días a las 08:30 h.

Los animales fueron adaptados a las condiciones experimentales durante 3 semanas, después de lo cual 10 cerdos recibieron inyecciones de LPS de Escherichia coli (LPS; O55:B5, Sigma–Aldrich) y seis cerdos de control se dejaron sin tratar. El LPS se administró a través del catéter en tres ocasiones (T1, T2 y T3), a intervalos de 48 h, con una dosis inicial de 30 µg/kg de peso corporal y aumentado en un 12% cada inyección posterior en un esfuerzo para mantener la respuesta inflamatoria (Ziegler -Heitbrock, 1995). El LPS fue diluido en solución salina estéril (400 µg/mL) y a cada cerdo se le

administró por vía intravenosa un volumen apropiado (entre 3,7 y 6,4 ml), en base a su peso corporal. Las inyecciones se realizaron lentamente (durante 5 min) a las 11:00 h. Las temperaturas rectales se midieron con un termómetro digital (Microlife Corporation) al inicio del estudio y 3 horas después de la T1 y T3.

Resultados y discusión

Temperatura rectal: Después de T1 los cerdos tratados con LPS mostraron evidencia de pirexia, con relación a la temperatura basal ($P < 0,001$). La exposición a LPS durante la infección con bacterias Gram-negativas provoca liberación de citoquinas pro-inflamatorias como parte de la respuesta inmune innata. Estas citoquinas actúan sistémicamente para inducir una respuesta febril y promover la síntesis de APPs desde el hígado (Eckersall, 2000).

Tasa de flujo salivar: No hubo una diferencia significativa comparando los grupos control y tratados con LPS en ningún punto de muestreo. Sin embargo, los cerdos tratados con LPS habían reducido las tasas de flujo salivar, en relación con la basal, después de T1 ($P < 0,01$), T2 ($P < 0,001$) y T3 ($P < 0,001$). En otros estudios se ha descrito una reducción en la secreción de la saliva después del tratamiento con LPS (Lomniczi et al., 2001; Fernández-Solari et al., 2010).

Análisis de las APPs en saliva: Teniendo en cuenta la edad de los cerdos, las concentraciones de Hp y CRP fueron más altas que los valores de referencia (Gutiérrez et al., 2009b) después de las dos primeras inyecciones de LPS, lo cual confirma que la respuesta inflamatoria puede ser detectada en la saliva. El aumento en las concentraciones de Hp (Fig. 4A) y CRP (Fig. 4B) salivar, en relación con el basal, fue mayor que la publicada por Frank et al. (2003) para muestras de suero después de administraciones similares de LPS.

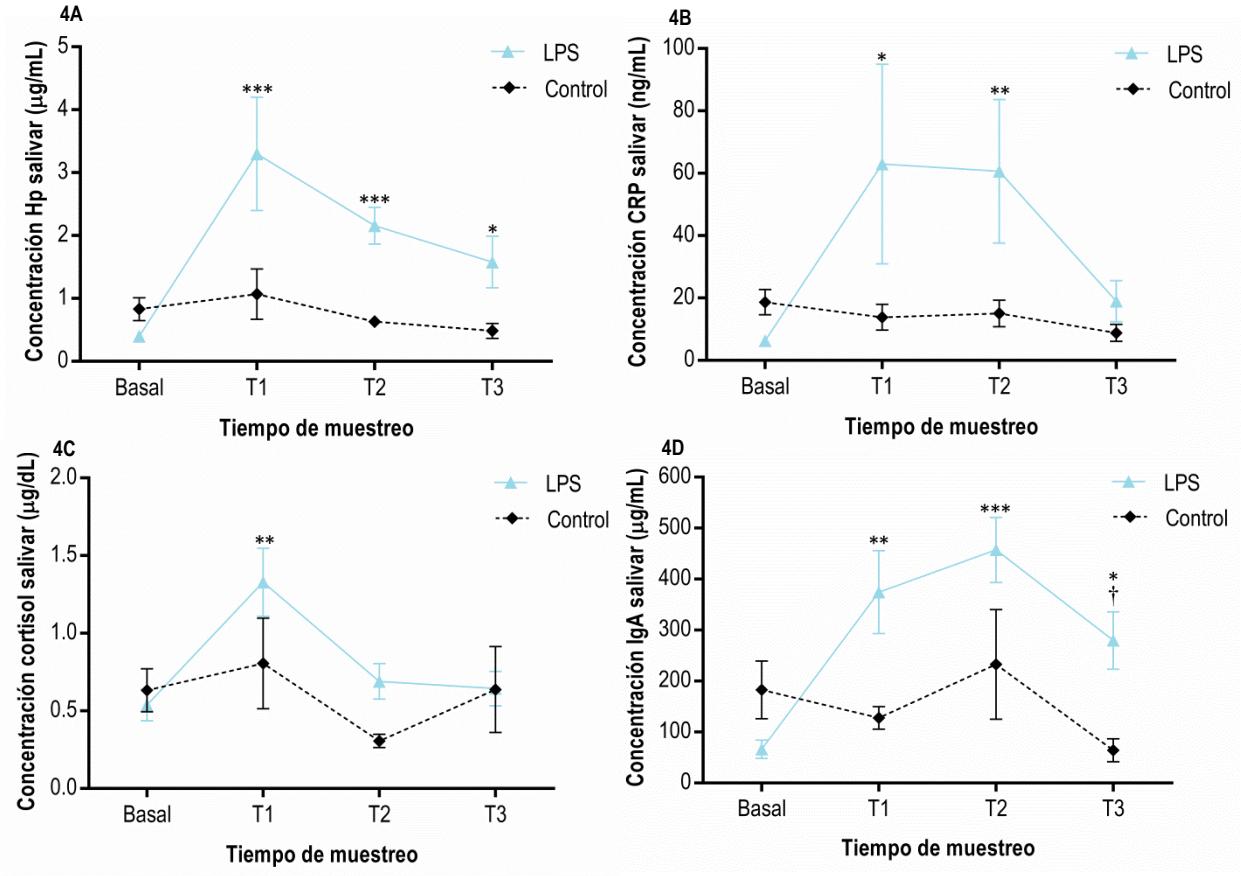


Fig. 4 (**A**) Concentraciones de haptoglobina (Hp), (**B**) proteína C-reactiva (CRP), (**C**) cortisol e (**D**) inmunoglobulina A (IgA) salivar en cerdos LPS tratados y controles antes (Basales), después de la primera (T1), la segunda (T2) y tercera (T3) administración de *Escherichia coli* lipopolysaccharide (LPS). Los valores mostrados son medias ± SEM. * P < 0,05; ** P < 0,01; *** P < 0,001 comparados con el basal de cada grupo. † P < 0,05 comparando cerdos LPS tratados y controles.

Análisis de los biomarcadores de estrés en saliva: El significativo aumento en el cortisol después de la primera administración de LPS (Fig. 4C) es consistente con que la endotoxemia induce la activación del eje HPA en los cerdos (Williams et al., 2009). La magnitud de la respuesta en el cortisol salivar vista en T1 fue en un rango similar al descrito anteriormente por Lay et al. (2011) y Koopmans et al. (2012) en los cerdos. En el presente estudio no hubo un cambio significativo en la CgA salivar después de la administración de LPS. Por otra parte, hemos demostrado que las concentraciones de IgA en la saliva también aumentan en respuesta a un desafío de LPS en cerdos (Fig. 4D). Alternativamente, la secreción salivar de IgA podría estar relacionada con la activación del sistema SAM, como se ha propuesto en humanos (Allgrove et al., 2008). Un aumento de IgA salivar ha sido descrito en cerdos como una respuesta al estrés agudo

durante la restricción (Muneta et al., 2010). Por lo tanto, un aumento de la IgA de la saliva no sólo se asocia con la activación del sistema inmune adaptativo, también puede producirse de forma no específica en respuesta al estrés.

Los resultados del estudio demostraron que la administración repetida de LPS puede inducir una elevación significativa en biomarcadores salivares de los sistemas neuroendocrino e inmunológico de los cerdos. La medición de Hp, CRP, IgA y cortisol en la saliva podría ser utilizado como una herramienta práctica, que es fácil de realizar y no invasiva, para la evaluación de la respuesta combinada de endotoxemia en los cerdos. En el caso de los estados inflamatorios crónicos, la Hp y la IgA salivar pueden ser marcadores más sensibles que la CRP y el cortisol.

4.4 Objetivo 4

El objetivo 4 fue cubierto por un experimento que aparece en al anexo.

4.4.1 Experimento. Cambios en los biomarcadores de estrés e inmunidad en saliva de cerdos domésticos expuestos a un estresor psicosocial

Diseño experimental

El diseño experimental empleado en el presente estudio es una adaptación de otros disponibles en la literatura para la evaluación del estrés psicosocial, basado en el "aislamiento" social (Tuchscherer et al., 2009; Soler et al., 2013) y el "reagrupamiento" o mezcla (Coutellier et al., 2007; Oster et al., 2014).

Cinco días antes del comienzo del periodo experimental, todos los animales fueron acostumbrados al contacto humano y a la toma de saliva durante 5 días. El diseño experimental se dividió en tres períodos: pre-estrés, aislamiento y reagrupación. En cada día, durante los tres periodos, se recogieron muestras de saliva a las 11:00 horas en ambos grupos, Test y Control, de estudio. Dos muestras de saliva adicionales se tomaron a los 30 min después del aislamiento y a los 30 min después de la reagrupación. En el estudio de aislamiento los comportamientos y las lesiones de cada animal fueron medidas cada día después del muestreo en base al etograma de comportamiento utilizado por Ruis et al. (2001). La acumulación de lesiones en la piel

después de la mezcla fueron medidas como un factor de predicción de la agresividad individual en cerdos a través de un método previamente descrito (Turner et al., 2006).

El período pre-estresante dura los primeros 5 días (días-1d a -5d; Fig. 5). Los cerdos Test (n = 7) y Control (n = 7) fueron mantenidos durante este período en las mismas condiciones de alojamiento (con una densidad de población de 1,1 m^2 por cerdo). En el día 6 de este periodo, se obtuvieron las muestras de referencia del aislamiento (0dI) para los grupos de Test y Control. Para el procedimiento de aislamiento, 7 cerdos fueron trasladados a un nuevo parque donde cada uno, en la misma sala, fue aislado en corrales individuales (7,78 m^2 por cerdo), donde tuvieron contacto olfativo y auditivo pero no físico con otros cerdos. Desde el día 0 del aislamiento (0dI; Fig. 5) hasta el día cinco de aislamiento (5dI; Fig. 5) los cerdos de prueba se mantuvieron en estas condiciones experimentales. En el día 6 de este periodo se tomaron las muestras iniciales de reagrupamiento (0DR) para los grupos de prueba y control. El período de reagrupación comenzó después del período de aislamiento. Los cerdos de prueba se reagruparon y permanecieron en estas condiciones durante 3 días (día 0 de la reagrupación, 0dR, hasta el día 3 de la reagrupación, 3dR; Fig. 5), volviendo a su grupo original con las condiciones iniciales (con una densidad ganadera de 1,1 m^2 por cerdo). El procedimiento general de aislamiento y reagrupamiento tomó alrededor de 3 min.

