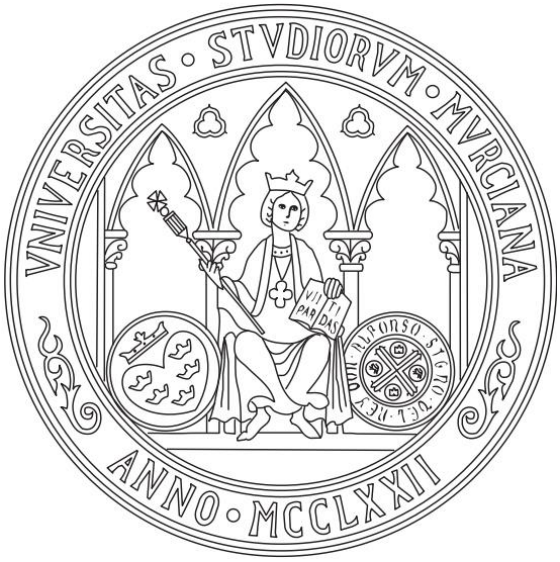



# UNIVERSIDAD DE MURCIA

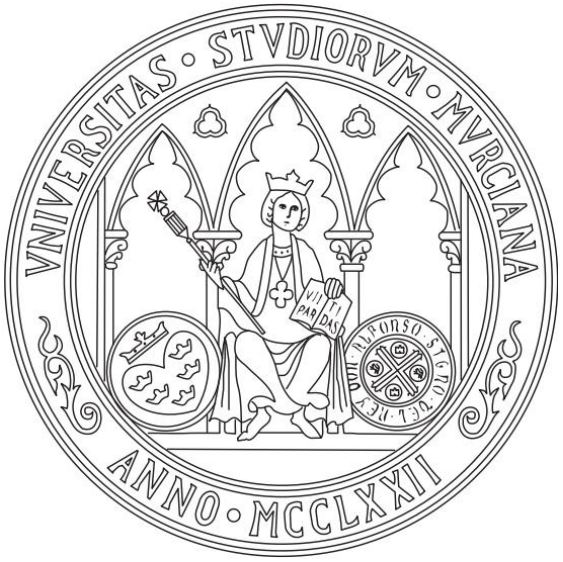
ESCUELA INTERNACIONAL DE DOCTORADO

Advances in biomarkers for welfare evaluation: oxytocin and corticoids

Avances en biomarcadores para la evaluación de bienestar: oxitocina y corticoides

**Dña. Marina López Arjona**

2021

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FACULTAD DE VETERINARIA

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## FACULTAD DE VETERINARIA

**“Advances in biomarkers for welfare evaluation: oxytocin and corticoids”**

**“Avances en biomarcadores para la evaluación de bienestar: oxitocina y corticoides”**

Memoria presentada por la graduada en veterinaria

**Marina López Arjona**

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

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*“Uno nunca se da cuenta de lo que ha hecho, solo puede ver lo que queda por hacer”*

*Marie Skłodowska-Curie*

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Mil gracias a todos, os llevo en mi corazón.

# INDEX

# DOCTORAL THESIS AS COMPENDIUM OF PUBLICATIONS

# DOCTORAL THESIS AS COMPENDIUM OF PUBLICATIONS

This Ph.D. Thesis, after the authorization of the directors of the Ph.D. Thesis and the Academic Commission responsible for the Veterinary Sciences Ph.D. Program, is presented as a compendium of nine studies previously published. Therefore, the Ph.D. Thesis is composed of the following article references:

I. **López-Arjona, M.,** Mateo, S.V., Manteca, X., Escribano, D., Cerón, J.J., Martínez-Subiela, S. (2020). Oxytocin in saliva of pigs: an assay for its measurement and changes after farrowing. *Domestic Animal Endocrinology*, 70, 106384.

II. **López-Arjona, M.,** Mateo, S.V., Escribano, D., Tecles, F., Cerón, J.J., Martínez-Subiela, S. (2021). Effect of reduction and alkylation treatment in three different assays used for the measurement of oxytocin in saliva of pigs. *Domestic Animal Endocrinology*, 74, 106498.

III. **López-Arjona, M.,** Padilla, L., Roca, J., Cerón, J.J., Martínez-Subiela, S. (2020). Ejaculate Collection Influences the Salivary Oxytocin Concentrations in Breeding Male Pigs. *Animals*, 10(8), 1268.

IV. **López-Arjona, M.,** Escribano, D., Mateo, S.V., Contreras-Aguilar, M.D., Rubio, C.P., Tecles, F., Cerón, J.J., Martínez-Subiela, S. (2020). Changes in oxytocin concentrations in saliva of pigs after a transport and during lairage at slaughterhouse. *Research in Veterinary Science*, 133, 26-30.

V. Padilla, L., **López-Arjona, M.,** Martínez-Subiela, S., Rodríguez-Martínez, H., Roca, J., Barranco, I. (2021). Oxytocin in pig seminal plasma is positively related with *in vivo* fertility of inseminated sows. *Journal of Animal Science and Biotechnology*, 12(1), 101.

VI. **López-Arjona, M.,** Mainau, E., Navarro, E., Contreras-Aguilar, M.D., Escribano, D., Mateo, S.V., Manteca, X., Cerón, J.J., Martínez-Subiela, S. (2021). Oxytocin in bovine saliva: validation of two assays and changes in parturition and at weaning. *BMC Veterinary Research*, 17(1), 1-8.

VII. **López-Arjona, M.,** Mateo, S.V., Cerón, J.J., Martínez-Subiela, S. (2021). Changes in salivary oxytocin after stroking in dogs: Validation of two assays for its assessment. *Research in Veterinary Science*, 136, 527-534.

VIII. **López-Arjona, M.,** Tecles, F., Mateo, S.V., Contreras-Aguilar, M.D., Martínez-Miró, S., Cerón, J.J., Martínez-Subiela, S. (2021). A Procedure for Oxytocin Measurement in Hair of Pig: Analytical Validation and a Pilot Application. *Biology*, 10(6), 527.

IX. **López-Arjona, M.,** Tecles, F., Mateo, S.V., Contreras-Aguilar, M.D., Martínez-Miró, S., Cerón, J.J., Martínez-Subiela, S. (2020). Measurement of cortisol, cortisone and 11β-hydroxysteroid dehydrogenase type 2 activity in hair of sows during different phases of the reproductive cycle. *The Veterinary Journal*, 259, 105458.

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

I. **López-Arjona, M.,** Cerón, J.J., Martínez-Subiela, S. Oxytocin assays in saliva: an update.

II. **López-Arjona, M.,** Mateo, S.V., Contreras-Aguilar, M.D., Cerón, J.J., Martínez-Subiela, S. Validation of two new immunoassays for oxytocin measurements in human saliva.

# ABBREVIATIONS

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**11β-HSD:** 11β-hydroxysteroid dehydrogenase

**AI:** artificial insemination

**BChE:** butyrylcholinesterase

**BSA:** bovine serum albumin

**CFA:** complete Freund's adjuvant

**Cn/C:** cortisone/cortisol

**CV:** coefficient of variation

**EIA:** enzyme immunoassay

**ELISA:** enzyme-linked immunosorbent assay

**FBS:** fetal bovine serum

**HAT:** hypoxanthine, aminopterin and thymidine

**IFA:** incomplete Freund's adjuvant

**KLH:** keyhole limpet hemocyanin

**LD:** limit of detection

**LDH:** lactate dehydrogenase

**LLOQ:** low limit of quantification

**mAb:** monoclonal antibody

**MS:** mass spectometry

**pAb:** polyclonal antibody

**PBS:** phosphate buffered saline

**PEG:** polyethylene glycol

**r**: coefficient of correlation

**R/A:** reduction/alkylation

**RIA:** radioimmunoassay

**RT:** room temperature

**sAA:** salivary alpha-amylase

**TEA:** total esterase activity

# INTRODUCTION

# INTRODUCTION

The use of non-invasive samples for stress or welfare evaluation in veterinary medicine provides an advantage over other samples such as blood, whose collection causes a stressful situation for the animals (Merlot et al., 2011). Two examples of non-invasive samples are saliva (Cook, 2012; Escribano et al., 2012), and hair, which has the particularity of evaluating the stress response during a long-term period (Bacci et al., 2014; Stubsjøen et al., 2018).

In saliva and hair, there are two fields in which it could be of interest to advance in the knowledge:

**1)** In general, the biomarkers used in saliva for evaluation of stress in farm animals (Contreras-Aguilar et al., 2020), such as cortisol, are "negative" biomarkers in the sense that they detect situations of inadequate animal health and welfare (Martínez-Miró et al., 2016; Ruis et al., 2001). However, there is a lack of use of "positive" biomarkers, such as oxytocin, that could increase in situations of improvement in welfare (Rault, 2017). This hormone, which traditionally has been associated with labor and lactation (Argiolas & Gessa, 1991), is involved in positive social interactions in humans (Carter et al., 2007; Uvnas-Moberg, 1998) and animals (MacLean et al., 2017; Ogi et al., 2020).

In addition, the studies about oxytocin measurement in domestic species are limited. There is a lack of assays that allow direct measurement of oxytocin in both animal and human species and poor knowledge about the form of oxytocin present in the different type of samples and its possible binding to other molecules. In general, there are many divergences about the technique that should be employed for oxytocin measurement. Although the mass spectrometry (MS) is considered the gold standard, it only recognizes the free oxytocin molecule, however, the immunoassays could recognize the oxytocin bound to other molecules or other forms of oxytocin (MacLean et al., 2019). Also, there are controversies regarding the sample processing, since some authors perform the sample extraction (de Jong et al., 2015; Geva et al., 2020) or lyophilization and concentration (Fujioka et al., 2020; White-Traut et al., 2009), while others indicate that these procedures are not necessary (MacLean et al., 2018). Moreover, there are no previous studies about the oxytocin measurement in hair samples, which could evaluate changes in oxytocin concentrations in a long-term way.

**2)** Cortisol in hair is widely studied in many animal species (Accorsi et al., 2008; Davenport et al., 2006; Sauveroche et al., 2020) and can reflect the mean levels in serum in the last 1-2 months (Bacci et al., 2014; Prims et al., 2019). Moreover, the hair samples can be obtained in a non-invasive, painless, and easy way, in addition to the compound concentrations are not affected by stress generated by restraining or handling the animals during sampling (Koren et al., 2002). The 11β-hydroxysteroid dehydrogenase (11β-HSD) isoenzyme type 2 converts cortisol into cortisone in hair (Raul et al., 2004), and it is currently of growing interest in humans. However, although there are some studies about cortisol measurement in the hair of pigs for stress evaluation (Bacci et al., 2014; Prims et al., 2019), the cortisol to cortisone conversion, through the 11β-HSD isoenzyme type 2 activity, has not been evaluated in this species.

This PhD will provide advance in the knowledge in these two topics, by the investigation of new assays that can provide simple and accurate measurements of oxytocin in saliva, hair, and other samples and by the development of methods that could evaluate the 11β-HSD isoenzyme type 2 activity.

# OBJECTIVES

# OBJECTIVES

The objectives of this Ph.D. Thesis were focused on providing advances in oxytocin, cortisol, and cortisone measurement in saliva, seminal plasma or hair. For this purpose, the specific aims were:

- **Objective 1.** To develop and validate immunoassays for oxytocin determination in the saliva of pigs and its use for evaluation as a biomarker of positive welfare in different situations of stress and positive experiences, as well as the development of an immunoassay for oxytocin measurement in seminal plasma of pigs and its relationship with sperm quality and *in vivo* fertility. The results of this objective are published in the papers (indicated in the section *Articles*) nº 1-5. In addition, a review of the literature about assays used for oxytocin measurements in saliva was made along this Thesis, described in *Annex* (Experiment 1).

- **Objective 2**. To develop and validate immunoassays for oxytocin determination in the saliva of other species, such as canine and bovine species, and study the changes in stress or positive situations. This objective led to the published papers (indicated in the section *Articles*) nº 6 and nº 7. In addition, the development and validation of immunoassays for oxytocin measurement in human saliva after physical and psychological stress is described in *Annex* (Experiment 2).

- **Objective 3**. To develop and validate an immunoassay for oxytocin determination in the hair of pigs, representing the first time in which oxytocin is measured in hair, covered by the published paper nº 8, indicated in the section *Articles*.

- **Objective 4.** To develop and validate immunoassays for cortisol and cortisone determination as stress biomarkers in the hair of pigs, covered by the published paper nº 9 indicated in the section *Articles*.

# EXTENDED SUMMARY

# EXTENDED SUMMARY

## 1. General review

### 1.1 Oxytocin

Oxytocin is a neuropeptide hormone composed of nine amino acids with a chemical structure in which it is joined by disulfide bonds between cysteine residues (Carter et al., 2007; Kocyigit, 2017). Oxytocin is synthesized in the supraoptic and paraventricular nucleus of the hypothalamus (Hruby et al., 1990). It has some similarities with arginine vasopressin, another neuropeptide synthesized in the hypothalamus, which shares with oxytocin seven of nine amino acid sequence, differing only in two amino acids. However, although arginine vasopressin is structurally similar to oxytocin, several known effects of arginine vasopressin are opposite to oxytocin (Carter et al., 2007). Both are transported along with the neurophysins, which are their carrier proteins, to the posterior lobe of the pituitary gland, and then they are released to the blood (Hruby et al., 1990). From the pituitary gland, oxytocin pathways are distributed in many brain regions associated with the stress response (Condés-Lara et al., 1994). Moreover, oxytocin receptors have been found in some limbic structures, such as the bed nucleus of the stria terminalis, central nucleus of the amygdala, septum, and hippocampus (Krémarik et al., 1993).

Traditionally, oxytocin has been known for its physiological effects on muscle contraction associated with labor (uterine contraction) and lactation (milk ejection) (Russell & Leng, 1998). However, in the last years, its association with psychology and behavior has been increasing, like a biomarker of positive emotions since it increases in calming and relaxing situations (Yeates & Main, 2008). This hormone plays an important role in social bonding (Ross & Young, 2009) with both short-term and long-term effects (Winslow et al., 2003) and also in childbirth, lactation, and maternal-filial relationship (Carter & Altemus, 1997), being in general released during positive interaction situations (Uvnas-Moberg, 1998). Moreover, it improves social memory, social recognition, and attention (Insel, 2010), and it also has an anxiolytic and stress-reducing effect (Veening & Olivier, 2013). Therefore, contrarily to most biomarkers used for evaluating welfare, such as cortisol, catecholamines, or alpha-amylase that usually are associated with stress and negative situations, oxytocin has the particularity of being associated with positive experiences.

### *1.1.1 Oxytocin in saliva*

In the last years, oxytocin has been measured in saliva samples (Carter et al., 2007; MacLean et al., 2018; White-Traut et al., 2009). Saliva originates mainly from the parotid, submandibular and sublingual glands (Soares Nunes et al., 2015), being the secretion controlled by autonomic nerves (Proctor & Carpenter, 2007). This fluid has various advantages compared to serum (Soares Nunes et al., 2015). It can be obtained in a simple and minimally invasive way with few risks and under natural and laboratory conditions (Carter et al., 2007; Guzik et al., 2006). In addition, saliva collection eliminates various sources of stress that are likely to interfere with oxytocin system activity, since it does not require medical care, a laboratory setting, or interaction with the researcher.

Initially, some authors did not recommend oxytocin measurement in saliva because its concentrations were very low, and therefore it would be difficult to be detected (Horvat-Gordon et al., 2005). Therefore, this author concluded that oxytocin is not a valid salivary biomarker when measured by the available immunoassays at that time. By contrast, Carter *et al.* (2007) used an enzyme-immunoassay (EIA) and concentrated samples, finding biologically relevant changes in salivary oxytocin. Some years later, other authors measured oxytocin in saliva with sensitive assays (Grewen et al., 2010; Holt-Lunstad et al., 2011) and demonstrated that oxytocin concentrations in saliva could change in stressful situations. In this line, Grewen *et al.* (2010) found a positive correlation between plasma and salivary oxytocin levels, and Holt-Lunstad *et al.* (2011) found higher oxytocin levels in patients with depressive symptoms, both in plasma and saliva.

### *1.1.2 Reduction/alkylation (R/A)*

Recently, a R/A method has been developed for sample processing (Brandtzaeg et al., 2016). Oxytocin in the blood can bind to larger protein and also, there is a free fraction. Brandtzaeg *et al.* (2016) developed a R/A procedure that breaks the bonds between oxytocin and plasma proteins enabling the detection of total oxytocin, not only the oxytocin that is free. When they applied R/A to plasma samples, they obtained significant increases in detectable oxytocin of human plasma compared with non-treated plasma. In addition, R/A plasma samples yielded excellent linearity and parallelism measured with commercial enzyme-linked immunosorbent assay (ELISA) kits. They indicated that total oxytocin

might, in many cases, be better suited as a biomarker than the free fraction of oxytocin, although the biological activity of the bound fraction is less clear.

### *1.1.3 Extraction*

The purpose of sample extraction is to eliminate potentially interfering molecules and reduce sample matrix effects (Leng & Sabatier, 2016). In the particular case of oxytocin, with the sample extraction procedure, the oxytocin bound to proteins and other molecules are removed. Therefore, with this procedure, the free fraction of oxytocin is measured.

There are different methods for sample extraction of oxytocin. One of them is solid-phase extraction (SPE) that uses C18 Sep-Pak columns (Cool & DeBrosse, 2003; Szeto et al., 2011) or Oasis PRiME cartridges (MacLean et al., 2018).

There is disagreement in the literature over whether oxytocin concentrations measured in samples without extraction are correlated with those in extracted samples and how accurate the use of non-extracted samples is (McCullough et al., 2013). Although there have been arguments suggesting that extraction is not necessary (McCullough et al., 2013), others such as Leng and Sabatier (2016) and Christensen *et al.* (2014) recommended extraction. Non-extracted samples could contain substances tagged as oxytocin in assays but are not oxytocin (Leng & Sabatier, 2016), although this fact would depend on the specificity of the assay used to detect only oxytocin. Also, some authors described a high risk of matrix interference if samples are not extracted (Christensen et al., 2014), but also it would depend on the susceptibility of the assay to matrix interference.

Some studies have described a direct relationship between oxytocin concentrations determined in extracted and non-extracted samples (Hoge et al., 2012). In some species, such as dogs, the high correlation between the extracted and non-extracted saliva samples and the high spiking recoveries found in non-extracted saliva samples make the extraction procedure unnecessary (MacLean et al., 2018). It could indicate that probably if the assay is adequate, sample extraction is not necessary, and this would avoid some risks associated with the extraction procedure, such as measuring only the free fraction. This free fraction measurement can, in many cases, be no convenient, since the free oxytocin concentration can be changed by factors such as age or by compounds/drugs that displace oxytocin from proteins (Bryson, 1983). In addition, the lower oxytocin values that are obtained when

extracted samples are measured in saliva samples could be due to the elimination of interfering substances but also due to the loss of the oxytocin of the sample during the procedure (Horvat-Gordon et al., 2005) and in addition, analytical extraction is expensive and time-consuming.

### *1.1.4 Assays*

The main types of assays used for oxytocin measurements are radioimmunoassay (RIA), EIA, and MS. The RIA was the method that was first used to measure oxytocin (Chard et al., 1970), although it has some limitations, such as low sensitivity in general to detect low oxytocin concentrations (Cyranowski et al., 2008), the use of radioactive material, and the need for special conditions in the laboratory for its performance. The EIA methods have some advantages over RIA kits, such as the longer shelf life of the reagents, the lack of radioactive materials, and a wider detection range. In a recent study by MacLean *et al.* (2018), oxytocin concentrations in dog saliva using MS and ELISA techniques were determined, demonstrating a high correlation of oxytocin levels in the same samples analyzed by these two assays. However, the oxytocin concentrations detected by ELISA were much higher than those for MS. By contrast, EIA may recognize not only the primary form of the target analyte but also structurally related molecules, including precursor forms and biologically-related metabolites (MacLean et al., 2018).

## 1.2 Cortisol and cortisone in hair

Compared to other types of samples such as serum or saliva, the main characteristic of hair is that it can provide information on possible endogenous or exogenous exposure to compounds over a medium to a long period. It also has the advantages that analytes are not affected by circadian rhythms and the samples can be easily transported and stored and have good traceability and stability (Gow et al., 2010). Hair sampling is easy, non-invasive, painless, and the concentrations of compounds are not affected by the stress generated by holding or handling the animals during sampling (Koren et al., 2002).

Cortisol is a physiologically active steroid hormone and plays a key role in the response of the body to stressors (Plenis et al., 2011). Cortisone is also a steroid hormone and is considered the inactive form of cortisol. It has been shown that cortisol is converted to cortisone in various tissues, such as the salivary gland and hair through the action of the 11β-HSD isoenzyme type 2.

The measurement of cortisol in hair is considered a valid system for measuring long-term stress, useful both in humans (Russell et al., 2012) and animals (Comin et al., 2011; Davenport et al., 2006). In humans, although most studies assessed only hair cortisol, in the last years, there is a trend to analyze both cortisol and cortisone, as the ratio between the two hormones to assess the activity of the 11β-HSD isoenzyme and is related to the level of stress and various psychological alterations (Vogeser et al., 2003).

In veterinary medicine, most studies have focused mainly on non-human primates (Davenport et al., 2006) and cows (Comin et al., 2011). In pigs, Bacci *et al.* (2014) observed that cortisol levels in hair taken at 70-75 days after farrowing reflect the previous stress that the animals had been subjected to during parturition, maternity, and weaning period, so pig hair is a useful tool for obtaining information about cortisol concentrations measured over a medium to a long period. Prims *et al.* (2019) found higher hair cortisol levels in the stressed group than the control group of piglets. In case of cortisone measurement in hair, there are two published works in veterinary medicine, one in dogs (Ouschan et al., 2013), where its usefulness for the diagnosis of hyperadrenocorticism is determined and another in sheep where it is related to chronic stress (Stubsjøen et al., 2015), not having been performed the estimation of 11β-HSD activity in any case.

## *2. General methods*

### 2.1 Ethics considerations

All experimental animal procedures and the protection of animals used for scientific purposes were performed following the current Spanish and European legislation: 'Real Decreto 53/2013, de 1 de febrero', and Directive 2010/63/EU of the European Parliament and of the Council, of 22 September 2010. The experiments related to handling animals were approved by Bioethical Committee (Comité Ético de Experimentación Animal, CEEA), under the protocol numbers 235/2016 (porcine species), 171/2015 (bovine species), and 288/2017 (canine species). In addition, owner consent was obtained for the participation of all animals. The boars used for ejaculate and saliva collection complied with the Spanish and European guidelines for animal health and welfare and the marketing of seminal Artificial Insemination (AI)-doses (Spanish: ES300130640127, August 2006; European: ES13RS04P, July 2012).

