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Monitoring stress hormone metabolites as a useful, non-invasive tool for welfare assessment in farm animals

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Abstract

A multitude of endocrine mechanisms are involved in coping with challenges. Glucocorticoids, secreted by the adrenal glands, are in the front line of the battle to overcome stressful situations. They are usually measured in plasma samples as parameters of adrenal activity and thus of disturbance. Unfortunately, collecting blood samples itself can disturb an animal. Thus, non-invasive methods for the determination of glucocorticoids or their metabolites have become increasingly popular. The pros and cons of various non-invasive sample materials (saliva, excreta, milk, hair/feathers and eggs) for glucocorticoid determination are given. Above all, faecal samples offer the advantage that they can be collected easily. In faecal samples, circulating hormone levels are integrated over a certain period of time and represent the cumulative secretion of hormones. Thus, the levels are less affected by short fluctuations or the pulse-like nature of hormone secretion. However, using this technique to assess an animal's adrenocortical activity is not especially simple. Whether frequent sampling is necessary or single samples will suffice depends upon the study's aim (whether one is examining the impact of acute or chronic stressors). Background knowledge of the metabolism and excretion of cortisol/corticosterone metabolites is required and a careful validation for each species and sex investigated is obligatory. The present review also addresses analytical issues regarding sample storage, extraction procedures and immunoassays and includes a comprehensive list of published studies (up to 2011) describing the use of such methods in farmed animals. Applied properly, non-invasive techniques to monitor glucocorticoid metabolites in faecal samples of various species are a useful tool for welfare assessment, especially as they are easily applied at farm or group level.

Keywords: *animal welfare, corticosterone, cortisol, faeces, farm animals, stress*

Introduction

In recent years, there has been growing interest in and concern about animal welfare. However, the assessment of animal well-being is a complex matter (Rushen *et al* 2011). Although good welfare is more than the absence of stress, stress plays an important part in welfare research (Broom 2001). The most often used nomenclature defines environmental stimuli that lead to an imbalance of homeostasis as 'stressors' and the corresponding defence reactions of an animal as 'stress responses' with the brain having the central role in linking stressors to responses (Möstl & Palme 2002). Responses include behavioural changes, changes to the immune system, and activation of the neuroendocrine system (hypothalamic-pituitary-adrenal [HPA] axis) and the autonomous nervous system (ANS), Moberg (2000). The range and complexity of changes can differ markedly between species, individuals and stressors and can vary according to prior experience and stage of life history (Cook *et al* 2000; Sheriff *et al* 2011). It is important to note that stress responses are not inherently bad as they help an organism to cope with its environment and challenging situations. However, if activated too much or for too long some may have detrimental effects on the organism, resulting in impaired biological functions (eg reproduction, immunity and growth; Moberg 2000).

Evaluating stress responses — HPA axis

As activation of stress responses is context dependent (eg the HPA axis may be activated during beneficial or detrimental circumstances), measurement of a single parameter alone may be misleading (Broom & Johnson 1993; Rushen 2000). Thus, it is common consensus that a combination of different measurements (eg physiological and behavioural) for evaluating stress should be considered (Rushen *et al* 2011). Catecholamines and glucocorticoids (secreted by the medulla and cortex of the adrenals, respectively) are released within seconds to minutes after a stressor. They are front-line hormones in the battle to overcome stressful situations. Both hormones are quickly metabolised and excreted via urine and faeces (El-Bahr *et al* 2005; Palme *et al* 2005; Lepschy *et al* 2008). Urinary catecholamine metabolites were measured in farm animals (eg Hay & Mormède 1998). However, activity of the ANS can be evaluated indirectly by heart rate (variability). This

measurement has proven a useful parameter for the ANS and is frequently applied (von Borell *et al* 2007).

The following focuses on glucocorticoids (GC), the end-products of the HPA axis. GC have been widely used in experimental welfare research in farm animals as an animal-based measure of welfare. Although GC represent a useful parameter, caution is advised in the interpretation of data as their concentrations may be influenced by a wide variety of factors (eg environmental factors such as temperature easily remain unrecognised). In this respect, also the individual animal and its sex, age, physiological stage and life history matters (for a more detailed review, see Mormède *et al* 2007; Sheriff *et al* 2011). Interestingly, for example, parturition in some species is triggered (foetus) and accompanied (mother) by an activation of the HPA axis (Möstl & Palme 2002).