Los cerdos de control se mantuvieron en las mismas condiciones de alojamiento durante todo el período experimental. La temperatura de los corrales en estos tiempo de muestreo fue de 21,2ºC ± 0,8 (media ± SD) y los animales se mantuvieron durante el período experimental con iluminación natural.

Resultados y discusión

Comportamiento animal después del aislamiento y lesiones corporales después de la reagrupación: El aislamiento causó un aumento significativo ($P < 0,05$) en el comportamiento de exploración (oler, tocar y caminar a través del corral) y en las vocalizaciones en el grupo de prueba en comparación con el grupo control, y también en relación con sus valores basales (0dI). Además, todos los cerdos tuvieron un mayor número de lesiones corporales después del reagrupamiento ($P < 0,05$) en comparación

con sus basales (0dR), y el grupo de prueba tuvo un mayor número de lesiones corporales ($P < 0,05$) en comparación con el grupo de control después de la reagrupación. El aumento de la vocalización que fue observado después del aislamiento y la agresión y el aumento significativo de lesiones de la piel que fueron encontradas después del reagrupamiento indicaron una activación de la respuesta al estrés en los cerdos (Ruis et al., 2001; Turner et al., 2006) después de la aplicación de nuestro modelo de estrés psicosocial.

Proteína total y tasa de flujo salivar: El flujo salivar y la concentración de proteína total salivar no ha cambiado en nuestro modelo de estrés. Por lo tanto, se puede descartar que los cambios significativos observados en nuestro estudio sean debidos a la posible dilución o concentración de los marcadores en la saliva por la falta/exceso de saliva o el espesor de la misma.

CgA e IgA salivar: La magnitud y el tiempo de aumento después del aislamiento y el reagrupado en la respuesta de la CgA salivar (Fig. 5A) fue similar al descrito previamente después de aplicar otros modelos de estrés, como la restricción con acial (Escribano et al., 2013) o el transporte y el alojamiento en el matadero (Escribano et al., 2014). Aunque se necesitan más investigaciones para aclarar este tema, la IgA en saliva mostró una respuesta tardía en comparación con la CgA después del aislamiento (Fig. 5B). Una hipótesis para la falta de aumento de IgA salivar después del reagrupamiento podría ser el hecho de la disminución de la respuesta inmune. Dado que, de Groot et al. (2001) han informado que el estrés social después de la mezcla podría dificultar la respuesta inmune en los cerdos y por lo tanto la producción de diferentes Igs.

Cortisol y testosterona salivar: En nuestro estudio, no se observaron cambios significativos en el cortisol y la testosterona salivar después del aislamiento (Fig. 5C; 5D). Estos resultados pueden indicar una adaptación rápida de los cerdos domésticos a las condiciones experimentales de alojamiento, como ha sido informado anteriormente por Jaskulke y Manteuffel (2011) para el cortisol salivar. En relación con los valores

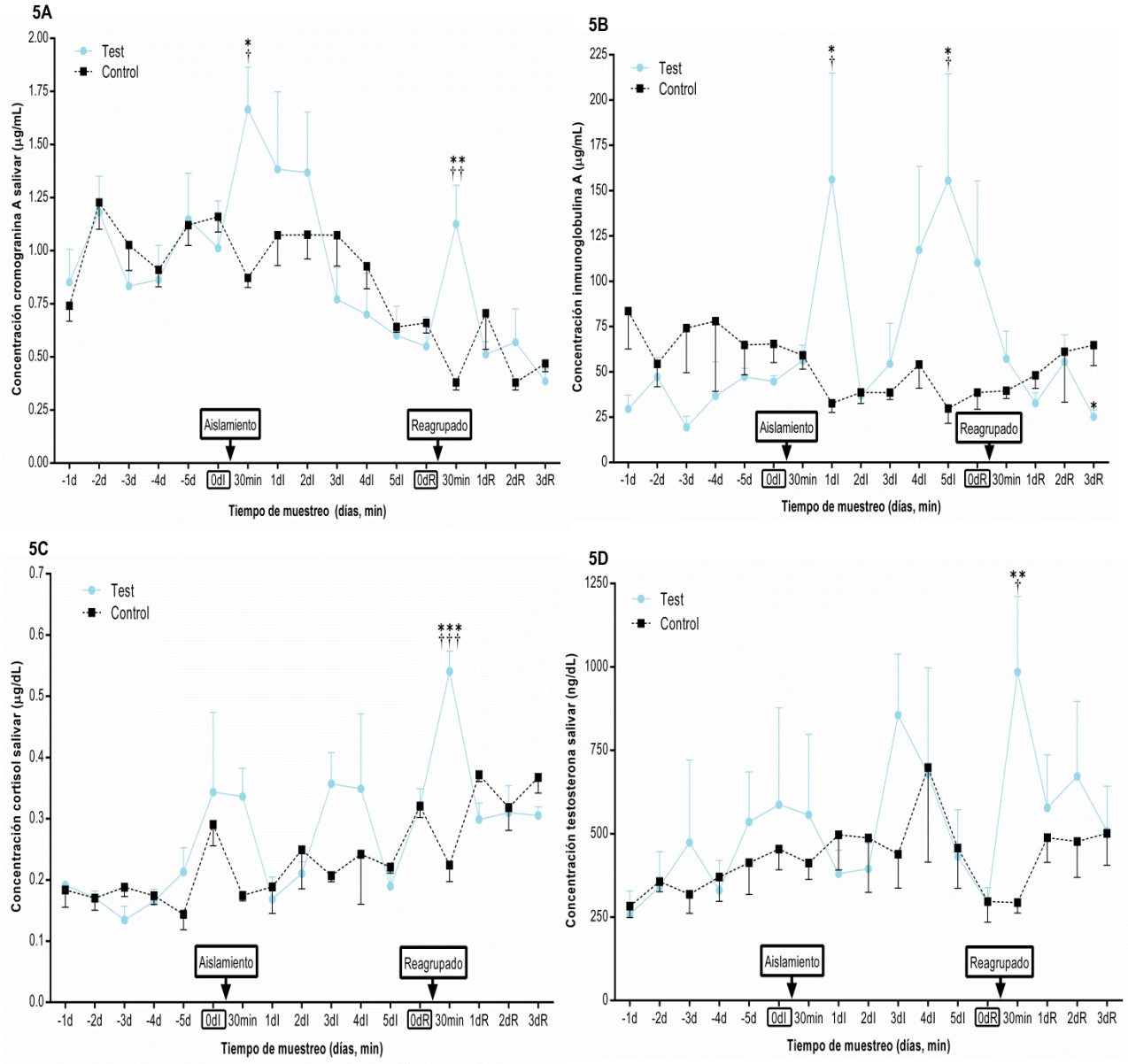


Fig. 5 (**A**) Concentraciones de cromogranina A (CgA), (**B**) inmunoglobulina A (IgA), (**C**) cortisol y (**D**) testosterona salivar en los cerdos test (n = 7) y controles (n = 7); que fue dividió en tres períodos: pre-estresor (día-1d a -5d), el aislamiento (0dI a 5dI) y reagrupamiento (0dR a 3dR). Los valores mostrados son medias ± SEM. * P < 0,05; ** P < 0,01; *** P <0,001 en comparación con los valores basales (0dI y 0dR) en los cerdos del test o los controles después del aislamiento y después de reagrupamiento para cada grupo. † P < 0,05; † † P < 0,01; † † † P < 0,001 comparando los cerdos del test y los controles.

basales del reagrupamiento (0dR) y en comparación con el grupo control de los cerdos, las concentraciones de cortisol y testosterona fueron mayores después de 30 min en los cerdos de prueba. La magnitud de incremento en el cortisol salivar fue de rango similar a lo publicado en estudios anteriores de mezcla, donde normalmente están involucrados cerdos no familiarizados (de Groot et al., 2001; Merlot et al., 2004; Coutellier et al., 2007). También ha sido descrito un resultado similar para la testosterona después de la reagrupación en el matadero (Escribano et al., 2014).

Hp y CRP salivar: Nuestros resultados indican que las concentraciones salivares de Hp y CRP no han cambiado significativamente en los modelos de estrés utilizados en nuestro estudio. Esto está en consonancia con un estudio previo en el que no se han encontraron cambios en la Hp tras el aislamiento social y el estrés del transporte corto por carretera (Soler et al., 2013). Luego, se debe reconocer que, en base a nuestros resultados, estos marcadores no parecen ser indicadores de estrés. Sin embargo, la Hp y la CRP salivar se pueden utilizar para evaluar el estado de salud y bienestar de las piaras de cerdos (Gómez-Laguna et al., 2010). En nuestro caso, teniendo en cuenta la edad de los cerdos, las concentraciones de Hp y CRP fueron siempre inferiores a los valores de referencia de corte internos del laboratorio para animales enfermos (Gutiérrez et al., 2009b), lo que indica que los animales utilizados en este estudio estaban sanos.

El uso de varios biomarcadores salivares podría ser esencial en la investigación de estrés ya que nuestros resultados demostraron que estos parecen reaccionar de manera diferente a diversos tipos de factores estresantes. En el caso de aislamiento, la CgA y la IgA salivar podrían ser marcadores más sensibles que el cortisol y la testosterona, con la CgA mostrando una respuesta más rápida que la IgA. Mientras que en el proceso de reagrupado, el cortisol, la testosterona y la CgA salivar podrían ser marcadores más sensibles que la IgA salivar. Por otra parte, la Hp, la CRP, las concentraciones de proteínas totales y el flujo en la saliva de los cerdos no cambiaron significativamente después de aplicar el factor de estrés psicosocial como modelo de estrés. La Hp y CRP permiten la discriminación entre animales sanos y enfermos, mientras que la proteína total y la tasa de flujo puede descartar que los cambios significativos observados se deban a alteraciones en el volumen o la concentración de

saliva. Por lo tanto, aunque se necesitan más estudios, estos biomarcadores podrían ser utilizados como una herramienta práctica y no invasiva para reflejar la actividad de los diferentes sistemas fisiológicos involucrados en la respuesta al estrés en los cerdos.