In the human species, procedures related to ethical considerations were approved by the Ethics Commission (Comisión Ética de Investigación, CEI) of Murcia University, with protocol number 1394/2016. The participants in human studies signed an informed consent after they were informed about the experiment, sampling performs and objectives.

## 2.2 Monoclonal and polyclonal antibody production and purification

### *2.2.1 Monoclonal antibody against oxytocin*

The monoclonal antibody production was carried out in the "Área Científica y Técnica de Investigación" (ACTI) of University of Murcia (Murcia, Spain). A summary of this protocol is represented in Figure 1. The procedures carried out in the experimental animals were performed in the "Servicio de Experimentación Animal" (SEA), while the procedures after the sacrifice of the animals, based on tissue culture, were performed in the "Servicio de Cultivo de Tejidos" (SCT) of University of Murcia (Murcia, Spain).

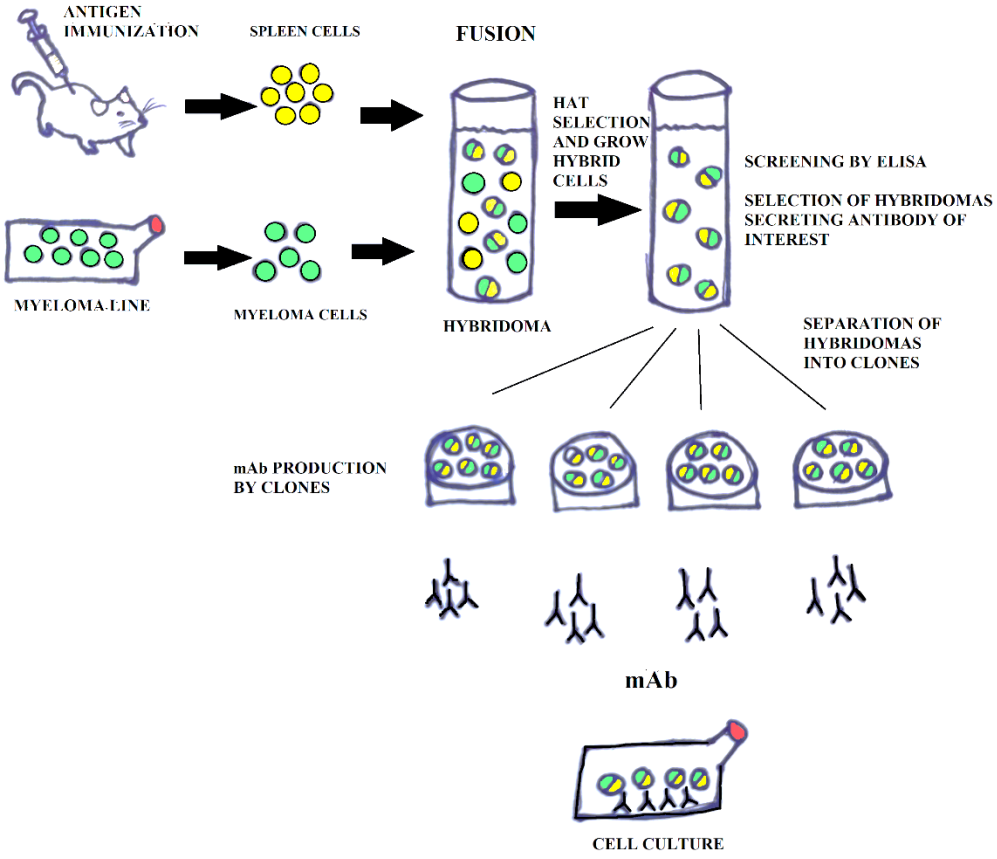


Figure 1. Summary of protocol for monoclonal antibody (mAb) production.

The standardized protocol used for monoclonal antibody production was described previously by Yokoyama (1999), as well as the follow-up of the guidelines of the Canadian Council on Animal Care (2002) (Clark et al., 2002). All the procedures were approved by Bioethical Committee (Comité Ético de Experimentación Animal, CEEA) and Ethics Commission (Comisión Ética de Investigación, CEI) of University of Murcia, following the European regulations on the monoclonal antibody production (ECVAM Workshop 23, 1997).

❖ Animals

The animals used for monoclonal antibody production were three BALB/c mice (three months old) (Figure 2). These were immunized with the antigen, which in this case was oxytocin conjugated to Keyhole limpet hemocyanin (KLH) (oxytocin-KLH, Cusabio, Houston, USA) because the oxytocin molecule alone would not generate a good immune response.

The immunization protocol consisted of a first injection intraperitoneally (Figure 2) per animal of 50 µg of oxytocin-KLH diluted in saline serum emulsified with Complete Freund's adjuvant (CFA) (Sigma Aldrich, St Louis, Mo, USA) and 14 days after the first immunization, a booster injection per animal administered intraperitoneally of 50 µg of oxytocin-KLH diluted in saline serum emulsified with Incomplete Freund's adjuvant (IFA) (Sigma Aldrich, St Louis, Mo, USA).

After ten days from the second immunization, the immune response against oxytocin of the three mice was evaluated. Blood was collected from the retro-orbital sinus (Figure 2) from each mouse, centrifuged at 3500 x *g* for 10 min to separate serum, and an ELISA screening (Figure 3) was performed. The following day, the mouse that resulted in the most potent immune response was intravenously tail vein immunized with 50 µg of oxytocin-KLH diluted in sterile Phosphate buffered saline (PBS) without adjuvant. Then, three days after the intravenous immunization, a procedure for fusing immune splenic B cells from mouse spleen with myeloma tumor cells to produce hybridomas was carried out. For this, the selected mouse was sacrificed by cervical dislocation in the SEA by qualified and trainer personal.

Figure 2. BALB/c mices used for monoclonal antibody production. Handling, intraperitoneally administration and blood extraction from the retro-orbital sinus.

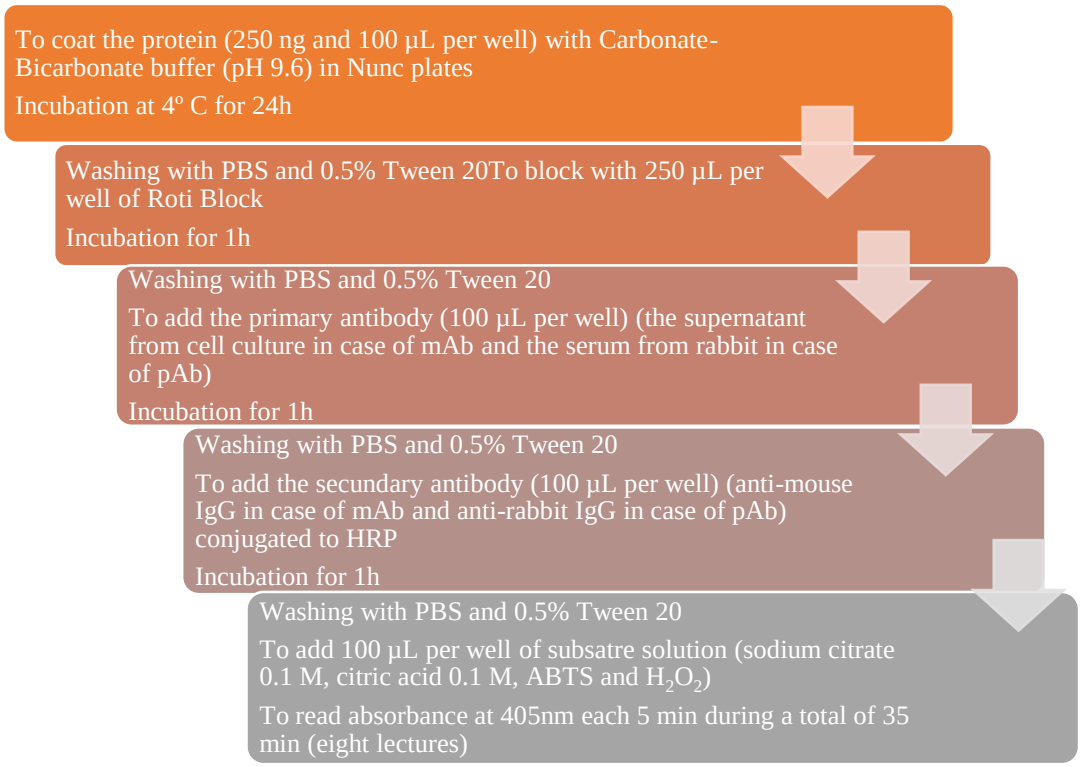


Figure 3. Protocol for ELISA screening (mAb: monoclonal antibody; pAb: polyclonal antibody).

❖ Cell fusion and selection of hybridomas

The cell fusion was performed between the immune splenic B cells from the spleen of the sacrificed mouse and the SP2/0-Ag14 myeloma cell line. Ten days before the fusion, the SP2/0-Ag14 myeloma cell line was thawed and prepared since it was stored in liquid nitrogen. These myeloma cells were plated into tissue culture plates with RPMI medium

supplemented with fetal bovine serum (FBS) (Thermo Fisher Scientific, Massachusetts, USA) glutamine and antibiotic until the conditions of concentration and growth were optimized for cell fusion. For the cells obtained from the spleen of mouse, three 75-$cm^2$ flasks with myeloma cells were necessary.

The cell fusion protocol was carried out in the SCT as follows:

1. The sacrificed mouse was placed in a biological safety cabinet level 2, the spleen was obtained by aseptic surgery and transferred to a sterile 100-mm-diameter petri dish filled with PBS and antibiotics for eliminating debris tissue (Figure 4).

Figure 4. Spleen extraction from sacrificed mice.

2. The spleen was placed in a Falcon 100 μm cell strainer, attached to a 50-mL conical centrifuge tube, and cells were dispersed and filtered with the plunger of a 2 mL syringe, filled with sterile RPMI medium.
3. The resulting suspension in the conical centrifuge tube was centrifuged at 200 x *g* for 5 min at room temperature (RT), the supernatant was discarded, 50 mL of RPMI medium were added and the cells were resuspended. This step was repeated two more times.
4. While spleen cells are being centrifuged and filtered, separately, the SP2/0-Ag14 myeloma cells were transferred to 50-mL conical centrifuge tubes, centrifuged at 200 x *g* for 5 min, and resuspended in RPMI medium. The myeloma cells were washed three times.
5. For fusion and hybridoma production, both SP2/0-Ag14 myeloma and splenic cells were counted using a Neubauer counting chamber and mixed at a 1:4 ratio in a 50-mL conical centrifuge tube. The mixed solution was centrifuged at 200 x *g* for 5 min, and the supernatant was discarded.
6. 1 mL 50% polyethylene glycol (PEG) was added to the mixed-cell pellet drop-by drop for 1 min, stirring the cells continuously. Then, the pellet was stirred for an additional minute and 7 mL RPMI at 37ºC were added to the cell mixture drop-by-drop for 3 min,

stirring continuously. Then, 14 mL RPMI were added over 3 min and centrifuged at 200 x *g* for 5 min, discarding the supernatant.

7. The corresponding volume of RPMI medium supplemented with glutamine, antibiotic, FBS and hypoxanthine, aminopterin and thymidine (HAT) was added at 1 mL of medium per 1.000.000 splenic cells.
8. In a 96-well flat-bottom plate, 100 µL of cell suspension were added to each well, and all the plates were incubated overnight in a humidified 37°C, 5% $CO_2$ incubator.

❖ Cloning, expansion and screening primary hybridoma supernatants

The day after the fusion procedure, each well was supplemented with 100 µL of the medium, and the hybridomas were monitored each day using an inverted microscope. The confluences of the wells were evaluated, and when most of the growing wells demonstrated 10-25% confluence, 14 days after the fusion, 100 µL from each well were collected for evaluating the antibody production by ELISA screening. Those hybridomas with a major antibody production against oxytocin were selected for cloning by limiting dilution technique. This technique consisted of diluting the hybridomas contained in the well-selected for incubation in a 96-well flat-bottom plate at approximately five viable cells per well. The ELISA screening for antibody evaluation and cloning were performed three times.

The expansion was performed when all clones were obtained from a single cell one and produced against oxytocin molecule evaluated by ELISA screening. For this, at first, the cells were transposed to 24-well microtiter plates when the growing hybridoma was 25-50% confluent, then they were transposed to 6-well plates, and when the growth rate was adequate, they were transposed to 25-$cm^2$ and 75- $cm^2$ flasks.

### *2.2.2 Polyclonal antibody against oxytocin*

A standardized protocol was carried out for polyclonal antibody production against oxytocin (University of California, 2014) in a New Zealand rabbit (female, 2.5 kg, 3-months old). The immunizing antigen used was oxytocin-KLH. For the first injection, 150 µg of oxytocin-KLH in saline serum were mixed with CFA and subcutaneously administered. For the second and successive injections (once a month), the oxytocin-KLH was mixed with IFA and subcutaneously administered. The blood samples were collected

from the marginal ear vein one week after each immunization, and for immune response evaluation, an ELISA screening was performed. The antibody purification was performed from each aliquot of serum after the blood centrifugation and frozen at -80ºC.

### *2.2.3 Polyclonal antibody against cortisone*

The protocol for polyclonal antibody against cortisone was carried out as the previous section 2.2.2 in other New Zealand rabbit (female, 2.5 kg, 3-months old), but the immunizing antigen was cortisone conjugated to bovine serum albumin (BSA) (cortisone-BSA, Cloud-Clone Corp., Wuhan, Hubei, China). The ELISA screening was performed for the immune response against cortisone, and antibodies were purified from serum.

### *2.2.4 Antibody purification*

❖ Monoclonal antibody purification

The supernatants from the cell culture of hybridomas obtained in section 2.2.1 were collected each week and stored at -80ºC. When the supernatant volume was enough, it was thawed and precipitated with ammonium sulfate (9.7 g per 25 mL of supernatant, 30 minutes under agitation, centrifuged at 4000 x *g* for 30 min, the supernatant is removed, and the precipitate is reconstituted with PBS) previous to antibody purification. After that, oxytocin monoclonal antibodies were purified with a HiTrap Protein G HP column (GE Healthcare Life Sciences, Munich, Germany) using a chromatography system (ÄKTA pure, GE Healthcare Life Sciences).

❖ Polyclonal antibody purification

The serum aliquots with polyclonal antibodies against oxytocin and cortisone were thawed, filtered using a 0.45 µm commercial filter (Millipore, Massachusetts, USA), and passed through a desalting column NAP-25 (NAP$^{TM}$-25 Columns Sephadex$^{TM}$ G-25 DNA Grade, GE Healthcare, UK) for medium change to PBS. In case of antibodies against cortisone, because of the structural similarity between cortisol and cortisone molecules, a previously described protocol was applied (Al-Dujaili et al., 2012), using a HiTrap NHS-activated HP affinity column (GE Healthcare Life Sciences, Munich, Germany) and batches of 1 mL of the cortisone antisera were passed through the column using a chromatography system (ÄKTA pure, GE Healthcare Life Sciences). In case of oxytocin antisera, the same protocol was used, but oxytocin-BSA (Cusabio, Houston, USA) was

coupled to the HiTrap NHS-activated HP affinity column, while in case of cortisone antisera, cortisone-KLH (Cloud-Clone Corp., Wuhan, Hubei, China) was coupled.

## 2.3 Assays development

The AlphaLISA® technology (PerkinElmer, Inc., Massachusetts, USA) was used to develop the immunoassays in this Thesis. The AlpahLISA is an amplified luminescent proximity homogenous assay that uses luminescent oxygen-channeling chemistry through two bead types, donor and acceptor. The binding of molecules captured on the beads leads to an energy transfer from one bead to the other, ultimately producing a luminescent/fluorescent signal. The AlphaLISA method used in this Thesis is based on the principle that, when a laser beam (680 nm) is shone on a donor bead, a single oxygen molecule is generated, producing a cascade of chemical events and excitation at an adjacent acceptor bead, resulting in a chemiluminescent emission (615 nm), which is detected and quantified.

For this Thesis, the formats used were a direct competitive assay for the monoclonal antibody against oxytocin and an indirect competitive assay for the polyclonal antibody against oxytocin, the polyclonal antibody against cortisone, and the commercial monoclonal antibody against cortisol. The difference between direct and indirect competitive assay is that the acceptor beads are directly coupled to the antibody in case of the direct assay, and the acceptor beads are not directly coupled to the antibody but they are coupled to protein G, and the antibody is captured on acceptor beads during an incubation step in case of the indirect assay. All AlphaLISA reagents were obtained from Perkin Elmer (Massachusetts, USA), and conjugation of the acceptor beads to the antibody was performed following manufacturer instructions. A graphic about direct and indirect competitive assays is shown in Figure 5.

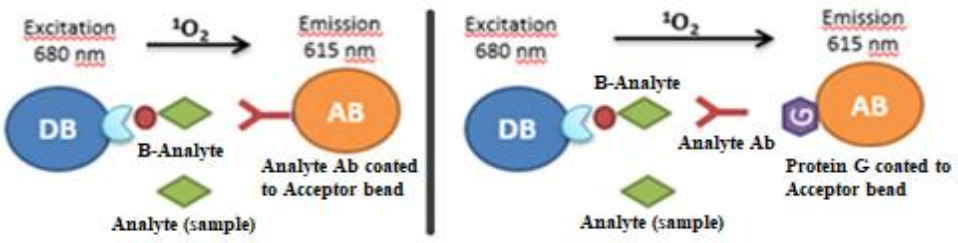


Figure 5. Direct (left) and indirect (right) competitive assays based on AlphaLISA technology (DB: donor beads; AB: acceptor beads; B-analyte: biotinylated analyte; analyte Ab: antibody against analyte).

In the development of the different assays in saliva, seminal plasma, and hair, the protocols were optimized for the amounts and concentrations of sample, acceptor beads coated to the antibody (20-40 µg/mL) (in case of direct assays), antibody (0-10 nM) and acceptor beads coated to protein G (20-40 µg/mL) (in case of indirect assays), biotinylated protein (0-20 nM) and donor beads (20-40 µg/mL). Each protein was labelled with the commercial biotin solution EZ-Link™ Micro Sulfo-NHS-Biotin, No-Weight™ Format (Thermo Scientific, USA). The optimization was carried out for oxytocin, cortisol and cortisone measurement in different type of samples, assay format or antibody (Table 1). The general steps of each format, adapting each one to the analyte, the species, and the type of sample, are shown in Figure 6.

| *ANALYTE* | *FORMAT* | *ANTIBODY* | *SAMPLE* | *SPECIES* |
|---|---|---|---|---|
| **Oxytocin** | Direct | Monoclonal | Saliva | Porcine |
| | | | | Bovine |
| | | | | Canine |
| | | | | Human |
| | | | Seminal plasma | Porcine |
| | | | Hair | Porcine |
| | Indirect | Polyclonal | Saliva | Porcine |
| | | | | Bovine |
| | | | | Canine |
| | | | | Human |
| **Cortisol** | Indirect | Monoclonal | Saliva | Porcine |
| | | | Hair | Porcine |
| **Cortisone** | Indirect | Polyclonal | Hair | Porcine |

Table 1. Different formats (direct or indirect) and antibodies (monoclonal or polyclonal) used for each type of sample, species and analyte in the development of AlphaLISA protocol.

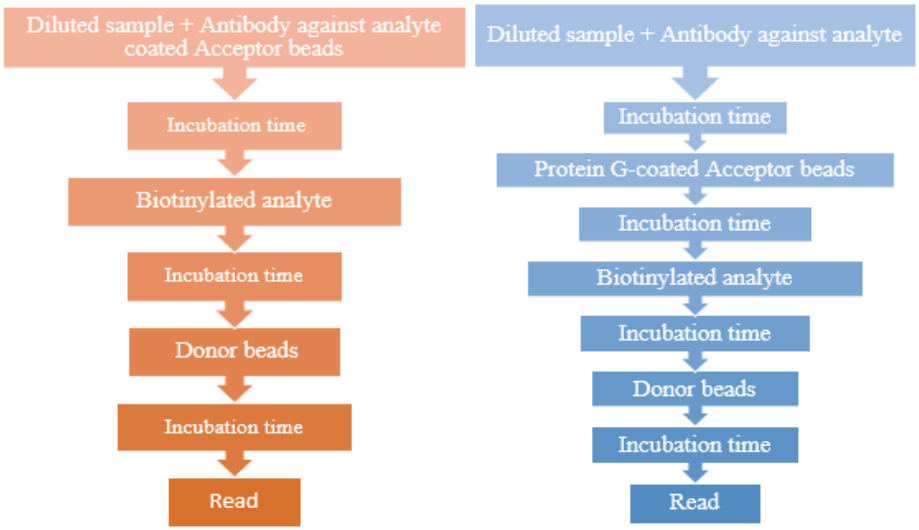


Figure 6. General AlphaLISA protocols for oxytocin, cortisol or cortisone measurement in saliva, seminal plasma or hair. Some modifications can be performed according to the analyte, species or type of sample. The direct competitive protocol is shown on the left and the indirect competitive protocol is shown on the right.

## 2.4 Saliva sampling and treatment

The saliva sampling procedure was carried out as follows in porcine, canine, and bovine species. The material used was a polypropylene sponge (Esponja Marina, La Griega E. Koronis, Madrid, Spain), which was cut into approximately 5 x 2 x 2 cm pieces and clipped to a flexible metal rod. The animals chewed the sponge during 1 min and each one was placed in a Salivette tube (Salivette, Sarstedt, Aktiengesellschaft & Co, Nümbrecht, Germany). These Salivette tubes were refrigerated until the arrival at the laboratory to be processed and centrifuged at 3000 x *g* for 10 min at 4 ºC. The collected volume from each animal was transferred in 1.5 mL Eppendorf tubes and stored at –80 ºC until analysis.

In human species, saliva samples were collected by passive flow for 1 min, using 5 mL standard micro-centrifuge polystyrene tubes. Samples were refrigerated until arrival at the laboratory. Then, the saliva samples were centrifuged (3500 x *g* for 10 min at 4 ºC), and the supernatant was transferred to 1.5 mL Eppendorf tubes. Saliva samples were stored at -80 ºC until analysis.

### *2.4.1. Extraction*

The procedure for oxytocin extraction from the saliva samples of pigs and humans was performed by SPE following the method described by MacLean *et al.* (2018) using the OasisPRiME cartridges (Waters Corporation, Massachusetts, USA). For SPE, saliva samples were diluted 1:2 with 0.1% trifluoroacetic acid (TFA), centrifuged at 4000 x *g* for 15 min, and collected the supernatant. Each cartridge was conditioned with 1 mL acetonitrile, followed by 1 mL of 0.1% TFA in $H_2O$ and then, each sample was loaded to each cartridge. Once the samples had flowed completely, cartridges were washed with 6 mL of 0.1% TFA in $H_2O$. Then, the samples were evaporated in a Centrifugal Vacuum Concentrator (Concentrator 5301, eppendorf, Hamburg, Germany), reconstituted with PBS, and frozen at -80ºC until assay.

