Stress and the measurement of glucocorticoids and their metabolites in different matrices

Erich Möstl
Veterinary University Vienna
Veterinärplatz 1
1210 Vienna
Focus of the presentation:

- Glucocorticoids are „anti-stress“ hormones
- Glucocorticoids are mainly excreted as metabolites. The concentration is a parameter for glucocorticoid production (activity of the HPA-axis)
- Some glucocorticoid metabolites are biologically active substances
Stress

….is the response of an organism to a stressor

Glucocorticoids help the organism to overcome stressors

- Regulating the metabolism
- Immunological effects
- Effects on reproduction, behaviour, food intake, wound healing…
- Transgenerational effects
- Involved in the hormonal cascade inducing parturition, developmental effects, cognition…….
Stress

- Welfare as absence of stress? (Yerkes-Dodson law). Arousal und Performance

- No standard definition of stress, but a need for additional biochemical or endocrine parameters to measure it.

- During stressful situations, often increased amounts of hormones are produced, but a lot of activities can cause a hormonal increase.
Frontline hormones of stress reactions are the

Catecholamines
Adrenaline (Epinephrine) /Noradrenaline

Glucocorticoids (GCs)
Glucocorticoids are by definition a class of steroid hormones that bind to the glucocorticoid receptor and have an effect.
Hypothalamus

CRH

Hypophysis

ACTH

Adrenal cortex

Glucocorticoids

Peripheral tissue

Extra-adrenal glucocorticoid production

Re-activation

Deactivation

Effects

Excretion

G. Talaber et al., Local glucocorticoid production in the thymus, Steroids (2015), http://dx.doi.org/10.1016/j.Steroids.201506.010

Cortisol and corticosterone independence in cortisol-dominant wildlife
L. Koren et al., Gen. Comp. Endo., 2012
Why do humans have two glucocorticoids: A question of intestinal fortitude
D. J. Morris, Steroids, 2015
Transport of GCs in the blood

Cortisol Binding Globulin (CBG)

Non-bound (free) GC

Albumine bound

Solubility of cortisol in water: 0.28 mg/ml

(http://www.vetpharm.uzh.ch/reloader.htm?wir/00000005/0237_01.htm?wir/00000005/0237_00.htm)
CBG influences local GC concentrations

- CBG – cortisol binding is decreased with increasing temperature
- A small increase in temperature (inflammation) causes a higher local proportion of „free“ cortisol

The tissue concentration of cortisol is context sensitive.

Cortisol

\[
\text{11\beta-hydroxysteroid dehydrogenase}
\]

\[
\text{11\beta-hydroxysteroid + NADP} \rightleftharpoons 11\text{-oxosteroid + NADPH}
\]
<table>
<thead>
<tr>
<th>Sample matrix</th>
<th>Now</th>
<th>past</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saliva</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Faeces</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hair</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Claw</td>
<td></td>
<td></td>
</tr>
<tr>
<td>horn</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Time post stressor**

*Dairycare, Bern, E. Möstl 2015*
Excretion of $^{14}$C-Cortisol via faeces (orange) and urine (yellow)

- Sheep: 72% (faeces), 28% (urine)
- Pig: 93% (faeces), 7% (urine)
- Rabbit: 92% (faeces), 8% (urine)
- Horse: 59% (faeces), 41% (urine)
- Dog: 77% (faeces), 23% (urine)

Source: DairyCare, Bern, E. Möstl 2015

Palme+Mostl-2001-KTBL 403,9-17
Advantages of faeces as sample material

What happened a certain time (e.g.: 12 h in ruminants) ago?

- not stressful for the animal
- even possible in zoo and wild animals
- simple and easy to collect
- enables large scale, longitudinal studies
Delay of faecal peak radioactivity after infusion of $^{14}$C-Steroids

Sheep

Pony

Pig

Dairycare, Bern, E. Möstl 2015

Palme et al., 1996
Anim. Reprod. Sci. 43, 43-63
Metabolic pattern of $^{14}$C glucocorticoids in faeces after i.v. infusion

There is a species specific metabolic pattern of glucocorticoid metabolism.
Influence of sampling time

With increasing time after infusion of $^{14}$C-cortisol, the excreted metabolites increased in polarity.

TLC:
Consecutive chromatography using chloroform/acetone
$14 + 6$
$16 + 4$
$18 + 2$

Days after infusion

Progesterone
Cortisol

Lexen et al., Vet.Med.Austria 95 (2008), 64-71
HPLC/MS of $^{14}$C-cortisol metabolites

Faeces of sheep

Straight Phase HPLC

Molecular weight (LC-MS)

MW 300 = C$_{19}$O$_3$

MW 350 = C$_{21}$O$_3$

Cortisol MW = 362.5
Side chain cleavage
11,17-dioxoandrostanes (11,17-DOA)

11-oxoaetiocholanolone
Formula and structure

The difference in the formulas is a small broken line instead of a straight line, but the shape of the two molecules differ!
How to measure those substances? The tools:

- The analytical repertoire used is similar as in doping control

- Mass spectrometry (combined with CG or HPLC)

- Immunoassays (RIA, EIA or FIA)

Mass spectrometry and immunoassays….
Specificity of immunoassays - a problem of comparing results between labs/assays

Antibodies „palpate“ parts of the molecules

they are group specific

Dairycare, Bern, E. Möstl 2015

Möstl et al., 2005 Ann. N. Y. Acad. Sci. 1046:17-34
Validity of the assay

Problem of „blank“-values

Serial dilution

Dairycare, Bern, E. Möstl 2015
Stimulation of the adrenal cortex

Cow

ACTH (16 µg, i.v.)