5. CONCLUSIONES

1. El ensayo desarrollado permite la cuantificación de CgA en muestras de saliva porcina de una forma fiable y su concentración aumenta después de una situación de estrés agudo. No ha sido detectado un patrón circadiano para la CgA salivar y este biomarcador fue bastante estable siendo posible su almacenamiento hasta un año a -20ºC o -80ºC.

2. Los inmunoensayos comerciales validados para las concentraciones de cortisol, testosterona e IgA pueden ser utilizados para su uso en muestras de saliva de cerdos con una buena precisión, sensibilidad y exactitud.

3. Una sola administración de LPS en cerdos produce incrementos de IgA salivar y cortisol. Sin embargo, administraciones repetidas solo producen incrementos en IgA mientras que el cortisol retorna a valores basales. No se observaron variaciones en las concentraciones de CgA ni con una sola administración ni con administraciones repetidas.

4. El uso de un panel de biomarcadores salivares podría ser esencial en la investigación de estrés, ya que estos reaccionan de manera diferente a diversos tipos de factores estresantes. En el caso del aislamiento, CgA e IgA parecen ser marcadores más sensibles que el cortisol y la testosterona, mientras que después del proceso de reagrupado, el cortisol, la testosterona y la CgA parecen ser marcadores más sensibles que la IgA.

REFERENCES

REFERENCES

Allgrove, J.E., Gomes, E., Hough, J., Gleeson, M., 2008. Effects of exercise intensity on salivary antimicrobial proteins and markers of stress in active men. Journal of Sports Sciences 6, 653–661.

Bushong, D.M., Friend, T.H., Knabe, D.A., 2000. Salivary and plasma cortisol response to adrenocorticotropin administration in pigs. Laboratory Animals 34, 171–181.

Chichinadze, K., Chichinadze, N., 2008. Stress-induced increase in testosterone: contributions of social and sympathetic reactivity. Physiology & Behavior 94, 596–603.

Choi, J.C., Chung, M.I., Lee, Y.D., 2012. Modulation of pain sensation by stress-related testosterone and cortisol. Anaesthesia 67, 1146–1151.

Chrousos, G.P., Torpy, D.J., Gold, P.W., 1998. Interactions between the hypothalamicpituitary-adrenal axis and the female reproductive system: clinical implications. Annals of Internal Medicine 129, 229–240.

Cook, N.J., Schaefer, A.L., Lepage, P.,Morgan Jones, S.D., 1996. Salivary cortisol for the assessment of adrenal activity in swine. Canadian Journal of Animal Science 76, 329–335.

Coutellier, L., Arnould, C., Boissy, A., Orgeur, P., Prunier, A., Veissier, I., Meunier-Salaün, M.C., 2007. Pig's responses to repeated social regrouping and relocation during the growing-finishing period. Applied Animal Behaviour Science 105, 102-114.

de Groot, J., Ruis, M.A.W., Scholten, J.W., Koolhaas, J.M., Boersma, W.J.A., 2001. Long-term effects of social stress on antiviral immunity in pigs. Physiology & Behavior 73, 145-158.

Eckersall, P.D., 2000. Acute phase proteins as markers of infection and inflammation: Monitoring animal health, animalwelfare and food safety. Irish Veterinary Journal 53, 307–311.

Eckersall, P.D., Duthie, S., Toussaint, M.J., Gruys, E., Heegaard, P., Alava, M., Lipperheide, C., Madec, F., 1999. Standardization of diagnostic assays for animal acute phase proteins. Advances in Veterinary Medicine 41, 643–655.

Escribano, D., Fuentes-Rubio, M., Cerón, J.J., 2014. Salivary testosterone measurements in growing pigs: validation of an automated chemiluminescent immunoassay and its possible use as an acute stress marker. Research in Veterinary Science, http://dx.doi.org/10.1016/j.rvsc.2014.04.001.

Escribano, D., Soler, L., Gutiérrez, A.M., Martínez-Subiela, S., Cerón, J.J., 2013. Measurement of chromogranin A in porcine saliva: validation of a time-resolved immunofluorometric assay and evaluation of its application as a marker of acute stress. Animal 7, 640–647.

Fernandez-Solari, J., Prestifilippo, J.P., Ossola, C.A., Rettori, V., Elverdin, J.C., 2010. Participation of the endocannabinoid system in lipopolysaccharide-induced inhibition of salivary secretion. Archives of Oral Biology 55, 583–590.

Frank, J.W., Carroll, J.A., Allee, G.L., Zannelli, M.E., 2003. The effects of thermal environment and spray-dried plasma on the acute-phase response of pigs challenged with lipopolysaccharide. Journal of Animal Science 81, 1166–1176.

Fuentes, M., Tecles, F., Gutiérrez, A., Otal, J., Martínez-Subiela, S., Cerón, J.J., 2011. Validation of an automated method for salivary alpha-amylase measurements in pigs (*Sus scrofa domesticus*) and its application as a stress biomarker. Journal of Veterinary Diagnostic Investigation 23, 282–287.

Gallagher, N.L., Giles, L.R., Wynn, P.C., 2002. The development of a circadian pattern of salivary cortisol secretion in the neonatal piglet. Biology of the Neonate 81, 113–118.

Geverink, N.A., Bühnemann, A., Van der Burgwal, J.A., Lambooij, E., Blokhuis, H.J., Wiegant, V.M., 1998. Responses of slaughter pigs to transport and lairage sounds. Physiology & Behavior 63, 667–673.

Geverink, N.A., Schouten, W.G.P., Gort, G., Wiegant, V.M., 2002. Individual differences in behavioral and physiological responses to restraint stress in pigs. Physiology & Behavior 77, 451–457.

Giltay, E.J., Enter, D., Zitman, F.G., Penninx, B.W., van Pelt, J., Spinhoven, P., Roelofs, K., 2012. Salivary testosterone: associations with depression, anxiety disorders, and antidepressant use in a large cohort study. Journal of Psychosomatic Research 72, 205–213.

Gómez-Laguna, J., Gutiérrez, A.M., Pallarés, F.J., Salguero, F.J., Cerón, J.J., Carrasco, L., 2010. Haptoglobin and C-reactive protein as biomarkers in the serum, saliva and meat juice of pigs experimentally infected with porcine reproductive and respiratory syndrome virus. The Veterinary Journal 185, 83-87.

Gutiérrez, A.M., Escribano, D., Fuentes, M., Cerón, J.J., 2013. Circadian pattern of acute phase proteins in the saliva of growing pigs. The Veterinary Journal 196, 167–170.

Gutiérrez, A.M., Martínez-Subiela, S., Cerón, J.J., 2009. Evaluation of an immunoassay for determination of haptoglobin concentration in various biological specimens from swine. American Journal of Veterinary Research 70, 691-696.

Gutiérrez, A.M., Martínez-Subiela, S., Cerón, J.J., 2011. Evaluation of changes in haptoglobin and C-reactive protein concentrations caused by freezing of saliva and meat juice samples collected from healthy and diseased pigs. American Journal of Veterinary Research 72, 11–17.

Gutiérrez, A.M., Martínez-Subiela, S., Soler, L., Pallarés, F.J., Cerón, J.J., 2009b. Use of saliva for haptoglobin and C-reactive protein quantifications in porcine respiratory and reproductive syndrome affected pigs in field conditions. Veterinary Immunology and Immunopathology 132, 218–223.

Hart, K.A., 2012. The use of cortisol for the objective assessment of stress in animals: pros and cons. The Veterinary Journal 192, 137-139.

Heintz, M.R., Santymire, R.M., Parr, L.A., Lonsdorf, E.V., 2011. Validation of a cortisol enzyme immunoassay and characterization of salivary cortisol circadian rhythm in chimpanzees (*Pan troglodytes*). American Journal of Primatology 73, 903–908.

Hillmann, E., Schrader, L., Mayer, C., Gygax, L., 2008. Effects of weight, temperature and behaviour on the circadian rhythm of salivary cortisol in growing pigs. Animal 2, 405–409.

Jaskulke, S., Manteuffel, G., 2011. No apparent effect of an experimental narrow confinement on heart activity and cortisol in domestic pigs. Animal 5, 433-438.

Kanai, K., Hino, M., Hori, Y., Nakao, R., Hoshi, F., Itoh, N., Higuchi, S., 2008. Short communication: Circadian variations in salivary chromogranin a concentrations during a 24-hour period in dogs. Journal of Veterinary Science 9, 421–423.

Kanno, T., Asada, N., Yanase, H., Iwanaga, T., Nishikawa, Y., Hoshino, M., Yanaihara, N., 1998. Autonomic control of submandibular chromogranin A secretion in the anaesthetized rat. Biomedical Research 19, 411–414.

Kennedy, B., Dillon, E., Mills, P.J., Ziegler, M.G., 2001. Catecholamines in human saliva. Life Sciences 69, 87–99.

Koopmans, S.J., van der Staay, F.J., Le Floc'h, N., Dekker, R., van Diepen, J.T., Jansman, A.J., 2012. Effects of surplus dietary L-tryptophan on stress, immunology, behavior, and nitrogen retention in endotoxemic pigs. Journal of Animal Science 90, 241–251.

Lay, D.C., Jr., Kattesh, H.G., Cunnick, J.E., Daniels, M.J., Kranendonk, G., McMunn, K.A., Toscano, M.J., Roberts, M.P., 2011. Effect of prenatal stress on subsequent response to mixing stress and a lipopolysaccharide challenge in pigs. Journal of Animal Science 89, 1787–1794.

Lee, T., Shimizu, T., Iijima, M., Obinata, K., Yamashiro, Y., Nagasawa, S., 2006. Evaluation of psychosomatic stress in children by measuring salivary chromogranin A. Acta Paediatrica 95, 935–939.

Lomniczi, A., Mohn, C., Faletti, A., Franchi, A., McCann, S.M., Rettori, V., Elverdin, J.C., 2001. Inhibition of salivary secretion by lipopolysaccharide: Possible role of prostaglandins. American Journal of Physiology. Endocrinology and Metabolism 281, E405–E411.

Melchior, D., Sève, B., Le Floc'h, N., 2004. Chronic lung inflammation affects plasma amino acid concentrations in pigs. Journal of Animal Science 82, 1091–1099.

Merlot, E., Meunier-Salaün, M.C., Prunier, A., 2004. Behavioural, endocrine and immune consequences of mixing in weaned piglets. Applied Animal Behaviour Science 85, 247–257.