### *2.4.2 R/A procedure*

The R/A procedure for oxytocin measurement was performed as Brandtzaeg *et al*. (2016) in saliva samples of pigs, cows, dogs, and humans. At first, 100 µL of each sample was diluted into 200 µL of 50 mM Tris-HCl (pH 8.0). Then, 5 µL of 0.5 M dithiothreitol (GE Healthcare Life Sciences, Munich, Germany) was added to the diluted saliva samples, mixed for 30 s, and incubated at 37ºC for 45 min, followed by cooling to RT (22ºC). After that, 15 µL of 0.5 M iodoacetamide (GE Healthcare Life Sciences, Munich, Germany) was added to each solution, mixed for 30 s, and incubated at 22ºC in the dark for 20 min. Then, ice-cold 80% acetonitrile in ultrapure water was added to the solution, mixed for 30 s, and centrifuged for 15 min at 4000 x *g*. After centrifugation, the supernatant was pipetted into a new 1.5 mL Eppendorf tube and was evaporated to dryness in a Centrifugal Vacuum Concentrator (Concentrator 5301, eppendorf, Hamburg, Germany) followed by reconstitution with PBS.

The treated samples were diluted at the respective proportion according to the species, with each buffer corresponding to each assay. The assay buffer was also processed using the R/A treatment and measured as an unknown sample to determine if the R/A procedure could affect the assays independently from the effect that it has on bound oxytocin.

## 2.5 Ejaculate collection and assessment of sperm function

Ejaculates from boars were collected using a semi-automatic collection method (Collectis®, IMV Technologies, L'Aigle, France), and the seminal plasma was harvested immediately after ejaculate collection. From each ejaculate, 5 mL of semen sample were centrifuged twice at RT at 1500 x *g* for 10 min. The seminal plasma was microscopically examined (Eclipse E400; Nikon, Tokyo, Japan) to check for sperm presence. The seminal plasma samples were stored in 3 mL-cryotubes at −80°C until the analysis.

The assessment of sperm function consisted of motility (the spermatozoa were microscopically analyzed, and a computer-assisted sperm analyzer was used), viability through the assessment of plasma and acrosomal membranes integrity (triple staining with Hoechst 33342, propidium iodide, and fluorescein-conjugated peanut agglutinin), intracellular generation of reactive oxygen species (Guthrie & Welch, 2006), and plasma membrane fluidity by a modified protocol (Koppers et al., 2008).

## 2.6 Hair sampling and cortisol and cortisone extraction from hair

The hair samples from pigs were cut manually with scissors, as close to the skin as possible, on the rump region, avoiding any contaminated areas. The samples were placed in small plastic bags, individually marked, and stored at RT until analysis. The hair samples from pigs were processed as Davenport *et al.* (2006) with some modifications. The hair of each pig was weighed (250 mg), placed in a polypropylene tube, and washed with isopropanol (5 mL) twice. During these washing steps, each tube was mixed at RT, centrifuged (1500 x *g*, 1 min), and the isopropanol discarded. The samples were dried at RT. Next, the fragments closest to the skin of hair samples were cut into small pieces, and 60 mg from each sample were weighed. The hair was placed in tubes with balls and pulverized to a fine powder in a homogenizer (Precellys Evolution homogeniser, Bertin Technologies, France). The pulverized hair was incubated with 1 mL of methanol for 18 h at RT with continuous gentle agitation. Samples were then centrifuged (2000 x *g*, 5 min), and 0.6 mL of each methanol extract was aliquoted into a new 1.5 mL Eppendorf tube. The samples were evaporated to dryness in a Centrifugal Vacuum Concentrator (Concentrator 5301, eppendorf, Hamburg, Germany) and the dry extracts were reconstituted with 0.1 mL of PBS and stored at –80ºC until analysis.

## 2.7 Analytical validation

For the analytical validation of the assays (Andreasson et al., 2015), the parameters of imprecision, accuracy, limit of detection (LD), and lower limit of quantification (LLOQ) were evaluated as:

- ***Imprecision.*** Inter- and intra-assay variations were calculated and expressed as coefficients of variation (CVs). For calculation of intra-assay precision of the method, five replicates of each sample (with low, medium, and high concentrations of the analyte) were analyzed simultaneously. To determine the inter-assay precision, five aliquots of each sample were stored in plastic vials at -80ºC, were thawed at RT each day, and measured in duplicate over five different days using freshly prepared calibration curves.
- ***Accuracy***. It was evaluated through the assessment of linearity under dilution and recovery experiments. For the linearity evaluation, two samples with high and medium values of the analyte were serially diluted from 1:2 to 1:128 with the assay buffer. For the recovery test, different amounts of analyte standard were added to a sample with a known concentration of the analyte, and the percentages of the measured concentrations to the expected concentrations were calculated.
- ***Sensitivity.*** The LD, defined as the lowest concentration of the analyte that could be differentiated from the zero value, was calculated from 12 replicate determinations of the zero standards (assay buffer) as mean value plus two standard deviations. For the LLOQ, which is the lowest amount of the analyte that can be determined with acceptable precision and accuracy, a sample was serially diluted with assay buffer, and each dilution was analyzed five times within the same run assay. The results were based on the lowest oxytocin concentration that could be measured with a precision of 20%.

# *3. Experimental design, results and discussion*

The experimental design, results, and discussion will be presented according to the different papers published from this Thesis.

## 3.1 Objective 1

The objective 1 was reflected by five studies corresponding to papers nº 1-5, in which new immunoassays were developed and validated for oxytocin measurement in the saliva and seminal plasma of pigs and the evaluation as a biomarker of positive welfare in different situations of stress and positive experiences, as well as its relationship with reproductive parameters. Moreover, a review about assays used for oxytocin measurements in saliva was made along with this Thesis, represented in experiment nº 1 (*Annex*).

### *3.1.1 Development and validation of a new immunoassay for oxytocin measurement in pig saliva samples with a monoclonal antibody (paper nº 1)*

❖ Aims and experimental design

For monoclonal antibody production, three BALB/c mice were immunized with Oxytocin-KLH conjugate as antigen. The assay developed consisted of a direct competitive assay based on AlphaLISA technology (Perkin Elmer, MA, USA), in which acceptor beads were coated to the monoclonal antibody against oxytocin developed.

Oxytocin concentrations were determined in the saliva of multiparous sows after farrowing collected on three days: the first day, 9th day, and 20th day after farrowing and during lactation. Moreover, saliva samples from 20 clinically healthy male pigs were divided into two aliquots; one of them for the measurement without extraction, whereas the other was measured after an extraction procedure described by MacLean *et al.* (2018).

❖ Results and discussion

*Analytical validation*. The developed assay had an intra- and inter-assay imprecision lower than 20% and low LD (112.9 pg/mL) and LLOQ (115.6 pg/mL). It showed good linearity in serially diluted saliva pools, with a linear regression equation mean of 0.97 and the average recovery was 104%.

*Influence of extraction procedure*. Non-extracted samples showed increased oxytocin concentrations (median: 1119.0 pg/mL) compared to extracted samples (median: 251.4 pg/mL). The correlation between samples with and without extraction was significant ($r = 0.79$; $P = 0.0003$). This high correlation, similar to described by MacLean *et al.* (2018) in dog saliva, could indicate that the extraction procedure is not necessary for saliva samples, so it is an advantage since the extraction procedure requires a high sample volume, toxic chemicals, technically difficult and a long process.

*Oxytocin concentrations at different times after farrowing*. Salivary oxytocin concentration was significantly increased at day 1 after farrowing (median: 1654.0 pg/mL) compared to day 9 (median: 1142.0 pg/mL) ($P < 0.01$), but there were no significant differences at day 20 after farrowing (median: 1477.0 pg/mL). These results were similar to the study of Okrasa *et al.* (1989), in which oxytocin concentrations in plasma of lactating sows increased at day 5 after farrowing, decreased at day 15, and increased again at day 25 after farrowing. This increase in oxytocin concentrations at the beginning of lactation could be due to the physiological suckling (Prevost et al., 2014).

### *3.1.2 Development and validation of a new immunoassay for oxytocin measurement with a polyclonal antibody and effect of R/A treatment in pig saliva samples for oxytocin measurement (paper nº 2)*

❖ Aims and experimental design

For polyclonal antibody production, a New Zealand rabbit was immunized with oxytocin-KLH as antigen. One week after each immunization, the blood samples were collected and an ELISA screening was performed to evaluate the antibody production. The assay with the polyclonal antibody was performed and validated with AlphaLISA technology, in which an indirect competitive assay was used.

Oxytocin concentrations were determined in the saliva of female pigs collected at three times (day 58 of gestation, day 7 after farrowing, and day 45 after farrowing) with the AlphaLISA polyclonal method developed and validated in this study, AlphaLISA monoclonal method developed and validated in paper nº 1 and a commercially available ELISA kit from Cayman Chemical (Ann Arbor, MI, USA).

The saliva samples were measured before and after the R/A procedure used by Brandtzaeg *et al.* (2016), which breaks the possible bonds to other molecules, with the three methods.

❖ Results and discussion

*Analytical validation*. The immunoassay with the polyclonal antibody showed intra-assay and inter-assay CVs lower than 15%. Dilution of saliva samples resulted in linear regression equations with a correlation coefficient between 0.98 and 0.99. The recovery results were between 80 and 116%, and the assay LD was 58.4 pg/mL.

*Effects of R/A treatment in saliva samples*. In case of the AlphaLISA monoclonal method, oxytocin concentrations showed no significant differences ($P = 0.0634$) between samples with (mean: 702.9 pg/mL) and without (mean: 882.1 pg/mL) the R/A procedure, so it could measure the oxytocin concentrations that would be liberated after R/A. Therefore, the R/A procedure does not significantly affect the oxytocin concentrations measured with this method and it would not be necessary to apply this procedure to the saliva samples.

When the AlphaLISA polyclonal method was used, samples with the R/A procedure (mean: 1308.0 pg/mL) were significantly lower ($P < 0.0001$) than those obtained without the R/A procedure (mean: 13492.0 pg/mL). Therefore, this method could detect oxytocin when it is bound to proteins and other structural-related forms or metabolites of oxytocin, due to the higher oxytocin concentrations obtained than the AlphaLISA monoclonal method and Cayman commercial kit.

Finally, with the Cayman kit, oxytocin concentrations were significantly higher ($P < 0.0001$) in samples with the R/A procedure (mean: 320.4 pg/mL) than those without the R/A procedure (mean: 11.8 pg/mL), although 24 saliva samples before R/A obtained low or undetectable values, so the high values obtained after the R/A could indicate that this kit can detect the oxytocin concentrations which are liberated from proteins after the R/A procedure (Brandtzaeg et al., 2016).

These results are shown in Figure 7.

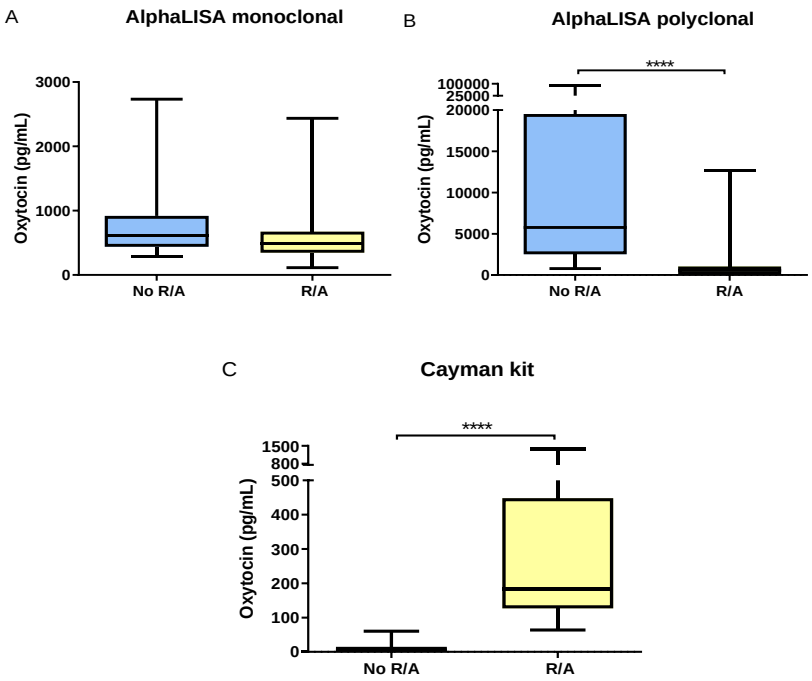


Figure 7. Salivary oxytocin concentrations without (No R/A) and with (R/A) R/A treatment obtained with AlphaLISA monoclonal method (A), AlphaLISA polyclonal method (B), and the Cayman kit (C). Asterisks indicate significant differences between with and without the R/A treatment. The plots show median (line within box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers).

*Biological response.* In case of the AlphaLISA monoclonal method without the R/A procedure, salivary oxytocin concentrations were significantly increased on day 7 after farrowing (mean: 1153.0 pg/mL) compared to day 58 of gestation (mean: 862.0 pg/mL) ($P = 0.0380$) and day 45 after farrowing (mean: 763.0 pg/mL) ($P = 0.0038$). When this method was applied with the R/A procedure, salivary oxytocin concentrations were significantly increased on day 7 after farrowing (mean: 701.4 pg/mL) compared to day 58 of gestation (mean: 524.1 pg/mL) ($P = 0.0159$) and day 45 after farrowing (mean: 552.1 pg/mL) ($P = 0.0138$). The magnitude of increase after farrowing was similar in samples with and without the R/A procedure, so this assay could not be affected by this procedure and it would not be necessary.

When the AlphaLISA polyclonal method was used without the R/A procedure, salivary oxytocin concentrations were significantly increased on day 7 after farrowing (mean: 25695.0 pg/mL) compared to day 58 of gestation (mean: 6896.0 pg/mL) ($P = 0.0007$) and day 45 after farrowing (mean: 5939.0 pg/mL) ($P = 0.0001$). When the R/A procedure was performed to saliva samples, oxytocin concentrations were significantly

increased on day 7 after farrowing (mean: 1677.0 pg/mL), compared to day 58 of gestation (mean: 534.8 pg/mL) (P = 0.0003) and day 45 after farrowing (mean: 906.5 pg/mL) (P = 0.0157). It showed a higher magnitude of increase on day 7 than the other assays, so this assay could be more sensitive detecting changes in oxytocin concentrations during the reproductive cycle in sows, as well as the detection of metabolites related to oxytocin that could increase during this period.

When the Cayman kit was used without the R/A procedure to the saliva samples, only 6 of the total samples analyzed were measured above the LD. In case of the Cayman kit with the R/A procedure, salivary oxytocin concentrations were significantly increased on day 7 after farrowing (mean: 448.6 pg/mL) compared to day 45 after farrowing (mean: 208.8 pg/mL) (P = 0.039), although there were no significant differences on day 58 of gestation (mean: 275.5 pg/mL) (P = 0.1999).

The biological response of oxytocin concentration with each assay is shown in Figure 8 and was similar with the three assays used, showing a significant increase on day 7 after farrowing, in agreement with studies in which oxytocin concentrations increase in plasma samples of pigs after parturition and during lactation (Forsling et al., 1979). Despite this, the magnitude of increase in oxytocin concentration depends on the assay used (Lefevre et al., 2017).

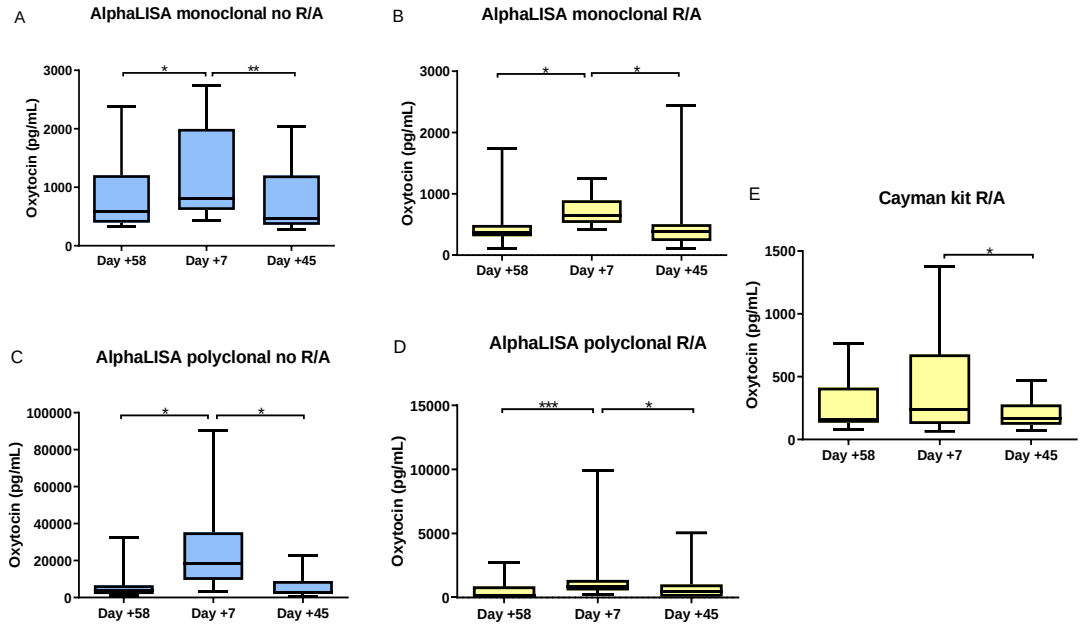


Figure 8. Salivary oxytocin concentrations at different times before and after farrowing: day 58 of gestation (Day +58), day 7 after farrowing (Day +7), and day 45 after farrowing (Day +45) with

AlphaLISA monoclonal method without (A) and with (B) R/A treatment, AlphaLISA polyclonal method without (C) and with (D) R/A treatment, and the Cayman kit with (E) R/A treatment. Asterisks indicate significant differences between days. The plots show median (line within box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers).

*Correlation between different methods*. When the R/A treatment was not performed on the samples, the AlphaLISA monoclonal method showed a significant moderate correlation with both the AlphaLISA polyclonal method ($r = 0.63$; $P < 0.001$) and the Cayman kit ($r = 0.51$; $P < 0.001$), but no significant correlation was found between the AlphaLISA polyclonal method and the Cayman kit. When the R/A treatment was performed, the AlphaLISA monoclonal method showed a significant moderate correlation with both the AlphaLISA polyclonal method ($r = 0.57$; $P < 0.001$) and the Cayman kit ($r = 0.53$; $P < 0.001$), but no significant correlation was found between the AlphaLISA polyclonal method and the Cayman kit. Within the same method, before and after the R/A treatment, the AlphaLISA monoclonal method showed a significant moderate correlation ($r = 0.65$; $P < 0.001$), the AlphaLISA polyclonal method showed a significant correlation ($r = 0.48$; $P < 0.001$) and the Cayman kit showed a significant low correlation ($r = 0.36$; $P < 0.05$).

### *3.1.3 Application of the oxytocin assays for stress and welfare evaluation in porcine species*

#### 3.1.3.1 Oxytocin changes in a positive situation (paper nº 3)

❖ Aims and experimental design

Saliva samples from 33 boars housed in a Spanish AI-center (AIM Ibérica; Topigs Norsvin Spain SLU, Calasparra, Murcia, Spain) were collected at three times: the day before ejaculate collection (DB), during the ejaculation time (T0), and two hours after ejaculate collection (T2), and oxytocin concentrations were measured with the AlphaLISA monoclonal and polyclonal method developed in papers nº 1 and nº 2, respectively. Moreover, differences in oxytocin concentrations were studied according to the age of boars (boars aged 12 to 24 months, aged 24 to 36 months, and aged more than 36 months) and the libido intensity (a three-point scale with a value of 1 for boars that showed little interest in the dummy sow and took more than 10 min to mount it; a value of 2 for the boars that did not show much interest but mounted it in less than 10 min; and a value of 3 for the boars that interacted with the dummy and quickly mounted it).

❖ Results and discussion

*Oxytocin concentrations with the AlphaLISA monoclonal method.* Salivary oxytocin concentrations were significantly higher ($P < 0.05$) at T0 (median: 1077.0 pg/mL) than at DB (median: 775.6 pg/mL), although there were no significant differences ($P > 0.05$) compared to T2 (median: 802.6 pg/mL). However, the boars did not show the same pattern of variation, since some boars showed increased oxytocin concentrations at T0 compared to DB (n = 21), while other boars showed decreased concentrations at T0 (n = 12). Oxytocin concentrations in boars of group that showed an increase at T0 were significantly higher ($P < 0.01$) at T0 (median: 1767.0 pg/mL) than at DB (median: 653.4 pg/mL) and T2 (median: 771.2 pg/mL). Oxytocin concentrations in the group that showed a decrease at T0 were lower ($P < 0.05$) at T0 (median: 574.8 pg/mL) than at DB (median: 1219.0 pg/mL) and T2 (median: 1022.0 pg/mL). These results are shown in Figure 9.

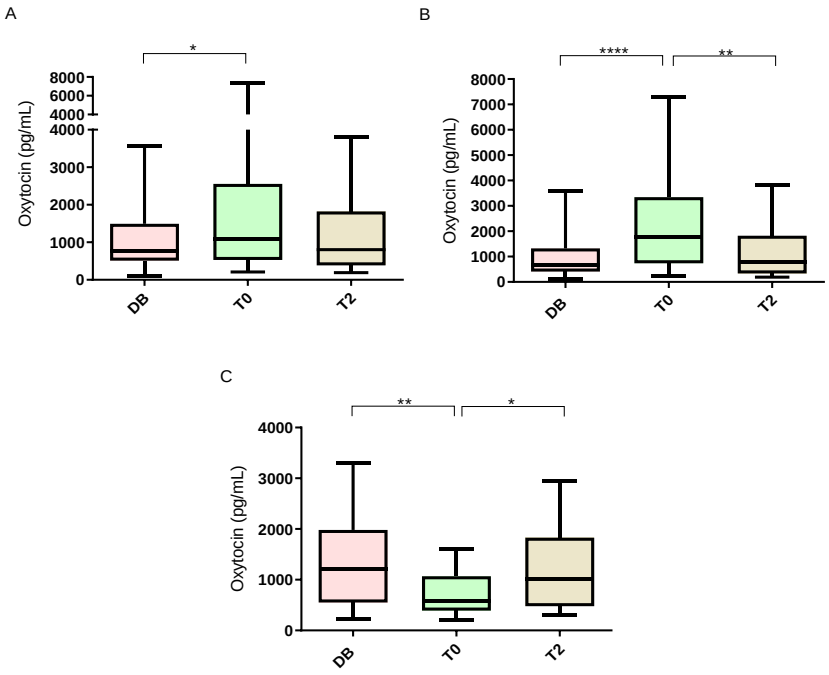


Figure 9. Salivary oxytocin concentrations measured with the AlphaLISA monoclonal method the day before ejaculate collection (DB), immediately after starting the ejaculation (T0) and two hours after ejaculation (T2) in all 33 boars (A), the 21 boars showing increased oxytocin concentration at ejaculation time (B) and the 12 boars that did not show increased oxytocin concentrations at ejaculation time (C). The plots show medians (line within box), 25th and 75th percentiles (boxes), min and max values (whiskers) and individual data points. Asterisks indicate significant differences between times.

