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Can non-invasive glucocorticoid measures be used as reliable indicators of stress in animals?

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Abstract

Refinement techniques are being increasingly employed in all fields of animal research to try to ensure that the highest standards of welfare are upheld. This review concerns one of the main emerging techniques for the assessment of welfare itself, namely the non-invasive measurement of glucocorticoids (GCs) as indicators of stress. The paper is divided into three sections. The first discusses the relationship between GCs and stress. The second section considers whether factors other than stress are linked to rises in GCs, eg exercise, oestrus cycle and diet. The final part examines the reliability of the non-invasive techniques that measure GCs from samples of saliva and faeces. Although it is important to take into account some caveats associated with the methodologies employed, it is concluded, nevertheless, that these techniques can give accurate and reliable information regarding the welfare status of an individual or group of animals without the procedures themselves causing any kind of distress to the subjects.

Keywords: *animal welfare, cortisol, faeces, non-invasive, refinement, stress*

Introduction

The assessment of welfare in animals is a developing field of study with new methods being devised and tested. It is now widely accepted that no single measure is sufficient and that in order to achieve an accurate and robust evaluation of the welfare of an animal a multifactorial approach should be taken, entailing the use of both behavioural and physiological parameters. Although there are many physiological indicators of a body under stress that can be measured non-invasively, it is usually not feasible to measure them all due to financial and/or temporal constraints. It is important, therefore, to determine which of the options are the most reliable, appropriate and accurate indicators of animal welfare.

It has been established for decades that stressful experiences cause the synthesis and release of glucocorticoids (GCs) (cortisol and/or corticosterone) from the adrenal gland (Seyle 1935). Although there is no dispute over this physiological event, the value of using levels of GCs in assessing the welfare of animals is sometimes overlooked as it is thought that these hormones are affected by too many other factors to be reliable stress indicators. This omission is especially unfortunate in the case of the non-invasive assessments of salivary and faecal GCs that have clear welfare benefits. The aim of this paper is to briefly discuss the most commonly raised questions that cast doubt on the validity of using GC levels to indicate animal stress; particularly measures from salivary and faecal samples.

Stress and glucocorticoids

When do stress parameters indicate poor welfare?

Stress is an integral part of all animals' lives and the body has developed many mechanisms to help it cope with both psychological and physical stressors (see Broom & Johnson 2001 for review). Hormones, such as GCs linked to the stress response, have beneficial and protective effects on the body under normal conditions and are vital for the normal functioning of the body on a day-to-day basis. Their functions include the production of glucose for energy, immune reactions and anti-inflammatory activity (Munck *et al* 1984). Their importance is demonstrated by the fact that the lack of these hormones, in conditions such as Addison's Disease, proves fatal, both in animals and man, without appropriate treatment. However, the extensive or prolonged production of GCs can cause wide-ranging and deleterious effects on the psychological and physiological health of animals, and in such cases the animals are said to be suffering from stress.

The definition of stress is difficult and contentious since the term is often used to indicate a whole range of symptoms and levels of suffering. The medical definition of stress is termed as "...reactions of the body to forces of a deleterious nature… that tend to disturb its normal physiological equilibrium" (Steadman's Medical Dictionary 1995). However, this is an oversimplification since disturbing homeostasis in the body does not necessarily lead to adverse effects. To clarify this point McEwen and Wingfield (2003) suggest the use of new nomenclature for stress biology. They argue that

the body's ability to cope should be the ultimate factor in deciding whether or not the animal is under 'stress' and that the terms 'allostasis' (the maintenance of homeostasis through change) and 'allostatic overload' (the state in which energy requirements exceed the capacity of the animal to replace that energy from environmental resources) should be employed. Furthermore they suggest that only in the latter of these cases should the term 'stress' be used. The logic behind this is quite simple; if the body can react to a stressor and retain homeostasis using reserves of biological resources without impacting on other biological functions, then the cost to the animal is minimal and using the term 'stress' to describe such events is confusing. However, if the energy required cannot be met from body stores and other biological functions (eg reproduction) need to be halted or reduced in order to provide the necessary resources, the persistence of this condition can be to the detriment of the animals' welfare. In this paper the term 'stress' is used only when adverse welfare is predicted due to disruption to the normal functioning of the animal.

The production of glucocorticoids

Stress activation of the different systems of the body includes the stimulation of the hypothalamic-pituitary-adrenal (HPA) axis. Initially this involves the synthesis of corticotrophin releasing hormone (CRH) from many areas of the brain (eg hypothalamus, amygdala, pre-frontal cortex) which is then secreted from the neurosecretory cells of the hypothalamus. This in turn stimulates the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland which acts directly on the cells of the zona fasciculata in the adrenal cortex to produce the GCs. This is all controlled by the GCs inhibiting ACTH and CRH secretion through a series of negative feedback loops.

Do the actions of glucocorticoids differ in acute versus chronic stress?

In most circumstances the effects of chronic stress are more likely to cause welfare problems than acute stress. Chronic stress is of particular concern since it can have major deleterious effects on the general health of the animal. For example, chronic stress can make the animal more prone to infections due to its suppression of the immune system; whereas acute stress actually enhances immune function leading to protection against disease. In addition, long-term elevation of GCs can have severe effects on the central nervous system. While acute stress enhances the memory of events that are potentially threatening to the organism, chronic stress causes adaptive plasticity in the brain whereby local neurotransmitters and systemic hormones interact to produce structural as well as functional changes (eg the suppression of ongoing neurogenesis in the dentate gyrus and the remodelling of dendrites in the Ammon's horn [Erickson & Drevets 2003]). The cells of the hippocampus are particularly responsive to GCs and high levels of these hormones can selectively reduce GC receptors in this part of the brain (Romero 2004) leading to dendritic atrophy. Such remodelling can result in impairment of functions and mood disorders (Beck *et al* 1994; Sapolsky 1996a). Chronic stress is, therefore, more likely than acute stress to lead to pathological conditions of major concern for studies of animal welfare.