Merlot, E., Mounier, A.M., Prunier, A., 2011. Endocrine response of gilts to various common stressors: A comparison of indicators and methods of analysis. Physiology and Behavior 102, 259–265.

Muneta, Y., Minagawa, Y., Nakane, T., Shibahara, T., Yoshikawa, T., Omata, Y., 2011. Interleukin-18 expression in pig salivary glands and salivary content changes during acute immobilization stress. Stress 14, 549–556.

Muneta, Y., Yoshikawa, T., Minagawa, Y., Shibahara, T., Maeda, R., Omata, Y., 2010. Salivary IgA as a useful non-invasive marker for restraint stress in pigs. The Journal of Veterinary Medical Science 72, 1295–1300.

Nakane, H., Asami, O., Yamada, Y., Harada, T., Matsui, N., Kanno, T., Yanaihara, N., 1998. Salivary chromogranin A as an index of psychosomatic stress response. Biomedical Research 19, 401–406.

Oster, M., Muráni, E., Ponsuksili, S., D'Eath, R.B., Turner, S.P., Evans, G., Thölking, L., Kurt, E., Klont, R., Foury, A., Mormède, P., Wimmers, K., 2014. Hepatic expression patterns in psychosocially high-stressed pigs suggest mechanisms following allostatic principles. Physiology & Behavior 128, 159-165.

Park, S., Lee, D.H., Park, J.G., Lee, Y.T., Chung, J., 2010. A sensitive enzyme immunoassay for measuring cotinine in passive smokers. Clinica Chimica Acta 411, 1238–1242.

Parra, M.D., Tecles, F., Martínez-Subiela, S., Cerón, J.J., 2005. C-reactive protein measurement in canine saliva. Journal of Veterinary Diagnostic Investigation 17, 139–144.

Ruis, M.A., te Brake, J.H., Engel, B., Buist, W.G., Blokhuis, H.J., Koolhaas, J.M., 2001. Adaptation to social isolation: Acute and long-term stress responses of growing gilts with different coping characteristics. Physiology & Behavior 73, 541–551.

Schönreiter, S., Huber, H., Lohmüller, V., Zanella, A.J., Unshelm, J., Henke, J., Erhardt, W., 1999. Salivary cortisol as a stress parameter in piglets. Tierärztliche Praxis. Ausgabe G, Grosstiere/Nutztiere 27, 175–179.

Schönreiter, S., Zanella, A.J., 2000. Assessment of cortisol in swine by saliva: new methodological approaches. Archiv Tierzucht 43, 165–170.

Schoofs, D., Wolf, O.T., 2011. Are salivary gonadal steroid concentrations influenced by acute psychosocial stress? A study using the Trier Social Stress Test (TSST). International Journal of Psychophysiology: official journal of the International Organization of Psychophysiology 80, 36-43.

Schwab, K.O., Heubel, G., Bartels, H., 1992. Free epinephrine, norepinephrine and dopamine in saliva and plasma of healthy adults. European Journal of Clinical Chemistry and Clinical Biochemistry 30, 541–544.

Smulders, D., Verbeke, G., Mormède, P., Geers, R., 2006. Validation of a behavioural observation tool to asses pig welfare. Physiology and Behavior 89, 438-447.

Soler, L., Gutiérrez, A., Escribano, D., Fuentes, M., Cerón, J.J., 2013. Response of salivary haptoglobin and serum amyloid A to social isolation and short road transport stress in pigs. Research in Veterinary Science 95, 298-302.

Soler, L., Gutiérrez, A., Martínez-Subiela, S., Cerón, J.J., 2011. Development and validation of a novel competitive ELISA for the detection of serum amyloid A in pigs. The Veterinary Journal 190, e7-11.

Takatsuji, K., Sugimoto, Y., Ishizaki, S., Ozaki, Y., Matsuyama, E., Yamaguchi, Y., 2008. The effects of examination stress on salivary cortisol, immunoglobulin A, and chromogranin A in nursing students. Biomedical Research 29, 221–224.

Tuchscherer, M., Kanitz, E., Puppe, B., Tuchscherer, A., Viergutz, T., 2009. Changes in endocrine and immune responses of neonatal pigs exposed to a psychosocial stressor. Research in Veterinary Science 87, 380-388.

Turner, S.P., Farnworth, M.J., White, I.M.S., Brotherstone, S., Mendl, M., Knap, P., Penny, P., Lawrence, A.B., 2006. The accumulation of skin lesions and their use as a predictor of individual aggressiveness in pigs. Applied Animal Behaviour Science 96, 245-259.

Vakharia, K., Hinson, J.P., 2005. Lipopolysaccharide directly stimulates cortisol secretion by human adrenal cells by a cyclooxygenase-dependent mechanism. Endocrinology 146, 1389–1402.

von Borrell, E.H., 2001. The biology of stress and its application to livestock housing and transportation assessment. Journal of Animal Science 79, E260–E267.

Williams, P.N., Collier, C.T., Carroll, J.A.,Welsh, T.H., Jr., Laurenz, J.C., 2009. Temporal pattern and effect of sex on lipopolysaccharide-induced stress hormone and cytokine response in pigs. Domestic Animal Endocrinology 37, 139–147.

Ziegler-Heitbrock, H.W., 1995. Molecular mechanism in tolerance to lipopolysaccharide. Journal of Inflammation 45, 13–26.

ANNEX

Changes in saliva biomarkers of stress and immunity in domestic pigs exposed to a psychosocial stressor

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Short title: Saliva markers in pigs after psychosocial stressor

Abstract

In this study changes in a panel of salivary biomarkers of stress and immunity including chromogranin A (CgA), immunoglobulin A (IgA), cortisol, testosterone, haptoglobin (Hp) and C-reactive protein (CRP) that evaluate the four different physiological systems (sympathoadrenal-medullary axis, hypothalamic-pituitary-adrenal axis, hypothalamus-pituitary-gonadal axis and immune system) induced in stress mechanism, were evaluated after applying a psychosocial stressor model in pigs based on isolation and regrouping. 14 growing pigs (7 pigs/pen) were adapted to group living for 5 days, after which time, pigs in one group (7 pigs) were subjected to the isolation procedure, where each animal had olfactory and auditory but not physical contact with other pigs during 5 days. After, test pigs were regrouped again during 3 days returning to the initial conditions. Seven control pigs were remained in the same housing condition during the entire experimental period. Saliva samples were taken daily at the same time, and one sampling was carried out at 30 min after of isolation and regrouping events. Our results show that after of isolation, there was a significant ($P < 0.05$) increase in salivary CgA and IgA. While after the process of regrouped, there was a significant increase ($P < 0.05$) in salivary cortisol, testosterone and CgA. Hp and CRP concentrations in saliva of pigs did not significantly changed after applying isolation and regrouping. It is concluded that including various salivary biomarkers could be used as a practical and non-invasive tool for reflecting the activity of different physiology systems involved in stress response in pigs.

Keywords: isolation; regrouping; salivary biomarkers; pigs; stress response.

Implications

Animal welfare is of great importance in animal production systems nowadays and can be evaluated by biochemical determination of biomarkers reflecting the physiological response of the animal to stress. The use of salivary biomarkers can be a suitable “non-invasive” way to assess animal welfare according to European guidelines on animal wellbeing. Currently there is a lack of studies where multiple biomarkers are globally evaluated in stress models. We have found that salivary biomarkers appear to react differently to various types of stressors, and therefore including a panel of various salivary biomarkers can be of useful for stress evaluation.

Introduction

Saliva sampling has the advantages of being non-invasive and cause minimal stress during collection, being an ideal tool for evaluating stress physiology in pigs (Muneta *et al*., 2010; Soler *et al*., 2013). Unlike blood sampling methods, it does not cause additional stress, which could be a confounding factor in stress models. Furthermore, in contrast the urine or faeces samples, the repeated sampling over short time intervals can be carried out which facilitates ongoing animal monitoring (Escribano *et al*., 2012a).

Stress can be defined as a state of threat to homeostasis, caused by psychological, environmental or physiological stressors (Chrousos and Gold, 1992). A complex system of physiological and behavioural responses is initiated under conditions of stress, which is known as the adaptive stress response (Johnson *et al*., 1992) and serves to re-establish the threatened body equilibrium. Main tools used to quantify stress in pigs include direct behavioural observation (Smulders *et al*., 2006)

and quantification of the adaptive responses to stress by means of “biomarkers” (Cook *et al*., 1996; Muneta *et al*., 2010, Escribano *et al*., 2013). Although widely used, the methods of observations can have some potential for error or misinterpretation in some situations due to the potential subjective interpretation of animal behavioural response or to the fact that wellbeing of an animal might be impaired even when signs of stress are not obviously visible (Hart, 2012). For this reason the quantification of physiological responses to a stimulus by biomarkers could be a complement to behavioural observation methods.

Endocrine, immune, and central nervous systems interact and respond to stressful stimuli in a coordinated manner (von Borrel, 2001). Several potential salivary stress biomarkers have been identified and used in pigs, in an effort to produce objective tools to evaluate the different pathways the stress response. Salivary cortisol is the most frequently employed biomarker to assess the hypothalamic-pituitary-adrenal axis (HPA) (Cook *et al*., 1996; Merlot *et al*., 2011). Salivary testosterone can be used to assess the hypothalamus pituitary gonadal (HPG) axis (Escribano *et al*., 2014). In addition, salivary chromogranin A (CgA) (Escribano *et al*., 2013) and salivary immunoglobulin A (IgA) (Muneta *et al*., 2010) have been used as biomarkers of sympathoadrenal-medullary system (SAM) whereas that salivary acute phase proteins (APPs) (Soler *et al*., 2013) have been used as markers of immune system response. However, to the author’ knowledge, there are no studies where biomarkers for all four pathways have been used simultaneously to study the stress response in pigs.