The oxytocin concentrations were also compared according to the age and the libido of the boars. When the boars were classified by age, no significant differences were found

(P > 0.05), however, boars with a libido intensity of 3 had significant (P < 0.05) higher oxytocin concentrations at T0 (median: 1250.0 pg/mL) than at DB (median: 834.9 pg/mL).

Depending on the breed, 83.4% of Pietrain boars showed higher oxytocin concentrations at T0 than at DB (P < 0.01) and T2 (P < 0.05), although only 46.1% of Duroc boars showed higher oxytocin concentrations at T0 without significant differences. The two Landrace boars did not show significant changes.

*Oxytocin concentrations with AlphaLISA polyclonal method.* Salivary oxytocin concentrations were significantly higher (P < 0.05) at T0 (median: 25.8 ng/mL) and T2 (median: 20.3 ng/mL) than at DB time (median: 13.3 ng/mL). Regarding the different pattern of variation, depending on the increase or decrease at T0, oxytocin concentrations in the group that showed an increase at T0 (n = 20) were significantly higher (P < 0.05) at T0 (median: 57.2 ng/mL) and T2 (median: 28.4 ng/mL) than DB (median: 8.7 ng/mL). Oxytocin concentrations in the group that showed a decrease at T0 (n = 13) were significantly higher (P < 0.05) at DB (median: 13.8 ng/mL) and T2 (median: 15.1 ng/mL) than T0 (median: 6.9 ng/mL). These results are shown in Figure 10.

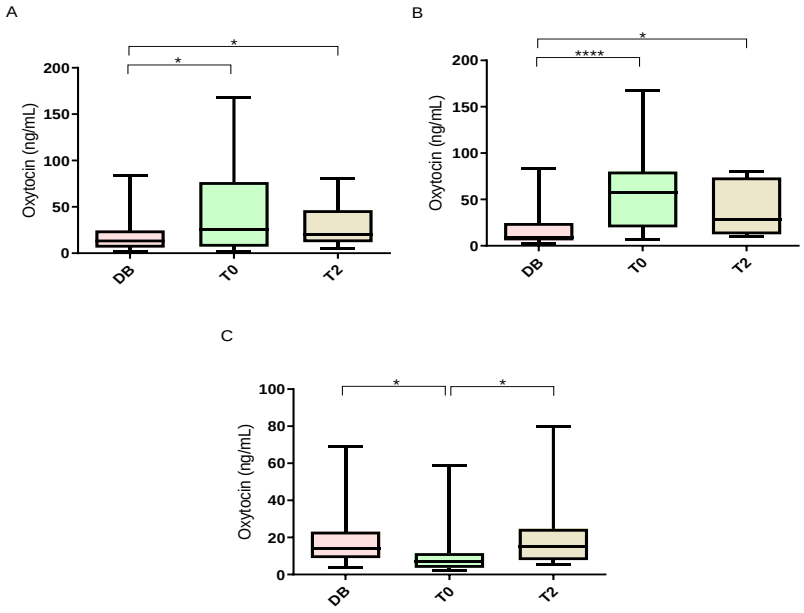


Figure 10. Salivary oxytocin concentrations measured with AlphaLISA polyclonal method the day before ejaculate collection (DB), immediately after starting the ejaculation (T0) and two hours after ejaculation (T2) in all 33 boars (A), the 20 boars that showed increased oxytocin concentrations at ejaculation time (B) and the 13 boars that did not show increased oxytocin concentrations at ejaculation time (C). The plots show medians (line within box), 25th and 75th percentiles (boxes), min and max values (whiskers) and individual data points. Asterisks indicate significant differences between times.

Although this increase could reflect its role in the ejaculation process (Thackare et al., 2006), as well as the modulation of sexual behavior (Ivell et al., 1997), and the control of male reproduction (Frayne & Nicholson, 1998), also, the oxytocin increase could be associated with positive emotions experienced by boars during ejaculation because of its association with social and physical feelings and emotions and sexual behavior (Ito et al., 2019) and the mechanisms mediating the well-being and anti-stress in positive social interactions in animals (Rault, 2017).

The polyclonal method detected changes of higher magnitude in salivary oxytocin concentrations than the monoclonal method, especially at two hours after ejaculate collection, so this could be related to the ability of polyclonal assays to detect different metabolites of the oxytocin molecule, as well as the oxytocin bound to other proteins that could remain two hours after ejaculation.

According to the age of boars, oxytocin concentrations in boars aged 12 to 24 months were significantly higher ($P < 0.05$) at T0 (median: 67.5 ng/mL) than at DB (median: 13.3 ng/mL). Moreover, oxytocin concentrations in boars aged 24 to 36 months were significantly higher ($P < 0.01$) at T2 (median: 32.9 ng/mL) than at DB (median: 13.9 ng/mL). It could be explained because those aged 18 to 24 months have a higher percentage of motile sperm than those aged more than 30 months, which also had higher percentages of sperm abnormalities (Jankevičiūtė & Žilinskas, 2002).

When the pigs were classified according to their libido, boars with a libido intensity of 3 showed significantly higher ($P < 0.05$) oxytocin concentrations at T0 (median: 25.8 ng/mL) and T2 (median: 24.5 ng/mL) than at DB (median: 9.8 ng/mL). Previous studies in humans found that blood oxytocin was positively related to the intensity of sexual behavior and couple interaction (Behnia et al., 2014).

Depending on the breed, 72.2% of Pietrain boars showed higher oxytocin concentrations at T0 than at DB ($P < 0.01$). However, 46.1% of Duroc boars showed an increase at T0 but without differences between DB and T0, although oxytocin concentrations at T2 were higher ($P < 0.05$) than at DB. The two Landrace boars did not show significant changes at different times. Genetic factors can influence the sexual behavior of boars at ejaculation (Oh et al., 2005). Most Pietrain boars showed increased oxytocin concentrations at ejaculation time, but Duroc boars did not show this increase, so that it could be due to the weaker libido of Duroc boars (Savić & Petrović, 2015) and the

lower total number of sperm and percentage of live sperm in the ejaculates compared to Pietrain boars (Kondracki, 2003).

### 3.1.3.2 Oxytocin changes in a stressful situation (paper nº 4)

❖ Aims and experimental design

The model used in this study was the stress evaluation after transport and during lairage at the slaughterhouse. For this, oxytocin concentrations were measured in saliva samples from 45 pigs, collected before the transport to the slaughterhouse (BT), at the time of arrival (T0), and 4 h after arrival to the slaughterhouse (T4). The methods used for oxytocin measurement were previously validated in papers nº 1 and nº 2 with monoclonal and polyclonal antibodies, respectively.

In addition, cortisol, salivary alpha-amylase (sAA), total esterase activity (TEA), butyrylcholinesterase (BChE), and lactate dehydrogenase (LDH), which are biomarkers associated with stress and pain in pigs, were measured and evaluated. For cortisol measurement, an AlphaLISA method used for cortisol measurement in pig hair in paper nº 9 was optimized and validated for saliva samples. SAA activity was measured by a colorimetric commercial kit (Alpha-Amylase, Beckman Coulter Inc.) (Fuentes et al., 2011), TEA was measured by a method based on the hydrolysis of the substrate 4-NA (Sigma-Aldrich) (Tecles et al., 2017), BChE was analyzed by an automated spectrophotometric assay (Tecles et al., 2016), and LDH was determined by a colorimetric commercial kit (Lactate Dehydrogenase, BioSystems S.A., Barcelona, Spain) (Escribano et al., 2019). These biomarkers, except cortisol, were assayed in the automated spectrophotometer Olympus AU400 (Olympus Diagnostica GmbH, Beckman Coulter, Ennis, Ireland).

❖ Results and discussion

*Oxytocin concentrations.* In case of the AlphaLISA monoclonal method, oxytocin concentrations decreased significantly at T4 (median: 1073.0 pg/mL) compared to BT (median: 1479.0 pg/mL) ($P = 0.0009$). Salivary concentrations measured with the AlphaLISA polyclonal method showed a significant decrease at T4 (median: 23.6 ng/mL) compared with BT (median: 40.8 ng/mL) ($P = 0.0304$). These results are shown in Figure 11. The oxytocin concentrations obtained with monoclonal and polyclonal method were significantly correlated ($r = 0.62$; $P < 0.0001$).

This decrease in oxytocin concentrations at 4 h after arrival to the slaughterhouse could indicate a decrease of positive feeling in pigs during the lairage at the slaughterhouse, that could be due to the new situation (Rioja-Lang et al., 2019) with various stressful stimuli such as the unloading process, mixing with unfamiliar pigs and stranger sounds (Goumon & Faucitano, 2017). Decreases in oxytocin concentrations during the lairage at the slaughterhouse were of higher magnitude when the polyclonal method was used than the monoclonal method, so this form of oxytocin could be more sensitive to these particular stressful conditions.

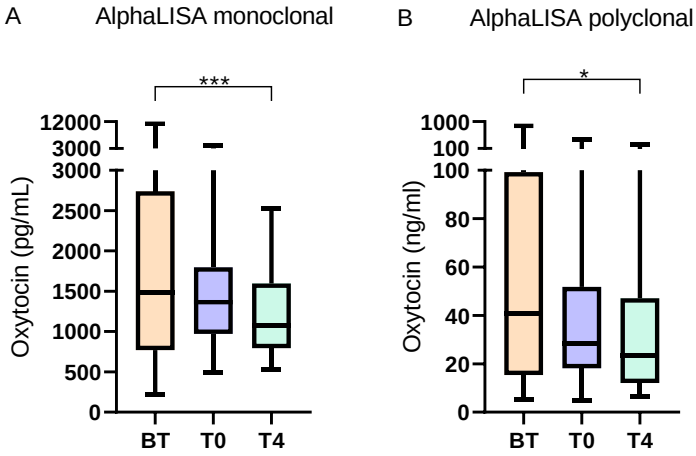


Figure 11. Salivary oxytocin concentrations at different times: before the transport (BT), at the time of arrival (T0) and 4 h after arrival to the slaughterhouse (T4) with AlphaLISA monoclonal (A) and polyclonal (B) method. The plots show median (line within box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers). Asterisks indicate significant differences between different times.

*Stress biomarkers.* Salivary concentrations of stress biomarkers measured in this study (cortisol, sAA, TEA, BChE, and LDH) showed a significant increase at T4 compared with T0 and BT ($P < 0.01$). Moreover, in case of cortisol, TEA and LDH, salivary concentrations showed a significant increase at T0 compared to BT ($P < 0.01$). These results are shown in Figure 12.

The pattern of variation of these stress biomarkers was opposed to oxytocin response since cortisol, sAA, TEA, LDH, and BChE showed an increase at 4 h of lairage, whereas oxytocin decreased. The increase in cortisol has been described after transportation and during the stay at the slaughterhouse (Escribano et al., 2012; Rey-Salgueiro et al., 2018). The increases found in the stress biomarkers in saliva at the slaughterhouse are agreed with previous studies that reported increases in these analytes in different stress

conditions (Contreras-Aguilar et al., 2018; Escribano et al., 2019; Tecles et al., 2016, 2017) and would support the concept that the pigs suffered stress in this situation.

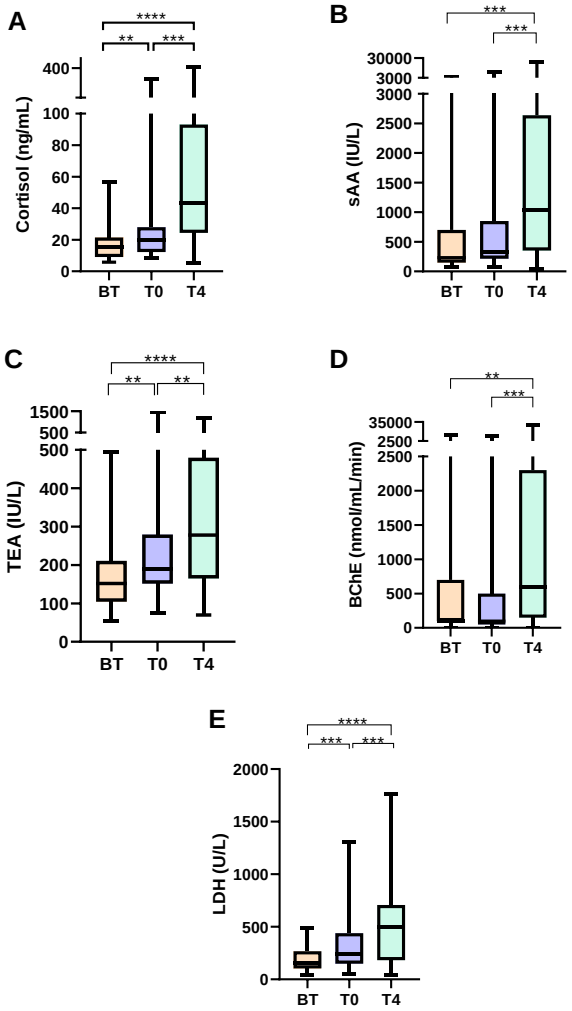


Figure 12. Cortisol (A), sAA (B), TEA (C), BChE (D) and LDH (E) concentrations at different times: before the transport (BT), at the time of arrival (T0) and 4 h after arrival to the slaughterhouse (T4). The plots show median (line within box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers). Asterisks indicate significant differences between different times.

### *3.1.4 Oxytocin measurement and its relationship with sperm quality and in vivo fertility (paper nº 5)*

❖ Aims and experimental design

This study aimed the evaluation of the relationship between concentrations of oxytocin (concentrations measured with an AlphaLISA method validated with a monoclonal antibody against oxytocin described in paper nº 1) in ejaculates from boars

used in AI programs and the sperm quality variables and *in vivo* fertility of their liquid-stored AI-semen. Seminal oxytocin concentrations were measured in 169 ejaculates from 61 boars housed in a Spanish AI-center (AIM Ibérica; Topigs Norsvin Spain SLU, Calasparra, Murcia, Spain). The parameters assessed were according to the ejaculate (ejaculate volume, sperm concentration, total sperm count) and the sperm (motility, viability, intracellular generation of reactive oxygen species, plasma membrane fluidity) at 0 h and 72 h in AI-semen samples stored at 17 ºC. The oxytocin concentrations in seminal plasma were evaluated according to the inter-breed, inter-boar, and intra-boar variability, and compared with boar ejaculate characteristics and *in vivo* fertility (percentage of farrowing sows over total inseminated and the total number of piglets born per litter).

❖ Results and discussion

*Validation of AlphaLISA method for oxytocin measurement in seminal plasma of pigs.* The intra-assay CV was below 7.5% and the inter-assay CV below 9.0%. The assay also showed high linearity under serial dilutions. The oxytocin concentrations were expressed in ng/mL.

*Oxytocin concentration in seminal plasma according to breed and boar variability.* There was not inter-breed variability, since the four breeds showing similar oxytocin concentrations (medians; Pietrain: 14.2 ng/mL; Duroc: 18.4 ng/mL; Landrace: 11.7 ng/mL; Large White: 20.9 ng/mL). There was significant inter-boar variability ($P < 0.001$) in oxytocin concentrations in seminal plasma from 3.4 to 47.9 ng/mL. Also, there was significant intra-boar variability ($P < 0.001$) in the concentration of oxytocin in seminal plasma between ejaculates from the same boar. According to these results, oxytocin can be measured in boar seminal plasma with AlphaLISA technology and is present at a higher concentration than men (Mostafa et al., 2015) and stallions (Watson et al., 1999), possibly because of the variations in oxytocin concentrations depending on the method used (Lefevre et al., 2017). The oxytocin concentrations in single seminal plasma samples varied between boars but not between breeds, such as other seminal plasma proteins (Barranco et al., 2020; Llavanera et al., 2020). Moreover, variability among individuals in seminal plasma oxytocin has also been reported in humans (Mostafa et al., 2015). One of the possible causes could be the libido, showing changes in salivary oxytocin concentrations depending on the libido of the boars in paper nº 3.

*Relationship between oxytocin concentration in seminal plasma and boar ejaculate characteristics.* The seminal plasma concentrations of oxytocin were correlated with ejaculate volume ($r = 0.46$; $P < 0.01$) and proportions of viable spermatozoa showing both high intracellular generation of reactive oxygen species ($r = -0.34$; $P < 0.05$) and high plasma membrane fluidity ($r = 0.35$; $P < 0.05$) in the samples analyzed at 0 h of liquid storage.

Semen samples were classified into two groups according to their oxytocin concentrations in seminal plasma, one of them with the highest oxytocin concentrations (27.8 - 61.0 ng/mL, n = 17) and the other with the lowest oxytocin concentrations (2.9 - 24.3 ng/mL, n = 19). According to the age, the ejaculates with the highest oxytocin concentrations ($P < 0.05$) came from younger boars (median: 16 months). Also, ejaculates with the highest oxytocin concentrations showed higher volume (median: 655 mL) than those with the lowest oxytocin concentrations (median: 608 mL) ($P < 0.05$). None of the assessed sperm parameters differed between the two groups, neither at 0 h nor at 72 h of storage at 17 ºC.

Oxytocin concentrations in seminal plasma of boars can be influenced by age, with the highest oxytocin concentrations in the youngest boars, agreed with those results in paper nº 3, with higher salivary oxytocin concentrations in the youngest boars and those reported by Elabd *et al.* (2014) in mice, that considered the oxytocin a hormone age-dependent in blood plasma. The positive relationship between oxytocin concentrations in seminal plasma and ejaculate volume could be due to its role in ejaculation (Maggi et al., 1987; Thackare et al., 2006). However, there was a lack of relationship between oxytocin concentrations and quality and sperm functionality parameters in semen samples, like previous studies in men (Goverde et al., 1998), although Mostafa *et al*. (2015) reported negative relationships of oxytocin concentrations in seminal plasma and total sperm count, motility and morphology in men.

*Relationship between seminal plasma oxytocin concentration and in vivo fertility.* The boars included in this section were classified into three sub-groups according to deviations concerning the breed means in farrowing rate: positive (from 1.81 to 7.54%, n = 6), without (from -0.74 to 1.44%, n = 7), and negative (from -1.53 to -2.79%, n = 5) deviation. Moreover, the boars were also classified in three sub-groups according to deviations concerning the breed means in total litter size: positive (from 0.22 to 0.83 piglets,

n = 6), without (from -0.15 to 0.11 piglets, n = 8), and negative (from -0.22 to -0.42 piglets, n = 4) deviation. Boars with positive farrowing rate deviation showed ejaculates with significantly higher ($P < 0.05$) oxytocin concentrations in seminal plasma (mean: 21.9 ng/mL) than boars with negative farrowing rate deviation (mean: 9.1 ng/mL). However, boars with positive and negative litter size deviation did not show significant differences in the oxytocin concentrations in seminal plasma (mean: 13.7 ng/mL vs. 12.8 ng/mL).

The positive relationship between oxytocin concentrations in seminal plasma and *in vivo* fertility could be supported by many studies that demonstrated the positive effects of supplementing semen AI-doses with oxytocin (Manjarín et al., 2019; Okazaki et al., 2014) since it activates endometrial and myometrial oxytocin receptors (Franczak et al., 2005), increasing the myometrial contractions and facilitating the passive transport of spermatozoa (Langendijk et al., 2003), and increasing the number of sperm in the utero-tubal junction reservoir, positively impacting fertility (Okazaki et al., 2014).

### *3.1.5 A review about oxytocin measurements (Experiment 1, Annex)*

The purpose of this review was to evaluate the advances and current status of the analytical techniques and sample management for the measurement of oxytocin in saliva. Despite the high interest that the measurement of oxytocin has gained in studies related to behavior and stress, its usually low concentration at different tissues and samples can represent a limitation for its use. For this reason, sensitive assays, large sample volumes, and even, on some occasions, a previous sample treatment is required for oxytocin quantification (White-Traut et al., 2009).

Oxytocin measurement in the saliva has been used in recent years (Carter et al., 2007). Although the oxytocin concentrations in saliva can be very low to be detected (Horvat-Gordon et al., 2005), Carter et al. (2007), using an EIA, detected reproducible changes in salivary oxytocin. Other authors measured oxytocin in saliva with sensitive assays (Grewen et al., 2010, Holt-Lunstad et al., 2011) and demonstrated that oxytocin concentrations in saliva could change in stressful situations. In animal species, dogs have higher values of oxytocin in saliva than reported in humans, although different values can be obtained depending on the assay used (MacLean et al., 2018).

Samples for oxytocin measurements can be analyzed without any processing or by doing a sample extraction and/or concentration. The purpose of sample extraction is to

eliminate potentially interfering molecules and reduce matrix effects (Leng and Sabatier, 2016). Due to the low oxytocin levels in human saliva, it has been described that for some methods, a sample concentration of at least 4-fold is necessary (Carter et al., 2007). In addition, recently, a R/A method has been developed for sample processing that broke the bond between oxytocin and plasma proteins enabling the detection of total oxytocin (Brandtzaeg et al., 2016)

This review offers an approach in methods used for oxytocin determination in saliva and the sample processing or not before the measurement.

## 3.2 Objective 2

The objective 2 was reflected by two studies corresponding to papers nº 6 and nº 7, and the experiment nº 2 (*Annex*), in which new immunoassays were developed and validated for oxytocin measurement in saliva of other species, such as canine, bovine and human species, as well as the study the changes in stress or positive situations.

### *3.2.1 Oxytocin as a marker for stress and welfare evaluation in bovine species (paper nº 6)*

❖ Aims and experimental design

Two AlphaLISA methods, with monoclonal antibody and polyclonal antibody, were developed and validated for oxytocin measurement in bovine saliva. The effect of the R/A procedure was evaluated in saliva from 20 cows. Moreover, changes in oxytocin concentrations were studied in two situations. One of them was evaluated in cows around the parturition, collecting saliva samples at three times: 7 days before the parturition (T-7), the day of parturition (T0), and 7 days after the parturition (T7). The other consisted of the saliva collection and evaluation of changes in oxytocin concentrations at three times in calves: 1 day before weaning (BW), 2 days after weaning (AW), and 4 days after calves were grouped (AG).