What is best: Area under the curve or absolute value?

Days before and after ACTH-administration
Transport – stress

11,17-Dioxoandrostanes (nmol/kg faeces)

Days after transport

Dairycare, Bern, E. Möstl 2015
Variation of cortisol concentrations in the blood of cows (n=10)

Max/Min: 35
Variation of faecal cortisol metabolites' concentrations in cows (n=10)

Max/Min: 3.8
"Free" glucocorticoids are excreted

This is known for urine, but in faeces?

Metabolism in other organs

Adrenals

Liver

Urine

Faeces

Dairycare, Bern, E. Möstl 2015
Immunisation of sheep against cortisol

Plasma cortisol (ng/ml)

FCM (immunoreactive 11-oxoetiocholanolone) ng/g wet faeces

Before imm. 8 wks after imm. 18 wks after imm.

Minutes after ACTH

Hours after ACTH
Correlation plasma – faeces

- **Plasma**
  - Most of the cortisol is protein bound
  - Low concentration of metabolites

- **Faeces**
  - **Metabolites** of the unbound fraction of the hormone

Do faecal cortisol metabolites and plasma cortisol tell the same story?

Sheriff et al., 2010: Gen.Comp. Endo. 166; 614-19
Concentration of immunoreactive FCM after defaecation (cows)


Boldenone formation.. Arioli et al., 2008, Rapid comunications in mass spectrometry

Dairycare, Bern, E. Möstl 2015
Are glucocorticoid metabolites anabolic or catabolic in cattle?

“Improved feed efficiency and FCM levels over the finishing phase”

Montanholi et al., Livestock Science 155 (2013), 130–136


An androgenic substance in feces from cattle as demonstrated by tests on the chick.
Cortisol and 11,17-DOA in the bile (cow)
Standardisation of results

- Wet or dry weight?
- Assay used (important for comparison between labs)

Reference substance like creatinine?

? Influence of food/microorganisms on faecal glucocorticoid metabolites in dairy cows

' Biological sensitivity of the test is important!

Using a corticosterone assay for measuring glucocorticoid metabolites in faeces of cows?
References of our group related to faecal glucocorticoid metabolites

- **Reviews**
**Ruminants**


Ruminants

Ruminants

**Ruminants**


**Ruminants**

Cortisol is also produced in the thymus, the skin and the intestine

- The skin has an equivalent of the HPA-axis
- Hair cycle of the species has to be considered
- Contamination of skin (social licking, excreta)
- Cortisol or cortison or corticosterone?
- UV-light
Matrices

**Blood**: invasive, episodic pattern can be followed
**Saliva**: less invasive, episodic pattern can be followed

**Urine**: non invasive, but not easy to collect, episodic pattern can not be followed
**Faeces**: non invasive, easy to collect, episodic pattern can not be followed
**Hair**: episodic pattern can not be followed, no chronologically assignment for a short term stressor
Cortisol metabolites are inactive?

Some cortisol metabolites are active substances

$5\alpha$-reduced glucocorticoids: a story of natural selection
Nixon et al., 2012, J. Endocrinol.212, 111-27
Cows faeces show androgenic activity (Riley and Hammond, 1942 Endocrinology, 31)

**Results**

**♀**

**♂**

*Fin tubercles*

**male phenotype (%)**

5α-Androstan-3,11,17-trion 11-Keto-testosterone

**Concentrations in tank water (µg/l)**

<table>
<thead>
<tr>
<th>Concentrations in tank water (µg/l)</th>
<th>0</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK</td>
<td>0</td>
<td>50</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>


Dairycare, Bern, E. Möstl 2015
What about the 5ß-androstanol metabolites?

(Credit: iStockphoto/Dave Brenner)

11-oxoetiocholanolone is a pheromone in the round goby

In humans, etiocholanolone causes fever

(Experimental etiocholanolone fever. Schulman et al., 1964, JCEM)
I hope I convinced you that:

- Glucocorticoids are „anti-stress“ hormones and are not inherently bad
- Glucocorticoids are excreted as metabolites and the concentration of those substances is a good parameter for glucocorticoid production
- Some glucocorticoid metabolites are biologically active substances

Validity and biological sensitivity of the assay are implicit presuppositions for non-invasive monitoring!!
Future

- Role of microorganisms and interaction with diet. Are different diets causing different metabolism of steroid hormones?

- Digestive tract and its role in the metabolome of vertebrates.

Analytical aspects:

1) High throughput analysis
2) HPLC-MS/MS
Catecholamines are mainly excreted via the urine as shown by radiometabolism studies. Those radioactive substances are of high molecular weight (adducts of catecholamines to proteins).

Catecholamines form adducts with thiol- or amino-groups.

Catecholamines are therefore incorporated in proteins.

The half-life of these adducts is much longer than that of the catecholamine itself.

Epinephrine

\[
\begin{align*}
\text{HNCH}_3 & \\
\text{CH}_2 & \\
\text{HCOH} & \\
\text{HO} & \\
\text{OH} & \\
\end{align*}
\]

5-Cystein-S-yl-epinephrine

\[
\begin{align*}
\text{HNCH}_3 & \\
\text{CH}_2 & \\
\text{HCOH} & \\
\text{HO} & \\
\text{OH} & \\
\text{S} & \\
\text{NH}_2 & \\
\text{COOH} & \\
\end{align*}
\]
Research group „Stress“