Sample material for assessing adrenocortical activity

Traditionally, GC are measured in blood samples but their use is often limited as the act of sample collection may stress an animal (but see Cook *et al* 2000 for special, automatic devices to overcome this problem). Alternatively, other sample matrixes such as saliva, milk, excreta (urine and faeces), hair/feathers or eggs are available. Particularly in horses that are used to being handled in their mouths (although other animals such as pigs can be easily trained to accept handling of this kind), saliva samples can readily be collected (eg Schmidt *et al* 2010a,b,c). In addition, only the biologically active, unbound (free) GC fraction from the blood is present in the saliva. Milk is restricted to lactating animals and urine samples are unpractical to collect. Hair and feathers have recently been advocated as a sample matrix for long-term evaluation of stress, but there is currently insufficient evidence to conclude that their GC concentrations accurately reflect long-term plasma GC levels in animals (Sheriff *et al* 2011). Instead, there is growing evidence that the skin expresses an independent equivalent of the HPA axis, resulting in the local production of GC in hair follicles (Taves *et al* 2011; Keckeis *et al* 2012). Thus, hair cortisol could act as a parameter for skin stress (eg UV radiation; Skobowiat *et al* 2011) rather than reflecting systemic GC levels. Measurement of corticosterone in eggs as stress indicator (eg Downing & Bryden 2008) has also been performed (though restricted to laying hens) but only a small portion of plasma corticosterone enters the egg and high amounts of potentially cross-reacting gestagens may confound results (Rettenbacher *et al* 2005, 2009). The pros and cons of all the different sample materials have been discussed in detail elsewhere (Mormède *et al* 2007; Sheriff *et al* 2011). The following review focuses on the measurement of faecal cortisol/corticosterone metabolites (FCM) especially in farm animals as a non-invasive method for evaluating adrenocortical activity (but see also Möstl *et al* 2005; Palme 2005; Touma & Palme 2005).

Faecal samples offer the advantage that they can be collected easily without stressing the animal (Möstl & Palme 2002) enabling repeated measurement in individuals (Touma & Palme 2005). Background knowledge of the metabolism and excretion of glucocorticoids has been gained by several radio-metabolism experiments performed in domestic livestock (Palme *et al* 1996; Rettenbacher *et al* 2004) and other farmed animals (Malmkvist *et al* 2011). This has been important for the development and application of methods for measuring FCM. In faecal samples, circulating hormone levels are integrated over a certain period of time and represent the cumulative secretion of hormones compared to point estimates obtained from blood samples. They are less affected by short episodic fluctuations or the pulsed nature of hormone secretion (Palme 2005; Touma & Palme 2005). For example, in ten cows (*Bos taurus*) sampled frequently over 24 h, variations (min to max) in plasma cortisol levels were almost ten times higher than those in FCM levels (Palme *et al* 2003). In addition, FCM concentrations were found to reflect adrenocortical reactivity better than plasma GC levels, as only the increase above baseline in FCM was correlated with the administered dose of ACTH in cattle (Palme *et al* 1999). Thus, FCM are more a measure of the total amount of GC released, which is a function of both the maximum and duration of the release and is regarded the biologically important variable (Sheriff *et al* 2011).

Collection, storage and extraction of faecal samples

There is a time delay between increased plasma GC levels and their reflection in the excreted FCM (gut passage time from the duodenum to the rectum; Palme *et al* 1996). As a consequence, faecal samples offer the advantage of a *post hoc* evaluation (Touma & Palme 2005). This time delay is species dependent but may be influenced by the individual and other factors such as feed intake (Morrow *et al* 2002; Palme *et al* 2005). Knowledge of delay times in combination with the aim of a study is crucial for the experimental set-up. Thus, monitoring an acute stressor requires more frequent sampling (eg claw trimming in cattle; Pesenhofer *et al* 2006) than evaluating baseline adrenocortical activity and possible chronic stress (eg comparing housing conditions; Palme *et al* 2003).

Besides time of collection, the conditions under which the samples are stored are critical, as further bacterial metabolism of the excreted FCM has been reported (Morrow *et al* 2002; Möstl *et al* 2005; Lexen *et al* 2008). Thus, it is recommended to collect fresh faecal samples and freeze them immediately (< 30 min) after defaecation. Storing faeces in a transportable ice box before transferring them into a deep freezer may also help reduce possible metabolism by bacterial enzymes. Keeping samples frozen (–20°C) until analysis is necessary. Thawing at higher temperature proved favourable as bacterial enzymes are destroyed and thus FCM levels remain unchanged (Möstl *et al* 2005). As FCM are not evenly distributed within the faeces (Palme *et al* 1996), samples should be homogenised. Wet faeces are normally used for extraction, especially in domestic livestock where animals are fed standardised feeds.

