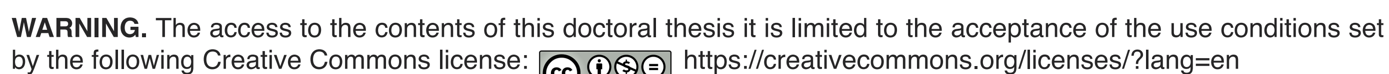
Universitat Autònoma de Barcelona

ADVERTIMENT. L'accés als continguts d'aquesta tesi queda condicionat a l'acceptació de les condicions d'ús establertes per la següent llicència Creative Commons: http://cat.creativecommons.org/?page_id=184

ADVERTENCIA. El acceso a los contenidos de esta tesis queda condicionado a la aceptación de las condiciones de uso establecidas por la siguiente licencia Creative Commons: http://es.creativecommons.org/blog/licencias/



WARNING. The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license: https://creativecommons.org/licenses/?lang=en

HAIR CORTISOL IN CATTLE AS A MEASURE OF LONG-TERM ADRENAL ACTIVITY

DISSERTATION TO OBTAIN THE DEGREE OF DOCTORBY:

Oriol Talló Parra

UNDER THE DIRECTION OF:

Dr. Manel López Béjar and **Dr. Xavier Manteca Vilanova**

PRESENTED TO THE DEPARTMENT OF ANIMAL AND FOOD SCIENCE,
VETERINARY FACULTY, UNIVERSITAT AUTÒNOMA DE BARCELONA

Bellaterra, 2016

FACULTAT DE VETERINÀRIA

Manel López Béjar, professor titular d'universitat del Departament de Sanitat i Anatomia Animals de la Universitat Autònoma de Barcelona, i **Xavier Manteca Vilanova**, catedràtic del Departament de Ciència Animal i dels Aliments de la Universitat Autònoma de Barcelona,

Certifiquen:

Que la memòria titulada "**Hair cortisol in cattle as a measure of long-term adrenal activity**", presentada per Oriol Talló Parra amb la finalitat d'optar al grau de Doctor en Veterinària, ha estat realitzada sota la seva direcció i, considerant-la acabada, autoritzen la seva presentació perquè sigui jutjada per la comissió corresponent.

I perquè consti als efectes oportuns, signen la present a Bellaterra, 9 de setembre de 2016.

Dr. Manel López Béjar   Dr. Xavier Manteca Vilanova

Oriol Talló Parra
(doctorand)

Per a la realització de la tesi l'autor va gaudir d'una beca pre-doctoral FI-DGR concedida per l'Agència de Gestió d'Ajuts Universitaris i de Recerca de la Generalitat de Catalunya (Ref. 2013FI_B 01118).

Als protagonistes d’aquesta història:

totes les vaques i vedells que han aguantat les nostres actuacions sense poder triar-ho ni saber el perquè.

Agraïments

Sempre he pensat que la ciència ens diu com som i l'art com ho portem. Després de passar la muntanya russa emocional que és el procés de fer una tesi doctoral, crec que els agraïments són una mica com l'art, i per això son tant importants.

En primer lloc vull parlar dels meus directors: el Manel i el Xavi. Quan fa cinc anys vaig decidir que volia dedicar-me a això, vaig preparar el meu millor estil i vaig escriure dos emails (*que he rellegit i són horribles*) un al Xavi i l'altre al Manel. Els explicava que em volia dedicar a la docència i a la recerca i que ells havien estat els meus referents. M'agradava el que feien i com ho feien. Vaig creuar els dits desitjant poder col·laborar d'alguna manera amb algú d'ells i quan els vaig desencreuar, ells ja m'havien ofert una cosa molt millor: treballar conjuntament amb els dos! Un màster i un doctorat després, segueixo amb l'esperança de poder seguir tenint-los a prop, col·laborant i aprenent-ne. **Manel**, gràcies per obrir-me la porta més difícil, la primera. Per lluitar per mi i creure-hi. Per fer-me sentir important. Per ensenyar-me però alhora escoltar-me. Per ser-hi sempre. Amb tu i aquella beca de col·laboració va començar tota aquesta aventura. **Xavi**, gràcies per tot el teu suport i atenció. Per la claredat i la senzillesa. Per haver-ho fet tot fàcil. Amb una reunió al bar ja m'estructuraves el cap. Tot el que he après de tu i tinc la sensació d'haver rascat només la punta de l'iceberg.

Si els directors son les veles del vaixell amb el qual he navegat en aquest impredictible oceà que és el doctorat, els companys i les companyes en són, sense cap mena de dubte, les provisions: impossible sobreviure'n sense.

A les amigues del despatx: heu fet d'aquest oceà un divertidíssim *aquaparc*. Gràcies per haver convertit tots els dilluns, dimarts, dimecres i dijous en divendres. Sou absolutament magnífiques! **Annaïs**, indiecita fea de la guarda (*no l'estic insultant, no patiu, coses nostres*), sapito imprescindible en el meu dia a dia. La teva amistat ha traspassat ELISA's, mostrejos, articles i medalles *fucking genious* a les millors idees. Tant, que has acabat sent clau en els moments personals més importants de la meva vida. Gràcies i benvinguda, d'aquí no te'n vas. **Laura**, ets tan única que porto 5 minuts intentant trobar un símil graciós i cap et supera. (*Ah, ara*) Ets tan genial que quan la rutina té malsons i mira a sota el llit, et busca a tu. Brindarem amb Kinderbuenos per cada au o ecosistema que salvis i per cada soroll que puguis imitar correctament... mentre intentem solventar el dilema ètic que portin oli de palma. Gràcies! **Maria**, has estat la meva constant en aquest viatge. Una barreja de germana gran i petita. Mira't, ara: doctora i a punt de parir (*substituir per /recent parida si la cosa s'avança!*). Gràcies! **Meritxell**, intento pensar en una forma graciosa de definir el teu somriure tímid i la teva presència agradable però no puc deixar de pensar en aquell menú vegetarià que ens vas cuinar l'altre dia... boníhim! Gràcies!

També voldria donar les gràcies al **Carlos**, ¡creo que eres mi amigo másmayor! (*el Sergi està així així*) Gracias por tu honesta amistad y compañía. Por el brutal conocimiento de tu trabajo y por estar siempre dispuesto a compartirlo. Sólo por aguantarnos ya deberían darte un *honoris causa*... I a l'**Encarna**: Gràcies! Has arribat com un miracle i el grup ha fet un salt qualitatiu enorme. Com una espècie de Mary Poppins científica... però sense marxar, vale? Tampoc voldria oblidar-me de totes les persones que han passat pel grup: doctorands, estudiants de màster, de TFG, investigadors realitzant estàncies... tots heu format part d'aquesta etapa. No poso tots els noms però us penso! Gràcies!

Durant la realització del doctorat, he tingut el privilegi de treballar amb dues investigadores (i persones) excepcionals: **Maria Devant** i **Elena Albanell**. **Maria**, moltíssimes gràcies per acollir-me com una espècie de fillol científic i fer-me sentir un més del grup. Gràcies per haver posat a la meva disposició tot el teu talent. De tu n'he après una barbaritat. I la de *tallaos* que ens queden! **Elena**, mil gracias por tus ganas, tu dedicación y tu atención. ¡Yo de mayor quiero ser como Elena Albanell!

Agrair també a l'**Eva Mainau**, la **Deborah Temple**, la **Lorena Castillejos** i a la **Bruna Quintana** la seva ajuda en la tesi sempre que l'he necessitat així com a la resta de companys i companyes de l'SNiBA, de la unitat de remugants de l'IRTA, al personal de la granja el Nial així com al Grup Alimentari Guissona. Gràcies! A la **Carme** i a tots els professors d'anatomia amb qui he compartit pràctiques: gràcies per haver-me ensenyat a ensenyar!

Tampoc podria oblidar-me del **Marçal** (amb tu sobren les paraules, ets *l'amic*), el **Sergi** (gran estadista i millor persona. Si ens veiem i no parem de riure, perquè deixar de veure'ns?), el **Nico** (un mestre de la vida) i la resta de companys i companyes del màster: **Imma**, **Gemma**, **Cris**, **Bryan**... a tots, gràcies per haver format part d'aquesta magnífica etapa.

Podria seguir i anomenar al **Miki**, la **Montse**, l'**Andreu**, l'**Albert** o la **Marina**, però això es faria etern. Intentaré evitar-ho agraint a totes les persones que han fet del meu dia a dia durant aquest doctorat, una etapa meravellosa: becaris de la facultat (ànims!), investigadors i investigadores que han destinat un moment o molts a resoldre'm algun dubte, personal del bar (i el seu cafè) i PAS de la facultat... a tots, gràcies!

Ah! Si heu flipat amb la portada i la maquetació de tant bé que estan, no és mèrit meu sinó de l'**Esther**, que sent més passió per la ciència que molts científics!

No surten com a coautors a cap article, però en la meva feina s'hi han de veure i sentir reflectits, encara que sigui mirant-me els cognoms: **papa** i **mama**, tot el que sóc, he fet i aconseguit és gràcies a vosaltres. El doctorat, també. Gràcies! **Laia**, gràcies per exercir de germana gran, cuidar-me i interessar-te per aquest mon estrany en el que em moc. **Marc**, gràcies per ser el meu còmplice en aquest *tinglado* que és el món de la recerca. Que orgullós n'estic! (*T'explico un secret? Encara que sembli impossible, el doctorat s'acaba!*). Gràcies a la resta de la meva família i especialment a les iaies, que han tingut el poder de fascinar-se pel que faig sense saber el que faig.

Els meus últims agraïments els voldria dedicar al **Fuet**, per recordar-me cada dia perquè val la pena el que faig i per donar-nos tant malgrat passar-se el dia dormint. I a tu, la bona, bonica, llesta i simpàtica **Alba**. Aquesta tesi és teva no només per tot el que has fet, sinó perquè de tu va tot això. Per a tu va això de voler un món millor i posar-hi el granet de sorra a través de la ciència. Per tu. *T'e.*

Gràcies.

Bé, ara ja ho puc escriure: Thesis, the end!!!

SUMMARY

In the last decades, social interest in animal welfare and welfare management has increased substantially. Stress is a decisive factor in animal welfare because of its impact and mainstreaming. Although stress response evolved to maintain animal's homeostasis, chronic stress is prejudicial and a serious threat to animal's well-being. The hypothalamic-pituitary-adrenal-axis is a key element in the stress response and ends with glucocorticoid secretion in the bloodstream by the adrenal gland. The measurement of glucocorticoid concentrations associated to adrenal activity is the most used methodology to assess animal's stress status. Cortisol, the main glucocorticoid in cattle, can be detected in blood (serum or plasma), saliva, urine, milk or faeces, but none of these matrixes is able to provide a measure of long-term adrenal activity. Hair is a matrix with the potential to provide an integrative value of long-term circulating cortisol levels and therefore, to be a measure of long-term adrenal activity. However, as a relatively new matrix, hair potential must be tested and validated. An accurate knowledge of hair as a matrix that accumulates cortisol is essential for its future use in chronic stress research associated to animal welfare and animal production. Consequently, the general objective of this thesis was to validate, evaluate and optimize the use of hair cortisol measurement as a tool to assess long-term adrenal activity in cattle. First, we validated a standardized protocol for the extraction of cortisol from cattle hair and the quantification of these cortisol concentrations by an enzyme immunoassay kit with excellent validation results. Second, we studied the feasibility of hair cortisol concentrations (HCC) to represent a long-term integrative value of adrenal activity by: 1) correlating HCC with faecal cortisol metabolites concentrations (FCMC), a validated measure of adrenal activity and 2) studying the effect on HCC of acute and non recurrent changes in circulating cortisol levels that did not represent the general status of the animal. Hair cortisol concentrations from dairy cows correlated well with the average value of FCMC, demonstrating indirectly that there exists a relationship between adrenal activity and HCC. Moreover, we evaluated the robustness of HCC from bull calves in front sporadic peaks of circulating cortisol induced by injections of adrenocorticotropic hormone and concluded that HCC are able to represent long-term adrenal activity. Third, we explored the potential uses of hair cortisol determination in dairy cattle production. This study investigated the relationships between HCC and physiological or productive data from a commercial dairy herd. High individual variability was found and greater HCC were associated to low milk yields during almost all lactation period demonstrating the sensitivity of HCC to certain long-term stressors affecting milk production. Fourth, we aimed to optimize hair cortisol detection by evaluating near infrared reflectance spectroscopy (NIRS) technique for hair cortisol quantification in cows. Although NIRS through the obtained equations was not able to replace current methodologies, it was able to detect accurately animals with high HCC and be used for general animal screening. Finally, we reported the effects of secondary characteristics associated to hair matrix with a presumptive influence in hair cortisol measurement. Hair colour and hair anatomical location affected significantly HCC. Although future research is necessary to understand confounding factors, cortisol concentrations in hair seem to represent long-term adrenal activity with potential to be used as a tool in dairy cattle studies of animal welfare and production.

RESUM

Durant les últimes dècades, l'interès social sobre el benestar animal i la seva gestió ha incrementat de manera notòria. L'estrès és un factor decisiu del benestar animal degut al seu impacte i a la seva transversalitat. Malgrat que la resposta d'estrès va evolucionar per a mantenir la homeòstasis de l'animal, l'estrès crònic és perjudicial i una amenaça seriosa pel benestar dels animals. L'eix hipotalàmic-hipofisari-adrenal és un element clau de la resposta d'estrès que finalitza amb la secreció de glucocorticoides al torrent sanguini per la glàndula adrenal. Mesurar les concentracions de glucocorticoides associades a l'activitat adrenal és la metodologia més usada per a l'avaluació de l'estat d'estrès d'un animal. El cortisol, el glucocorticoid principal en vaquí, pot detectar-se en sang (sèrum o plasma), saliva, orina, llet o femta, però cap d'aquestes matrius és capaç de proporcionar una mesura a llarg termini de l'activitat adrenal. El pèl és una matriu amb el potencial de proporcionar un valor integrador dels nivells de cortisol circulants a llarg termini i, per tant, de ser una mesura de l'activitat adrenal també a llarg termini. No obstant, al ser una matriu relativament nova, tot el potencial del pèl ha de ser testat i validat. Un coneixement exacte del pèl com a matriu que acumula cortisol és essencial per al seu futur us en estudis d'estrès crònic associats a benestar i producció animals. Per tant, l'objectiu general d'aquesta tesi era validar, avaluar i optimitzar l'ús del cortisol en pèl com a eina per a valorar l'activitat adrenal a llarg termini en vaquí. Primerament, es va validar un protocol estandarditzat per a l'extracció de cortisol provinent de pèl de vaquí i la seva quantificació mitjançant l'ús d'un enzim immunoassaig comercial amb excel·lent resultats de validació. Seguidament, es va verificar la capacitat de les concentracions de cortisol en pèl (HCC per les seves sigles en anglès) per representar un valor integrador de l'activitat adrenal a llarg termini a través de: 1) la correlació de les HCC amb concentracions de metabòlits del cortisol en femta, una mesura ja validada d'activitat adrenal i 2) l'estudi de l'efecte de canvis no recurrents i aguts (que no representen l'estat general de l'animal) dels nivells de cortisol circulant en les HCC. Les HCC de vaques lleteres van correlacionar bé amb la seva mitjana dels valors de metabòlits de cortisol fecals, demostrant indirectament que existeix una relació entre l'activitat adrenal i les HCC. A més a més, es va avaluar la robustesa de les HCC de vedells en front pics esporàdics en els nivells de cortisol circulant induïts per injeccions d'hormona adrenocorticotròpica i es va concloure que les HCC eren capaces de representar l'activitat adrenal a llarg termini. Després es va explorar les aplicacions potencials de la determinació de cortisol en pèl en la producció de vaca lletera. En aquest estudi es van investigar les relacions entre les HCC i dades fisiològiques o productives en una granja comercial de vaques lleteres. Es va trobar una alta variabilitat individual i HCC elevades es van associar a una menor producció de llet durant tota la lactació, demostrant així la sensibilitat de les HCC a certs estressors a llarg termini que afecten la producció lletera. En quart lloc i amb l'objectiu d'optimitzar la detecció de cortisol en pèl, es va avaluar la tècnica de l'espectroscòpia de l'infraroig proper (NIRS per les seves sigles en anglès) per a la quantificació del cortisol en pèl de vaques. Malgrat que el NIRS (a través de les equacions obtingudes) no va resultar capaç de reemplaçar les metodologies actualment utilitzades, si que va demostrar ser capaç de detectar amb exactitud animals amb altes HCC i ser útil per exàmens inicials. Finalment, es van reportar característiques secundàries descobertes durant la tesi associades al pèl amb una presumpta influència en les seves concentracions de cortisol. Malgrat que es necessita futura recerca per entendre alguns factors de confusió, les concentracions

de cortisol en pèl semblen representar l'activitat adrenal a llarg termini amb el potencial de ser usades en estudis de benestar i producció animal en vaquí lleter.

RESUMEN

Durante las últimas décadas, el interés social sobre el bienestar y su gestión ha incrementado de forma notoria. El estrés es un factor decisivo del bienestar animal debido a su impacto y su transversalidad. A pesar de que la respuesta de estrés evolucionó para mantener la homeóstasis del animal, el estrés crónico es perjudicial y una amenaza seria para el bienestar de los animales. El eje hipotalámico-hipofisario-adrenal es un elemento clave de la respuesta de estrés que finaliza con la secreción de glucocorticoides en el torrente sanguíneo por la glándula adrenal. Medir las concentraciones de glucocorticoides asociadas a la actividad adrenal es la metodología más usada para la evaluación del estado de estrés de un animal. El cortisol, el glucocorticoide mayoritario en vacuno, puede detectarse en sangre (suero o plasma), saliva, orina, leche o heces, pero ninguna de estas matrices es capaz de proporcionar una medida a largo plazo de la actividad adrenal. El pelo es una matriz con el potencial de proporcionar un valor integrador de los niveles de cortisol circulantes a largo plazo y, por lo tanto, de ser una medida de la actividad adrenal también a largo plazo. No obstante, al ser una matriz relativamente nueva, todo el potencial del pelo debe ser testado y validado. Un conocimiento exacto del pelo como matriz que acumula cortisol es esencial para su futuro uso en estudios de estrés crónico asociados a bienestar y producción animales. Por lo tanto, el objetivo general de esta tesis era validar, evaluar y optimizar el uso del cortisol en pelo como herramienta para valorar la actividad adrenal a largo plazo en vacuno. En primer lugar se validó un protocolo estandarizado para la extracción de cortisol proveniente de pelo de vacuno y su cuantificación mediante el uso de un enzimoinmunoensayo con excelentes resultados de validación. Seguidamente, se verificó la capacidad de las concentraciones de cortisol en pelo (HCC por sus siglas en inglés) para representar un valor integrador de la actividad adrenal a largo plazo a través de: 1) la correlación de las HCC con concentraciones de metabolitos del cortisol en heces, una medida ya validada de actividad adrenal y 2) el estudio del efecto de cambios no recurrentes y agudos (que no representan el estado general del animal) de los niveles de cortisol circulante en las HCC. Las HCC de vacas lecheras se correlacionaron bien con la media de los valores de los metabolitos de cortisol fecales, demostrando indirectamente que existe una relación entre la actividad adrenal y las HCC. Además, se evaluó la robustez de las HCC en terneros frente a picos esporádicos en los niveles de cortisol circulante inducidos por inyecciones de hormona adrenocorticotropicay se concluyó que las HCC eran capaces de representar la actividad adrenal a largo plazo. Después se exploró las aplicaciones potenciales de la determinación de cortisol en pelo en la producción de vaca lechera. Se investigó las relaciones entre las HCC y datos fisiológicos o productivos en una granja comercial de vacas lecheras. Se encontró una alta variabilidad individual y HCC elevadas se asociaron a una menor producción de leche durante toda la lactación, demostrando así la sensibilidad de las HCC frente a ciertos estresores a largo plazo que afectan la producción lechera. En cuarto lugar y con el objetivo de optimizar la detección de cortisol en pelo, se evaluó la técnica de la espectroscopia del infrarrojo cercano (NIRS por sus siglas en inglés) para la cuantificación de cortisol en pelo de vacas. Pese que el NIRS (a través de las ecuaciones obtenidas) no resultó capaz de reemplazar las metodologías actualmente utilizadas, sí que demostró ser capaz de detectar con exactitud animales con altas HCC y ser útil para un examen inicial. Finalmente, se reportaron las características secundarias descubiertas durante la tesis asociadas al pelo con una presunta influencia en sus concentraciones de cortisol. A demás, la influencia de la tasa de crecimiento del pelo y la

relación entre los estresores y el período de actividad adrenal monitorizado por el pelo parecen ser otros aspectos cruciales en la detección de cortisol en pelo. Pese a que se necesita más investigación para entender algunos factores de confusión, las concentraciones de cortisol en pelo parecen representar la actividad adrenal a largo plazo con el potencial de ser usadas en estudios de bienestar y producción animal en vacuno lechero.

PREFACE

"The only feelings we can feel are our own. When it comes to the feelings of others,

we can only infer them"

StevanHarnad

Animal welfare is one of the scientific fields closer to ethics and philosophy. The whole concept of animal welfare science relies on the study of an entity considered individual because of its unified cognition, own consciousness and capacity to feel. All these concepts are still being unknown mysteries for humanity despite they have been approached from very different fields such as philosophy, neurobiology or even artificial intelligence.

Researchers in animal welfare do not only try to study the emotional states and conscious experience of others but we do it in other species rather than humans (none of them able to talk and most of them very different from our own) and try to find ways to measure and judge it!

Consequently, the nature of animal welfare science is to be controversial and in constant discussion and the best example of this is the absence of a unified animal welfare definition. However, this situation should be accepted as an intrinsic and wonderful characteristic of this field.

After all, animal welfare science is one of the toughest scientific fields if someone is looking for certainties but at the same time it is one of their most altruistic and fascinating ones.

Oriol Talló Parra

TABLE OF CONTENT

SUMMARY ..... **IX**
RESUM ..... **X**
RESUMEN ..... **XII**
PREFACE ..... **XV**
TABLE OF CONTENT ..... **XVII**
INDEX OF TABLES ..... **XXIII**
INDEX OF FIGURES ..... **XXV**
ABBREVIATIONS ..... **XXVII**
LIST OF SPECIES MENTIONED ..... **XXIX**

INTRODUCTION 1

LITERATURE REVIEW 5
1. ANIMAL WELFARE 7
1.1. Importance of animal welfare 7
1.2. General description of animal welfare 8
1.3. Welfare in dairy cattle 10
2. STRESS 10
2.1. Stress definition 11
2.2. The stress system: stressor, stressor perception and stress response 12
2.2.1.Stressor and stressor perception 12
2.2.2. Stress response 13
2.3. General implications 15
2.4. The role of stress in animal welfare 17
2.5. Stress in cattle 18
3. STRESS ASSESSMENT 19
3.1. Activity of Hypothalamic-Pituitary-Adrenal axis 19
3.1.1. Glucocorticoids 20
3.2. Other 20
3.2.1. Behaviour 20
3.2.2. Other stress indicators 21
3.3. Stress assessment in cattle 21
4. MATRIXES FOR CORTISOL DETECTION 22
4.1. Single-point matrixes 23
4.1.1. Blood 24
4.1.2. Saliva 24
4.2. Intermediate matrixes 25
4.2.1. Faeces 25
4.2.2. Urine 25

4.2.3. Milk 26
4.3. Cumulative matrixes 26
4.4. Cortisol detection in cattle 27
5. HAIR MATRIX FOR CORTISOL DETECTION 27
5.1. Hair characteristics 27
5.1.1. Mechanisms for cortisol incorporation in hair 28
5.2. Hair cortisol analysis: background, pros and cons 29
5.2.1. Background 29
5.2.2. Pros and cons 30
5.2.3. State of the art of hair cortisol detection in cattle 31
6. CORTISOL DETECTION METHODS 31
6.1. Immunoassays 32
6.1.1. Radioimmunoassay 32
6.1.2. Enzyme immunoassay and enzyme-linked immunosorbent assay 32
6.2. Others 33
6.2.1. Near infrared reflectance spectroscopy 33
7. VALIDATION OF CORTISOL DETECTION METHODOLOGIES 35
7.1. The importance of validation 35
7.2. Validation test for immunoassays 35
7.2.1. Specificity 35
7.2.1. Linearity 36
7.2.1. Accuracy 36
7.2.1. Precision 36
7.2.1. Range 36
7.2.1. Sensitivity 37
7.3. Validation of near infrared reflectance spectroscopy 37
8. REFERENCES 38

OBJECTIVES 55

Chapter I. Hair cortisol detection in dairy cattle by using EIA: protocol validation and correlation with faecal cortisol metabolites 59
1. ABSTRACT 62
2. IMPLICATIONS 63
3. INTRODUCTION 63
4. MATERIAL AND METHODS 64
4.1. Animals, housing conditions, diet and handling 64
4.2. Faecal sampling and steroid extraction 64
4.3. Hair sampling and hormone extraction 65

4.4. Steroid analysis and validation tests ... 66
4.5. Statistical analysis ... 66
5. RESULTS AND DISCUSSION ... 67
5.1. Validation tests ... 67
5.2. Hair cortisol concentrations ... 68
5.3. Hair cortisol and faecal cortisol metabolites ... 69
6. CONCLUSION ... 70
7. REFERENCES ... 71

Chapter II. Progesterone in hair from dairy cattle by EIA: protocol validation ... 77
1. ABSTRACT ... 80
2. INTRODUCTION ... 81
3. MATERIAL AND METHODS ... 81
3.1. Animals and hair sampling ... 81
3.2. Hair wash and progesterone extraction ... 81
3.3. Progesterone analysis and validation tests ... 82
4. RESULTS AND DISCUSSION ... 83
5. CONCLUSION ... 84
6. REFERENCES ... 85

Chapter III. Acute ACTH-induced elevations of circulating cortisol do not affect hair cortisol concentrations in calves ... 87
1. ABSTRACT ... 90
2. INTRODUCTION ... 91
3. MATERIAL AND METHODS ... 91
3.1. Animals and study design ... 91
3.2. Housing conditions, diet and performance ... 92
3.3. ACTH administration and blood sampling ... 92
3.4. Hair sampling ... 93
3.5. Hair cortisol extraction ... 93
3.6. Cortisol analysis and EIA validation tests ... 94
3.7. Statistical analysis ... 95
4. RESULTS AND DISCUSSION ... 95
4.1. ACTH administration and serum cortisol levels ... 95
4.2. ACTH administration and hair cortisol levels ... 95
4.3. Correlation between hair samples ... 98
5. CONCLUSION ... 98
6. REFERENCES ... 99

Chapter IV. Hair cortisol and progesterone detection in dairy cattle: interrelation with physiological status and milk production ... 103
1. ABSTRACT ... 106
2. INTRODUCTION ... 107
3. MATERIAL AND METHODS ... 108
3.1. Study design ... 108
3.2. Animals ... 108
3.3. Housing and handling conditions ... 109
3.4. Hair sampling and storage ... 109
3.5. Hair washing and steroid extraction ... 110
3.6. Hormone detection and validation tests ... 110
3.7. Animal's data collection ... 111
3.8. Statistical analysis ... 112
4. RESULTS ... 113
4.1. Hair steroid concentrations ... 113
4.2. Hair steroid concentrations and physiological data ... 114
4.3. Hair steroid concentrations and milk data from the two previous months ... 114
4.4. Hair steroid concentrations and milk data from the whole lactation ... 115
5. DISCUSSION ... 115
5.1. Hair cortisol concentrations and physiological data ... 115
5.2. Hair cortisol concentrations and milk production and composition ... 117
5.3. Hair progesterone concentrations and physiological data ... 118
5.4. Interrelation between hair steroid concentrations ... 119
6. CONCLUSION ... 120
7. REFERENCES ... 121

Chapter V. Prediction of cortisol and progesterone concentrations in hair from cow by near infrared reflectance spectroscopy (NIRS) ... 127
1. ABSTRACT ... 130
2. INTRODUCTION ... 131
3. MATERIAL AND METHODS ... 132
3.1. Study design ... 132
3.2. Animals and hair sampling ... 132
3.3. Hair washing ... 133
3.4. Hair trimming and NIRS analysis ... 133
3.5. Steroid extraction ... 133
3.6. Steroid determination by EIA ... 134
3.7. Spectral data analysis ... 134
3.8. Data analysis ... 135

4. RESULTS 135
4.1. Description of hair spectrum by NIRS 135
4.2. NIRS calibration and validation 136
4.3. Comparison of HSC and quartile distribution between EIA analyses and NIRS prediction in trimmed hair 137
5. DISCUSSION 138
6. CONCLUSION 139
7. REFERENCES 140

GENERAL DISCUSSION 145
1. Biochemical validation of standardized protocol for hair cortisol detection 147
2. Feasibility of hair cortisol concentrations to representan integrative value of long-term adrenal activity 150
3. Potential uses of hair cortisol determination in dairy cattle production 154
4. Evaluation of the near infrared reflectance spectroscopy technique for hair cortisol quantification in cows 157
5. Effects of hair secondary characteristics on hair cortisol measurement 158
6. Findings on hair progesterone detection in cattle 162
7. Hair cortisol detection in cattle: present and future. A personal view 163
8. REFERENCES 164

FINAL CONCLUSIONS 175

INDEX OF TABLES

General introduction

Table 1. Central and peripheral functions of the stress response 15

Table 2. The five major systems involved in the stress response and its effects 16

Table 3. Comparative of main mammal matrixes' attributes regarding their cortisol detection ... 23

Chapter I. Hair cortisol detection in dairy cattle by using EIA: protocol validation and correlation with faecal cortisol metabolites

Table 1. Cortisol concentrations (pg cortisol/mg hair) from hair sampled at the beginning (d_0) and at the end (d_{25}) of the study 68

Chapter III. Acute ACTH-induced elevations of circulating cortisol do not affect hair cortisol concentrations in calves

Table 1. Hair cortisol concentrations (HCC, pg/mg) from forehead and hip depending on treatment (control and ACTH-injected) at D_0 (initial) and D_{14} (final) of the study 96

Chapter IV. Hair cortisol and progesterone detection in dairy cattle: interrelation with biological status and milk production and composition

Table 1. Number of parturitions of the cows used in the study 109

Table 2. Milking control data from the same month (17 November) and the previous months (17 October) of hair sampling 111

Table 3. Number of pen changes during the two months previous to the hair sampling 111

Table 4. Milk production parameters obtained for the whole lactation (until 124 days after hair sampling) 112

Table 5. Hair cortisol and progesterone concentrations from milking cows by enzyme immunoassay 113

Chapter V. Prediction of cortisol and progesterone concentrations in hair from cow by near infrared reflectance spectroscopy (NIRS)

Table 1. Physiological and productive data of the cows used in this study 132

Table 2. Reference values of cortisol and progesterone concentrations in cow hair samples determined by enzyme immunoassay 134

Table 3. NIRS calibration. Spectral pre-treatments and statistics of the best model obtained for predicting the concentrations of cortisol and progesterone in cow hair samples...................... 136

Table 4. Relationship of data distribution in quartiles between a reference method (EIA) and trimmed NIRS predictions for hair cortisol concentrations 137

Table 5. Relationship of data distribution in quartiles between a reference method (EIA) and trimmed NIRS predictions for hair progesterone concentrations 137

INDEX OF FIGURES

General introduction

Figure 1. Hypothalamic-pituitary-adrenal axis 14

Figure2. Mechanisms for cortisol incorporation in hair 28

Chapter I. Hair cortisol detection in dairy cattle by using EIA: protocol validation and correlation with faecal cortisol metabolites

Figure 1. Parallelismbetweenlinesfromthe $\log_{10}$ of thestandardandsample pool curves 67

Figure2. Simple linear regressionbetween $\log_{10}$transformedvalues of whitehairsamplesat d_{25}andtheaverage of all faecalsamples ($R^2 = 0.58$, $P < 0.001$) 69

Chapter II. Progesterone detection in hair from dairy cattle by EIA: protocol validation

Figure 1. Linearity of dilution of progesteroneconcentrationsfromhairextracts 83

Figure2. Parallelismbetweenlinesfromthestandardandsample pool curves 84

Chapter III. Acute ACTH-induced elevations of circulating cortisol do not affect hair cortisol concentrations in calves

Figure 1. Parallelismbetweenlinesfrom $\log_{10}$ of thestandardandsample pool curvesshowingimmunologicalsimilaritiesbetweenstandardand pool hormones 94

Figure2. Serum cortisol concentrations (ng cortisol/ml serum; presented as a mean + standard error) immediately prior to ACTH administration (b_{0h}), onehour post administration (b_{1h}) andthreehours post administration (b_{3h}) from control groupand ACTH-administratedgroupat $D_0$and D_7. 97

Chapter V. Prediction of cortisol and progesterone concentrations in hair from cow by near infrared reflectance spectroscopy (NIRS)

Figure 1. Average of spectrafromtrimmed (greyline) and non-trimmed (blackline) hairsamplesby NIRS 135

Figure2. Linear relationshipbetweennearinfraredreflectancespectroscopy (NIRS) predicted data andchemicalreference data, determinedby EIA, for cortisol (a) andprogesterone (b) concentrations in trimmedcowhairsamples. 136

ABBREVIATIONS

ACTH	adrenocorticotropic hormone
AV	avoidance
AVP	arginine vasopressin
BW	body weight
CNS	central nervous system
CRH	corticotropin-releasing hormone
CV	coefficient of variation
DIM	days in milk
DNA	deoxyribonucleic acid
DT	detrend
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
FAWC	Farm Animal Welfare Council
FCMC	faecal cortisol metabolites concentrations
HCC	hair cortisol concentrations
HPA	hypothalamic–pituitary– adrenal
HPC	hair progesterone concentrations
HPLC	high-performance liquid chromatography
HSC	hair steroid concentrations
MPLS	modified partial least square
MSC	multiple scatter correction
NIR	near infrared reflectance
NIR	near infrared reflectance
NIRS	near infrared reflectance spectroscopy
PVN	hypothalamic paraventricular nucleus
R	reflectance
RER	range error ratio
RIA	radio immunoassay
RPD	ratio of performance to deviation
SC	somatic cell
SCC	serum cortisol concentrations
SD	standard deviation
SEC	standard error of calibration
SEP	standard error of prediction
SNS	sympathetic nervous system
SNV	standard normal variate

LIST OF SPECIES MENTIONED

Name used	Scientific name
cattle	*Bostaurus*
cat	*Feliscatus*
dog	*Canis lupus familiaris* or *Canisfamiliaris*
ewes (sheep)	*Ovisaries*
giant panda	*Ailuropodamelanoleuca*
grizzly bears	*Ursusarctos*
humans	*Homo sapiens sapiens*
red deer	*Cervuselaphus*
caribou, reindeer	*Rangifer tarandus*
rhesus macaques	*Macacamulatta*
western lowland gorilla	*Gorilla gorillagorilla*
wolves	*Canis lupus*

INTRODUCTION

In the last decades, social and political interest in animal welfare and welfare management has increased substantially (de Vries et al., 2011). Once we have realised and recognised that animals are sentient beings, society is starting to face this moral challenge considering that our lifestyle use or condition all of them. In this context, animal welfare scientists have been given the task of defining objective and quantifiable parameters of an animal's welfare status under given conditions and providing solutions to animal welfare problems identified by society(Ohl and van der Staay, 2012). Animal welfare is a complex concept that includes animal's normal biological functioning but also animal's emotional state and their possibility to express certain normal behaviours (Fraser et al., 1997). Stress is a decisive factor in animal welfare because of its impact and mainstreaming. Although stress response evolved to maintain animal's homeostasis (Moberg and Mench, 2000), chronic stress is prejudicial and a serious threat to animal's well-being. Welfare-threatening stress appears when inadequate, inappropriate, or excess activation of the compensatory stress systems does not maintain homeostasis and resistance fails. In this condition, somatic and psychological damage results as the biological cost of these responses can ultimately affect the growth, health and reproduction of the animal, among others (Barnett and Hemsworth, 1990). Moreover, they are often associated with negative emotional experiences, such as anxiety, fear or anger (Clark et al., 1997).The hypothalamic-pituitary-adrenal-axis is a key element in the stress response and ends with glucocorticoid secretion in the bloodstream by the adrenal gland (Buchanan, 2000). The measurement of glucocorticoid concentrations associated to adrenal activity is the most used methodology to assess animal's stress status. Cortisol, the main glucocorticoid in cattle, can be detected in blood (serum or plasma), saliva, urine, milk or faeces, but none of these matrixes is able to provide a measure of long-term adrenal activity. In contrast, hair matrix for cortisol detection has gained much attention in the last years. Hair is a keratin matrix and accumulates hormones during all its growth. Consequently, it is a matrix with the potential to provide an integrative value of long-term circulating cortisol levels becoming a measure of long-term adrenal activity (Stalder and Kirschbaum, 2012). Moreover, hair is present in almost all mammals, it is abundant and its sampling is non-invasive, safeand easy. Other advantage of hair matrix is their stability: hair does not have special storage requirements and can be stored at

room temperature (Macbeth et al., 2010). Despite their promising capacity of long-term adrenal activity monitoring and practical advantages, hair is a relatively new matrix and an accurate knowledge of hair as a matrix that accumulates cortisol is essential for its future use in chronic stress research. Consequently, several studies are necessary to validate hair biologically before confirming what it looks like: hair is perhaps the best matrix for cortisol detection in studies of long-term stress assessmentassociated to animal welfare and animal production.Over the recent decades the dairy industry in Europe has undergone profound changes and has been demonstrated to be particularly supportive of welfare research, perhaps conscious of the importance of good welfare standards for their productivity and consumer's view.There exists the need for evaluating the use of hair cortisol measurements as a potential tool to assess welfare in cattle.

LITERATURE REVIEW

1. ANIMAL WELFARE

1.1. Importance of animal welfare

The human being has an exceptional ability to know and study the other species (Harnad, 2016) but at the same time to understand and empathise with them (Rizzolatti and Craighero, 2005). The union of these two capacities is what initially motivated the idea of animal welfare science. The capacity to empathise with animals is inherent to the human being and some cultures acquired an exceptional sensitivity to the life of other species thousands of years ago. However, in a global perspective, humanity reached recently consensus that non-human animals (at least some) are also sentient beings. Regarding farm animals, mentioning the importance of Ruth Harrison and her book *Animal Machines: The New Farming Industry* in 1964 cannot be avoided. Therefore, since 1960s society is starting to face a fact: once we have realised and recognised that animals are sentient beings, we had to choose how to deal with this reality and this moral challenge considering that our lifestyle use or condition all of them.

In the last decades, social and political interest in animal welfare and welfare management has increased substantially (de Vries et al., 2011). Whenever we act upon the principle of respect for the intrinsic value of an animal, independent of its extrinsic value (to us), we make a moral judgement (Webster, 2001). Therefore, deciding how to treat animals requires ethical decision-making. However, the confidence about an ethical conclusion increases if the arguments that lead to it are supported convincingly by facts, scientific deductions, reason and ethical argument, and arise from a process of genuine discussion (Rushen et al., 2008). Consequently, animal welfare scientists have been given the task of defining objective and quantifiable parameters of an animal's welfare status under given conditions and providing solutions to animal welfare problems identified by society (Ohl and van der Staay, 2012). In other words, animal welfare science must provide the framework of knowledge in which society is based to perform its decisions about the level of welfare that should be afforded to animals within his charge.

The quality of life of millions of animals will depend on the result of these decisions, which at the same time are defining us as a society and as a species.

1.2. General description of animal welfare

Animal welfare is an abroad, complex concept in constant evolution that uses a multidimensional approach. Although there is not a single or ultimate definition, it is generally accepted that animal welfare includes these three interrelated elements (Fraser et al., 1997):

- Animal's normal biological functioning: especially related to good health and nutritional status
- Animal's emotional state: it includes the absence of negative emotions such as pain, fear and distress
- Animal's possibility to express certain normal behaviours: the feasibility to satisfy the behavioural needs

One of the most famous and useful standpoint to animal welfare is the "Five freedoms" principle created by the currently renamed Farm Animal Welfare Council (FAWC). The principle states that an animal's welfare is ensured when the five conditions are met (Farm Animal Welfare Council, 1993, 1992). Following, the five conditions are presented with five provisions also provided by the FAWC:

- Freedom from thirst, hunger and malnutrition (by ready access to a diet to maintain full health and vigour)
- Freedom from thermal and physical discomfort (by providing a suitable environment including shelter and a comfortable resting area)
- Freedom from pain, injury and disease (by prevention or rapid diagnosis and treatment)
- Freedom from fear and distress (by providing sufficient space, proper facilities and the company of the animal's own kind)
- Freedom to express normal behaviour (by ensuring conditions which avoid mental suffering)

The five freedoms (which are actually eleven) were especially designed for farm animals although it has been adapted to the assessment of welfare in other animals (Bayne and Turner, 2014).

The interrelation between all aspects of animal welfare is crucial to understand its whole concept (Fraser, 2008). For instance, a disease can cause suffering to an animal and impair the expression of some normal behaviours. However, although the emotional state of the animal is a key factor in animal welfare, the absence of negative emotions does not ensure a good animal welfare. Even without suffering pain or any negative feeling, the welfare of an animal with any impairment related to any other mentioned element should be considered poor or diminished (Broom, 1991a).

Finding other ways to define animal welfare, Broom (1986) stated that the welfare of an individual is its state as regards its attempts to cope with its environment. Interestingly, this definition interrelates very well with most of stress definitions (Section **2. STRESS**) and allows the proposal that good animal welfare is the biological state in which an animal would desire to be investing its biological resources (e.g. behavioural, physiological, immunological) to reach it.

Importantly, welfare is a characteristic of an animal, not something that is given to it (Broom, 1991a); and consequently it is an individual issue or characteristic (Wickins-Dražilová, 2006). Despite the obvious influence of factors such as species, gender or age, similar animals can be in different welfare status under the same conditions, as its behavioural, physiological and cognitive capacities to face an environment are different. This is easy to understand with an example using animal nutrition, where cows (*Bostaurus*) from the same characteristics under the same conditions can need different amounts of feed to satisfy their energy needs. However, this is also happening with other aspects less clear such as behavioural needs or stress. Therefore, this situation should be cautiously considered when performing animal welfare studies and future generalisations with them.

It should be noted that animal welfare is a continuous concept that ranges from very poor (or very bad) to very good and that can be scientifically measured (Broom and Johnson, 1993). Ethical decision on what should be considered as acceptable or not for the animal must be taken after welfare has been scientifically evaluated (Broom, 1991b).

Despite all above mentioned, the study of animal welfare continues to struggle with the problem about how to define animal welfare but also with another problem interrelated: how to determine which measures should be used to evaluate it (Moberg and Mench, 2000).

Behavioural, physiological, immunological, injury, disease, mortality risk, growth and reproduction measures are all of value in assessing welfare (Broom, 1991b). However, the definitive parameter or indicator of animal welfare does not exist because of its high complexity and mainstreaming. Although the scientific community is widely discussing the best measures for animal welfare, their characteristics, strengths and weaknesses, the most repeated recommendation is to use several (Botreau et al., 2007; Broom, 1991a; Fraser, 2009).

Therefore, it is evident that animal welfare assessment is truly complicated. For example, evaluating the severity or physiological impact of a disease can be relatively easy by using biomedical indicators (e.g. blood analysis) and it is evident that the welfare of the diseased animal is diminished. However, determine if the welfare of an animal with a mild disease is better or worse than the welfare of an animal with severe malnutrition could be extremely difficult. This question about animal welfare is indeed a question about the animal's subjective experience, but in these cases scientists are unable to provide empirical answers (Fraser et al., 1997). This inability arises because there is not yet any generally accepted method to combine different negative states into overall indices but neither to quantify the pleasure experienced by an animal or to balance suffering against enjoyment. Interestingly, Fraser (1995) considered that instead of attempting to measure animal welfare, the role of science should be seen as identifying, rectifying and preventing welfare problems. However, different approaches have proposed with the aim to face the challenge of global animal welfare assessment, being the Welfare Quality® project one of the most interesting and ambitious (Botreau et al., 2009). Moreover, some new research lines are focusing in markers of positive emotions to assess animal welfare (Boissy et al., 2007).

1.3. Welfare in dairy cattle

Over the recent decades the dairy industry in Europe has undergone profound changes: economic pressures, technological innovations, demographic shifts, consumer expectations, and an evolving regulatory framework have contributed to the impetus for changes in the global dairy industry (Barkema et al., 2015; Jacobs and Siegford, 2012; von Keyserlingk et al., 2009). All these changes affected the welfare of dairy cows and calves. For instance, the number of farms has decreased considerably, whereas herd size has increased (Barkema et al., 2015). The management and housing conditions of big size herds threat the animal welfare in a different way (not necessarily better or worse) than small or medium herds (Phillips, 2002). This underlines that dairy cattle research is also entering in a period of changes in which the main spotlights of its attention may vary. Fortunately, the dairy industry has been demonstrated to be particularly supportive of welfare research (Walker et al., 2014).

Some of most important current issues concerning animal welfare in dairy cattle production related with animal handing and management are: tail docking, absence or insufficient anaesthesia or analgesia in processes such as dehorning, castration or postpartum, timing of cow-calf separation and individual housing of calves, among others (Barkema et al., 2015; Stafford and Mellor, 2011; von Keyserlingk et al., 2009; Weary et al., 2006). Regarding health, laminitis, mastitis, dystocia in cows and diarrhoea and high levels of mortality in suckling calves are some of the pathological problems with great impact in animal welfare from cattle (Rushen et al., 2008; Stull and Reynolds, 2008). Periods of dairy cattle productive cycle like peripartum or the dry period (and the dry-off) are also threatening cow's animal welfare (Rushen et al., 2008). Moreover, the debate about the impact of the lack of access to pasture is increasing (Arnott et al., 2016; Legrand et al., 2009) and the effect of heat stress in dairy cows still being a hot topic (Roland et al., 2016; Staples and Thatcher, 2000).

Other aspects of animal welfare in dairy cattle not mentioned previously must be considered as potential key factors deserving scientific attention. For example, considerable research in pain has been done but not much in fear, anxiety or distress (von Keyserlingk et al., 2009; Weary et al., 2006) and it seems reasonable that future efforts will be focus in these welfare issues.

Finally, the effect of ordinary citizens on the welfare of dairy cattle should not be forgotten: the pressure to governments for producing laws ensuring some animal welfare standards or the tendency of consumers during the last years to increase their interest in the welfare of the animals producing their bought milk or cheese, for instance, influence enormously dairy cattle's animal welfare.

2. STRESS

"*I have been so stressed trying to finish my thesis on time that I need holidays again*". This sentence, including the concept of stress, could be easily understood for most people regardless whether they have scientific background in stress or not. Curiously, the term "stress" had a strong wide acceptation in popular

culture. Mason (1975) suggested that this may be because stress concept solved intuitively the need for describing a biological phenomena not adequately covered by other generic terms. Unfortunately, the difficulty to define scientifically the concept of stress has been proportionally to its wide use and popular acceptance. This paradox also exists within scientific community, which has not been able to reach an agreement in stress definition but this situation has not stopped stress from being one of the most cited constructions in biomedical literature in recent years (Le Moal, 2007; Levine, 2005).

There is some controversy over if Walter B. Cannon or Hans Seyle were the first to use the term stress in a physiological or biomedical context (Levine, 2005). However, it seems clear that Seyle was the responsible for stress popularisation after mentioning in 1936 a "biologic stress syndrome" in the definition of the so-called "General Adaptation Syndrome" what could be considered one of the first scientific descriptions of the stress response (Clark et al., 1997; Mason, 1975).

2.1. Stress definition

Uncountable attempts to define stress have been made. McEwen (2007) defined stress as a real or interpreted threat to the physiological or psychological integrity of an individual that results in physiological and/or behavioural responses. Moberg(2000) defined it as the biological response elicited when an individual perceives a threat to its homeostasis. Finally, Chrousos(2009) described stress as a state in which homeostasis is actually threatened or perceived to be so.

Interestingly, although these three definitions are similar, they are defining stress as a "threat that cause responses", as a "response" and as a "state", respectively. One of the main reasons why stress definition is still being subject of debate in scientific community is because we use stress for defining a composite, multidimensional concept (Le Moal, 2007; Levine, 2005; Moberg, 2000; Romero et al., 2015). It includes at least three components that can be identified, are interrelated and interact:

- The input or threat (stress stimulus or "stressor")
- The processing systems (the interpretation, including the subjective experience of stress)
- The output (stress responses)

A fourth element should be included as another key component in the understanding of stress concept: "homeostasis". Used as a more complex and encompassing concept than "integrity", homeostasis refers to the complex dynamic equilibrium that all living organisms try to maintain with the aim to live and reach its biological potential (Chrousos, 2009; Levine, 2005).

Again, several authors emphasized the ambiguity and circularity of the definition of stress in terms of a threat to homeostasis in general as virtually all activities of an organism directly or indirectly concern the defence of homeostasis (Koolhaas et al., 2011; Levine, 2005; Romero et al., 2009). McEwen et al. (2010) exemplify this problem by arguing that biological processes like the beginning of lactation for a cow undergo morphological, physiological and behavioural changes (involving regulation of gene expression) and although homeostatic set points will have changed from pre-lactation levels, none of this is essential for the

maintenance of cow's homeostasis."*Of course the cow lactating must do so to reproduce successfully. But the adjustments in homeostasis that occur during this life cycle events are to accommodate changed physiology as part of the predictable life cycle, not simply responses to deviations from some set point that maintains life processes*" stated.

Romero and colleagues (2009) presented a different terminology and a new model for conceptualizing and describing the impact of stress on the body, combining traditional notions of stress and homeostasis with the more recent terminology of "allostasis" and "allostatic load". Recent discussions are published regarding the new terminology to solve (or at least improve) the problem with ambiguities and rigidity in homeostasis and stress definitions and to include a framework allowing the incorporation of perturbations in major events of the predictable life cycle (such as reproduction, migration etc.) also as potential sources of stress (Koolhaas et al., 2011; McEwen and Wingfield, 2010; Romero et al., 2009). As this discussion still ongoing and a deeper understanding of these new concepts are not essential in the comprehension of this thesis, the concepts of allostasis and allostatic load will not be presented here. However, the lectures of Romero et al. (2009), McEwen and Wingfield (2010) and Koolhaas et al. (2011) are recommended if lector wants to obtain deeper information. Interestingly, Kopin et al. (1988) described many years ago stress as a state in which expectations—whether genetically programmed or acquired—do not match current or anticipated perceptions of the internal or external environment. They probably used "expectations" with the aim to encompass not only pure homeostatic or survival needs but also life cycle processes.

Therefore, in this thesis stress could be considered an heterogeneous assortment of phenomena that appears when a cow or calf perceive (being real or not) a potential negative alteration of its life expectations (genetic and acquired, including survival and life cycle) and respond to it by changing their state into a most appropriate one to face the threat and reach expectations again.

Finally it should be noted that, despite it is a source of confusion and an obstacle to precise scientific communication, the controversy over the definition of stress should not affect the validity of the underlying scientific and clinical observations or concepts (Clark 1997). The stress research is complex, wide and sometimes contradictory, but there are not many other fields in animal and human research with such degree of mainstreaming and thus, potential positive impact if research on it is performed.

2.2. The stress system: stressor, stressor perception and stress response

Before starting the following section, it should be noted that the aim of this review is to provide a brief overview of the stress system and not to provide a deep review. The neuroendocrine factors involved in the stress response presented here are only the main ones but lector should be aware that the stress system is more complex than presented below.

2.2.1. Stressor and stressor perception

The stressor is the input or supposed threat perceived by the individual that activate the stress response and is also called stress stimulus. Strictly speaking, the only characteristic in common between all stressors is that

activate the stress response. The description of the stressor is extremely connected with stressor perception because it's the central nervous system (CNS) which gives to a stimulus the status of stressor. Therefore, the identification of a stressor can be only performed through the detection of stress response and never inversely. Interestingly, and this is a key point in the understanding of the stress, whether or not the stimulus is actually a threat in the homeostasis is not important; it is only the perception of a threat that is critical (Levine, 2005; Moberg, 2000). This is why psychological stressors can be so devastating and the influence of individual perception in front potential stress stimuli so determinant (Buchanan, 2000; Grandin and Shivley, 2015). The importance and impact of a stressor (even if the stimulus can be considered a stressor or not) is so depending on individual factors that the classification and extrapolation of stressors from one individual to other should be done very carefully. For instance, genetic, temperament, passed experiences or age are some of several factors identified that affects the perception of a stimulus as a stressor or not and its severity (Grandin and Shivley, 2015; Moberg, 2000).