❖ Results and discussion

*Validation of AlphaLISA methods*. In case of the AlphaLISA monoclonal method, the intra-assay CV was lower than 9%, and the inter-assay CV was lower than 14%. The linear regression equation resulted in a correlation coefficient between 0.98 and 0.99. The recovery was between 93 and 120%. The assay LD and LLOQ were 7.1 and 56.8 pg/mL,

respectively. In case of the AlphaLISA polyclonal method, the intra-assay CV was lower than 16%, and the inter-assay CV was lower than 17%. The linear regression equation resulted in a correlation coefficient between 0.98 and 0.99. The recovery obtained was between 99 and 120%. The assay LD and LLOQ were 4.7 and 2.4 ng/mL, respectively. These assays can measure oxytocin concentrations in bovine saliva with good precision and accuracy, offering some advantages compared to ELISA kits, such as a lower sample volume and it is not necessary any washing step.

*Effect of R/A procedure*. In case of the AlphaLISA monoclonal method, no significant differences (P = 0.6237) were found between saliva samples with (median: 358.6 pg/mL) and without (median: 337.0 pg/mL) the R/A procedure. In case of the AlphaLISA polyclonal method, no differences (P = 0.0707) were found between saliva samples with (median: 75.8 ng/mL) and without (median: 62.9 ng/mL) the R/A procedure (Figure 13). The similar oxytocin concentrations obtained with the monoclonal method before and after the R/A procedure show that it could evaluate the oxytocin liberated after the R/A procedure, as well as the oxytocin measured before this treatment, while the polyclonal method, differently to pig species in paper nº 2, did not show significant changes before and after the R/A procedure, and could not measure the oxytocin bound to proteins or the  link cannot be broken by the R/A procedure in this species. The higher oxytocin concentrations detected by the polyclonal method could indicate that it measures other different forms of oxytocin, while the monoclonal method obtained a similar range of concentrations to a recent study in the saliva of cattle (Lürzel et al., 2020).

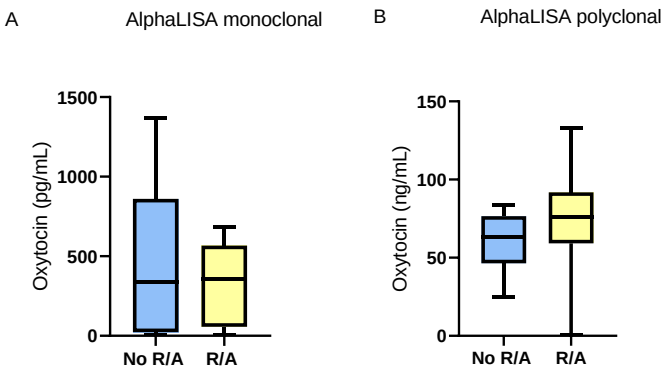


Figure 13. Oxytocin concentrations in saliva of cows before (No R/A) and after (R/A) the R/A treatment to the saliva samples with AlphaLISA monoclonal (A) and polyclonal (B) method. The plots show median (line within box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers).

The Spearman correlation showed a significant positive correlation between oxytocin concentrations with and without R/A procedure ($r = 0.873$; $P < 0.0001$) with the monoclonal method, while the polyclonal method showed a no significant correlation with and without R/A procedure ($r = 0.162$; $P = 0.495$). The monoclonal method without R/A procedure showed a significant positive correlation with polyclonal method without R/A procedure ($r = 0.565$; $P = 0.009$) and a no significant correlation with polyclonal method with R/A procedure ($r = 0.056$; $P = 0.821$).

*Oxytocin changes in the saliva of cows around parturition.* With the AlphaLISA monoclonal method, a significant increase in oxytocin concentrations was found on the day of parturition (median: 1105.0 pg/mL) compared to 7 days before (median: 69.1 pg/mL) and 7 days after (median: 7.1 pg/mL) ($P = 0.0375$ and $P = 0.0306$, respectively) the parturition. In case of the AlphaLISA polyclonal method, a significant increase was found on the day of parturition (median: 40.5 ng/mL) compared to 7 days after the parturition (median: 30.1 ng/mL) ($P = 0.0189$) (Figure 14). This increase in oxytocin concentrations on the day of parturition was higher with the monoclonal method than the polyclonal method. The increase on the day of parturition could be due to the involvement of oxytocin in the mechanism of parturition (Fuchs et al., 1992; Landgraf et al., 1983). However, the oxytocin concentrations were decreased in lactation because the oxytocin secreted at nursing could be lower than at parturition (Aurich et al., 1993).

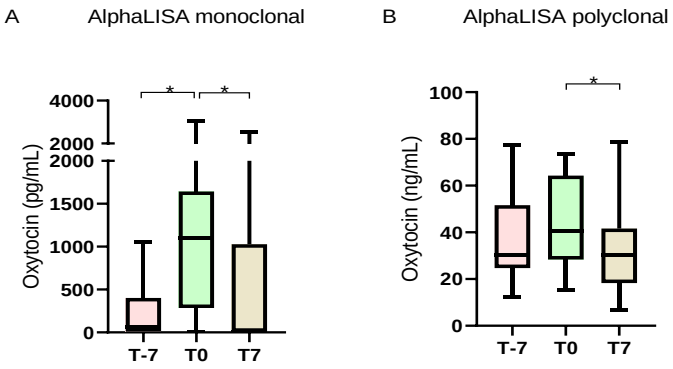


Figure 14. Oxytocin concentrations in saliva of cows at 7 days before the parturition (T-7), the day of parturition (T0) and 7 days after the parturition (T7) with AlphaLISA monoclonal (A) and polyclonal (B) method. The plots show median (line within box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers). Asterisks indicate significant differences between different times.

*Oxytocin changes in calves after weaning and grouping.* With the AlphaLISA monoclonal method, a significant increase in oxytocin concentrations was found at BW (median: 780.0 pg/mL) and AW (median: 795.0 pg/mL) compared to AG time (median: 18.6 pg/mL) ($P < 0.0001$). In case of the polyclonal method, a significant increase in oxytocin concentrations was found at BW (median: 107.2 ng/mL) and AW (median: 88.8 ng/mL) compared to AG time (median: 64.6 ng/mL) ($P = 0.0016$ and $P = 0.0068$, respectively) (Figure 15). The stressful situation suffered by the calves after grouping (Bøe & Færevik, 2003) is reflected in the decrease in oxytocin concentrations found in calves at this time, so oxytocin could be a marker of welfare in cows.

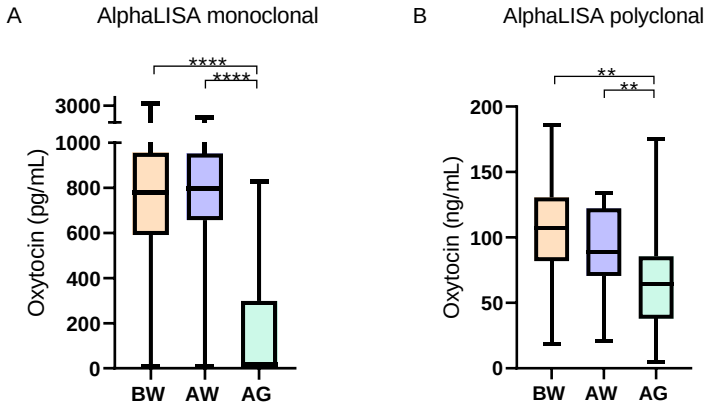


Figure 15. Oxytocin concentrations measured with monoclonal (A) and polyclonal (B) method in saliva of calves at 1 day before weaning (BW), 2 days after weaning or milk withdrawal (AW) and 4 days after grouping calves (AG). Plots show median (line within box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers). Asterisks indicate significant differences between times.

### *3.2.2 Oxytocin as a marker for stress and welfare evaluation in canine species (paper nº 7)*

❖ Aims and experimental design

This study aimed to develop and validate two new methods for oxytocin measurement in the saliva of dogs. For this, AlphaLISA technology was employed with a monoclonal antibody against oxytocin in one of the assays and a polyclonal antibody against oxytocin in the other assay. These assays were used for oxytocin measurement in the saliva of dogs before and after the R/A procedure and compared with a commercial ELISA kit from Cayman Chemical (Ann Arbor, MI, USA).

In addition, the AlphaLISA methods were used for detecting changes in oxytocin concentrations in the saliva from 17 dogs that were stroked by their owners and were sampled three times: a basal sample (TB), just after 10 min of affiliative interaction with their owners (T0) and 15 min after the end of affiliative interaction (T15). The dogs were divided into two groups based on a questionnaire answered by the owners, consisting of a Likert-type scale from 1 to 10 depending on the acceptance of the sponge and the response to the stroking.

❖ Results and discussion

*Validation of AlphaLISA methods*. When the monoclonal method was used, the intra-assay CV was lower than 8%, and the inter-assay CV was lower than 13%. The linear regression equation resulted in a correlation coefficient of 0.99. The recovery was between 92 and 118%. The assay LD and LLOQ were 28.3 and 87.7 pg/mL, respectively. When the polyclonal method was used, the intra-assay CV was lower than 15%, and the inter-assay CV was lower than 11%. The linear regression equation resulted in a correlation coefficient between 0.99. The recovery obtained was between 84 and 108%. The assay LD and LLOQ were 4.0 and 3.0 ng/mL, respectively.

These assays allow the measurement of oxytocin concentrations in the saliva of dogs without the need for extraction procedure (MacLean et al., 2018), which is recommended by other authors (Leng & Sabatier, 2016; Szeto et al., 2011), although the extraction procedure could discard oxytocin linked to others proteins (Brandtzaeg et al., 2016).

*Effect of R/A procedure*. Oxytocin concentrations showed no significant differences (P = 0.9680) between samples with (median: 303.3 pg/mL) and without the R/A procedure (median: 301.5 pg/mL) in case of the monoclonal method.

When the polyclonal method was used, oxytocin concentrations with the R/A procedure (median: 12.8 ng/mL) were significantly lower (P = 0.0143) than concentrations obtained without the R/A procedure (median: 25.2 ng/mL).

When the Cayman kit was used, oxytocin concentrations with the R/A procedure to the samples (median: 208.8 pg/mL) were significantly higher ($P < 0.0001$) than those obtained without the R/A procedure (median: 58.4 pg/mL) (Figure 16).

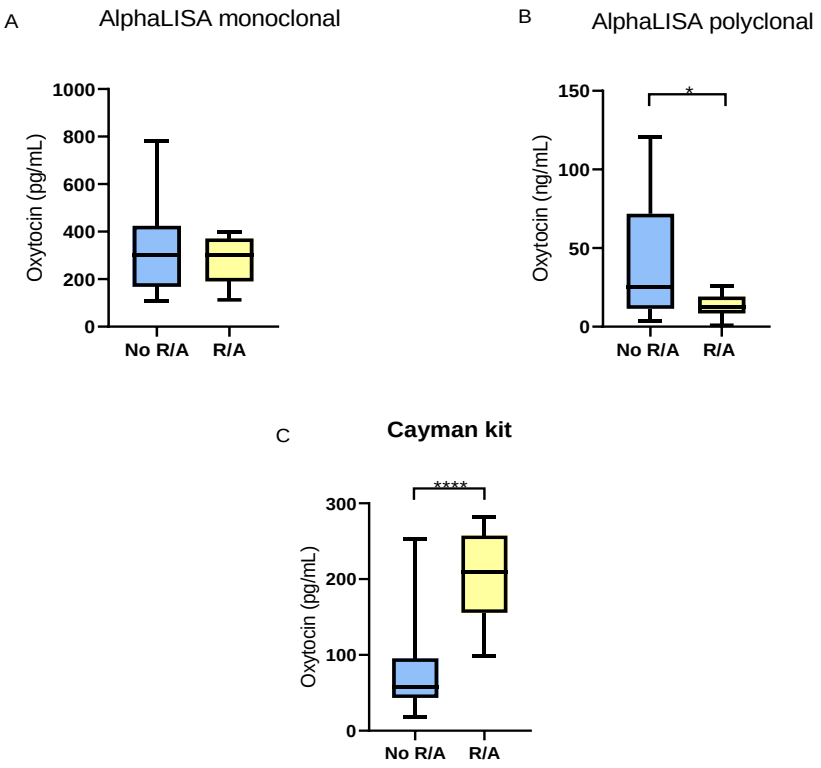


Figure 16. Oxytocin concentrations in saliva of dogs before (No R/A) and after (R/A) the R/A treatment to the saliva samples with AlphaLISA monoclonal method (A), AlphaLISA polyclonal method (B) and Cayman kit (C). The plots show median (line within box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers). Asterisks indicate significant differences between before and after the R/A treatment.

Regarding the correlations between methods with and without the R/A procedure, a significant positive correlation was found with the monoclonal method between concentrations with and without the R/A procedure ($r = 0.688$; $P = 0.001$), while the polyclonal method showed no significant positive correlation with and without R/A procedure ($r = 0.229$; $P = 0.322$). The monoclonal method without R/A procedure showed a significant positive correlation with polyclonal method with and without R/A procedure ($r = 0.539$; $P = 0.017$ and $r = 0.647$; $P = 0.003$, respectively). The monoclonal method with R/A procedure showed a significant positive correlation with polyclonal method with and without R/A procedure ($r = 0.626$; $P = 0.004$ and $r = 0.489$; $P = 0.033$, respectively). In case of the Cayman kit, a significant positive correlation was found between concentrations with and without the R/A procedure ($r = 0.507$; $P = 0.023$), while the correlations with the monoclonal method and polyclonal method were no significant ($P > 0.05$).

These changes in oxytocin concentrations with the two assays before and after the R/A treatment were similar to those obtained in pigs (paper nº 2), with no significant differences before and after the R/A procedure with the monoclonal method (it could detect oxytocin concentrations liberated after the R/A) and a significant decrease in oxytocin concentrations after the R/A procedure with the polyclonal method and higher oxytocin concentration than the monoclonal, so this method would not measure the oxytocin liberated by the R/A procedure, in addition to the measurement of other forms of oxytocin or metabolites (MacLean et al., 2019). The different affinities for the oxytocin molecule depending on the antibody can explain these divergences and the poor correlations with the commercial kit.

*Changes in oxytocin concentrations after stroking.* The dogs were separated into two groups based on a questionnaire by the owners: group 1 (n = 8) (the dogs chewed the sponge without trying to remove and the stroking induced a good and relaxing response on them detecting by the owners) and group 2 (n = 9) (the dogs had not a good acceptance to the sponge and/or the stroking did not induce a relaxing response on them). When oxytocin concentrations were measured with the monoclonal method, in case of the group 1, a significant increase was found after stroking at T0 (median: 607.7 pg/mL) (P = 0.0041) and T15 (median: 904.3 pg/mL) (P = 0.0079) compared to TB (median: 335.5 pg/mL), while in the group 2, higher significant concentrations were found at TB (median: 993.4 pg/mL) compared to T0 (median: 582.4 pg/mL) (P = 0.0398) and T15 (median: 452.7 pg/mL) (P = 0.0236). No significant changes in oxytocin concentrations were found between the sampling times when the polyclonal method was used (Figure 17).

These higher oxytocin concentrations after stroking with the monoclonal method in dogs that had a positive response to the stroking and also a good acceptance of the sponge could indicate its relation to trigger positive emotions in the dog, like previous changes in oxytocin concentration in dogs after an affiliative situation (MacLean et al., 2017; Ogi et al., 2020). Dogs that showed increased oxytocin concentration after stroking had lower initial oxytocin values than those that did not increase their concentration, so it could indicate a stronger need for interaction to increase their oxytocin concentrations, similar to previous studies in humans (Petersson et al., 2017).

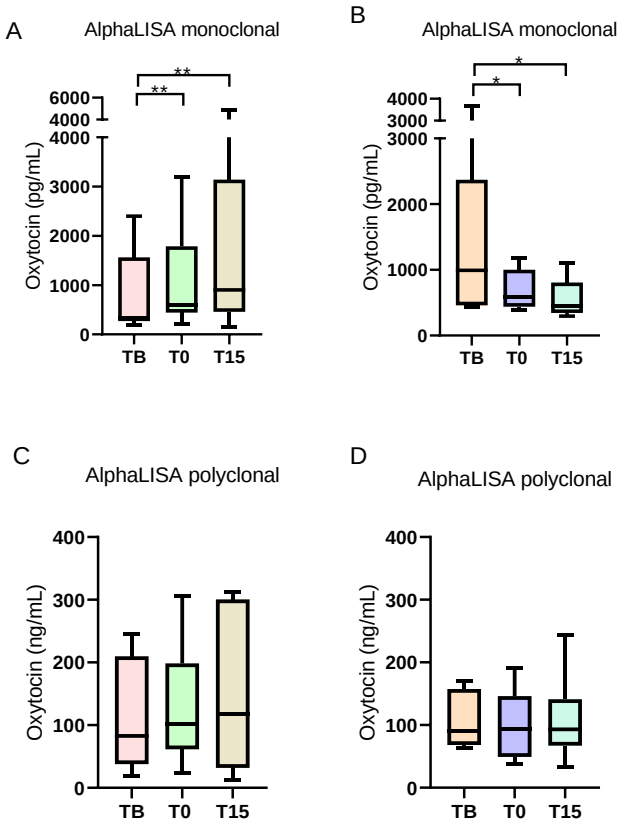


Figure 17. Oxytocin concentrations in saliva of dogs at a basal time (TB), after 10 min (T0) of affiliative interaction with their owners and 15 min (T15) after the end of affiliative interaction with AlphaLISA monoclonal method in group 1 (A) and group 2 (B) and AlphaLISA polyclonal method in group 1 (C) and group 2 (D). The plots show median (line within box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers). Asterisks indicate significant differences between times.

### *3.2.3 Oxytocin as a marker for stress and welfare evaluation in humans (Experiment 2, Annex)*

❖ Aims and experimental design

The purpose of this study was to validate two new assays for oxytocin measurement in human saliva, one using a monoclonal and the second using a polyclonal antibody, and the effect of extraction and R/A treatments to saliva samples were evaluated in both methods and compared with a commercial kit (Cayman Chemical, Ann Arbor, MI, USA). In addition, to evaluate the ability of these assays to detect physiological changes in oxytocin, these assays were applied to two situations that have previously been described to produce an increase in salivary oxytocin (de Jong et al., 2015): a physical effort (CrossFit) and induced psychological stress, such as the Trier Social Stress Test (TSST). Saliva samples of the physical effort model were obtained 5 min before the exercise (TBe),

after completion of the exercise (T+0e), and 10 min after the exercise (T+10e). In case of the psychological stress model, the saliva samples were collected in the isolation room 5 min before the interview (TBs), just after the arithmetic task (that lasted 10 min) (T+0s), and 15 min later (T+15s).

❖ Results and discussion

*Validation of AlphaLISA methods.* The immunoassay with the monoclonal antibody showed intra-assay CV lower than 13% and inter-assay CV lower than 16%. The linear regression equation resulted in a correlation coefficient between 0.96 and 0.97. The results of recovery obtained were between 81 and 120%. The assay LD was 54.8 pg/mL, and the LLOQ was 72.5 pg/mL. The immunoassay with the polyclonal antibody showed intra-assay CV lower than 12% and inter-assay CV lower than 15%. The linear regression equation resulted in a correlation coefficient between 0.96 and 0.99. The results of recovery obtained were between 91 and 120%. The assay LD was 0.5 ng/mL, and the LLOQ was 11.5 ng/mL. These methods were precise and accurate with the advantages of using a lower sample volume than most of the EIA assays currently commercially available and the lack of washing steps. In addition, these methods were sensitive enough to detect oxytocin in saliva without the need for lyophilization, which is need for some assays that are not sensitive enough to detect oxytocin in saliva (White-Traut et al., 2009; Fujioka et al., 2020).

*Effect of extraction and R/A procedure.* In case of AlphaLISA monoclonal method, the oxytocin concentrations obtained in samples without treatment (median: 965.4 pg/mL) were significantly higher than those with extraction (median: 175.2 pg/mL) ($P = 0.0384$). The concentrations obtained in the samples without treatment were higher but did not show significant differences with the values obtained with the R/A procedure (median: 326.0 pg/mL) ($P = 0.3293$).

In case of AlphaLISA polyclonal method, the oxytocin concentrations obtained in samples without treatment (median: 20.3 ng/mL) were significantly higher than those with extraction (median: 1.6 ng/mL) ($P = 0.0005$). The R/A treatment to the samples produced a significant decrease in oxytocin concentrations (median: 6.6 ng/mL) ($P = 0.0445$).

In case of Cayman kit, the oxytocin concentrations obtained in samples without treatment (median: 18.6 pg/mL) were significantly lower than those with extraction (median: 40.6 pg/mL) ($P = 0.0084$). The R/A treatment to the samples showed a significant

increase in oxytocin values (median: 97.7 pg/mL) compared to samples without treatment ($P < 0.0001$) (Figure 18).

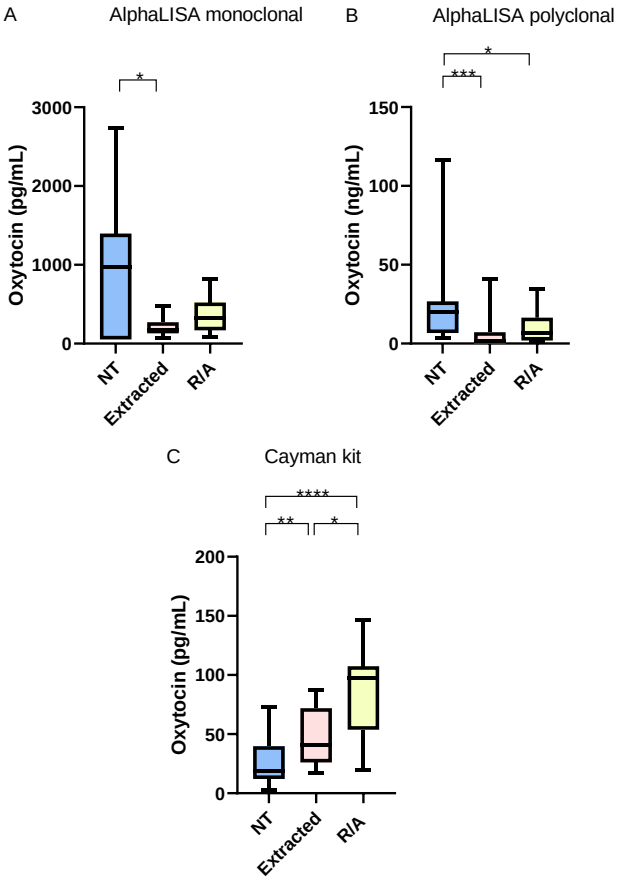


Figure 18. Oxytocin concentrations with AlphaLISA monoclonal (A), polyclonal (B) method and Cayman kit (C) in saliva samples of humans without previous treatment (NT), after extraction (Extracted) and after R/A treatment (R/A). Plots show median (line within box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers). Asterisks indicate significant differences between treatments to the samples.