Our objective was to study a panel of salivary markers of stress including CgA, IgA, cortisol, testosterone, haptoglobin (Hp) and C-reactive protein (CRP) to evaluate the four different physiological systems (HPA, HPG, SAM and immune system) that

101 are taking part in stress mechanism, after applying a psychosocial stressor model in
102 pigs based on isolation and regrouping.
103
104 **Materials and methods**
105 *Animal and housing*
106 A total of 14 non-castrated crossbred Duroc × (Landrace × Large White) males
107 were used for the experiment. The procedures were conducted on a high
108 sanitary/health-status farm in the southeast of Spain under general commercial
109 housing, feeding and husbandry conditions conforming to European Union
110 Guidelines (Directive 2010/63/EU[1]), and had access to a nutritionally-balanced diet
111 (commercial dry diets) and water ad libitum (from nipple drinkers). Pen size
112 employed along this experiment was of 2.73 × 2.85 (7.78 m^2) m and with a slatted-
113 floor. The 14 pigs were housed in slatted-floor pens (stocking density of 1.1 m^2 per
114 pig) in two groups (test and control) of 7 animals each group. All animals selected
115 belonged to the same stock of pigs, with 190 days of age, and they were subjected to
116 a clinical examination prior to and throughout the study and no clinical signs of
117 disease were detected. Furthermore, the concentrations of salivary CRP and Hp
118 obtained in the present study were always lower than the internal reference cut-off
119 values of the laboratory for diseased age-matched animals (Gutiérrez *et al*., 2009c).
120
121 *Experimental design*
122 Five days before the beginning of the experimental period, all animals were
123 accustomed to human contact and saliva collection methodology for 5 days to avoid
124 stress. The experimental design was divided into three periods: pre-stressor,

[1] See: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:en:PDF

125 isolation and regrouping. On each day, during the three periods, saliva samples were
126 collected at 1100 h in both test and control groups. Two additional saliva samples
127 were collected at 30 min after isolation and at 30 min after regrouping. In the study of
128 isolation the behaviour and lesion scores of each animal were measured each day
129 after sampling based on ethogram of behavioural used by Ruis *et al*. (2001). After
130 mixing the accumulation of skin lesions as a predictor of individual aggressiveness in
131 pigs were measured by a previously described method (Turner *et al*., 2006).
132 The pre-stressor period lasts the first 5 days (day -1d to -5d; Figure 1). Test (n =
133 7) and control (n = 7) pigs remained during this period in the same housing
134 conditions (stocking density of 1.1 m^2 per pig). In the day 6 of this period baseline
135 sample of isolation (0dI) for test and control groups were obtained. For the isolation
136 procedure, 7 pigs were relocated to a new barn where each one in the same room
137 was isolated in individual pens (7.78 m^2 each), where they had olfactory and auditory
138 but not physical contact with other pigs. From day 0 of isolation (0dI; Figure 1) till day
139 five of isolation (5dI; Figure 1) the test pigs were kept in this experimental condition.
140 In the day 6 of this period were taken the baseline samples of regrouping (0dR) for
141 test and control groups. The regrouping period started after the isolation period. Test
142 pigs were regrouped and remaining in these conditions during 3 days (day 0 of
143 regrouping, 0dR, to day 3 of regrouping, 3dR; Figure 1), returning to their original
144 group with the initial conditions (stocking density of 1.1 m^2 per pig). The overall
145 isolation and regrouping procedure took about 3 min.
146 The control pigs were remained in the same housing conditions during all the
147 experimental period. The temperature of the pens at these sampling time-points was
148 21.2 ºC ± 0.8 (mean ± SD) and animals were maintained during experimental period
149 with natural lighting. Pigs of both groups were slaughtered at the end of the

experiment in the local slaughterhouse. Procedures involving animal handling were approved by the University of Murcia Ethics Committee and followed the recommendations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council of Europe, ETS Number 123).

Saliva collection and salivary analysis

Saliva was collected from all animals using saliva collection tubes (Sarstedt, Aktiengesellschaft & Co., Nümbrecht, Germany) containing a sponge, as reported previously (Gutiérrez *et al*., 2009a). Each pig was allowed to gently chew on a sponge, which was clipped to a flexible thin metal rod, exactly 1 min and without forcing. The sponges were then placed in the tubes and centrifuged at 3,000 × *g* for 10 min. Saliva samples were collected and stored at –80 °C until analysis. Saliva volume obtained for each pig was measured using a micropipette and saliva flow rate (mL/min) determined by dividing the volume of saliva by the collection time.

Salivary cortisol and salivary testosterone were analysed using an automated chemiluminescence immunoassay (Immulite 1000 cortisol, Siemens Medical Solutions Diagnostics) previously validated for its use in pigs (Escribano *et al*., 2012a; Escribano *et al*., 2014). To cortisol the intra- and inter-assay coefficients of variations (CVs) were below 16% and the limit of detection was 0.016 µg/dL whereas that in testosterone CVs were below 10% and the limit of detection was 13.60 ng/dL. The concentration of salivary Hp, CgA and CRP were determined by time-resolved immunofluorometry assays (TR-IFMA), developed and validated in our laboratory as previously described (Gutiérrez *et al*., 2009a, b; Escribano *et al*., 2013). The assays showed intra- and inter-assay CVs below 10% and 15%, respectively, and lower

limits of detection of 4.27 × 10-3 µg/mL for CgA, 0.52 ng/mL for Hp and 0.47 ng/mL for CRP. Salivary IgA was analysed using a commercially-available enzyme-linked immunosorbent assay (IgA ELISA Quantitation Kit, Bethyl Laboratories). Intra- and inter-assay CVs were below 13% and the limit of detection was 4.29 ng/mL (Escribano *et al*., 2012b). Total protein concentration in saliva was determined using a pyrogallol red colorimetric method (Protein in urine and CSF, Spinreact, Spain) at 598 nm employing an automated biochemical analyser (AU400, Olympus, Minneapolis, USA). Intra- and inter-assay CVs were below 10% and the limit of detection was 0.005 mg/mL.

Statistical analysis

Data analyses were performed using a commercial statistics package (GraphPad Prism 6, GraphPad Software Inc., La Jolla, CA). The different salivary biomarkers concentrations were evaluated for normality of distribution, using the Kolmogorov–Smirnov test. As the results did not meet the normal distribution criteria, data were log transformed. Data are presented as means ± standard error of means (SEM). To test the effect of isolation and regrouping on the concentration of the biomarkers in the saliva of both trials, two datasets were used. Both datasets were tested using a two-way ANOVA mixed model of repeated measured with a Tukey multiple comparison between groups (test and control) post hoc test. The model included the main effects effects of treatment; day and the interaction between these effects were included as factor. The effect of isolation on behavioural and regrouping on total number of body lesions were tested in a separate two-way ANOVA mixed model of repeated measured with a Tukey multiple comparison post hoc test. The model included the main effects of treatment and experimental period (pre-stressor,

post-mixing and post-deprivation) and the interaction between these effects. For comparison within each group (control and test group, independently), the concentrations of each analyte at 0dI, as baseline for isolation, and at 0dR, as baseline for regrouping, were used. A value of $P < 0.05$ was used to indicate significance in all analyses. To get an indication about the interrelationships between the biomarkers, Spearman correlations were calculated on pooled data of all pigs and sampling days because no treatment and day effects were found.

Results

Animal behaviour after isolation and body lesions after regrouping

Before isolation, patterns of different behaviour did not differ between test and control group. Isolation caused a significant ($P < 0.05$) increase in exploratory behavioural (sniffing, touching and walking through the pen) and vocalizations in test group compared to control group and also in relation to its baseline values (0dI). This exploratory behaviour was maintained in test group the first day of isolation (1dI), after, an inactive behavioural was observed until the regrouping period. All pigs had a higher number of body lesions after regrouping ($P < 0.05$) compared to its baseline values (0dR), and the test group had a higher number of body lesions ($P < 0.05$) compared to the control group after regrouping. The number of lesions did not increase after 1dR.

Salivary total protein and flow rate

Salivary total protein did not change significantly over sampling, neither during the isolation nor during the regrouping (Figure 1a). There was no significant difference between control and test pigs at same time point. In addition, salivary total

protein concentrations did not differ significantly from its baseline values (0dI and 0dR) in test or control pigs after isolation and after the regrouping. Salivary flow rate there were no significant differences between control and test groups in the same day (Figure 1b). No significant differences in salivary flow rate after isolation and after the regrouping compared to baseline levels (0dI and 0dR) for both groups, were obtained.

Salivary chromogranin A and immunoglobulin A

The interaction between sampling and treatment was significant for salivary CgA ($P < 0.001$). Relative to baseline (0dI and 0dR), CgA concentrations were higher 30 min after of isolation ($P < 0.05$) and 30 min after of the regrouping ($P < 0.01$) in test pigs (Figure 1c). In addition, test pigs had significantly greater salivary CgA concentrations compared to control pigs at 30 min after isolation ($P < 0.05$) and 30 min after the regrouping ($P < 0.01$). The interaction between sampling and treatment was significant on salivary IgA ($P < 0.001$; Figure 1d). In test pigs, relative to baseline (0dI and 0dR), salivary IgA was significantly increased at first day and fifth day of isolation ($P < 0.05$) and was significantly decreased ($P < 0.05$) after three days of the regrouping. Test pigs had significant ($P < 0.05$) higher values of salivary IgA compared with controls at 1dI and 5dI.

Salivary cortisol and testosterone

The interaction between sampling and treatment was significant for salivary cortisol ($P < 0.001$). Relative to regrouping baseline (0dR), cortisol concentrations were higher after 30 min ($P < 0.001$) in test pigs (Figure 1e). In addition, test pigs had significantly greater concentrations compared to control pigs at 30 min after the

regrouping ($P < 0.001$). The interaction between sampling and treatment was significant for salivary testosterone ($P < 0.05$). In test pigs, salivary testosterone was elevated ($P < 0.01$), relative to regrouping baseline (0dR) after 30 min (Figure 1f). In addition, test pigs had significantly greater testosterone concentrations compared to control pigs at 30 min after the regrouping ($P < 0.05$).

Salivary haptoglobin and C-reactive protein

There was no significant difference comparing control and test groups at any sampling time (Figure 1g). Salivary Hp concentrations after isolation and after the regrouping were not significantly different compared to baseline levels (0dI and 0dR) in both groups.There was no significant difference between control and test pigs at any sampling time (Figure 1h). In addition, salivary CRP concentrations did not differ significantly from its baseline values (0dI and 0dR) in test or control pigs after isolation and after the regrouping.

Correlations analysis

Several biomarkers showed a significant positive relationship between them and also a significant negative relationship (except with cortisol) in relation to volume of saliva were observed, but correlations were weak to moderate (Table 1). Salivary CgA showed the highest positive correlation with salivary Hp ($r = 0.51$, $P < 0.0001$).