When exposed to a stimulus, several brain centres can be activated, including, among others, the locus coeruleus in the brain stem, the paraventricular nucleus in the hypothalamus, and amygdala and hippocampus in the limbic system (Charmandari et al., 2005; Fink, 2007). If these brain centres activates, the stimulus is considered as a stressor and the stress response triggers. After several neuroendocrine processes, the activation of these centres leads to increase activity of sympathetic nervous system (SNS) and hypothalamic-pituitary-adrenal* (HPA) axis (Fink, 2007; Sapolsky et al., 2000; Sheriff et al., 2011). Both SNS and HPA axis are homeostatic mechanisms that induce behavioural, autonomic, neuroendocrine and/or immunological responses with the aim to allow an individual to cope with a stressor and eventually turn off the stress response through negative feedback (Fink, 2007; Moberg, 2000).

2.2.2. Stress response

As introduced previously, the stress response is mediated by the stress system, which is located in both the central nervous system and peripheral organs (Chrousos, 2009; Habib et al., 2001). Although the main central effectors of the stress system are highly interconnected and complex (Chrousos, 2009), the neuroendocrinology of stress response has been historically separated in the commonly called Fight-or-Flight response (associated to the increase of SNS activity) and the HPA-axis response, which does not have specific name and is usually called simply as stress response (Habib et al., 2001; Le Moal, 2007; Romero and Butler, 2007). In the same way, although there are many hormones that have been identified as playing a role in the stress response, two categories of hormones are thought to form the central components of the endocrine response: the catecholamines (epinephrine and norepinephrine, also known as adrenalin and noradrenalin, respectively) and the glucocorticoids (cortisol and corticosterone). These are the main hormones associated to the SNS and HPA-axis activity, respectively (Charmandari et al., 2005; Romero and Butler, 2007).

The Fight-or-Flight is the first response and appears few seconds after a stress stimulus (McEwen and Wingfield, 2010). After a neuroendocrinological cascade, catecholamines are released by both the adrenal medulla and nerve terminals of the sympathetic nervous system. The suite of responses mediated by catecholamines is designed to help the animal survive an acute threat such as an attack by a predator or

* also called hypothalamic-pituitary-adrenocortical

conspecific competitor. They not only activate beneficial responses such as increasing alertness and providing energy to muscles, but also inhibit processes, such as digestion, that can be superfluous during an acute emergency (McCarty, 2016; Romero and Butler, 2007).

On the other hand, the response mediated by the HPA axis is less rapid than the latter presented. Although it started at the same moment of stressor perception, it last few minutes – three to five minutes in vertebrates (Sapolsky et al., 2000; Sheriff et al., 2011) - to finish the hormonal cascade with the final result of glucocorticoid secretion into bloodstream by adrenal cortex (Sapolsky et al., 2000). Briefly, when an animal perceives a stressor, the hypothalamic paraventricular nucleus increases the release of corticotropin-releasing hormone (CRH) and other secretagoges such as arginine vasopressin (AVP) or analogues. These hormones stimulate the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland into the bloodstream (Sheriff et al., 2011). Circulating ACTH is the key regulator of glucocorticoid secretion as it stimulates the adrenal cortex to secrete large quantities of them (Habib et al., 2001). Other hormones, cytokines, and neuronal information from the autonomic nerves of the adrenal cortex may also participate in the regulation of cortisol secretion (Charmandari et al., 2005; Tsigos and Chrousos, 2002), however, it should be noted that HPA-axis activity and adrenal activity can be used as synonyms in stress context. The suite of glucocorticoid effects is believed to help the animal recover from a stressor, shut down those systems that can profitably be delayed until the danger has passed, and prepare the animal for potential subsequent stressors (Romero and Butler, 2007). A schematic model of the HPA axis is shown in the Figure 1.

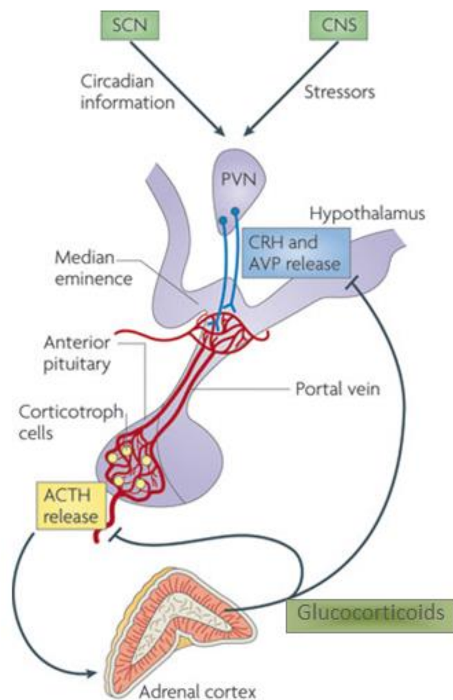


Figure 1. Hypothalamic-pituitary-adrenal axis. *Source*: Lightman and Conway-Campbell (2010).

One of the main differences in the responses mediated by catecholamnes and glucocorticoid hormones is that glucocorticoids have the capacity to promote or inhibit gene transcription and thus, to stimulate or inhibit protein production. Thus, the delay of glucocorticoid secretion after the onset of a stressor and the effects of newly produced proteins allow the stress response to create both an immediate and a longer-term response to stressors by the effects of chatecolamines and glucocorticoids, respectively (Romero and Butler, 2007). The central and peripheral functions of the stress response are summarised in Table 1.

Table 1. *Central and peripheral functions of the stress response*

Functions of the central nervous system	Peripheral functions
- Facilitation of arousal, alertness, vigilance, cognition, attention and aggression - Inhibition of vegetative functions (e.g. reproduction, feeding, growth) - Activation of counter-regulatory feedback loops	- Increase of oxygenation - Nutrition of brain, heart and skeletal muscles - Increase of cardiovascular tone and respiration - Increase of metabolism (catabolism, inhibition of reproduction and growth) - Increase of detoxification of metabolic products and foreign substances - Activation of counter-regulatory feedback loops (includes immunosuppression)

Source: Chrousos(2009).

Until now, the stress response has been presented as a non-specific response that appears in a similar way in front all stressors. This assumption, that stress causes only a non-specific response to stimulation, has often led researchers to use the word stress to describe virtually any type of stimulation that was applied to their subjects, as if all forms of stimulation were functionally equivalent (Clark et al., 1997). However, later research has documented that each form of stimulation has nonspecific but also specific effects on the animal. It is demonstrated that the stress response has some specificity toward the stressor that generates them (Clark et al., 1997; Tsigos and Chrousos, 2002). For instance, the CNS can adapt the behavioural aspect of the stress response to the stressor. Consequently, although the activation of the HPA axis is the most widely recognized nonspecific indicator of stress, other neuroendocrine responses occur depending on specific characteristics of the stressor and the individual responding to it (Carlstead et al., 1993).

2.3. General implications

In previous sections the stress response have been presented as an advantageous mechanism in natural selection (Table 1), providing means to anticipate and react rapidly to threats to the survival or well-being of the organism, preserving homeostasis by short-term adjustments in activities of several systems (Chrousos et al., 1988; Nesse et al., 2016). Facing stressors is a part of life and stress-response mechanisms evolved to help organisms to cope with them (Moberg, 2000). Therefore, some logical questions rise at this point: why it is stress so commonly considered as something negative? Why is stress associated to animal welfare? Perhaps the most appropriate question should be: when the stress response stops being something beneficial and becomes something prejudicial?

The key to differentiate beneficial or “positive” stress from prejudicial or “negative” stress is the biological cost of the stress (Moberg, 2000). When the biological cost of the stress response is met by reserves with no long-term impact on other biological functions, this is positive stress. In contrast, welfare-threatening stress appears when stress response does not maintain homeostasis and resistance fails due to inadequate, inappropriate, or excess activation of the compensatory systems. In this condition, somatic and psychological damage results as the biological cost of these responses can ultimately affect the growth, health and reproduction of the animal, among others (Barnett and Hemsworth, 1990). Moreover, they are often associated with negative emotional experiences, such as anxiety, fear or anger (Clark et al., 1997). Thus, stress jeopardizes the animal’s welfare only if the stress results in some significant biological change that

places that animal's well-being at risk (Moberg, 2000). It occurs when an animal cannot successfully adapt, cope or habituate to the stress stimulus (Clark et al., 1997). A list of major systems involved in the stress response and its effects is shown in table 2. It should be noted that the table only provide an overall view of the main effectors and effects of the stress response.

Table 2. *The five major systems involved in the stress response and its effects*

Physiological system	Physiological mediator (stress effectors)	Non-stress physiological adjustments (circadian or seasonal changes)	Acute and mild stress effects (eustress)	Long-term or high intensity stress effects (distress)	Extreme of long-term or high intensity stress effects (distress)
Central nervous system	Neurogenesis Dendritic arborisation Neutrotransmit-ter concentrations Cytokines	Life-history changes in neural networks Learning and memory	Increase neurotransmis-sion (titers or receptors) Increase learning and memory	Neuronal atrophy/death Depression Decrease learning and memory	Post traumatic stress disorder
Hypotalamic-pituitari-adrenal axis	Glucocorticoids Adrenocortico-tropic hormone	Daily or seasonal life-story needs: - Energetic needs - Behavioral needs - Preparative needs	Inhibit immune system Energy mobilization Change behaviour Inhibit reproduction Inhibit growth	Immunosupres-sion Diabetes Muscle breakdown Reproductive suppression Decreased survival	Energy disregulation Water balance failure Catecholamine insufficiency Decreased survival
Sympathetic nervous system	Catecholamines	Life-story energy needs	Fight-or-flight Energy mobilization	Hypertension Myocardial infarction Muscle breakdown	Hypotension Lethargy Decreased survival
Behaviour	Foraging/feeding Locomotion Migration Conspecific aggression	Life-history changes: Energy needs Energy availability Predator presence Mate access	Fleeing behaviour Freezing behaviour Increase/decrease foraging Increase food intake Increase vigilance Conspecific fighting	Tonic immobility Obesity Anxiety Fear Aggressiveness	
Immune	Prostaglandin T-cell activation Antibody titers Cytokines	Seasonal ability to fight infection	Mobilization of immune system	Immunosupres-sion Autoimmune problems	Immune failure

Source: modified from Romero et al. (2007).

Research has always struggled with this dual nature of the stress response. As the concept of stress is confusing and controversial enough, several terms were created with the aim to differentiate between beneficial and prejudicial stress. Among others, eustress and distress, neutral stress, overstress, adaptive and maladaptive stress were proposed. While "eustress" is used to describe positive or beneficial stress, "distress" is a term used to define this prejudicial stress or stress with maladaptive consequences on the organisms resulted in impaired biological functions and well-being (Clark et al., 1997; Koolhaas et al., 2011; Moberg, 2000).

The intensity and length of the stress are probably the most important factors of distress but not the only. Although distress can result from both acute and chronic stress, acute stress is usually considered to be a relatively brief exposure to a single stressor and thus, the stress response is usually adequate and beneficial. Only severe stress situations would disrupt critical biological events or divert biological resources. In contrast, chronic stress (whether it is associated to continuous stress or to frequent series of acute stressors) is easy to become in distress. First, because the stress response evolved to maintain homeostasis in front acute threats and second, because of the high demand of biological resources of the stress response under chronic conditions (Moberg, 2000). Thus, although acute and chronic stresses are not synonyms of eustress and distress, this associations are commonly done, especially the assumption that chronic stress is always threat to animal well-being (Dantzer et al., 2014; Ralph and Tilbrook, 2016; Sheriff et al., 2011).

Despite the concepts of eustress and distress have been applied in this section to present the negative effects of stress, their use is still controversial. The dual condition of the stress response (that can be beneficial or prejudicial for the organism) is generally accepted (Chrousos, 2009; McEwen, 2007; Moberg and Mench, 2000; Möstl and Palme, 2002), however, several authors discuss the extreme difficulty to dissociate these "two sides of the coin" and argue that performing this separation may lead to a certain degree of interpretation bias of the experimental results in both directions (Koolhaas et al., 2011; Kupriyanov and Zhdanov, 2014). Furthermore, in many of the classical behavioural and physiological measures used to evaluate stress it is impossible to know if such meaningful biological changes are occurring or have occurred (Moberg, 2000). Thus, the knowledge of whether the stress response detected is affecting animal well-being (distress) or not is usually unknown. For all previously stated, distress concept will not be further employed in the present thesis and stress will be used instead.

2.4. The role of stress in animal welfare

Stress plays a key role in animal welfare because affects it transversally. Stress is at the same time an indicator of poor welfare and a threat to animal's welfare by itself. When an animal is suffering hungry, thirst, pain or the impairment of performing behavioural needs (all situations affecting welfare), the stress response can activate (Broom and Johnson, 1993; Ralph and Tilbrook, 2016). Moreover, the negative impact of stress can lead to the rising or aggravation of other problems associated to animal's well-being (Barnett and Hemsworth, 1990; Rushen et al., 2008). For example, the immunosuppressive effects of the stress response could favour the development or aggravation of an infectious disease in a cow chronically stressed.

Moreover, as mentioned in section **1.2.** (**General description of animal welfare**), one of the five freedoms is "freedom from fear and distress". Both terms fear and distress are referencing the emotional state of the animals and the need to be free of, at least, these negative emotions. It is generally accepted that negative feelings are experienced by animals (at least mammals) under stress (Dawkins, 2015; Duncan, 2005; Moberg and Mench, 2000). Ultimately, it is how the animal feels about its bodily state, how it perceives its environment and how aware it is of these feelings and perceptions that are crucial for its well-being (Clark et al., 1997).

Despite the huge advantages of stress monitoring as an indicator of animal welfare, it should be noted that the assumptions that an animal that was not showing a physiological stress response would have good welfare and inversely, an animal that was showing a significant stress response would have poor welfare are not always true. Sometimes animals that appear to be distressed show no stress response and sometimes animals show a stress response when engaging in a rewarding activity. Therefore, the early hope of being able to assess welfare simply by taking some measurement of the physiological stress response has not been fulfilled (Duncan, 2005).

However it has been suggested that the total biological cost to the animal of mounting a stress response is the most important determinant of welfare because it affects the emotional state of the animal and when such costs are great the animal can enter a "pre-pathological" state, meaning that the animal comes under increased risk of developing some clinical pathology (Moberg, 1985).

2.5. Stress in cattle

Regarding stress in cattle, almost all welfare-threatening situations mentioned in the section **1.3.** (**Welfare in dairy cattle**) are also involving stress. For instance, the absence of analgesics in some procedures produces pain and the stress response has been widely associated to pain (Charmandari et al., 2005; Stafford and Mellor, 2011). However, there are other situations or procedures that cause stress to the animals (and impair their welfare) but are not so evident. Tail docking, high stock density, social conflicts between cows or rude human handling can be examples of them (Phillips, 2002; Rushen et al., 2008; von Keyserlingk et al., 2009).

In the case of the dairy cow, which is either lactating, heavily pregnant, or both, it is clear that this imposes a metabolic burden that can induce stress. The management of milking or the transition period are other situations that need to be cautiously evaluated in a stress perspective (Rushen et al., 2008). In parallel to public awareness on climate change, heat stress have also raised in the last years as other important stressor especially for dairy cows.

Although in some cases the impact of stress in cattle productivity can be difficult to measure, there are other situations in which stress has been identified as a key factor affecting productivity. For instance, the relationship between stress and diminished fertility has been demonstrated (Dobson et al., 2001; von Borell et al., 2007) as well as the impact of stress on the incidence of bovine respiratory disease (Rushen et al., 2008) or dark-cutting meat (Lacourt and Tarrant, 1985).

3. STRESS ASSESSMENT

Due to the nature of stress, the stress assessment cannot be done by identifying a presumable stressor and unfortunately, the knowledge and assessment on CNS of cognitive processes and stress-related feelings are still extremely limited or impossible. Thus, stress assessment has always been performed through the identification of the stress response. As mentioned in its definition, stress response includes an abroad variety of changes the complexity and suitability of those can differ markedly from species to species, individual to individual and stressor to stressor, and can vary according to prior experience and physiological status (Cook et al., 2000). Perhaps the greatest problem in measuring stress is inter-animal variations in the stress response (Cockrem, 2013; Moberg, 2000). Thus, although the detection of the stress response can be possible, the comparison of the magnitude of this stress between animals should be done cautiously because changes in a stress indicator (including behaviour) are not equally proportional in all animals and are not always proportional to stress perception.

Unfortunately, stress and physiology of stress response are too complex that the perfect or ultimate stress indicator does not exist. Ideally, more than one stress indicator should be monitored in order to ensure a good stress assessment (Moberg and Mench, 2000). The most appropriate method for assessing stress will be dependent largely on the experimental design and the overall characteristics of stress pretending to evaluate.

3.1. Activity of Hypothalamic-Pituitary-Adrenal axis

Undoubtedly, the assessment of HPA-axis activity has been one of the most (if not the most) used ways to assess stress. Hypothalamic-pituitary-adrenal axis is a physiological system almost always activated by stress and is not stressor-specific (Ralph and Tilbrook, 2016). Moreover, the HPA axis is responsible of most of the harmful effects of stress. The assessment of HPA-axis activity have been done through the quantification of glucocorticoid hormones whose secretion in bloodstream by the adrenal gland can be considered the last step in HPA-axis hormonal cascade. Increases in the circulation of the adrenal glucocorticoids (cortisol and corticosterone) have long been equated with stress (Buchanan, 2000; Moberg and Mench, 2000). The secretion of other hormones associated to HPA axis like prolactin and somatotropin has proven to be also sensitive to stress. Finally, other methods of studying HPA function involve assessing changes in pituitary-adrenocortical reactivity by using ACTH and CRH challenges or dexamethasone suppression tests. However, quantification of glucocorticoid levels is by far the most generalised way to assess HPA-axis activity.

Although monitoring HPA-axis activity is currently one of the best ways to assess stress, it is not exempt of problems and difficulties. First, not every type of stressor is mediated via increased activity of the HPA axis (Hodges et al., 2010). Second, HPA axis is not only activated under stress conditions. A physiological circadian rhythm associated to HPA axis has been demonstrated in several mammals, including cattle (Lightman and Conway-Campbell, 2010; Thun et al., 1981). Moreover, increases in HPA-axis activity have been also associated to non-stressful situations or activities involving emotional arousal (e.g. courtship,

sexual and play behaviour) and parturition (Broom and Johnson, 1993; Touma and Palme, 2005). Finally, several biological factors such as season reproductive and body condition, sex, age, social status or and diet can influence glucocorticoid secretion, requiring the exercise of caution when interpreting HPA-axis activity and stress.

3.1.1.Glucocorticoids

Glucocorticoids are steroid hormones (Sultan and Raza, 2015). As the final hormone secreted in the HPA-axis cascade, glucocorticoids are responsible of most of effects associated to HPA axis. Glucocorticoids have glucoregulatory actions and widespread effects to mobilize energy stores throughout the body. They influence the expression of approximately 10% of the genome and targets include genes controlling metabolism, growth, repair, reproduction and the management of resource allocation (Ralph and Tilbrook, 2016). Although glucocorticoids have been extensively studied and are considered key elements in stress response, there is still being controversies about their actions. Sapolsky et al. (2000) reported permissive, suppressive, stimulatory and preparative actions of glucocorticoids, what underlines the complexity of glucocorticoid actions and the importance of adequate interpretations in stress studies analysing them (Hodges et al., 2010; Touma and Palme, 2005).

3.2. Other

3.2.1. Behaviour

Behaviour analysis for the stress assessment is widely used and has some clear advantages. It is non-invasive and in many cases non-intrusive. Moreover, behaviour it is the result of animal's own decision-making processes and changes in behaviour associated to stress are the result of all stress-response mediators (e.g. cognitive CNS, SNS and HPA-axis).

Unfortunately, despite the previous advantages presented regarding behaviour analysis for the assessment of stress, it is still presenting most of problems associated to HPA-axis monitoring and others. For instance, behaviour analysis is still presenting high inter-individual variability, with temperament and other individual characteristics conditioning in a significant way the behavioural response to stress (Barnett and Hemsworth, 1990; Rushen, 2000). Other example is that animal behaviour is complex and its interpretation is not exempt of possible inter-evaluator bias (Dantzer, 2016; Hart, 2012). Finally, several different behavioural stress responses can appear after the same stressor but at the same time are in most of cases stressor-dependent. This implies the assumption that the stress stimuli is identified and known, which could be problematic as mentioned in section **2.2.1.(Stressor and stressor perception**). Thus, although valid and widely used, each stress-reactive behavioural must be careful validated, at least, for species, stressor and context (Dawkins, 2004).

3.2.2.Other stress indicators

Together with HPA axis, the activity of SNS is the other key component of the neuroendocrine stress response. However, the detection of catecholamines is not extensively used because increases in catecholamine concentrations associated to a stress response occur within seconds and last very short (Charmandari et al., 2005; Romero and Butler, 2007). Thus, their measurement is complicated and the interpretation of catecholamine levels are difficult (Buchanan, 2000; McEwen and Wingfield, 2010). Moreover, compared to the steroid hormones, there is relatively little known about the function and dynamics of these peptides (Fink, 2007). As an alternative to catecholamines detection, activity of SNS can be evaluated indirectly by changes in heart rate. Although simple and subject to several confounding factors, its measurement has proven a useful parameter for the SNS and is frequently applied (von Borell et al., 2007).

The heterophil:lymphocyte ratio is an historical stress indicator whose use has been decreasing in mammals but increasing in avian research (Davis et al., 2008).

Recently, other indicators have been studied with the aim to be used in stress assessment. Acute phase proteins, for example, are proteins involved in physiological response to infections and injuries (Ceciliani et al., 2012). Although some authors suggested that could be stress indicators in some context (Ceciliani et al., 2012; Monteverde et al., 2016; Saco et al., 2008), their use as a generalised stress indicator seems complicated (Ott et al., 2014; Saco et al., 2008). Telomere length and telomerase activity have been presented as other potential stress indicators: telomeres are repetitive DNA sequences that cap and protect the ends of chromosomes while telomerase is an enzyme that can rebuild and restore telomere length. In humans, some findings suggest a link between stress and shorter telomeres or low telomerase activity (Epel et al., 2010, 2004; Mathur et al., 2016; Parks et al., 2009). Unfortunately, future research is necessary to generate enough knowledge about these potential stress indicators before obtaining the certainty of its reliability in stress assessment.

3.3. Stress assessment in cattle

Stress assessment in cattle has not many particularities. Stress assessment is a common practice in cattle research and the amount of literature is notable. Although several stress indicators have been used and tested, glucocorticoid detection (HPA-axis activity monitoring) is the most used way to assess stress in cattle. Cortisol is the major glucocorticoid in cattle (Mormède et al., 2007) and thus is the most measured parameter for stress assessment. Circulating cortisol levels of cattle is subjected to a circadian rhythm. However, it is weak and these low cortisol variations have led to controversial studies, with some detecting circadian rhythm and others not doing so (Mormède et al., 2007). In cattle, the response of adrenal activity to intravenous or intramuscular administrations of exogenous doses of ACTH has been demonstrated.

Numerous studies performed during decades have validated the association of cortisol increases and stress. Therefore, the analysis of cortisol concentrations is a generalised practice in cattle research when stress is pretended to be assessed.

4. MATRIXES FOR CORTISOL DETECTION

Until now, the analysis of cortisol concentrations has been presented as one of the best methods for stress assessment: the HPA axis is one of the main effectors of the stress response and, after a hormonal cascade, HPA axis finishes with the releasing of glucocorticoids (mainly cortisol in cattle) into the bloodstream. Thus, analysing cortisol concentrations in blood would provide valuable information regarding the stress status of the sampled animal. However, blood is not the only matrix available for cortisol and most other steroids detection. Circulating cortisol can reach saliva through salivary gland (Kirschbaum and Hellhammer, 1994) or faeces through liver and bile excretion (Palme et al., 1996; Taylor, 1971), for example. In this point, a question can rise: why the detection of cortisol in other matrixes has been developed, validated and used?

Intuitively, it is easy to assume that the development of cortisol detection in other matrixes has been motivated for practical reasons. Obviously, it is easier to collect faeces from an animal than perform blood samplings, for instance. However, the influence of cortisol matrix in stress assessment goes much further than just practical advantages: depending on the matrix used for cortisol analysis, the information of stress response provided by its concentration is different. Specifically, cortisol concentrations are representing HPA-axis activity in different frame and length of time depending on the matrix analysed (Mormède et al., 2007; Russell et al., 2012). The use of different matrixes multiplies the possibilities and versatility of cortisol detection for stress monitoring. Following, a classification of matrixes for cortisol detection is proposed and main characteristics provided. A summary can be found in table 3.

Table 3. *Comparative of main mammal matrixes' attributes regarding their cortisol detection*

	Blood (serum/ plasma)	**Saliva**	**Urine**	**Faeces**	**Hair**
Correlation between ↑[cortisol] and stressful situations	Demonstrated	Demonstrated	Demonstrated	Demonstrated	Demonstrated
Period of time of adrenal activity representing its [cortisol]	Short (minutes)	Short (minutes)	Short/Middle (minutes or hours)	Middle (hours)	Long (weeks to months)
Potential influence of handling and sampling in [cortisol]	High	High	Moderate	Low	Low
Influence of physiological factors not stress-related in [cortisol]	High (circadian rhythm)	High (circadian rhythm)	High (glomerular and tubular function)	Moderate (intestinal transit, others)	Low (differences in hair growth rate)
Type of cortisol analysed	Total cortisol (free and protein-bound)	Free cortisol	Free cortisol and cortisol metabolites	Cortisol metabolites	Free cortisol
Invasiveness of sampling	High	Low	Low-High (external vs. catheter)	Low	Low
Difficulty of sampling (and problems of re-samplings)	High (technical and/or ethical)	Moderate	High (technical and/or ethical)	Low	Low
Storage needs	High (refrigeration, freezing, anticoagulant)	Moderate (refrigeration, freezing)	Moderate (refrigeration, freezing)	Moderate (refrigeration, freezing)	Low (room temperature, dark)

[cortisol] = cortisol concentrations
Source: author

4.1. Single-point matrixes

These matrixes provide a measurement of cortisol concentration at a single point in time, what means that their cortisol concentrations are representing a single moment of HPA-axis activity. According to Cook (2012), measurement of cortisol in blood and saliva (single-point matrixes) give a "snap shot" picture of adrenocortical activity at the time the sample was taken. They also allow on-time monitoring of HPA-axis activity and the stress status of the animal (with minimal degree of delay in case of saliva). Cortisol concentration in single-point matrixes can be used to detect acute changes in circulating cortisol levels but they cannot represent long term adrenal activity (Thun *et al.*, 1981; Negrão *et al.*, 2004; Hernandez *et al.*, 2014). Consequently, they are excellent matrixes for acute stress assessment and concrete monitoring of stress status but not for the study of chronic stressors or the evaluation of long-term stress status.

4.1.1.Blood

Blood is the matrix most directly connected to HPA-axis activity as adrenal glands secrete cortisol directly to bloodstream (Charmandari et al., 2005; Habib et al., 2001; Sapolsky et al., 2000). Thus, the delay between stress response and reactivity of blood ortisol concentrations is virtually inexistent as the secretion of cortisol in bloodstream is considered part of stress response. The detection of cortisol in serum or plasma (from now generalised as serum cortisol) has been extensively used and validated for stress assessment (Mormède et al., 2007; Romero and Butler, 2007). Moreover, blood is the "mother" matrix from which cortisol arrives to the others. However, several confounding factors are associated to this matrix:

First, in species with circadian rhythm in HPA-axis activity (like cattle), blood cortisol vary physiologically along the day. It difficulties or impedes comparisons between blood samplings performed in different moments of the day (Palme et al., 2005; Thun et al., 1981).

Second, the rapid reaction of HPA-axis to a stressor (3-5 minutes) carry over a problem: the act of blood sampling can be stressful (human presence, animal restraining, venipuncture, among others) and can mask the real blood cortisol levels existing before sampling (Beerda et al., 1996; Cook et al., 2000; Willemse et al., 1993). Thus, in some circumstances cortisol detected in serum cannot be representing the stress status of the animal before the process of sampling.

Third, measuring cortisol in serum samples assesses total serum cortisol. This includes both protein-bound and free (bioactive) cortisol (Russell et al., 2012). Consequently, total serum cortisol is affected by changes in levels of cortisol-binding globulin which are not related with stress status (Cook, 2012; Turpeinen and Hämäläinen, 2013).

Fourth, blood sampling is invasive and potentially painful. It implies a health risk for both the animal and the person who perform it. Moreover, blood or serum has special storage requirements (anticoagulant, temperature) that can affect quality of samples and thus, reliability of final cortisol concentrations detected (Mormède et al., 2007; Sheriff et al., 2011).

4.1.2.Saliva

A direct relationship exists between cortisol in blood and cortisol in saliva with a few-minutes delay (Aardal-Eriksson et al., 1998; Landon et al., 1984; Negrão et al., 2004). This delay is the time spent between cortisol is secreted by the adrenal gland and it is filtered by the salivary gland and diffused into the saliva (Sheriff et al., 2011). Cortisol detection in saliva was developed with the aim to solve some of problems or limitations associated to blood cortisol detection with an excellent acceptance. For instance, saliva sampling is safer and less invasive and stressful than blood collection. Furthermore, only free (unbound, bioactive) cortisol arrives to saliva, what facilitates analysis interpretation because is not affected by changes in cortisol-binding globulin levels (Bigert et al., 2005; Hofman, 2001; Kirschbaum and Hellhammer, 1994). However, as a single-point matrix, saliva is still affected by circadian rhythm and potential stress for sampling. Moreover, saliva contains enzymes able to affect cortisol (Sheriff et al., 2011) and can be contaminated easily with food or blood (D'Anna-Hernandez et al., 2011; Inder et al., 2012).

4.2. Intermediate matrixes

Intermediate matrixes are definitively not single-point matrixes. They cannot represent on-time HPA-axis activity because they accumulate circulating cortisol for some time. Consequently, the cortisol analysed in these matrixes is not representative of the activity of HPA-axis in a single moment with a certain delay. Instead, cortisol concentrations in these matrixes are able to represent HPA-axis activity for a determined window of time providing a more integrative measure of stress status of the animal within a period of time.

The difference with cumulative matrixes is that intermediate matrixes still being subjected to an important degree of replacement in its production. Therefore, the frame of time of HPA-axis activity able to be monitored in a single sampling is dependent on it and this situation limits the capacity to represent circulating cortisol levels from a few to several hours.

4.2.1. Faeces

Free blood cortisol is metabolised by the liver and excreted to the duodenum by the bile duct (Morrow et al., 2002; Möstl and Palme, 2002; Palme et al., 1996; Taylor, 1971). Thus, HPA-axis activity in faeces is measured indirectly by detecting cortisol metabolites (Möstl et al., 1999; Touma and Palme, 2005). It has been extensively used and validated for stress assessment (Möstl et al., 2002, 1999; Palme et al., 1999) because of several reasons. First, faecal sampling is simple, safe and non-stressful. It can even be done non-intrusive if faeces are collected after defecation. Second, the detection of cortisol metabolites in faeces attenuates the effect of circadian rhythm allowing comparisons between samples collected in different times (Palme, 2012; Palme et al., 2005). Finally, until the development of cumulative matrixes, faecal matrix was the one with the capability to represent the longest period of HPA-axis activity with a single sampling. In cattle, a single faecal sampling could represent several hours (Palme et al., 1999).

Unfortunately, faeces as a matrix for HPA-axis assessment still present some problems. Although the basic structure of the cortisol metabolite does not degrade, cortisol and other steroid metabolites in faeces can be subjected to enterohepatic circulation and bacterial metabolism (Macdonald et al., 1983; Taylor, 1971). There also exist controversial results regarding the impact of feed characteristics, intestinal transit or bacterial activity in concentrations of faecal cortisol metabolites (Morrow et al., 2002, 2001; Möstl and Palme, 2002; Rabiee et al., 2001) or whether they are uniformly distributed within the faecal samples or not (Bennett and Hayssen, 2010; Buchanan and Goldsmith, 2004). Moreover, the delay between faecal collecting and putting the samples in appropriate storage conditions is crucial, with significant alterations in faecal cortisol metabolites concentrations if faeces are not frozen immediately (Buchanan and Goldsmith, 2004; Mormède et al., 2007).

4.2.2. Urine

Urine is the main elimination route of glucocorticoids and has an extensive background in human endocrinology (Davenport et al., 2006; Mormède et al., 2007). In contrast, cortisol and cortisol metabolites from animals are not extensively analysed in urine. Probably this is because urine collection is extremely easy in humans but much complicated in animals: as renal cortisol secretion is dependent on glomerular and tubular function, the measured daily secretion rate depends on a correct 24-hour collection of urine

(Aardal and Holm, 1995). Thus, it requires installing urinary catheters or alternatives in animals for 24-hour urine collection. Despite the possibility to obtain a daily measure of adrenal activity is high desirable, the use of mechanisms for the collection of urine for 24 hours without disturbing normal routines or stress status is almost impossible. This situation together with the high risk of sample contamination limit the use of urinary cortisol for stress assessment in animals (Creel et al., 1992; Koren et al., 2002; Sheriff et al., 2011).

4.2.3.Milk

Milk can also be used for cortisol detection (Fukasawa et al., 2008; Tucker and Schwalm, 1977; Verkerk et al., 1998). Although the relationship between circulating and milk cortisol was demonstrated (Mormède et al., 2007; Verkerk et al., 1998), it is a matrix rarely used with an evident limitation: cortisol in milk can be assessed only to females during the milking period. However, for dairy cows and other dairy animals, milk cortisol concentration could be the most easily accessible and practical indicator for assessment of management systems and individual adaptability as milking is a daily procedure. The integration of milk cortisol analysis as another parameter to analyse in milking controls could be of great utility.

Unfortunately, there are some uncertainties in milk cortisol physiology, especially regarding cortisol accumulation in milk and the influence of milk distribution within the udder prior to milking (Mormède et al., 2007; Verkerk et al., 1998). Thus, although highly applicable in dairy production, more research is necessary to understand the nature of the link between HPA-axis activity and milk cortisol concentrations.

4.3. Cumulative matrixes

In recent years, cumulative matrixes for cortisol (and other steroids) analysis has appeared and gained much attention. The main characteristic of these matrixes is that accumulate cortisol in a continuous way for long periods of time. Consequently, its cortisol values are representative of long-term HPA-axis activity. Although these matrixes are not sensitive to short fluctuations in circulating cortisol levels, they have the potential to provide an integrative value of long-term retrospective circulating cortisol levels with a single sampling. The major contribution of these matrixes is that they redefine the limits of stress monitoring as virtually amplify them from several hours to months in a single sampling.

During all their growth, cumulative matrixes incorporate cortisol levels proportionally to their concentrations in blood. Thus, the concentrations of cortisol in these matrixes have the potential to represent an “average” value of cortisol for all the growth period. Moreover, most of cumulative matrixes are keratin matrixes, what gives them a great stability in front degradation and thus the capacity to be stored at room temperature without special storage conditions. This situation facilitates significantly their use in research and diminishes potential problems in transport or storage that could affect cortisol measurements.

Perhaps the most relevant cumulative matrixes are hair (a deep description is presented in next section) or feathers (e.g. Bortolotti et al., 2009, 2008; Carbajal et al., 2013; Romero and Fairhurst, 2016), whose have gained much attention last years. However, in different species cortisol has been also detected in claws (Comin et al., 2014; Matas et al., 2016a; Veronesi et al., 2015), nails(Ben Khelil et al., 2011; Matas et al.,

2016b; Warnock et al., 2010), vibrissae(Tallo-Parra et al., 2015), baleen plates (Hunt et al., 2014; Tallo-Parra et al., 2015) and even shed skin (Berkvens et al., 2013; Tallo-Parra et al., 2015). Moreover, cortisol has been detected in mineralised matrixes like fish scales (Aerts et al., 2015) or cerumen-based matrixes like earplug in whales (Trumble et al., 2013) and could be also considered cumulative matrixes.

All these matrixes are still being developed, studied and need several biological validations to know their characteristics and evaluate all their potential capacities prior to confirm that can be classified as cumulative matrixes and their usefulness as stress indicators. However, their potential is high as well as their expectations regarding their future use in long-term HPA-axis activity assessment.

4.4. Cortisol detection in cattle

In cattle, almost all matrixes have been used and it is one of the species with greater number of matrix tested. Aside from blood, saliva or urine (e.g. Chacón et al., 2004; Huzzey et al., 2011; Negrão et al., 2004), there is a deep knowledge of cortisol metabolites detection in faeces (e.g. Kahrer et al., 2006; Möstl et al., 2002; Palme, 2012; Palme et al., 1999) and milk cortisol analysis(e.g. Fukasawa et al., 2008; Tucker and Schwalm, 1977; Verkerk et al., 1998).

Moreover, several studies have been performed analysing cortisol in hair (next section) and even claws (Comin et al., 2014) in cattle, being dairy cows the most typical used.

5. HAIR MATRIX FOR CORTISOL DETECTION

Hair is the perfect representative of cumulative matrixes and their advantages in mammals. It is a keratin matrix and accumulates hormones during all its growth. Moreover, hair is present in almost all mammals, it is abundant and easy accessible. With this scenario, it is not surprising the attention gained in all fields using hormonal analyses in the last years. However, as a new matrix, several studies are necessary to validate hair biologically before confirming what it looks like: hair is perhaps the best matrix for cortisol detection in studies of long-term stress assessment and animal welfare research.

5.1. Hair characteristics

The information of this section is obtained mainly from Balíková(2005), Harkey(1993), Pragst et al. (2006) and Robbins et al. (1988).

Although hair could appear to be a uniform structure differing between individuals only in colour, length or amount, it is a very complex structure whose biology and physiology is only partially understood. Hair is an

annex of skin originated from the hair follicle and consists of keratinized cells glued by the cell membrane complex that together form three concentric structures: cuticle, cortex and medulla. The cortex is pigmented and is responsible for the stretching stability and colour composition, whereas the layers of shingle-like cells of the cuticle are responsible for high chemical and physical resistance and shine. The most important components of hair are fibrous proteins (keratins), melanin and lipids.

Hair follicles are located a few millimetres below the surface of the skin and are surrounded by a rich capillary system that provides the growing hair with necessary metabolic material. The apocrine, sebaceous and sweat glands are associated with the hair follicle. The secretions of the first two glands bathe the hair shaft in the follicle and the one of the sweat gland bathe it above the surface of the skin.

The hair fibre can be divided into three distinct zones along its axis. The zone of biological synthesis and orientation resides at (or around) the bulb of the hair, in which the germination centre is formed by matrix cells (keratinocytes and melanocytes) present on the basement membrane. Rapid mitosis forces a migration of the upper zones into the direction of the hair root mouth. The next zone in an outward direction along the hair shaft is the zone of keratinization, where the genes for formation of keratin are expressed providing stability into the hair structure. Finally, the third zone that eventually emerges through the skin surface is the region of the permanent hair fibre. It consists of dehydrated cornified cells and intercellular binding material. In the root, cells are in active proliferation, whereas within the hair shaft above the skin the metabolism is negligible.

Regarding growth, hair grows in a cycle composed of the anagen (active growing), catagen (transition) and telogen (resting) stages. The individual length of hair depends on growth rate which is affected by hair growth velocity and the duration of each cycle stage. Although in human it is accepted that at any time, approximately 85% of hair is in the growing phase (anagen) with the remaining 15% in the resting phase (telogen), this has not confirmed in other species.

5.1.1.Mechanisms for cortisol incorporation in hair

The exact mechanism whereby cortisol is incorporated into hair is not yet known (Meyer and Novak, 2012; Stalder and Kirschbaum, 2012). Consequently, because cortisol and other steroid hormones are lipophilic substances, the theories of steroid incorporation are based on forensic knowledge about routes of incorporation of lipophilic drugs into the growing hair shaft (Meyer and Novak, 2012). The generally accepted theory for lipophilic substances incorporation in hair is the model proposed by Henderson (1993), which has

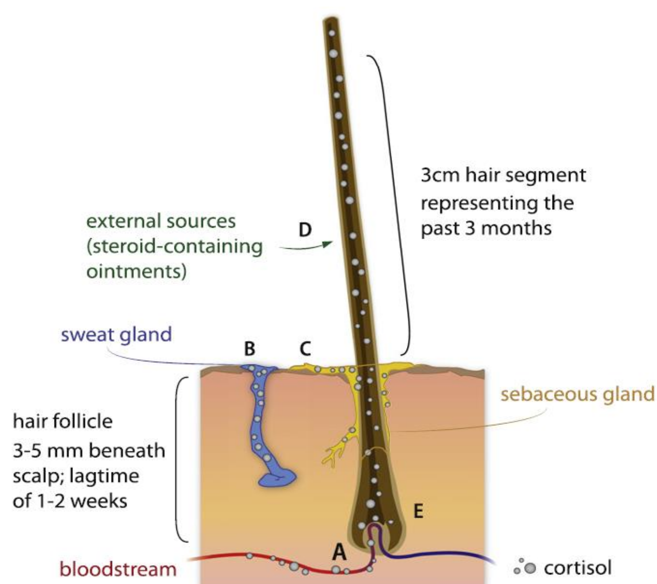


Figure 2. Mechanisms for cortisol incorporation in hair. *Source:*Stalder and Kirschbaum(2012).

been also called "multicompartment model" (Balíková, 2005; Boumba et al., 2006; Stalder and Kirschbaum, 2012) and is represented in Figure 2. According this model, cortisol enters hair primarily at the level of the medulla of the hair shaft via passive diffusion from blood. Most authors argue that this is the major route of cortisol incorporation in hair (Cone, 1996; Gow et al., 2010; Henderson, 1993) and that in this case only free cortisol would be incorporating to hair (Russell et al., 2012; Stalder and Kirschbaum, 2012), with the same associated benefits described in saliva. In this scenario, free cortisol would be incorporated proportionally to its concentrations in blood during hair formation. Moreover, the model also suggests that cortisol from sweat and sebum can be incorporated after hair formation. Finally, the contamination with external (environmental) sources of cortisol once hair has emerged from the skin is also considered in the model. Although the multicompartment model from Henderson has not been fully demonstrated, it has been accepted by almost all scientists in the field of hair cortisol analysis (e.g. Davenport et al., 2006; Gow et al., 2010; Koren et al., 2002; Meyer and Novak, 2012; Stalder and Kirschbaum, 2012) but with the addition of an extra source of cortisol: the locally produced cortisol by hair follicle and/or skin. A peripheral HPA-like system contained in hair follicles and steroideogenesis process in skin have been demonstrated (Ito et al., 2005; Jozic et al., 2014; Sharpley et al., 2012). Although most of authors consider their influence in HCC negligible or secondary, some others state that local production and regulation can the main source of hair cortisol concentrations (HCC) (Keckeis et al., 2012; Sharpley et al., 2009).

5.2. Hair cortisol analysis: background, pros and cons

5.2.1.Background

The use of hair as a matrix for the analysis of endogenous and exogenous compounds is increasingly widespread (Gow et al., 2010). Although hair has been extensively used to extract DNA (Foran et al., 1997; Woodruff, 1993), is in long-term retrospective analysis where hair becomes an exceptional matrix and gained special scientific recognition (Kirschbaum et al., 2009). Hair is being extensively used in various toxicological fields, forensic sciences, doping control or clinical diagnostics, among others (Boumba et al., 2006; Harkey, 1993; Kintz, 2004; Vogliardi et al., 2014).

Regarding detection of glucocorticoids, Cirimele et al. (1999) were the first to detect synthetic glucocorticoids in human hair (prednisone) while Raul et al. (2004) demonstrated the detection of endogenous concentrations of cortisol and cortisone also in human hair. Two years later, Davenport et al. (2006) published the methodological details and validation of a simpler protocol for measurements of endogenous cortisol concentrations in rhesus monkey (*Macacamulatta*) hair with a special focus on the appropriate wash procedure. The work from Davenport and colleagues opened the door for hair cortisol measurement in animals as a tool for stress monitoring. In cattle, the first published study analysing HCC was done by Comin et al. (2008) in hair of neonatal calves and cows with the aim to determine its significance as biomarker of neonatal stress.

5.2.2.Pros and cons

As mentioned previously in the description of cumulative matrixes, hair presents some advantages and characteristics that make it a potential excellent matrix for long-term retrospective cortisol analysis. The most important characteristic is based on a simple fact: hair grow while blood, saliva, faeces and other matrixes do not.

In hair, the act of growing is associated to its capacity to accumulate hormones. Hair incorporates cortisol during all its growth and consequently, hair monitor retrospectively the HPA-axis activity for the amount of time that has been growing. As hair growth can last from weeks to months (Davenport et al., 2006; Meyer and Novak, 2012), it gives the researchers the possibility to monitor a period of time impossible to do by the other matrixes developed until now. The possibility of long-term cortisol monitoring provided by hair analysis is particularly relevant as chronic stress has been a hot topic in stress and animal welfare research in the recent years (Möstl and Palme, 2002; Walker et al., 2014).

Furthermore, when hair is cut or lost, it has de capacity to start its growth again (by activating the anagen phase) (LeBeau et al., 2011). This situation allows the possibility to synchronise all individual hairs from one area to start the growing phase and accumulate hormones from a controlled period of time (Ashley et al., 2011). This is another exceptional advantage of hair matrix over the other matrixes as it gives to researchers the possibility to define the exact period of time in which free cortisol from blood is being accumulated in their samples. In case of the other matrixes, researchers cannot interfere with their physiological timing and are limited by their characteristics, needing to perform several samplings when trying to monitor large periods of time or the global status of an animal under certain conditions. The need of several samplings and analysis increase the impact of intra-assay variability errors and the costs of the experiment, as well as the animal handling.

Therefore, hair provides an unprecedented range of time able to be monitored for adrenal activity and at the same time it is versatile to allow researchers to define the period monitored. Overall, this process results in a matrix with the potential to supply an integrative value of long-term retrospective cortisol levels within a voluntary defined period of time by a single sampling.

Aside from conceptual advantages in adrenal activity monitoring, hair characteristics also provide practical advantages: first, hair cortisol concentrations are not affected by the process of sampling or, if existing, circadian rhythm (Comin et al., 2013; Koren et al., 2002; Russell et al., 2012); second, hair collection is easy, safe and non invasive; and finally, as a keratin matrix hair does not have storage requirements being stable over time at room temperature (González-de-la-Vara et al., 2011; Macbeth et al., 2010).

Despite all the potential advantages, hair cortisol detection has also defects. The most important is that, as a relatively new matrix, all its potential must be tested and validated. An accurate knowledge of hair as a matrix that accumulates cortisol is essential for its future use in chronic stress research associated to animal welfare and animal production. The theoretical capacity of hair to represent adrenal activity for long-time still needs more scientific studies to confirm it. There is a scarcity of information available on potential confounding factors affecting hair cortisol incorporation and the relationship between adrenal activity and

HCC. For instance, some accumulation mechanisms are still incompletely understood (Henderson 1993; Cone 1996; Boumba *et al.*, 2006) and theimpact of local steroidogenesis in skin is still unknown (Taves *et al.*, 2011; Slominski *et al.*, 2013 and 2014). Moreover, recent studies suggested that the influence of light could be affecting HCC (Grass et al., 2016; Wester et al., 2016) which could hinder comparisons between animals in different light conditions and force hair to be stored in a dark place. Finally, it should be noted that hair growth rate do not allow the use of hair for acute stress.

5.2.3. State of the art of hair cortisol detection in cattle

Aside from human, cattle is one of the species with larger number of studies published analysing hair cortisol concentrations. However, it does not mean that there exists wide knowledge and literature regarding HCC in cattle. Hair cortisol detection in animals it is still being a recent developed methodology and the number of articles published could be considered negligible in comparison with the amount of literature published in cattle for cortisol detection using blood, saliva or faeces.

The profile of literature published until the presentation of this thesis regarding HCC analysis in cattle is in concordance with the current context of new matrix appearance. Several articles attempted to understand intra-animal sources of HCC variability (Burnett et al., 2014; Cerri et al., 2012; Maiero et al., 2005; Moya et al., 2013) and confirm the relationship between HCC and HPA-axis activity (González-de-la-Vara et al., 2011; Moya et al., 2013). However, and despite the current gaps in hair cortisol detection, some other studies have already used HCC as a biomarker of stress and studied its association with health (Burnett et al., 2015; Comin et al., 2013), reproduction (Burnett et al., 2015; Comin et al., 2013, 2008), breed (Peric et al., 2013), cow's environmental conditions (Comin et al., 2011), stocking density (Silva et al., 2016) and reproductive treatments (Biancucci et al., 2016). It should be noted that in many of them the lack of deeper knowledge on hair cortisol in cattle is mentioned as a factor that hindered the development of discussions and conclusions.

6. CORTISOL DETECTION METHODS

The determination of a substance in biological fluid usually consists of at least two steps—reaction and detection. The nature of the reaction and the detection steps can be physical, chemical, biological, or immunological (Chan, 1987). Several methodologies can be used for the analysis of cortisol concentrations in hair. In this section, a brief description of most used methods is provided.

6.1. Immunoassays

Immunoassays have become the most valuable analytical tool of medicinal in vitro diagnostics and are routinely employed for the detection of a wide range of analytes including among others hormones, peptides, proteins, viruses or pharmaceuticals (Skrzipczyk and Verdier, 2013; Stepaniak et al., 2002). Briefly, immunoassays can be defined as quantitative binding reactions between antibodies and target antigens, in our case, cortisol. They can precisely quantify the concentration of cortisol in hair's extract that must be in liquid phase by using the specific antibodies to select and retain cortisol hormone and the reaction of conjugated enzymes or radioactively labeled compounds to generate chemiluminiscent or radioactive tracers in proportion to number of cortisol molecules present (Ngo, 1991; Stepaniak et al., 2002). Depending on the way to generate a measurable signal, immunoassays can be classified as radioimmunoassay (RIA) if they use radioactivity or enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA) if they use enzymes using chromogenic substrates (Chan, 1987; Chernesky and Mahony, 1996; Porstmann and Kiessig, 1992). Some of most relevant advantages of immunoassays are their great sensitivity, good specificity and their easy handle, allowing high throughput in most of laboratories and potential development of semi-or fully automated immunoassay systems. In contrast, the main disadvantages of immunoassays are related with the time of antibodies production, cross-reactivity with other analytes (especially metabolites) and the influence of other compounds from the matrix that, in high concentrations, could increase the error in measurement (Skrzipczyk and Verdier, 2013).

Importantly, it should be noted that when using immunoassays for cortisol quantification, all molecules bound to anti-cortisol antibodies are considered cortisol. Some metabolites of cortisol is still being able to interact with antibodies and in this scenario, some authors prefer to use "immunoreactive cortisol" when detecting cortisol concentrations by immunoassays as a more abroad but correct concept.

6.1.1.Radioimmunoassay

Radioimmunoassay was the first formulated immunoassay. It was developed by Yalow and Berson in 1959 for the determination of insulin. They combined for the first time the high sensitivity of a radioactively labelled compound with the high specificity of an immunological reaction by the formation of a thermodynamically stable antigen-antibody complex (Skrzipczyk and Verdier, 2013). The use of a radioactive label provides RIA of an excellent sensitivity, flexibility and precision of radiochemical labelling and high robustness with regard to interferences because of the "hard" signal. However, some the disadvantages of radioactivity are evident: it is danger and a health risk for scientist and thus RIA use requires handling licenses, adapted facilities and has high disposal costs. Moreover, RIA assays have a very limited signal emission during the measuring time (usually only 1 min) and their commercial kits a short half-life (Lequin, 2005; Ngo, 1991; Skrzipczyk and Verdier, 2013).

6.1.2.Enzyme immunoassay and enzyme-linked immunosorbent assay

Some years after the development of RIA (1060s), and motivated by the problems associated to work with radioactivity, non-isotopic immunoassays were developed. The most relevant ones were the EIA and the

ELISA although there were others (Skrzipczyk and Verdier, 2013). Since 1980s, these techniques are the most used immunoassays.

Although both EIA and ELISA are based on the same principle of an immunoassay with an enzyme as the reporter label, they were developed independently at the same time by two different research groups (Perlmann and Engvall for EIA and Schuurs and van Weemen for ELISA). Perhaps due to this situation the use of EIA and ELISA words is still controversial, with some researchers arguing that there are synonyms while others considering that EIA is a somewhat more general term, including ELISA but also other techniques (Stepaniak et al., 2002).

Theoretically, EIA and ELISA have the potential to be more sensitive than RIA because enzymes produce a lot of signal-generating species. In practice, but, the sensitivity is determined by the signal to noise ratio and current EIA and ELISA still showing slightly lower sensitivity than RIA (Kurstak, 1986). However, the sensitivity of EIA and ELISA are more than good for cortisol analysis in biological matrixes including hair (Chan, 1987).

6.2. Others

Despite the many advantages and general application of immunoassay methodology, other detection methods like high-performance liquid chromatography (HPLC) have been used (although in much lower number) for cortisol detection (Makin and Newton, 1988; Palme and Möstl, 1997) and new ones are emerging as potential practical tools for cortisol analysis.