Table 1 Validation and application of methods for measuring faecal cortisol/corticosterone metabolites in farmed animals: a comprehensive literature survey.

Species	Validated method used	Application (described)
Cattle	Palme *et al* (1999)[a]	Transport (Palme *et al* 2000)
		Social stress (Mülleder *et al* 2003)
		Claw trimming (Pesenhofer *et al* 2006)
		Milking system (Hopster *et al* 2002; Weiss *et al* 2004, 2005; Belo *et al* 2009; Lexer *et al* 2009)
		Housing (Palme *et al* 2003; Rouha-Mülleder *et al* 2010)
		Feed efficiency (Montanholi *et al* 2010)
		Pre-post partum period (Huzzey *et al* 2011)
	Möstl *et al* (2002)[b]	Transport/regrouping/manipulation (Möstl *et al* 2002)
	Morrow *et al* (2002)[c]	Feeding/housing/transport (Morrow *et al* 2002; Fisher *et al* 2003; Tucker *et al* 2007b; González *et al* 2008a,b, 2009; Webster *et al* 2008; Faleiro *et al* 2011)
		Milking (Tucker *et al* 2007a)
		Post-partum period (Alvarez-Rodriguez *et al* 2010)
Sheep	Palme *et al* (1999)[a]	Shearing (Lexen *et al* 2008)
Goat	Kleinsasser *et al* (2010)[b]	Feed barrier design (Nordmann *et al* 2011)
Horse	Möstl *et al* (1999)[a]	Pain (Merl *et al* 2000)
		Management/artificial insemination (Berghold *et al* 2007)
		Training (Gorgasser *et al* 2007; Jakubowska *et al* 2010)
		Husbandry system (Hoffman *et al* 2009)
	Flauger *et al* (2010)[b]	Transport (Schmidt *et al* 2010a,b,c)
Rabbit	Monclús *et al* (2006)[d]	Stocking density/enrichment/transport (Buijs *et al* 2011)
Mink	Malmkvist *et al* (2011)[e]	Housing/parturition (Hansen *et al* 2007; Malmkvist & Palme 2008)
		Stereotypic behaviour (Svendsen *et al* 2007; Malmkvist *et al* 2011)
Chicken	Rettenbacher *et al* (2004)[f]	Feed restriction (Janczak *et al* 2007)
		Transport/handling (Rettenbacher & Palme 2009)
		Stocking density (Buijs *et al* 2009)
		Animal welfare inspection (Kjaer *et al* 2011)

[a] 11-oxoaetiocholanolone EIA-I;

[b] 11-oxoaetiocholanolone EIA-II;

[c] corticosterone RIA;

[d] 5α-pregnane-3ß,11ß,21-triol-20-one EIA;

[e] 11ß-hydroxyaetiocholanolone EIA;

[f] cortisone EIA.

(NB In birds, faeces and urine are excreted together in the form of droppings).

However, one has to keep in mind that large differences in feed may alter FCM levels (Dantzer *et al* 2011).

For extraction (more precisely, suspension) most authors add mixtures of methanol (or ethanol) and water to the faeces (eg 0.5 g plus 5 ml 80% methanol; Palme 2005). After shaking (by hand- or multi-vortex) and centrifugation, an aliquot of the supernatant (sometimes after further dilution) is transferred to the immunoassay. If low amounts of FCM are present in the faeces an additional concentration step may be necessary (eg Merl *et al* 2000).

Analysis of FCM

As GCs are extensively metabolised prior to excretion, native GC are not present in the faeces. Instead their metabolites are found and it is these that are commonly measured by immunoassays (Möstl *et al* 2005). Thus, commercially available cortisol or corticosterone immunoassays also depend upon antibody cross-reactions to measure some of the metabolites. To date, only one radioimmunoassay (ICN corticosterone RIA, MP Biomedicals, Costa Mesa, CA, USA) has been validated and used in domestic livestock (cattle: Morrow *et al* 2002; but see also Table 1). Alternatively,