Within each method, the Spearman correlation showed a positive significant correlation in AlphaLISA monoclonal method between samples without treatment and with extraction ($r = 0.697$; $P = 0.031$) or with R/A treatment ($r = 0.503$; $P = 0.024$). However, with the other methods, only the polyclonal method showed a significant correlation between samples without treatment and R/A ($r = 0.442$; $P = 0.039$). When the methods were compared without sample treatment, significant positive correlations were found between monoclonal method and polyclonal method ($r = 0.688$; $P = 0.001$), between monoclonal method and Cayman kit ($r = 0.633$; $P = 0.046$) and between polyclonal method and Cayman kit ($r = 0.700$; $P = 0.043$).

Both developed assays (monoclonal and polyclonal) showed a different behavior when the R/A procedure was applied to the samples, so that it could indicate a different affinity of the two antibodies for the oxytocin molecule and/or that each antibody recognizes different oxytocin forms. However, the correlation between monoclonal and polyclonal method without sample treatment was positive and significant, similar to previous studies in porcine (paper nº 2), bovine (paper nº 6), and canine (paper nº 7) species. The polyclonal method could have more affinity for the oxytocin bound to proteins since it showed a significant decrease after the R/A procedure, while the monoclonal method did not show differences. Similar results were obtained in porcine and canine species (paper nº 2 and nº 7, respectively). Regarding the commercial kit, the decrease in oxytocin concentrations when the R/A procedure is performed could indicate that it measures the oxytocin liberated by the R/A procedure.

In case of the monoclonal assay, the values before and after the extraction procedure were correlated, as previously described in the saliva of pigs (paper nº 1) and dogs (MacLean et al., 2018), whereas with the polyclonal assay, there was no correlation between extracted and non-extracted samples, a fact which has been reported with a different assay in human serum (Szeto et al., 2011). It could be due to the possible loss of oxytocin bound to proteins or other forms of oxytocin that could be eliminated during the extraction procedure.

*Oxytocin changes after physical effort and psychological stress.* These results are shown in Figure 19. When AlphaLISA monoclonal method was applied to the samples from the physical effort model, oxytocin concentrations showed a significant increase at T+0e (median: 4664.0 pg/mL) compared to TBe (median: 1184.0 pg/mL) (P = 0.0406) and T+10e (median: 1469.0 pg/mL) (P = 0.0469). When the polyclonal method was applied, a significant increase in oxytocin concentrations was detected at T+0e (median: 84.8 ng/mL) compared to TBe (median: 70.4 ng/mL) (P = 0.0448) and T+10e (median: 52.8 ng/mL) (P = 0.0029). These changes in salivary oxytocin concentrations found in the physical effort model are according to previous studies that measured oxytocin in saliva after physical efforts (de Jong et al., 2015).

The results obtained with AlphaLISA monoclonal method in saliva samples from the psychological stress model showed a significant increase in oxytocin concentrations at T+0s (median: 1103.0 pg/mL) compared to TBs (median: 492.8 pg/mL) (P = 0.0200) but

did not show significant differences with T+15s (median: 718.8 pg/mL) (P = 0.3927). The results obtained with AlphaLISA polyclonal method showed a significant increase at T+0s (median: 64.1 ng/mL) compared to T+15s (median: 49.5 ng/mL) (P = 0.0310) but did not show significant differences with TBs (median: 53.4 ng/mL) (P = 0.3279). These changes in oxytocin in saliva after the psychological stress are also in agreement with the previous studies (Jong et al., 2015).

The magnitude of increase was higher in case of the monoclonal method, so it would be recommended to evaluate oxytocin changes in these situations since it appears to be more sensitive than the polyclonal assay.

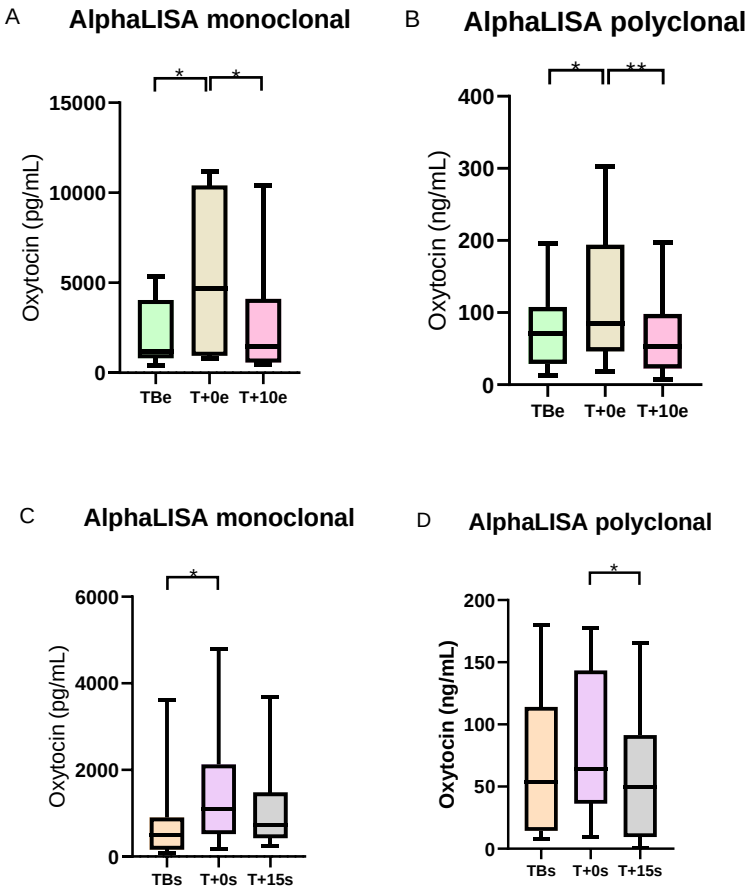


Figure 19. Oxytocin concentrations in saliva samples of the participants during the physical effort and the psychological stress. The oxytocin concentrations were measured 5 min before the exercise (TBe), after completion of the exercise (T+0e), and 10 min after the exercise (T+10e) with AlphaLISA monoclonal (A) and polyclonal (B) method, and 5 min before the interview (TBs), just after the arithmetic task (T+0s), and 15 min later (T+15s) with AlphaLISA monoclonal (C) and polyclonal (D) method. Plots show median (line within box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers). Asterisks indicate significant differences between different times.

## 3.3 Objective 3

The objective 3 was reflected by one study corresponding to paper nº 8, in which a new immunoassay was developed and validated for oxytocin measurement in the hair of pigs.

### *3.3.1 Oxytocin measurement in the hair of pigs (paper nº 8)*

❖ Aims and experimental design

For oxytocin measurement in the hair of pigs, the samples were collected from 20 sows in two reproductive cycles: 10 of them were sampled from April to September 2018 (spring-summer period: S–S), and the other 10 sows were samples from January to June 2019 (winter-spring period: W–S). The sampling times in each reproductive cycle were 5 days before expected farrowing (-5), at 23 after farrowing (23), and 59 days after farrowing (59). Two methods for oxytocin extraction from hair were tested: methanol and acetonitrile, adding a standard solution of purified oxytocin to four different pools of hair samples after the pulverization. For oxytocin measurement in the extract of hair, a new immunoassay based on AlphaLISA technology was developed and validated with a monoclonal antibody against oxytocin (paper nº 1).

The correlation between oxytocin concentrations in hair of sows with concentrations of cortisol, cortisone, and 11β-HSD type 2 activity obtained in paper nº 9 was also evaluated.

❖ Results and discussion

*Validation of AlphaLISA method*. The intra-assay CV was lower than 4%, and the inter-assay CV was lower than 10%. The linear regression equation resulted in a correlation coefficient of 0.99. The recovery was between 80 and 103%. The assay LD was 30.9 pg/mL, and the LLOQ was 0.2 pg/mg of hair. The high precision and accuracy and the low LD and LLOQ allow the measurement of oxytocin in hair, with some advantages, such as the low volume of sample, it is not necessary washing steps, and a shorter processing time, as well as the use of hair over other sample types, with easy transport and storage.

*Selection of the extractor solvent*. The extraction with methanol showed an efficiency average of 103.4 ± 19.9, while it was 57.3 ± 19.7 with acetonitrile. According to

these results, methanol would be the best solvent for oxytocin extraction from hair and does not produce conformational changes (Belec et al., 2005) and has also been used to study oxytocin extraction from milk (Mishra et al., 2013). Moreover, other studies do not recommend acetonitrile as an extractor solvent for selected drugs because of the low efficiency of extraction (Madry et al., 2018).

*Changes in oxytocin concentrations in hair of sows and correlation with cortisol, cortisone, and 11β-HSD type 2 activity*. In the S–S period, no significant differences were detected in oxytocin concentrations at different days of the reproductive cycle ($P > 0.05$). However, in the W–S period, oxytocin concentrations were higher at day 23 after farrowing (median: 3.4 pg/mg) and day 59 after farrowing (median: 3.5 pg/mg) than at day 5 before farrowing (median: 1.1 pg/mg) ($P = 0.0001$ and $P = 0.0002$, respectively). When each day was compared between seasons, at day 5 before farrowing, oxytocin concentrations in hair showed significantly higher concentrations in the S–S period compared with W–S (median: 4.3 pg/mg vs. median: 1.1 pg/mg) ($P < 0.0001$), but no significant differences were found depending on the season at day 23 or 59 ($P > 0.05$) (Figure 20).

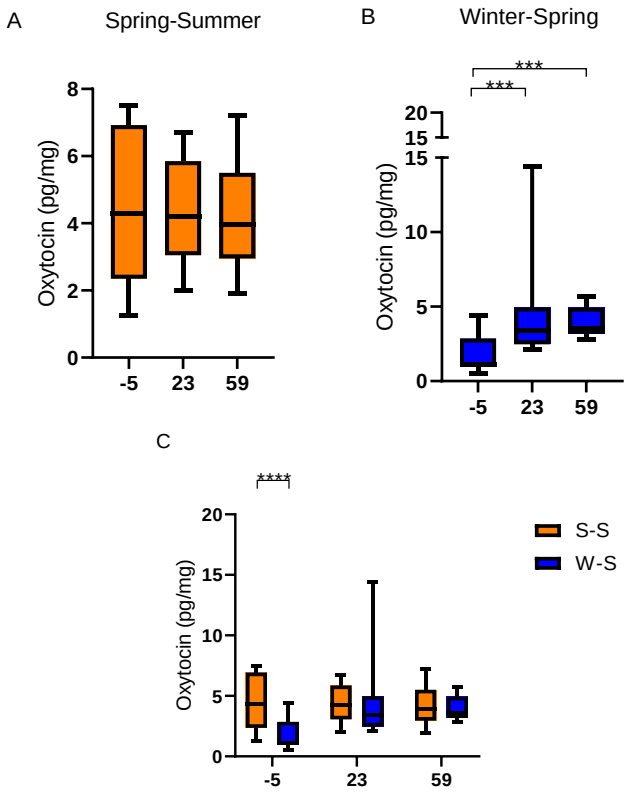


Figure 20. Oxytocin concentrations in hair (pg/mg) of sows at 5 days before farrowing (-5), 23 days after farrowing (23), and 59 days after farrowing (59) in spring-summer (A); winter-spring (B); and

in both periods (S–S, spring-summer; W–S, winter-spring) on the same sampling day per period (C). Plots show median (line within box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers). Asterisks indicate significant differences between times and periods.

When oxytocin concentrations were correlated with cortisol, cortisone, and 11β-HSD type 2 activity, there was a significant moderate correlation of oxytocin concentrations with cortisone concentrations in hair ($r = 0.663$, $P < 0.0001$), a significant low correlation with cortisol concentrations ($r = 0.316$, $P = 0.008$) and a significant moderate correlation with cortisone/cortisol (Cn/C) ratio (11β-HSD type 2 activity) ($r = 0.509$, $P < 0.0001$).

Despite the fact that oxytocin has been traditionally related to psychological and social well-being (Rault, 2017), these increases after a stressor stimulus could be a reaction by the organisms to stress, improving the stress tolerance in farm animals (Chen & Sato, 2016). Oxytocin could have an inhibitory action on the hypothalamic-pituitary-adrenal axis (Quintana & Guastella, 2020).

The higher oxytocin concentrations during the spring-summer period, with higher external temperatures, could be related to the secretion of vasopressin in cases of externally high temperatures to reduce water loss increases (Dreiling et al., 1991). The increase in oxytocin concentrations after the farrowing and the lactation period in the W–S period could be associated with cortisone in hair due to the moderate correlation and increases in cortisone concentrations in these samples in the same period in paper nº 9. Moreover, the significant correlation with the increase in the activity of 11β-HSD isoenzyme type 2, which is expressed close to oxytocin in the hypothalamus (Bisschop et al., 2013), could indicate a relation between both analytes.

## 3.4 Objective 4

The objective 4 was reflected by one study corresponding to paper nº 9, in which two new immunoassays were developed and validated for cortisol and cortisone measurement as stress biomarkers in the hair of pigs and the evaluation of the 11β-HSD type 2 activity.

### *3.4.1 Stress evaluation through the cortisol and cortisone measurement in the hair of pigs (paper nº 9)*

❖ Aims and experimental design

The objective of this study was to evaluate the 11β- HSD type 2 activity in the hair of 32 sows, through the measurement of cortisol and cortisone, during different phases of the reproductive cycle. The hair samples from 16 sows were collected in spring-summer period (S–S) (from April to September 2018), and the hair samples of the other 16 sows were collected in winter-spring period (W–S) (from January to June 2019). The different temperatures recorded are shown in Figure 21. The times of sampling in each reproductive cycle were 5 days before expected farrowing (-5), at 23 after farrowing (23), and 59 days after farrowing (59). The extraction of cortisol and cortisone from the hair of sows was performed in a similar way to Davenport *et al.* (2006). For cortisol and cortisone measurements in the extracts of hair, two new assays were developed and validated, using a commercial monoclonal antibody for cortisol assay and a polyclonal antibody developed during this Thesis for cortisone assay. The estimation of 11β-HSD type 2 activity was calculated as the Cn/C ratio.

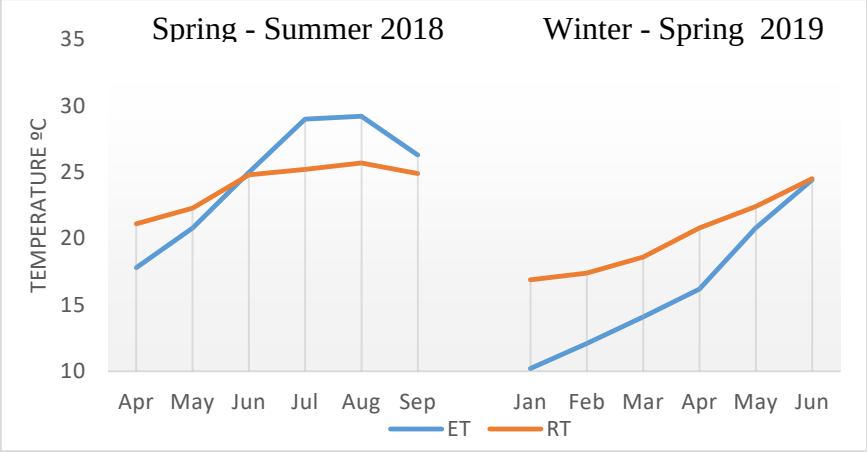


Figure 21. Average between the max and min environmental temperature (ET; blue line) and sow housing room temperature at time of sampling (RT; orange line) during the two reproductive cycles: spring-summer 2018 and winter-spring 2019.

❖ Results and discussion

*Validation of AlphaLISA methods*. The intra-assay CV was lower than 11%, and the inter-assay CV was lower than 12% in both methods. The linear regression equations resulted in a correlation coefficient of 0.99. The recovery was between 83-115% in case of the cortisol assay and 80-112% in case of the cortisone assay. The assay LD was 1.4 and 0.06 ng/mL in case of cortisol and cortisone assay, respectively. The LLOQ was 6.9 and 2.8 pg/mg of hair in case of cortisol and cortisone assay, respectively. These assays allow to measure cortisol and cortisone in pig hair samples in an accurate way with some

advantages over the blood samples in this species since it is non-invasive, easy to transport, and can indicate the changes in concentrations during a long period (Bacci et al., 2014; Burnett et al., 2014).

*Hair cortisol and cortisone concentrations, and hair Cn/C ratio (11β- HSD type 2 activity).*

Cortisone concentrations. In the S–S period, cortisone concentrations increased significantly on day 23 after farrowing (median: 527.2 pg/mg) compared to day 5 before farrowing (median: 232.0 pg/mg) ($P = 0.0260$) and day 59 after farrowing (median: 140.9 pg/mg) ($P < 0.0001$). In the W–S period, cortisone concentrations increased on day 59 after farrowing (median: 136.1 pg/mg) compared to day 23 after farrowing (median: 53.2 pg/mg) ($P = 0.0027$) and day 5 before farrowing (median: 7.8 pg/mg) ($P < 0.0001$). When cortisone concentrations were compared between seasons, significantly higher concentrations were found on the S–S period compared with W–S on day 5 before farrowing (median: 232.0 pg/mg vs. median: 7.8 pg/mg) ($P < 0.0001$) and on day 23 after farrowing (median: 527.2 pg/mg vs. median: 53.2 pg/mg) ($P < 0.0001$) but not on day 59 after farrowing between seasons (S–S and W–S) (median: 140.9 pg/mg vs. median: 136.1 pg/mg) ($P = 0.8352$) (Figure 22).

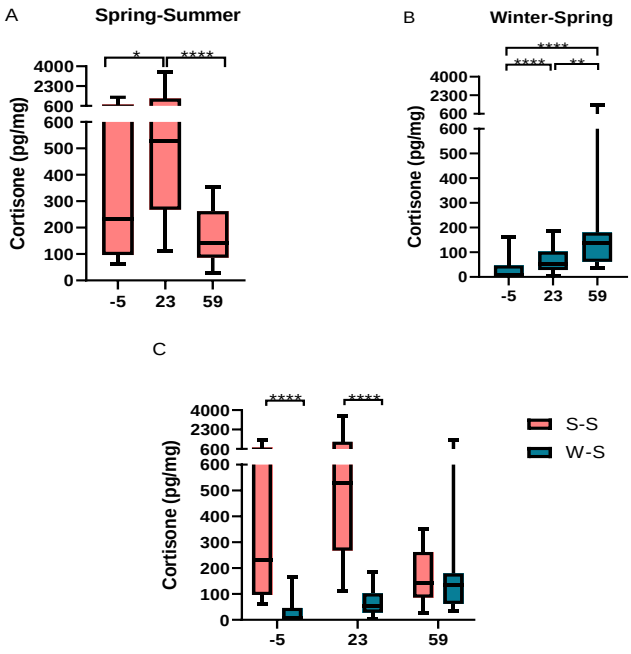


Figure 22. Cortisone concentrations in hair (pg/mg) of sows at 5 days before farrowing (-5), 23 days after farrowing (23) and 59 days after farrowing (59) in spring-summer (A), winter-spring (B) and

in both periods (S–S, spring-summer; W–S, winter-spring) (C). Plots show median (line within box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers). Asterisks indicate significant differences between times and periods.

Cortisol concentrations. In the S–S period, there were no significant differences between the different days. In the W–S period, cortisol concentrations increased on day 59 after farrowing (median: 33.7 pg/mg) compared to day 5 before farrowing (median: 31.9 pg/mg) ($P = 0.0127$) and day 23 after farrowing (median: 29.5 pg/mg) ($P = 0.0101$). When cortisol concentrations were compared between seasons, there were no significant differences between the S–S and W–S period in each sampling time (Figure 23).

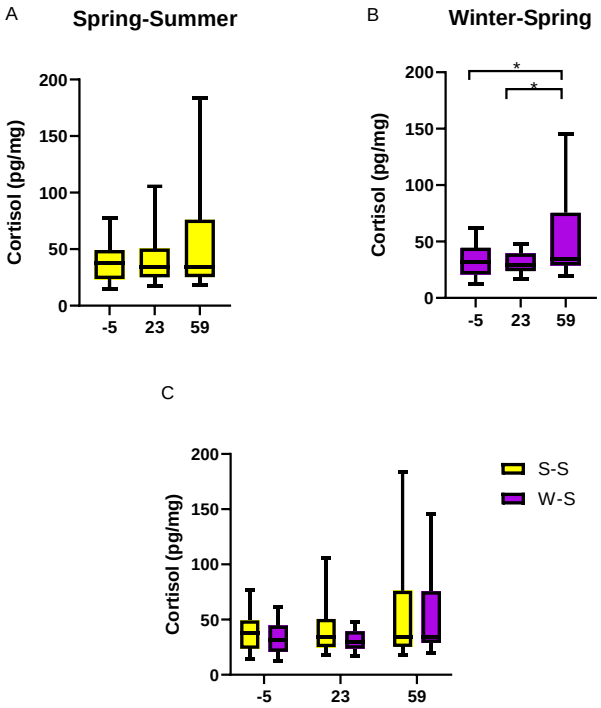


Figure 23. Cortisol concentrations in hair (pg/mg) of sows at 5 days before farrowing (-5), 23 days after farrowing (23) and 59 days after farrowing (59) in spring-summer (A), winter-spring (B) and in both periods (S–S, spring-summer; W–S, winter-spring) (C). Plots show median (line within box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers). Asterisks indicate significant differences between times and periods.

Cn/C ratio (11β-HSD type 2 activity). In the S–S period, the Cn/C ratio was significantly increased on day 23 after farrowing, with a median ratio of 12.12, compared to day 5 before farrowing, with a median ratio of 7.97 ($P = 0.0466$), and day 59 after farrowing, with a median ratio of 4.67 ($P < 0.0001$). In the W–S period, the Cn/C ratio was significantly increased on day 59 after farrowing, with a median ratio of 3.94, compared to day 5 before farrowing, with a median ratio of 0.19 ($P = 0.0006$), and day 23 after

farrowing, with a median ratio of 1.55 (P = 0.0338). When each day was compared between seasons, Cn/C ratios were significantly higher in the S–S period compared with the W–S on day 5 before farrowing (median: 7.97 vs. median: 0.19) (P < 0.0001) and day 23 after farrowing (median: 12.12 vs. median: 1.55) (P < 0.0001). There were no significant differences on day 59 after farrowing between S–S and W–S periods (median: 4.67 vs. median: 3.94) (P = 0.7317) (Figure 24).