6.2.1.Near infrared reflectance spectroscopy

Although light in the near infrared region was discovered in 1800 and the use of NIRS for analysing complex compounds started in 1970s, it is in the past two decades that the development of improved electronic and optical components and the advent of computers capable of effectively processing the information contained in NIR spectra facilitated the expansion of this technique (Blanco and Villarroya, 2002). Near infrared reflectance spectroscopy is a multi-component predictor technique characterized as rapid, non destructive and reagent free. It is based on the interaction of electromagnetic waves (near infrared radiation) and matter (mainly C-H, N-H, O-H and C-O bonds) (Cen and He, 2007; Foley et al., 1998; Scarff et al., 2006). Briefly, when irradiated by different near infrared frequencies, the bonds between atoms or functional groups of atoms are subject to vibrational energy changes (stretching and bending). As any organic compound is different in type and amount of atomic and functional group bonds, the type of vibrational wave is in a frequency characteristic of each organic molecule. When the frequencies of incident light match the frequencies of the vibrational waves are absorbed whereas the rest of frequencies are reflected or transmitted. Therefore, the spectrum of light that is reflected from the sample contains information on the chemical composition of that sample (Blanco and Villarroya, 2002; Foley et al., 1998; Osborne, 2000).

Unfortunately, the complexity of absorptions and light interactions within compounds presents in the sample makes impossible to find a direct relationship between absorbance and specific compounds. Therefore, for each analyte wanted to detect NIRS needs to be calibrated and validated with an independent laboratory assay by applying statistical models that test the intensity of the relationship between a particular absorbance and the concentrations obtained with the reference assay (Nduwamungu et al., 2009; Walker and Tolleson, 2010). As described by Foley et al. (1998): "*NIRS is an indirect method that estimates chemical composition from a sample by comparing spectra with samples of known composition"*.

Regarding the analyses of cortisol and other steroids in hair, the advantages and disadvantages of NIRS over immunoassays are evident. First, NIRS is able to analyse several compounds with one single lecture while immunoassays need to run at least one different assay kit for each target hormone. Moreover, once the spectrum of the sample is obtained, it can be used in the future for the quantification of analytes even whether calibration and validation are done afterwards. Second, NIRS is non-destructive technique which means that is not necessary any pretreatment of the sample for the analysis. After the lecture, the sample remains unchanged and can be used for further uses without sample losses (negligible). Although in several cases the sample is trimmed with the aim to homogenise it, this is a completely different scenario from immunoassays, in which solid matrixes for hormonal detection are processed with the aim to obtain a liquid solution with their hormones extracted. Furthermore, in immunoassays the amount of hormone extract used cannot be restored. Third, in relation to hair cortisol analysis by immunoassay, the process for obtaining hair hormonal extracts lasts several days and the assay is also time-consuming (3-4 hours). In contrast, the no need for hair processing (perhaps only hair trimming) and the fast sample lecture by NIR spectrophotometer, make it a faster technique. Finally, as NIRS is reagent-free, the laboratory requirements are few and its environmental impact minimal.

Regarding the disadvantages, NIRS is a predictor technique and this involves some limitations. All predictor techniques need an independent reference-method or laboratory assay for the calibration and validation of their analysis and statistical models. Therefore predictor techniques cannot be better than their reference-method in terms of pure analysis (not considering practical aspects) (Deaville and Flinn, 2000; Foley et al., 1998; Osborne, 2000). It should be noted that using a good reference-method do not ensure a similar capacity of NIRS in terms of accuracy of its detection. Furthermore, unlike immunoassays, NIRS cannot be selective. This provides to NIRS its capacity of multi-component analysis but also increases its difficulties in detecting analytes in biological and complex matrixes like hair.

7. VALIDATION OF CORTISOL DETECTION METHODOLOGIES

7.1. The importance of validation

In the field of cinema, there is a saying that states: " *With a good screenplay you can make a good or a bad movie, but with a bad screenplay you can only make a bad one*". The same happens with analytical science: only with reliable results you can try to obtain true knowledge. Analytical method validation is the process of demonstrating that an analytical procedure is suitable for its intended purpose (Food and Drug Administration, 2015). Therefore, independent of a "true" value, a "good" assay should fulfill some criteria, like to provide appropriate specificity and sensitivity, to be precise, to be robust, to have a "working range" adequate for the study and to be compatible with the environment (Möstl et al., 2005). In other words, the objective of the analytical procedures should be clearly defined and understood before initiating the validation of a methodology because the same validation results can be acceptable or not depending on the final purpose of the analysis.

The detection of cortisol concentrations in hair is the methodological base of the present thesis and thus, the validation of this procedure acquire transcendental importance. Moreover, the absence of specific immunoassays designed for hair matrix force the researchers to validate commercial or self-prepared EIA for the quantification of cortisol levels. The validation results cannot be generalised and at least for every matrix, hormone and species, the validation of the methodology used for the quantification of the target hormone should be performed (Buchanan and Goldsmith, 2004; Touma and Palme, 2005).

7.2. Validation test for immunoassays

In immunoassays, the typical validation characteristics evaluated with validation tests are specificity, linearity, accuracy, precision, range and sensitivity (Food and Drug Administration, 2015). It should be noted that the validation characteristics are not completely independent from one to another. For instance, precision influence the sensitivity of an assay and the specificity its accuracy. Consequently, some validation tests performed with the aim to validate an immunoassay are testing more than one characteristic.

7.2.1.Specificity

Specificity (also called selectivity) is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present (Crowther and Walker, 2001; ICH, 2005). Regarding hair cortisol detection, these components might include any compound from hair matrix that can be extracted with cortisol in the hair extraction protocol. The most relevant ones are other hormones and metabolites because in antibody-based assays it is important to consider the possible cross-reactivity of the antibody with molecules that are structurally similar to cortisol (Mormède et al., 2007; Sheriff et al., 2011). Therefore, the specificity of the technique is estimated by calculating the percentage cross-reaction with different steroids and is something usually provided by the kit or antibody manufacturer (Chacón et al.,

2004). Although parallelism of diluted samples with a standard curve is not exactly a marker of specificity (but a proof of a dose-response relationship) (Möstl et al., 2005), it is usually used as a complementary tool to assess specificity as most of cross-reacting compounds do not yield correct dilution curves.

7.2.2.Linearity

The linearity (or linearity of dilution) of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of analyte in the sample (NCCLS, 2003; U.S. Department of Health and Human, 1999). A linear relationship should be evaluated across the range of the analytical procedure, for instance, with the aim to ensure that differences in HCC between animals with high and low HCC will be correctly detected (ICH, 2005). In other words regarding HCC, linearity provides confirmation that cortisol in hair extracts interacted with the assay antibody in a dose-dependent manner and supports the assumption that the antibody-binding characteristics of standard cortisol and cortisol from hair samples are similar.

7.2.3.Accuracy

Accuracy is a measure of the exactness of an analytical method. It describes the closeness of its measurement to the true concentration of the analyte(Food and Drug Administration, 2013). Accuracy can be affected by all components of an assay. Generally, accuracy has to be determined by comparing results to a reference method. However, in most cases, only an indirect assessment is possible, and several methods like calibration standards, recovery studies and parallelism are performed (Crowther and Walker, 2001).

7.2.4.Precision

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly from the same sample (Food and Drug Administration, 2013). Precision may be considered at three levels: repeatability (also termed intra-assay precision), intermediate precision (also termed inter-assay) and reproducibility (precision between laboratories) (ICH, 2005; U.S. Department of Health and Human, 1999).

In this thesis, in which all hormonal quantifications were performed in the same laboratory and using the same equipments and EIA kit models, precision were subdivided only into intra-assay (which assesses precision within the same EIA plate) and inter-assay (which assesses precision between different EIA plates).

7.2.5.Range

The range of an analytical procedure is the interval between the upper and lower concentration of analyte for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity (ICH, 2005; U.S. Department of Health and Human, 1999). In commercial immunoassay kits, the range is provided by the manufacturer and there is an important variability between brands and kit types.

7.2.6. Sensitivity

The sensitivity of an assay is defined as the smallest value that can be reliably discriminated from zero values with a 95% probability (two standard deviations from the signal given by the zero blanks) (Crowther and Walker, 2001; Möstl et al., 2005). As it is defined statistically, sensitivity is dependent on the precision and therefore, in practice the required sensitivity depends on a balance: it may be advantageous to reduce the sensitivity for certain assays to improve both accuracy and specificity (Crowther and Walker, 2001). The antigen-antibody interactions together with features inherent in assays also affect sensitivity.

Regarding sensitivity, sometimes it is separated in two concepts: detection limit and quantitation limit (U.S. Department of Health and Human, 1999). The detection limit is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value. In contrast, the quantitation limit is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. When analysing HCC using commercial immunoassay kits, sensitivity is provided and usually defined by the lowest standard on the calibration curve (the quantitation limit).

7.3. Validation of near infrared reflectance spectroscopy

There exist differences in validation processes between NIRS and immunoassays. Due to the predictive nature of NIRS, most of validation characteristics (e.g. range, specificity) are associated to the reference method as the process of calibrating involves all characteristics of the reference ones (Walker and Tolleson, 2010). However, before being able to validate NIRS for the detection of an analyte, it should be first calibrated. Calibrate means that specific predictive equations have been developed by relating absorbance of chemical bounds and values of analyte concentrations provided by the reference method. Once calibrated, the accuracy of NIRS is validated usually with an external set of samples (Marten et al., 1989). It should be noted that regarding NIRS, what is validated is not de detection of an analyte by NIRS but the detection of this analyte by NIRS through specific predictive equations (Stuth et al., 2003; Windham et al., 1989). Consequently, these equations can change and the same equipment can show completely different validation results.

8. REFERENCES

Aardal, E., Holm, A.C., 1995. Cortisol in saliva - Reference ranges and relation to cortisol in serum. Eur. J. Clin. Chem. Clin. Biochem. 33, 927–932.

Aardal-Eriksson, E., Karlberg, B.E., Holm, A.C., 1998. Salivary cortisol--an alternative to serum cortisol determinations in dynamic function tests. Clin. Chem. Lab. Med. 36, 215–22. doi:10.1515/CCLM.1998.037

Aerts, J., Metz, J.R., Ampe, B., Decostere, A., Flik, G., De Saeger, S., 2015. Scales Tell a Story on the Stress History of Fish 1–17. doi:10.1371/journal.pone.0123411

Arnott, G., Ferris, C.P., O'Connell, N.E., 2016. Review: welfare of dairy cows in continuously housed and pasture-based production systems. Animal 1–13. doi:10.1017/S1751731116001336

Ashley, N.T., Barboza, P.S., Macbeth, B.J., Janz, D.M., Cattet, M.R.L., Booth, R.K., Wasser, S.K., 2011. Glucocorticosteroid concentrations in feces and hair of captive caribou and reindeer following adrenocorticotropic hormone challenge. Gen. Comp. Endocrinol. 172, 382–391. doi:10.1016/j.ygcen.2011.03.029

Balíková, M., 2005. Hair analysis for drugs of abuse. Plausibility of interpretation. Biomed. Pap. Med. Fac. Univ. Palacky. Olomouc. Czech. Repub. 149, 199–207.

Barkema, H.W., von Keyserlingk, M.A.G., Kastelic, J.P., Lam, T.J.G.M., Luby, C., Roy, J.-P., LeBlanc, S.J., Keefe, G.P., Kelton, D.F., 2015. Invited review: Changes in the dairy industry affecting dairy cattle health and welfare. J. Dairy Sci. 98, 7426–7445. doi:10.3168/jds.2015-9377

Barnett, J.L., Hemsworth, P.H., 1990. The validity of physiological and behavioural measures of animal welfare. Appl. Anim. Behav. Sci. 25, 177–187. doi:10.1016/0168-1591(90)90079-S

Bayne, K., Turner, P. V, 2014. American College of Laboratory Animal Medicine Series: Laboratory animal welfare.

Beerda, B., Schilder, M.B.H., Janssen, N.S.C.R.M., Mol, J.A., 1996. The Use of Saliva Cortisol, Urinary Cortisol, and Catecholamine Measurements for a Noninvasive Assessment of Stress Responses in Dogs. Horm. Behav. 30, 272–279. doi:10.1006/hbeh.1996.0033

Ben Khelil, M., Tegethoff, M., Meinlschmidt, G., Jamey, C., Ludes, B., Raul, J.-S., 2011. Simultaneous measurement of endogenous cortisol, cortisone, dehydroepiandrosterone, and dehydroepiandrosterone sulfate in nails by use of UPLC-MS-MS. Anal. Bioanal. Chem. 401, 1153–1162. doi:10.1007/s00216-011-5172-3

Bennett, A., Hayssen, V., 2010. Measuring cortisol in hair and saliva from dogs: Coat color and pigment differences. Domest. Anim. Endocrinol. 39, 171–180. doi:10.1016/j.domaniend.2010.04.003

Berkvens, C.N., Hyatt, C., Gilman, C., Pearl, D.L., Barker, I.K., Mastromonaco, G.F., 2013. Validation of

a shed skin corticosterone enzyme immunoassay in the African House Snake (Lamprophis fuliginosus) and its evaluation in the Eastern Massasauga Rattlesnake (Sistrurus catenatus catenatus). Gen. Comp. Endocrinol. 194C, 1–9. doi:10.1016/j.ygcen.2013.08.011

Biancucci, A., Sbaragli, T., Comin, A., Sylla, L., Monaci, M., Peric, T., Stradaioli, G., 2016. Reducing treatments in cattle superovulation protocols by combining a pituitary extract with a 5% hyaluronan solution: Is it able to diminish activation of the hypothalamic pituitary adrenal axis compared to the traditional protocol? Theriogenology 85, 914–921. doi:10.1016/j.theriogenology.2015.10.041

Bigert, C., Bluhm, G., Theorell, T., 2005. Saliva cortisol – a new approach in noise research to study stress effects. Int. J. Hyg. Environ. Health 208, 227–230. doi:10.1016/j.ijheh.2005.01.014

Blanco, M., Villarroya, I., 2002. NIR spectroscopy: A rapid-response analytical tool. TrAC - Trends Anal. Chem. 21, 240–250. doi:10.1016/S0165-9936(02)00404-1

Boissy, A., Manteuffel, G., Jensen, M.B., Moe, R.O., Spruijt, B., Keeling, L.J., Winckler, C., Forkman, B., Dimitrov, I., Langbein, J., Bakken, M., Veissier, I., Aubert, A., 2007. Assessment of positive emotions in animals to improve their welfare. Physiol. Behav. 92, 375–397. doi:10.1016/j.physbeh.2007.02.003

Bortolotti, G.R., Marchant, T.A., Blas, J., German, T., 2008. Corticosterone in feathers is a long-term, integrated measure of avian stress physiology. Funct. Ecol. 22, 494–500. doi:10.1111/j.1365-2435.2008.01387.x

Bortolotti, G.R., Marchant, T., Blas, J., Cabezas, S., 2009. Tracking stress: localisation, deposition and stability of corticosterone in feathers. J. Exp. Biol. 212, 1477–1482. doi:10.1242/jeb.022152

Botreau, R., Veissier, I., Butterworth, A., Bracke, M.B.M., Keeling, L.J., 2007. Definition of criteria for overall assessment of animal welfare. Anim. Welf. 16, 225–228.

Botreau, R., Veissier, I., Pern, P., 2009. Overall assessment of animal welfare: Strategy adopted in Welfare Quality. Anim. Welf. 18, 363–370.

Boumba, V.A., Ziavrou, K.S., Vougiouklakis, T., 2006. Hair as a biological indicator of drug use, drug abuse or chronic exposure to environmental toxicants. Int. J. Toxicol. 25, 143–63. doi:10.1080/10915810600683028

Broom, D.M., 1986. Indicators of poor welfare. Br. Vet. J. 142, 524–526. doi:10.1016/0007-1935(86)90109-0

Broom, D.M., 1991a. Animal welfare: concepts and measurement. J. Anim. Sci. 69, 4167–4175.

Broom, D.M., 1991b. Assessing welfare and suffering. Behav. Processes 25, 117–123. doi:10.1016/0376-6357(91)90014-Q

Broom, D.M., Johnson, K.G., 1993. Stress and Animal Welfare. Springer Netherlands, Dordrecht.

doi:10.1007/978-94-024-0980-2

Buchanan, K.L., 2000. Stress and the evolution of condition-dependant signals. Trends Ecol. Evol. 15, 156–160.

Buchanan, K.L., Goldsmith, A.R., 2004. Noninvasive endocrine data for behavioural studies: the importance of validation. Anim. Behav. 67, 183–185. doi:10.1016/j.anbehav.2003.09.002

Burnett, T.A., Madureira, A.M.L., Silper, B.F., Tahmasbi, A., Nadalin, A., Veira, D.M., Cerri, R.L.A., 2015. Relationship of concentrations of cortisol in hair with health, biomarkers in blood, and reproductive status in dairy cows. J. Dairy Sci. 98, 1–13. doi:10.3168/jds.2014-8871

Burnett, T.A., Madureira, A.M.L.L., Silper, B.F., Nadalin, A., Tahmasbi, A., Veira, D.M., Cerri, R.L.A., 2014. Short communication: Factors affecting hair cortisol concentrations in lactating dairy cows. J. Dairy Sci. 97, 7685–7690. doi:10.3168/jds.2014-8444

Carbajal, A., Tallo-Parra, O., Sabes-Alsina, M., Mular, I., Lopez-Bejar, M., 2013. Feather corticosterone evaluated by ELISA in broilers : A potential tool to evaluate broiler welfare. Poult. Sci. 93, 1–3. doi:10.3382/ps.2014-04092

Carlstead, K., Brown, J.L., Strawn, W., 1993. Behavioral and physiological correlates of stress in laboratory cats. Appl. Anim. Behav. Sci. 38, 143–158. doi:10.1016/0168-1591(93)90062-T

Ceciliani, F., Ceron, J.J., Eckersall, P.D., Sauerwein, H., 2012. Acute phase proteins in ruminants. Spec. Issue Farm Anim. Proteomics 75, 4207–4231. doi:10.1016/j.jprot.2012.04.004

Cen, H., He, Y., 2007. Theory and application of near infrared reflectance spectroscopy in determination of food quality. Trends Food Sci. Technol. 18, 72–83. doi:10.1016/j.tifs.2006.09.003

Cerri, R.L.A., Tabmasbi, A.M., Veira, D.M., 2012. Hair cortisol concentrations -Influence of color and location in Holstein cows. J. Dairy Sci. 95, S574.

Chacón, G., García-Belenguer, S., Illera-del-Portal, J.C., Palacio, J., 2004. Validation of an EIA technique for the determination of salivary cortisol in cattle. Spanish J. Agric. Res. 2, 45–51.

Chan, D.W., 1987. General Principle of Immunoassay, in: Immunoassay. ACADEMIC PRESS, INC., pp. 1–23. doi:10.1016/B978-0-12-167635-3.50004-3

Charmandari, E., Tsigos, C., Chrousos, G., 2005. Endocrinology of the stress response. Annu. Rev. Physiol. 67, 259–84. doi:10.1146/annurev.physiol.67.040403.120816

Chernesky, M.A., Mahony, J.B., 1996. 7 – Immunoassays: Principles and assay design, in: Virology Methods Manual. pp. 123–141. doi:10.1016/B978-012465330-6/50008-7

Chrousos, G.P., 2009. Stress and disorders of the stress system. Nat. Rev. Endocrinol. 5, 374–381. doi:10.1038/nrendo.2009.106

Chrousos, G.P., Loriaux, D.L., Gold, P.W., 1988. Mechanisms of physical and emotional stress, in: Advances in Experimental Medicine and Biology. Springer science. doi:10.1007/978-1-4899-2064-5

Cirimele, V., Tracqui, A., Kintz, P., Ludes, B., 1999. First Identification of Prednisone in Human Hair by Liquid Chromatography-Ionspray Mass Spectrometry. J. Anal. Toxicol. 23, 225–226. doi:10.1093/jat/23.3.225

Clark, J.D., Rager, D.R., Calpin, J.P., 1997. Special Topic Overview Animal Well-Being II . Stress and Distress. Lab. Anim. Sci. 47, 571–579.

Cockrem, J.F., 2013. Individual variation in glucocorticoid stress responses in animals. Gen. Comp. Endocrinol. 181, 45–58. doi:10.1016/j.ygcen.2012.11.025

Comin, A., Peric, T., Corazzin, M., Veronesi, M.C.C., Meloni, T., Zufferli, V., Cornacchia, G., Prandi, A., 2013. Hair cortisol as a marker of hypothalamic-pituitary-adrenal axis activation in Friesian dairy cows clinically or physiologically compromised. Livest. Sci. 152, 36–41. doi:10.1016/j.livsci.2012.11.021

Comin, A., Peric, T., Magrin, L., Corazzin, M., Cornacchia, G., Prandi, A., 2014. Study of progesterone and cortisol concentrations in the Italian Friesian claw. J. Dairy Sci. 97, 5491–6. doi:10.3168/jds.2014-7943

Comin, A., Prandi, A., Peric, T., Corazzin, M., Dovier, S., Bovolenta, S., 2011. Hair cortisol levels in dairy cows from winter housing to summer highland grazing. Livest. Sci. 138, 69–73. doi:10.1016/j.livsci.2010.12.009

Comin, A., Tidu, L., Cornacchia, G., Cappa, A., Renaville, B., Prandi, A., 2008. Neonatal period and hair cortisol in cattle as a marker of stress., in: XVI Congress of the Mediterranean Federation for Health and Production of Ruminants (FeMeSPrum). 23-26 april, Zadar, Croatia, pp. 221–225.

Cone, E.J., 1996. Mechanisms of Drug Incorporation into Hair. Ther. Drug Monit. 18, 438–443. doi:10.1097/00007691-199608000-00022

Cook, C.J., Mellor, D.J., Harris, P.J., Ingram, J.R., Matthews, L.R., 2000. Hands-on and hands-off measurement of stress. Biol. Anim. Stress basic Princ. Implic. Anim. Welf. 123–146.

Cook, N.J., 2012. Review: Minimally invasive sampling media and the measurement of corticosteroids as biomarkers of stress in animals. Can. J. Anim. Sci. 92, 227–259. doi:10.4141/cjas2012-045

Creel, S., Creel, N., Wildt, D.E., Monfort, S.L., 1992. Behavioural and endocrine mechanisms of reproductive suppression in Serenge dwarf mongooses. Anim. Behav. 43, 231–245. doi:10.1016/S0003-3472(05)80219-2

Crowther, J., Walker, J., 2001. The ELISA guidebook. Humana Press Inc., New Jersey.

D'Anna-Hernandez, K.L., Ross, R.G., Natvig, C.L., Laudenslager, M.L., 2011. Hair cortisol levels as a retrospective marker of hypothalamic-pituitary axis activity throughout pregnancy: Comparison to

salivary cortisol. Physiol. Behav. 104, 348–353. doi:10.1016/j.physbeh.2011.02.041

Dantzer, B., Fletcher, Q.E., Boonstra, R., Sheriff, M.J., 2014. Measures of physiological stress: a transparent or opaque window into the status, management and conservation of species? Conserv. Physiol. 2, 1–18. doi:10.1093/conphys/cou023.

Dantzer, R., 2016. Behavior: Overview, Stress: Concepts, Cognition, Emotion, and Behavior. Elsevier Inc. doi:10.1016/B978-0-12-800951-2.00006-6

Davenport, M.D., Tiefenbacher, S., Lutz, C.K., Novak, M.A., Meyer, J.S., 2006. Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. Gen. Comp. Endocrinol. 147, 255–261. doi:10.1016/j.ygcen.2006.01.005

Davis, A.K., Maney, D.L., Maerz, J.C., 2008. The use of leukocyte profiles to measure stress in vertebrates: A review for ecologists. Funct. Ecol. 22, 760–772. doi:10.1111/j.1365-2435.2008.01467.x

Dawkins, M., 2004. Using behaviour to assess animal welfare. Anim. Welf. 13, 3–7.

Dawkins, M., 2015. Animal welfare and the paradox of animal consciousness, Advances in the Study of Behavior. Elsevier Ltd. doi:10.1016/bs.asb.2014.11.001

Deaville, E., Flinn, P., 2000. Near-infrared (NIR) spectroscopy: an alternative approach for the estimation of forage quality and voluntary intake, Forage evaluation in ruminant nutrition. doi:10.1079/9780851993447.0301

de Vries, M., Bokkers, E.A.M., Dijkstra, T., van Schaik, G., de Boer, I.J.M., 2011. Invited review: Associations between variables of routine herd data and dairy cattle welfare indicators. J. Dairy Sci. 94, 3213–3228. doi:10.3168/jds.2011-4169

Dobson, H., Tebble, J.E., Smith, R.F., Ward, W.R., 2001. Is stress really all that important? Theriogenology 55, 65–73. doi:10.1016/S0093-691X(00)00446-5

Duncan, I.J.H., 2005. Science-based assessment of animal welfare: farm animals. Rev. Sci. Tech. 24, 483–92.

Epel, E.S., Blackburn, E.H., Lin, J., Dhabhar, F.S., Adler, N.E., Morrow, J.D., Cawthon, R.M., 2004. Accelerated telomere shortening in response to life stress. Proc. Natl. Acad. Sci. U. S. A. 101, 17312–5. doi:10.1073/pnas.0407162101

Epel, E.S., Lin, J., Dhabhar, F.S., Wolkowitz, O.M., Puterman, E., Karan, L., Blackburn, E.H., 2010. Dynamics of telomerase activity in response to acute psychological stress. Brain. Behav. Immun. 24, 531–539. doi:10.1016/j.bbi.2009.11.018

Farm Animal Welfare Council, 1992. FAWC updates the five freedoms. Vet. Rec. 17, 357.

Farm Animal Welfare Council, 1993. Second Report on Priorities for Research and Development in Farm Animal Welfare, in: London: DEFRA.

Fink, G., 2007. Encyclopedia of Stress, Second edi. ed. Academic press.

Foley, W.J., McIlwee, A., Lawler, I., Aragones, L., Woolnough, A.P., Berding, N., 1998. Ecological applications of near infrared reflectance spectroscopy - A tool for rapid, cost-effective prediction of the composition of plant and animal tissues and aspects of animal performance. Oecologia. doi:10.1007/s004420050591

Food and Drug Administration, 2013. Guidance for industry. Bioanalytical method validation.

Food and Drug Administration, 2015. Analytical Procedures and Methods Validation for Drugs and Biologics. Guid. Ind. 1–15.

Foran, D.R., Minta, S.C., Heinemeyer, K.S., Forans, D.R., 1997. DNA-Based species and analysis of hair for to identify individuals and monitoring. Wildl. Soc. Bull. 25, 840–847.

Fraser, D., 1995. Science, Values and Animal Welfare: Exploring the “Inextricable Connection.” Anim. Welf. 4, 103–117.

Fraser, D., 2008. Understanding animal welfare. Acta Vet. Scand. 50 Suppl 1, S1–S12. doi:10.1186/1751-0147-50-S1-S1

Fraser, D., 2009. Assessing animal welfare: different philosophies, different scientific approaches. Zoo Biol. 28, 507–18. doi:10.1002/zoo.20253

Fraser, D., Weary, D., Pajor, E.A., Milligan, B.N., 1997. A scientific conception of animal welfare that reflects ethical concerns. Anim. Welf. 187–205.

Fukasawa, M., Tsukada, H., Kosako, T., Yamada, A., 2008. Effect of lactation stage, season and parity on milk cortisol concentration in Holstein cows. Livest. Sci. 113, 280–284. doi:10.1016/j.livsci.2007.05.020

González-de-la-Vara, M.R.D.R., Valdez, R.A., Lemus-Ramirez, V., Vázquez-Chagoyán, J.C., Villa-Godoy, A., Romano, M.C., 2011. Effects of adrenocorticotropic hormone challenge and age on hair cortisol concentrations in dairy cattle. Can. J. Vet. Res. 75, 216–221.

Gow, R., Thomson, S., Rieder, M., Van Uum, S., Koren, G., 2010. An assessment of cortisol analysis in hair and its clinical applications. Forensic Sci. Int. 196, 32–37. doi:10.1016/j.forsciint.2009.12.040

Grandin, T., Shivley, C., 2015. How Farm Animals React and Perceive Stressful Situations Such As Handling, Restraint, and Transport. Animals 5, 1233–1251. doi:10.3390/ani5040409

Grass, J., Miller, R., Carlitz, E.H.D., Patrovsky, F., Gao, W., Kirschbaum, C., Stalder, T., 2016. In vitro influence of light radiation on hair steroid concentrations. Psychoneuroendocrinology 73, 109–116. doi:10.1016/j.psyneuen.2016.07.221

Habib, K.E., Gold, P.W., Chrousos, G.P., 2001. Neuroendocrinology of stress, in: Neuroendocrinology. pp. 695–728. doi:10.1007/978-0-387-30405-2_14

Harkey, M.R., 1993. Forensic Science Internihnal Anatomy and physiology of hair. Forensic Sci. Int. 63, 9–18.

Harnad, S., 2016. Animal sentience : The other-minds problem. Anim. sentience 1–11.

Hart, K.A., 2012. The use of cortisol for the objective assessment of stress in animals: Pros and cons. Vet. J. 192, 137–139. doi:10.1016/j.tvjl.2012.03.016

Henderson, G.L.L., 1993. Mechanisms of drug incorporation into hair. Forensic Sci. Int. 63, 19–29. doi:10.1016/0379-0738(93)90256-A

Hernandez, C.E., Thierfelder, T., Svennersten-Sjaunja, K., Berg, C., Orihuela, A., Lidfors, L., 2014. Time lag between peak concentrations of plasma and salivary cortisol following a stressful procedure in dairy cattle. Acta Vet. Scand. 56, 61. doi:10.1186/s13028-014-0061-3

Hodges, K., Brwon, J., Heistermann, M., 2010. Endocrine Monitoring of Reproduction and Stress, in: Wild Mammals in Captivity: Principles and Techniques for Zoo Management. The University of Chicago, pp. 447–468.

Hofman, L.F., 2001. Human saliva as a diagnostic specimen. J. Nutr. 131, 1621S–1625S.

Hunt, K.E., Stimmelmayr, R., George, C., Hanns, C., Suydam, R., Jr, H.B., Rolland, R.M., 2014. Themed Issue Article : Stress in Vertebrates Baleen hormones : a novel tool for retrospective assessment of stress and reproduction in bowhead whales (Balaena mysticetus). Conserv. Physiol. 2, 1–12. doi:10.1093/conphys/cou030.

Huzzey, J.M., Nydam, D. V, Grant, R.J., Overton, T.R., 2011. Associations of prepartum plasma cortisol, haptoglobin, fecal cortisol metabolites, and nonesterified fatty acids with postpartum health status in Holstein dairy cows. J. Dairy Sci. 94, 5878–5889. doi:10.3168/jds.2010-3391

ICH, 2005. ICH Topic Q2 (R1) Validation of Analytical Procedures: Text and Methodology. Int. Conf. Harmon. 17.

Inder, W.J., Dimeski, G., Russell, A., 2012. Measurement of salivary cortisol in 2012 - Laboratory techniques and clinical indications. Clin. Endocrinol. (Oxf). 77, 645–651. doi:10.1111/j.1365-2265.2012.04508.x

Ito, N., Ito, T., Kromminga, A., Bettermann, A., Takigawa, M., Kees, F., Straub, R.H., Paus, R., 2005. Human hair follicles display a functional equivalent of the hypothalamic-pituitary-adrenal (HPA) axis and synthesize cortisol. Faseb J. 19, 1332–+. doi:10.1096/fj.04-1968fje

Jacobs, J.A., Siegford, J.M., 2012. Invited review: The impact of automatic milking systems on dairy cow management, behavior, health, and welfare. J. Dairy Sci. 95, 2227–47. doi:10.3168/jds.2011-4943

Jozic, I., Stojadinovic, O., Kirsner, R.S., Tomic-Canic, M., 2014. Stressing the steroids in skin: paradox or fine-tuning? J. Invest. Dermatol. 134, 2869–2872. doi:10.1038/jid.2014.363

Kahrer, E., Möstl, E., Baumgartner, W., 2006. Measurement of cortisol metabolites in faeces of transported cows with abomasal displacement. Bull. Vet. Inst. Pulawy 50, 105–106.

Keckeis, K., Lepschy, M., Schöpper, H., Moser, L., Troxler, J., Palme, R., 2012. Hair cortisol: a parameter of chronic stress? Insights from a radiometabolism study in guinea pigs. J. Comp. Physiol. B. 182, 985–96. doi:10.1007/s00360-012-0674-7

Kintz, P., 2004. Value of hair analysis in postmortem toxicology. Forensic Sci. Int. 142, 127–134. doi:10.1016/j.forsciint.2004.02.027

Kirschbaum, C., Hellhammer, D.H., 1994. Salivary cortisol in psychoneuroendocrine research: recent developments and applications. Psychoneuroendocrinology 19, 313–333. doi:10.1016/0306-4530(94)90013-2

Kirschbaum, C., Tietze, A., Skoluda, N., Dettenborn, L., 2009. Hair as a retrospective calendar of cortisol production—Increased cortisol incorporation into hair in the third trimester of pregnancy. Psychoneuroendocrinology 34, 32–37. doi:10.1016/j.psyneuen.2008.08.024

Koolhaas, J.M., Bartolomucci, a, Buwalda, B., de Boer, S.F., Flügge, G., Korte, S.M., Meerlo, P., Murison, R., Olivier, B., Palanza, P., Richter-Levin, G., Sgoifo, a, Steimer, T., Stiedl, O., van Dijk, G., Wöhr, M., Fuchs, E., 2011. Stress revisited: a critical evaluation of the stress concept. Neurosci. Biobehav. Rev. 35, 1291–301. doi:10.1016/j.neubiorev.2011.02.003

Kopin, I.J., Eisenhofer, G., Goldstein, D., 1988. Sympathoadrenal medullary system and stress, in: Mechanisms of Physical and Emotional Stress. pp. 11–23. doi:10.1007/978-1-4899-2064-5_2

Koren, L., Mokady, O., Karaskov, T., Klein, J., Koren, G., Geffen, E., 2002. A novel method using hair for determining hormonal levels in wildlife. Anim. Behav. 63, 403–406. doi:10.1006/anbe.2001.1907

Kupriyanov, R., Zhdanov, R., 2014. The Eustress Concept : Problems and Outlooks. World J. Med. Sci. 11, 179–185. doi:10.5829/idosi.wjms.2014.11.2.8433

Kurstak, E., 1986. Enzyme Immunodiagnosis, Enzyme Immunodiagnosis. ACADEMIC PRESS, INC. doi:10.1016/B978-0-12-429745-6.50008-8

Lacourt, A., Tarrant, P. V, 1985. Glycogen depletion patterns in myofibres of cattle during stress. Meat Sci. 15, 85–100. doi:10.1016/0309-1740(85)90049-X

Landon, J., Smith, D.C., Perry, L.A., Al-Ansari, A.A.K., 1984. The assay of salivary cortisol., in: Ltd., C.A.O.P. (Ed.), Immunoassays of Steroids in Saliva. Proceedings of the Ninth Tenovus Workshop, 1982. Cardiff: Alpha Omega Publishing Ltd., Cardiff: Alpha Omega Publishing Ltd., pp. 300–307.

Le Moal, M., 2007. Historical approach and evolution of the stress concept: A personal account. Psychoneuroendocrinology 32, S3–S9. doi:10.1016/j.psyneuen.2007.03.019

LeBeau, M.A., Montgomery, M.A., Brewer, J.D., 2011. The role of variations in growth rate and sample collection on interpreting results of segmental analyses of hair. Forensic Sci. Int. 210, 110–116. doi:10.1016/j.forsciint.2011.02.015

Legrand, A.L., von Keyserlingk, M.A.G., Weary, D.M., 2009. Preference and usage of pasture versus free-stall housing by lactating dairy cattle. J. Dairy Sci. 92, 3651–3658. doi:10.3168/jds.2008-1733

Lequin, R.M., 2005. Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). Clin. Chem. 51, 2415–8. doi:10.1373/clinchem.2005.051532

Levine, S., 2005. Stress: an historical perspective, in: Steckler, T., Kalin, N.H., Reul, J.M.H.M. (Eds.), Handbook of Stress and the Brain. Elsevier B.V., pp. 3–23.

Lightman, S.L., Conway-Campbell, B.L., 2010. The crucial role of pulsatile activity of the HPA axis for continuous dynamic equilibration. Nat. Rev. Neurosci. 11, 710–718.

Macbeth, B.J., Cattet, M.R.L.R.L., Stenhouse, G.B., Gibeau, M.L., Janz, D.M., 2010. Hair cortisol concentration as a noninvasive measure of long-term stress in free-ranging grizzly bears (Ursus arctos): Considerations with implications for other wildlife. Can. J. Zool. 88, 935–949. doi:10.1139/Z10-057

Macdonald, I.A., Bokkenheuser, V.D., Winter, J., Mclernon, A.M., Mosbach, E.H., 1983. Degradation of Steroids in the Human Gut. J. Lipid Res. 24, 675–700.

Maiero, S., Marchini, E., Comin, A., Renaville, B., Prandi, A., 2005. Determination of cortisol in hair as indicator of long-term stress in Simmenthal dairy cows. Reprod. Domest. Anim. 40, 396.

Makin, H.L.J., Newton, R., 1988. High-Performance Liquid Chromatography in Endocrinology. doi:10.1007/978-3-642-83467-7

Marten, G., Shenk, J., Barton, F., 1989. Near infrared reflectance spectroscopy (NIRS): analysis of forage quality. U.S. Dep. Agric. Agric. Handb. 643, 1–110.

Mason, J.W., 1975. A historical view of the stress field (Part I). J. Human Stress 1, 6–12. doi:10.1080/0097840X.1975.9940405

Matas, D., Keren-Rotem, T., Koren, L., 2016a. A method to determine integrated steroid levels in wildlife claws. Gen. Comp. Endocrinol. 230–231, 26–28. doi:10.1016/j.ygcen.2016.03.020

Matas, D., Koren, L., Mina, T., Gan, R., 2016b. A simple method for measuring long-term integrated testosterone levels in men 1–13.

Mathur, M.B., Epel, E., Kind, S., Desai, M., Parks, C.G., Sandler, D.P., Khazeni, N., 2016. Perceived Stress and Telomere Length: A Systematic Review, Meta-Analysis, and Methodologic Considerations for Advancing the Field. Brain. Behav. Immun. doi:10.1016/j.bbi.2016.02.002

McCarty, R., 2016. The Fight-or-Flight Response, Stress: Concepts, Cognition, Emotion, and Behavior. Elsevier Inc. doi:10.1016/B978-0-12-800951-2.00004-2

McEwen, B.S., 2007. Stress, Definitions and Concepts of, in: Fink, G. (Ed.), Encyclopedia of Stress. pp. 653–653. doi:10.1016/B978-012373947-6.00364-0

McEwen, B.S., Wingfield, J.C., 2010. What is in a name? Integrating homeostasis, allostasis and stress. Horm. Behav. 57, 105–111. doi:10.1016/j.yhbeh.2009.09.011

Meyer, J.S., Novak, M.A., 2012. Minireview: Hair cortisol: a novel biomarker of hypothalamic-pituitary-adrenocortical activity. Endocrinology 153, 4120–4127. doi:10.1210/en.2012-1226

Moberg, G., 1985. Animal Stress. Springer New York, New York, NY. doi:10.1007/978-1-4614-7544-6

Moberg, G., 2000. Biological response to stress: implications for animal welfare, in: Moberg, G.P., Mench, J.A. (Eds.), The Biology of Animal Stress: Basic Principles and Implications for Animal Welfare. CABI, Wallingford, Oxon, pp. 1–21.

Moberg, G.P., Mench, J.A., 2000. The biology of animal stress: basic principles and implications for animal welfare. CABI, Wallingford. doi:10.1079/9780851993591.0000

Monteverde, V., Perillo, L., Dara, S., Vazzana, I., Zumbo, A., Piccone, G., 2016. Relationship between different environmental and housing conditions on some acute phase markers in dairy cows. Large Anim. Rev. 22, 65–69.

Mormède, P., Andanson, S.S., Aupérin, B., Beerda, B., Guémené, D., Malmkvist, J., Manteca, X., Manteuffel, G., Prunet, P., van Reenen, C.G., Richard, S., Veissier, I., Mormede, P., Auperin, B., Guemene, D., 2007. Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. Physiol. Behav. 92, 317–339. doi:10.1016/j.physbeh.2006.12.003

Morrow, C.J., Kolver, E.S., Macdonald, K.A., Verkerk, G.A., Matthews, L.R., 2001. Monitoring adrenal activity in dairy cows under various feeding regimens using faecal glucocorticoid metabolites. Proc. New Zeal. Soc. Anim. Prod. 61, 52–55.

Morrow, C.J., Kolver, E.S., Verkerk, G.A., Matthews, L.R., 2002. Fecal glucocorticoid metabolites as a measure of adrenal activity in dairy cattle. Gen. Comp. Endocrinol. 126, 229–241. doi:10.1006/gcen.2002.7797

Möstl, E., Maggs, J.L., Schro, G., Schrötter, G., Besenfelder, U., Palme, R., 2002. Measurement of cortisol metabolites in faeces of ruminants. Vet. Res. Commun. 26, 127–139. doi:10.1023/A:1014095618125

Möstl, E., Messmann, S., Bagu, E., Robia, C., Palme, R., 1999. Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock. J. Vet. Med. Ser. A-Physiology Pathol. Clin. Med. 46, 621–631.

Möstl, E., Palme, R., 2002. Hormones as indicators of stress. Domest. Anim. Endocrinol. 23, 67–74. doi:10.1016/S0739-7240(02)00146-7

Möstl, E., Rettenbacher, S., Palme, R., 2005. Measurement of corticosterone metabolites in birds'

droppings: An analytical approach. Ann. N. Y. Acad. Sci. 1046, 17–34. doi:10.1196/annals.1343.004

Moya, D., Schwartzkopf-Genswein, K.S., Veira, D.M., 2013. Standardization of a non-invasive methodology to measure cortisol in hair of beef cattle. Livest. Sci. 158, 138–144. doi:10.1016/j.livsci.2013.10.007

NCCLS, 2003. EP6-A- Evaluation of the Linearity of Quantitative Measurement Procedures : A Statistical Approach ; Approved Guideline. National Committee for Clinical Laboratory Standards, West Valley Road, Suite 1400, Wayne, Pennsylvania, USA.

Nduwamungu, C., Ziadi, N., Parent, L.-É., Tremblay, G.F., Thuriès, L., 2009. Opportunities for, and limitations of, near infrared reflectance spectroscopy applications in soil analysis: A review. Can. J. Soil Sci. 89, 531–541. doi:10.4141/CJSS08076

Negrão, J.A., Porcionato, M.A., Passillé, A.M. de, Rushen, J., 2004. Cortisol in saliva and plasma of cattle after ACTH administration and milking. J. Dairy Sci. 87, 1713–8. doi:10.3168/jds.S0022-0302(04)73324-X

Nesse, R.M., Bhatnagar, S., Ellis, B., 2016. Evolutionary Origins and Functions of the Stress Response System, in: Stress: Concepts, Cognition, Emotion, and Behavior. pp. 95–101. doi:http://dx.doi.org/10.1016/B978-0-12-800951-2.00011-X

Ngo, T.T., 1991. Immunoassay. Curr. Opin. Biotechnol. 2, 102–109.

Ohl, F., van der Staay, F.J., 2012. Animal welfare: at the interface between science and society. Vet. J. 192, 13–19. doi:10.1016/j.tvjl.2011.05.019

Osborne, B.G., 2000. Near-infrared spectroscopy in food analysis. Encycl. Anal. Chem. 1–14. doi:10.1002/9780470027318.a1018

Ott, S., Soler, L., Moons, C.P.H., Kashiha, M.A., Bahr, C., Vandermeulen, J., Janssens, S., Gutiérrez, A.M., Escribano, D., Cerón, J.J., Berckmans, D., Tuyttens, F.A.M., Niewold, T.A., 2014. Different stressors elicit different responses in the salivary biomarkers cortisol, haptoglobin, and chromogranin A in pigs. Res. Vet. Sci. doi:10.1016/j.rvsc.2014.06.002

Palme, R., 2012. Monitoring stress hormone metabolites as a useful, non-invasive tool for welfare assessment in farm animals. Anim. Welf. 21, 331–337. doi:10.7120/09627286.21.3.331

Palme, R., Fischer, P., Schildorfer, H., Ismail, M.N., 1996. Excretion of infused 14C-steroid hormones via faeces and urine in domestic livestock. Anim. Reprod. Sci. 43, 43–63. doi:10.1016/0378-4320(95)01458-6

Palme, R., Möstl, E., 1997. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. Zeitschrift Fur Saugetierkunde-International J. Mamm. Biol. 62, 192–197.

Palme, R., Rettenbacher, S., Touma, C., El-Bahr, S.M., Möstl, E., 2005. Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion, and noninvasive measurement in fecal samples. Ann. N. Y. Acad. Sci. 1040, 162–171. doi:10.1196/annals.1327.021

Palme, R., Robia, C., Messmann, S., Hofer, J., Möstl, E., 1999. Measurement of faecal cortisol metabolites in ruminants: a non-invasive parameter of adrenocortical function. Wien. Tierarztl. Monatsschr. 86, 237–241.

Parks, C.G., Miller, D.B., McCanlies, E.C., Cawthon, R.M., Andrew, M.E., DeRoo, L. a., Sandler, D.P., 2009. Telomere Length, Current Perceived Stress, and Urinary Stress Hormones in Women. Cancer Epidemiol. Biomarkers Prev. 18, 551–560. doi:10.1158/1055-9965.EPI-08-0614

Peric, T., Comin, A., Corazzin, M., Montillo, M., Cappa, A., Campanile, G., Prandi, A., 2013. Short communication: hair cortisol concentrations in Holstein-Friesian and crossbreed F1 heifers. J. Dairy Sci. 96, 3023–7. doi:10.3168/jds.2012-6151

Phillips, C.J.C., 2002. Cattle behaviour and welfare, Second Edi. ed. Blackweel Science Ltd.

Porstmann, T., Kiessig, S.T., 1992. Enzyme immunoassay techniques an overview. J. Immunol. Methods 150, 5–21. doi:10.1016/0022-1759(92)90061-W

Pragst, F., Balikova, M.A., 2006. State of the art in hair analysis for detection of drug and alcohol abuse. Clin. Chim. Acta. 370, 17–49. doi:10.1016/j.cca.2006.02.019

Rabiee, A.R., Macmillan, K.L., Schwarzenberger, F., 2001. The effect of level of feed intake on progesterone clearance rate by measuring faecal progesterone metabolites in grazing dairy cows. Anim. Reprod. Sci. 67, 205–214. doi:10.1016/S0378-4320(01)00130-0

Ralph, C.R., Tilbrook, A.J., 2016. INVITED REVIEW : The usefulness of measuring glucocorticoids for assessing animal welfare. J. Anim. Sci. 94, 457–470. doi:10.2527/jas2015-9645

Raul, J.S., Cirimele, V., Ludes, B., Kintz, P., 2004. Detection of physiological concentrations of cortisol and cortisone in human hair. Clin. Biochem. 37, 1105–1111. doi:10.1016/j.clinbiochem.2004.02.010

Rizzolatti, G., Craighero, L., 2005. Mirror neuron: a neurological approach to empathy, in: Neurobiology of Human Values. pp. 107–123. doi:10.1007/3-540-29803-7_9

Robbins, C.R., 1988. Chemical and physical behavior of human hair, 4th Editio. ed, Chemical and Physical Behaviour of Human Hair. Springer. doi:10.1007/978-3-642-25611-0

Roland, L., Drillich, M., Klein-Jöbstl, D., Iwersen, M., 2016. Invited review: Influence of climatic conditions on the development, performance, and health of calves. J. Dairy Sci. 1–15. doi:10.3168/jds.2015-9901

Romero, L.M., Butler, L.K., 2007. Endocrinology of Stress. Int. J. Comp. Psychol. 20, 89–95.

Romero, L.M., Dickens, M.J., Cyr, N.E., 2009. The reactive scope model - A new model integrating

homeostasis, allostasis, and stress. Horm. Behav. 55, 375–389. doi:10.1016/j.yhbeh.2008.12.009

Romero, L.M., Fairhurst, G.D., 2016. Measuring corticosterone in feathers: Strengths, limitations, and suggestions for the future. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. doi:10.1016/j.cbpa.2016.05.002

Romero, L.M., Platts, S.H., Schoech, S.J., Wada, H., Crespi, E., Martin, L.B., Buck, C.L., 2015. Understanding stress in the healthy animal – potential paths for progress. Stress 18, 491–497. doi:10.3109/10253890.2015.1073255

Rushen, J., 2000. Some issues in the interpretation of behavioral responses to stress. Biol. Anim. Stress basic Princ. Implic. Anim. Welf. 23–42. doi:10.1079/9780851993591.0023

Rushen, J., de Passillé, A.M., von Keyserlingk, M.A.G., Weary, D.M., Passillé, A. de, Keyserlingk, M. von, 2008. The welfare of cattle. Springer. doi:10.1007/978-1-4020-6558-3

Russell, E., Koren, G., Rieder, M., Van Uum, S., 2012. Hair cortisol as a biological marker of chronic stress: Current status, future directions and unanswered questions. Psychoneuroendocrinology 37, 589–601. doi:10.1016/j.psyneuen.2011.09.009

Saco, Y., Fina, M., Giménez, M., Pato, R., Piedrafita, J., Bassols, A., 2008. Evaluation of serum cortisol, metabolic parameters, acute phase proteins and faecal corticosterone as indicators of stress in cows. Vet. J. 177, 439–441. doi:10.1016/j.tvjl.2007.05.019

Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21, 55–89.

Scarff, M., Arnold, S.A., Harvey, L.M., McNeil, B., 2006. Near Infrared Spectroscopy for bioprocess monitoring and control: Current status and future trends. Crit. Rev. Biotechnol. 26, 17–39. doi:Doi 10.1080/07388550500513677

Sharpley, C.F., Kauter, K.G., McFarlane, J.R., 2009. An Initial Exploration of in vivo Hair Cortisol Responses to a Brief Pain Stressor: Latency, Localization and Independence Effects. Physiol. Res. 58, 757–761.

Sharpley, C.F., McFarlane, J.R., Slominski, A., 2012. Stress-linked cortisol concentrations in hair: what we know and what we need to know. Rev. Neurosci. 23, 111–121. doi:10.1515/RNS.2011.058

Sheriff, M.J., Dantzer, B., Delehanty, B., Palme, R., Boonstra, R., 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia 166, 869–87. doi:10.1007/s00442-011-1943-y

Silva, P.R.B., Lobeck-Luchterhand, K.M., Cerri, R.L.A., Haines, D.M., Ballou, M.A., Endres, M.I., Chebel, R.C., 2016. Effects of prepartum stocking density on innate and adaptive leukocyte responses and serum and hair cortisol concentrations. Vet. Immunol. Immunopathol. 169, 39–46. doi:10.1016/j.vetimm.2015.11.007

Skrzipczyk, H.J., Verdier, P., 2013. Bioanalytical Assays: RIA/EIA. Drug Discov. Eval. Saf. Pharmacokinet. Assays 869–886. doi:10.1007/978-3-642-25240-2

Slominski, A., Zbytek, B., Nikolakis, G., Manna, P.R., Skobowiat, C., Zmijewski, M., Li, W., Janjetovic, Z., Postlethwaite, A., Zouboulis, C.C., Tuckey, R.C., 2013. Steroidogenesis in the skin: implications for local immune functions. J. Steroid Biochem. Mol. Biol. 137, 107–23. doi:10.1016/j.jsbmb.2013.02.006

Slominski, A.T., Manna, P.R., Tuckey, R.C., 2014. Cutaneous glucocorticosteroidogenesis: securing local homeostasis and the skin integrity. Exp. Dermatol. 23, 369–74. doi:10.1111/exd.12376

Stafford, K.J., Mellor, D.J., 2011. Addressing the pain associated with disbudding and dehorning in cattle. Appl. Anim. Behav. Sci. 135, 226–231. doi:10.1016/j.applanim.2011.10.018

Stalder, T., Kirschbaum, C., 2012. Analysis of cortisol in hair – State of the art and future directions. Brain. Behav. Immun. 26, 1019–29. doi:10.1016/j.bbi.2012.02.002

Staples, C.R., Thatcher, W.W., 2000. Stress, heat, in dairy cattle. Effects on milk production and composition, in: Encyclopedia of Dairy Science. pp. 2592–2597.

Stepaniak, L., Sorhaug, T., Jedrychowski, L., 2002. ANALYSIS/Immunochemical, in: Encyclopedia of Dairy Sciences. pp. 62–67.