Discussion

The experimental design employed in the present study was adapted from others available in the literature for psychosocial stress assessment, based in social "isolation" (Tuchscherer *et al*., 2009; Soler *et al*., 2013) and "regrouping" or mixing

(Coutellier *et al*., 2007; Oster *et al*., 2014). The increase of vocalization that was observed after isolation and the aggression and the significant increase of skin lesions that was found after regrouping would indicate an activation of the stress response in pigs (Ruis *et al*., 2001; Turner *et al*., 2006) after applying our psychosocial stressor model. Salivary flow rate and salivary total protein concentration did not change in our stress model. Therefore, it can rule out that the significant changes observed in our study are due to the possible dilution or concentration of markers in saliva because of the lack/excess of saliva or the thickness thereof. In human, salivary IgA appears to be affected by salivary flow rate (Klentrou *et al*., 2002), while no impact of salivary flow rate have been found on salivary cortisol, CgA and testosterone (Arregger *et al*., 2007; Obayashi, 2013).

Recent studies have suggested that salivary CgA (Escribano *et al*., 2013) and immunoglobulin A (IgA; Muneta *et al*., 2010) might be sensitive and specific biomarkers of the acute stress response during restraint in pigs, reflecting SAM activity, as suggested in humans (Allgrove *et al*., 2008; Gallina *et al*., 2011). Significant increases were found in salivary CgA and IgA levels post-isolation in our study. The magnitude and time of increase in salivary CgA response was similar to previously described after applying others models of stress, as restriction with nose-snare (Escribano *et al*., 2013) or transport and accommodation in slaughterhouse (Escribano *et al*., 2014), with a mean increase around of 39% of baseline concentration after 30 min of isolation. No increase in salivary IgA after 30 min of isolation was observed in our study. However, significant increases were obtained in IgA after one and five days post-isolation and, although more investigations are needed to clarify this topic, the salivary IgA showed a delayed response compared with CgA after isolation.

Salivary cortisol has been commonly measured to indicate activity of HPA axis, in response to different stressor in pigs (Cook *et al*., 1996; Merlot *et al*., 2011), while salivary testosterone levels (HPG axis) also can be used as a possible marker of acute stress (Escribano *et al*., 2014). In our study, no significant changes on salivary cortisol and testosterone post-isolation were observed. These results may indicate a quickly adaptation of the domestic pigs to the housing experimental conditions and, as has been reported before for salivary cortisol by Jaskulke and Manteuffel (2011). The domestication has adapted domestic pigs to narrow housing conditions without impairing physiological homeostasis. However, this does not exclude mental suffering, and cortisol and testosterone may not be enough sensible to indicate psychic stress. Thus, the combinations of multiple salivary biomarkers, including markers of SAM and HPA, should be assessed at the same time has been recommended (Obayashi, 2013).

Significant increases in salivary CgA levels were found with a mean increase around of 51% of baseline concentration after 30 min of regrouping, similar results for CgA 30 min after the regrouping in the slaughterhouse has been described (Escribano *et al*., 2014). In contrast, no increase was found in salivary IgA but a significant decrease was observed after 3 days of regrouping. A hypothesis for this lack of increase of IgA could be the fact of the decrease of the immune response. Since, de Groot *et al*. (2001) have reported that social stress after mixing could hinder the immune response in pigs and therefore the production of different immunoglobulins.

The magnitude of the increase in salivary cortisol found in the present study after 30 min of regrouping was in a similar range as reported in previous studies on mixing, where typically are involved unfamiliar pigs (de Groot *et al*., 2001; Merlot *et*

al., 2004; Coutellier *et al*., 2007), with a mean increase of 40% of baseline concentration. Also significant increases, around of 70% of baseline concentration, in salivary testosterone levels were found after 30 min of regrouping, similar results for testosterone after regrouping in the slaughterhouse has been found (Escribano *et al*., 2014). The mechanism for the increase in testosterone to acute stress remains unclear, but Chichinadze and Chichinadze (2008) suggested that could be due to increased sensitivity of the testes to LH as result of the activation of the SAM axis by norepinephrine, which has been shown to stimulate production of LH in both men and women (Chrousos *et al*., 1998). SAM and HPA axis are activated after mixing of unfamiliar pigs (de Groot *et al*., 2001; Otten *et al*., 2002) due to factors as uncertainty, social pressure and fear, that post-regrouping can have induced a "fight-flight" response in pigs (von Borrell, 2001).

During stress, it has been proposed that the activation of the SAM and HPA axis may lead to the activation and release of APPs in the liver (Murata, 2007; Aninat *et al*., 2008). Our results indicate that salivary Hp and CRP concentrations did not significantly change in the stress models used in our study. This is in line with a previous report in which no changes in Hp were found after social isolation and short road transport stress (Soler *et al*., 2013). Then, it should be recognised that, based on our results, these markers do not appears to be indicators of stress. However, salivary Hp and CRP can be used to assess the health status and well-being of pig herds (Gómez-Laguna *et al*., 2010). In our case, considering the age of the pigs, the concentrations of Hp and CRP were always lower than the internal reference cut-off values of the laboratory for diseased animals (Gutiérrez *et al*., 2009c), indicating that animal used in this study were healthy. Therefore, these APPs could be used within

the panel as salivary markers in studies of stress when required a clear differentiation between stressed and diseased animals.

Although the correlations were weak to moderate, the salivary biomarkers used in our study to evaluate the same axis or system showed a significant positive relationship between them (CgA-IgA for SAM axis; Cort-Tes for HPA and HPG axis; CRP-HP for immune system) and also between immune system and SAM axis (Hp-CgA and Hp-IgA). These results suggest that a connection between the four response pathways (SAM, HPA, HPG and immune system) may exist. In any case, further studies are needed because it seems that the psychosocial stress response is a rather complex web of interactions in which the interrelationships between metabolites, SAM, HPA, HPG axis and immune system, are not exactly known.

Conclusions

The use of several salivary biomarkers could be essential in stress research since our results demonstrated that salivary biomarkers appear to react differently to various types of stressors. In the case of isolation, salivary CgA and IgA might be more sensitive markers than cortisol and testosterone, with CgA showing a faster response than IgA. While in the process of regrouped, salivary cortisol, testosterone and CgA might be more sensitive markers than salivary IgA. Moreover, Hp, CRP and total protein concentrations and flow rate in saliva of pigs did not significantly changed after applying this psychosocial stressor as stress model. However, Hp and CRP allow to discrimination between healthy and diseased animals while total protein and flow rate can rule out that the significant changes observed are due to alterations in volume or concentration of saliva. Therefore, although further studies are needed, these salivary biomarkers could be used as a practical and non-invasive tool for

374 reflecting the activity of different physiology systems involved in stress response in
375 pigs.
376
377 **Conflict of interest statement**
378 None of the authors of this paper has a financial or personal relationship with
379 other people or organizations that could inappropriately influence or bias the content
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386 **References**
387 Allgrove JE, Gomes E, Hough J and Gleeson M 2008. Effects of exercise intensity on
388 salivary antimicrobial proteins and markers of stress in active men. Journal of Sports
389 Sciences 6, 653-661.
390 Aninat C, Seguin P, Descheemaeker PN, Morel F, Malledant Y and Guillouzo A 2008.
391 Catecholamines induce an inflammatory response in human hepatocytes. Critical Care
392 Medicine 36, 848–854.
393 Arregger AL, Contreras LN, Tumilasci OR, Aquilano DR and Cardoso EM 2007. Salivary
394 testosterone: a reliable approach to the diagnosis of male hypogonadism. Clinical
395 Endocrinology (Oxford) 67, 656-662.
396 Chichinadze K and Chichinadze N 2008. Stress-induced increase in testosterone:
397 Contributions of social and sympathetic reactivity. Physiology & Behavior 94, 596-603.
398 Chrousos GP and Gold PW 1992. The concepts of stress and stress system disorders.
399 Overview of physical and behavioural homeostasis. The journal of the American
400 Medical Association 267, 1244-1252.

401 Chrousos GP, Torpy DJ and Gold PW 1998. Interactions between the hypothalamic-pituitary-
402 adrenal axis and the female reproductive system: clinical implications. Annals of
403 Internal Medicine 129, 229-240.
404 Cook NJ, Schaefer AL, Lepage P and Morgan Jones SD 1996. Salivary vs. serum cortisol for
405 the assessment of adrenal activity in swine. Canadian Journal of Animal Science 76,
406 329–335.
407 Coutellier L, Arnould C, Boissy A, Orgeur P, Prunier A, Veissier I and Meunier-Salaün MC
408 2007. Pig's responses to repeated social regrouping and relocation during the growing-
409 finishing period. Applied Animal Behaviour Science 105, 102-114.
410 de Groot J, Ruis MAW, Scholten JW, Koolhaas JM and Boersma WJA 2001. Long-term
411 effects of social stress on antiviral immunity in pigs. Physiology & Behavior 73, 145-
412 158.
413 Escribano D, Fuentes M and Cerón JJ 2012a. Validation of an automated chemiluminescent
414 immunoassay for salivary cortisol measurements in pigs. Journal of Veterinary
415 Diagnostic Investigation 24, 918-923.
416 Escribano D, Fuentes-Rubio M and Cerón JJ 2014. Salivary testosterone measurements in
417 growing pigs: validation of an automated chemiluminescent immunoassay and its
418 possible use as an acute stress marker. Research in Veterinary Science,
419 http://dx.doi.org/10.1016/j.rvsc.2014.04.001.
420 Escribano D, Gutiérrez AM, Martínez-Subiela S, Tecles F and Cerón JJ 2012b. Validation of
421 three commercially available immunoassays for quantification of IgA, IgG, and IgM in
422 porcine saliva samples. Research in Veterinary Science 93, 682-687.
423 Escribano D, Soler L, Gutiérrez AM, Martínez-Subiela S and Cerón JJ 2013. Measurement of
424 chromogranin A in porcine saliva: validation of a time-resolved immunofluorometric
425 assay and evaluation of its application as a marker of acute stress. Animal 7, 640-647.
426 Gallina S, Di Mauro M, D´Amico MA, D´Angelo E, Sablone A, Di Fonso A, Bascelli A,
427 Izzicupo P and Di Baldassarre A 2011. Salivary chromogranin A, but not α-amylase,

428 correlates with cardiovascular parameters during high-intensity exercise. Clinical
429 Endocrinology 75, 747-752.
430 Gómez-Laguna J, Gutiérrez AM, Pallarés FJ, Salguero FJ, Cerón JJ and Carrasco L 2010.
431 Haptoglobin and C-reactive protein as biomarkers in the serum, saliva and meat juice
432 of pigs experimentally infected with porcine reproductive and respiratory syndrome
433 virus. The Veterinary Journal 185, 83-87.
434 Gutiérrez AM, Martínez-Subiela S and Cerón JJ 2009a. Evaluation of an immunoassay for
435 determination of haptoglobin concentration in various biological specimens from swine.
436 American Journal of Veterinary Research 70, 691-696.
437 Gutiérrez AM, Martínez-Subiela S, Ekersall PD and Cerón JJ 2009b. C-reactive protein in
438 porcine saliva: A minimally invasive test for pig health monitoring. The Veterinary
439 Journal 181, 261-265.
440 Gutiérrez AM, Martínez-Subiela S, Soler L, Pallarés FJ and Cerón JJ 2009c. Use of saliva for
441 haptoglobin and C-reactive protein quantifications in porcine respiratory and
442 reproductive syndrome affected pigs in field conditions. Veterinary Immunology and
443 Immunopathology 132, 218-223.
444 Hart KA 2012. The use of cortisol for the objective assessment of stress in animals: pros and
445 cons. The Veterinary Journal 192, 137-139.
446 Jaskulke S and Manteuffel G 2011. No apparent effect of an experimental narrow
447 confinement on heart activity and cortisol in domestic pigs. Animal 5, 433-438.
448 Johnson EO, Kamilaris TC, Chrousos GP and Gold PW 1992. Mechanisms of stress: a
449 dynamic overview of hormonal and behavioral homeostasis. Neuroscience
450 Biobehavioral Reviews 16, 115-130.
451 Klentrou P, Cieslak T, MacNeil M, Vintinner A and Plyley M 2002. Effect of moderate
452 exercise on salivary immunoglobulin A and infection risk in humans. European Journal
453 of Applied Physiology 87, 153-158.