enzyme immunoassays (EIAs) have been developed that utilise so called 'group-specific' antibodies. Such antibodies have been specifically designed to measure groups of metabolites, so they have several advantages and have found a broad application (Möstl *et al* 2005; Touma & Palme 2005). The first EIA of this type was an 11-oxoaetiocholanolone EIA (I) that measures 11,17-dioxoandrostanes (11,17-DOA; Palme & Möstl 1997), which has been validated and successfully applied in ruminants (cattle, sheep [*Ovis ammon f. aries*] and goats [*Capra aegagrus hircus*]) and horses (*Equus caballus*). Another 11-oxoaetiocholanolone EIA (II; Möstl *et al* 2002), measuring faecal metabolites with a 5ß-3α-hydroxy-11-oxo structure, proved similarly suitable for use in the same species (for details see Table 1). In chicken (*Gallus domesticus*), mink (*Mustela vison*) and rabbit (*Oryctolagus cuniculus*) faeces, other group-specific EIAs (see Table 1) were found to be best suited for evaluating adrenocortical activity. A 5α-pregnane-3ß,11ß,21-triol-20-one EIA (first developed for mice [*Mus musculus*]; Touma *et al* 2003) has been used with success in rabbits and in a large number of studies on laboratory rodents (see Touma *et al* 2004 and Lepschy *et al* 2007 for a validation in mice and rats [*Rattus* spp], respectively), some of which dealt with animal welfare issues (eg Akre *et al* 2011; Kolbe *et al* 2012).

Whatever immunoassay is used, besides an analytical validation (including cross-reactions, sensitivity, precision etc) of the assay, it is necessary to provide a successful physiological validation before a method can reliably be applied (Palme 2005; Touma & Palme 2005). The preferred technique for this is a hormonal challenge (ACTH stimulation) test. The resulting pattern of sharply increasing (and later decreasing again) plasma GC levels should be clearly reflected in the concentration of FCM after a certain lag time (Touma & Palme 2005). Due to differences in GC metabolism and excretion between species and sometimes even between sexes (Palme *et al* 2005), this must be performed for each species and sex investigated. It is also important to recognise that concentrations of FCM measured by immunoassays are, due to the diverse mixture of GC metabolites present in the different species, always relative measures. Thus, comparisons within the same species (sometimes even sex) are only possible if exactly the same method (including extraction) is used. As a consequence, it is not possible to give general reference values of FCM and thus threshold levels for stressful situations. Nevertheless, FCM levels between different situations can easily be compared as long as they were measured by the same method (see examples below).

Applications of FCM analysis

An increasing number of studies deal with animal welfare issues in farm animals. In addition to other parameters, such studies utilise FCM analysis, many of them highlighting the advantages of this non-invasive method. For example, a large-scale on-farm study in cows (n = 207) compared two different devices for restraint during functional claw trimming (acute stressor). FCM concentrations were significantly higher in cows trimmed with a mobile walk-in crush than with a tilt table. This fact, together with a higher evasion score and a longer time needed to trim the cattle in the walk-in crush, renders the tilt table less disturbing and therefore better suited for claw trimming (Pesenhofer *et al* 2006). Cortisol metabolites analysed in frequently collected samples during and after different modes of road transport proved useful in several studies in cattle and horses (Palme *et al* 2000; Möstl *et al* 2002; Schmidt *et al* 2010a,b,c). In the latter salivary cortisol and heart rate was also measured.

As an integrated measure of adrenocortical activity, FCM are especially useful and frequently used to compare different housing conditions (chronic stressors). For example, cows on a concrete yard were reported to have lower bodyweight and higher FCM than cows on woodchip pad (Fisher *et al* 2003). Cattle (seven farms) housed on straw yards had significantly lower FCM concentrations compared to standard cubicle-housed herds (22 farms; Palme *et al* 2003). Tucker *et al* (2007b) reported that during winter weather, cows exposed to cold and wet conditions (outdoor) spent less time lying down and had higher FCM concentrations than when they were kept indoors (see also Webster *et al* 2008).

Table 1 gives a comprehensive overview of all respective studies reported in the literature (up to the end of 2011). They have been performed in cattle, sheep, goats, horses, chickens, rabbits and farmed mink. Although an increase of FCM after ACTH injection has been found in some pigs, it was not observed in others (Möstl *et al* 1999). Thus, pigs are the only species investigated, to date, where an FCM measurement has not been achieved, although salivary cortisol may instead be used as a parameter of HPA activity. Recently, the advantages of measuring FCM have also been acknowledged in studies dealing with newly farmed animals such as deer species (Christofoletti *et al* 2010; Konjević *et al* 2011).

Conclusion

Responses to stressors are complex and context dependent and therefore a combination of different measurements (eg physiological and behavioural) for evaluating stress should be considered. Applied properly, non-invasive techniques for monitoring glucocorticoid metabolites in faecal samples are a useful tool for welfare assessment in various species, especially as they are easily applied at farm or group level. Inter-disciplinary approaches using such methods can advance our understanding of the biology of stress and related animal well-being.

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