Stull, C., Reynolds, J., 2008. Calf Welfare. Vet. Clin. North Am. - Food Anim. Pract. 24, 191–203. doi:10.1016/j.cvfa.2007.12.001

Stuth, J., Jama, A., Tolleson, D., 2003. Direct and indirect means of predicting forage quality through near infrared reflectance spectroscopy. F. Crop. Res. 84, 45–56. doi:10.1016/S0378-4290(03)00140-0

Sultan, A., Raza, A.R., 2015. Steroids: A Diverse Class of Secondary Metabolites. Med. Chem. (Los. Angeles). 5, 310–317. doi:10.4172/2161-0444.1000279

Tallo-Parra, O., Crespo-Picazo, J.L., García-Parraga, D., Monclús, L., Carbajal, A., Sabes-Alsina, M., Lopez-Bejar, M., 2015. Cortisol detection in new matrixes from several marine mammals: vibrissae, baleen plates and shed skin, in: International Conference on Diseases of Zoo and Wild Animals. Barcelona, Spain.

Taves, M.D., Gomez-Sanchez, C.E., Soma, K.K., 2011. Extra-adrenal glucocorticoids and mineralocorticoids: evidence for local synthesis, regulation, and function. Am. J. Physiol. Endocrinol. Metab. 301, E11-24. doi:10.1152/ajpendo.00100.2011

Taylor, W., 1971. The excretion of steroid hormone metabolites in bile and feces. Vitam.Horm 29, 201–285.

Thun, R., Eggenberger, E., Zerobin, K., Luscher, T., Vetter, W., 1981. 24-Hour Secretory Pattern of Cortisol in the Bull - Evidence of Episodic Secretion and Circadian-Rhythm. Endocrinology 109,

2208–2212.

Touma, C., Palme, R., 2005. Measuring Fecal Glucocorticoid Metabolites in Mammals and Birds: The Importance of Validation. Ann. N. Y. Acad. Sci. 1046, 54–74. doi:10.1196/annals.1343.006

Trumble, S.J., Robinson, E.M., Berman-Kowalewski, M., Potter, C.W., Usenko, S., 2013. Blue whale earplug reveals lifetime contaminant exposure and hormone profiles. Proc. Natl. Acad. Sci. U. S. A. 110, 16922–6. doi:10.1073/pnas.1311418110

Tsigos, C., Chrousos, G.P., 2002. Hypothalamic–pituitary–adrenal axis, neuroendocrine factors and stress. J. Psychosom. Res. 53, 865–871. doi:10.1016/S0022-3999(02)00429-4

Tucker, H.A., Schwalm, J.W., 1977. Glucocorticoids in mammary tissue and milk. J. Anim. Sci. 46, 627–634.

Turpeinen, U., Hämäläinen, E., 2013. Determination of cortisol in serum, saliva and urine. Best Pract. Res. Clin. Endocrinol. Metab. 27, 795–801. doi:10.1016/j.beem.2013.10.008

U.S. Department of Health and Human, 1999. Guidance for Industry: VALIDATION OF ANALYTICAL PROCEDURES: DEFINITION AND TERMINOLOGY.

Verkerk, G.A., Phipps, A.M., Carragher, J.F., Matthews, L.R., Stelwagen, K., 1998. Characterization of Milk Cortisol Concentrations as a Measure of Short-Term Stress Responses in Lactating Dairy Cows. Anim. Welf. 7, 77–86.

Veronesi, M.C., Comin, A., Meloni, T., Faustini, M., Rota, A., Prandi, A., 2015. Coat and claws as new matrices for non-invasive long-term cortisol assessment in dogs from birth up to 30 days of age. Theriogenology. doi:10.1016/j.theriogenology.2015.05.013

Vogliardi, S., Tucci, M., Stocchero, G., Ferrara, S.D., Favretto, D., 2014. Sample preparation methods for determination of drugs of abuse in hair samples: A review. Anal. Chim. Acta. doi:10.1016/j.aca.2014.06.053

von Borell, E., Dobson, H., Prunier, A., 2007. Stress, behaviour and reproductive performance in female cattle and pigs. Horm. Behav. 52, 130–138. doi:10.1016/j.yhbeh.2007.03.014

von Keyserlingk, M.A.G., Rushen, J., de Passillé, A.M., Weary, D.M., 2009. The welfare of dairy cattle—Key concepts and the role of science. J. Dairy Sci. 92, 4101–4111. doi:10.3168/jds.2009-2326

Walker, J., Tolleson, D., 2010. Shining Light on Manure Improves Livestock and Land Management, First edit. ed, Texas AgriLife Research and Society for Range Management. Texas AgriLife Research and Society for Range Management, Jefferson City, Missouri.

Walker, M., Diez-Leon, M., Georgia, M., 2014. Animal Welfare Science: Recent Publication Trends and Future Research Priorities. Int. J. Comp. Psychol. 27, 80–100.

Warnock, F., McElwee, K., Seo, R.J., McIsaac, S., Seim, D., Ramirez-Aponte, T., Macritchie, K.A.N.,

Young, A.H., 2010. Measuring cortisol and DHEA in fingernails: A pilot study. Neuropsychiatr. Dis. Treat. 6, 1–7.

Weary, D.M., Niel, L., Flower, F.C., Fraser, D., 2006. Identifying and preventing pain in animals. Appl. Anim. Behav. Sci. 100, 64–76. doi:10.1016/j.applanim.2006.04.013

Webster, A.J.F., 2001. Farm animal welfare: the five freedoms and the free market. Vet. J. 161, 229–237. doi:10.1053/tvjl.2000.0563

Wester, V.L., van der Wulp, N.R.P., Koper, J.W., de Rijke, Y.B., van Rossum, E.F.C., 2016. Hair cortisol and cortisone are decreased by natural sunlight. Psychoneuroendocrinology. doi:10.1016/j.psyneuen.2016.06.016

Wickins-Dražilová, D., 2006. Zoo Animal Welfare. J. Agric. Environ. Ethics 19, 27–36. doi:10.1007/s10806-005-4380-2

Willemse, T., Vroom, M.W., Mol, J.A., Rijnberk, A., 1993. Changes in Plasma Cortisol,corticotropin, and Alpha-Melanocyte-Stimulating Hormone Concentrations in Cats before and After Physical Restraint and Intradermal Testing. Am. J. Vet. Res. 54, 69–72.

Windham, W.R., Mertens, D.R., Barton, F.E., 1989. Protocol for NIRS Calibration: Sample Selection and Equation Development and Validation, in: Marten, G.C., Shenk, J.S., Barton, F.E. (Eds.), Near Infrared Reflectance Spectroscopy (NIRS): Analysis of Forage Quality. pp. 96–104.

Woodruff, D.S., 1993. Non-invasive genotyping of primates. Primates 34, 333–346. doi:10.1007/BF02382629

OBJECTIVES

The general objective of this thesis is to validate, evaluate and optimize the use of hair cortisol measurement as a tool to assess long-term adrenal activity in cattle.

The specific objectives are as follows:

1. To validate a standardized protocol for the extraction of cortisol from cattle's hair and the quantification of these cortisol concentrations by an enzyme immunoassay kit.

2. To study the feasibility of hair cortisol concentrations to represent a long-term integrative value of adrenal activity.

3. To evaluate the potential uses of hair cortisol determination in dairy cattle production and its relationship with hair progesterone concentrations.

4. To evaluate near infrared reflectance spectroscopy technique for hair cortisol quantification in cows.

5. To study secondary characteristics of hair matrix with an influence in hair cortisol measurement.

With the aim to reach these objectives, four different studies were performed. The first study was entitled "*Hair cortisol detection in dairy cattle by using EIA: protocol validation and correlation with faecal cortisol metabolites*" (Chapter I) and influenced in the assessment of specific objectives 1 and 2. The second study, named "*Acute ACTH-induced elevations of circulating cortisol do not affect hair cortisol concentrations in calves*" (Chapter III), was designed for the specific objective 2. The third study was "*Hair cortisol and progesterone detection in dairy cattle: interrelation with biological status and milk production and composition*" (Chapter IV) and allowed us to reach specific objective 3. Finally, in order to optimise hair steroid detection, near infrared reflectance spectroscopywas tested in the study "*Prediction of cortisol and progesterone concentrations in hair from cow by near infrared reflectance spectroscopy (NIRS)*" (Chapter V) for trying to achieve specific objective 4. The specific objective 5 was established at the beginning of this thesis as we were plenty conscious that hair is a new matrix for cortisol assessment and the report of any resulting knowledge from the conducted studies could be relevant to achieve the general objective. All studies carried out were useful for the achievement of specific objective 5.

Taking advantage of hair steroid extraction for hair cortisol analysis, progesterone in hair was also quantified in studies performed in Chapter IV and V and its biochemical validation is also presented in Chapter II. The results and discussions regarding hair progesterone analysis are included in these specific chapters but only a specific section was given to it in the general discussion as hair progesterone analysis was considered complementary to this thesis.

Chapter I

Hair cortisol detection in dairy cattle by using EIA: protocol validation and correlation with faecal cortisol metabolites

Hair cortisol detection in dairy cattle by using EIA: protocol validation and correlation with faecal cortisol metabolites

O. Tallo-Parra[1,2], X. Manteca[1], M. Sabes-Alsina[2], A. Carbajal[2] and M. Lopez-Bejar[2]

[1] *Department of Animal and Food Science, Faculty of Veterinary, UniversitatAutònoma de Barcelona,* Edifici V, Campus UAB, 08193 Bellaterra, Spain

[2] *Department of Animal Health and Anatomy, Faculty of Veterinary, UniversitatAutònoma de Barcelona,* Edifici V, Campus UAB, 08193 Bellaterra, Spain

This chapter has been published in *Animal*

(*Animal*. 2015.9:(6) 1059–1064)

1. ABSTRACT

Hair may be a useful matrix to detect cumulative cortisol concentrations in studies of animal welfare and chronic stress. The aim of this study was to validate a protocol for cortisol detection in hair from dairy cattle by enzyme immunoassay (EIA). Seventeen adult Holstein-Friesian dairy cows were used during the milking period. Hair cortisol concentration was assessed in 25-day-old hair samples taken from the frontal region of the head, analyzing black and white coloured hair separately. Concentrations of cortisol metabolites were determined in faeces collected twice a week during the same period of time. There was a high correlation between cortisol values in faeces and cortisol in white colour hair samples but such correlation was not significant with the black colour hair samples. The intra- and inter-assay coefficients of variation were 4.9 % and 10.6 %, respectively. The linearity showed R^2 = 0.98 and mean percentage error of -10.8 ± 1.55 %. The extraction efficiency was 89.0 ± 23.52 % and the parallelism test showed similar slopes. Cortisol detection in hair by using EIA seems to be a valid method to represent long term circulating cortisol levels in dairy cattle.

2. IMPLICATIONS

The main implication of this study is the validation of a methodology for hair cortisol detection by EIA in dairy cattle. Hair cortisol detection is a promising methodology able to give information on long term retrospective cortisol levels, which cannot be provided by any other established matrix. This study opens the possibility to use this methodology in chronic stress studies in dairy cattle welfare research, which is nowadays a hot topic.

3. INTRODUCTION

The importance of welfare in dairy cattle production is increasing not only as a consequence of society demands, but also due to the relationship between animal welfare and performance (von Keyserlingk et al., 2009). Different methodologies have been developed to objectively assess cattle welfare, including quantification of cortisol response to stressors (Morrow et al., 2002; Probst et al., 2014; Rigalma et al., 2010).

Depending on the matrix where cortisol is analysed, the information provided by its concentration is different (Russell et al., 2012). Cortisol concentration in serum and saliva can be used to detect acute changes in circulating cortisol concentrations although they do not represent long term circulating cortisol levels (Hernandez et al., 2014; Negrão et al., 2004; Thun et al., 1981). Cortisol in urine should be collected over 24 hours to provide an integral daily cortisol value and this may be rather complicated in farm animals (Aardal and Holm, 1995; Russell et al., 2012). Cortisol in faeces is measured indirectly by detecting its metabolites (Möstl et al., 1999; Touma and Palme, 2005) and despite it has been extensively used and validated (Möstl et al., 2002, 1999; Palme et al., 1999) this matrix still have some limitations. Cortisol metabolites concentrations could be modified by changes in diet, intestinal transit and/or bacterial activity (Möstl et al., 2002; Möstl and Palme, 2002) and have storage requirements (Morrow et al., 2002; Palme et al., 2013). However, faecal collection is less invasive and stressful than saliva, blood or urine and cortisol metabolites detection has been demonstrated as a valid tool to assess 12h to 24h retrospective cortisol levelsin cows (Möstl et al., 1999; Touma and Palme, 2005).

Detection of cortisol concentrations in hair seems to provide an integrated value of retrospective circulating cortisol levels during the hair growth (Meyer and Novak, 2012). This is due to the hair growth physiology, hair vascularisation and hormone accumulation mechanisms that are associated (Gow et al., 2010; Koren et al., 2002; Stalder and Kirschbaum, 2012). No other matrix seems to have the same potential to evaluate long-term hypothalamic–pituitary–adrenal (HPA)-axis activity and thereby, long term or chronic stress (Macbeth et al., 2012; Russell et al., 2012; Van Uum et al., 2008). In spite of the clear advantages and potential applications of hair cortisol detection, several gaps need to be clarified, especially regarding the origin of the cortisol and the factors that modulate its accumulation. Some accumulation mechanisms are still incompletely understood (Boumba et al., 2006; Cone, 1996; Henderson, 1993) and thepresence of

local steroidogenesis in skin has been demonstrated (Slominski et al., 2013, 2014; Taves et al., 2011). As a consequence, some studies discuss the main source of cortisol accumulated in hair by highlighting the importance of local cortisol production in the final hair cortisol concentration and its different regulation from adrenal-origin cortisol (Ito et al., 2005; Keckeis et al., 2012; Sharpley et al., 2012). However, several studies have associated elevations of hair cortisol concentrations with stressful situations (Comin et al., 2013, 2008; Dettmer et al., 2012), providing that hair cortisol concentrations (regardless of cortisol origin and regulation) can be sensitive to the presence of certain stressors.

Although enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA) methodologies have been used to detect hair cortisol in cows and beef (Burnett et al., 2014; Cerri et al., 2012; Moya et al., 2013), a complete validation of the methodology has only been done in beef cattle (Moya et al., 2013). Other studies have detected cortisol in hair from dairy cows using radioimmunoassay (RIA) with validation tests (Comin et al., 2012, 2011; González-de-la-Vara et al., 2011). Nevertheless, validation tests cannot be generalised or extrapolated from one technique to another or after using different extraction protocols (Buchanan and Goldsmith, 2004; Gatti et al., 2009; Stalder and Kirschbaum, 2012).

The aims of this study were to validate a protocol and the use of an EIA test to measure cortisol concentrations in hair from Holstein-Friesian cows and to evaluate if a single hair cortisol value can represent retrospective cortisol concentrations during a specific period of time in milking cows. This evaluation was done by correlating hair cortisol levels with average values of faecal cortisol metabolites measured repeatedly during the same period of time.

4. MATERIAL AND METHODS

4.1. Animals, housing conditions, diet and handling

Seventeen adult Holstein-Friesian dairy cows (mean age 4.0 years old; range 2.1 – 7.2 y.o) were used during the milking period (mean days in milk 146.5; range 13 – 338 days). All animals were under the same conditions on a covered farm with cubicles (1 lying stall/cow) located in Caldes de Malavella (Girona, Spain, 41º 49' 34.4", 2º 46' 19.19"). Milking was done robotically twice a day. All animals received the same diet (as-fed basis: 20 kg of corn silage; 4 kg of dry alfalfa; 0.5 kg of straw; 10 kg of unifeed composed mainly of corn and soybean; and 3 kg of concentrate equilibrated per cow and day in milk in the robot). Diet and concentrated formulation were established according to the NRC recommendations (2001). Animals were handled and sampled benefiting the restraining time to eat implemented in the cows' routine between 0830h and 1030h in the morning.

4.2. Faecal sampling and steroid extraction

Faeces were individually collected twice a week for four weeks (days 0, 4, 7, 11, 14, 17, 21, 23). The number of faecal samples required to provide comparable repeatability to that of a single hair sample was

based on study of Bryan et al. (2013) performed in dogs (*Canis lupus familiaris* or *Canisfamiliaris*). Faeces were collected by direct extraction from the rectum and stored in a zip-lock plastic bag at -20 ºC until steroid extraction.

For steroid extraction, DetectX™ Steroid Solid Extraction Protocol (Arbor Assays®, MI, USA) was followed with modifications based on de Lima et al. (2013) and Sabés-Alsina et al. (2015) studies. Faecal samples were put into an oven (Heraeus model T6, Kendro® Laboratory Products, Langenselbold, Germany) at 60 ºC for approximately 48 hours in order to evaporate the water. Once dried, faecal samples were manually ground by using a mortar and 300 mg faecal powder were weighted and put into a 15-ml conical tube (Deltalab, S.L., Rubí, Spain). After that, 2.5 ml distilled water and 3 ml methanol (methanol reagent grade 99.9 %, Scharlab S.L., Sentmenat, Spain) were added to each sample and vortexed (Vortex Mixer S0200-230V-EU; Labnet International Inc., NJ, USA) for 30 minutes. Then, the samples were centrifuged (Hermle Z300K, Hermle® Labortechnik, Wehingen, Germany) at 1750 g for 15 minutes and 1 ml of the supernatant was transferred into a 1.5-ml eppendorf tube (Scharlab S.L., Sentmenat, Spain) and immediately stored at -20 ºC until analysis.

4.3. Hair sampling and hormone extraction

Hair samples were collected on the first day (d_0) and last day (d_{25}) of the study. The hair collected at d_{25} was only the new hair regrowing after d_0 collection. White and black hair samples were collected from each animal and analysed separately. White hair samples were collected from the frontal region of the head (forehead). Black hair samples were collected from the frontal region of the head and also from the occipital crest area in order to obtain enough amount of sample. All samples were collected using the same electric hair clipper (X3 ceramic-titanium hair clipper, Palson® Trading España S.L., Collbató, Spain) and trying to acquire the longest possible hair sample while at the same time avoiding to injure the skin or to take out the hair follicles. The clipper blade was disinfected with alcohol 70 % after each animal recollection. Each hair sample weighed around 5 g and was stored into zip-lock plastic bag at room temperature and darkness until washing and cortisol extraction.

For the hair cortisol extraction, a modified protocol from that described by Davenport et al. (2006)and developed by our lab for other species was followed (Tallo-Parra et al., 2013). Two hundred and fifty milligrams of hair from each sample were weighed and placed into a 15-ml conical tube. Each sample was washed by adding 2.5 ml of isopropanol (2-propanol 99.5%, Scharlab S.L., Sentmenat, Spain) and vortexed at 1800 rpm for 2.5 minutes in order to remove external steroid sources but avoiding the loss of internal steroids as suggested by Davenport (2006). The supernatant was separated by decantation and the process was repeated twice (three washes in total). The hair samples were left to dry completely for approximately 36 hours at room temperature. Then, the hair was minced into < 2 mm length fragments by using a peeler and 50 mg of trimmed hair were carefully weighted and placed into a 2-ml eppendorf tube. For each sample, 1.5 ml pure methanol was added and the samples were shaken at 100 rpm for 18 hours at 30ºC (G24 Environmental Incubator Shaker, New Brunswick Scientific CO Inc., Edison, NJ, USA) to steroid extraction. Following extraction, samples were centrifuged at 7 000 × g for 2 minutes. Subsequently, 0.750

ml of supernatant were transferred into a new 2-ml eppendorf tube and then placed in an oven at 38 ºC. Once the methanol was completely evaporated (approximately after 24 hours), the dried extracts were reconstituted with 0.2 ml EIA buffer provided by the EIA assay kit (Cortisol ELISA KIT; Neogen® Corporation, Ayr, UK) and shaken for 30 seconds. Then the samples were immediately stored at -20 ºC until analysis.

4.4. Steroid analysis and validation tests

The cortisol concentrations from hair extracts, the metabolites concentrations from faecal extracts and all the validation tests were determined per duplicate by using cortisol EIA detection kits (Neogen® Corporation Europe, Ayr, UK), with a sensitivity of 0.32 pg cortisol/mg of hair and 7.3 10^{-4} ng cortisol/mg of dry faeces.

All the validation tests used different pool of samples constituted by five hair extracts each one with high, low and medium final concentrations. Each final solution was analysed by duplicate. The precision within test was assessed by calculating intra-assay coefficients of variation from all duplicated samples analysed. The inter-assay coefficients of variation was calculated from ten pool samples with markedly different concentrations and analysed per duplicate in each EIA kit. The linearity of dilution was determined by using 1:1, 1:2, 1:5 and 1:10 dilutions of pools with EIA buffer. Accuracy was assessed through the spike-and-recovery test, calculated by adding to 50, 100 and 200 µl of pool, volumes of 200, 100 and 50 µl of pure standard cortisol solution, respectively. Combinations were repeated with three different pure standard cortisol solutions (20, 2 and 0.2 ng/ml). Although cross-reactivity was provided by the EIA manufacturer, specificity was also evaluated comparing slopes from the straight lines resulting from the application of common logarithm (log_{10}) to the values from the standard curve ($m_{standard}$) and from new pool curve (m_{pool}) created with the same serial dilutions (1:1, 1:5, 1:10, 1:25, 1:50 and 1:100). According to the manufacturer, cross-reactivity of the EIA antibody with other steroids is as follows: prednisolone 47.4 %, cortisone 15.7 %, 11-deoxycortisol 15.0 %, prednisone 7.83 %, corticosterone 4.81 %, 6ß-hydroxycortisol 1.37 %, 17-hydroxyprogesterone 1.36 %, deoxycorticosterone 0.94 %. Steroids with a cross-reactivity lower than 0.06 % are not presented.

4.5. Statistical analysis

All data was processed and analysed using the SAS software (Statistical Analysis System, version V.8; SAS Institute, Cary, NC, USA) and a p-value < 0.05 was considered significant. The values are presented as means ± standard deviation. A Shapiro-Wilk test was carried out to check normality. Data with non-normality distribution were transformed in log_{10} and normality was evaluated again. All parameters transformed in log_{10} were normally distributed and used in statistical analysis. A parametric test (two tails paired data t-test, PROC TTEST with PAIRED option in SAS) was done to compare the cortisol concentrations between white and black hair samples and between d_0 and d_{25} hair samples. A different

parametric test (Pearson's correlation analysis, PROC CORR in SAS) was done to detect correlations within cortisol parameters. A simple linear regression was performed in order to analyse statistically the relations between hair cortisol and faecal metabolites of cortisol concentrations.

5. RESULTS AND DISCUSSION

5.1. Validation tests

Intra- and inter-assay coefficients of variation were 4.9 ± 2.39 % and 10.6 ± 1.77 %, respectively. The linearity of dilution showed a R^2 = 0.98 and a mean percentage error of -10.8 ± 1.55 %, providing confirmation that pool samples interacted with the assay antibody in a dose-dependent manner and supporting the assumption that the antibody-binding characteristics of standard and pool samples are similar. The average recovery percentage from spike-and-recovery test was 89.0 ± 23.52 % which indicate that no other components of the hair extracts interfere acutely with the estimation of cortisol hormone. The lines from the $\log_{10}$ of the standard curve values and the $\log_{10}$ of the pool curve values showed similar slopes ($m_{standard}$ = 0.33, m_{pool} = 0.35; Figure 1) detecting immunological similarities between the standard and sample hormones.

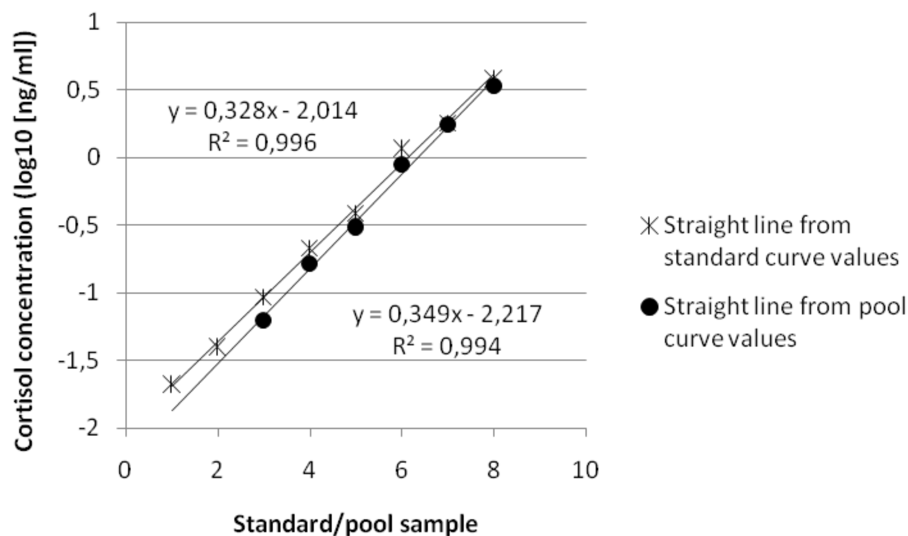


Figure 1. Parallelism between lines from the $\log_{10}$ of the standard and sample pool curves.

These results indicate that hair cortisol detection in dairy cows through this methodology is successful even using an EIA kit no designed specifically for hair cortisol detection. According to Stalder and Kirschbaum(2012) or Meyer and Novak (2012), the major source of cortisol presented in hair should be free or unbound cortisol, which is the cortisol detected by the EIA kit used. However, further research is

necessary to describe the type of cortisol present in hair and the role of hair follicle and local production in the final hair concentration.

5.2. Hair cortisol concentrations

As is shown in Table 1, the average cortisol concentrations in white and black hair differed significantly at d_0 and at d_{25} (*$P < 0.01$*).

Table 1. *Cortisol concentrations (pg cortisol/mg hair) from hair sampled at the beginning (d_0) and at the end (d_{25}) of the study*

		Hair colour	
		White	**Black**
Sampling day	**d_0**	2.1 ± 1.10^a	3.9 ± 1.44^b
	d_{25}	1.4 ± 0.73^c	2.5 ± 1.61^d

[a,b,c,d] Means with different superscripts are different (two tails paired data t-test between colour samples and between day of sampling, P < 0.01).

The colour in hair is produced by melanocytes located in the basal layer of the hair (Pragst and Balikova, 2006). The presence of higher cortisol concentrations in black samples in relation with the white ones is in concordance with studies that suggest that number of melanocytes and pigmentation favour the incorporation of lipophilic substances from the bloodstream into the hair (Pötsch et al., 1997; Pragst and Balikova, 2006). However, other studies found higher cortisol concentrations in white hair than in the black ones (Burnett et al., 2014; Cerri et al., 2012; González-de-la-Vara et al., 2011). Our contradictory results may be because while the white hair samples were harvested only from the frontal region of the head, the black hair ones were a mixture of hair from frontal region of head and also from the occipital crest. Thus, black hair samples were homogeneous in colour but not in location and so, hair type. The authors did not find any study comparing cortisol concentrations from these two close regions but other studies found differences in hair cortisol levels among other locations (Burnett et al., 2014; Cerri et al., 2012; Moya et al., 2013). Hair from the occipital crest in Holstein-Friesian cows is longer and thicker than hair from frontal region and studies performed by Burnett et al. (2014) and Moya et al. (2013) found higher cortisol concentrations in locations where hair type was also longer and thicker (especially the tail switch). The growth rate and hormone accumulation or production rate could be different between types of hair (Burnett et al., 2014; Moya et al., 2013) and these differences could be the cause of the presence of higher cortisol levels in the heterogeneous black hair samples.

Mean values for the same hair colour samples at d_0 and d_{25} showed also significant differences (Table 1, *$P < 0.01$*). The authors suggest that these results could be explained by the different lifetime between d_0 samples (unknown lifetime, probably months-old samples) and d_{25} samples (25-day-old). Hair from d_0 could have accumulated, in terms of cortisol variations, unknown stressful or physiologically compromising experiences (such as parturition, disease or hierarchy reestablishment). Comin et al. (2011) cautiously

suggested hair cortisol variations due to seasonal differences in hair growth rate in dairy cows. This fact could also add differences between d_0 and d_{25} samples. Additionally, the different hair growth phases present in samples from regions not previously harvested has been considered as a cause of hair cortisol variations, especially in short hair types (Carlitz et al., 2014; Moya et al., 2013; Peric et al., 2013).

5.3. Hair cortisol and faecal cortisol metabolites

A significant correlation was detected between faecal cortisol metabolites (average value of all faecal samples 25.27 ± 4.16 ng/g dry sample, range 10.14 – 54.83) and cortisol in white colour hair samples from d_{25} (r = 0.75926, P <0.001). A simple lineal regression was done between white hair samples at $d_{25}$and average of all faecal samples (Figure 2) in order to better understand the relationship between cortisol and cortisol metabolites found in these matrixes.

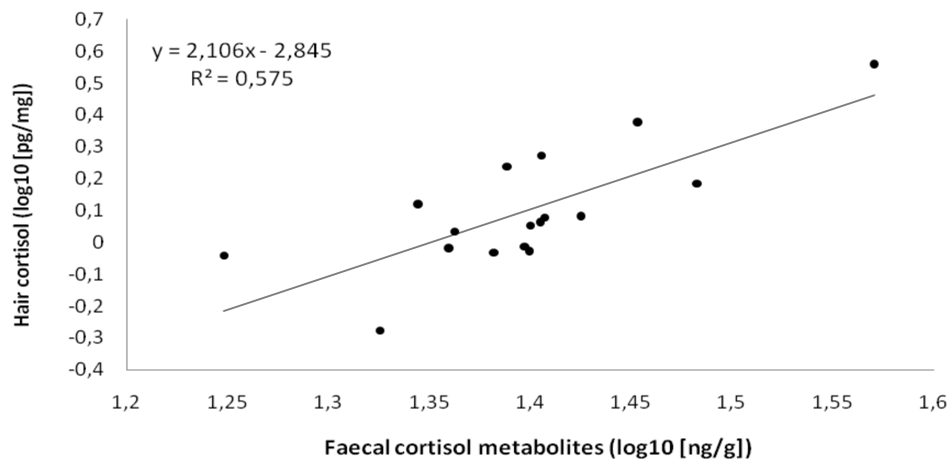


Figure 2. Simple linear regression between log_{10} transformed values of white hair samples at d_{25} and the average of all faecal samples (R^2 = 0.58, P <0.001).

The good correlation between white colour hair samples from d_{25} and the average of all faecal samples confirms the possibility to use hair as a matrix to assess retrospective concentrations of cortisol. No correlation was found between faecal cortisol metabolites and black colour hair samples from d_{25} (P > 0.05). The absence of correlation between black hair samples at $d_{25}$and average faecal levels could be explained also by the lack in homogeneity of location of these hair samples and so, a lack in hair type homogeneity.

6. CONCLUSION

In conclusion, the validation tests confirm that it is possible to detect cortisol concentrations in hair with an acceptable repeatability and reliability by using EIA. The correlations between concentrations in hair and faeces verify, indirectly, the use of hair cortisol as a parameter to represent retrospective circulating cortisol levels and consequently, long-term HPA axis activity. Although hair sampling is simple, non-invasive and fast, collected hair must be homogeneous in colour, sampling region and days old in order to be used in dairy cattle welfare studies.

7. REFERENCES

Aardal, E., Holm, A.C., 1995. Cortisol in saliva - Reference ranges and relation to cortisol in serum. Eur. J. Clin. Chem. Clin. Biochem. 33, 927–932.

Boumba, V.A., Ziavrou, K.S., Vougiouklakis, T., 2006. Hair as a biological indicator of drug use, drug abuse or chronic exposure to environmental toxicants. Int. J. Toxicol. 25, 143–63. doi:10.1080/10915810600683028

Bryan, H.M., Adams, A.G., Invik, R.M., Wynne-Edwards, K.E., Smits, J.E.G., 2013. Hair as a meaningful measure of baseline cortisol levels over time in dogs. J. Am. Assoc. Lab. Anim. Sci. 52, 189–96.

Buchanan, K.L., Goldsmith, A.R., 2004. Noninvasive endocrine data for behavioural studies: the importance of validation. Anim. Behav. 67, 183–185. doi:10.1016/j.anbehav.2003.09.002

Burnett, T.A., Madureira, A.M.L.L., Silper, B.F., Nadalin, A., Tahmasbi, A., Veira, D.M., Cerri, R.L.A., 2014. Short communication: Factors affecting hair cortisol concentrations in lactating dairy cows. J. Dairy Sci. 97, 7685–7690. doi:10.3168/jds.2014-8444

Carlitz, E.H.D., Kirschbaum, C., Stalder, T., van Schaik, C.P., 2014. Hair as a long-term retrospective cortisol calendar in orang-utans (Pongo spp.): new perspectives for stress monitoring in captive management and conservation. Gen. Comp. Endocrinol. 195, 151–6. doi:10.1016/j.ygcen.2013.11.002

Cerri, R.L.A., Tabmasbi, A.M., Veira, D.M., 2012. Hair cortisol concentrations -Influence of color and location in Holstein cows. J. Dairy Sci. 95, S574.

Comin, A., Peric, T., Corazzin, M., Veronesi, M.C.C., Meloni, T., Zufferli, V., Cornacchia, G., Prandi, A., 2013. Hair cortisol as a marker of hypothalamic-pituitary-adrenal axis activation in Friesian dairy cows clinically or physiologically compromised. Livest. Sci. 152, 36–41. doi:10.1016/j.livsci.2012.11.021

Comin, A., Prandi, A., Peric, T., Corazzin, M., Dovier, S., Bovolenta, S., 2011. Hair cortisol levels in dairy cows from winter housing to summer highland grazing. Livest. Sci. 138, 69–73. doi:10.1016/j.livsci.2010.12.009

Comin, A., Tidu, L., Cornacchia, G., Cappa, A., Renaville, B., Prandi, A., 2008. Neonatal period and hair cortisol in cattle as a marker of stress., in: XVI Congress of the Mediterranean Federation for Health and Production of Ruminants (FeMeSPrum). 23-26 april, Zadar, Croatia, pp. 221–225.

Comin, A., Veronesi, M.C., Montillo, M., Faustini, M., Valentini, S., Cairoli, F., Prandi, A., 2012. Hair cortisol level as a retrospective marker of hypothalamic–pituitary–adrenal axis activity in horse foals. Vet. J. 194, 131–132. doi:10.1016/j.tvjl.2012.04.006

Cone, E.J., 1996. Mechanisms of Drug Incorporation into Hair. Ther. Drug Monit. 18, 438–443. doi:10.1097/00007691-199608000-00022

Davenport, M.D., Tiefenbacher, S., Lutz, C.K., Novak, M.A., Meyer, J.S., 2006. Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. Gen. Comp. Endocrinol. 147, 255–261. doi:10.1016/j.ygcen.2006.01.005

de Lima, V., Piles, M., Rafel, O., López-Béjar, M., Ramón, J., Velarde, A., Dalmau, A., 2013. Use of infrared thermography to assess the influence of high environmental temperature on rabbits. Res. Vet. Sci. 95, 802–10. doi:10.1016/j.rvsc.2013.04.012

Dettmer, A.M., Novak, M.A., Suomi, S.J., Meyer, J.S., 2012. Physiological and behavioral adaptation to relocation stress in differentially reared rhesus monkeys: Hair cortisol as a biomarker for anxiety-related responses. Psychoneuroendocrinology 37, 191–199. doi:10.1016/j.psyneuen.2011.06.003

Gatti, R., Antonelli, G., Prearo, M., Spinella, P., Cappellin, E., De Palo, E.F., 2009. Cortisol assays and diagnostic laboratory procedures in human biological fluids. Clin. Biochem. 42, 1205–17. doi:10.1016/j.clinbiochem.2009.04.011

González-de-la-Vara, M.R.D.R., Valdez, R.A., Lemus-Ramirez, V., Vázquez-Chagoyán, J.C., Villa-Godoy, A., Romano, M.C., 2011. Effects of adrenocorticotropic hormone challenge and age on hair cortisol concentrations in dairy cattle. Can. J. Vet. Res. 75, 216–221.

Gow, R., Thomson, S., Rieder, M., Van Uum, S., Koren, G., 2010. An assessment of cortisol analysis in hair and its clinical applications. Forensic Sci. Int. 196, 32–37. doi:10.1016/j.forsciint.2009.12.040

Henderson, G.L.L., 1993. Mechanisms of drug incorporation into hair. Forensic Sci. Int. 63, 19–29. doi:10.1016/0379-0738(93)90256-A

Hernandez, C.E., Thierfelder, T., Svennersten-Sjaunja, K., Berg, C., Orihuela, A., Lidfors, L., 2014. Time lag between peak concentrations of plasma and salivary cortisol following a stressful procedure in dairy cattle. Acta Vet. Scand. 56, 61. doi:10.1186/s13028-014-0061-3

Ito, N., Ito, T., Kromminga, A., Bettermann, A., Takigawa, M., Kees, F., Straub, R.H., Paus, R., 2005. Human hair follicles display a functional equivalent of the hypothalamic-pituitary-adrenal (HPA) axis and synthesize cortisol. Faseb J. 19, 1332–+. doi:10.1096/fj.04-1968fje

Keckeis, K., Lepschy, M., Schöpper, H., Moser, L., Troxler, J., Palme, R., 2012. Hair cortisol: a parameter of chronic stress? Insights from a radiometabolism study in guinea pigs. J. Comp. Physiol. B. 182, 985–96. doi:10.1007/s00360-012-0674-7

Koren, L., Mokady, O., Karaskov, T., Klein, J., Koren, G., Geffen, E., 2002. A novel method using hair for determining hormonal levels in wildlife. Anim. Behav. 63, 403–406. doi:10.1006/anbe.2001.1907

Macbeth, B.J., Cattet, M.R.L., Obbard, M.E., Middel, K., Janz, D.M., 2012. Evaluation of hair cortisol concentration as a biomarker of long-term stress in free-ranging polar bears. Wildl. Soc. Bull. 36, 747–758. doi:10.1002/wsb.219

Meyer, J.S., Novak, M.A., 2012. Minireview: Hair cortisol: a novel biomarker of hypothalamic-pituitary-

adrenocortical activity. Endocrinology 153, 4120–4127. doi:10.1210/en.2012-1226

Morrow, C.J., Kolver, E.S., Verkerk, G.A., Matthews, L.R., 2002. Fecal glucocorticoid metabolites as a measure of adrenal activity in dairy cattle. Gen. Comp. Endocrinol. 126, 229–241. doi:10.1006/gcen.2002.7797

Möstl, E., Maggs, J.L., Schro, G., Schrötter, G., Besenfelder, U., Palme, R., 2002. Measurement of cortisol metabolites in faeces of ruminants. Vet. Res. Commun. 26, 127–139. doi:10.1023/A:1014095618125

Möstl, E., Messmann, S., Bagu, E., Robia, C., Palme, R., 1999. Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock. J. Vet. Med. Ser. A-Physiology Pathol. Clin. Med. 46, 621–631.

Möstl, E., Palme, R., 2002. Hormones as indicators of stress. Domest. Anim. Endocrinol. 23, 67–74. doi:10.1016/S0739-7240(02)00146-7

Moya, D., Schwartzkopf-Genswein, K.S., Veira, D.M., 2013. Standardization of a non-invasive methodology to measure cortisol in hair of beef cattle. Livest. Sci. 158, 138–144. doi:10.1016/j.livsci.2013.10.007

Negrão, J.A., Porcionato, M.A., Passillé, A.M. de, Rushen, J., 2004. Cortisol in saliva and plasma of cattle after ACTH administration and milking. J. Dairy Sci. 87, 1713–8. doi:10.3168/jds.S0022-0302(04)73324-X

NRC, 2001. Nutrient requirements of dairy cattle, 7th rev. ed, 7th rev. ed. National Academy Press, Washington, DC.

Palme, R., Robia, C., Messmann, S., Hofer, J., Möstl, E., 1999. Measurement of faecal cortisol metabolites in ruminants: a non-invasive parameter of adrenocortical function. Wien. Tierarztl. Monatsschr. 86, 237–241.

Palme, R., Touma, C., Arias, N., Dominchin, M.F., Lepschy, M., 2013. Steroid extraction : Get the best out of faecal samples. Wiener Tierärztliche Monatsschrift – Vet. Med. Austria 100.

Peric, T., Comin, A., Corazzin, M., Montillo, M., Cappa, A., Campanile, G., Prandi, A., 2013. Short communication: hair cortisol concentrations in Holstein-Friesian and crossbreed F1 heifers. J. Dairy Sci. 96, 3023–7. doi:10.3168/jds.2012-6151

Pötsch, L., Skopp, G., Moeller, M.R., 1997. Influence of pigmentation on the codeine content of hair fibers in guinea pigs. J. Forensic Sci. 42, 1095–8.

Pragst, F., Balikova, M.A., 2006. State of the art in hair analysis for detection of drug and alcohol abuse. Clin. Chim. Acta. 370, 17–49. doi:10.1016/j.cca.2006.02.019

Probst, J.K., Spengler Neff, A., Hillmann, E., Kreuzer, M., Koch-Mathis, M., Leiber, F., 2014. Relationship between stress-related exsanguination blood variables, vocalisation, and stressors imposed on cattle

between lairage and stunning box under conventional abattoir conditions. Livest. Sci. 164, 154–158. doi:10.1016/j.livsci.2014.03.013

Rigalma, K., Duvaux-Ponter, C., Barrier, A., Charles, C., Ponter, A.A., Deschamps, F., Roussel, S., 2010. Medium-term effects of repeated exposure to stray voltage on activity, stress physiology, and milk production and composition in dairy cows. J. Dairy Sci. 93, 3542–3552. doi:10.3168/jds.2009-2903

Russell, E., Koren, G., Rieder, M., Van Uum, S., 2012. Hair cortisol as a biological marker of chronic stress: Current status, future directions and unanswered questions. Psychoneuroendocrinology 37, 589–601. doi:10.1016/j.psyneuen.2011.09.009

Sabés-Alsina, M., Planell, N., Torres-Mejia, E., Taberner, E., Maya-Soriano, M.J., Tusell, L., Ramon, J., Dalmau, A., Piles, M., Lopez-Bejar, M., 2015. Daily exposure to summer circadian cycles affects spermatogenesis, but not fertility in an in vivo rabbit model. Theriogenology 83, 246–52. doi:10.1016/j.theriogenology.2014.09.013

Sharpley, C.F., McFarlane, J.R., Slominski, A., 2012. Stress-linked cortisol concentrations in hair: what we know and what we need to know. Rev. Neurosci. 23, 111–121. doi:10.1515/RNS.2011.058

Slominski, A., Zbytek, B., Nikolakis, G., Manna, P.R., Skobowiat, C., Zmijewski, M., Li, W., Janjetovic, Z., Postlethwaite, A., Zouboulis, C.C., Tuckey, R.C., 2013. Steroidogenesis in the skin: implications for local immune functions. J. Steroid Biochem. Mol. Biol. 137, 107–23. doi:10.1016/j.jsbmb.2013.02.006

Slominski, A.T., Manna, P.R., Tuckey, R.C., 2014. Cutaneous glucocorticosteroidogenesis: securing local homeostasis and the skin integrity. Exp. Dermatol. 23, 369–74. doi:10.1111/exd.12376

Stalder, T., Kirschbaum, C., 2012. Analysis of cortisol in hair – State of the art and future directions. Brain. Behav. Immun. 26, 1019–29. doi:10.1016/j.bbi.2012.02.002

Tallo-Parra, O., Carbajal, A., Sabes-Alsina, M., Almagro, V., Fernandez-Bellon, H., Enseñat, C., Quevedo, M.A., Manteca, X., Abaigar, T., Lopez-Bejar, M., 2013. Preliminary results on hair cortisol detection as a tool to evaluate chronic stress in Sahrawi dorcas gazelle (Gazella dorcas neglecta), in: 9th International Conference on Behaviour, Physiology and Genetics of Wildlife. Berlin, Germany, p. 193.

Taves, M.D., Gomez-Sanchez, C.E., Soma, K.K., 2011. Extra-adrenal glucocorticoids and mineralocorticoids: evidence for local synthesis, regulation, and function. Am. J. Physiol. Endocrinol. Metab. 301, E11-24. doi:10.1152/ajpendo.00100.2011

Thun, R., Eggenberger, E., Zerobin, K., Luscher, T., Vetter, W., 1981. 24-Hour Secretory Pattern of Cortisol in the Bull - Evidence of Episodic Secretion and Circadian-Rhythm. Endocrinology 109, 2208–2212.

Touma, C., Palme, R., 2005. Measuring Fecal Glucocorticoid Metabolites in Mammals and Birds: The

Importance of Validation. Ann. N. Y. Acad. Sci. 1046, 54–74. doi:10.1196/annals.1343.006

Van Uum, S.H.M., Sauvé, B., Fraser, L.A., Morley-Forster, P., Paul, T.L., Koren, G., 2008. Elevated content of cortisol in hair of patients with severe chronic pain: A novel biomarker for stress. Stress 11, 483–488. doi:10.1080/10253890801887388

von Keyserlingk, M.A.G., Rushen, J., de Passillé, A.M., Weary, D.M., 2009. The welfare of dairy cattle—Key concepts and the role of science. J. Dairy Sci. 92, 4101–4111. doi:10.3168/jds.2009-2326

Chapter II

Progesterone detection in hair from dairy cattle by EIA: protocol validation

Progesterone detection in hair from dairy cattle by EIA: protocol validation

O. Tallo-Parra[1,2], A. Carbajal[2], L. Monclús[2], M. Sabes-Alsina[2], X. Manteca[1]and M. Lopez-Bejar[2]

[1]*Department of Animal and Food Science, Faculty of Veterinary, UniversitatAutònoma de Barcelona,* Edifici V, Campus UAB, 08193 Bellaterra, Spain

[2]*Department of Animal Health and Anatomy, Faculty of Veterinary, UniversitatAutònoma de Barcelona,* Edifici V, Campus UAB, 08193 Bellaterra, Spain

The abstract of this chapter has been accepted for the *20th Annual European Society for Domestic Animal Reproduction (ESDAR) Conference 2016*, whose abstracts are published in *Reproduction in Domestic Animals* and it is presented in a short communication format for this thesis.

1. ABSTRACT

Hair accumulates hormones during all its growth. Therefore, it provides an integrative measure of long-term retrospective hormonal levels that can comprise from days to months. This study validated the use of a methanol-based extraction protocol and a commercial enzyme immunoassay kit (progesterone ELISA KIT; Neogen® Corporation) for the quantification of progesterone levels in hair from dairy cattle. Hair samples (250 mg) from 25 cows were used. Three washes of 2.5 ml of isopropanol and 2.5 min of vortex each one were applied to each sample. Then, hair samples were left to dry for 36h at room temperature. Next, samples were powdered using a ball mill (22 Hz; 5min) and 50 mg of powder were weighed and 1.5 ml of methanol was added. Samples were incubated under moderate shaking for 18h at 30 ºC. Afterward, extracted samples were centrifuged at 7000 × g for 2min at 25 ºC and 0.750 ml of supernatant was placed in an oven at 38ºC. Once the methanol was evaporated, the dried extracts were reconstituted with 1 ml of buffer. The extracts were stored at -20 ºC until analysis. All extracts were pooled and used for biochemical validation. Intra-assay coefficient of variation was 8.64 ± 6.41 %. The linearity of dilution showed a R^2= 0.98. The recovery percentage from spike-and-recovery test was 103.80 ± 10.37 %. The standard and pool curves showed parallel displacements. According the results, hair progesterone detection in cows is successful using this protocol. The use of hair could open a new window in long-term hormonal monitoring for future endocrinology or reproductive research.

2. INTRODUCTION

Progesterone is essential in the establishment and maintenance of pregnancy and it is produced mainly by the corpus luteum in the ovary or the placenta (Mann and Lamming, 1999; Spencer, 2004a, 2004b; Wiltbank et al., 2014). Therefore, progesterone detection has been used for pregnancy diagnosis or as indicator of gonadal activity and reproductive status (Crowe, 2008; Kornmatitsuk et al., 2007; Robinson et al., 2008).

In recent years, the use of hair as a matrix for steroid detection has gained much attention due to its supposed capacity to represent long-term circulating steroid levels and practical advantages (Bichon et al., 2012; Duffy et al., 2009; Meyer and Novak, 2012; Yang et al., 1998), especially in hair cortisol detection (Burnett et al., 2014; Comin et al., 2011; Moya et al., 2013). Surprisingly, only two articles published decades ago analysing hair progesterone concentrations (HPC) in cattle were found by the authors. Liu et al., (1988) aimed to diagnose pregnancy by using HPC in samples collected 24 days after artificial insemination and Gleixner and Meyer (1997) compared HPC between cows and bulls and between hair colours. The promising results obtained in hair cortisol analysis and the development of improved immunoassays in the last decades (Lequin, 2005; Smiley et al., 2016) reopened the interest for the evaluation of the potential utilities of hair progesterone analysis. However, previous the biological validation of HPC, a validated protocol for hair progesterone analysis must be performed.

The objective of this study was to validate a protocol and a commercial EIA kit to measure progesterone concentrations in hair from Holstein-Friesian cows as the first necessary step allowing future studies evaluating potential uses of hair in long-term progesterone monitoring.

3. MATERIAL AND METHODS

3.1. Animals and hair sampling

Twenty-five Holstein-Friesian adult cows from a commercial farm were used for the validation of hair progesterone detection. Between 300 and 500 mg of homogenized white hair from the ventrolateral region of the neck were collected per animal. Hair was cut as closest to the skin as possible but preventing skin damage and hair follicle removal. An electric hair clipper (X3 ceramic-titanium hair clipper; Palson® Trading España S.L., Collbató, Spain) was used and the peeler blade was cleaned between animals using a brush. Each hair sample was stored into properly identified zip-lock plastic bags in a dark place at room temperature.

3.2. Hair wash and progesterone extraction

For hair washing and progesterone extraction, the same validated protocol for hair cortisol detection in dairy cattle (Chapter I, Tallo-Parra et al., 2015) was followed. Two hundred and fifty milligrams of hair were weighed from each sample and placed into a 15-ml conical tube. Three washes consisting of 2.5 ml of isopropanol (2-propanol

99.5 %, Scharlab S.L., Sentmenat, Spain) and 2.5 minutes of vortex each one were applied to all the samples. The isopropanol was eliminated by decantation after each wash. Once washing finished, hair samples were left to dry for 36 hours at room temperature. Then, dried hair samples were trimmed using a ball mill for 5 minutes at 22 Hz (MM200, Retsch, Haan, Germany; 10-ml stainless-steel grinding jars; two 12-mm stainless-steel grinding balls). For progesterone extraction, 50 mg of trimmed hair were carefully weighed, placed into a 2-ml eppendorf tube and 1.5 ml of pure methanol was added. Samples were incubated for 18 hours at 30 ºC (G24 Environmental Incubator Shaker, New Brunswick Scientific CO Inc., Edison, NJ, USA) under moderately shaking. After incubation, extracted samples were centrifuged at 7 000 × g for 2 minutes at 25 ºC and 0.750 ml of supernatant was transferred into a new 2-ml eppendorf tube. The supernatant was then placed in an oven (Heraeus model T6; Kendro® Laboratory Products, Langenselbold, Germany) at 38 ºC to evaporate the methanol. Once the methanol was completely evaporated, the dried extracts were reconstituted with 0.250 ml of buffer provided by the enzyme immunoassay (EIA) kit (Progesterone ELISA KIT; Neogen® Corporation, Ayr, UK) and shaken for 30 seconds. Preliminary results showed concentrations of progesterone in these reconstituted hormone extracts reaching the upper limit of progesterone detection range of EIA kit. Thus, 1:4 dilution was applied to each hair extract in order to analyse concentrations within the detection range of the progesterone EIA kits. Then, all hormone extracts were stored at -20 ºC until analysis.

3.3. Progesterone analysis and validation tests

A progesterone EIA detection kit (Neogen® Corporation Europe, Ayr, UK) was used for progesterone analysis with a sensitivity of 0.016 ng progesterone/mg hair. All the validation tests used different pool of samples constituted by five hair extracts each one with high, low and medium final concentrations. Each final solution was analysed at least by duplicate. The precision within test was assessed by calculating intra-assay coefficients of variation (CV) from all duplicated or tripled samples analysed. The linearity of dilution was determined by using 1:1, 1:2, 1:5 and 1:10 dilutions of pools with EIA buffer. Accuracy was assessed through the spike-and-recovery test, calculated by adding to 40, 100 and 160 µl of pool, volumes of 160, 100 and 40 µl of pure standard cortisol solution, respectively. Combinations were repeated with two different pure standard progesterone solutions (20 and 2 ng/ml). A seventh combination of 240µl of pool with 10µl of high-concentrated progesterone standard (1µ/ml) was also done. Although cross-reactivity was provided by the EIA manufacturer, specificity was also evaluated comparing curves from progesterone standards and from new pool curve created with the same serial dilutions (1:1, 1:5, 1:10, 1:25, 1:50 and 1:100). According to the manufacturer, cross-reactivity of the EIA antibody with other steroids is as follows: deoxycorticosterone 2.5 %, cortisone 2.0 %, pregnenolone 2.0 %, androstenedione 1.0 %, 17-hydroxyprogesterone 0.4 %. Steroids with a cross-reactivity lower than 0.3 % are not presented.