454 Merlot E, Meunier-Salaün MC and Prunier A 2004. Behavioural, endocrine and immune
455 consequences of mixing in weaned piglets. Applied Animal Behaviour Science 85,
456 247–257.
457 Merlot E, Mounier AM and Prunier A 2011. Endocrine response of gilts to various common
458 sressors: A comparison of indicators and methods of analysis. Physiology & Behavior
459 102, 259-265.
460 Muneta Y, Yoshikawa T, Minagawa Y, Shibahara T, Maeda R and Omata Y 2010. Salivary
461 IgA as a useful non-invasive marker for restraint stress in pigs. The Journal of
462 Veterinary Medical Science 72, 1295-1300.
463 Murata H 2007. Stress and acute phase protein response: an inconspicuous but essential
464 linkage. The Veterinary Journal 173, 473–474.
465 Obayashi K 2013. Salivary mental stress proteins. Clinica Chimica Acta 425, 196-201.
466 Oster M, Muráni E, Ponsuksili S, D'Eath RB, Turner SP, Evans G, Thölking L, Kurt E, Klont
467 R, Foury A, Mormède P and Wimmers K 2014. Hepatic expression patterns in
468 psychosocially high-stressed pigs suggest mechanisms following allostatic principles.
469 Physiology & Behavior 128, 159-165.
470 Otten W, Puppe B, Kanitz E, Schön PC, Stabenow B and 2002. Physiological and behavioral
471 effects of different success during social confrontation in pigs with prior dominance
472 experience. Physiology & Behavior 75, 127-133.
473 Ruis MA, te Brake JH, Engel B, Buist WG, Blokhuis HJ and Koolhaas JM 2001. Adaptation to
474 social isolation: Acute and long-term stress responses of growing gilts with different
475 coping characteristics. Physiology & Behavior 73, 541–551.
476 Smulders D, Verbeke G, Mormède P and Geers R 2006. Validation of a behavioral
477 observation tool to asses pig welfare. Physiology & Behavior 89, 438-447.
478 Soler L, Gutiérrez A, Escribano D, Fuentes M and Cerón JJ 2013. Response of salivary
479 haptoglobin and serum amyloid A to social isolation and short road transport stress in
480 pigs. Research in Veterinary Science 95, 298-302.

Tuchscherer M, Kanitz E, Puppe B, Tuchscherer A and Viergutz T 2009. Changes in endocrine and immune responses of neonatal pigs exposed to a psychosocial stressor. Research in Veterinary Science 87, 380-388.

Turner SP, Farnworth MJ, White IMS, Brotherstone S, Mendl M, Knap P, Penny P and Lawrence AB 2006. The accumulation of skin lesions and their use as a predictor of individual aggressiveness in pigs. Applied Animal Behaviour Science 96, 245-259.

von Borrell EH 2001. The biology of stress and its application to livestock housing and transportation assessment. Journal of Animal Science 79, E260-E267.

Table 1

Spearman correlation coefficients among salivary biomarkers (Chromogranin A, CgA; Immunoglobulin A, IgA; Cortisol, Cort; Testosterone, Tes; Haptoglobin, Hp; protein C-reactive, CRP and total protein, TP) and the volume of saliva (Vol) for all sampling days and pigs (n = 238).

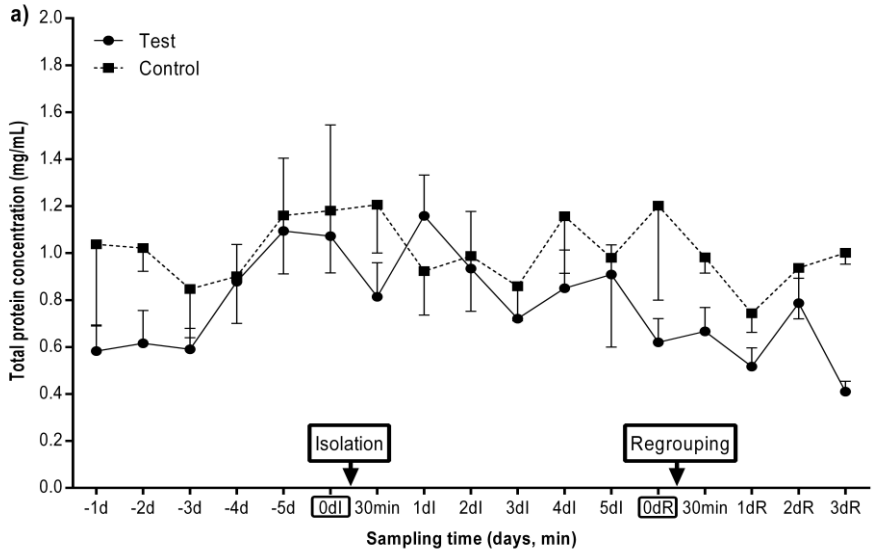
	CgA	IgA	Cort	Tes	Hp	CRP	TP	Vol
CgA		0.22**	NS	NS	0.51***	NS	0.29***	-0.50***
IgA	0.22**		NS	NS	0.36***	NS	0.41***	-0.28***
Cort	NS	NS		0.32***	NS	NS	NS	0.32***
Tes	NS	NS	0.32***		NS	NS	0.20**	NS
Hp	0.51***	0.36***	NS	NS		0.27***	0.49***	-0.43***
CRP	NS	NS	NS	NS	0.27***		NS	NS
TP	0.29***	0.41***	NS	0.20**	0.49***	NS		-0.28***
Vol	-0.50***	-0.28***	0.32***	NS	-0.43***	NS	-0.28***	

NS = not significant; ***P*< 0.01; ****P*< 0.0001

528 **Figure legends**

529 Figure 1. (a) Salivary total protein, (b) salivary flow rate, (c) chromogranin A (CgA), (d)
530 immunoglobulin A (IgA), (e) cortisol, (f) testosterone, (g) haptoglobin (Hp) and (h) C-reactive
531 protein (CRP) concentrations in test (n = 7) and control pigs (n = 7); that was divided into
532 three periods: pre-stressor (days -1d to -5d), isolation (0dI to 5dI) and regrouping (0dR to
533 3dR). Values shown are means ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to
534 baseline values (0dI and 0dR) in test or control pigs after isolation and after regrouping for
535 each group. †*P* < 0.05, ††*P* < 0.01, †††*P* < 0.001 comparing test and control pigs.

536



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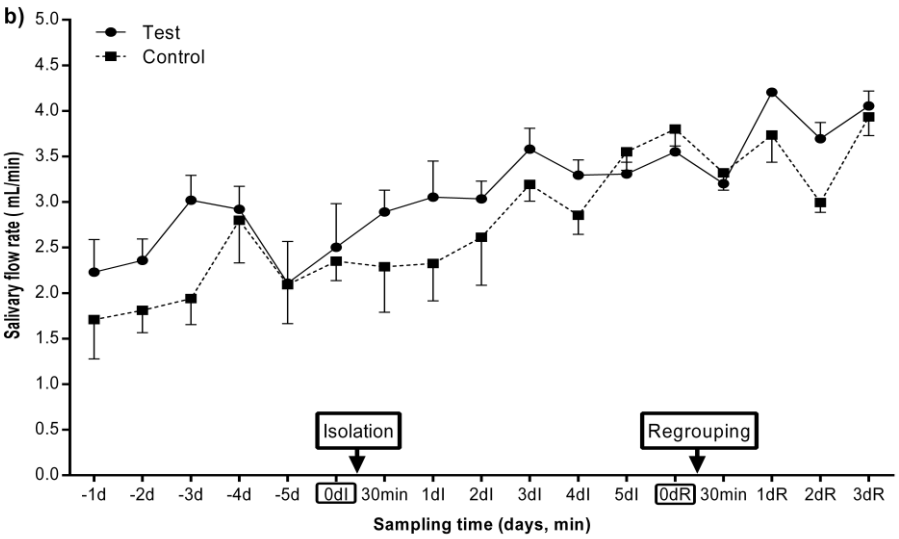