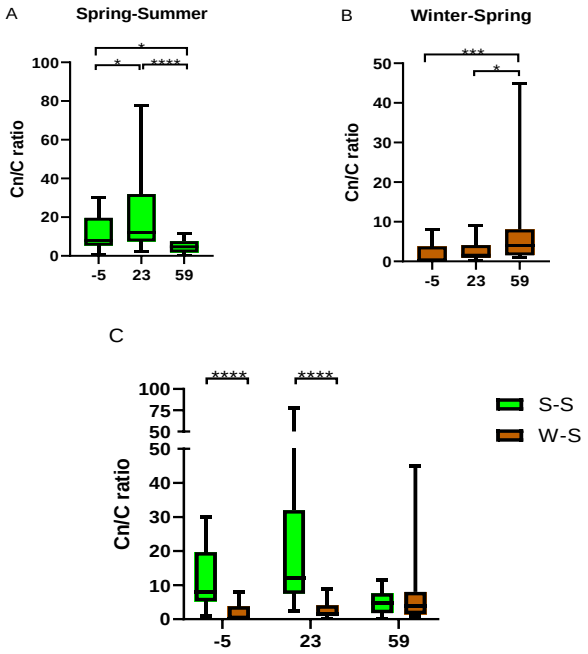


Figure 24. Cortisone/cortisol (Cn/C) ratios in hair samples of sows at 5 days before farrowing (-5), 23 days after farrowing (23) and 59 days after farrowing (59) in spring-summer (A), winter-spring (B) and in both periods (S–S, spring-summer; W–S, winter-spring) (C). Plots show median (line within box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers). Asterisks indicate significant differences between times and periods.

The trend of higher cortisol concentrations at 59 days after farrowing, especially in the winter-spring period, was also found by Bacci *et al.* (2014) in sows at 70–75 days after farrowing. In humans, it was also reported higher cortisone concentrations than cortisol in hair (Staufenbiel et al., 2015), such as the finding in this study. Moreover, the cortisone concentrations obtained are similar to studies in sheep (Stubsjøen et al., 2018).

A higher increase in cortisone concentrations after farrowing could be the explained by the higher activity of 11β- HSD isoenzyme type 2 at this time because of the stress of placement in farrowing stalls and the previous lactation (Kemp & Soede, 2012). The

influence of the season in cortisol and cortisone concentrations, with higher increases in cortisone in warmer weather, was found in studies in humans (Wester et al., 2016). The higher cortisone concentrations during periods of higher temperatures could be due to an increase in the activity of 11β-HSD type 2 (increase in the Cn/C ratio found in the spring-summer period) because of the high temperatures, leading to an increase in the transformation of cortisol to cortisone.

# ARTICLES

## *OBJECTIVE 1*

**To develop and validate immunoassays for oxytocin determination in the saliva of pigs and its use for evaluation as biomarker of positive welfare in different situations of stress and positive experiences, as well as the development of an immunoassay for oxytocin measurement in seminal plasma of pigs and its relationship with sperm quality and *in vivo* fertility.**


## ARTICLE **1** (Published)

### *Oxytocin in saliva of pigs: an assay for its measurement and changes after farrowing*

**Journal:** Domestic Animal Endocrinology

**Abstract:** Oxytocin is a hormone of interest in reproduction, but also in the field of psychology and behavior, being considered as a biomarker of positive emotions. Saliva can be a noninvasive way to measure oxytocin, which is very useful in species such as the pig where blood collection can produce a high degree of stress. In this study, a new assay for oxytocin measurement was developed, analytically validated, and used to measure possible changes in oxytocin in saliva of female pigs at different days after farrowing. The assay showed an adequate accuracy and precision and does not need a previous extraction step. In addition, oxytocin concentrations were significantly higher at day 1 of lactation than at day 9 after farrowing, but levels increased at day 20 again. This assay can contribute to a wider use of oxytocin measurements in pigs as it is a noninvasive sampling procedure that minimizes stress.

**URL:**https://www.sciencedirect.com/science/article/abs/pii/S0739724019300554?via%3Dihub


## ARTICLE **2** (Published)

### *Effect of reduction and alkylation treatment in three different assays used for the measurement of oxytocin in saliva of pigs*

**Journal:** Domestic Animal Endocrinology

**Abstract:** Oxytocin is a hormone that is increasingly being used for welfare evaluation in animals. Although several types of samples have been used for oxytocin measurement, saliva can be a suitable option for pigs producing less stress than blood sampling. In this study, 3 different methods for oxytocin measurements, 2 based on alphaLISA technology (one with a monoclonal and other with a polyclonal antibody) and one commercially available kit, were compared in saliva of pigs. These methods were used in saliva samples obtained from female pigs at 3 different days during gestation and lactation, with and without a reduction/alkylation (R/A), which is a procedure for breaking the links between oxytocin and proteins of the sample. The assays showed a different behavior after the R/A procedure, with no significant changes in the oxytocin results in case of the alphaLISA monoclonal method, a significant decrease with the alphaLISA polyclonal method, and a significant increase with the commercial kit. Although all assays showed a similar tendency in detecting the changes in oxytocin during gestation and lactation, they showed changes of different magnitude and statistical signification. This report indicates that different assays can measure different forms of oxytocin present in saliva and can have a different behavior after R/A of the sample and when are used to measure oxytocin in gestation and lactation.

**URL:** https://www.sciencedirect.com/science/article/abs/pii/S0739724020300655

## ARTICLE **3** (Published)

***Ejaculate collection influences the salivary oxytocin concentrations in breeding male pigs***

**Journal:** animals

**Abstract:** The objective of the present study was to evaluate the possible changes of oxytocin concentrations in saliva during and after ejaculate collection in breeding boars usually used in artificial insemination programs. Saliva samples of 33 boars were collected the day before ejaculate collection (DB), during the ejaculation time (T0) and two hours after ejaculate collection (T2). Free oxytocin and oxytocin linked to proteins concentrations were measured by two methods previously developed and validated for saliva of pigs. Younger boars, boars with higher libido intensity and boars of the Pietrain breed showed higher values of oxytocin in saliva during ejaculation than the day before. In addition, boars with higher libido showed higher concentrations two hours after ejaculate collection than during the day before. These changes were of higher magnitude and significance when oxytocin linked to proteins was measured. In conclusion, this study demonstrated for the first time that ejaculation influences the salivary oxytocin concentrations in breeding boars, although this influence varies according to age, libido and breed.

**URL:** https://www.mdpi.com/2076-2615/10/8/1268

## ARTICLE **4** (Published)

### *Changes in oxytocin concentrations in saliva of pigs after a transport and during lairage at slaughterhouse*

**Journal:** Research in Veterinary Science

**Abstract:** Oxytocin is associated with reproductive physiology but also with welfare and positive emotions. In this study, oxytocin was measured in saliva samples of 45 pigs that were collected before being transported to the slaughterhouse, at the time of arrival and 4 h after arrival to the slaughterhouse. Two previously validated assays, one that measures free oxytocin and other that measures oxytocin linked to proteins, were used. In addition, cortisol, salivary alpha-amylase (sAA), total esterase activity (TEA), butyrylcholinesterase (BChE) and lactate dehydrogenase (LDH), which are biomarkers associated with stress and pain in pigs, were measured. The results showed a decrease in free and protein-linked oxytocin concentrations at 4 h after transport compared with the time before transport, while cortisol, sAA, TEA, BChE and LDH showed an increase at 4 h after transport compared with the time before transport. Based on these results it can be concluded that the transport and lairage at slaughterhouse in the conditions of this study produce a decrease in oxytocin in the saliva of pigs that could indicate a reduced emotional well-being.

**URL:**https://www.sciencedirect.com/science/article/abs/pii/S0034528820309875?via%3Dihub


## ARTICLE 5 (Published)

### *Oxytocin in pig seminal plasma is positively related with in vivo fertility of inseminated sows*

**Journal:** Journal of Animal Science and Biotechnology

**Abstract:** Identification of relevant *in vivo* biomarkers for fertility remains a challenge for the livestock industry. Concentrations of the small peptide hormone oxytocin (OXT), involved in male reproductive function and present in the seminal plasma (SP) of several species could be a robust one. This study characterized concentrations of SPOXT in ejaculates from boars used in artificial insemination (AI) programs aiming to evaluate its relationship with sperm quality variables and in vivo fertility of their liquidstored AI-semen. Seminal OXT concentrations (ng/mL) were measured in 169 ejaculates from 61 boars of the Duroc, Pietrain, Landrace and Large White breeds using a direct competitive immunoassay test based on AlphaLISA ® technology. Ejaculate (ejaculate volume, sperm concentration, total sperm count) and sperm parameters (motility, viability, intracellular generation of reactive oxygen species, plasma membrane fluidity) were assessed at 0 h and 72 h in AI-semen samples stored at 17 ºC. In vivo fertility included only 18 Large White and Landrace boars whose AI-semen was used to inseminated > 100 sows and evaluated both farrowing rate and litter size of 3,167 sows. The results showed that SP-OXT differed between boars and between ejaculates within boar ($P < 0.05$) but not between breeds (Duroc, Pietrain, Landrace and Large White). Ejaculates with higher SP-OXT concentration/mL (hierarchically grouped; $P < 0.001$) had larger volume and came from younger boars ($P < 0.05$). Ejaculates of boars showing positive farrowing rate deviation exhibited higher ($P < 0.05$) SP-OXT concentration/mL than those with negative farrowing rate deviation. The SP concentrations of OXT are boar, ejaculate and age dependent, and positively related with ejaculate volume and farrowing rates of liquid-stored semen AI-doses.

**URL:**https://jasbsci.biomedcentral.com/articles/10.1186/s40104-021-00620-z

## *OBJECTIVE 2*

**To develop and validate immunoassays for oxytocin determination in the saliva of other species, such as canine and bovine species, and study the changes in stress or positive situations.**


## ARTICLE **6** (Published)

### *Oxytocin in bovine saliva: validation of two assays and changes in parturition and at weaning*

**Journal:** BMC Veterinary Research

**Abstract:** The possible use of oxytocin in saliva as an indicator of positive emotions in bovine species has been poorly investigated. In the present study, two new assays (one using a monoclonal antibody and the other using a polyclonal antibody) for the measurement of oxytocin in bovine saliva were developed and validated. Also, the changes in oxytocin in saliva were explored in two different situations. One was around parturition, and for this purpose, saliva samples from 13 cows were collected at three different times: 7 days before the parturition, the day of parturition and 7 days after the parturition. The second situation was weaning and grouping of calves, and for this purpose, saliva from 25 calves was collected at three different times: 1 day before weaning, 2 days after weaning or milk withdrawal and 4 days after grouping calves. In cows, oxytocin concentrations showed an increase on the day of parturition with both assays, while in calves, oxytocin concentrations showed a decrease 4 days after the grouping. The assays validated in this report could be used for the measurement of oxytocin in bovine saliva and detect changes in this analyte that can occur in different physiological or productive situations such as parturition and weaning.

**URL:** https://bmcvetres.biomedcentral.com/articles/10.1186/s12917-021-02838-5

## ARTICLE 7 (Published)

### *Changes in salivary oxytocin after stroking in dogs: Validation of two assays for its assessment*

**Journal:** Research in Veterinary Science

**Abstract:** Oxytocin is currently of high interest as a biomarker of welfare and stress in humans and animals. The purpose of this study was to validate two new assays (one using a monoclonal antibody and the other using a polyclonal antibody) for the oxytocin measurement in the saliva of dogs. For this purpose, an analytical validation was performed, and these assays were applied in an experimental trial in which dogs were stroked by their owners. In the experimental trial, saliva samples of 17 dogs were collected by the owners at three different times: a basal sample, at the end of 10 min of an affiliative interaction with their owners consisting of stroking and 15 min after the end of the affiliative interaction. The dogs were separated into two groups (group 1, $n = 8$ and group 2, $n = 9$) according to the acceptance of the sponge and the response to the stroking. Significant differences in the response of salivary oxytocin after stroking in the two groups were found when the assay with the monoclonal antibody was used. This assay showed a significant increase just after the end of affiliative interaction ($P < 0.01$) and 15 min after ($P < 0.01$) in those dogs that had a good acceptance of the sponge and the stroking induced a positive response on them (based in a Likert-type scale from 1 to 10). These data reflect that the assays used in this study can lead to different results when quantifying oxytocin in the saliva of dogs after stroking.

**URL:**https://www.sciencedirect.com/science/article/abs/pii/S0034528821001065?via%3Dihub

## *OBJECTIVE 3*

**To develop and validate an immunoassay for oxytocin determination in the hair of pigs, representing the first time in which oxytocin is measured in hair**

ARTICLE **8** (Published)

## *A procedure for oxytocin measurement in hair of pig: Analytical validation and a pilot application*

**Journal:** biology

**Abstract:** There is growing interest in oxytocin as a biomarker of stress and welfare. The objective of this study was to develop and validate a procedure based on a highly sensitive immunoassay to measure oxytocin in the hair of pigs. In addition, a pilot study to apply this procedure to evaluate possible changes in concentrations of oxytocin in hair during the reproductive cycle of pigs at different periods of the year was conducted. This procedure used methanol for sample extraction, since it offered better recoveries than acetonitrile, and the immunoassay developed was precise and accurate for the quantification of the oxytocin in the hair. When this procedure was applied to hair collected at different times of the reproductive cycle and season, higher values were found at days 23 and 59 after farrowing in the winter–spring period. In addition, higher oxytocin values in the spring–summer period were found in hair collected 5 days before farrowing compared to winter–spring. Oxytocin in hair showed moderate and low correlations with cortisone and cortisol in hair, respectively. This study represents the first report in which oxytocin was measured in hair and could open new lines for future research about the measurement of oxytocin in pigs and other biological species as a biomarker of stress.

**URL:** https://www.mdpi.com/2079-7737/10/6/527

# *OBJECTIVE 4*

**To develop and validate immunoassays for cortisol and cortisone determination as stress biomarkers in the hair of pigs**


## ARTICLE **9** (Published)

### *Measurement of cortisol, cortisone and 11β-hydroxysteroid dehydrogenase type 2 activity in hair of sows during different phases of the reproductive cycle*

**Journal:** The Veterinary Journal

**Abstract:** Two sensitive assays based on AlphaLISA technology were developed and validated for the measurement of cortisol and cortisone in hair of pigs, that also enabled estimation of 11β-hydroxysteroid dehydrogenase type 2 activity. These assays were applied to hair samples from sows (n = 32) collected at 5 days before, and at 23 and 59 after farrowing, in reproductive cycles in two different periods: spring– summer (n = 16) and winter–spring (n = 16). The assays were precise (imprecision <12%) and accurate (recovery range, 80–115%) for cortisol and cortisone determination. Hair cortisone concentrations and the cortisone/cortisol ratio (an estimate of 11β-hydroxysteroid dehydrogenase isoenzyme type 2 activity) increased after farrowing more than cortisol, being these changes of higher magnitude during periods of higher atmospheric temperature. The measurement of hair cortisone concentrations and estimations of the activity of the 11β-hydroxysteroid dehydrogenase isoenzyme type 2, measured by the assays developed in this study, are complementary biomarkers to hair cortisol, and can increase at periods associated with stress, such as farrowing and lactation, especially at high atmospheric temperatures.

**URL:**https://www.sciencedirect.com/science/article/abs/pii/S1090023320300356?via%3Dihub

# CONCLUSIONS

# CONCLUSIONS

1. Oxytocin can be measured in the saliva of porcine, canine, bovine, and human species, being a useful tool for the welfare evaluation, especially of positive emotions, in these species. With the assays developed in this Thesis, oxytocin can be measured directly without the need of the sample processing treatments that other assays require, such as extraction or lyophilization. Oxytocin concentrations can also be quantified in the seminal plasma of pigs, being related to some reproductive parameters.

2. The different assays developed in this Thesis for oxytocin measurement resulted in a different range of magnitudes and can also show a diverse behavior when applied to evaluate salivary oxytocin after different physiological states. It is possible because they measure different forms or metabolites of oxytocin.

3. Oxytocin can be measured in the hair of pigs, showing changes along the reproductive cycle and due to environmental temperature.

4. The 11β-HSD isoenzyme type 2 activity can be evaluated in the hair of pigs through the measurement of cortisol and cortisone, showing changes depending on the productive state and environmental temperature.

# RESUMEN

# RESUMEN

El uso de muestras no invasivas resulta más adecuado que la sangre para la evaluación de estrés o bienestar en medicina veterinaria, ya que su obtención no provoca una situación de estrés para los animales (Merlot et al., 2011). Dos ejemplos de muestras no invasivas son la **saliva** (Cook, 2012; Escribano et al., 2012), y el **pelo**, que tiene la particularidad de evaluar la respuesta al estrés durante un periodo prolongado (Bacci et al., 2014; Stubsjøen et al., 2018).

En saliva y pelo, hay dos aspectos en los que sería interesante aumentar su conocimiento:

**1)** En general, los biomarcadores utilizados en saliva para la evaluación de estrés en animales de producción (Contreras-Aguilar et al., 2020), como el cortisol, son biomarcadores "negativos" en el sentido de que detectan situaciones de salud y bienestar animal inadecuados (Martínez-Miró et al., 2016; Ruis et al., 2001). Sin embargo, hay una falta del uso de biomarcadores "positivos", como la **oxitocina**, que podría aumentar en situaciones de mejora en bienestar (Rault, 2017). Esta hormona, que tradicionalmente se ha asociado al parto y lactanción (Argiolas y Gessa, 1991), está implicada en las interacciones sociales positivas en humanos (Carter et al., 2007; Uvnas-Moberg, 1998) y en animales (MacLean et al., 2017; Ogi et al., 2020).

Además, hay varias limitaciones en los estudios sobre la medición de la oxitocina en especies domésticas. Faltan ensayos que permitan la medición directa de oxitocina tanto en especies animales como en humana, y hay un escaso conocimiento sobre la forma de oxitocina presente en la muestra y su posible unión a otras moléculas. En general, existen muchas divergencias sobre la técnica que debe emplearse para la medición de la oxitocina. Aunque la espectrometría de masas se considera la prueba estándar, los inmunoensayos podrían reconocer la oxitocina unida a otras moléculas o a otras formas de oxitocina (MacLean et al., 2019). Asimismo, existen controversia respecto al procesado de la muestra, ya que algunos autores realizan la extracción de la misma (de Jong et al., 2015; Geva et al., 2020) o la liofilización y concentración (Fujioka et al., 2020; White-Traut et al., 2009), mientras que otros indican que estos procedimientos no son necesarios (MacLean et al., 2018). Además, no existen estudios previos sobre la medición de oxitocina

en muestras de pelo, que pueda evaluar cambios en las concentraciones de oxitocina a largo plazo.

**2)** El **cortisol** en pelo está ampliamente estudiado en muchas especies animales (Accorsi et al., 2008; Davenport et al., 2006; Sauveroche et al., 2020) y puede reflejar los niveles medios en suero en los últimos 1-2 meses (Bacci et al., 2014; Prims et al., 2019). Además, el muestreo de pelo es no invasivo, indoloro y fácil, además de que las concentraciones de la molécula no se ven afectadas por el estrés generado por la sujeción o manipulación de los animales durante el muestreo (Koren et al., 2002). La isoenzima **11β-hidroxiesteroide deshidrogenasa (11β-HSD) tipo 2** convierte el cortisol en **cortisona** en el pelo (Raul et al., 2004), y actualmente, es de creciente interés en la especie humana. Sin embargo, aunque existen algunos estudios sobre la medición del cortisol en pelo de cerdos para la evaluación del estrés (Bacci et al., 2014; Prims et al., 2019), la conversión de cortisol en cortisona, a través de la actividad de la isoenzima 11β-HSD tipo 2, no ha sido evaluada en esta especie.

Esta Tesis Doctoral proporcionará un avance en el conocimiento de estos dos aspectos, mediante la investigación de nuevos ensayos que puedan proporcionar mediciones simples y precisas de oxitocina en saliva, pelo, y otras muestras, y mediante el desarrollo de métodos que puedan evaluar la actividad de la isoenzima 11β-HSD tipo 2.

Los **objetivos** de esta Tesis Doctoral se centraron en proporcionar avances en la medición de oxitocina, cortisol y cortisona en saliva, plasma seminal o pelo. Para ello, los objetivos específicos fueron:

- **Objetivo 1.** Desarrollar y validar inmunoensayos para la determinación de oxitocina en saliva de la especie porcina y aplicarlos en diferentes situaciones de estrés y experiencias positivas, así como el desarrollo de un inmunoensayo para la medición de oxitocina en plasma seminal de la especie porcina y su relación con la calidad espermática y la fertilidad *in vivo*. Los resultados de este objetivo se publicaron en los artículos (indicados en la sección '*Articles'*) nº 1-5. Además, la realización de una revisión bibliográfica sobre los ensayos utilizados para la medición de oxitocina en saliva, descrita en '*Annex*' (experimento nº 1).

- **Objetivo 2.** Desarrollar y validar inmunoensayos para la determinación de oxitocina en saliva de otras especies, como canina y bovina, y aplicarlos en

diferentes situaciones de estrés o positivas. Este objetivo dio lugar a los trabajos publicados (indicados en el apartado *'Articles'*) nº 6 y 7. Además, en el apartado '*Annex*' (experimento nº 2) se describe el desarrollo y la validación de inmunoensayos para la medición de oxitocina en saliva de la especie humana tras el estrés físico y psicológico.

- **Objetivo 3.** Desarrollar y validar un inmunoensayo para la determinación de oxitocina en pelo de la especie porcina, representando la primera vez en la que se mide oxitocina en pelo, y recogido en el trabajo publicado nº 8, indicado en el apartado *'Articles'*.

- **Objetivo 4.** Desarrollar y validar un inmunoensayo para la determinación de cortisol y cortisona como biomarcadores de estrés en pelo de la especie porcina, recogido en el trabajo publicado nº 9 indicado en la sección *'Articles'*.

Para la realización de los experimentos llevados a cabo durante esta Tesis, los estudios recibieron la aprobación del **Comité Ético** de Experimentación Animal (CEEA) (235/2016; 171/2015; 288/2017), de la Comisión Ética de Investigación (CEI) de la Universidad de Murcia (1394/2016) y se siguieron las directrices de la Directiva 2010/63/EU del Parlamento Europeo. Se siguieron las normas de bienestar y salud animal española (ES300130640127, agosto 2006) y europea (ES13RS04P, julio 2012). Además, se obtuvieron los consentimientos firmados de todos los dueños y participantes de los estudios, en los cuales se ofrecía información sobre los objetivos y procedimientos.