4. RESULTS AND DISCUSSION

Intra-assay CV showed a precision of 8.64 ± 6.41 %, revealing an acceptable repeatability within the assay. The linearity of dilution presented a R^2 = 0.98 (Figure 1) and indicate that a correct dose-dependent interaction between progesterone from hair extracts and EIA antibodies exists.

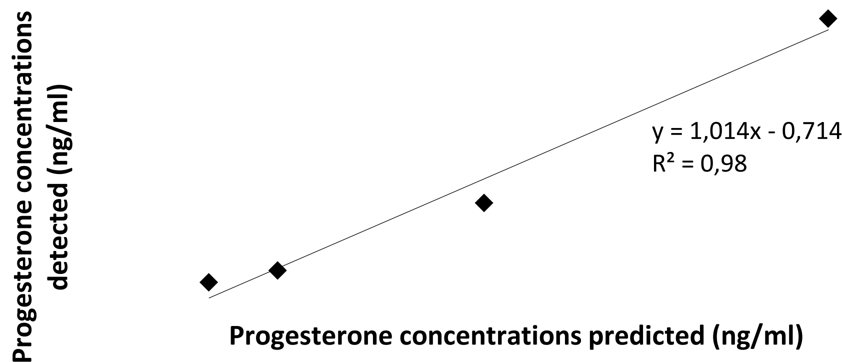


Figure 1. Linearity of dilution of progesterone concentrations from hair extracts.

The recovery percentage from spike-and-recovery test was 103.80 ± 10.37 % demonstrating that no other components of the hair extracts interfere acutely with the estimation of progesterone hormone regardless the concentrations of these potentially-confounding components.

Finally, immunological similarities between standard progesterone provided by the EIA kit and progesterone extracted from cows' hair were observed as the standard and pool curves showed parallel displacements and similar slopes (Figure 2).

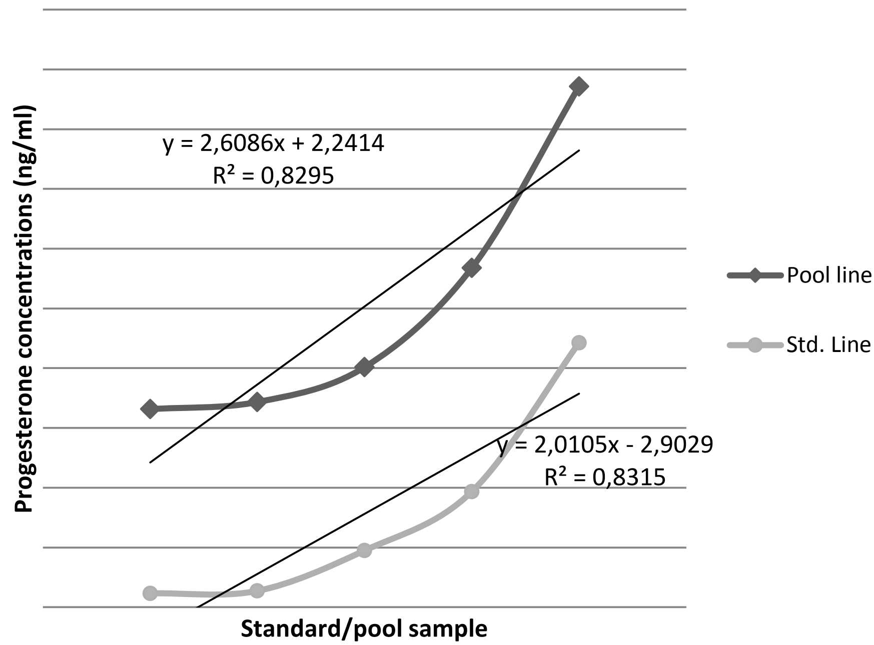


Figure 2. Parallelism between lines from the standard and sample pool curves.

Taking together all the validation results and the information provided by the EIA manufacturer (sensitivity and cross-reactivity), it can be concluded the detection of progesterone in hair by the commercial EIA kit used is successful and reliable despite the EIA kit was not initially designed for progesterone detection in hair or cows. The use of hair progesterone measurement as a tool for long-term progesterone monitoring seems to be possible through the protocol and EIA kit used. Although more research should be done with the aim to understand the relationship between blood and hair progesterone concentrations, this study provides a validated methodology to perform hair progesterone quantifications.

5. CONCLUSIONS

Hair progesterone detection in cows is successful using the protocol described in this study. The use of hair could open a new window in long-term hormonal monitoring for future endocrinological or reproductive research.

6. REFERENCES

Bichon, E., Béasse, A., Prevost, S., Christien, S., Courant, F., Monteau, F., Le Bizec, B., 2012. Improvement of estradiol esters monitoring in bovine hair by dansylation and liquid chromatography/tandem mass spectrometry analysis in multiple reaction monitoring and precursor ion scan modes. Rapid Commun. Mass Spectrom. 26, 819–27. doi:10.1002/rcm.6160

Burnett, T.A., Madureira, A.M.L.L., Silper, B.F., Nadalin, A., Tahmasbi, A., Veira, D.M., Cerri, R.L.A., 2014. Short communication: Factors affecting hair cortisol concentrations in lactating dairy cows. J. Dairy Sci. 97, 7685–7690. doi:10.3168/jds.2014-8444

Comin, A., Prandi, A., Peric, T., Corazzin, M., Dovier, S., Bovolenta, S., 2011. Hair cortisol levels in dairy cows from winter housing to summer highland grazing. Livest. Sci. 138, 69–73. doi:10.1016/j.livsci.2010.12.009

Crowe, M.A., 2008. Resumption of ovarian cyclicity in post-partum beef and dairy cows. Reprod. Domest. Anim. 43, 20–28. doi:10.1111/j.1439-0531.2008.01210.x

Duffy, E., Mooney, M.H., Elliott, C.T., O'Keeffe, M., 2009. Studies on the persistence of estradiol benzoate and nortestosterone decanoate in hair of cattle following treatment with growth promoters, determined by ultra-high-performance liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1216, 8090–5. doi:10.1016/j.chroma.2009.04.053

Gleixner, A., Meyer, H.H.D., 1997. Non-invasive monitoring of endocrine function by analysis of progesterone, testosterone and estradiol in hair of adult cattle. Zeitschrift fur Saugetierkd. - Int. J. Mamm. Biol. 62, 71–74.

Kornmatitsuk, B., Thitaram, C., Kornmatitsuk, S., 2007. Measurement of faecal progesterone metabolites and its application for early screening of open cows post-insemination. Reprod. Domest. Anim. 42, 238–242. doi:10.1111/j.1439-0531.2006.00758.x

Lequin, R.M., 2005. Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). Clin. Chem. 51, 2415–8. doi:10.1373/clinchem.2005.051532

Liu, X., Chen, F., Guo, D., Song, X., Zhong, Y., 1988. Early pregnancy diagnosis in dairy cows based on hair progesterone analysis. Int. J. Anim. Sci. 3, 123–127.

Mann, G.E., Lamming, G.E., 1999. The Influence of Progesterone During Early Pregnancy in Cattle. Reprod. Domest. Anim. 34, 269–274. doi:10.1111/j.1439-0531.1999.tb01250.x

Meyer, J.S., Novak, M.A., 2012. Minireview: Hair cortisol: a novel biomarker of hypothalamic-pituitary-adrenocortical activity. Endocrinology 153, 4120–4127. doi:10.1210/en.2012-1226

Moya, D., Schwartzkopf-Genswein, K.S., Veira, D.M., 2013. Standardization of a non-invasive methodology to measure cortisol in hair of beef cattle. Livest. Sci. 158, 138–144. doi:10.1016/j.livsci.2013.10.007

Robinson, R.S., Hammond, a. J., Wathes, D.C., Hunter, M.G., Mann, G.E., 2008. Corpus Luteum-Endometrium-Embryo Interactions in the Dairy Cow: Underlying Mechanisms and Clinical Relevance.

Reprod. Domest. Anim. 43, 104–112. doi:10.1111/j.1439-0531.2008.01149.x

Smiley, R.D., Stam, C.N., Dwivedi, H.P., 2016. Immunoassays: Principles, in: Encyclopedia of Food and Health. pp. 398–403. doi:10.1016/B978-0-12-384947-2.00386-X

Spencer, T.E., 2004a. Conceptus signals for establishment and maintenance of pregnancy. Anim. Reprod. Sci. 15, 1–15. doi:10.1016/S0378-4320(04)00070-3

Spencer, T.E., 2004b. Progesterone and Placental Hormone Actions on the Uterus: Insights from Domestic Animals. Biol. Reprod. 71, 2–10. doi:10.1095/biolreprod.103.024133

Tallo-Parra, O., Manteca, X., Sabes-Alsina, M., Carbajal, A., Lopez-Bejar, M., 2015. Hair cortisol detection in dairy cattle by using EIA: protocol validation and correlation with faecal cortisol metabolites. Animal 9, 1059–1064. doi:10.1017/S1751731115000294

Wiltbank, M.C., Souza, A.H., Carvalho, P.D., Cunha, A.P., Giordano, J.O., Fricke, P.M., Baez, G.M., Diskin, M.G., 2014. Physiological and practical effects of progesterone on reproduction in dairy cattle. Animal 8 Suppl 1, 70–81. doi:10.1017/s1751731114000585

Yang, H.Z., Lan, J., Meng, Y.J., Wan, X.J., Han, D.W., 1998. A preliminary study of steroid reproductive hormones in human hair. J. Steroid Biochem. Mol. Biol. 67, 447–50. doi:10.1016/S0960-0760(98)00120-4

Chapter III

Acute ACTH-induced elevations of circulating cortisol do not affect hair cortisol concentrations in calves

Acute ACTH-induced elevations of circulating cortisol do not affect hair cortisol concentrations in calves

O. Tallo-Parra [1,2], M. Lopez-Bejar[1], A. Carbajal [1], L. Monclús[1], X. Manteca [2] and M. Devant[3]

[1] Department of Animal Health and Anatomy, Veterinary Faculty, UniversitatAutònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.

[2] Department of Animal and Food Science, Veterinary Faculty, UniversitatAutònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.

[3]Department of Ruminant Production, Institut de RecercaiTecnologiaAgroalimentàries (IRTA), Torre Marimon, 08140 Caldes de Montbui, Barcelona, Spain.

This chapter has been submitted to *General and Comparative Endocrinology*

(currently in second revision)

1. ABSTRACT

In recent times, the detection of cortisol in hair is intended to be used as an animal-based indicator for the assessment of chronic stress. However, the relationship between the ability to report average values of long-term circulating cortisol concentrations and the sensitivity to acute peaks of cortisol is still unclear. To gain insight into this relationship, 24 Holstein-Friesian bull calves under the same management conditions were used in this study. Two injections of ACTH (at D_0 and D_7) were administrated to twelve animals with the aim to create two acute increases of serum cortisol concentrations. Blood samples were taken in order to determine the duration of serum cortisol peaks and to confirm a mediated response by the administration of ACTH. Cortisol concentrations from 14-day-old white hair samples collected from forehead and hip were analysed separately by EIA and compared with those from the control group. Serum cortisol analyses revealed an acute increase of cortisol concentrations for approximately three hours after each ACTH administration. Concentrations of hair cortisol from forehead and hip locations showed no differences between ACTH-administrated and control animals. Hair cortisol concentrations from 14-day old samples were not altered by two acute elevations of serum cortisol suggesting that hair cortisol is not masked by short and non-recurrent moments of stress. These results are a step forward in the validation of hair cortisol detection as a robust integrative measure of serum cortisol concentrations from an extended period of time.

2. INTRODUCTION

Detection of cortisol in hair is being evaluated as a potential animal-based measurement for the assessment of long-term or chronic stress (Davenport et al., 2006; Meyer and Novak, 2012; Burnett et al., 2015). As a cumulative matrix, hair incorporates hormones and other circulating substances during its growth period (Gow et al., 2010; Meyer and Novak, 2012; Stalder and Kirschbaum, 2012). This process results in a matrix with the potential to supply an integrative value of long-term retrospective cortisol levels. Hair cortisol concentrations (HCC) could be useful as an indicator of the overall effect of the environment or management conditions on the physiological homeostasis of the animal. An accurate knowledge of hair as a matrix that accumulates cortisol is essential for its future use in chronic stress research associated to animal welfare and animal production. There is a scarcity of information available on the influence of acute and short elevations of serum cortisol or corticosterone concentrations on their final concentration in "cumulative" matrixes like hair, feathers or shed skin (Bortolotti et al., 2008; Meyer and Novak, 2012; Berkvens et al., 2013). Thus, testing the influence of acute and short elevations of cortisol in the HCC is relevant in order to evaluate its value as an integrative measure of long-term adrenal activity in studies of chronic stress. Adrenocorticotropic hormone (ACTH) challenges have been used in cattle in order to test the reactiveness of the adrenal gland to produce cortisol through stimulation of the hypothalamic-pituitary-adrenal (HPA)-axis activity (Gwazdauskas et al., 1972; Lay et al., 1996; Veissier et al., 1999). The ACTH challenge provokes a physiological immediate and transient peak of cortisol. Some studies used administrations of ACTH in order to demonstrate a relationship between HPA-axis activity and HCC in dairy cattle (González-de-la-Vara et al., 2011) andother mammals(Mastromonaco et al., 2014; Terwissen et al., 2013). However, these studies administrated repeated doses of ACTH that increased circulating cortisol levels for an extended period of time. To the authors' knowledge, only one study has been published on this topic, analysing HCC after a single intramuscular dose of ACTH in caribou and reindeer (*Rangifer tarandus*) (Ashley et al., 2011).

The aim of this study was to assess the effect of sporadic and non-recurrent peaks o circulating cortisol on HCC. To this end, we tested the effect of two ACTH-induced acute elevations of cortisol on 14-day HCC in calves. This study is a step forward in the comprehensive understanding of the use of hair cortisol detection as an integrative measure of long-term retrospective cortisol concentrations by testing the robustness of its cortisol concentrations against acute and sporadic perturbations.

3. MATERIALS AND METHODS

3.1. Animals and study design

Twenty-four Holstein-Friesian bull calves with an initial body weight (BW) of 190.1 ± 17.42 kg and ages of 137 ± 10 days old were used in this study. Animals were randomly allocated into two groups, depending on the treatment (ACTH-administrated n = 12 vs. Control n = 12). One administration of ACTH at the start

(D_0) and another administration on day seven of the study (D_7) were performed on ACTH-administrated animals. The aim of this design was to temporarily increase serum cortisol concentrations (SCC) and to evaluate this effect on cortisol concentrations in 14-day-old hair samples in comparison with control animals.

3.2. Housing conditions, diet and performance

All animals were housed in the same facility owned by GrupAlimentariGuissona in Guissona (Lleida, Spain). Animals were housed in individually slatted pens (1.2 x 1.45 m) with open partitions that allowed visual, olfactory and limited physical contact. During the study, animals received commercial concentrate, formulated according to the NRC recommendations (NRC, 2001) and wheat straw. Animals were fed *ad libitum* and fresh water was available at automatic drinkers at all times. Feeding management and hygiene was under human supervision.

Animals were weighed individually at the start (D_0), middle (D_7) and end (D_{14}) of the study at the same time of the day. At D_0 and D_7, animals were weighed within the hour prior to each ACTH administration for individual dose adjustment.

3.3. ACTH administration and blood sampling

Two intravenously administrations of porcine ACTH (Sigma-Aldrich,St. Louis, MO, USA) were performed to stimulate the secretion of cortisol from the adrenal glands. The dose administrated to each calf was 1 IU of ACTH/Kg $BW^{0.75}$ (dissolved in 1.1 ml saline solution). This dose was selected to increase circulating cortisol levels for a period of three to four hours (Lay et al., 1996). In order to avoid differences in SCC associated with the circadian rhythm, ACTH was always injected at 1100h. Simultaneous to each ACTH administration, three blood samples (10-ml blood sampling; BD Vacutainer Nonadditive Tube) were collected by jugular venipuncture in both treatment and control animals: one blood sample immediately before ACTH administration (b_{0h}) and two blood samples collected one and three hours after administration (b_{1h} and b_{3h}). These samples served to confirm that ACTH administration was correct and established an elevation of serum cortisol concentrations between 0 and 3h post-injection. No saline solution was administrated to control group animals as no effect of product injection was seen by González-de-la-Vara et al. (2011) in HCC from dairy cattle. Furthermore, as both treatment and control groups were sampled for blood b_{0h}, the administration of ACTH did not suppose a substantial difference in animal manipulation between both groups.

Injections of ACTH and blood samplings were carried out by qualified personnel to minimise the time of animal handling and any potential pain or stress associated with the procedure. Blood samples were centrifuged at 1500 × g at 4 °C for 15 min within one hour after sampling. Serum was transferred into a 1.5-ml Eppendorf tube and stored at −20 °C until cortisol analysis.

3.4. Hair sampling

In all the animals, hair was collected from two locations (forehead and the hip regions) and analysed separately. Only white hair was collected in order to maximise hair homogeneity and avoid HCC differences caused by differences in hair colour (Cerri et al., 2012; Burnett et al., 2014; Tallo-Parra et al., 2015). Two hair samplings were performed during this study, at D_0 and D_{14}. With the aim to obtain hair samples with the same age (14-day-old hair) at D_0 and at D_{14}, a previous shaving was performed at D_{14}(fourteen days before the start of the study). Hair collected at D_0 and at D_{14} corresponded to new hair regrowing after the last shaving and therefore, the cortisol accumulated was considered representative of the SCC during the 14 days previous to the hair collection.

All samples were collected using the same electric hair clipper (Golden A5 M5-55J, Oster, Sunbeam Products Inc., Boca Raton, FL, USA) aiming to cut the hair as close to the skin as possible. Hair harvesting was performed carefully to prevent hair follicle removal and avoid skin damage and potential pain. The peeler blade was cleaned between each hair collection with a brush. Each hair sample weighed approximately 300 mg (forehead) and 750 mg (hip). All hair samples were stored into individually identified zip-lock plastic bags kept in a dark container at room temperature until cortisol extraction.

3.5. Hair cortisol extraction

For hair cortisol extraction, a methanol-based extraction protocol described by Davenport et al. (2006) modified and validated for dairy cattle (Tallo-Parra et al., 2015) was followed. Two hundred and fifty milligrams of hair were weighed from each sample and placed into a 15-ml conical tube. Each sample was washed by adding 2.5 ml of isopropanol (2-propanol 99.5 %, Scharlab S,L., Sentmenat, Spain) and vortexed for five seconds. The supernatant was separated by decantation and the process was repeated twice (three washes in total).

After washing, hair samples were left to dry completely for approximately 36 hours at room temperature. Once dried, hair was minced into < 2 mm length fragments by using the electric hair clipper and 50 mg of trimmed hair were weighed and placed into a 2-ml Eppendorf tube. Then, 1.5 ml of methanol was added to each sample and they were incubated for 18 hours at 30ºC (G24 Environmental Incubator Shaker, New Brunswick Scientific CO Inc., Edison, NJ, USA) under moderate shaking for steroid extraction. Following extraction, samples were centrifuged at 7 000 × g for 2 minutes at 25 ºC and 0.750 ml supernatant was transferred into a new 2-ml Eppendorf tube. The supernatant was then placed in an oven at 38ºC in order to evaporate the methanol. Once the samples were completely evaporated, the dried extracts were reconstituted with 0.2 ml buffer provided by the enzyme immunoassay (EIA) kit (Cortisol ELISA KIT; Neogen® Corporation, Ayr, UK) and shaken for 30 seconds. Samples were then immediately stored at -20 ºC until analysis.

3.6. Cortisol analysis and EIA validation tests

Cortisol concentrations from serum samples and hair extracts were determined in duplicate using cortisol EIA detection kits (Neogen® Corporation Europe, Ayr, UK), with a sensitivity of 0.04 ng cortisol/ml for serum and 0.32 pg cortisol/mg for hair.

For the validation of assays, precision, linearity and accuracy were assessed (Tallo-Parra et al., 2015). Intra-assay and inter-assay coefficients of variation were 8.35 % and 10.51 %, respectively. The linearity test showed a R^2 = 0.98 and a mean percentage error of -15.5 ± 6.92 %. The average of the recovery percentage from spike-and-recovery test was 88.6 ± 29.75 %. This percentage improved when the samples included in the calculation were exclusively those that encompassed the range of values of hair extracts and not all the range of detection of the kit (102.9 ± 18.12 %). The parallelism test showed similar slopes between the standard and the pool lines (0.36 and 0.38, respectively, Figure 1).

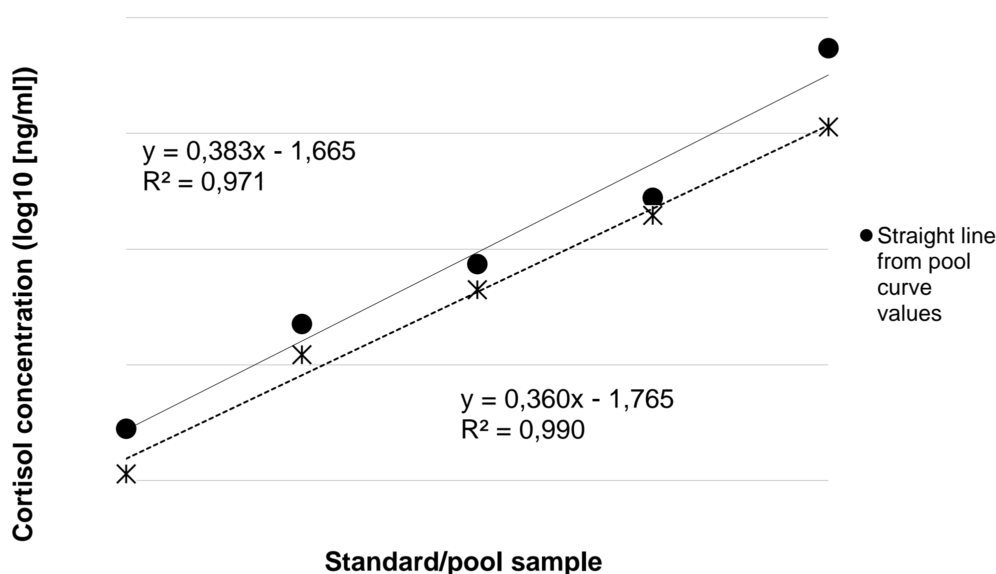


Figure 1. Parallelism between lines from log_{10} of the standard and sample pool curves showing immunological similarities between standard and pool hormones.

According to the manufacturer, cross-reactivity of the EIA antibody with other steroids is as follows: prednisolone 47.4 %, cortisone 15.7 %, 11-deoxycortisol 15.0 %, prednisone 7.83 %, corticosterone 4.81 %, 6ß-hydroxycortisol 1.37 %, 17-hydroxyprogesterone 1.36 %, deoxycorticosterone 0.94 %. Steroids with a cross-reactivity lower than 0.06 % are not shown.

3.7. Statistical analysis

The experimental unit for all statistical analysis was each calf (n = 12 calves per treatment; 2 treatments). A Shapiro-Wilk test was performed to test normality of biological variables. Hair cortisol concentrations from D_0 and D_{14} for both locations were not normally distributed. These non-normality parameters were transformed into $\log_{10}$ and evaluated for normality again. All parameters transformed into $\log_{10}$ were normally distributed. Data were processed and analysed using SAS software version 9.3 (SAS Inst. Inc., Cary, NC). In order to evaluate differences in SCC a mixed-effects model with repeated measures (PROC MIXED with SAS) was carried out. The model included time (b_{0h}, b_{1h} and b_{3h}), treatment (ACTH-injected vs. control) and the interaction of these two factors as fixed effects and the calf as a random effect. Time was considered a repeated factor. Three covariance structures were tested with the aim to create the best mixed-effects model with repeated measures: compound symmetry, autoregressive order 1 and unstructured. The covariance structure that yielded the smallest Schwarz's Bayesian information criterion was used. F-tests were performed to check for equality of variances of HCC variables. After that, t-tests (two tails) were performed using TTEST procedure of SAS in order to detect differences in HCC between treatments (ACTH-injected vs. control) at D_0 and D_{14}. Simple lineal correlations using the CORR procedure of SAS were carried out to detect relations of HCC between sampling day (D_0 and D_{14}) and between hair locations (forehead and hip). The Pearson's correlation coefficient was considered. All the values are presented as means ± standard deviation.

4. RESULTS AND DISCUSSION

4.1. ACTH administration and serum cortisol levels

The increase of SCC after the injection of ACTH (Figure 2, $P < 0.0001$) demonstrated that the intravenous administration of ACTH was successful in generating an adrenal response. According the similarities in SCC between b_{0h} and b_{3h} and the evolution of SCC after ACTH administration (Lay et al., 1996; Veissier et al., 1999), animals returned to the baseline values during the third hour post-administration on both injection days (D_0 and D_7).

4.2. ACTH administration and hair cortisol levels

Initial homogeneity of HCC between ACTH-administrated and control groups prior to the start of the study was confirmed by the lack of differences between treatments at D_0 for forehead and hip hair samples (Table 1. $P > 0.05$). Housing, handling and environmental conditions, as well as health status, remained constant during the study and no stressful events or situations were detected by the farm personnel or the researchers. The only difference between the two treatment groups during the study period corresponded

to the two ACTH injections. No differences were found in HCC between ACTH-injected and control groups from forehead or hip samples at D_{14} (Table 1. $P > 0.05$).

Table 1. *Hair cortisol concentrations (HCC, pg/mg) from forehead and hip depending on treatment (control and ACTH-injected) at D_0 (initial) and D_{14} (final) of the study*

Item	Control group	ACTH group	*P* value
Forehead HCC at D_0, log_{10}pg/mg	0.49 ± 0.222	0.53 ± 0.306	0.9034
Forehead HCC at D_{14}, log_{10}pg/mg	0.47 ± 0.313	0.48 ± 0.304	0.8554
Hip HCC at D_0, log_{10}pg/mg	0.78 ± 0.190	0.84 ± 0.444	0.7909
Hip HCC at D_{14}, log_{10}pg/mg	0.66 ± 0.555	0.56 ± 0.284	0.9904

The absence of differences between treatments at D_{14} could be explained because hair accumulated serum cortisol for 14 days and the alterations of SCC by the ACTH only lasted a short time. Six hours of extra elevations of SCC would not be representative of cortisol concentrations from the whole 14-day studied period. Our results are supported by the multicompartment model of Henderson (1993), the most accepted theory of substance incorporation into the hair shaft (Meyer and Novak, 2012; Russell et al., 2012; Stalder and Kirschbaum, 2012). According to this model, free hormones are incorporated into hair mainly via passive diffusion from blood, although several complementary incorporation routes may exist. Despite the SCC after ACTH injections were significantly greater (Figure 2, $P < 0.0001$), the period of increased SCC in injected animals only represented 1.8% of total time of cortisol incorporation into the sampled hair at D_{14}.

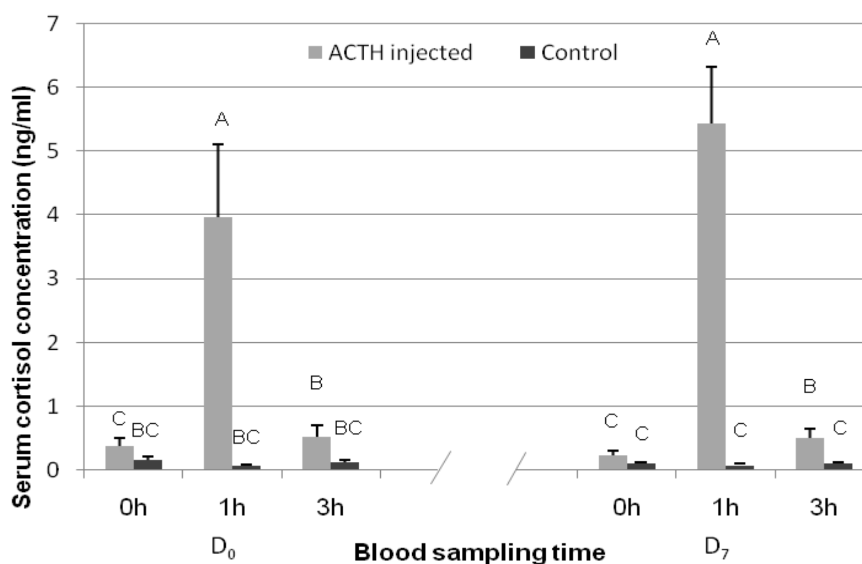


Figure 2. Serum cortisol concentrations (ng cortisol/ml serum; presented as a mean + standard error) immediately prior to ACTH administration (b_{0h}), one hour post administration (b_{1h}) and three hours post administration (b_{3h}) from control group and ACTH-administrated group at D_0 and D_7.
A, B, C Different letters indicate statistical significance ($P < 0.01$).

Our results are in concordance with the study performed by Ashley et al., (2011). They administrated a single dose of ACTH (2 IU/kg BW in the first experiment and 8 IU/kg BW in the second) to caribou and reindeer and did not find effects in three-week-old hair samples. Unfortunately, the magnitude and time of increased circulating cortisol as a consequence of the ACTH injections were not controlled and the authors considered that HPA stress-responses were partially unclear in the reindeer. Thus, this study could not precisely monitor the impact of ACTH on SCC. Other studies that used ACTH challenges in order to validate hair cortisol for other species (Mastromonaco et al., 2014; Terwissen et al., 2013) or corticosterone in shed skin (Berkvens et al., 2013) also considered a single or few doses of ACTH unable to increase the concentration of cortisol or corticosterone in the target matrix. Terwissen et al. (2013) and Mastromonaco et al. (2014) aimed to confirm a relationship between injected ACTH and HCC by designing experiments that increased circulating cortisol concentrations for a long time. The doses used were significantly higher (20 IU/kg BW and 10 IU/kg BW, respectively) than those used in our experiment (1 IU of ACTH/Kg $BW^{0.75}$) and the frequency or number of administrations was also greater. Thus, although these studies confirmed the relationship between ACTH administration and HCC for the first time, they did not asssess the potential influence of short and non-recurrent elevations of SCC on HCC. Furthermore, these studies did not monitor the time perod or the magnitude of increased circulating cortisol as a consequence of the ACTH injections.

Surprisingly, our results are in discordance with those of González-de-la-Vara et al. (2011). In a point of their experiment, the authors reported a significant increase of HCC in 14-day-old hair samples from adult pregnant dairy cattle that accumulated cortisol as a response to only two ACTH injections. They administered three ACTH doses (0.15 UI/kg BW) at days 0, 7 and 14 of their study and collected hair samples at days 0, 14, 28 and 42. Thus, at day 14, hair samples accumulated cortisol as a response to the ACTH doses from days 0 and 7 but not from the same day 14. The portion of hair that received the increase of cortisol as a consequence of the third ACTH injection was presumptively under the skin at the moment of hair collection.

Although the animals used in our study were Holstein-Friesian too, age and sex differed significantly (pregnant adult females vs. bull calves). This could be the main cause for the differences between these results as differences of sex, age and physiological status on HPA-axis activity have been detected in several mammals (Moberg and Mench, 2000; Mormède et al., 2007). Another possible explanation could be that SCC of cows in the study of González-de-la-Vara et al. (2011) remained high for a longer time after each ACTH injection. Blood samples were taken at 0 min, 60 min and 90 min after each ACTH administration and revealed elevated SCC in samples collected at both 60 and 90 min. Thus, the moment in which SCC returned to basal levels remained unclear in their study. However, Lay et al. (1996) studied ACTH dose response on pregnant Brahman cattle and after an administration of a similar dose of ACTH used in the study of González-de-la-Vara et al. (2011), SCC returned to basal levels between 150 and 180 min after ACTH injection. Future work is required to better understand the differences between the present study and that of González-de-la-Vara et al. (2011). Experimental designs using animals of different age groups and sexes under the same experimental conditions and long-term monitoring of SCC after ACTH administration would be useful steps in this direction.

The results of our study showed that HCC from bull calves were not affected by acute elevations of serum cortisol as these elevations were short lasting and not frequent. This is the first study that tested and confirmed the usefulness of hair as a matrix that is not influenced by occasional elevations of SCC. These findings are relevant in order to validate the use of HCC as a measure to provide an integral or global value of SCC covering an extended period of time that is not affected by short and non-recurrent moments of stress.

4.3. Correlation between hair samples

Hair samples showed a significant low correlation in HCC between hip and forehead hair at D_0 (R^2 = 0.51; P= 0.0117) and a strong tendency at D_{14} (R^2 = 0.40; P= 0.0543). Another low correlation was also found in HCC from hip at D_0 and D_{14} (R^2 = 0.41; P= 0.0463) but not between HCC in forehead at D_0 and D_{14} (P= 0.1808). The absence of strong correlations between HCC from different locations have been reported previously (Burnett et al., 2014; Cerri et al., 2012; Moya et al., 2013). Moya et al. (2013) suggested that the differences in hair growth rate depending on hair locations could be the main cause. Ashley et al.(2011) considered the variability in the onset and timing of the annual body moult or the differences in local glandular secretions containing cortisol as other possible confounding factors. Recently, it has been suggested that differences in skin blood flow could be another factor affecting cortisol incorporation into the hair shaft depending on the body location (Carlitz et al., 2015). The effect on HCC of natural sunlight exposure (Wester et al., 2016), extensive skin brushing or dexamethasone local administration (Salaberger et al., 2016) have also been demonstrated. However, the locations selected in this study (hip and forehead) were probably exposed to similar sunlight regimes and no extensive skin brushings were applied or observed during the experiment. Future studies are needed in order to gain a better understanding on the main causes of HCC differences between body locations and its influence on serum cortisol period represented by collected hair samples. However, hair location should be carefully considered in experimental designs.

5. CONCLUSIONS

This study demonstrates that cortisol determination from 14-day old hair samples is not affected by two elevations of cortisol, simulating acute and non-recurrent moments of stress. When analysing HCC, hair samples should be collected from the same anatomical area as differences exist in HCC depending on the body region. Although more research is required in order to definitively validate the HCC as an integrative measure of long-term retrospective HPA-axis activity, this study proves the robustness of HCC against occasional changes in SCC.

6. REFERENCES

Ashley, N.T., Barboza, P.S., Macbeth, B.J., Janz, D.M., Cattet, M.R.L., Booth, R.K., Wasser, S.K., 2011. Glucocorticosteroid concentrations in feces and hair of captive caribou and reindeer following adrenocorticotropic hormone challenge. Gen. Comp. Endocrinol. 172, 382–391. doi:10.1016/j.ygcen.2011.03.029

Berkvens, C.N., Hyatt, C., Gilman, C., Pearl, D.L., Barker, I.K., Mastromonaco, G.F., 2013. Validation of a shed skin corticosterone enzyme immunoassay in the African House Snake (Lamprophis fuliginosus) and its evaluation in the Eastern Massasauga Rattlesnake (Sistrurus catenatus catenatus). Gen. Comp. Endocrinol. 194C, 1–9. doi:10.1016/j.ygcen.2013.08.011

Bortolotti, G.R., Marchant, T.A., Blas, J., German, T., 2008. Corticosterone in feathers is a long-term, integrated measure of avian stress physiology. Funct. Ecol. 22, 494–500. doi:10.1111/j.1365-2435.2008.01387.x

Burnett, T.A., Madureira, A.M.L., Silper, B.F., Tahmasbi, A., Nadalin, A., Veira, D.M., Cerri, R.L.A., 2015. Relationship of concentrations of cortisol in hair with health, biomarkers in blood, and reproductive status in dairy cows. J. Dairy Sci. 98, 1–13. doi:10.3168/jds.2014-8871

Burnett, T.A., Madureira, A.M.L.L., Silper, B.F., Nadalin, A., Tahmasbi, A., Veira, D.M., Cerri, R.L.A., 2014. Short communication: Factors affecting hair cortisol concentrations in lactating dairy cows. J. Dairy Sci. 97, 7685–7690. doi:10.3168/jds.2014-8444

Carlitz, E.H.D., Kirschbaum, C., Miller, R., Rukundo, J., van Schaik, C.P., 2015. Effects of body region and time on hair cortisol concentrations in chimpanzees (Pan troglodytes). Gen. Comp. Endocrinol. doi:10.1016/j.ygcen.2015.09.022

Cerri, R.L.A., Tabmasbi, A.M., Veira, D.M., 2012. Hair cortisol concentrations -Influence of color and location in Holstein cows. J. Dairy Sci. 95, S574.

Davenport, M.D., Tiefenbacher, S., Lutz, C.K., Novak, M.A., Meyer, J.S., 2006. Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. Gen. Comp. Endocrinol. 147, 255–261. doi:10.1016/j.ygcen.2006.01.005

González-de-la-Vara, M.R.D.R., Valdez, R.A., Lemus-Ramirez, V., Vázquez-Chagoyán, J.C., Villa-Godoy, A., Romano, M.C., 2011. Effects of adrenocorticotropic hormone challenge and age on hair cortisol concentrations in dairy cattle. Can. J. Vet. Res. 75, 216–221.

Gow, R., Thomson, S., Rieder, M., Van Uum, S., Koren, G., 2010. An assessment of cortisol analysis in hair and its clinical applications. Forensic Sci. Int. 196, 32–37. doi:10.1016/j.forsciint.2009.12.040

Gwazdauskas, F.C., Thatcher, W.W., Wilcox, C.J., 1972. Adrenocorticotropin alteration of bovine peripheral plasma concentrations of cortisol, corticosterone, and progesterone. J. Dairy Sci. 55, 1165–1169. doi:10.3168/jds.S0022-0302(72)85640-6

Henderson, G.L.L., 1993. Mechanisms of drug incorporation into hair. Forensic Sci. Int. 63, 19–29. doi:10.1016/0379-0738(93)90256-A

Lay, D.C., Friend, T.H., Randel, R.D., Jenkins, O.C., Neuendorff, D.A., Kapp, G.M., Bushong, D.M., 1996. Adrenocorticotropic hormone dose response and some physiological effects of transportation on pregnant Brahman cattle. J. Anim. Sci. 74, 1806–1811.

Mastromonaco, G.F., Gunn, K., Edwards, D.B., 2014. Validation and use of hair cortisol as a measure of chronic stress in eastern chipmunks (Tamias striatus). Conserv. Physiol. 2, 1–12. doi:10.1093/conphys/cou055

Meyer, J.S., Novak, M.A., 2012. Minireview: Hair cortisol: a novel biomarker of hypothalamic-pituitary-adrenocortical activity. Endocrinology 153, 4120–4127. doi:10.1210/en.2012-1226

Moberg, G.P., Mench, J.A., 2000. The biology of animal stress: basic principles and implications for animal welfare. CABI, Wallingford. doi:10.1079/9780851993591.0000

Mormède, P., Andanson, S.S., Aupérin, B., Beerda, B., Guémené, D., Malmkvist, J., Manteca, X., Manteuffel, G., Prunet, P., van Reenen, C.G., Richard, S., Veissier, I., Mormede, P., Auperin, B., Guemene, D., 2007. Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. Physiol. Behav. 92, 317–339. doi:10.1016/j.physbeh.2006.12.003

Moya, D., Schwartzkopf-Genswein, K.S., Veira, D.M., 2013. Standardization of a non-invasive methodology to measure cortisol in hair of beef cattle. Livest. Sci. 158, 138–144. doi:10.1016/j.livsci.2013.10.007

NRC, 2001. Nutrient requirements of dairy cattle, 7th rev. ed, 7th rev. ed. National Academy Press, Washington, DC.

Russell, E., Koren, G., Rieder, M., Van Uum, S., 2012. Hair cortisol as a biological marker of chronic stress: Current status, future directions and unanswered questions. Psychoneuroendocrinology 37, 589–601. doi:10.1016/j.psyneuen.2011.09.009

Salaberger, T., Millard, M., Makarem, S. El, Möstl, E., Grünberger, V., Krametter-Frötscher, R., Wittek, T., Palme, R., 2016. Influence of external factors on hair cortisol concentrations. Gen. Comp. Endocrinol. doi:10.1016/j.ygcen.2016.05.005

Stalder, T., Kirschbaum, C., 2012. Analysis of cortisol in hair – State of the art and future directions. Brain. Behav. Immun. 26, 1019–29. doi:10.1016/j.bbi.2012.02.002

Tallo-Parra, O., Manteca, X., Sabes-Alsina, M., Carbajal, A., Lopez-Bejar, M., 2015. Hair cortisol detection in dairy cattle by using EIA: protocol validation and correlation with faecal cortisol metabolites. Animal 9, 1059–1064. doi:10.1017/S1751731115000294

Terwissen, C. V., Mastromonaco, G.F., Murray, D.L., 2013. Influence of adrenocorticotrophin hormone challenge and external factors (age, sex, and body region) on hair cortisol concentration in Canada

lynx (Lynx canadensis). Gen. Comp. Endocrinol. 194, 162–167. doi:10.1016/j.ygcen.2013.09.010

Veissier, I., van Reenen, C.G., Andanson, S., Leushuis, I.E., 1999. Adrenocorticotropic hormone and cortisol in calves after corticotropin-releasing hormone. J.Anim Sci. 77, 2047–2053.

Wester, V.L., Noppe, G., Savas, M., Stolk, R.P., Akker, E.L.T. van den, Rijke, Y.B. de, Rossum, E.F.C. van, 2016. Long-Term Glucocorticoids Measured in Hair Are Influenced by Local Corticosteroid Treatment: the LifeLines Cohort Study, in: Endocrine Society's 98th Annual Meeting and Expo. Boston, p. FRI-405.

Chapter IV

Hair cortisol and progesterone detection in dairy cattle: interrelation with physiological status and milk production

Hair cortisol and progesterone detection in dairy cattle: interrelation with physiological status and milk production

O. Tallo-Parra[1,2], A. Carbajal[1], L. Monclús[1], X. Manteca[2] and M. Lopez-Bejar[1],

[1]*Department of Animal Health and Anatomy, Veterinary Faculty, UniversitatAutònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain*

[2]*Department of Animal and Food Science, Veterinary Faculty, UniversitatAutònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain*

1. ABSTRACT

Cortisol is a key factor in the stress response and has been widely used in studies of dairy cattle stress and welfare. Progesterone is produced mainly by the corpus luteum and has been used as an indicator of gonadal activity and reproductive status andfor pregnancy diagnosis. Hair cortisol concentrations (HCC) and hair progesterone concentrations (HPC) allow monitoring long-term retrospective steroid levels because hair seems to accumulate circulating steroids during all its period of growth. However, there are still gaps in the knowledge of the mechanisms of steroids deposition in hair and its potential application in dairy cattle. This study aimed to evaluate the potential uses of hair steroid determinations by studying the interrelations between HCC, HPC, physiological data from cows and their milk production and composition. Cortisol and progesterone concentrations were analysed in hair from 101 milking Holstein-Friesian cows in a commercial farm. Physiological and productive data were recorded at the moment of sampling from the two closest milking controls and also 124 days after from the same lactation. Slight correlations were found between HCC and HPC and between HPC and age. High HCC were associated to low milk yields and also to high somatic cell count. No effect of HCC on fat or protein content was detected. No correlations were detected between hair steroid concentrations and pregnancy status, days of gestation, parturitioncategory (primiparous vs. multiparous), number of lactations or days in milk. The relationship between physiological variables and HCC or HPC could depend on the period of time in which hair samples accumulated hormones. Steroid concentrations in hair present high variability between individuals but are a potential tool for dairy cattle welfare, reproductive and production research by providing a useful and practical tool for long-term steroid monitoring.

2. INTRODUCTION

Steroid hormones play a key role in several physiological processes. Cortisol, the main glucocorticoid in most of mammals including bovine species, is a key factor in the stress response mediated by the hypothalamic–pituitary–adrenal (HPA) axis (Charmandari et al., 2005; Mormède et al., 2007; Ralph and Tilbrook, 2016). Thus, cortisol has been widely used in studies of dairy cattle stress and welfare (Arnott et al., 2016; Jacobs and Siegford, 2012; von Keyserlingk et al., 2009). On the other hand, progesterone is produced mainly by the corpus luteum in the ovary or the placenta and it is essential in the establishment and maintenance of pregnancy (Mann and Lamming, 1999; Spencer, 2004a, 2004b; Wiltbank et al., 2014). Progesterone detection has been used as an indicator of gonadal activity and reproductive status, including pregnancy diagnosis (Crowe, 2008; Kornmatitsuk et al., 2007; Robinson et al., 2008).

Although literature is wide regarding research in steroid hormones and dairy cattle production, reproduction and welfare, most of studies have analysed steroids in blood, but also in saliva, faeces or milk (Duncan, 2005; Mormède et al., 2007; Palme, 2012). All of these matrixes have different characteristics but none of them is able to represent long-term retrospective steroid levels (Davenport et al., 2006; Russell et al., 2012; Stalder and Kirschbaum, 2012).

The development of hair as a matrix for steroid detection allowed researchers to monitor long-term retrospective levels because hair seems to accumulate circulating steroids during all its growth period, providing an integrative value of them (Comin et al., 2011; Koren et al., 2002; Meyer and Novak, 2012; Stalder and Kirschbaum, 2012). The measurement of hair cortisol concentrations (HCC) or hair progesterone concentrations (HPC) makes possible to assess long-term adrenal or gonadal activity, respectively, without the need of serial and continuous sampling. This opens new possibilities in the study of the impact of chronic stress over milk production.

In dairy cattle, some studies attempted to understand intra-animal sources of HCC variability (Burnett et al., 2014; Cerri et al., 2012) and confirmed the relationship between HCC and HPA-axis activity (González-de-la-Vara et al., 2011; Tallo-Parra et al., 2015). Although hair cortisol measurement is considered a relatively new practice, HCC have already been used in dairy cattle research as a biomarker of stress by studying its association with health (Burnett et al., 2015; Comin et al., 2013), reproduction (Burnett et al., 2015; Comin et al., 2013, 2008), breed (Peric et al., 2013), cow's environmental conditions (Comin et al., 2011), stocking density (Silva et al., 2016) or with reproductive treatments (Biancucci et al., 2016).

In contrast to hair cortisol analysis, only two studies using hair progesterone detection in cattle were found by the authors, additionally to the protocol validation for hair progesterone detection performed by our laboratory (Tallo-Parra et al., 2016). Gleixner and Meyer (1997) compared

HPC between cows and bulls and between hair colours and Liu et al., (1988) aimed to diagnose pregnancy by using HPC in samples collected 24 days after artificial insemination.

Despite its promising results, there are still gaps in the knowledge of hair steroid determination and its real applications in applied research still needs evaluation (Buchanan and Goldsmith, 2004; Salaberger et al., 2016; Sharpley et al., 2012).

This study aimed to evaluate the potential uses of hair steroid determinations (HCC and HPC, separately) in dairy cattle production. It focused on the study of the interrelations between HCC, HPC, physiological data from cows and, especially, between HCC and milk production and quality.

3. MATERIALS AND METHODS

3.1. Study design

Hair sampling from dairy cows were performed on 6th November, 2014.From the two months previous to hair sampling, data from the milking controls and clinical history were obtained. Moreover, 124 days after hair sampling (10/03/2015), milk production data from the same lactation was obtained. Although most cows were still lactating at this second data collection, the collected data represented a wide range of lactation period and was considered representative of long-term milk production.The relationship between long-term circulating cortisol or progesterone levels and status, history or milk production and composition was studied.

3.2. Animals

One hundred and one Holstein-Friesian cows were used in this study. All cows were in the milking period and their average age was 3.5 ± 1.3 years old (range: 2.1 - 8.2). The distribution of number parturition is shown in Table 1. The 59.8 % (N = 58) of the studied cows were pregnant, with an average of 79.07 ± 52.09 days of gestation (range: 1 - 164).

Table 1. *Number of parturitions of the cows used in the study*

Number of parturition	N	%
1	45	44.5
2	33	32.7
3	11	10.9
4	9	8.9
5	2	2.0
6	1	1.0
Primiparous	45	44.5
Multiparous	56	55.5

3.3. Housing and handling conditions

All cows were housed in a commercial farm located in Girona, northeast of Spain, under commercial conditions. The farm had 819 Friesian lactating cows feed with total mixed ration twice a day (23.5 kg of dry matter per cow) with water *ad libitum*. The milking routine consisted of three successive milkings per day: at 0500h, 1300h and 2100h. The farm average milk yield was 37.7 litres per cow and day. This study aimed to assess the effects of chronically elevated steroids levels on milk production and quality in cows under commercial conditions. Thus, the selected farm passed a Welfare Quality Protocol® evaluation before the starting of the study to confirm no extreme poor welfare conditions were present.

3.4. Hair sampling and storage

All hair samples were taken at the same day. Between 300 and 500 mg of hair from the ventrolateral region of the neck were collected per animal. During the hair sampling, animals were headlocked individually at the feeder and released after each sampling. In order to avoid differences in HSC related to hair colour (Burnett et al., 2014; Cerri et al., 2012; Gleixner and Meyer, 1997; Tallo-Parra et al., 2015), homogenized white hair samples were collected. Hair was cut as closest to the skin as possible but preventing skin damage and hair follicle removal. An electric hair clipper (X3 ceramic-titanium hair clipper; Palson® Trading España S.L., Collbató, Spain) was used and the peeler blade was cleaned between animals using a brush. Each hair sample was stored into properly identified zip-lock plastic bags in a dark place at room temperature. Although the specific hair growth rate for the ventrolateral region of the neck is not known, the hair sampled was considered to accumulate circulating hormones during approximately the last two months before hair sampling. This assumption was based on the studied hair growth rate from gluteal region, shoulder or forehead performed previously (Burnett et al., 2014; Comin et al., 2013; Martin et al., 1969). These regions have a similar hair length and

diameter than from the neck. In contrast, hair from tail switch has a greater growth rate (Burnett et al., 2014; Moya et al., 2013).

3.5. Hair washing and steroid extraction

For hair washing and steroid extraction, a validated protocol for hair cortisol detection in dairy cattle was followed (Tallo-Parra et al., 2015). Two hundred and fifty milligrams of hair were weighed from each sample and placed into a 15-ml conical tube. Three washes consisting of 2.5 ml isopropanol (2-propanol 99.5 %, Scharlab S.L., Sentmenat, Spain) and 2.5 minutes of vortex each one were applied to all the samples. The isopropanol was eliminated by decantation after each wash. Once washing finished, hair samples were left to dry for 36 hours at room temperature. Then, dried hair samples were trimmed using a ball mill for 5 minutes at 22 Hz (MM200, Retsch, Haan, Germany; 10-ml stainless-steel grinding jars; two 12-mm stainless-steel grinding balls). For the steroid extraction, 50 mg of trimmed hair were carefully weighed, placed into a 2-ml eppendorf tube and 1.5 ml of pure methanol was added. Samples were incubated for 18 hours at 30 ºC (G24 Environmental Incubator Shaker, New Brunswick Scientific CO Inc., Edison, NJ, USA) under moderate shaking. After incubation, extracted samples were centrifuged at 7 000 × g for 2 minutes at 25 ºC and 0.750 ml of supernatant was transferred into a new 2-ml eppendorf tube. The supernatant was then placed in an oven (Heraeus model T6; Kendro® Laboratory Products, Langenselbold, Germany) at 38 ºC to evaporate the methanol. Once the methanol was completely evaporated, the dried extracts were reconstituted with 0.250 ml of buffer provided by the enzyme immunoassay (EIA) kit (Cortisol ELISA KIT; Neogen Corporation, Ayr, UK) and shaken for 30 seconds. Cortisol concentrations were assessed from these reconstituted hormonal extracts. However, preliminary results showed high concentrations of progesterone. Thus, 75 μl from each hormonal extract were separated and diluted with 225 μl of EIA buffer only for progesterone detection. This 1:4 dilution was applied in order to analyse concentrations within the detection range of the progesterone EIA kit. Then, all hormone extracts were stored at -20 ºC until analysis.

3.6. Hormone detection and validation tests

Hair steroid concentrations (HSC) were determined using two cortisol and two progesterone EIA detection kits (Neogen Corporation Europe, Ayr, UK). Only intra and inter-assay Coefficient of variation (CV) were calculated in order to assess precision as the hair hormone extraction protocol and hormone analyses were previously validated for the same species, matrix and EIA

kit for both cortisol (Tallo-Parra et al., 2015) and progesterone(Tallo-Parra et al., 2016). The intra-assay CV for cortisol and progesterone were 7.11 % and 8.64 %, respectively. The inter-assay CV was 4.44 % for cortisol and 10.53 % for progesterone.

3.7. Animal’s data collection

All data from animals were obtained from the Herd Management Software Dairy Plan C21 (GEA Farm Technologies, Inc., Bönen, Germany). Physiological data obtained was age, pregnancy status, days of gestation and number of previous parturitions. Data from the milking controls from the two months previous to hair sampling included days in milk (DIM), daily milk yield, fat content, protein content and somatic cell (SC) count (Table 2). The clinical history of each cow from the last two months was recorded by the veterinarian personnel and included diagnosis of lameness (n = 5), fever (n = 7), abortion (n = 2), mastitis (n = 4), metritis (n = 1), diarrhea (n = 2), lameness (n = 7), indigestion (n = 2). The number of pen changes within the milking period was also individually recorded (Table 3).