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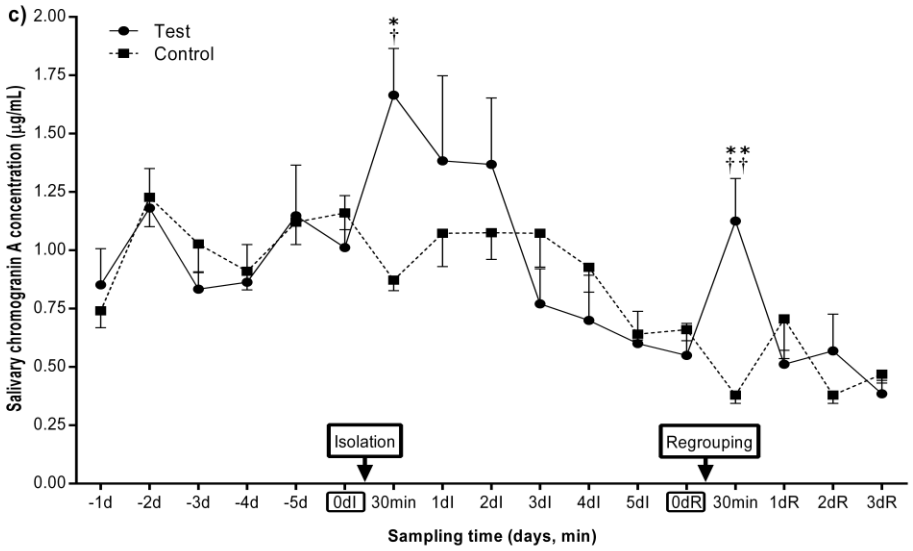
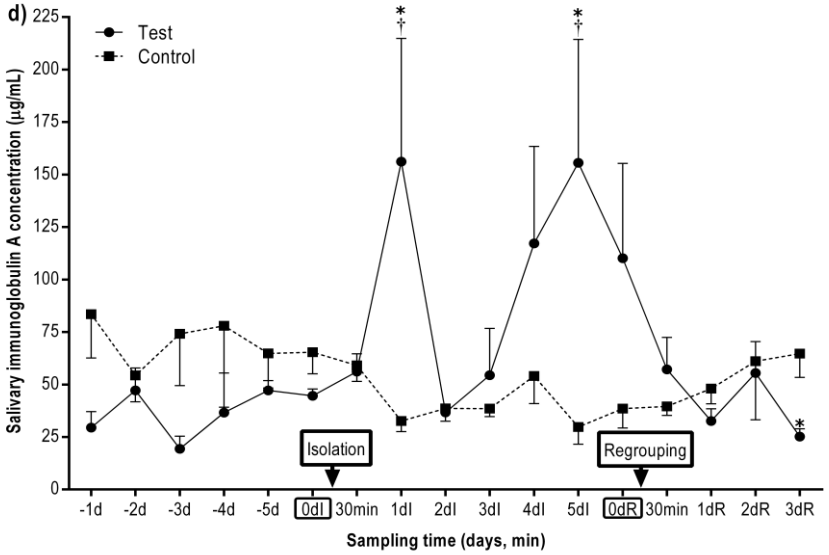
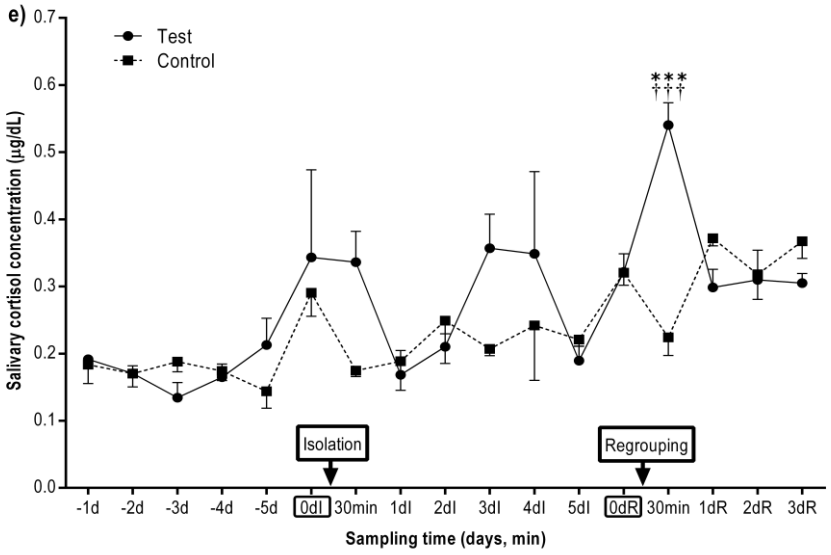

c)
Test
Control
Salivary chromogranin A concentration (µg/mL)
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Isolation
Regrouping
-1d -2d -3d -4d -5d 0dI 30min 1dI 2dI 3dI 4dI 5dI 0dR 30min 1dR 2dR 3dR
Sampling time (days, min)

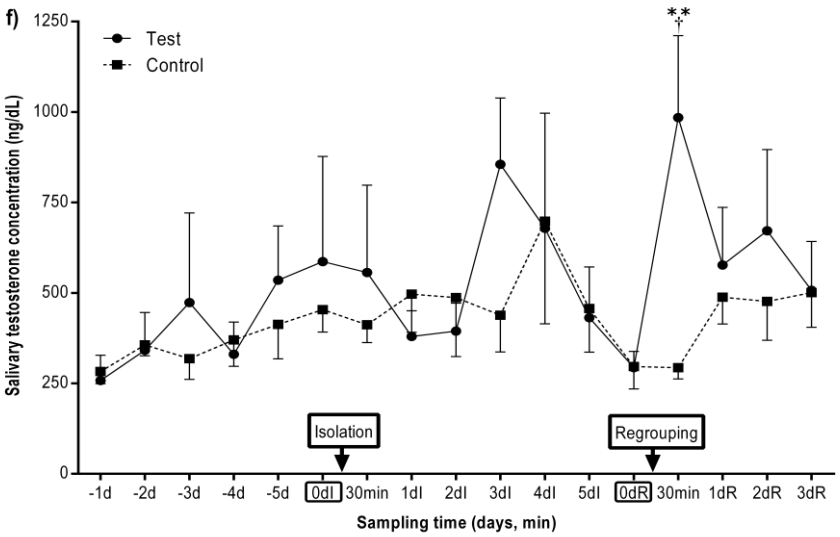
539


d)
Test
Control
Salivary immunoglobulin A concentration (µg/mL)
225
200
175
150
125
100
75
50
25
0
Isolation
Regrouping
-1d -2d -3d -4d -5d 0dI 30min 1dI 2dI 3dI 4dI 5dI 0dR 30min 1dR 2dR 3dR
Sampling time (days, min)

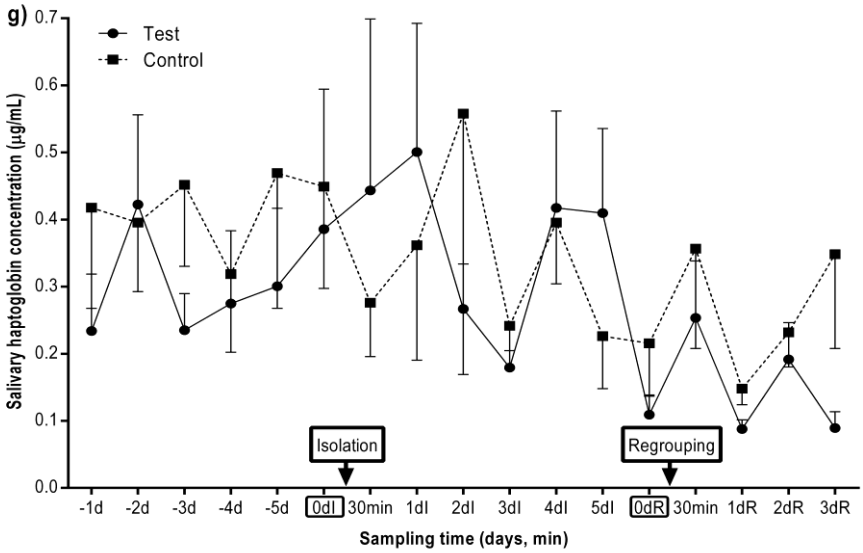
540


e)
Test
Control
Salivary cortisol concentration (µg/dL)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Isolation
Regrouping
-1d -2d -3d -4d -5d 0dI 30min 1dI 2dI 3dI 4dI 5dI 0dR 30min 1dR 2dR 3dR
Sampling time (days, min)

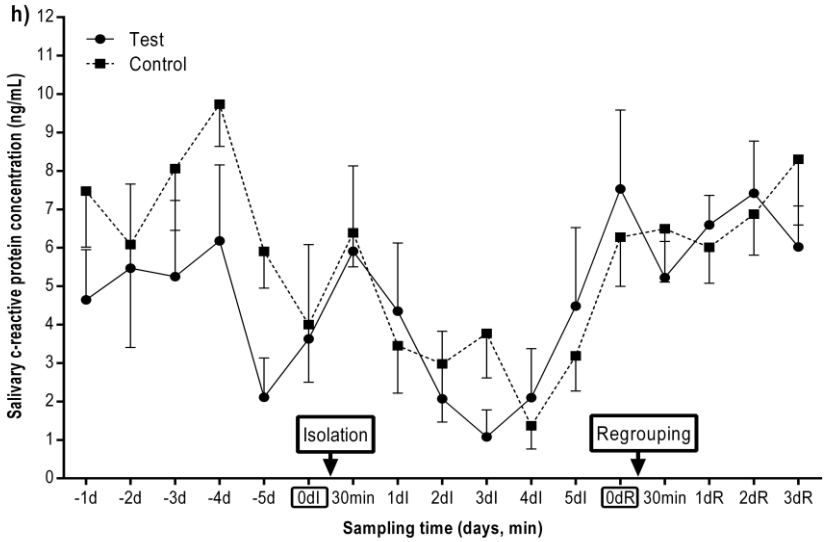
541


f)
Test
Control
Salivary testosterone concentration (ng/dL)
1250
1000
750
500
250
0
**
Isolation
Regrouping
-1d -2d -3d -4d -5d 0dI 30min 1dI 2dI 3dI 4dI 5dI 0dR 30min 1dR 2dR 3dR
Sampling time (days, min)

542


g)
Test
Control
Salivary haptoglobin concentration (µg/mL)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Isolation
Regrouping
-1d -2d -3d -4d -5d 0dI 30min 1dI 2dI 3dI 4dI 5dI 0dR 30min 1dR 2dR 3dR
Sampling time (days, min)

543


h)
Test
Control
Salivary c-reactive protein concentration (ng/mL)
12
11
10
9
8
7
6
5
4
3
2
1
0
Isolation
Regrouping
-1d -2d -3d -4d -5d 0dI 30min 1dI 2dI 3dI 4dI 5dI 0dR 30min 1dR 2dR 3dR
Sampling time (days, min)

544

APPENDIX

APPENDIX

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Journal Citation Reports®

2012 JCR Science Edition

MARKED JOURNAL LIST

Sorted by: Journal Title

Abbreviated Journal Title	ISSN	2012 Total Cites	Impact Factor	5-Year Impact Factor	Immediacy Index	2012 Articles	Cited Half-life	*Eigenfactor*® Score	*Article Influence*® Score
ANIMAL	1751-7311	1894	1.648	1.761	0.392	227	3.3	0.00885	0.498
J VET DIAGN INVEST	1040-6387	3497	1.181	1.556	0.125	192	8.1	0.00626	0.425
RES VET SCI	0034-5288	4447	1.774	1.722	0.268	366	9.9	0.00669	0.444
VET J	1090-0233	4990	2.424	2.656	0.569	357	4.6	0.01358	0.676

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