La **toma de muestras** se realizó para la determinación de oxitocina, cortisol o cortisona. En las especies porcina, canina y bovina, las muestras de **saliva** se obtuvieron de forma activa mediante una esponja de polipropileno sujeta a una varilla de metal flexible que se introduce en la boca del animal, y la cual el animal mastica durante un minuto. Una vez humedecida la esponja, se introduce en un tubo Salivette (Salivette, Sarstedt, Aktiengesellschaft & Co, Nümbrecht, Germany). Estos tubos con la esponja en su interior, se almacenan refrigeradas durante su transporte al laboratorio y una vez allí, se centrifugan, y el sobrenadante obtenido se transfiere a tubos Eppendorf de 1.5 mL, almacenándolos en un congelador a –80 ºC para su posterior análisis. En caso de saliva humana, la diferencia es que la obtención de saliva se realiza mediante flujo pasivo, en el que el participante

deposita la saliva a través de una pajita en un tubo de 5 mL. Posteriormente, se centrifuga y se procesa igual que en el caso de las especies animales.

Las muestras de **saliva** se sometieron a dos tipos de tratamientos: uno de extracción (en las especies porcina y humana, artículo nº 1 y experimento nº 2, respectivamente) y otro de reducción alquilación (R/A) (en las especies porcina, bovina, canina, y humana, artículos nº 2, nº 6, nº 7 y experimento nº 2, respectivamente). El procedimiento de extracción de muestras de saliva se realizó siguiendo el método descrito por MacLean *et al*. (2018) utilizando las columnas OasisPRiME (Waters Corporation, Massachusetts, Estados Unidos), mientras que el procedimiento de R/A se realizó según Brandtzaeg *et al*. (2016).

El **semen** de los verracos, para el análisis en plasma seminal, se obtuvo utilizando un método de recogida semiautomático (Collectis®, IMV Technologies, L'Aigle, Francia) y el plasma seminal se recogió inmediatamente después de la recogida del eyaculado. De cada eyaculado, se centrifugaron 5 mL de muestra de semen dos veces. Las muestras de plasma seminal se almacenaron en criotubos de 3 mL a -80°C hasta el análisis.

En el caso del **pelo**, cuyo muestreo se llevó a cabo en la especie porcina, las muestras se cortaron manualmente con tijeras, lo más cerca posible de la piel, en la región de la grupa, evitando las zonas contaminadas. Las muestras se colocaron en pequeñas bolsas de plástico, se identificaron individualmente y se almacenaron a temperatura ambiente hasta su análisis. Las muestras de pelo de los cerdos se procesaron siguiendo el protocolo de Davenport *et al.* (2006) con algunas modificaciones. Los extractos secos se reconstituyeron con 0.1 mL de PBS y se almacenaron a -80ºC hasta su análisis.

Para alcanzar los objetivos de la presente Tesis, se desarrollaron **anticuerpos monoclonales y policlonales**. En el caso de la oxitocina, se desarrolló un anticuerpo monoclonal (Yokoyama, 1999), por el que se obtuvieron clones y se seleccionó uno de ellos en base a su reactividad con la molécula de oxitocina mediante un screening ELISA, así como un anticuerpo policlonal (University of California, 2014) contra oxitocina. En ambos casos se utilizó como inmunógeno oxitocina conjugada a KLH. En el caso de la cortisona, se desarrolló un anticuerpo policlonal, utilizando como inmunógeno la cortisona conjugada a BSA. En todos los casos, tanto anticuerpos monoclonales como policlonales, se purificaron mediante un sistema de cromatografía (ÄKTA pure, GE Healthcare Life Sciences), usando la columna HiTrap Protein G HP (GE Healthcare Life Sciences, Munich, Germany) en el caso del anticuerpo monoclonal contra oxitocina y la columna HiTrap

NHS-activated HP affinity (GE Healthcare Life Sciences) en el caso de los anticuerpos policlonales contra oxitocina y cortisona.

Para la determinación de analitos en saliva, extractos de pelo y plasma seminal, se desarrollaron inmunoensayos basados en la tecnología **AlphaLISA**® (Perkin Elmer, MA, USA). Esta tecnología consta de un ensayo homogéneo de proximidad luminiscente amplificado que utiliza una química de canalización de oxígeno luminiscente, a través de dos tipos de esferas, donantes y aceptoras. La unión de las moléculas capturadas en las esferas conduce a una transferencia de energía de una esfera a la otra, produciendo finalmente una señal luminiscente/fluorescente. El principio del ensayo se basa en que, cuando un rayo láser (680 nm) incide sobre una esfera donante, se genera una única molécula de oxígeno que produce una cascada de eventos químicos y excitación en una esfera aceptora adyacente, lo que da lugar a una emisión quimioluminiscente (615 nm), que se detecta y cuantifica. Para esta tesis, los formatos utilizados fueron un ensayo competitivo directo para el anticuerpo monoclonal contra oxitocina en saliva de las especies porcina, canina, bovina y humana, y en pelo y plasma seminal de la especie porcina, y un ensayo competitivo indirecto para el anticuerpo policlonal contra oxitocina en saliva de la especies porcina, canina, bovina y humana, el anticuerpo policlonal contra cortisona en pelo de la especie porcina y el anticuerpo monoclonal comercial contra cortisol en saliva y pelo de la especie porcina.

Todos los ensayos desarrollados fueron **optimizados y validados analíticamente** para cada especie y tipo de muestra mediante parámetros de precisión (inter- e intra-ensayo), exactitud (linealidad bajo dilución y recovery) y sensibilidad (límite de detección y límite bajo de cuantificación), siguiendo los protocolos descritos previamente (Andreasson et al., 2015). Todos los métodos desarrollados fueron precisos, exactos y sensibles para cada especie y tipo de muestra.

A continuación, se expone un resumen de los estudios realizados durante esta Tesis y publicados en revistas científicas de alto impacto y reconocidas internacionalmente (9 artículos), así como los dos experimentos llevados a cabo durante el desarrollo de la Tesis para su posible publicación, que permitieron alcanzar los objetivos de esta Tesis.

*OBJETIVO Nº 1:*

Artículo nº **1**: Se desarrolló y validó un nuevo inmunoensayo basado en la tecnología de AlphaLISA utilizando un anticuerpo monoclonal para la determinación de

oxitocina en saliva de la especie porcina y se utilizó para medir los posibles cambios en las concentraciones de oxitocina en saliva de cerdas en diferentes días después del parto. Además, se realizó un proceso de extracción en las muestras de saliva para comparar las concentraciones de oxitocina antes y después de la extracción. El inmunoensayo mostró una adecuada exactitud y precisión y no necesita un paso previo de extracción. Las concentraciones de oxitocina fueron significativamente más elevadas en el día 1 que en el día 9 de lactación, pero los niveles volvieron a aumentar en el día 20. Este ensayo puede contribuir a un mayor uso de las determinaciones de oxitocina en saliva de cerdos la especie porcina, ya que es un procedimiento de muestreo no invasivo que minimiza el estrés.

Artículo nº **2**: En este estudio se utilizaron tres métodos diferentes para medir oxitocina en saliva de la especie porcina, dos de ellos basados en la tecnología AlphaLISA (uno con un anticuerpo monoclonal, desarrollado y validado en el artículo nº 1, y otro con un anticuerpo policlonal, desarrollado y validado en el presente artículo) y un kit comercial (Cayman Chemical, Ann Arbor, MI, USA). Estos métodos se utilizaron para medir oxitocina en muestras de saliva obtenidas de cerdas en 3 días diferentes durante la gestación y la lactancia, a cuyas muestras se les aplicó un tratamiento de reducción/alquilación (R/A). En cuanto a la aplicación de tratamiento de R/A en las muestras de saliva, no se detectaron cambios significativos en las concentraciones de oxitocina antes y después del tratamiento en el caso del método AlphaLISA monoclonal, mientras que se observó una disminución significativa con el método AlphaLISA policlonal, y un aumento significativo con el kit comercial después del tratamiento de R/A. Todos los ensayos mostraron una tendencia similar en la detección de los cambios en la oxitocina durante la gestación y la lactancia, aunque con diferente magnitud y significancia estadística. Por lo tanto, dependiendo del ensayo utilizado, se pueden medir diferentes formas de oxitocina presentes en saliva de cerdas durante la gestación y lactancia y pueden tener un comportamiento diferente después del tratamiento de R/A de la muestra.

Artículo nº **3**: El objetivo del presente estudio fue evaluar los posibles cambios en las concentraciones de oxitocina en saliva de verracos durante y después de la recogida de semen en programas de inseminación artificial. Se tomaron muestras de saliva de 33 verracos el día anterior a la recogida de semen, durante el tiempo de eyaculación y dos horas después de la recogida de semen. Las concentraciones de oxitocina se determinaron mediante dos métodos previamente desarrollados y validados en saliva de cerdos (con el anticuerpo monoclonal del artículo nº 1 y el policlonal del artículo nº 2). Los verracos más

jóvenes, los de mayor intensidad de libido y los de la raza Pietrain mostraron valores más elevados de oxitocina en saliva durante la eyaculación comparado con el día anterior. Además, los verracos con mayor libido mostraron concentraciones más altas dos horas después de la recogida de semen que en el día anterior. Estos cambios fueron de mayor magnitud y significancia cuando se midió con el método del anticuerpo policlonal, es decir, la oxitocina ligada a proteínas. En conclusión, este estudio demostró por primera vez que la extracción de semen y la eyaculación influye en las concentraciones de oxitocina salival en los verracos, aunque esta influencia varía según la edad, libido y raza.

Artículo nº **4**: Se determinaron las concentraciones de oxitocina en muestras de saliva de 45 cerdos que se recogieron antes de ser transportados al matadero, en el momento de la llegada y 4 h después de la llegada al matadero. Para esta determinación, se utilizaron dos inmunoensayos previamente validados, uno de ellos validado en el artículo nº 1 con un anticuerpo monoclonal, y otro con el anticuerpo policlonal validado en el artículo nº 2. Además, se midieron las concentraciones de cortisol, alfa-amilasa salival, la actividad de la esterasa total, butirilcolinesterasa y lactato deshidrogenasa, que son biomarcadores asociados al estrés y al dolor en la especie porcina. Los resultados mostraron una disminución de las concentraciones de oxitocina libre y ligada a proteínas a las 4 horas después del transporte durante la estancia en matadero, mientras que el cortisol, alfa-amilasa salival, la actividad de la esterasa total, butirilcolinesterasa y lactato deshidrogenasa aumentaron a las 4 horas después del transporte durante la estancia en matadero. Por lo tanto, el transporte y la estancia en el matadero producen una disminución de la oxitocina y un aumento en biomarcadores de estrés en saliva de la especie porcina, lo que podría indicar una disminución del bienestar.

Artículo nº **5**: Un nuevo inmunoensayo basado en tecnología de AlphaLISA (utilizando el anticuerpo monoclonal contra oxitocina) se desarrolló y validó para la determinación de las concentraciones de oxitocina en plasma seminal de verracos utilizados en programas de inseminación artificial, con el objetivo de evaluar su relación con las variables de calidad del esperma y la fertilidad *in vivo* de sus dosis seminales. Los parámetros del eyaculado (volumen, concentración y recuento total de espermatozoides) y espermáticos (motilidad, viabilidad, generación intracelular de especies reactivas de oxígeno y fluidez de la membrana plasmática) se evaluaron a las 0 h y a las 72 h en muestras de semen almacenadas a 17 ºC. La fertilidad *in vivo* se evaluó mediante la tasa de partos y el tamaño de la camada en las cerdas inseminadas. Los resultados mostraron que las

concentraciones de oxitocina en plasma seminal difieren entre verracos y entre eyaculados dentro de un mismo verraco, pero no entre razas. Además, los eyaculados con mayor concentración de oxitocina tenían mayor volumen de eyaculado y procedían de verracos más jóvenes. Los eyaculados de verracos con desviación positiva de la tasa de partos presentaron una mayor concentración de oxitocina que los que presentaban una desviación negativa de la tasa de partos. Por lo tanto, las concentraciones de oxitocina en plasma seminal son dependientes del verraco, del eyaculado, y de la edad, y están positivamente relacionadas con el volumen del eyaculado y la tasa de partos.

Experimento nº **1**: Los principales objetivos de esta revisión fueron describir el estado actual de las técnicas analíticas y la gestión de las muestras para las mediciones de oxitocina, en la especie humana y animal. Además, esta revisión proporciona algunas recomendaciones generales para su medición. Recientemente, la oxitocina se utiliza como biomarcador de experiencias "positivas" en psicología y comportamiento. Para la medición de la oxitocina, se han utilizado diferentes tipos de muestras, principalmente plasma, pero también saliva, orina o líquido cefalorraquídeo, así como diferentes tipos de ensayos. A pesar del gran interés que ha cobrado la medición de oxitocina en los estudios relacionados con el comportamiento y el estrés, su concentración, generalmente baja, en diferentes tejidos y muestras puede representar una limitación para su uso. Por esta razón, para la cuantificación de la oxitocina se requieren ensayos sensibles e incluso un tratamiento previo de la muestra. Además, la falta de información exhaustiva y de directrices sobre cómo deben manejarse las muestras y el conocimiento limitado de los ensayos disponibles también pueden representar una limitación para el uso de la oxitocina.

*OBJETIVO Nº 2:*

Artículo nº **6**: Se desarrollaron dos nuevos inmunoensayos basados en tecnología AlphaLISA (uno con un anticuerpo monoclonal y otro con un anticuerpo anticuerpo policlonal) para la medición de oxitocina en saliva de la especie bovina. Se evaluaron los cambios en las concentraciones de oxitocina después del tratamiento de la muestra mediante reducción/alquilación en saliva de vacas y los cambios en las concentraciones de oxitocina en saliva en dos situaciones diferentes. Una de ellas consistió en la toma de muestras de saliva en vacas 7 días antes del parto, el día del parto y 7 días después del parto. La otra consistió en la toma de muestras de saliva en terneros 1 día antes del destete, 2 días después del destete y 4 días después de agruparlos. En el caso de las vacas, las

concentraciones de oxitocina mostraron un aumento el día del parto con ambos ensayos, mientras que, en el caso de los terneros, las concentraciones de oxitocina mostraron una disminución 4 días después del agrupamiento. Estos ensayos puedes utilizarse para la medición de oxitocina en saliva de la especie bovina y detectar cambios en diferentes situaciones fisiológicas o productivas.

Artículo nº **7**: El objetivo de este estudio fue validar dos nuevos inmunoensayos (uno con un anticuerpo monoclonal y otro con un anticuerpo policlonal) para medir la oxitocina en la saliva de la especie canina. Estos ensayos se aplicaron en un modelo en el que los perros tuvieron una relación afiliativa, en la que fueron acariciados por sus dueños, además de comparar las concentraciones de oxitocina en muestras de saliva antes y después de aplicar un tratamiento de reducción/alquilación. Los propietarios recogieron muestras de saliva de sus perros en tres momentos diferentes: una muestra basal, después de 10 minutos de interacción afiliativa con sus ellos (consistente en caricias) y 15 minutos después del final de la interacción afiliativa. Los perros fueron separados en dos grupos según la aceptación de la esponja para la toma de muestras y la respuesta a las caricias. En los resultados con el método del anticuerpo monoclonal, se detectó un aumento significativo en las concentraciones de oxitocina justo después del final de la interacción afiliativa y 15 minutos después del final de la interacción en los perros que tuvieron buena aceptación a la esponja y las caricias indujeron una respuesta positiva en ellos (basada en una escala de 1 a 10). Estos datos reflejan que los ensayos utilizados pueden conducir a resultados diferentes al cuantificar la oxitocina en la saliva de los perros después de las caricias.

Experimento nº **2**: El objetivo de este estudio fue desarrollar y validar dos nuevos inmunoensayos (uno de ellos utilizando un anticuerpo monoclonal y el otro utilizando un anticuerpo policlonal contra la oxitocina) para la medición de oxitocina en saliva humana, y comparar los resultados con un kit comercial. Además, estos ensayos se utilizaron para evaluar los cambios en las concentraciones de oxitocina salival después de un tratamiento de extracción y otro de reducción/alquilación. Además, se evaluaron los cambios en las concentraciones de oxitocina después de un esfuerzo físico (CrossFit) y un estrés psicológico (TSST). Las muestras de saliva se recogieron 5 minutos antes del ejercicio, tras la finalización del mismo y 10 minutos después de la finalización en el caso del esfuerzo físico, y 5 minutos antes de la entrevista, justo después de la entrevista y 15 minutos después de su finalización en el caso del estrés psicológico. Cada uno de estos ensayos midió

diferentes formas de oxitocina. Las concentraciones de oxitocina mostraron aumentos significativos después del estímulo en los modelos físico y psicológico. Este estudio indica que la oxitocina se puede medir en saliva humana mediante los ensayos desarrollados sin tratamiento previo, y que ambos son suficientemente sensibles para detectar cambios en la concentración de oxitocina tras un esfuerzo físico y un estrés psicológico.

*OBJETIVO Nº 3:*

Artículo nº **8**: Se desarrolló y validó un inmunoensayo basado en tecnología AlphaLISA para medir concentraciones de oxitocina en pelo de la especie porcina. Además, se realizó un estudio piloto para aplicar este ensayo y evaluar los posibles cambios en las concentraciones de oxitocina en pelo durante el ciclo reproductivo de cerdas en diferentes períodos del año: día 5 antes del parto, día 23 después del parto y día 59 después del parto durante la estación de temperaturas altas (primavera-verano) y temperaturas más bajas (invierno-primavera). Se utilizó metanol para la extracción de oxitocina de la muestra, ya que ofrecía mejores tasas de recuperación que el acetonitrilo. Se encontraron concentraciones de oxitocina más altas en los días 23 y 59 después del parto en el periodo de invierno-primavera. Además, los valores de oxitocina fueron más altos en el día 5 antes del parto en el periodo de primavera-verano que en el mismo día de invierno-primavera. Las concentraciones de oxitocina en pelo mostraron correlaciones moderada y baja con la cortisona y el cortisol en el pelo, respectivamente. Este estudio demuestra la posible medición de oxitocina en pelo y los posibles cambios durante el ciclo reproductivo de la cerda.

*OBJETIVO Nº 4:*

Artículo nº **9**: Se desarrollaron y validaron dos inmunoensayos basados en la tecnología AlphaLISA para la medición de cortisol y cortisona en pelo de la especie porcina, que también permitieron estimar la actividad de la enzima 11β-HSD tipo 2, la cual transforma el cortisol en cortisona. Estos ensayos se aplicaron a muestras de pelo de cerdas recogidas a los 5 días antes, y a los 23 y 59 después del parto, en ciclos reproductivos en dos periodos diferentes: primavera-verano e invierno-primavera. Las concentraciones de cortisona en el pelo y la relación cortisona/cortisol (estimación de la actividad 11β-HSD tipo 2) aumentaron después del parto más que el cortisol, siendo estos cambios de mayor magnitud durante los periodos de mayor temperatura. Por lo tanto, la medición de las concentraciones de cortisona en el pelo y las estimaciones de la actividad de la isoenzima

11β-HSD tipo 2 podrían ser biomarcadores complementarios al cortisol en pelo, y pueden aumentar en periodos asociados al estrés, como el parto y la lactancia, especialmente durante altas temperaturas.

Las **conclusiones** obtenidas de este Tesis Doctoral fueron las siguientes:

1. Se pueden determinar las concentraciones de oxitocina en saliva de las especies porcina, canina, bovina, y humana, siendo una herramienta útil para la evaluación de bienestar, especialmente de emociones positivas, en estas especies. Con los ensayos desarrollados en esta Tesis, la oxitocina se puede medir directamente sin necesidad de los tratamientos de procesado de muestras que requieren otros ensayos, como la extracción o la liofilización. La oxitocina se puede medir también en el plasma seminal de la especie porcina y se relaciona con algunos parámetros reproductivos.

2. Los diferentes ensayos desarrollados en esta Tesis para la medición de oxitocina pueden dar resultados de diferente rango de magnitud y también pueden mostrar un distinto comportamiento cuando se aplican para evaluar la oxitocina en saliva en diferentes situaciones fisiológicas. Esto es posible debido a que miden diferentes formas o metabolitos de la oxitocina.

3. Las concentraciones de oxitocina pueden ser determinadas en pelo de la especie porcina, mostrando cambios a lo largo del ciclo reproductivo y también debido a la temperatura ambiental.

4. La actividad de la isoenzima 11β-HSD tipo 2 puede ser evaluada en pelo de la especie porcina mediante la determinación de cortisol y cortisona, y muestra cambios en función del estado productivo y de la temperatura ambiental.

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# ANNEX

# ANNEX

## *EXPERIMENT 1*

**Planned to be submitted for possible publication.**

### *Oxytocin assays in saliva: an update.*

*Abstract*: Oxytocin has traditionally been known for its physiological effects on muscle contraction associated with childbirth and lactation. In recent years, oxytocin is widely used as a biomarker of "positive" experiences in psychology and behavior. Different kinds of samples have been used for oxytocin measurements, mainly plasma, but also saliva, urine or cerebrospinal fluid. Despite the high interest that the measurement of oxytocin has gained in studies related to behavior and stress, its usually low concentration at different tissues and samples can represent a limitation for its use. For this reason, sensitive assays and even previous sample treatment are required for oxytocin quantification. In addition, the lack of comprehensive information and guidelines about how the samples should be managed and the limited knowledge about the available assays can also represent a limitation for the use of oxytocin. The main objectives of this review were to describe the current status of the analytical techniques and sample management for the measurements of oxytocin, in human and animal species. In addition, this report will provide some general recommendations for its measurement. It is expected that this information can contribute to more accurate and wider use of oxytocin measurements in the future.

# *EXPERIMENT 2*

**Planned to be submitted for possible publication.**

## *Validation of two new immunoassays for oxytocin measurements in human saliva*

*Abstract:* The objective of this research was to develop and validate two new immunoassays for oxytocin measurement in human saliva. These assays were used to compare oxytocin concentrations with a commercial kit before and after the extraction or reduction/alkylation treatments to the samples and to evaluate the changes in salivary oxytocin concentrations in human after a physical effort and an induced psychological stress. The samples were collected 5 min before the exercise, after completion of the exercise, and 10 min after the completion in case of physical effort, and 5 min before the interview, just after the arithmetic task, and 15 min later in case of the psychological stress. Oxytocin concentrations showed significant increases after the physical and psychological models. This study indicates that oxytocin can be measured in human saliva by these assays without previous treatment and both are enough sensitive to detect changes after a physical effort and psychological stress.