Table 2. *Milking control data from the same month (17 November) and the previous months (17 October) of hair sampling*

	17 OCTOBER				17 NOVEMBER			
	AVERAGE	**SD**	**MIN**	**MAX**	**AVERAGE**	**SD**	**MIN**	**MAX**
Days in milk	157.85	102.19	16	521	188.85	102.19	47	552
Milk yield (kg)	36.62	8.83	19.00	61.80	37.87	9.45	16.30	64.74
Milk Fat (%)	3.39	0.72	0.16	5.38	3.64	0.64	2.09	5.53
Milk Protein (%)	3.38	0.32	2.66	4.15	3.36	0.30	2.69	4.07
Milk SC count (x 1000/ml)	183.23	437.46	0	3590	143.17	216.62	11	1073

Table 3. *Number of pen changes during the two months previous to the hair sampling*

Number of pen changes	N	%
0	72	71.29
1	21	20.79
2	7	6.93
3	1	0.99

The second set of data collection was collected approximately four months after hair sampling from all the animals that were still at the same lactation. Data included: DIM, average daily milk yield, and the sum of milk produced in the last three milkings (Table 4). As it can be seen in Table 4, the second set of data collection represented the milk production of cows from the beginning of lactation until the day of data collection (the whole lactation until data collection). The average of lactating days encompassed in this period was about 270 days. Consequently, it can be considered almost representative of an entire lactation. Cows that were eliminated for medical reasons between the hair sampling and the second data collection (n=7) were identified and classified as "eliminated".

Table 4. *Milk production parameters obtained for the whole lactation (until 124 days after hair sampling)*

	N	AVERAGE	SD	MIN	MAX
Days in milk	101	269.71	100.54	76	603
Daily average (kg of milk)	84	33.54	9.56	13.12	65.88
Sum 3 last milkings (kg)	101	31.27	11.05	9.8	66.3

3.8. Statistical analyses

Data were processed and analysed using SAS software version 9.3 (SAS Inst. Inc., Cary, NC). Cow was considered the experimental unit for all statistical analysis. The number of animals varied among statistical analyses because of some individual missing data in Dairy Plan C21 software. A Shapiro-Wilk test was performed to test normality of variables. Several Pearson's correlation analyses (PROC CORR) were performed in order to find relationships within HSC (between hair cortisol and hair progesterone) and between HSC (HCC and HPC, separately) and age, DIM, days of gestation and number of pen changes. A new health parameter was created as there were not enough animals to analyse the effect of each individual disease in HCC and milk production and composition. Thus, cows were categorically classified as Healthy and Non-healthy history depending on the presence or absence of at least one disease in the clinical history from the last two months previous hair sampling. A two tails parametric test (PROC TTEST) was done to compare HCC between healthy and non-healthy cows and between eliminated and no eliminated. The same test was performed to detect differences in HSC between pregnant and non-pregnant and between primiparous and multiparous cows. The influence of parturition category (primiparous or multiparous) in the relationship between HSC and DIM, pregnancy status, days of gestation and number of pen changes were also evaluated as some differences were detected previously (Burnett et al., 2015). Thus, correlations were done again but separating primiparous and multiparous cows. The average from the milking controls from the two previous

months to hair sampling were used in order to study the effect of long term cortisol and progesterone levels on milk production and composition. Different multiple linear regression (PROC GLM) were used to assess the influence of HSC and health status in milk production and composition. In order to identify the subset of independent variables for the best statistical model, a forward selection test (PROC REG; selection FOWARD with a slentry value of 0.25), a backward selection test (PROC REG; selection BACKWARD with a slstay value of 0.10) and a stepwise selection test (PROC REG; selection STEPWISE with a slstay value of 0.10 and slentry value of 0.25) were applied for each parameter. For milk yield prediction, DIM and primiparous/multiparous category were considered as the independent parameters for the best model. For milk fat prediction, milk yield and primiparous/multiparous category were the independent parameters selected. Days in milk parameter was considered the only independent variable of the best model for milk protein prediction. For SC count, age was selected as the independent variable. For the milk production values obtained for the whole lactation, DIM and primiparous/multiparous category were used as independent parameters. A quadratic adjustment for DIM was tested in order to improve all the models for the prediction of milk production parameters. However, better models appeared when using linear adjustments as a linear relationship existed between milk production and DIM data. All the values are presented as means ± standard deviation.

4. RESULTS

4.1. Hair steroid concentrations

Hair concentrations of cortisol and progesterone hormones are shown in Table 6. A slight correlation between HCC and HPC existed ($R^2 = 0.25$, $P < 0.0001$).

Table 5. *Hair cortisol and progesterone concentrations from milking cows by enzyme immunoassay*

	AVERAGE	SD	MIN	MAX
Hair cortisol concentrations (pg/mg)	4.52	1.42	1.66	7.82
Hair progesterone concentrations (ng/mg)	0.897	0.328	0.292	1.862

4.2. Hair steroid concentrations and physiological data

No correlations existed between HCC and age ($P > 0.10$). In contrast, a very slight positive correlation was found between HPC and age ($R^2 = 0.06$, $P = 0.0133$). No differences in HCC or HPC were found between primiparous and multiparous cows ($P > 0.10$) or among cows with different lactation number ($P > 0.10$). Likewise, no differences in HCC or HPC were found between pregnant and non-pregnant cows ($P > 0.10$) and no relationships were detected between the same parameters and days of gestation ($P > 0.10$). Animals with or without any clinical problem during the last two months previous to hair sampling (healthy vs. non-healthy history) did not present differences in HCC or HPC ($P > 0.10$). In the same way, animals eliminated for medical reasons between hair sampling and the second data collection (124 days after) had not statistically elevated HCC or HPC ($P > 0.10$).

The number of pen changes within the last two months previous the hair sampling did not affect HCC, or HPC ($P > 0.10$).

The correlations between HSC and DIM, pregnancy status, days of gestation and number of pen changes did not differ between primiparous and multiparous cows.

4.3. Hair steroid concentrations and milk data from the two previous months

Data from the two milking controls were averaged and used as integrative values of milk production and composition from the two months previous hair sampling. No correlations were found between HCC or HPC and DIM. In the statistical model for milk yield prediction ($R^2 = 0.57$, $P < 0.0001$) with DIM and primiparous/multiparous category as the other independent variables, HCC influenced and affected negatively milk production (Partial $R^2 = 0.023$, $P = 0.0396$). Hair progesterone concentrations did not affect milk yield prediction ($P > 0.10$). For milk fat content ($R^2 = 0.39$, $P < 0.0001$) or milk protein content ($R^2 = 0.37$, $P < 0.0001$) no influence of HCC or HPC ($P > 0.10$). Finally, an effect of HCC ($P = 0.0241$) but not HPC ($P > 0.10$) on SC count was observed although the model for SC count prediction was very limited ($R^2 = 0.12$, $P < 0.0053$). Cows with greater HCC had greater SC count in its milk.

4.4. Hair steroid concentrations and milk data from the whole lactation

Hair progesterone concentration did not affect any milk parameter obtained for the whole lactation until the second data collection (average daily milk yield and sum of three last milkings; $P > 0.10$).

However, the average milk yield prediction ($R^2 = 0.24$, $P < 0.0001$) and the sum of the three last milkings ($R^2 = 0.30$, $P < 0.0001$) were lower in cows with high HCC ($P = 0.0247$ and $P = 0.0208$, respectively) although the influence of HCC was slight (Partial $R^2 = 0.057$ and 0.074, respectively).

5. DISCUSSION

The present study evaluated the utility of a single sampling of hair for the detection of cortisol and progesterone levels with the objective to study long-term retrospective steroid hormonal levels and its relationship with physiological status and milk productive parameters in dairy cattle.

5.1. Hair cortisol concentrations and physiological data

High individual variability was found between cows in HCC as mentioned previously in hair (Comin et al., 2013) and other matrixes and species (Cockrem, 2013; Moberg and Mench, 2000; Mormède et al., 2007).

This study did not detect the effect of age in HCC within the range of age studied although differences in HCC between adult dairy cows and calves were observed (Comin et al., 2008; González-de-la-Vara et al., 2011; Maiero et al., 2005). Likewise, no relationship existed between HCC and lactation number. The absence of correlations between HCC and age could be explained because all cows were adult at the moment of sampling. These results also suggest that older cows with greater number of lactations are not necessarily more adapted to the handling and herd routine and conditions as they did not show lower HCC.

This study was not able to detect differences in HCC between primiparous and multiparous cows, contrary to observed in previous studies (Burnett et al., 2015; Cerri et al., 2012), where multiparous cows showed greater HCC than primiparous cows. In the study performed by Burnett et al., (2015), HCC was greater in multiparous cows except for hair samples collected at

the moment of calving. In their study, cows were at the same DIM, all hair samples were only 21 days-old and they analysed hair samples from cows at 0, 21, 42, 84 and 126 DIM. In contrast, our study encompassed cows with a wide range of DIM, most of them > 126 DIM and hair samples were approximately 2 months-old. Supporting our results but using a more acute cortisol matrix, Fukusawa et al.(2008) did not find differences in milk cortisol concentrations between primiparous and multiparous cows at any of these DIM ranges: 7-90, 91-180, 181-271 and ≥ 271. The present study hypothesized that primiparous cows could be more stressed than multiparous as a consequence of the novelty of the environment, milking routine and all changes associated, as suggested previously (Comin et al., 2013; Szentléleki et al., 2015). The absence of HCC variations could be explained because average DIM of primiparous cows was 153 ± 96 days and these cows should have passed the hypothesized adaptation period several weeks ago. Thus, although this study assumed that cortisol accumulated in hair samples reflected a period of two months, our sampling was unable to cover the first days under milking conditions of most of primiparous cows.

In relation to pregnancy parameters, no differences were detected in HCC between pregnant and non-pregnant cows or among days of gestation in case of pregnant cows.Burnett et al., (2015) did not detect differences of cortisol levels in 21-day-old hair between pregnant and non-pregnantcows 26 days after insemination. However, they found an interesting relationship: multiparous cows that were pregnant at 100 DIM showed lower HCC than multiparous cows that did not get pregnant in samples collected at previous 42 and 84 DIM. In contrast, Comin et al., (2008) detected greater cortisol concentrations in hair collected from cows at parturition when compared with non-pregnant dry cows. This increase of HCC was related with the third trimester of pregnancy (when hair collected at parturition was growing and accumulating hormones) and has also been observed in women (D'Anna-Hernandez et al., 2011; Kirschbaum et al., 2009). In the present study, however, no cows in the third trimester of pregnancy were used and a significant linear relationship between HCC and days of gestation for the two first trimesters of pregnancy was not observed.

The present study was not able to detect differences between healthy and non healthy cows in HCC. Our results are in contradiction with results obtained in the literature that found greater cortisol concentrations in hair from cows recently suffering a disease (Burnett et al., 2015; Comin et al., 2013). However, the information is contradictory regarding cortisol concentrations and diseases in cows depending on severity, specific disease and matrix in which cortisol is detected (Forslund et al., 2010; Galvão et al., 2010; Lavon et al., 2010; Walker et al., 2010). As our study was conducted under commercial conditions, all diseases diagnosed were treated by the farm's veterinarians as soon as possible. Thus, cows classified as non-healthy not necessarily had increased their HPA axis activity for several days. No specifications regarding medical treatments to diseased cows were mentioned in Comin et al., (2013) and Burnett et al., (2015) articles.

Moreover, hair samples from our study and the study from Comin et al., (2013) were assumed to represent circulating hormonal levels for an approximate period of two month but hair from cows collected by Burnett et al., (2015) were allowed to growth only for 21 days. In the present study, the absence of elevated HCC in non healthy cows could be explained by the sum of these three effects: the absence of a more sensitive classification in relation to type of disease and severity; the short-time effect of diseases in HPA-axis activity due to the quickly administration of medical treatments; the dilution of the period under disease in two-months old hair samples. Together with literature, our study reveals the importance the period of time that researchers decide to monitor with the hair sampling design. The balance between providing an integrative value of long-period HPA-axis activity and the sensitivity of the samples to specific potential stressors within this period should be studied and considered when using hair hormonal detection in future research.

The present study failed at demonstrating the use of hair cortisol measurement as a tool to predict the evolution of a disease into a critical stage that induced animal to its elimination. Likewise, HCC was not sensitive to stress caused by pen changes and the subsequent new social environment. Although the stress for pen change is demonstrated (Boe and Gry, 2003; Chebel et al., 2016; Phillips, 2002) it probably not lasted enough to increase significantly HCC in moved cows as most of moved cows suffered only one pen's change in the last two months.

5.2. Hair cortisol concentrations and milk production and composition

The present study demonstrated a negative relationship between HCC and milk yield using data from the period of time monitored by the hair samples (two months) but also using the average milk yield data for the whole lactation obtained until 124 days after hair sampling and therefore encompassing a variable but wide period of lactation. The relationship was also observed using the sum of the three last milkings at the same point. The negative influence of elevated HCC in milk yield was mentioned before (Burnett et al., 2015) and highlights the importance of stress and HPA-axis activity in milk production. Several factors related to cortisol functions could explain its impact in milk production as cortisol is involved in many physiological processes (Chrousos, 2009; Mormède et al., 2007; Ralph and Tilbrook, 2016). For example, the decrease of milk production could be due to the negative impact of cortisol in milk ejection (Bruckmaier, 2005; Wellnitz and Bruckmaier, 2001) or due to a disruptive effect of cortisol on energy mobilization (Galvão et al., 2010; Hall, 2015; Moberg and Mench, 2000), especially important in high-producing dairy cows. However, the influence of elevated endocrine markers on milk production is still equivocal and partially unknown (Reading, 1999).

Although the high individual variability impairs the standardisation of a cut-off value for presumptively stressed or non stressed cows, it seems clear that there are several sources of stress in the herd affecting cows in a different magnitude with consequences in their welfare and their milk production. Hair cortisol measurement seems to be sensitive to at least some of these factors, providing a new way for cortisol monitoring and amplifying scientific tools for the study of potential factors affecting HPA-axis activity, especially the chronic ones.

The association between HCC and milk yield data collected for the whole lactation (encompassing a long period of lactation) suggests that a single sampling of hair could be representative of the general HPA-axis activity of the cow for probably the whole lactation. Our results indicate that unless an unexpected stressor like a disease happens, the stress status of the cow is probably maintained stable along the lactation, especially after the early lactation period. Potential stress factors affecting HCC during the whole lactation (e.g. hierarchy) could be studied in the future using hair hormonal analysis in order to improve dairy cattle welfare and production.

No effect of DIM in HCC was detected. The period of circulating cortisol accumulated in hair was too long to be sensitive to the decrease in HCC associated to DIM between 21 and 42 as observed previously (Burnett et al., 2015, 2014). Changes in milk cortisol concentrations among lactation stages were also detected, with animals encompassed in the stage 1 (7 to 90 DIM) having greater cortisol concentrations than animals in the rest of stages (91-180, 181-271 and ≥ 271) (Fukasawa et al., 2008).

The present study found a limited but interesting connection between SC count and HCC although high HCC variability was present in cows with low SC count. A relationship between SC count and milk cortisol concentrations was detected by Sgorlon et al., (2015) only in the group of cows with the highest SC count. Thus, the slightly relationship between HCC and SC count found in our study could be due to the same effect. Other studies did not found this relationship between SC count and cortisol concentrations in hair (Comin et al., 2011)or milk (Fukasawa et al., 2008). However, future research should be done to evaluate if HCC could be a good indicator of the severity of sublinicalmastitis in dairy cattle.

5.3. Hair progesterone concentrations and physiological data

Hair progesterone concentrations did not correlate with days of gestation. These results are expected as progesterone concentrations remain high since the first days of pregnancy until calving (Mukasa-Mugerwa and Tegegne, 1989; Spencer, 2004a; Stabenfeldt et al., 1970). However, although progesterone is considered the hormone of pregnancy and its increase in pregnant cows are well documented (Forde et al., 2009; Mann and Lamming, 1999; Wiltbank et

al., 2014). We did not find differences in HPC between pregnant and non-pregnant cows. This could be explained because under commercial conditions, the period between calving and the next successful insemination is as short as possible. Considering that hair samples accumulated hormones during the last two months, hair samples taken from some of non-pregnant cows with low DIM could accumulate progesterone levels from their last parturition. Moreover, the increase of progesterone in non-pregnant cows associated to the cyclical appearance of the corpus luteum (Wiltbank et al., 2014) could also increase progesterone accumulated in non-pregnant hair samples. Although the benefits of hair steroid measurement are clear, the present study visualise that HPC could not be sensitive to short or medium-term progesterone changes when analysing hair samples encompassing a broad period of time. Future work with specific hair sampling design should be done in order to evaluate the ability of HPC to detect changes in progesterone levels associated to gonadal activity or pregnancy.

This study showed a correlation between HPC and age, but in a very slight association. The assumption that progesterone production increase with age should be interpreted cautiously as the decision of cow's elimination in a commercial farm is based in medical but also productive and reproductive factors and older cows kept in farms could be a biased population. Moreover, this study failed to associate HPC and lactation number. Appropriate studies should be performed in the future with the aim to understand and confirm the relationship between age and long-term progesterone levels in dairy cows.

5.4. Interrelation between hair steroid concentrations

Hair steroid concentrations were correlated positively for cortisol and progesterone. Although this correlation has been reported previously in blood for cows and woman (Echternkamp, 1984; Herrera et al., 2016), the relationship between these two steroids is still unclear. The demonstrated influence of stress status and adrenocorticotropic (ACTH) hormone in the secretion of progesterone by the adrenal gland (Gwazdauskas et al., 1972; Yoshida and Nakao, 2005), as well as the skin steroidogenesis (Slominski et al., 2013) could be some of factors explaining the correlations between HCC and HPC. However, future research is necessary to explore the relationship between these two hormones in hair.

6. CONCLUSIONS

This study is a step forward in the use of hair steroid measurements in dairy cattle research. Cortisol concentrations in hair are presenting the same high individual variability previously reported in serum, milk and other matrixes. Hair cortisol concentrations influenced negatively milk yield demonstrating the potential of this tool to be used in dairy cattle welfare and production research. The sensitivity of hair steroid measurements to acute, medium or long-term changes in circulating steroid concentrations is conditioned by the period of hair samples are allowed to accumulate steroids. Thus, hair steroid determination is “versatile” and adaptable to different situations and conditions to be tested, providing a useful and practical tool for long-term steroid monitoring and chronic stress research.

7. REFERENCES

Arnott, G., Ferris, C.P., O'Connell, N.E., 2016. Review: welfare of dairy cows in continuously housed and pasture-based production systems. Animal 1–13. doi:10.1017/S1751731116001336

Biancucci, A., Sbaragli, T., Comin, A., Sylla, L., Monaci, M., Peric, T., Stradaioli, G., 2016. Reducing treatments in cattle superovulation protocols by combining a pituitary extract with a 5% hyaluronan solution: Is it able to diminish activation of the hypothalamic pituitary adrenal axis compared to the traditional protocol? Theriogenology 85, 914–921. doi:10.1016/j.theriogenology.2015.10.041

Boe, K.E., Gry, F., 2003. Grouping and social preferences in calves , heifers and cows. Appl. Anim. Behav. Sci. 80, 175–190.

Bruckmaier, R.M., 2005. Normal and disturbed milk ejection in dairy cows. Domest. Anim. Endocrinol. 29, 268–273. doi:10.1016/j.domaniend.2005.02.023

Buchanan, K.L., Goldsmith, A.R., 2004. Noninvasive endocrine data for behavioural studies: the importance of validation. Anim. Behav. 67, 183–185. doi:10.1016/j.anbehav.2003.09.002

Burnett, T.A., Madureira, A.M.L., Silper, B.F., Tahmasbi, A., Nadalin, A., Veira, D.M., Cerri, R.L.A., 2015. Relationship of concentrations of cortisol in hair with health, biomarkers in blood, and reproductive status in dairy cows. J. Dairy Sci. 98, 1–13. doi:10.3168/jds.2014-8871

Burnett, T.A., Madureira, A.M.L.L., Silper, B.F., Nadalin, A., Tahmasbi, A., Veira, D.M., Cerri, R.L.A., 2014. Short communication: Factors affecting hair cortisol concentrations in lactating dairy cows. J. Dairy Sci. 97, 7685–7690. doi:10.3168/jds.2014-8444

Cerri, R.L.A., Tabmasbi, A.M., Veira, D.M., 2012. Hair cortisol concentrations -Influence of color and location in Holstein cows. J. Dairy Sci. 95, S574.

Charmandari, E., Tsigos, C., Chrousos, G., 2005. Endocrinology of the stress response. Annu. Rev. Physiol. 67, 259–84. doi:10.1146/annurev.physiol.67.040403.120816

Chebel, R.C., Silva, P.R.B., Endres, M.I., Ballou, M. a., Luchterhand, K.L., 2016. Social stressors and their effects on immunity and health of periparturient dairy cows. J. Dairy Sci. 1–12. doi:10.3168/jds.2015-10369

Chrousos, G.P., 2009. Stress and disorders of the stress system. Nat. Rev. Endocrinol. 5, 374–381. doi:10.1038/nrendo.2009.106

Cockrem, J.F., 2013. Individual variation in glucocorticoid stress responses in animals. Gen. Comp. Endocrinol. 181, 45–58. doi:10.1016/j.ygcen.2012.11.025

Comin, A., Peric, T., Corazzin, M., Veronesi, M.C.C., Meloni, T., Zufferli, V., Cornacchia, G., Prandi, A., 2013. Hair cortisol as a marker of hypothalamic-pituitary-adrenal axis activation in Friesian dairy cows clinically or physiologically compromised. Livest. Sci. 152, 36–41. doi:10.1016/j.livsci.2012.11.021

Comin, A., Prandi, A., Peric, T., Corazzin, M., Dovier, S., Bovolenta, S., 2011. Hair cortisol levels in dairy cows from winter housing to summer highland grazing. Livest. Sci. 138, 69–73. doi:10.1016/j.livsci.2010.12.009

Comin, A., Tidu, L., Cornacchia, G., Cappa, A., Renaville, B., Prandi, A., 2008. Neonatal period and hair cortisol in cattle as a marker of stress., in: XVI Congress of the Mediterranean Federation for Health and Production of Ruminants (FeMeSPrum). 23-26 april, Zadar, Croatia, pp. 221–225.

Crowe, M.A., 2008. Resumption of ovarian cyclicity in post-partum beef and dairy cows. Reprod. Domest. Anim. 43, 20–28. doi:10.1111/j.1439-0531.2008.01210.x

D'Anna-Hernandez, K.L., Ross, R.G., Natvig, C.L., Laudenslager, M.L., 2011. Hair cortisol levels as a retrospective marker of hypothalamic-pituitary axis activity throughout pregnancy: Comparison to salivary cortisol. Physiol. Behav. 104, 348–353. doi:10.1016/j.physbeh.2011.02.041

Davenport, M.D., Tiefenbacher, S., Lutz, C.K., Novak, M.A., Meyer, J.S., 2006. Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. Gen. Comp. Endocrinol. 147, 255–261. doi:10.1016/j.ygcen.2006.01.005

Duncan, I.J.H., 2005. Science-based assessment of animal welfare: farm animals. Rev. Sci. Tech. 24, 483–92.

Echternkamp, S.E., 1984. Relationship between LH and cortisol in acutely stressed beef cows. Theriogenology 22, 305–311. doi:10.1016/0093-691X(84)90487-4

Forde, N., Carter, F., Fair, T., Crowe, M. a., Evans, a. C.O., Spencer, T.E., Bazer, F.W., McBride, R., Boland, M.P., O'Gaora, P., Lonergan, P., Roche, J.F., 2009. Progesterone-Regulated Changes in Endometrial Gene Expression Contribute to Advanced Conceptus Development in Cattle. Biol. Reprod. 81, 784–794. doi:10.1095/biolreprod.108.074336

Forslund, K.B., Ljungvall, O. a, Jones, B. V, 2010. Low cortisol levels in blood from dairy cows with ketosis: a field study. Acta Vet. Scand. 52, 31. doi:10.1186/1751-0147-52-31

Fukasawa, M., Tsukada, H., Kosako, T., Yamada, A., 2008. Effect of lactation stage, season and parity on milk cortisol concentration in Holstein cows. Livest. Sci. 113, 280–284. doi:10.1016/j.livsci.2007.05.020

Galvão, K.N., Flaminio, M.J.B.F., Brittin, S.B., Sper, R., Fraga, M., Caixeta, L., Ricci, A., Guard, C.L., Butler, W.R., Gilbert, R.O., 2010. Association between uterine disease and indicators of neutrophil and systemic energy status in lactating Holstein cows. J. Dairy Sci. 93, 2926–37. doi:10.3168/jds.2009-2551

Gleixner, A., Meyer, H.H.D., 1997. Non-invasive monitoring of endocrine function by analysis of progesterone, testosterone and estradiol in hair of adult cattle. Zeitschrift fur Saugetierkd. - Int. J. Mamm. Biol. 62, 71–74.

González-de-la-Vara, M.R.D.R., Valdez, R.A., Lemus-Ramirez, V., Vázquez-Chagoyán, J.C., Villa-Godoy, A., Romano, M.C., 2011. Effects of adrenocorticotropic hormone challenge and age on hair cortisol concentrations in dairy cattle. Can. J. Vet. Res. 75, 216–221.

Gwazdauskas, F.C., Thatcher, W.W., Wilcox, C.J., 1972. Adrenocorticotropin alteration of bovine peripheral plasma concentrations of cortisol, corticosterone, and progesterone. J. Dairy Sci. 55, 1165–1169. doi:10.3168/jds.S0022-0302(72)85640-6

Hall, J.E., 2015. Guyton and Hall textbook of medical physiology, 13th ed. Elsevier Inc., Phyladelphia.

Herrera, A.Y., Nielsen, S.E., Mather, M., 2016. Stress-induced increases in progesterone and cortisol in naturally cycling women. Neurobiol. Stress Accepted manuscript. doi:10.1016/j.ynstr.2016.02.006

Jacobs, J.A., Siegford, J.M., 2012. Invited review: The impact of automatic milking systems on dairy cow management, behavior, health, and welfare. J. Dairy Sci. 95, 2227–47. doi:10.3168/jds.2011-4943

Kirschbaum, C., Tietze, A., Skoluda, N., Dettenborn, L., 2009. Hair as a retrospective calendar of cortisol production—Increased cortisol incorporation into hair in the third trimester of pregnancy. Psychoneuroendocrinology 34, 32–37. doi:10.1016/j.psyneuen.2008.08.024

Koren, L., Mokady, O., Karaskov, T., Klein, J., Koren, G., Geffen, E., 2002. A novel method using hair for determining hormonal levels in wildlife. Anim. Behav. 63, 403–406. doi:10.1006/anbe.2001.1907

Kornmatitsuk, B., Thitaram, C., Kornmatitsuk, S., 2007. Measurement of faecal progesterone metabolites and its application for early screening of open cows post-insemination. Reprod. Domest. Anim. 42, 238–242. doi:10.1111/j.1439-0531.2006.00758.x

Lavon, Y., Leitner, G., Voet, H., Wolfenson, D., 2010. Naturally occurring mastitis effects on timing of ovulation, steroid and gonadotrophic hormone concentrations, and follicular and luteal growth in cows. J. Dairy Sci. 93, 911–921. doi:http://dx.doi.org/10.3168/jds.2009-2112

Liu, X., Chen, F., Guo, D., Song, X., Zhong, Y., 1988. Early pregnancy diagnosis in dairy cows based on hair progesterone analysis. Int. J. Anim. Sci. 3, 123–127.

Maiero, S., Marchini, E., Comin, A., Renaville, B., Prandi, A., 2005. Determination of cortisol in hair as indicator of long-term stress in Simmenthal dairy cows. Reprod. Domest. Anim. 40, 396.

Mann, G.E., Lamming, G.E., 1999. The Influence of Progesterone During Early Pregnancy in Cattle. Reprod. Domest. Anim. 34, 269–274. doi:10.1111/j.1439-0531.1999.tb01250.x

Martin, Y.G., Miller, W.J., Blackmon, D.M., 1969. Wound healing, hair growth and biochemical measures as affected by subnormal protein and energy intake in young cattle. Am. J. Vet. Res. 30, 355–364.

Meyer, J.S., Novak, M.A., 2012. Minireview: Hair cortisol: a novel biomarker of hypothalamic-pituitary-adrenocortical activity. Endocrinology 153, 4120–4127. doi:10.1210/en.2012-1226

Moberg, G.P., Mench, J.A., 2000. The biology of animal stress: basic principles and implications for animal

welfare. CABI, Wallingford. doi:10.1079/9780851993591.0000

Mormède, P., Andanson, S.S., Aupérin, B., Beerda, B., Guémené, D., Malmkvist, J., Manteca, X., Manteuffel, G., Prunet, P., van Reenen, C.G., Richard, S., Veissier, I., Mormede, P., Auperin, B., Guemene, D., 2007. Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. Physiol. Behav. 92, 317–339. doi:10.1016/j.physbeh.2006.12.003

Moya, D., Schwartzkopf-Genswein, K.S., Veira, D.M., 2013. Standardization of a non-invasive methodology to measure cortisol in hair of beef cattle. Livest. Sci. 158, 138–144. doi:10.1016/j.livsci.2013.10.007

Mukasa-Mugerwa, E., Tegegne, A., 1989. Peripheral plasma progesterone concentration in zebu (Bos indicus) cows during pregnancy. Reprod. Nutr. Dev. 29, 303–308. doi:10.1051/rnd:19890308

Palme, R., 2012. Monitoring stress hormone metabolites as a useful, non-invasive tool for welfare assessment in farm animals. Anim. Welf. 21, 331–337. doi:10.7120/09627286.21.3.331

Peric, T., Comin, A., Corazzin, M., Montillo, M., Cappa, A., Campanile, G., Prandi, A., 2013. Short communication: hair cortisol concentrations in Holstein-Friesian and crossbreed F1 heifers. J. Dairy Sci. 96, 3023–7. doi:10.3168/jds.2012-6151

Phillips, C.J.C., 2002. Cattle behaviour and welfare, Second Edi. ed. Blackweel Science Ltd.

Ralph, C.R., Tilbrook, A.J., 2016. INVITED REVIEW : The usefulness of measuring glucocorticoids for assessing animal welfare. J. Anim. Sci. 94, 457–470. doi:10.2527/jas2015-9645

Reading, F., 1999. Stress, management induced, in dairy cattle. Effects on Health and Milk Production, in: STRESS , MANAGEMENT INDUCED , IN DAIRY CATTLE. pp. 2604–2611.

Robinson, R.S., Hammond, a. J., Wathes, D.C., Hunter, M.G., Mann, G.E., 2008. Corpus Luteum-Endometrium-Embryo Interactions in the Dairy Cow: Underlying Mechanisms and Clinical Relevance. Reprod. Domest. Anim. 43, 104–112. doi:10.1111/j.1439-0531.2008.01149.x

Russell, E., Koren, G., Rieder, M., Van Uum, S., 2012. Hair cortisol as a biological marker of chronic stress: Current status, future directions and unanswered questions. Psychoneuroendocrinology 37, 589–601. doi:10.1016/j.psyneuen.2011.09.009

Salaberger, T., Millard, M., Makarem, S. El, Möstl, E., Grünberger, V., Krametter-Frötscher, R., Wittek, T., Palme, R., 2016. Influence of external factors on hair cortisol concentrations. Gen. Comp. Endocrinol. doi:10.1016/j.ygcen.2016.05.005

Sgorlon, S., Fanzago, M., Guiatti, D., Gabai, G., Stradaioli, G., Stefanon, B., 2015. Factors affecting milk cortisol in mid lactating dairy cows. BMC Vet. Res. 1–8. doi:10.1186/s12917-015-0572-9

Sharpley, C.F., McFarlane, J.R., Slominski, A., 2012. Stress-linked cortisol concentrations in hair: what we know and what we need to know. Rev. Neurosci. 23, 111–121. doi:10.1515/RNS.2011.058

Silva, P.R.B., Lobeck-Luchterhand, K.M., Cerri, R.L.A., Haines, D.M., Ballou, M.A., Endres, M.I., Chebel, R.C., 2016. Effects of prepartum stocking density on innate and adaptive leukocyte responses and serum and hair cortisol concentrations. Vet. Immunol. Immunopathol. 169, 39–46. doi:10.1016/j.vetimm.2015.11.007

Slominski, A., Zbytek, B., Nikolakis, G., Manna, P.R., Skobowiat, C., Zmijewski, M., Li, W., Janjetovic, Z., Postlethwaite, A., Zouboulis, C.C., Tuckey, R.C., 2013. Steroidogenesis in the skin: implications for local immune functions. J. Steroid Biochem. Mol. Biol. 137, 107–23. doi:10.1016/j.jsbmb.2013.02.006

Spencer, T.E., 2004a. Conceptus signals for establishment and maintenance of pregnancy. Anim. Reprod. Sci. 15, 1–15. doi:10.1016/S0378-4320(04)00070-3

Spencer, T.E., 2004b. Progesterone and Placental Hormone Actions on the Uterus: Insights from Domestic Animals. Biol. Reprod. 71, 2–10. doi:10.1095/biolreprod.103.024133

Stabenfeldt, G.H., Osburn, B.I., Ewing, L.L., 1970. Peripheral plasma progesterone levels in the cow during pregnancy and parturition. Am. J. Physiol. 218, 571–5.

Stalder, T., Kirschbaum, C., 2012. Analysis of cortisol in hair – State of the art and future directions. Brain. Behav. Immun. 26, 1019–29. doi:10.1016/j.bbi.2012.02.002

Szentléleki, A., Nagy, K., Széplaki, K., Kékesi, K., Tőzsér, J., 2015. Behavioural Responses of Primiparous and Multiparous Dairy Cows to The Milking Process over an Entire Lactation. Ann. Anim. Sci. 15, 185–195. doi:10.2478/aoas-2014-0064

Tallo-Parra, O., Carbajal, A., Monclús, L., Sabés-Alsina, M., Manteca, X., Lopez-Bejar, M., 2016. Progesterone detection in hair from dairy cattle by EIA: protocol validation, in: 20th ESDAR Conference and the 13th AREA Conference. Lisbon, Portugal.

Tallo-Parra, O., Manteca, X., Sabes-Alsina, M., Carbajal, A., Lopez-Bejar, M., 2015. Hair cortisol detection in dairy cattle by using EIA: protocol validation and correlation with faecal cortisol metabolites. Animal 9, 1059–1064. doi:10.1017/S1751731115000294

von Keyserlingk, M.A.G., Rushen, J., de Passillé, A.M., Weary, D.M., 2009. The welfare of dairy cattle—Key concepts and the role of science. J. Dairy Sci. 92, 4101–4111. doi:10.3168/jds.2009-2326

Walker, S.L., Smith, R.F., Jones, D.N., Routly, J.E., Morris, M.J., Dobson, H., 2010. The effect of a chronic stressor, lameness, on detailed sexual behaviour and hormonal profiles in milk and plasma of dairy cattle. Reprod. Domest. Anim. 45, 109–117. doi:10.1111/j.1439-0531.2008.01263.x

Wellnitz, O., Bruckmaier, R.M., 2001. Central and peripheral inhibition of milk ejection. Livest. Prod. Sci. 70, 135–140. doi:10.1016/S0301-6226(01)00206-8

Wiltbank, M.C., Souza, A.H., Carvalho, P.D., Cunha, A.P., Giordano, J.O., Fricke, P.M., Baez, G.M., Diskin, M.G., 2014. Physiological and practical effects of progesterone on reproduction in dairy cattle. Animal 8 Suppl 1, 70–81. doi:10.1017/s1751731114000585

Yoshida, C., Nakao, T., 2005. Response of plasma cortisol and progesterone after ACTH challenge in ovariectomized lactating dairy cows. J. Reprod. Dev. 51, 99–107. doi:10.1262/jrd.51.99

Chapter V

Prediction of cortisol and progesterone concentrations in hair from cow by near infrared reflectance spectroscopy (NIRS)

Prediction of cortisol and progesterone concentrations in hair from cow by near infrared reflectance spectroscopy (NIRS)

O. Tallo-Parra[1,2], E. Albanell[2], A. Carbajal[1], L. Monclús[1], X. Manteca[2], M. Lopez-Bejar[1]

[1] Department of Animal Health and Anatomy, Veterinary Faculty, UniversitatAutònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

[2] Department of Animal and Food Science, Veterinary Faculty, UniversitatAutònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

This chapter has been submitted to *Livestock Science*

1. ABSTRACT

Concentrations of different steroid hormones have been used in cows as a measure of adrenal or gonadal activity and thus, as indicators of stress or reproductive state. Detecting cortisol and progesterone in cow's hair provides a long-term integrative value of retrospective adrenal or gonadal/placental activity, respectively. Current techniques for steroid detection require a hormone-extraction procedure that involves time, several equipments, management of reagents and some assay procedures that are also time-consuming and can destroy the samples. In contrast, near infrared reflectance spectroscopy (NIRS) is a multi-component predictor technique characterized as rapid, non destructive for the sample and reagent free. However, as a predictor technique, NIRS needs to be calibrated and validated for each matrix, hormone and species. The main objective of this study was to evaluate the predictive value of the NIRS technique for hair cortisol and progesterone quantification in cows by usingspecific enzyme immunoassay as the methodology of reference. Hair samples from fifty-two adult Friesian lactating cows from a commercial dairy farm were used. Reflectance spectra of hair samples were determined with NIR spectrophotometer before and after trimming them. Although similar results were obtained, a slightly better relationship between the reference data and NIRS predicted values was found using trimmed samples. Near infrared reflectance spectroscopy demonstrated its ability to predict cortisol and progesterone concentrations with accuracy (R^2 = 0.83 for cortisol and R^2 = 0.89 for progesterone) but with lower validation values (r_{cv}^2= 0.65 and 0.69 respectively). Despite NIRS is far from being a complete alternative to current methodologies, the proposed equations can offer screening capability. Considering the advantages of both fields, our results open the possibility of future work on the combination of hair steroid measurement and NIRS methodology.

2. INTRODUCTION

The concentrations of different steroid hormones have been widely used as a measure of adrenal or gonadal activity. In most mammals including bovine species, glucocorticoid hormones have been linked to the hypothalamus-pituitary-adrenal axis activity and used as an indicator of the stress response (Matteri et al., 2000; Mormède et al., 2007). On the other hand, progesterone has been associated to gonadal/placental activity and pregnancy in females (Spencer et al., 2007; Walsh et al., 2011).

The use of hair as a matrix for the detection of steroid hormones is relatively new in bovine. It has been mainly used in dairy cattle for cortisol analysis (Burnett et al., 2015; Comin et al., 2008; O. Tallo-Parra et al., 2015), the main glucocorticoid in cattle(Mormède et al., 2007). Contrary to the most typical matrixes used (blood, saliva, urine or faeces), hair accumulates hormones during all its growth period. Thus, hair provides a long-term integrative value of retrospective adrenal or gonadal/placental activity, which can comprise a time period from days to months (Meyer and Novak, 2012; Russell et al., 2012; Stalder and Kirschbaum, 2012). Hair steroid concentrations (HSC) are not affected by the proper process of sampling or, if existing, circadian rhythm (Comin et al., 2013; Koren et al., 2002; Russell et al., 2012). Moreover, hair collection is easy, non invasive and, as a keratin matrix, it can be stored at room temperature being stable over time (González-de-la-Vara et al., 2011; Macbeth et al., 2010). Thus, HSC is a potential tool for monitoring long-term steroid hormonal levels, which is usually the main objective when studying chronic stress or the effects of steroid hormones on animal production.

Enzyme immunoassay (EIA) and radio immunoassay (RIA) have been the most used techniques for steroid quantification regardless the matrix used because they are practical and they have a good sensitivity and specificity (Turpeinen and Hämäläinen, 2013). High-performance liquid chromatography (HPLC) has also been used but in less frequency. Both immunoassay techniques and HPLC require a hormone-extraction procedure that involves time, several equipments and the management of reagents. Moreover, the assay procedures are also time-consuming and the samples cannot be further used.

Near infrared reflectance spectroscopy (NIRS) is a multi-component predictor technique characterized as rapid, non destructivefor the sample and reagent free. It is based on the interaction of electromagnetic waves (near infrared radiation) and matter (mainly C-H, N-H, O-H and C-O bonds) (Cen and He, 2007; Foley et al., 1998; Scarff et al., 2006). Because its characteristics, NIRS has the potential to become a useful methodology for hormone quantification but needs to be calibrated and validated for each matrix, compound and species. As a predictor technique, NIRS needs statistical models that test the intensity of the relationship between the mentioned interactions (absorbance) and an independent reference-method or laboratory assay (Deaville and Flinn, 2000; Foley et al., 1998).

Near infrared reflectance spectroscopy was evaluated for the prediction of glucocorticoid metabolites levels in faeces from red deer (*Cervuselaphus*) (Santos et al., 2014) and western lowland gorilla (*Gorilla gorilla gorilla*) (Tallo-Parra et al., 2015a) but no studies have been performed in cows. In contrast, prediction of

progesterone concentrations by NIRS in cows was attempted in plasma (Tolleson et al., 2003) and faeces (Gandy, 2001; Tolleson et al., 2001a, 2001b, 2001c).

The main objective of this study was to evaluate the predictive value of the NIRS technique for hair cortisol and progesterone quantification in cows by using specific EIA as the methodology of reference. To the author's knowledge, this is the first study to analyse and validate the potential of NIRS for hair steroid detection in any species.

3. MATERIAL AND METHODS

3.1. Study design

Reflectance spectra of hair samples cleaned from external sources of steroids were determined with NIR spectrophotometer before and after trimming them. After that, a methanol-based hormone extraction protocol was applied to the trimmed samples and hair cortisol (HCC) and progesterone concentrations (HPC) were detected using two respective EIA kits. The cortisol and progesterone resultant values were used as reference values for NIRS calibration and validation.

3.2. Animals and hair sampling

Hair samples from fifty-two adult Friesian lactating cows (mean age 3.7 years old; 38 % pregnant; Table 1) from a commercial dairy farm located at the north-east of Spain were collected at the same day. Homogenous white-colour hair from the ventrolateral region of the neck was taken (25 samples from the right side, 27 samples from the left side). Hair was collected using an electric hair clipper (Palson® Trading España S.L., Collbató, Spain) as closest to the skin as possible but preventing skin damage and hair follicle removal. A brush was used to clean the peeler blade between animals in order to avoid cross contamination. Hair samples were individually stored into properly identified zip-lock plastic bags in a dark place at room temperature.

Table 1. *Physiological and productive data of the cows used in this study*

	Average	SD	Minimum	Maximum
Age (days)	1324.4	465.8	753	2987
Days in milk	143.7	98.6	11	391
Average milk yield (kg/d)	33.7	11.6	9.7	65.9
Total milk yield (kg)	8839.4	4613.0	110	21821
Days of gestation	77.2	59.2	2	200

SD = Standard deviation.

3.3. Hair washing

In order to remove external sources of steroids and other contaminants, a washing procedure included in a validated protocol for hair cortisol detection in dairy cattle was performed (O. Tallo-Parra et al., 2015). Two hundred and fifty milligrams of hair were weighed from each sample and placed into a 15-ml conical tube. Three washes consisting of 2.5 ml of isopropanol (2-propanol 99.5 %, Scharlab S.L., Sentmenat, Spain) and 2.5 minutes of vortex each one were applied to all the samples. At the end of each washing, the isopropanol was eliminated by decantation. After the third washing, hair samples were left to dry at room temperature.

3.4. Hair trimming and NIRS analysis

Cleaned and dried hair samples were packed into 35 mm diameter circular cups with quartz glass windows and were scanned from 1100 to 2500 nm using a NIRSystems 5000 scanning monochromator (FOSS, Hillerød, Denmark). Reflectance (R) was recorded at 2 nm intervals as log (1/R), resulting in 692 data points for each sample. Each sample was scanned two times, rotating the sample cup 90° between scans. Then, hair samples were trimmed into a < 2 mm length fragments using a ball mill at 22 Hz for 5 minutes (MM200, Retsch, Haan, Germany;10-ml stainless-steel grinding jars; single 12-mm stainless-steel grinding ball). Trimmed hair samples were packed again into the same circular cups but introducing a micro-sample insert, a black ring with a circular slit in the middle that reduced the diameter of the cup up to 18 mm.The use of this diameter-reducer accessory was necessary because the volume of trimmed hair samples decreased in relation with the whole, non-trimmed ones. Trimmed hair samples were also scanned twice with 90° rotation between scans. All trimmed and non-trimmed hair samples were scanned by the same operator.

3.5. Steroid extraction

After NIRS analysis, steroid extraction was done following the validated protocol for hair cortisol detection in dairy cattle by our laboratory (O. Tallo-Parra et al., 2015). Fifty milligrams of trimmed hair previously analysed with NIRS were carefully weighed and placed into a 2-ml eppendorf tube. One and a half millilitre of pure methanol (methanol reagent grade 99.9 %, Scharlab S.L., Sentmenat, Spain) was added and samples were incubated under moderate shaking for 18 hours at 30 ºC (G24 Environmental Incubator Shaker, New Brunswick Scientific CO Inc., Edison, NJ, USA). After incubation, extracted samples were centrifuged at 7000 × g for 2 minutes at 25 ºC and 0.75 ml supernatant was transferred into a new 2-ml eppendorf tube. The supernatant was then placed in an oven (Heraeus model T6; Kendro® Laboratory Products, Langenselbold, Germany) at 38 ºC in order to evaporate the methanol. Once the methanol was completely evaporated, the dried extracts were reconstituted with 0.25 ml of buffer provided by the EIA kit and shaken for 30 seconds. Finally, hormone extracts were stored at -20 ºC until analysis.

3.6. Steroid determination by EIA

Cortisol and progesterone concentrations from hair extracts were determined using a cortisol and a progesterone EIA detection kit, respectively (Neogen® Corporation Europe, Ayr, UK). Preliminary results obtained in our laboratory showed high concentrations of progesterone in the original hormone extracts. Thus, a 1:4 dilution was applied only for progesterone detection in order to analyse concentrations within the optimal detection range of the progesterone EIA kit. The hair steroid extraction protocol and analyses were previously validated for the same species, matrix and EIA kit for both cortisol (O. Tallo-Parra et al., 2015) and progesterone(Tallo-Parra et al., 2016). Intra-assay Coefficients of variation (CV) were calculated in order to assess precision within the used EIA plates (4.83 % for cortisol and 8.64 % for progesterone). The descriptive statistics for cortisol and progesterone concentrations of cow hair samples obtained by EIA and used as a reference values are shown in Table 2.

Table 2.*Reference values of cortisol and progesterone concentrations in cow hair samples determined by enzyme immunoassay*

	N	Average	SD	Minimum	Maximum
Cortisol (pg/mg)	52	4.48	1.47	1.66	8.65
Progesterone (ng/mg)	52	1.019	0.407	0.356	2.162

N = number of samples used for NIRS calibration; SD = Standard deviation.

3.7. Spectral data analysis

A WinISI III (v. 1.6) software program was employed for spectra data analysis and development of chemometric models. Prior to calibration, log 1/R spectra were corrected for the effects of scatter using the standard normal variate (SNV), detrend (DT) and multiple scatter correction (MSC) and transformed into first or second derivative using different gap size (nm) and smoothing interval. For each sample, the mean of the spectra from the two lectures were used. Modified partial least square (MPLS) was the regression method used for calibration development and cross-validation was applied to optimize calibration models and to detect outliers. The optimum calibration model was selected on the basis of minimum standard error of calibration (SEC) and of greatest coefficient of determination of calibration (R^2) and cross validation (r_{cv}^2). These coefficients were used as indicators of precision. Further, performance of calibrations was evaluated using the ratio of performance to deviation (RPD) described as the ratio of standard deviation for the validation samples to the standard error of prediction (SEP), and the range error ratio (RER) described as the ratio of the range in the reference data (validation set) to the SEP (Williams, 2014; Williams and Sobering, 1996).

3.8. Data analysis

Data were analysed using SAS software analysis (Statistical Analysis System, version V.8; SAS Institute, Cary, NC, USA) and a P-value < 0.05 was considered significant. A Shapiro-Wilk test was carried out to check normality before performing the following analyses. Simple linear regressions (PROC REG in SAS) were performed in order to study the relationship between steroid concentrations predicted by NIRS in trimmed and non-trimmed hair samples. With the aim to evaluate the discriminatory or screening ability of NIRS for the prediction of steroid concentrations, samples were classified into four quartiles according to its steroid concentrations (Q1 < Q2 < Q3 < Q4). Each quartile encompassed 25 % of total samples. These classifications were done separately for cortisol and progesterone concentrations and for each type of sample measurement (EIA and trimmed samples by NIRS). Several contingency analyses were performed to compare the distribution of the data in the quartiles between EIA measurements and trimmed hair predictions.

4. RESULTS

4.1. Description of hair spectrum by NIRS

The average spectra from both trimmed and non-trimmed hair sample are shown in Figure 1. Irrespective of their hormonal status, all hair samples showed local peaks at 1190, 1506, 1696, 1736, 1940, 2056, 2174, 2284, 2348, and 2470 nm. Notice that, the raw spectra showed increasing absorbance with greater wavelengths and the influence of particle size on the spectra (trimmed vs. non-trimmed) is evident from the distance between the lines.

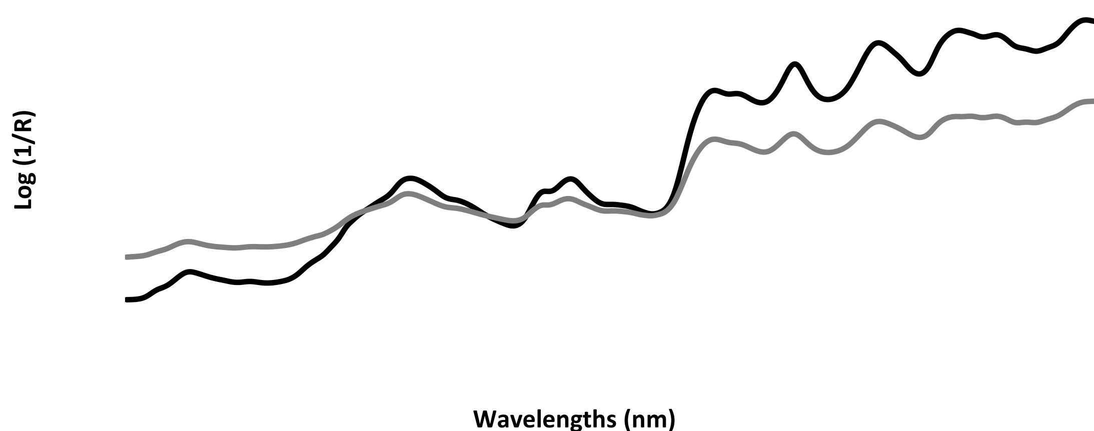


Figure 1. Average spectra from trimmed (grey line) and non-trimmed (black line) hair samples by NIRS (R = reflectance).

4.2. NIRS calibration and validation

The characteristics of the best models obtained for the prediction of steroid concentrations in hair are shown in Table 3. The coefficient of determination obtained by NIRS in this study showed an $R^2 \geq 0.82$ and $r_{cv}^2 \geq 0.63$ for hair cortisol and progesterone prediction. We found similar predictive power with both non-trimmed and trimmed hair calibrations. The R^2, r_{cv}^2, RER and RPD from the trimmed samples were higher except the R^2 for cortisol prediction (Table 3). The analysis of the linear relationship between trimmed and non-trimmed predicted values showed R^2 of 0.77 for cortisol and 0.88 for progesterone (P-value < 0.0001).

Table 3. *NIRS calibration. Spectral pre-treatments and statistics of the best model obtained for predicting the concentrations of cortisol and progesterone in cow hair samples*

	Math [a] treatment	Scatter [b] correction	R^2	SEC	r_{cv}^2	SECV	RER	RPD
Non-trimmed hair samples								
Cortisol	2,4,4,1	MSC	0.84	0.636	0.63	0.910	7.7	1.6
Progesterone	1,10,10,1	MSC	0.82	0.170	0.67	0.230	7.8	1.8
Trimmed hair samples								
Cortisol	2,4,4,1	MSC	0.83	0.605	0.65	0.802	8.7	1.8
Progesterone	1,10,10,1	MSC	0.89	0.138	0.69	0.208	8.7	2.0

[a]Mathematical treatment: derivative order, gap, first smoothing, second smoothing.
[b]Spectra correction algorithm: MSC = multiplicative scatter correction.
R^2 = coefficient of determination of calibration; SEC = standard error of calibration; r_{cv}^2 = coefficient of determination of cross validation; SECV = standard error of cross-validation; RER = range error ratio (Max - Min/SECV); RPD = ratio of performance deviation (SD/SECV).

The relationships between reference values assessed by EIA and predictive values obtained by NIRS of trimmed samples for HCC and HPC are shown in Figure 2.

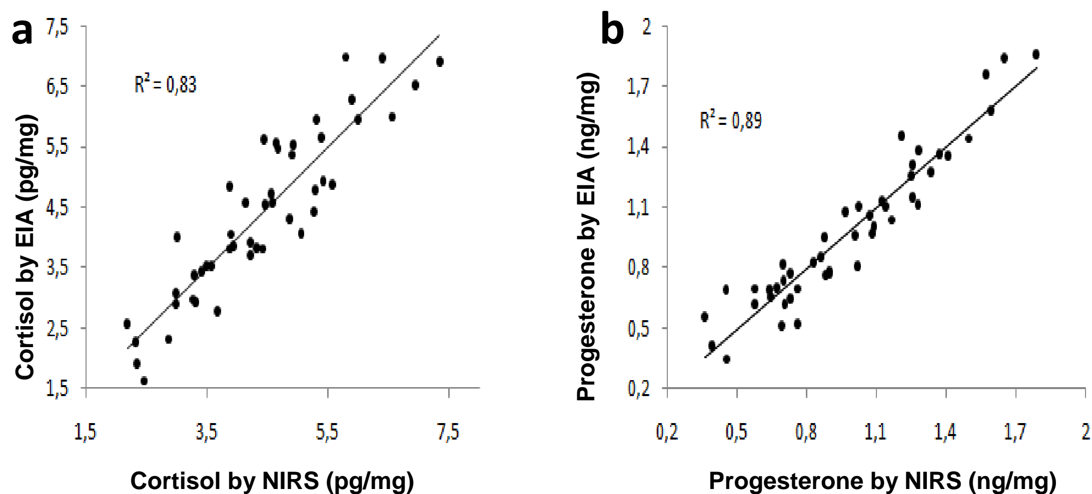


Figure 2. Linear relationship between near infrared reflectance spectroscopy (NIRS) predicted data and chemical reference data, determined by EIA, for cortisol (a) and progesterone (b) concentrations in trimmed cow hair samples.

4.3. Comparison of HSC and quartile distribution between EIA analyses and NIRS prediction in trimmed hair

Contingency analysis evaluated the discriminatory or screening ability of NIRS for the prediction of steroid concentrations. The coincidences and differences in quartile classification of data between the reference method (EIA) and NIRS are shown for hair cortisol (Table 4) and progesterone values (Table 5). The success rate of NIRS for classifying samples in the right quartile was greater in samples with extreme concentrations (Q1 and Q4) than in medium concentrations (Q2 and Q3) for both cortisol and progesterone prediction.

Table 4. *Relationship of data distribution in quartiles between a reference method (EIA) and trimmed NIRS predictions for hair cortisol concentrations*

% Col/Row (n)	REFERENCE EIA Q1	REFERENCE EIA Q2	REFERENCE EIA Q3	REFERENCE EIA Q4
NIRS Q1	83.3 (10)	16.7 (2)	0.00 (0)	0.00 (0)
NIRS Q2	16.7 (2)	66.6 (8)	16.7 (2)	0.00 (0)
NIRS Q3	0.00 (0)	16.7 (2)	58.3 (7)	25.0 (3)
NIRS Q4	0.00 (0)	0.00 (0)	25.0 (3)	75.0 (9)

Q1, Q2, Q3 and Q4 = quartile ascendant classification considering hair cortisol concentrations, encompassing 25 % of total data each one.
EIA = enzyme immunoassay.
n = number of samples.

Table 5. *Relationship of data distribution in quartiles between a reference method (EIA) and trimmed NIRS predictions for hair progesterone concentrations*

% Col/Row (n)	REFERENCE EIA Q1	REFERENCE EIA Q2	REFERENCE EIA Q3	REFERENCE EIA Q4
NIRS Q1	75.0 (9)	25.0 (3)	0.00 (0)	0.00 (0)
NIRS Q2	25.0 (3)	66.7 (8)	8.3 (1)	0.00 (0)
NIRS Q3	0.00 (0)	8.3 (1)	75.0 (9)	16.7 (2)
NIRS Q4	0.00 (0)	0.00 (0)	16.7 (2)	83.3 (10)

Q1, Q2, Q3 and Q4 = quartile ascendant classification considering hair progesterone c oncentrations, encompassing 25 % of total data each one.
EIA = enzyme immunoassay.
n = number of samples.

5. DISCUSSION

This study describes for the first time the use of NIRS for hair steroid hormone prediction in cattle. The NIR spectra showed peaks at 1190, 1506, 1696, 1736, 2056, 2174, 2284, 2348, and 2470 nm, which can be ascribed to the absorption of functional groups related to proteins and absorptions at 1940 nm could arise from functional groups related to water (Osborne and Fearn, 1986). These absorptions are similar to those reported previously by Andueza et al. (2014), where from blood plasma, NIRS can discriminate between pregnant and non-pregnant ewes by detecting presumptively progesterone (*Ovisaries*). In addition to chemical features of a sample, physical attributes, notably particle size, also affect NIR spectra by creating scatter. Scatter is the dispersion of reflected light from the surface of sample particles without penetrating the sample, and can be a significant portion of the observed variation in NIR spectra (Stuth et al., 2003). Thus, the spectra of trimmed and non-trimmed hair samples presented the expected differences associated to the particle size of hair: in trimmed hair samples, the log (1/R) signal has its intensity reduced as a consequence of the increasing specular reflectance due to the reduction of the size of sample fragments (Aucott et al., 1988; Pasquini, 2003; Pellicer and Bravo, 2011).

The data set, used as a reference data for NIRS calibration and validation, included samples of Friesian lactating cows with widely range of age, pregnant and non-pregnant status and different days in milk. The characteristics of the set of animals used for calibration are in concordance with the profile of a dairy cows herd from a typical intensive milking farm in northeast of Spain (DARP, 2008; MAGRAMA, 2014). For cortisol prediction, the spectrum pre-treatment of the best model was the second derivative, combined with MSC. Santos *et al.* (2014) also concluded that this math treatment was the best for the prediction of faecal glucocorticoid metabolites in red deer. For progesterone prediction, in contrast, the spectrum pre-treatment of the best model was the first derivative with MSC, which was also used in previous studies detecting progesterone in plasma from sheep (Andueza et al., 2014).

Although the validity and capacity of steroid hormones' prediction by NIRS are very similar for both trimmed and non-trimmed hair samples, minor differences were detected between these two types of hair formulations. A slightly better relationship between the reference data and NIRS predicted values was found when trimmed samples were used for both cortisol (R^2 = 0.8; r_{cv}^2 = 0.7;RD = 1.8) and progesterone (R^2 = 0.9; r_{cv}^2 = 0.7;RD = 2.0) and could be considered adequate for screening. The values obtained by NIRS in this study for cortisol and progesterone prediction (Table 3) demonstrated the ability of the model to predict steroid concentrations (Fig. 2 and 3). However, the r_{cv}^2 values were much lower than the R^2. The cross-validation involves sequentially withholding samples from the calibration set and using the withdrawn samples to validate the developed model (Walker and Tolleson, 2010). Moreover, it is generally accepted that RER should be greater than 10 and RPD greater than 3 for being considered an acceptable equation (Williams and Sobering, 1996). Thus, NIRS methodology through these equations seems not to be able to replace current hormone-quantification methodologies for the prediction of both cortisol and progesterone concentration in cows' hair. However, NIRS is able to predict steroid concentrations in hair with an acceptable accuracyfor screening, considering that the predictive performance decrease with materials that are more complex (Andrés et al., 2005; Williams, 2010). Our calibration and validation results are relatively

similar with other studies that aimed to predict steroid concentrations (progestagens, estrogens or glucocorticoids) from complex matrixes like faeces or urine by NIRS (Gandy, 2001; Kinoshita et al., 2015, 2012; Santos et al., 2014; O Tallo-Parra et al., 2015; Tolleson et al., 2001b). Increasing the number of samples in the calibration set may achieve more robust calibration as predictive equations would be less affected by the number of samples withdrawn for the validation. The use of an independent set of samples for external validation should also be beneficial for a better validation of NIRS equations and thus, a better assessment of the NIRS methodology for the prediction of HCC and HPC. Moreover, it is important to consider that limitations and errors present in EIA analyses (used as a reference method) like cross-reactivity or the intra-assay variability were associated to NIRS and affected negatively the validation results. The use of more precise and accurate methodologies as the reference could also be useful.

Our results are promising because NIRS is able to discriminate between high and low values for both cortisol and progesterone in hair, especially when considering that NIRS technology is still being explored, developed and improved. The contingency analysis (Tables 4 and 5) revealed that, when classifying HCC and HPC into quartiles, NIRS classify most of the samples at same quartile as EIA. Samples classified by NIRS in a different quartile were always categorised in a contiguous one. This discriminatory ability of NIRS equations for the prediction of steroid concentrations has been used in previous research. For example, some studies aimed to develop NIRS as a tool for the diagnosis of oestrus in urine (Kinoshita et al., 2015, 2012, 2010) or gender or pregnancy in faeces (for review, see Dixon and Coates, 2009; Wiedower et al., 2012) by detecting high levels of specific steroids. However, diet has been considered an important factor affecting faecal NIRS steroid prediction (Dixon and Coates, 2009; Wiedower et al., 2012). Hair accumulates hormones more chronically and its growth and composition are probably less affected by diet and other factors than faeces. Thus, our results encourage future studies with the aim to evaluate if progesterone detection in hair improves the precocity or the reliability of pregnancy diagnosis by NIRS.

In terms of using HCC as an indicator of chronic stress in cows, the advantages of cortisol quantifications associated to NIRS technique could potentially amplify and facilitate its use in stress and welfare research or farm assessment. For example, the speed and relative low cost of NIRS could make feasible a routine control of cortisol levels with the aim to detect individuals or herds with elevated HCC.

6. CONCLUSIONS

This study evaluates for the first time the ability of NIRS to predict the concentrations of cortisol and progesterone in cow hair. Although NIRS can perform it with certain accuracy, it is far from being a complete alternative to current methodologies. However, taking in consideration the advantages of hair steroid measurement and NIRS methodology, our results open the possibility of future work on the combination of these two promising fields.

7. REFERENCES

Andrés, S., Calleja, A., López, S., González, J.S., Rodríguez, P.L., Giráldez, F.J., 2005. Prediction of gas production kinetic parameters of forages by chemical composition and near infrared reflectance spectroscopy. Anim. Feed Sci. Technol. 123–124 Pa, 487–499. doi:10.1016/j.anifeedsci.2005.04.043

Andueza, D., Alabart, J.L., Lahoz, B., Muñoz, F., Folch, J., 2014. Early pregnancy diagnosis in sheep using near-infrared spectroscopy on blood plasma. Theriogenology 81, 509–13. doi:10.1016/j.theriogenology.2013.10.016

Aucott, L.S., Garthwaite, P.H., Buckland, S.T., 1988. Transformations to reduce the effect of particle size in near-infrared spectra. Analyst 113, 1849–1854. doi:10.1039/an9881301849

Burnett, T.A., Madureira, A.M.L., Silper, B.F., Tahmasbi, A., Nadalin, A., Veira, D.M., Cerri, R.L.A., 2015. Relationship of concentrations of cortisol in hair with health, biomarkers in blood, and reproductive status in dairy cows. J. Dairy Sci. 98, 1–13. doi:10.3168/jds.2014-8871

Cen, H., He, Y., 2007. Theory and application of near infrared reflectance spectroscopy in determination of food quality. Trends Food Sci. Technol. 18, 72–83. doi:10.1016/j.tifs.2006.09.003

Comin, A., Peric, T., Corazzin, M., Veronesi, M.C.C., Meloni, T., Zufferli, V., Cornacchia, G., Prandi, A., 2013. Hair cortisol as a marker of hypothalamic-pituitary-adrenal axis activation in Friesian dairy cows clinically or physiologically compromised. Livest. Sci. 152, 36–41. doi:10.1016/j.livsci.2012.11.021

Comin, A., Tidu, L., Cornacchia, G., Cappa, A., Renaville, B., Prandi, A., 2008. Neonatal period and hair cortisol in cattle as a marker of stress., in: XVI Congress of the Mediterranean Federation for Health and Production of Ruminants (FeMeSPrum). 23-26 april, Zadar, Croatia, pp. 221–225.

DARP, 2008. Les explotacions de vaques de llet a catalunya. Doss. Tècnic. Núm. 33 24.

Deaville, E., Flinn, P., 2000. Near-infrared (NIR) spectroscopy: an alternative approach for the estimation of forage quality and voluntary intake, Forage evaluation in ruminant nutrition. doi:10.1079/9780851993447.0301

Dixon, R., Coates, D., 2009. Review: Near infrared spectroscopy of faeces to evaluate the nutrition and physiology of herbivores. J. Near Infrared Spectrosc. 17, 1–31. doi:10.1255/jnirs.822

Foley, W.J., McIlwee, A., Lawler, I., Aragones, L., Woolnough, A.P., Berding, N., 1998. Ecological applications of near infrared reflectance spectroscopy - A tool for rapid, cost-effective prediction of the composition of plant and animal tissues and aspects of animal performance. Oecologia. doi:10.1007/s004420050591

Gandy, B.S., 2001. An evaluation of the early conception factor (ECF) test and near infrared reflectance spectroscopy (NIRS) for determining pregnancy status in dairy cattle. Tesis. Mississippi State University. doi:10.16953/deusbed.74839

González-de-la-Vara, M.R.D.R., Valdez, R.A., Lemus-Ramirez, V., Vázquez-Chagoyán, J.C., Villa-Godoy, A., Romano, M.C., 2011. Effects of adrenocorticotropic hormone challenge and age on hair cortisol concentrations in dairy cattle. Can. J. Vet. Res. 75, 216–221.

Kinoshita, K., Kuze, N., Kobayashi, T., Miyakawa, E., Narita, H., Inoue-Murayama, M., Idani, G., Tsenkova, R., 2015. Detection of urinary estrogen conjugates and creatinine using near infrared spectroscopy in Bornean orangutans (Pongo Pygmaeus). Primates. doi:10.1007/s10329-015-0501-3

Kinoshita, K., Miyazaki, M., Morita, H., Vassileva, M., Tang, C., Li, D., Ishikawa, O., Kusunoki, H., Tsenkova, R., 2012. Spectral pattern of urinary water as a biomarker of estrus in the giant panda. Sci. Rep. 2, 856. doi:10.1038/srep00856

Kinoshita, K., Morita, H., Miyazaki, M., Hama, N., Kanemitsu, H., Kawakami, H., Wang, P., Ishikawa, O., Kusunoki, H., Tsenkova, R., 2010. Near infrared spectroscopy of urine proves useful for estimating ovulation in giant panda (Ailuropoda melanoleuca). Anal. Methods 2, 1671. doi:10.1039/c0ay00333f

Koren, L., Mokady, O., Karaskov, T., Klein, J., Koren, G., Geffen, E., 2002. A novel method using hair for determining hormonal levels in wildlife. Anim. Behav. 63, 403–406. doi:10.1006/anbe.2001.1907

Macbeth, B.J., Cattet, M.R.L.R.L., Stenhouse, G.B., Gibeau, M.L., Janz, D.M., 2010. Hair cortisol concentration as a noninvasive measure of long-term stress in free-ranging grizzly bears (Ursus arctos): Considerations with implications for other wildlife. Can. J. Zool. 88, 935–949. doi:10.1139/Z10-057

MAGRAMA, 2014. Resultados técnico-económicos del Ganado Vacuno de leche en 2014.

Matteri, R., Carroll, J., Dyer, C., 2000. Neuroendocrine responses to stress, in: Moberg, G., Mench, J. (Eds.), The Biology of Animal Stress: Basic Principles and Implications for Animal Welfare. CABI Publishing, pp. 43–76.

Meyer, J.S., Novak, M.A., 2012. Minireview: Hair cortisol: a novel biomarker of hypothalamic-pituitary-adrenocortical activity. Endocrinology 153, 4120–4127. doi:10.1210/en.2012-1226

Mormède, P., Andanson, S.S., Aupérin, B., Beerda, B., Guémené, D., Malmkvist, J., Manteca, X., Manteuffel, G., Prunet, P., van Reenen, C.G., Richard, S., Veissier, I., Mormede, P., Auperin, B., Guemene, D., 2007. Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. Physiol. Behav. 92, 317–339. doi:10.1016/j.physbeh.2006.12.003

Osborne, B.G., Fearn, T., 1986. Near infrared spectroscopy in food analysis. Longman Scientific & Technical, Harlow, Essex, U.K.

Pasquini, C., 2003. Near infrared spectroscopy: Fundamentals, practical aspects and analytical applications. J. Braz. Chem. Soc. 14, 198–219. doi:10.1590/S0103-50532003000200006

Pellicer, A., Bravo, M.D.C., 2011. Near-infrared spectroscopy: A methodology-focused review. Semin. Fetal Neonatal Med. 16, 42–49. doi:10.1016/j.siny.2010.05.003

Russell, E., Koren, G., Rieder, M., Van Uum, S., 2012. Hair cortisol as a biological marker of chronic stress: Current status, future directions and unanswered questions. Psychoneuroendocrinology 37, 589–601. doi:10.1016/j.psyneuen.2011.09.009

Santos, J.P. V, Vicente, J., Villamuelas, M., Albanell, E., Serrano, E., Carvalho, J., Fonseca, C., Gortázar, C., López-Olvera, J.R., 2014. Near infrared reflectance spectroscopy (NIRS) for predicting glucocorticoid metabolites in lyophilised and oven-dried faeces of red deer. Ecol. Indic. 45, 522–528. doi:10.1016/j.ecolind.2014.05.021

Scarff, M., Arnold, S.A., Harvey, L.M., McNeil, B., 2006. Near Infrared Spectroscopy for bioprocess monitoring and control: Current status and future trends. Crit. Rev. Biotechnol. 26, 17–39. doi:Doi 10.1080/07388550500513677

Spencer, T.E., Johnson, G.A., Bazer, F.W., Burghardt, R.C., Palmarini, M., 2007. Pregnancy recognition and conceptus implantation in domestic ruminants: Roles of progesterone, interferons and endogenous retroviruses. Reprod. Fertil. Dev. 19, 65–78. doi:10.1071/RD06102

Stalder, T., Kirschbaum, C., 2012. Analysis of cortisol in hair – State of the art and future directions. Brain. Behav. Immun. 26, 1019–29. doi:10.1016/j.bbi.2012.02.002

Stuth, J., Jama, A., Tolleson, D., 2003. Direct and indirect means of predicting forage quality through near infrared reflectance spectroscopy. F. Crop. Res. 84, 45–56. doi:10.1016/S0378-4290(03)00140-0

Tallo-Parra, O., Albanell, E., Carbajal, A., Monclús, L., Sabes-Alsina, M., Riba, C., Martin, M., Abelló, M., Lopez-Bejar, M., 2015. Prediction of faecal cortisol metabolites from western lowland gorilla (Gorilla gorilla gorilla) by near infrared reflectance spectroscopy (NIRS), in: Proceedings of the 5th ISWE CONFERENCE. Berlin, Germany, p. 61.

Tallo-Parra, O., Carbajal, A., Monclús, L., Sabés-Alsina, M., Manteca, X., Lopez-Bejar, M., 2016. Progesterone detection in hair from dairy cattle by EIA: protocol validation, in: 20th ESDAR Conference and the 13th AREA Conference. Lisbon, Portugal.

Tallo-Parra, O., Manteca, X., Sabes-Alsina, M., Carbajal, A., Lopez-Bejar, M., 2015. Hair cortisol detection in dairy cattle by using EIA: protocol validation and correlation with faecal cortisol metabolites. Animal 9, 1059–1064. doi:10.1017/S1751731115000294

Tolleson, D., Rabbe, D., Randel, R., Stuth, J., Busch, K., 2003. Prediction of blood plasma progesterone via near infrared transmittance spectroscopy. J. Anim. Sci. 81, 16.

Tolleson, D., Randel, R., Stuth, J., 2001a. Detection of pregnancy in cattle using near infrared reflectance spectroscopy of feces. J. Anim. Sci. 79, 21.

Tolleson, D., Willard, S., Gandy, B., Stuth, J., 2001b. Determination of reproductive status in dairy cattle using near infrared reflectance spectroscopy of feces. J. Anim. Sci. 79, 21.

Tolleson, D., Wilson, T., Randel, R., Neuendorff, D., Lewis, A., Stuth, J., 2001c. Near infrared reflectance spectroscopy of feces did not reliably predict serum progesterone (P4) in cows. J. Anim. Sci. 79, 21.

Turpeinen, U., Hämäläinen, E., 2013. Determination of cortisol in serum, saliva and urine. Best Pract. Res. Clin. Endocrinol. Metab. 27, 795–801. doi:10.1016/j.beem.2013.10.008

Walker, J., Tolleson, D., 2010. Shining Light on Manure Improves Livestock and Land Management, First edit. ed, Texas AgriLife Research and Society for Range Management. Texas AgriLife Research and Society for Range Management, Jefferson City, Missouri.

Walsh, S.W., Williams, E.J., Evans, A.C.O., 2011. A review of the causes of poor fertility in high milk producing dairy cows. Anim. Reprod. Sci. 123, 127–38. doi:10.1016/j.anireprosci.2010.12.001

Wiedower, E.E., Kouba, A.J., Vance, C.K., Hansen, R.L., Stuth, J.W., Tolleson, D.R., 2012. Fecal near infrared spectroscopy to discriminate physiological status in giant pandas. PLoS One 7. doi:10.1371/journal.pone.0038908

Williams, P., 2010. The Analysis of Wheat by Near-Infrared Spectroscopy, in: Handbook of Vibrational Spectroscopy. doi:10.1002/0470027320.s8950

Williams, P., 2014. Tutorial: The RPD statistic: a tutorial note. NIR news 25, 22–26. doi:10.1255/nirn.1419

Williams, P.C., Sobering, D.C., 1996. How we do it?: a brief summary of the methods we use in developing near infrared calibrations, in: Davies, A.M.C., Williams, P.C. (Eds.), Infrared Spectroscopy: The Future Waves. NIR Publications, Chichester, pp. 185–188.

GENERAL DISCUSSION

This thesis started with the aim to explore the following hypothesis: the measurement of hair cortisol concentrations can be an indicator of long-term adrenal activity in cattle. Three specific studies (Chapters I, III and V) and a more exploratory one (Chapter IV) were carried out with the aim to confirm or reject this hypothesis. The structure of the following general discussion is based on the specific objectives.

1. Biochemical validation of standardized protocol for hair cortisol detection

The biochemical validation of the protocol for hair cortisol detection was the first step needed to start the present project. For its chemical characteristics, steroids tend to be highly soluble in lower alcohols (such as methanol, the most solvent used in hair steroid extraction), less soluble in higher alcohols (such as isopropanol, used for hair washes) and even less soluble in water (Pötsch and Moeller, 1996). The protocol for hair cortisol quantification is first defined as an alcohol-based extraction protocol as it uses the major affinity of steroid hormones for being solubilised in an alcohol (mainly methanol) than remaining in the hair matrix. Thus, when a hair extract is created for cortisol analysis, the extract is containing not only a proportional amount of cortisol but also a proportional amount of the rest of steroids present in hair. This situation opens the possibility to analyse more than one steroid hormone from the same hair extract.

The extraction protocol applied in this thesis was based on the protocol presented by Davenport et al. (2006) for the analysis of cortisol in hair from rhesus macaques (*Macacamulatta*) with slight modifications. In our protocol, we presented a lower ratio ml isopropanol/mg hair in the wash process (2.5 ml vs. 5 ml of isopropanol), as cow hair needs less isopropanol for each wash to be cleaned from external sources of steroids. The use of less volume of wash solvent reduced the risk of internal steroid losses during the washing procedure(Davenport et al., 2006; Vogliardi et al., 2014). Furthermore, our protocol was simplified because it was not used a stream of nitrogen gas for the evaporation of methanol, what could facilitate hair steroid measurement in laboratories without that equipment. Other studies analysing hair cortisol in cattle applied a protocol based on that described byAccorsi et al. (2008), that was a modified protocol from the described by Koren et al. (2002). Although these protocols do not present any wash step, the remainder

studies analysing hair cortisol in cattle included the wash step presented by Davenport et al. (2006). Overall, all protocols published until now for hair cortisol analysis in cattle were quite similar. They consisted in a wash procedure, a process of hair trimming, alcohol incubation for several hours and the evaporation of methanol and subsequent reconstitution with EIA or RIA buffer.

In cattle, some authors studied factors affecting hair cortisol extraction and analysis. Moya et al. (2013) found differences in hair cortisol concentrations (HCC) between hair samples collected by clipping and plucking suggesting clipping as a better collection method. Cerri et al. (2012) and Burnett et al. (2014) studied the effect of the hair particle size before methanol incubation on final HCC. They found about two times greater cortisol concentrations in hair extracts from hair trimmed with a ball mill in comparison with hair minced with scissors. Our protocol paid special attention to avoid follicle removal and to obtain trimmed hair with small particles as suggested by these studies.

In the present thesis, all studies used the same commercial enzyme immunoassay (EIA) kits (Neogen® Corporation, Ayr, UK) for hormonal detection, including the validation tests. Neogen® anti-cortisol (cortisol-immunoreactive) EIA kits were selected because of their broad assay range of cortisol detection (from 0.04 to 10 mg of cortisol/ml of hair extract) and species immunoreactivity. Furthermore, our laboratory had previous positive experience with this EIA kit and it was also validated for the detection of cortisol metabolites in cattle's faeces in previous works.

Surprisingly, although no specific EIA or radioimmunoassay (RIA) kits for hair cortisol measurements in cattle exist in the market, only Moya et al. (2013) performed a complete biochemical validation of hair cortisol analysis by using EIA. A few other authors performed biochemical validations for cortisol analysis in hair cattle by using RIA (Comin et al., 2013; González-de-la-Vara et al., 2011; Peric et al., 2013).

The test battery presented in this thesis for the evaluation of hair cortisol quantification is one of the most complete validations for hair cortisol analysis in cattle published until now. We performed a test battery with the aim to evaluate precision, accuracy, specificity and sensitivity additionally to the information provided by the EIA manufacturer. We did it for the whole detection range of the assay and with a greater number of dilutions in comparison with other published validations. For example, the number of concentrations used for the detection of parallelism between the standard line and the pool line were five in our study in comparison to other published validations that used three (Comin et al., 2013; González-de-la-Vara et al., 2011; Peric et al., 2013). Moya et al. (2013) performed a deep test battery for hair cortisol analysis in beef cattle but they did not test parallelism. Another example: for the spike-and-recovery test, we tested a total of nine spiked samples while Moya et al. (2013) tested six. Moreover, Gozalez-de-la-Vara et al. (2011) do not mention how the test was performed and Peric et al. (2013) and Comin et al. (2013) did not tested accuracy.

Overall, our validation results for hair cortisol measurement (performed in Chapters I and III) were similar to all those found in above-mentioned literature. Precision tests showed intra-assay coefficient of variation (CV) around 5 % and inter-assay CV around 10%. Coefficients of variation lower than 15 % are considered acceptable precision (Crowther and Walker, 2001; Food and Drug Administration, 2015, 2013, 2011). The accuracy of our assay was acceptable but slightly lower than the assessed by Moya et al. (2013) (89.0 % vs.

101.0 %) with greater standard deviation. However, it improved when samples included in the calculation were exclusively those that encompassed the range of values of hair extracts and not all the range of detection of the kit. Surprisingly, González-de-la-Vara et al. (2011) observed a recovery efficiency of 43.5 %. Parallelism and linearity of dilution were also confirmed with similar results in theliterature. Sensitivity was in all cases much lower than the cortisol concentrations from hair extracts. The cross-reactivity was provided by the assay manufacturers in all studies and some differences were found between manufacturers. Neogen® anti-cortisol EIA kits had the greater cross-reactivity of validated commercial kits although it was acceptable.

Taking together, the validation tests confirmed that hair cortisol detection in cattle through the presented protocol and the commercial Neogen® EIA kit is reliable and can be used for research (CENAM and EMA, 2008; Crowther and Walker, 2001; Food and Drug Administration, 2015, 2013, 2011).

The magnitude of hair cortisol levels detected through our validated protocol in all the studies conducted in this thesis was similar to most studies published analysing HCC in cattle (Burnett et al., 2014; Cerri et al., 2012; Comin et al., 2012, 2011, 2008; Maiero et al., 2005; Moya et al., 2013; Peric et al., 2013) and slightly lower than some others (Burnett et al., 2015; González-de-la-Vara et al., 2011; Silva et al., 2016). Differences in extraction protocols, detection methodologies, age, breed, physiological and welfare status of animals, among others, could explain these differences in HCC detected (Buchanan and Goldsmith, 2004; Moberg and Mench, 2000; Mormède et al., 2007). However, even when comparing studies using the same protocol, the comparison of hormonal levels between studies should be done very carefully. There are no official reference laboratories, methodologies or protocols in hair cortisol detection and several other factors could affect hair cortisol quantification. In immunoassays, of instance, the reader plate type, the commercial kit or even the production lot of the kit could affect hair cortisol measurement, among others. Thus, the experimental design of studies using hair cortisol detection should consider this reality.

When conducting this thesis, the implementation of steroid extraction protocols with non-validated modifications and the use of EIA or RIA kits not validated for the matrix or species analysed has been commonly observed in the literature. These situations have been seen especially in new matrixes like hair. However, validation tests cannot be generalised or extrapolated from one technique to another or after using different extraction protocols (Buchanan and Goldsmith, 2004; Gatti et al., 2009; Stalder and Kirschbaum, 2012; Touma and Palme, 2005). Interestingly, Buchanan and Goldsmith (2004) suggested that the decrease of the publications of new procedure validations might influence authors to not fully appreciate the importance of assay validation procedures. The difficulty of publishing studies validating a protocol and methodology for the detection of one or several hormones in a new matrix and or species has been also experienced by the author of this thesis although the importance of validation is constantly mentioned in international congresses and expert meetings.

2. Feasibility of hair cortisol concentrations to represent an integrative value of long-term adrenal activity

The potential benefits of hair cortisol measurement are several: hair collection is easy, non invasive, non stressful and painless. Moreover, it is safe for both animal sampled and person who collect the hair. However, the main contribution or characteristic of hair cortisol measurement in animal welfare research is their supposed capacity to represent an integrative value of long-term adrenal activity. To date, no other matrix developed for glucocorticoid analysis in cattle or other mammals has the potential to represent global adrenal activity encompassing a frame of time from days to months. The potential of hair cortisol measurement in studies of chronic stress or long-term hormonal levels has been the main cause of its development and increasing interest in welfare and endocrinology research.

This thesis evaluated the feasibility of hair cortisol concentrations to represent an integrative value of long-term adrenal activity through two experiments: 1) the study of the relationship between HCC and faecal cortisol metabolites concentration (FCMC) (Chapter I) and 2) the study of the robustness of HCC against acute and sporadic peaks in serum cortisol concentrations (SCC) (Chapter III). These both experiments could be considered part of the biological validation of hair matrix as they aimed to answer two questions biologically meaningful (Buchanan and Goldsmith, 2004; Touma and Palme, 2005): are HCC related with hypothalamic-pituitary-adrenal (HPA)-axis activity? How is this relationship?

In the experiment presented in Chapter I, cortisol concentrations found in hair from dairy cows correlated well with cortisol metabolites in faeces (r = 0.76). The coefficient of determination obtained in the simple linear regression between HCC and FCMC (R^2 = 0.58) was expected to be greater. However, we associated a value of a continuous cortisol accumulation (one-month old hair) with an average value of punctual samplings (faeces, eight samplings during the same month). It seems reasonable to think that this R^2 would be greater by increasing the number of faecal samples used for monitoring that period. Faeces are a matrix whose concentrations of cortisol metabolites have been widely associated to adrenal activity and thus, HPA axis regulation (Hellhammer et al., 2009; Kirschbaum and Hellhammer, 1994; Mormède et al., 2007; Möstl et al., 2002, 1999; Palme et al., 1999). The relationship between HCC and FCMC detected in our study indirectly associate HCC with HPA-axis activity as shows that the production or accumulation of cortisol in hair is determined or regulated by the same mechanisms as cortisol metabolites accumulation in faeces.

Furthermore, these results are in concordance with the multicompartment model described by Henderson (1993) for hair substance accumulation which hypothesized that the main source of substance in hair shaft comes from the bloodstream. According this model, the amount of cortisol incorporated in hair would be proportional to its concentrations in bloodstream (produced mainly by the adrenal cortex and regulated by the HPA axis). The relationship between HCC and FCMC could be explained as faecal cortisol metabolites have been demonstrated to come also from bloodstream before being metabolised in the liver and excreted by the bile duct (Morrow et al., 2002; Möstl and Palme, 2002; Palme et al., 1996; Taylor, 1971). In this case, the HPA axis would be the main regulatory mechanism for both faecal and hair cortisol and

metabolites concentrations. Overall, our results are in concordance with the literature. Moya et al. (2013) correlated HCC with cortisol concentrations in saliva and faeces from beef cattle with poorer coefficient of correlations, probably because the number of saliva and faecal samples collected was lower than in our experiment (five samplings in a month). Accorsi et al. (2008) found a similar correlation between HCC and FCMC in dogs by performing faecal collection approximately every three days and higher correlation in cats (*Feliscatus*) after sporadic collection of faeces. Bennett and Hayssen(2010) detected correlation between HCC and salivary cortisol levels in dogs with similar coefficients of correlations to Moya et al. (2013). Short et al. (2016) monitored cortisol almost continuously in saliva and urine in order to correlate them with HCC in humans. They found a relationship between hair and salivary cortisol but not with urinary cortisol and concluded that HCC provides a reliable estimate of long-term integrated free cortisol production.

The use of adrenocorticotropic hormone (ACTH)-challenge tests is other way to study the relationship between HPA axis, adrenal activity and cortisol concentrations in a target matrix. It consists in the administration of ACTH hormone with the aim to increase adrenal activity and cortisol production and detect this increase in cortisol concentrations on the target matrix (Kersey and Dehnhard, 2014; Palme, 2005). Some studies were published using ACTH challenges in hair cortisol research in several mammals focusing on the confirmation of the relationship between HPA activity and HCC through ACTH administration (Ashley et al., 2011; González-de-la-Vara et al., 2011; Mastromonaco et al., 2014; Terwissen et al., 2013).Most studies succeed increasing HCC after ACTH injections by designing experiments that increased circulating cortisol concentrations for a long time.

Although we used ACTH administrations, increasing HCC was not the objective of the experiment presented in Chapter III of this thesis. As mentioned above, previous research related HCC and HPA-axis activity. However, the statement that HCC represents an integrative value of long-term retrospective adrenal activity assumed by many authors (e.g. Gow et al., 2010; Meyer and Novak, 2012; Stalder and Kirschbaum, 2012; Steudte-Schmiedgen et al., 2016)needed to be demonstrated. In other words, HCC could be regulated or determined by HPA axis without being able to provide an integrative value of long-term adrenal activity. With the objective to explore it, the influence on HCC of acute and short elevations of SCC that do not represent the overall state of the animal was studied. The experiment performed in Chapter III was motivated by the consideration that hair could not be established as a matrix that represents the global adrenal activity of an animal for a certain period of time if punctual and non-recurrent increases of circulating cortisol concentrations could interfere with its final concentrations. No previous literature was found trying to assess this objective.

In our experiment, the pattern applied for ACTH administration was designed with the aim to alter SCC for a short time (approximately 3 hours after each ACTH injection) and in a frequency considered non-recurrent (two injections in a 14-day period). Overall, the period of increased SCC in injected animals only represented 1.8 % of total time of cortisol incorporation into the hair sampled at the end of the study. No differences were found in HCC between ACTH-injected and control groups demonstrating that HCC from bull calves were not affected by elevations of SCC when these elevations were short lasting and not frequent.

Although our results were the first scientific confirmation reported, the incapacity of a single or few injections of ACTH to alter glucocorticoid concentrations in hair or other cumulative matrixes was previously discussed and assumed (Ashley et al., 2011; Berkvens et al., 2013; Mastromonaco et al., 2014; Terwissen et al., 2013). However, in a point of their experiment, González-de-la-Vara et al. (2011) reported a significant increase of HCC in 14-day-old hair samples from adult pregnant dairy cattle that accumulated cortisol as a response to two ACTH injections with lower doses. This experimental situation was very similar to our experimental design in the study presented at Chapter III because we also performed two ACTH administrations and analysed its impact in 14-day-old hair samples but from bull calves (same breed). Biological differences in HPA-axis regulation has been associated to age, sex or physiological status (Moberg and Mench, 2000; Mormède et al., 2007) and could explain the differences in HCC-reactivity to ACTH between both studies. However, the author has not found literature of hyperreactivity adrenal after ACTH administration in adult cattle. The most possible explanation according the author of this thesis would be that SCC of cows in that study remained high for a long time after each ACTH injection. According Lay et al. (1996) the dose used in the study of González-de-la-Vara et al. (2011) should increase four to five times SCC for less than 3 hours after each administration. If SCC did not remain elevated for a long time, it seems hardly acceptable that after six hours of elevated SCC, cortisol concentrations from 14-day-old hair samples remained four times greater in ACTH-administered group than in the control group. Unfortunately, this contradiction cannot be solved due to the impossibility to know the time that SCC remained high after each ACTH administration and the lack of deep knowledge of biological factors affecting adrenal reactivity to ACTH. Although future studies should focus on it, this situation underlines the importance and difficulties of performing complete biological validations of steroid matrixes, especially for the recently developed ones.

Regarding hair cortisol detection, some confounding factors need to be deeply studied in order to fully understand the relationship between HCC and long-term adrenal activity. The most controversial factors are related with the influence of hair follicle and skin steroidogenesis on final HCC and some studies used it to question hair cortisol capability to represent adrenal activity (Keckeis et al., 2012; Palme, 2012). Other confounding factors to consider when using hair cortisol measurement are discussed in section **5.(Effects of hair secondary characteristics on hair cortisol measurement)**. In the most accepted theory of substance incorporation in hair, the multicompartment of Henderson (1993), cortisol is incorporated into hair mainly via passive diffusion from blood but also by sweat, sebum and from external sources. The hair wash process applied by Davenport et al. (2006) was created with the aim to eliminate part of cortisol not provided from bloodstream. However, some accumulation mechanisms are still incompletely understood (Boumba et al., 2006; Cone, 1996; Henderson, 1993) and the presence of local steroidogenesis in skin has been demonstrated (Slominski et al., 2013, 2014; Taves et al., 2011). In a review, Sharpley et al. (2012) concluded that there are enough data to confirm the existence of a HPA-like axis in hair follicles and that glucocorticoids can be synthesised there. The impact of local glucocorticoid production and its regulation in final HCC is still unknown despite some studies demonstrated the influence of some topic treatments in HCC in humans and other mammals (Salaberger et al., 2016; Sharpley et al., 2009; Wester et al., 2016). The treatments that influenced HCC were the use of topical corticosteroids (Wester et al., 2016), the execution of a cold pressor test consisting in 1 minute of cold (0 to 4 ºC) in contact with skin (Sharpley

et al., 2009) and the application of extensive brushing or dexamethasone fluid (Salaberger et al., 2016). Although more research is needed in this field, the sensitivity or regulation of HCC to local skin incidences should not necessarily be considered a problem in the evaluation of HCC as an indicator of long-term HPA-axis activity. The stressors or treatments that locally influenced HCC are avoidable or controllable in most cases and some studies suggest that its influence in final HCC is marginal(Ouschan et al., 2013; Russell et al., 2012; Stalder and Kirschbaum, 2012). Avoiding hair sampling in anatomical areas with skin submitted to local stressors (skin problems or lesions, receiving local treatments or suffering extensive brushing) could solve the problem. In contrast, sensitivity of HCC to local incidences could open new research lines in the study of HCC as a measure of local inflammation or infection, for instance.

Despite the existence of controversial studies previously mentioned, other evidences support the hypothesis that hair cortisol concentrations can represent an integrative value of long-term adrenal activity. Several studies found a close interrelation between HCC and well-defined conditions that caused changes in HPA axis. In cattle, increases of HCC have been related to health status and reproduction, with cows suffering diseases or in peripartum period having greater HCC (Burnett et al., 2015; Comin et al., 2013, 2008). Diseases and the neonatal period are considered situations that induce the stress-response (Charmandari et al., 2005; Comin et al., 2008; Tsigos and Chrousos, 2002). In other mammals, changes in HCC have been associated with other stressful situations like severe chronic pain (Van Uum et al., 2008), relocation (Comin et al., 2011; Dettmer et al., 2012; Yamanashi et al., 2016), subordinate position in social hierarchy and frequency of aggressions received (Feng et al., 2016; Yamanashi et al., 2013), high population density (Dettmer et al., 2014), decrease in environment quality (Bechshøft et al., 2013) or exposure to stressful and traumatic events (Schreier et al., 2015; Steudte-Schmiedgen et al., 2016), among others. In a study conducted in ewes, GhassemiNejad et al. (2014) concluded that hair (wool) cortisol was a better indicator of stress than blood cortisol in ewes exposed to heat stress and water restriction. Bryan et al. (2013) concluded in dogs that for practitioners and researchers interested in long-term cortisol levels, a single hair sample could be collected rather than multiple samples of saliva or faeces.

Another favourable argument for the capacity of HCC to represent long-term adrenal activity is present in the experiment performed in Chapter IV in which we analysed cortisol but also progesterone from dairy cattle hair. A correlation between HCC and HPC was found. Although it was obviouslyslight (they have different functions and regulations), this relationship was observed previously in blood and could be explained by the secretion of progesterone or progesterone precursors by the adrenal gland and its regulation by HPA axis (Gwazdauskas et al., 1972; Yoshida and Nakao, 2005). Thus, although the main producer of progesterone is the corpus luteum (and placenta during pregnancy) (Spencer, 2004a; Wiltbank et al., 2014), the observed relationship could be an indicator of the influence of HPA-axis in hair steroid accumulation.

Overall, the experiments conducted in this thesis together with data from theliterature seem to confirm the hypothesis that HCC can be representative of long-term adrenal activity. However, the present work was only able to confirm partially this hypothesis as other variables and local factors affecting HCC are still unknown, controversial and should be studied in the future. The potential positive impact of hair cortisol

measurement in welfare research and those consequences on the welfare of cattle and other mammals do definitely worth these efforts.

3. Potential uses of hair cortisol determination in dairy cattle production

Once demonstrated that HCC were able to be correctly analysed through our protocol and to represent long-term adrenal activity, the next step in this thesis was to test its potential uses under commercial conditions (Chapter IV). A commercial dairy farm was chosen as intensive dairy cattle production is one of most potentially animal-welfare threatening of bovine production (Oltenacu and Broom, 2010; von Keyserlingk et al., 2009). The analysis of HCC in a commercial dairy farm provided a wide sample of quite homogeneous animals (all females in the milking period, adult, Holstein-Friesian, etc.) but with different ages, days in milk (DIM), pregnancy status and productive performance. This allowed us to control some variables and study others in a within-herd context, what hindered the extrapolation of our results to other productions or phases, but facilitated a deeper understanding of the potential of hair cortisol quantification in dairy cattle. Previously, some studies analysed the relationship between cortisol (as indicator of adrenal activity) and biological parameters, milk production and milk composition in dairy cattle (e.g. Duncan, 2005; Hasegawa et al., 1997; Palme, 2012; Trevisi et al., 2005). However, they used other matrixes rather than hair for cortisol analysis. In our study, hair analysis allowed us to study the impact of adrenal activity on milk production and composition in a long-term perspective.

The first remarkable point of the results was the high individual variability in HCC. This situation has been commonly observed in cortisol concentrations from all the other matrixes as it is a characteristic of HPA-axis and the stress response (Cockrem, 2013; Moberg and Mench, 2000; Mormède et al., 2007). Although this high individual variability not necessarily demonstrates the relationship between HCC and adrenal activity, it is another favourable argument.

Interestingly, we did not observe any relationships between HCC and the following physiological variables: age, pregnancy status (pregnant vs. non-pregnant) and parturition condition (primiparous vs. multiparous). Our results were apparently in contradiction with the few articles published analysing HCC in dairy cattle in which HCC differences were associated to age (Comin et al., 2008; González-de-la-Vara et al., 2011; Maiero et al., 2005), pregnancy status (Comin et al., 2008) and cow vs. heifer condition(Burnett et al., 2015; Cerri et al., 2012). However, the presumed contradictions of our results could be explained whether the experimental designs and hair-sampling pattern are analysed.

First, in our study the effect of age on HCC were analysed between adult cows while in all the other studies the effect of age was studied comparing HCC between adult cows and heifers or calves. This is the first study demonstrating that age does not affect HCC in adult cows, at least within the range of cow's age in a usual dairy farm under intensive conditions. In cattle and other mammals, animal welfare evaluations and

studies avoid comparisons between production stages or types (adult vs. young, dairy vs. beef, etc.) as the internal (physiology) and external (handling and housing) conditions of the animals are too different. Thus, this discovery is relevant because it discards age as a biological factor affecting HCC in dairy cows. Regarding pregnancy status, Comin et al. (2008) found that cows had greater HCC at third trimester of pregnancy but in our study, there were no pregnant cows in the third trimester of pregnancy. Thus, only cows in the third trimester of pregnancy should deserve special attention when performing comparisons between pregnant and non-pregnant cows in relation with HCC. Finally, the absence of significant differences in HCC between primiparous and multiparous cows could be explained by the differences of moment and time monitored by each hair sample. While our samples encompassed a period of two months and they did not show significant differences, Burnett et al. (2015) collected hair that was in growth for "only" 21 days and they observed greater HCC in multiparous only at 42 and 84 DIM. The differences in hair age could affect also the sensitivity of their hair samples in front changes in HPA-axis. Moreover, the DIM was homogeneous for all their cows (the mentioned 42 and 84 DIM) while it was not in our case (153 ± 96 DIM). This could indicate that there is an effect of previous parturition but only in the early lactation. However, literature is contradictory when using other matrix for cortisol analysis, with some studies observing no differences and others observing greater cortisol levels in primiparouscows (Fukasawa et al., 2008; Galvão et al., 2010; Goff et al., 1989). Future research should be done in order to clarify the impact of parturition in HCC especially because the mixture of primiparous and multiparous cows is the most common situation in dairy herds.

Analysing all this supposed contradictions together, it should be noted the importance to understand the limitations of each experimental design and the need of cautious extrapolations and generalisations. For instance, the effect of age on HCC had a completely different meaning between studies. This caution should be maximised in the case of hair for cortisol analysis because of the possibility to compare hair with different age (accumulating cortisol for different amounts of time) and perhaps different capacity to represent changes in HPA axis. This situation does not happen with the other established matrixes and is deeper discussed in section **5.(Effects of hair secondary characteristics on hair cortisol measurement)**.

Although other studies found an increase of HCC in non healthy cows (Burnett et al., 2015; Comin et al., 2013), our study did not find it. As we were working under commercial conditions, the veterinary treatment was rapidly applied after diagnosing cows with any pathological problem. Consequently, the increase in SCC associated to disease and pain could be short and therefore "diluted" in the two-months-old hair samples. Comin et al. (2013) and Burnett et al. (2015) performed excellent experiments studying the relationship between HCC and health status using a large number of animals and both found that diseased animals had statistically greater HCC. Unfortunately, high HCC variability was also observed in both studies in the two healthy and diseased groups, even when analysing diseased cows separated for pathology type. This degree of intra-group variability would impede the early implementation of HCC as non-specific indicator of cow's health status.

We also tried to evaluate for the first time HCC as a predictor of the disease evolution (cow heals vs. cow is eliminated for medical reasons). We did not found any relationship between HCC and diseased animals

with critical evolution. Not many animals were eliminated during our study and future and deeper studies should be performed. Moreover, several eliminated cows suffered acute pathologies. Perhaps the usefulness of HCC for the prediction of the disease critical evolution should be studied focusing specifically in chronic diseases with possibilities for critical evolution and, once diagnosed, the utility of HCC as a prognostic indicator.

The other main objective of the experiment performed in Chapter IV was studying the relationship between HCC and milk production and composition. Our study found a negative relationship between HCC and milk yield, that was previously described by Burnett et al.(2015). Even with the difference between ourstudies (study design and time monitored by hair samples) and despite the high individual variability, both thestudy from Burnett et al. (2015)and our found that cows with greater HCC produced less amount of milk. Although several hypothesis exist, the influence of elevated cortisol and other endocrine markers on milk production is still equivocal and partially unknown (Reading, 1999). In any case, the importance of stress and HPA-axis activity in milk production seems demonstrated again (von Keyserlingk et al., 2009) with HCC being sensitive to certain stressors that should be identified in future studies.

Interestingly, we found the same relationship between HCC and milk yield when averaged daily milk yield produced from the beginning of the lactation to 124 days after hair sampling. This result suggests that the use of hair representing two months of the milking period could provide an integrative value representative of the general HPA-axis activity of the cow for probably the whole lactation if no unexpected circumstances like a disease happened. This situation may help future research trying to identify mild chronic stressors affecting milk production for the whole lactation as they should be consistent enough in intensity, length and frequency to alter at least two-months HCC.

Finally, the same study found a relationship between somatic cell (SC) count and HCC. As suggested by Sgorlon et al. (2015) using milk cortisol, the slightly relationship between HCC and SC count could be due to an effect of the animals with the higher SC count as high cortisol levels were also present in cows with low SC count. Furthermore, other studies did not found any relationship (Comin et al., 2011; Fukasawa et al., 2008). Hair cortisol concentrations seems to not be useful indicators of somatic cell counts in healthy animals, but future research could focus in the study of the interrelation between HCC and somatic cell count exclusively in cows suffering mastitis.

Overall, the exploratory nature of the experiment presented in Chapter IV can be noted on the number of ideas for future studies that appeared. Evaluate the potential uses of hair cortisol determination in dairy cattle production was one of the specific objectives of this thesis and the relationship between HCC and milk production is an especially interesting discovery. However, the potential uses of HCC in dairy cattle production still need more exploration and the proposed applications should be further studied and validated.

4. Evaluation of the near infrared reflectance spectroscopy technique for hair cortisol quantification in cows

The optimisation of hair cortisol detection is a wide concept that can include, for instance: improving hair extraction protocol with the aim to need less sample amount, solvent, time or equipment or also establishing easier ways to create an own anti-cortisol EIA plate, which would decrease costs. However, in the Chapter V of the present thesis we tried to optimise hair cortisol detection by focusing in the evaluation of near infrared reflectance spectroscopy (NIRS), a completely different technique from immunoassays, for the quantification of steroids in cow's hair. Enzyme immunoassays were developed in 1970s and its impact and use in science is highly remarkable (Lequin, 2005; Skrzipczyk and Verdier, 2013; Stepaniak et al., 2002). However, new methodologies appeared recently with different characteristics and quantification methods. The potential of NIRS and its practical characteristics (extremely rapid analysis, reagent free, non destructive for the sample, safe, capability to quantify multiple analytes in a single analysis, etc.) make NIRS a methodology extremely interesting to be evaluated for hair cortisol analysis. Unfortunately, results of our study demonstrated that the NIRS methodology through the developed equations were not able to replace current hormone-quantification methodologies for the quantification of cortisol levels in cows' hair. The validation parameters established that hair cortisol prediction by NIRS through these equations was only useful for screening. However, our results should be considered encouraging for several reasons.

First, a more robust calibration could be achieved by increasing the number of samples used for validation. Fifty-two hair samples used in our study allowed the creation of good NIRS equations for the prediction of cortisol concentrations in hair. However, the withdrawal of some samples used for the cross-validation affected negatively the validation parameters. Therefore, increasing the number of samples used for calibration or the use of an independent set of samples for an external validation could help to improve calibration and validation results. Some studies validated NIRS equations using a similar number of samples, however, the nature of each population, matrix and analyte defines the number of samples necessary for NIRS calibration and validation (Windham et al., 1989). Increasing the number of samples used would be an option to confirm or improve our results.

Second, our results could also improve if the error of HCC measurement from our reference method diminished. As NIRS is a predictive technique that needs a reference method to be calibrated, the measurement errors from the reference method are carried over to NIRS calibration (Deaville and Flinn, 2000; Foley et al., 1998). In our experiment with NIRS (Chapter V), we used a commercial EIA kit as the methodology of reference. Although the error of measurement associated to EIA methodology and to our laboratory (equipment, personnel, etc.) are easily acceptable in endocrinology research, several actions could be done in order to reduce it: increasing the number of determinations for each hair sample, testing several EIA brands or analysing the same samples in different laboratories are some of them. Furthermore, the use of more accurate and precise methodologies like HPLC could also be beneficial.

Third, NIRS spectroscopy is still being developed, especially in relation to endocrinology and other physiological analysis (Pellicer and Bravo, 2011; Vance et al., 2016). Within the field of analytical

biochemistry, the use of NIRS has been mainly focused on bioprocessing industry (Broad et al., 2001; Scarff et al., 2006), agricultural product analysis and feed quality (Deaville and Flinn, 2000; Pasquini, 2003; Walker and Tolleson, 2010) or food analysis (Cen and He, 2007; Osborne, 2000; Rodriguez-Otero et al., 1997). In contrast, its use in biomedical research or analysis has just started (Pasquini, 2003) and improvements of the NIRS methodology focusing on hormonal analysis are expected in the coming years.

Finally, the confirmation that NIRS methodology can be used for screening in the assessment of HCC opens new possibilities even if validation results would not improve. In our study, when classifying our cows into quartiles by HCC, the 25 % of cows with greater HCC were correctly classified by NIRS into highest HCC quartile (Q4) three out of four times. The rest of samples wrongly classified by NIRS were always categorised in the contiguous quartile (Q3). Thus, NIRS equations were quite good identifying the animals with greater HCC. This capability of NIRS for differentiating at least between cows with high and low HCC could be exploited. With the aim to detect animals with the stress response frequently or permanently activated, the combination of hair (a matrix whose sampling is non-invasive and easy) with NIRS (a fast, safe and reagent-free methodology) could be established and useful. This combination would facilitate a preliminary identification of animals potentially under chronic stress despite the low accuracy of hair cortisol prediction by NIRS. Moreover, portable NIR spectrophotometers exist and have been used successfully for faecal analysis of Giant panda (*Ailuropodamelanoleuca*) in the wild (Vance et al., 2013). Thus, the possibility of performing HCC analysis in the future by portable NIRS through direct contact with cow's coat is theoretically possible.

Hair cortisol detection and NIRS are two fields with great potential and scope for improvement in the medium to long-term. Furthermore, they both share an important common characteristic: potential applicability. This means the possibility to exceed its use in research and to be used for routine welfare assessment in farms, slaughterhouses or others. In fact, on-farm welfare assessment for regulatory purposes seems to be the future and animal-based indicators with feasible on-farm measurements (relevant, reliable, economically affordable, etc.) are the most difficult to obtain (Sørensen and Fraser, 2010).

5. Effects of hair secondary characteristics on hair cortisol measurement

All kind of matrixes used for cortisol detection have their own characteristics and subsequent confounding factors. Hair is not an exception and during the course of the present thesis, some characteristics of hair affecting its cortisol concentrations were identified. The effect of these characteristics needs to be always taken into account as their impact in HCC can be really important. Confounding factors related with hair follicle and skin steroidogenesis have been mentioned in section **2.**of this general discussion (**Feasibility of hair cortisol concentrations to represent an integrative value of long-term adrenal activity**) and will not be discussed again.

In our first experiment (Chapter I), black and white hair samples were collected separately but black hair was collected from two regions (frontal region of head and occipital crest). In sampled cows, most hair from the frontal region (called forehead from now) was white so with the aim to reach a considerable amount of black hair sample, black hair from occipital crest was also collected. Although occipital crest is contiguous to forehead, its hair is longer and thicker. Fortunately, white hair samples were homogeneous regarding anatomical location and the study could be continued. However, this situation spotlights two important characteristics of hair to be aware of when analysing HCC: colour and location.

All cows and calves used in the present thesis were Holstein-Friesian. This breed is typically characterised by having coat with homogeneous areas with white and black colour patterns. Although their colour patterns can also be red and white, all animals used in this thesis were white and black. In our first experiment (Chapter I), HCC were different between white and black hair samples and despite there was a confounding effect between hair colour and sampling location, it seems clear that there exist differences in HCC associated to hair colour in cattle as it has been reported previously in other studies (Burnett et al., 2014; Cerri et al., 2012; González-de-la-Vara et al., 2011). The influence of hair colour in HCC has been also described in dogs (Bennett and Hayssen, 2010). In contrast, differences in HCC between hair colours were not found in grizzly bears (*Ursusarctos*) (Macbeth et al., 2010) nor wolves (*Canis lupus*)(Bryan, 2013). However, in these both studies hair comparisons were done between individuals and not within individuals as performed in cattle and dogs. Furthermore, they created a subjective classification for the range of colouration as samples were not homogeneous. In humans, contradictory results have been published (Rippe et al., 2015; Sauvé et al., 2007) although the effect of dye, other hair treatments and wash frequency could be confounding the real effect of hair colour (Abell et al., 2015; Manenschijn et al., 2011).

The causes why cortisol accumulates differently depending on hair colour are unknown. Some studies performed in hair drug monitoring suggested that greater number of melanocytes and pigmentation favoured the incorporation of lipophilic substances from the bloodstream into the hair (Pötsch et al., 1997; Pragst and Balikova, 2006). Surprisingly, although steroids are lipophilic (Sultan and Raza, 2015), white hair samples showed about two times greater cortisol concentrations than black ones (Bennett and Hayssen, 2010; Burnett et al., 2014; Cerri et al., 2012; González-de-la-Vara et al., 2011). In dogs, Bennett and Haysen (2010) hypothesized two possible causes for the observed differences in HCC depending on hair colour. First, these differences were caused by the demonstrated interrelation between glucocorticoids, stress-response and hair growth inhibition as well as melanocyte development. Second, these differences could be due to a more simple cause: cortisol competes with pigment for hair room and thus, as more pigment is present less cortisol can be accumulated. No studies have proved these two hypotheses. However, the literature used for the first possible explanation (Botchkarev, 2003; Roulin et al., 2008; Slominski, 2004) do not reference differences depending on hair colour and their associations are hardly transferable to the condition of studied dogs.

As introduced previously, HCC are also conditioned by anatomical location of hair (Burnett et al., 2014; Carlitz et al., 2015, 2014; Macbeth et al., 2010; Moya et al., 2013; Sauvé et al., 2007). Unlike influence of hair colour in HCC, the effect of hair location can be less extrapolated and compared from the literature as

not all studies compared hair from same locations. Moreover, species, breed or even individual could potentially vary the characteristics of hair present in each anatomical location.

In our first experiment (Chapter I), hair location has such a degree of influence on HCC that black heterogeneous hair samples had greater HCC than homogeneous white ones despite white hair is supposed to accumulate more cortisol. This contradictory situation could be explained because hair from the occipital crest (longer and thicker) had probably greater cortisol concentrations than hair from forehead. No other studies analysed hair cortisol from occipital crest location but Moya et al. (2013) and Burnett et al. (2014) found the greatest cortisol concentrations in cow's tail switch which is characterised by having the longest and thickest hair type in Holstein-Friesian cow's body.

Like in hair colour, the causes of different cortisol accumulation in hair depending on anatomical location are unknown. Moya et al. (2013) suggested that hair growth rate including growth/rest cycles could explain these differences. In cattle and other mammals, hair from different locations has different length, thickness and growth rate. In our experiment presented in Chapter III, homogeneous white hair samples were collected from forehead and hip in bull calves. In concordance with literature(Burnett et al., 2014; Moya et al., 2013), hair from hip showed greater cortisol concentrations than hair from forehead. However, contrary to what we expected, only low correlations were found between hip and forehead HCC. Although no other studies tried to understand the relationship between hair locations, in relation to HCC, we expected greater correlations. Hair growth rate could cause these differences as hair from hip were longer than hair from forehead at the moment of sampling despite hair from both locations were allowed to grow for the same 14 days. As there exists a delay between hair cortisol accumulation in the follicle and the arrival of this portion of hair at the skin surface, hair collected from two locations could be representing adrenal activity in a slightly different manner due to their different growth rate (LeBeau et al., 2011).

Although not specific research has been done regarding hair growth rate and cortisol accumulation, it seems generally accepted as the main source of HCC variation among anatomical locations in animals (Burnett et al., 2014; Fourie et al., 2016; Macbeth et al., 2010; Moya et al., 2013; Peric et al., 2013; Yamanashi et al., 2013). The studies conducted in cattle showed that hair with the greater growth rate (tail switch) had also the greater hair size and HCC (Burnett et al., 2014; Moya et al., 2013). The interrelation between growth rate, hair morphology and HCC should be studied in the future. It would be interesting to know if HCC from different anatomical regions but similar growth rate and hair morphology can be comparable under controlled circumstances. For instance, with the aim to obtain solutions when trying to compare HCC from animals that presents different hair colour in the same anatomical location.

In this general discussion, the existence of growth rate has been negatively associated to differences in HCC between anatomical locations so far. However, it should be noted that if other positive and useful advantages of hair matrix in hormonal detection exist is also due to the capacity "to grow" of hair matrix. Its versatility to monitor HPA activity for long periods of time but allowing (by performing shaves) the control of the beginning and the end of cortisol accumulation (the hair's age) is what makes hair an exceptional matrix for cortisol detection. In this point, the minimum and maximum frame of time able to be monitored by hair cortisol analysis are discussed as they are defined by different factors.

The smallest hair age collected or, in other words, the minimum period of time assessed through hair for cortisol analysis is defined by the interrelation of several factors: the growth rate of hair, the capacity of hair collection (the minimum hair length able to be cut and collected), the area of hair available (same anatomic location and homogeneous hair type) and finally, the amount of hair needed for cortisol analysis. Thus, in experimental designs using hair for adrenal monitoring for a few days, technical aspects are important. The other important factor to take cautiously is the delay of time between cortisol accumulation in hair (in hair follicle) and arrival of this portion of hair at the skin surface. There always exist a portion of hair that has already incorporated cortisol but it is inaccessible because remains between the hair follicle and the skin surface. This hair portion causes a desynchronization between the period of time within hair shaves and the real period of time of cortisol incorporated to the portion of hair sampled. Researchers should be aware that cortisol concentrations in hair collected with a previous shave are always representing HPA-axis activity from few days prior the first shave until few days prior the final shave. In the experiment using ACTH presented in Chapter III we had to deal with this kind of situation: we collected 14-days-old hair and as we did not know exactly the mentioned delay, we designed a pattern of ACTH injections to ensure that changes in SCC associated to both ACTH administrations would be accumulated in a portion of hair collected at the end of the study.

In contrast, the greatest period of time that hair could be accumulating cortisol is not so dependent on technical aspects or hair's delay but on hair growth physiology. It depends on the duration of hair growth cycle and its subsequent hair shed and replacement. Unfortunately, hair growth cycle has been only deeply studied in humans (Harkey, 1993; Robbins, 1988; Webb et al., 2015). In our experiment with cows from a commercial farm presented in Chapter IV, we were not able to perform a previous hair shave. Thus, when we collected hair samples we needed to make some assumptions regarding the period of time that was represented in those samples. These assumptions were based on the studied hair growth rate from other anatomical locations presenting similar type of hair (Burnett et al., 2014; Comin et al., 2013; Martin et al., 1969).

In any case, the decision of time allowing hair to grow should not be exclusively conditioned by the length of the study and the above-mentioned time limits of hair cortisol monitoring. The nature of potential stressors (if controlled or known) and its impact on the stress response of the studied animals (frequency, length, intensity, etc.) should be considered. As seen in Chapter IV, the sensitivity of hair to certain stressors depends on the characteristics of the induced stress response (intensity and length of stress) but also on the length of time monitored by hair. Although it is not demonstrated, it seems reasonable the existence of a "dilution-on-time" effect if the stress-response is not enough intense or frequent in comparison with the total time of adrenal activity monitored by hair. Once demonstrated that HCC is not altered by acute and non-recurrent peaks of SCC (Chapter III), the establishment of HCC sensitivity to specific stressors depending on hair age could be of interest.

6. Findings on hair progesterone detection in cattle

As mentioned in section **1. (Biochemical validation of standardized protocol for hair cortisol detection)**, the hair extract performed by our protocol was able to extract other steroids apart from cortisol. Some of most commonly analysed steroids in animal research (besides from cortisol or corticosterone) are testosterone, progesterone and estradiol. As some of our studies were performed in cow hair, testosterone was discarded and progesterone and estradiol remained as interesting hormones to be analysed. However, physiological changes in estradiol concentrations are too acute for long-term monitoring (Ball and Peters, 2004; Crowe, 2002) and our experimental design (in Chapter IV hair was considered to represent a period of two months of circulating hormones). Furthermore, progesterone is a key hormone in the establishment and maintenance of pregnancy (Mann and Lamming, 1999; Spencer, 2004a, 2004b; Wiltbank et al., 2014) and the interest of its monitoring in cumulative matrixes is increasing (Comin et al., 2014). For all these reasons, progesterone was selected to be analysed in hair extracts from experiments presented in chapters IV and V.

Previously, a biochemical validation for hair progesterone detection by a commercial EIA kit was performed (Chapter II). The results were similar to those obtained for hair cortisol analysis and demonstrated that hair progesterone concentrations (HPC) from cow hair could be correctly quantified through the same protocol and a progesterone-immunoreactiveNeogen® EIA kit.

In the same way, calibration and validation of NIRS for hair progesterone prediction showed similar results (slightly better) than for hair cortisol prediction: our resultant equations allowed the use of NIRS for animal screening. The discussion presented in section **4.(Evaluation of NIRS technique for hair cortisol quantification in cows)** regarding the potential of NIRS and hair cortisol measurement could be extrapolated to hair progesterone detection. However, the physiological fluctuation of progesterone due to the oestrus cycle in non-pregnant cows (Allrich, 1994; Ball and Peters, 2004; Crowe, 2002) could add difficulties when using screening capacity of NIRS for pregnancy diagnose in cattle as it was seen in our study presented in Chapter IV.

In that study, HPC were not significantly related with pregnancy status in cows. This initially surprising result could be explained because of two factors previously mentioned in this general discussion: 1) the characteristics of the cow oestrus cycle and 2) the sensitivity of hair to the changes in steroids pretended to be monitored. Progesterone remains high since the beginning of pregnancy until the end (Mukasa-Mugerwa and Tegegne, 1989; Spencer, 2004a; Stabenfeldt et al., 1970). However, in non pregnant cows luteal phase of oestrus cycle is also characterised by an increase of progesterone concentrations (Ball and Peters, 2004; Wiltbank et al., 2014). Oestrus cycle of cows is characterised for lasting 21 days and progesterone could remain elevated two thirds of the cycle duration. In our experiment with hair samples accumulating two months of circulating progesterone (Chapter IV), HPC were not sensitive enough to differentiate between pregnant and non-pregnant cows, at least at the initial months of pregnancy. Future studies with experimental designs focused on the possibilities of HPC for pregnancy diagnosis should be done. However, hair steroid detection for reproductive research seems to be a very interesting field to explore.

7. Hair cortisol detection in cattle: present and future. A personal view

Regarding hair cortisol analysis, it seems that this thesis and the rest of literature provide enough evidences to start using it as a complementary tool for long-term stress monitoring in dairy cattle welfare research. I am convinced that most of unknown factors affecting HCC can be avoided by performing an adequate experimental design, hair sampling and analysis. However, I also think that hair cortisol detection needs specific studies with the aim to resolve its uncertainties. The use of hair cortisol detection in human and animal research is increasing year after year and it seems reasonable to think that some of the mentioned unknown factors will be identified while few others will appear as it happened with all the other matrixes. Finally, I believe that hair matrix has come to stay as another matrix for cortisol detection with an exceptional application niche.

A list of some possible future studies resulting from the present thesis is shown below:

- Study of the interrelation between growth rate, hair morphology and HCC: Can we understand differences in HCC between anatomical locations?
- Study of the influence of pigments and melanocytes on HCC.
- Establishment of HCC sensitivity to specific stressors depending on hair's time allowed to grow.
- Study of biological factors affecting adrenal reactivity to ACTH in cows.
- Study of the impact of parturition on HCC.
- Usefulness of HCC for the prediction of disease critical evolution should be studied focusing specifically in chronic diseases with possibilities for critical evolution and, once diagnosed, the utility of HCC for the prognostic.
- Dairy farm stressors affecting two-months-old hair cortisol levels and milk production.
- Study of the interrelation between HCC and somatic cell count in cows suffering mastitis.
- Sensitivity of HCC to local incidences or pathologies: HCC as a measure of local inflammation or infection.
- Potential applicability of hair cortisol detection by NIRS in on-farm animal welfare assessment.

8. References

Abell, J., Kirschbaum, C., Ferrie, J., Shipley, M., Kivimäki, M., Kumari, M., 2015. Assessing cortisol from hair samples in a large observational cohort: Whitehall II. Psychoneuroendocrinology 61, 66. doi:10.1016/j.psyneuen.2015.07.570

Accorsi, P.A., Carloni, E., Valsecchi, P., Viggiani, R., Gamberoni, M., Tamanini, C., Seren, E., 2008. Cortisol determination in hair and faeces from domestic cats and dogs. Gen. Comp. Endocrinol. 155, 398–402. doi:10.1016/j.ygcen.2007.07.002

Allrich, R.D., 1994. Endocrine and Neural Control of Estrus in Dairy Cows. J. Dairy Sci. 77, 2738–2744. doi:10.3168/jds.S0022-0302(94)77216-7

Ashley, N.T., Barboza, P.S., Macbeth, B.J., Janz, D.M., Cattet, M.R.L., Booth, R.K., Wasser, S.K., 2011. Glucocorticosteroid concentrations in feces and hair of captive caribou and reindeer following adrenocorticotropic hormone challenge. Gen. Comp. Endocrinol. 172, 382–391. doi:10.1016/j.ygcen.2011.03.029

Ball, P.J.H., Peters, A.R., 2004. Reproduction in Cattle: Third Edition. Blackwell Publishing. doi:10.1002/9780470751091

Bechshøft, T.Ø., Sonne, C., Rigét, F.F., Letcher, R.J., Novak, M.A., Henchey, E., Meyer, J.S., Eulaers, I., Jaspers, V.L.B., Covaci, A., Dietz, R., 2013. Polar bear stress hormone cortisol fluctuates with the North Atlantic Oscillation climate index. Polar Biol. 36, 1525–1529. doi:10.1007/s00300-013-1364-y

Bennett, A., Hayssen, V., 2010. Measuring cortisol in hair and saliva from dogs: Coat color and pigment differences. Domest. Anim. Endocrinol. 39, 171–180. doi:10.1016/j.domaniend.2010.04.003

Berkvens, C.N., Hyatt, C., Gilman, C., Pearl, D.L., Barker, I.K., Mastromonaco, G.F., 2013. Validation of a shed skin corticosterone enzyme immunoassay in the African House Snake (Lamprophis fuliginosus) and its evaluation in the Eastern Massasauga Rattlesnake (Sistrurus catenatus catenatus). Gen. Comp. Endocrinol. 194C, 1–9. doi:10.1016/j.ygcen.2013.08.011

Botchkarev, V.A., 2003. Stress and the hair follicle: exploring the connections. Am. J. Pathol. 162, 709–12. doi:10.1016/S0002-9440(10)63866-7

Boumba, V.A., Ziavrou, K.S., Vougiouklakis, T., 2006. Hair as a biological indicator of drug use, drug abuse or chronic exposure to environmental toxicants. Int. J. Toxicol. 25, 143–63. doi:10.1080/10915810600683028

Broad, N.W., Jee, R.D., Moffat, A.C., Smith, M.R., 2001. Application of transmission near-infrared spectroscopy to uniformity of content testing of intact steroid tablets. Analyst 126, 2207–2211. doi:10.1039/b106741a

Bryan, H.M., 2013. Parasites and Hormones as Non-invasive Bioindicators of Ecophysiology in Carnivores.

University of Calgary.

Bryan, H.M., Adams, A.G., Invik, R.M., Wynne-Edwards, K.E., Smits, J.E.G., 2013. Hair as a meaningful measure of baseline cortisol levels over time in dogs. J. Am. Assoc. Lab. Anim. Sci. 52, 189–96.

Buchanan, K.L., Goldsmith, A.R., 2004. Noninvasive endocrine data for behavioural studies: the importance of validation. Anim. Behav. 67, 183–185. doi:10.1016/j.anbehav.2003.09.002

Burnett, T.A., Madureira, A.M.L., Silper, B.F., Tahmasbi, A., Nadalin, A., Veira, D.M., Cerri, R.L.A., 2015. Relationship of concentrations of cortisol in hair with health, biomarkers in blood, and reproductive status in dairy cows. J. Dairy Sci. 98, 1–13. doi:10.3168/jds.2014-8871

Burnett, T.A., Madureira, A.M.L.L., Silper, B.F., Nadalin, A., Tahmasbi, A., Veira, D.M., Cerri, R.L.A., 2014. Short communication: Factors affecting hair cortisol concentrations in lactating dairy cows. J. Dairy Sci. 97, 7685–7690. doi:10.3168/jds.2014-8444

Carlitz, E.H.D., Kirschbaum, C., Miller, R., Rukundo, J., van Schaik, C.P., 2015. Effects of body region and time on hair cortisol concentrations in chimpanzees (Pan troglodytes). Gen. Comp. Endocrinol. doi:10.1016/j.ygcen.2015.09.022

Carlitz, E.H.D., Kirschbaum, C., Stalder, T., van Schaik, C.P., 2014. Hair as a long-term retrospective cortisol calendar in orang-utans (Pongo spp.): new perspectives for stress monitoring in captive management and conservation. Gen. Comp. Endocrinol. 195, 151–6. doi:10.1016/j.ygcen.2013.11.002

Cen, H., He, Y., 2007. Theory and application of near infrared reflectance spectroscopy in determination of food quality. Trends Food Sci. Technol. 18, 72–83. doi:10.1016/j.tifs.2006.09.003

CENAM, EMA, 2008. Guía para la validación y la verificación de los procedimientos de examen cuantitativos empleados por el laboratorio clínico. Centro Nacional de Metrología, México DF, MEX.

Cerri, R.L.A., Tabmasbi, A.M., Veira, D.M., 2012. Hair cortisol concentrations -Influence of color and location in Holstein cows. J. Dairy Sci. 95, S574.

Charmandari, E., Tsigos, C., Chrousos, G., 2005. Endocrinology of the stress response. Annu. Rev. Physiol. 67, 259–84. doi:10.1146/annurev.physiol.67.040403.120816

Cockrem, J.F., 2013. Individual variation in glucocorticoid stress responses in animals. Gen. Comp. Endocrinol. 181, 45–58. doi:10.1016/j.ygcen.2012.11.025

Comin, A., Peric, T., Corazzin, M., Veronesi, M.C.C., Meloni, T., Zufferli, V., Cornacchia, G., Prandi, A., 2013. Hair cortisol as a marker of hypothalamic-pituitary-adrenal axis activation in Friesian dairy cows clinically or physiologically compromised. Livest. Sci. 152, 36–41. doi:10.1016/j.livsci.2012.11.021

Comin, A., Peric, T., Magrin, L., Corazzin, M., Cornacchia, G., Prandi, A., 2014. Study of progesterone

and cortisol concentrations in the Italian Friesian claw. J. Dairy Sci. 97, 5491–6. doi:10.3168/jds.2014-7943

Comin, A., Prandi, A., Peric, T., Corazzin, M., Dovier, S., Bovolenta, S., 2011. Hair cortisol levels in dairy cows from winter housing to summer highland grazing. Livest. Sci. 138, 69–73. doi:10.1016/j.livsci.2010.12.009

Comin, A., Tidu, L., Cornacchia, G., Cappa, A., Renaville, B., Prandi, A., 2008. Neonatal period and hair cortisol in cattle as a marker of stress., in: XVI Congress of the Mediterranean Federation for Health and Production of Ruminants (FeMeSPrum). 23-26 april, Zadar, Croatia, pp. 221–225.

Comin, A., Veronesi, M.C., Montillo, M., Faustini, M., Valentini, S., Cairoli, F., Prandi, A., 2012. Hair cortisol level as a retrospective marker of hypothalamic–pituitary–adrenal axis activity in horse foals. Vet. J. 194, 131–132. doi:10.1016/j.tvjl.2012.04.006

Cone, E.J., 1996. Mechanisms of Drug Incorporation into Hair. Ther. Drug Monit. 18, 438–443. doi:10.1097/00007691-199608000-00022

Crowe, M.A., 2002. Oestrus cycles: Characteristics, in: Roginski, H. (Ed.), Encyclopedia of Dairy Sciences. Elsevier, pp. 2152–2157.

Crowther, J., Walker, J., 2001. The ELISA guidebook. Humana Press Inc., New Jersey.

Davenport, M.D., Tiefenbacher, S., Lutz, C.K., Novak, M.A., Meyer, J.S., 2006. Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. Gen. Comp. Endocrinol. 147, 255–261. doi:10.1016/j.ygcen.2006.01.005

Deaville, E., Flinn, P., 2000. Near-infrared (NIR) spectroscopy: an alternative approach for the estimation of forage quality and voluntary intake, Forage evaluation in ruminant nutrition. doi:10.1079/9780851993447.0301

Dettmer, A.M., Novak, M.A., Meyer, J.S., Suomi, S.J., 2014. Population density-dependent hair cortisol concentrations in rhesus monkeys (Macaca mulatta). Psychoneuroendocrinology 42, 59–67. doi:10.1016/j.psyneuen.2014.01.002

Dettmer, A.M., Novak, M.A., Suomi, S.J., Meyer, J.S., 2012. Physiological and behavioral adaptation to relocation stress in differentially reared rhesus monkeys: Hair cortisol as a biomarker for anxiety-related responses. Psychoneuroendocrinology 37, 191–199. doi:10.1016/j.psyneuen.2011.06.003

Duncan, I.J.H., 2005. Science-based assessment of animal welfare: farm animals. Rev. Sci. Tech. 24, 483–92.

Feng, X., Wu, X., Morrill, R.J., Li, Z., Li, C., Yang, S., Li, Z., Cui, D., Lv, L., Hu, Z., Zhang, B., Yin, Y., Guo, L., Qin, D., Hu, X., 2016. Social correlates of the dominance rank and long-term cortisol levels in adolescent and adult male rhesus macaques (Macaca mulatta). Sci. Rep. 6, 25431. doi:10.1038/srep25431

Foley, W.J., McIlwee, A., Lawler, I., Aragones, L., Woolnough, A.P., Berding, N., 1998. Ecological applications of near infrared reflectance spectroscopy - A tool for rapid, cost-effective prediction of the composition of plant and animal tissues and aspects of animal performance. Oecologia. doi:10.1007/s004420050591

Food and Drug Administration, 2011. Guidance for Industry Process Validation : General Principles and Practices Guidance for Industry Process Validation : General Principles and Practices 22.

Food and Drug Administration, 2013. Guidance for industry. Bioanalytical method validation.

Food and Drug Administration, 2015. Analytical Procedures and Methods Validation for Drugs and Biologics. Guid. Ind. 1–15.

Fourie, N.H., Brown, J.L., Jolly, C.J., Phillips-Conroy, J.E., Rogers, J., Bernstein, R.M., 2016. Sources of variation in hair cortisol in wild and captive non-human primates. Zoology 119, 119–125. doi:10.1016/j.zool.2016.01.001

Fukasawa, M., Tsukada, H., Kosako, T., Yamada, A., 2008. Effect of lactation stage, season and parity on milk cortisol concentration in Holstein cows. Livest. Sci. 113, 280–284. doi:10.1016/j.livsci.2007.05.020

Galvão, K.N., Flaminio, M.J.B.F., Brittin, S.B., Sper, R., Fraga, M., Caixeta, L., Ricci, A., Guard, C.L., Butler, W.R., Gilbert, R.O., 2010. Association between uterine disease and indicators of neutrophil and systemic energy status in lactating Holstein cows. J. Dairy Sci. 93, 2926–37. doi:10.3168/jds.2009-2551

Gatti, R., Antonelli, G., Prearo, M., Spinella, P., Cappellin, E., De Palo, E.F., 2009. Cortisol assays and diagnostic laboratory procedures in human biological fluids. Clin. Biochem. 42, 1205–17. doi:10.1016/j.clinbiochem.2009.04.011

Ghassemi Nejad, J., Lohakare, J.D., Son, J.K., Kwon, E.G., West, J.W., Sung, K.I., 2014. Wool cortisol is a better indicator of stress than blood cortisol in ewes exposed to heat stress and water restriction. Animal 8, 128–32. doi:10.1017/S1751731113001870

Goff, J.P., Kehrli, M.E., Horst, R.L., 1989. Periparturient hypocalcemia in cows: prevention using intramuscular parathyroid hormone. J. Dairy Sci. 72, 1182–7. doi:10.3168/jds.S0022-0302(89)79222-5

González-de-la-Vara, M.R.D.R., Valdez, R.A., Lemus-Ramirez, V., Vázquez-Chagoyán, J.C., Villa-Godoy, A., Romano, M.C., 2011. Effects of adrenocorticotropic hormone challenge and age on hair cortisol concentrations in dairy cattle. Can. J. Vet. Res. 75, 216–221.

Gow, R., Thomson, S., Rieder, M., Van Uum, S., Koren, G., 2010. An assessment of cortisol analysis in hair and its clinical applications. Forensic Sci. Int. 196, 32–37. doi:10.1016/j.forsciint.2009.12.040

Gwazdauskas, F.C., Thatcher, W.W., Wilcox, C.J., 1972. Adrenocorticotropin alteration of bovine

peripheral plasma concentrations of cortisol, corticosterone, and progesterone. J. Dairy Sci. 55, 1165–1169. doi:10.3168/jds.S0022-0302(72)85640-6

Harkey, M.R., 1993. Forensic Science Internihnal Anatomy and physiology of hair. Forensic Sci. Int. 63, 9–18.

Hasegawa, N., Nishiwaki, A., Sugawara, K., Iwao, I., 1997. The effects of social exchange between two groups of lactating primiparous heifers on milk production, dominance order, behavior and adrenocortical response. Appl. Anim. Behav. Sci. 51, 15–27. doi:10.1016/S0168-1591(96)01082-9

Hellhammer, D.H., Wüst, S., Kudielka, B.M., 2009. Salivary cortisol as a biomarker in stress research. Psychoneuroendocrinology 34, 163–71. doi:10.1016/j.psyneuen.2008.10.026

Henderson, G.L.L., 1993. Mechanisms of drug incorporation into hair. Forensic Sci. Int. 63, 19–29. doi:10.1016/0379-0738(93)90256-A

Keckeis, K., Lepschy, M., Schöpper, H., Moser, L., Troxler, J., Palme, R., 2012. Hair cortisol: a parameter of chronic stress? Insights from a radiometabolism study in guinea pigs. J. Comp. Physiol. B. 182, 985–96. doi:10.1007/s00360-012-0674-7

Kersey, D.C., Dehnhard, M., 2014. The use of noninvasive and minimally invasive methods in endocrinology for threatened mammalian species conservation. Gen. Comp. Endocrinol. 203, 296–306. doi:10.1016/j.ygcen.2014.04.022

Kirschbaum, C., Hellhammer, D.H., 1994. Salivary cortisol in psychoneuroendocrine research: recent developments and applications. Psychoneuroendocrinology 19, 313–333. doi:10.1016/0306-4530(94)90013-2

Koren, L., Mokady, O., Karaskov, T., Klein, J., Koren, G., Geffen, E., 2002. A novel method using hair for determining hormonal levels in wildlife. Anim. Behav. 63, 403–406. doi:10.1006/anbe.2001.1907

Lay, D.C., Friend, T.H., Randel, R.D., Jenkins, O.C., Neuendorff, D.A., Kapp, G.M., Bushong, D.M., 1996. Adrenocorticotropic hormone dose response and some physiological effects of transportation on pregnant Brahman cattle. J. Anim. Sci. 74, 1806–1811.

LeBeau, M.A., Montgomery, M.A., Brewer, J.D., 2011. The role of variations in growth rate and sample collection on interpreting results of segmental analyses of hair. Forensic Sci. Int. 210, 110–116. doi:10.1016/j.forsciint.2011.02.015

Lequin, R.M., 2005. Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). Clin. Chem. 51, 2415–8. doi:10.1373/clinchem.2005.051532

Macbeth, B.J., Cattet, M.R.L.R.L., Stenhouse, G.B., Gibeau, M.L., Janz, D.M., 2010. Hair cortisol concentration as a noninvasive measure of long-term stress in free-ranging grizzly bears (Ursus arctos): Considerations with implications for other wildlife. Can. J. Zool. 88, 935–949. doi:10.1139/Z10-057

Maiero, S., Marchini, E., Comin, A., Renaville, B., Prandi, A., 2005. Determination of cortisol in hair as indicator of long-term stress in Simmenthal dairy cows. Reprod. Domest. Anim. 40, 396.

Manenschijn, L., Koper, J.W., Lamberts, S.W.J., van Rossum, E.F.C., 2011. Evaluation of a method to measure long term cortisol levels. Steroids 76, 1032–1036. doi:10.1016/j.steroids.2011.04.005

Mann, G.E., Lamming, G.E., 1999. The Influence of Progesterone During Early Pregnancy in Cattle. Reprod. Domest. Anim. 34, 269–274. doi:10.1111/j.1439-0531.1999.tb01250.x

Martin, Y.G., Miller, W.J., Blackmon, D.M., 1969. Wound healing, hair growth and biochemical measures as affected by subnormal protein and energy intake in young cattle. Am. J. Vet. Res. 30, 355–364.

Mastromonaco, G.F., Gunn, K., Edwards, D.B., 2014. Validation and use of hair cortisol as a measure of chronic stress in eastern chipmunks (Tamias striatus). Conserv. Physiol. 2, 1–12. doi:10.1093/conphys/cou055

Meyer, J.S., Novak, M.A., 2012. Minireview: Hair cortisol: a novel biomarker of hypothalamic-pituitary-adrenocortical activity. Endocrinology 153, 4120–4127. doi:10.1210/en.2012-1226

Moberg, G.P., Mench, J.A., 2000. The biology of animal stress: basic principles and implications for animal welfare. CABI, Wallingford. doi:10.1079/9780851993591.0000

Mormède, P., Andanson, S.S., Aupérin, B., Beerda, B., Guémené, D., Malmkvist, J., Manteca, X., Manteuffel, G., Prunet, P., van Reenen, C.G., Richard, S., Veissier, I., Mormede, P., Auperin, B., Guemene, D., 2007. Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. Physiol. Behav. 92, 317–339. doi:10.1016/j.physbeh.2006.12.003

Morrow, C.J., Kolver, E.S., Verkerk, G.A., Matthews, L.R., 2002. Fecal glucocorticoid metabolites as a measure of adrenal activity in dairy cattle. Gen. Comp. Endocrinol. 126, 229–241. doi:10.1006/gcen.2002.7797

Möstl, E., Maggs, J.L., Schro, G., Schrötter, G., Besenfelder, U., Palme, R., 2002. Measurement of cortisol metabolites in faeces of ruminants. Vet. Res. Commun. 26, 127–139. doi:10.1023/A:1014095618125

Möstl, E., Messmann, S., Bagu, E., Robia, C., Palme, R., 1999. Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock. J. Vet. Med. Ser. A-Physiology Pathol. Clin. Med. 46, 621–631.

Möstl, E., Palme, R., 2002. Hormones as indicators of stress. Domest. Anim. Endocrinol. 23, 67–74. doi:10.1016/S0739-7240(02)00146-7

Moya, D., Schwartzkopf-Genswein, K.S., Veira, D.M., 2013. Standardization of a non-invasive methodology to measure cortisol in hair of beef cattle. Livest. Sci. 158, 138–144. doi:10.1016/j.livsci.2013.10.007

Mukasa-Mugerwa, E., Tegegne, A., 1989. Peripheral plasma progesterone concentration in zebu (Bos

indicus) cows during pregnancy. Reprod. Nutr. Dev. 29, 303–308. doi:10.1051/rnd:19890308

Oltenacu, P.A., Broom, D.M., 2010. The impact of genetic selection for increased milk yield on the welfare of dairy cows. Anim. Welf. 19, 39–49.

Osborne, B.G., 2000. Near-infrared spectroscopy in food analysis. Encycl. Anal. Chem. 1–14. doi:10.1002/9780470027318.a1018

Ouschan, C., Kuchar, A., Möstl, E., 2013. Measurement of cortisol in dog hair: a noninvasive tool for the diagnosis of hypercortisolism. Vet. Dermatol. 24, 428–31, e93-4. doi:10.1111/vde.12043

Palme, R., 2005. Measuring fecal steroids: guidelines for practical application. Ann. N. Y. Acad. Sci. 1046, 75–80. doi:10.1196/annals.1343.007

Palme, R., 2012. Monitoring stress hormone metabolites as a useful, non-invasive tool for welfare assessment in farm animals. Anim. Welf. 21, 331–337. doi:10.7120/09627286.21.3.331

Palme, R., Fischer, P., Schildorfer, H., Ismail, M.N., 1996. Excretion of infused 14C-steroid hormones via faeces and urine in domestic livestock. Anim. Reprod. Sci. 43, 43–63. doi:10.1016/0378-4320(95)01458-6

Palme, R., Robia, C., Messmann, S., Hofer, J., Möstl, E., 1999. Measurement of faecal cortisol metabolites in ruminants: a non-invasive parameter of adrenocortical function. Wien. Tierarztl. Monatsschr. 86, 237–241.

Pasquini, C., 2003. Near infrared spectroscopy: Fundamentals, practical aspects and analytical applications. J. Braz. Chem. Soc. 14, 198–219. doi:10.1590/S0103-50532003000200006

Pellicer, A., Bravo, M.D.C., 2011. Near-infrared spectroscopy: A methodology-focused review. Semin. Fetal Neonatal Med. 16, 42–49. doi:10.1016/j.siny.2010.05.003

Peric, T., Comin, A., Corazzin, M., Montillo, M., Cappa, A., Campanile, G., Prandi, A., 2013. Short communication: hair cortisol concentrations in Holstein-Friesian and crossbreed F1 heifers. J. Dairy Sci. 96, 3023–7. doi:10.3168/jds.2012-6151

Pötsch, L., Moeller, M.R., 1996. On pathways for small molecules into and out of human hair fibers. J. Forensic Sci. 41, 121–125.

Pötsch, L., Skopp, G., Moeller, M.R., 1997. Influence of pigmentation on the codeine content of hair fibers in guinea pigs. J. Forensic Sci. 42, 1095–8.

Pragst, F., Balikova, M.A., 2006. State of the art in hair analysis for detection of drug and alcohol abuse. Clin. Chim. Acta. 370, 17–49. doi:10.1016/j.cca.2006.02.019

Reading, F., 1999. Stress, management induced, in dairy cattle. Effects on Health and Milk Production, in: STRESS , MANAGEMENT INDUCED , IN DAIRY CATTLE. pp. 2604–2611.

Rippe, R.C.A., Noppe, G., Windhorst, D.A., Tiemeier, H., van Rossum, E.F.C., Jaddoe, V.W.V., Verhulst, F.C., Bakermans-Kranenburg, M.J., van IJzendoorn, M.H., van den Akker, E.L.T., 2015. Splitting hair for cortisol? Associations of socio-economic status, ethnicity, hair color, gender and other child characteristics with hair cortisol and cortisone. Psychoneuroendocrinology. doi:10.1016/j.psyneuen.2015.12.016

Robbins, C.R., 1988. Chemical and physical behavior of human hair, 4th Editio. ed, Chemical and Physical Behaviour of Human Hair. Springer. doi:10.1007/978-3-642-25611-0

Rodriguez-Otero, J.L., Hermida, M., Centeno, J., 1997. Analysis of Dairy Products by Near-Infrared Spectroscopy: A Review. J. Agric. Food Chem. 45, 2815–2819. doi:10.1021/jf960744p

Roulin, A., Almasi, B., Rossi-Pedruzzi, A., Ducrest, A.L., Wakamatsu, K., Miksik, I., Blount, J.D., Jenni-Eiermann, S., Jenni, L., 2008. Corticosterone mediates the condition-dependent component of melanin-based coloration. Anim. Behav. 75, 1351–1358. doi:10.1016/j.anbehav.2007.09.007

Russell, E., Koren, G., Rieder, M., Van Uum, S., 2012. Hair cortisol as a biological marker of chronic stress: Current status, future directions and unanswered questions. Psychoneuroendocrinology 37, 589–601. doi:10.1016/j.psyneuen.2011.09.009

Salaberger, T., Millard, M., Makarem, S. El, Möstl, E., Grünberger, V., Krametter-Frötscher, R., Wittek, T., Palme, R., 2016. Influence of external factors on hair cortisol concentrations. Gen. Comp. Endocrinol. doi:10.1016/j.ygcen.2016.05.005

Sauvé, B., Koren, G., Walsh, G., Tokmakejian, S., Van Uum, S.H.M., 2007. Measurement of cortisol in human hair as a biomarker of systemic exposure. Clin. Investig. Med. 30, E183–E191.

Scarff, M., Arnold, S.A., Harvey, L.M., McNeil, B., 2006. Near Infrared Spectroscopy for bioprocess monitoring and control: Current status and future trends. Crit. Rev. Biotechnol. 26, 17–39. doi:Doi 10.1080/07388550500513677

Schreier, H.M.C., Bosquet Enlow, M., Ritz, T., Coull, B.A., Gennings, C., Wright, R.O., Wright, R.J., 2015. Lifetime Exposure to Traumatic and Other Stressful Life Events and Hair Cortisol in a Multi-Racial/Ethnic Sample of Pregnant Women. Stress 3890, 1–30. doi:10.3109/10253890.2015.1117447

Sgorlon, S., Fanzago, M., Guiatti, D., Gabai, G., Stradaioli, G., Stefanon, B., 2015. Factors affecting milk cortisol in mid lactating dairy cows. BMC Vet. Res. 1–8. doi:10.1186/s12917-015-0572-9

Sharpley, C.F., Kauter, K.G., McFarlane, J.R., 2009. An Initial Exploration of in vivo Hair Cortisol Responses to a Brief Pain Stressor: Latency, Localization and Independence Effects. Physiol. Res. 58, 757–761.

Sharpley, C.F., McFarlane, J.R., Slominski, A., 2012. Stress-linked cortisol concentrations in hair: what we know and what we need to know. Rev. Neurosci. 23, 111–121. doi:10.1515/RNS.2011.058

Short, S.J., Stalder, T., Marceau, K.P., Entringer, S., Moog, N.K., Shirtcliff, E. a., Wadhwa, P.D., Buss, C.,

2016. Correspondence between hair cortisol concentrations and 30-day integrated daily salivary and weekly urinary cortisol measures. Psychoneuroendocrinology. doi:10.1016/j.psyneuen.2016.05.007

Silva, P.R.B., Lobeck-Luchterhand, K.M., Cerri, R.L.A., Haines, D.M., Ballou, M.A., Endres, M.I., Chebel, R.C., 2016. Effects of prepartum stocking density on innate and adaptive leukocyte responses and serum and hair cortisol concentrations. Vet. Immunol. Immunopathol. 169, 39–46. doi:10.1016/j.vetimm.2015.11.007

Skrzipczyk, H.J., Verdier, P., 2013. Bioanalytical Assays: RIA/EIA. Drug Discov. Eval. Saf. Pharmacokinet. Assays 869–886. doi:10.1007/978-3-642-25240-2

Slominski, A., 2004. Melanin Pigmentation in Mammalian Skin and Its Hormonal Regulation. Physiol. Rev. 84, 1155–1228. doi:10.1152/physrev.00044.2003

Slominski, A., Zbytek, B., Nikolakis, G., Manna, P.R., Skobowiat, C., Zmijewski, M., Li, W., Janjetovic, Z., Postlethwaite, A., Zouboulis, C.C., Tuckey, R.C., 2013. Steroidogenesis in the skin: implications for local immune functions. J. Steroid Biochem. Mol. Biol. 137, 107–23. doi:10.1016/j.jsbmb.2013.02.006

Slominski, A.T., Manna, P.R., Tuckey, R.C., 2014. Cutaneous glucocorticosteroidogenesis: securing local homeostasis and the skin integrity. Exp. Dermatol. 23, 369–74. doi:10.1111/exd.12376

Sørensen, J.T., Fraser, D., 2010. On-farm welfare assessment for regulatory purposes: Issues and possible solutions. Livest. Sci. 131, 1–7. doi:10.1016/j.livsci.2010.02.025

Spencer, T.E., 2004a. Conceptus signals for establishment and maintenance of pregnancy. Anim. Reprod. Sci. 15, 1–15. doi:10.1016/S0378-4320(04)00070-3

Spencer, T.E., 2004b. Progesterone and Placental Hormone Actions on the Uterus: Insights from Domestic Animals. Biol. Reprod. 71, 2–10. doi:10.1095/biolreprod.103.024133

Stabenfeldt, G.H., Osburn, B.I., Ewing, L.L., 1970. Peripheral plasma progesterone levels in the cow during pregnancy and parturition. Am. J. Physiol. 218, 571–5.

Stalder, T., Kirschbaum, C., 2012. Analysis of cortisol in hair – State of the art and future directions. Brain. Behav. Immun. 26, 1019–29. doi:10.1016/j.bbi.2012.02.002

Stepaniak, L., Sorhaug, T., Jedrychowski, L., 2002. ANALYSIS/Immunochemical, in: Encyclopedia of Dairy Sciences. pp. 62–67.

Steudte-Schmiedgen, S., Kirschbaum, C., Alexander, N., Stalder, T., 2016. An integrative model linking traumatization, cortisol dysregulation and posttraumatic stress disorder: insight from recent hair cortisol findings. Neurosci. Biobehav. Rev. doi:10.1016/j.neubiorev.2016.07.015

Sultan, A., Raza, A.R., 2015. Steroids: A Diverse Class of Secondary Metabolites. Med. Chem. (Los. Angeles). 5, 310–317. doi:10.4172/2161-0444.1000279

Taves, M.D., Gomez-Sanchez, C.E., Soma, K.K., 2011. Extra-adrenal glucocorticoids and mineralocorticoids: evidence for local synthesis, regulation, and function. Am. J. Physiol. Endocrinol. Metab. 301, E11-24. doi:10.1152/ajpendo.00100.2011

Taylor, W., 1971. The excretion of steroid hormone metabolites in bile and feces. Vitam.Horm 29, 201–285.

Terwissen, C. V., Mastromonaco, G.F., Murray, D.L., 2013. Influence of adrenocorticotrophin hormone challenge and external factors (age, sex, and body region) on hair cortisol concentration in Canada lynx (Lynx canadensis). Gen. Comp. Endocrinol. 194, 162–167. doi:10.1016/j.ygcen.2013.09.010

Touma, C., Palme, R., 2005. Measuring Fecal Glucocorticoid Metabolites in Mammals and Birds: The Importance of Validation. Ann. N. Y. Acad. Sci. 1046, 54–74. doi:10.1196/annals.1343.006

Trevisi, E., Lombardelli, R., Bionaz, M., Bertoni, G., 2005. Plasma cortisol level in relation to welfare conditions in dairy farms, in: 56th EAAP Annual Meeting. Uppsala, Sweden, pp. 1–6.

Tsigos, C., Chrousos, G.P., 2002. Hypothalamic–pituitary–adrenal axis, neuroendocrine factors and stress. J. Psychosom. Res. 53, 865–871. doi:10.1016/S0022-3999(02)00429-4

Van Uum, S.H.M., Sauvé, B., Fraser, L.A., Morley-Forster, P., Paul, T.L., Koren, G., 2008. Elevated content of cortisol in hair of patients with severe chronic pain: A novel biomarker for stress. Stress 11, 483–488. doi:10.1080/10253890801887388

Vance, C., Kouba, A., Ouyang, X., Willard, S., 2013. Giant panda physiology from faeces: sampling strategies optimised for a different kind of question. NIR news 24, 13. doi:10.1255/nirn.1405

Vance, C.K., Tolleson, D.R., Kinoshita, K., Foley, W.J., 2016. Near infrared spectroscopy in wildlife and biodiversity 25, 1–25. doi:10.1255/jnirs.1199

Vogliardi, S., Tucci, M., Stocchero, G., Ferrara, S.D., Favretto, D., 2014. Sample preparation methods for determination of drugs of abuse in hair samples: A review. Anal. Chim. Acta. doi:10.1016/j.aca.2014.06.053

von Keyserlingk, M.A.G., Rushen, J., de Passillé, A.M., Weary, D.M., 2009. The welfare of dairy cattle—Key concepts and the role of science. J. Dairy Sci. 92, 4101–4111. doi:10.3168/jds.2009-2326

Walker, J., Tolleson, D., 2010. Shining Light on Manure Improves Livestock and Land Management, First edit. ed, Texas AgriLife Research and Society for Range Management. Texas AgriLife Research and Society for Range Management, Jefferson City, Missouri.

Webb, E.C., White, C.D., Van Uum, S., Longstaffe, F.J., 2015. Integrating cortisol and isotopic analyses of archeological hair: Reconstructing individual experiences of health and stress. Am. J. Phys. Anthropol. 156, 577–594. doi:10.1002/ajpa.22673

Wester, V.L., Noppe, G., Savas, M., Stolk, R.P., van den Akker, E.L.T., de Rijke, Y.B., van Rossum,

E.F.C., 2016. Long-Term Glucocorticoids Measured in Hair Are Influenced by Local Corticosteroid Treatment: the LifeLines Cohort Study, in: Endocrine Society's 98th Annual Meeting and Expo. Boston, p. FRI-405.

Wiltbank, M.C., Souza, A.H., Carvalho, P.D., Cunha, A.P., Giordano, J.O., Fricke, P.M., Baez, G.M., Diskin, M.G., 2014. Physiological and practical effects of progesterone on reproduction in dairy cattle. Animal 8 Suppl 1, 70–81. doi:10.1017/s1751731114000585

Windham, W.R., Mertens, D.R., Barton, F.E., 1989. Protocol for NIRS Calibration: Sample Selection and Equation Development and Validation, in: Marten, G.C., Shenk, J.S., Barton, F.E. (Eds.), Near Infrared Reflectance Spectroscopy (NIRS): Analysis of Forage Quality. pp. 96–104.

Yamanashi, Y., Morimura, N., Mori, Y., Hayashi, M., Suzuki, J., 2013. Cortisol analysis of hair of captive chimpanzees (Pan troglodytes). Gen. Comp. Endocrinol. 194C, 55–63. doi:10.1016/j.ygcen.2013.08.013

Yamanashi, Y., Teramoto, M., Morimura, N., Hirata, S., Inoue-Murayama, M., Idani, G., 2016. Effects of Relocation and Individual and Environmental Factors on the Long-Term Stress Levels in Captive Chimpanzees (Pan troglodytes): Monitoring Hair Cortisol and Behaviors. PLoS One 11, e0160029. doi:10.1371/journal.pone.0160029

Yoshida, C., Nakao, T., 2005. Response of plasma cortisol and progesterone after ACTH challenge in ovariectomized lactating dairy cows. J. Reprod. Dev. 51, 99–107. doi:10.1262/jrd.51.99

FINAL CONCLUSIONS

The studies conducted in the present thesis with the aim to validate, evaluate and optimize the use of hair cortisol measurement as a tool to assess long-term adrenal activity in cattle allowed us to reach the following conclusions:

Specific objective 1:

To validate a standardized protocol for the extraction of cortisol from cattle's hair and the quantification of these cortisol concentrations by an enzyme immunoassay kit

CONCLUSION 1: **It is possible to perform a correct quantification of cortisol concentrations in hair from cattle through the developed protocol and enzyme immunoassayused.**

Specific objective 2:

To study the feasibility of hair cortisol concentrations to represent a long-term integrative value of adrenal activity

CONCLUSION 2.1: **Hair cortisol concentrations in cattle are associated to long-term hypothalamic-pituitary-adrenal-axis activity despite the existence of local confounding factors.**

CONCLUSION 2.2: **Hair cortisol concentrations from bull calves are not affected by short and non-recurrent increases of serum cortisol concentrations that do not represent the general and continuous adrenal activity of the animal.**

Specific objective 3:

To evaluate the potential uses of hair cortisol determination in dairy cattle production and its relationship with hair progesterone concentrations

CONCLUSION 3.1: **Despite showing high individual variability, hair cortisol concentrations in cows are sensitive to long-term stressors affecting milk production and composition demonstrating the potential of this tool to be used in dairy cattle welfare and production research.**

CONCLUSION 3.2: **Cortisol and progesterone in hair show similar relationship as found in other matrixes providing a useful tool for long-term endocrinology research / for the study of long-term interactions between these two steroids.**

Specific objective 4:

To evaluate near infrared reflectance spectroscopy technique for hair cortisol quantification in cows

CONCLUSION 4: **Although it is far from being a complete alternative to current methodologies, near infrared reflectance spectroscopy is able to predict hair cortisol concentrations from cattle with accuracy for screening.**

Specific objective 5:

To study secondary characteristics of hair matrix with an influence in hair cortisol measurement

CONCLUSION 5.1: **Hair colour and anatomical location affects hair cortisol concentrations in cattle.**

CONCLUSION 5.2: **The sensitivity of hair cortisol measurements to changes in adrenal activity is conditioned by the characteristics of induced stress and its interrelation with the amount of time allowing hair samples to grow.**