However, acute stress cannot be ignored because it may cause major welfare problems depending on the severity and timing of the event. For example, acute stress is known to disrupt ovulation (Rivier & Vale 1984; Rivier *et al* 1986), which could have major implications in animals that only have a single oestrus event. Similarly an acute stressor in pregnancy can cause spontaneous abortion or premature parturition (Sapolsky *et al* 2000). The effect of an acute stressor can also have long-term psychological effects on an animal resulting in symptoms more classically associated with chronic stress. For example, dogs wearing shock collars exhibit stress-related behaviours that persist even after removal, with animals continuing to show outward signs of stress for long periods (presumably due to the unpredictability of the painful stimulus [Polsky 1994; Schilder & van der Borg 2004]). Confounding factors can also mean that acute stress events lead to pathological conditions. This is particularly apparent in animals with a heightened immune system (eg fighting infection), which are then subjected to an acute stressor. Laugero and Moberg (2000) demonstrated that mice injected with lipopolysaccharide (as a model of mild infection) and then subjected to a restraint stress showed suppressed growth and metabolic reduction to a far greater extent than either stressor acting alone.

Since both acute and chronic stress can lead to adverse effects and pathological conditions, the key to using GCs in assessing welfare must lie in ensuring that repeated sampling is used wherever possible; a single stand-alone measure of a GC can be misleading. Repeated measures of these hormones provide a more accurate assessment of whether the welfare of the animal is being compromised.

How are the functions of glucocorticoids mediated by different receptors?

GCs have two very different modes of action depending on the types of receptor through which the effects are mediated (Romero 2004). The receptors are located in the limbic system of the brain. The immediate response of the body to a stressor is via the Type-1 or mineralocorticoid receptors (MR) that mediate the behavioural, sympathetic and HPA response in the short term. The effects of GCs in this instance are to directly stimulate and facilitate other systems (eg catecholamines) associated with the coping mechanisms of the body (eg cardiovascular system) in order to retain homeostasis (de Kloet 2003). The MRs have a high affinity for GCs and are found in relatively low numbers. Therefore they become quickly saturated at peak circadian levels and are not available in chronically stressed situations. In these instances the GCs bind to the Type II or Glucorticoid Receptors (GR). These have a lower affinity for GCs and hence GCs will only bind to them when the MRs are fully saturated. GRs are associated with the accepted effects of

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GCs that include mobilisation of energy resources, gluconeogenesis and the inhibition of inflammatory responses. In addition GRs prepare the body for future stressful events by mediating behavioural adaptations and promoting the storage of energy (Sapolsky *et al* 2000). The balance between these two types of systems is thought to be essential for homeostasis and imbalance due to chronic stress can have implications for health as well as adverse effects on mental well-being (Romero 2004). In essence, if only MRs are activated there is no likelihood of adverse effects from GCs being released. Although the study of receptor numbers and dynamics is not a practical consideration in animal welfare studies, the activation of GRs can be presumed when levels of GCs exceed those normally found under basal conditions (Romero 2004). It is for this reason that whenever GCs are being used as a research tool in the measurement of stress, hormone basal levels are known for the species (or, preferably, the individual) in question.

Are circadian rhythms of glucocorticoids important?

Like most hormones circulating in the body GCs are produced in a circadian manner under basal conditions (Fulkerson & Tang 1979; Cavigelli *et al* 2005). This circadian rhythm is ACTH-dependent and is demonstrated with peak levels pre- and post-wakening and a nadir immediately prior to the main sleep period. In diurnally active animals this translates as a peak early in the morning with a plateau phase until a fall at night time; this pattern is thought to be linked to gearing the body for action in the morning with the drop at night time facilitating uninterrupted sleep. This rhythm can be disrupted by chronically stressful conditions or events (Fulkerson & Tang 1979). Stress-induced changes to the rhythm normally manifest themselves through a blunted morning peak and a higher plateau phase, thus resulting in an overall flatter pattern. Alterations of this kind have been demonstrated in both man and animals suffering from chronic stress, for example post-traumatic stress disorder (PTSD) in man (Yehuda *et al* 2005) and pigs housed under barren conditions for prolonged periods (Ruis *et al* 1997). Although short-term stress (eg transportation) leads to an immediate increase in GCs it does not seem to affect the overall pattern of circadian output (Becker *et al* 1985). Similarly, under basal conditions the GC circadian rhythm remains relatively stable, although young and adolescent animals can show a less robust rhythm (Ekkel *et al* 1996).

Why should glucocorticoids be measured in preference to other stress-linked hormones?

Many hormones are linked to the stress response and all of these have been investigated thoroughly. Stress has been shown to be a huge factor in disease aetiology and it is for this reason that much of the research that underpins stress physiology has its origins in clinical studies. Cortisol is routinely used in clinical evaluations and is medically termed 'the stress hormone'. The study of anxiety and stress-related illnesses remains complex and relatively poorly understood and is well beyond the scope of this paper. However, the importance of GCs in relation to certain depressive states does demonstrate the robust connection these hormones have with the perception and response of the body to stressful events even though they are by no means the definitive indicators of these conditions. For example, cortisol has been shown to be chronically elevated in anxiety illnesses such as depression (eg Bakke *et al* 2004; Tse 2004; van Praag 2004) and is also used as a measure of post-traumatic stress disorder (Sher 2004). Moreover it has been shown that very high levels of cortisol are present in people who are suicidal (Westrin *et al* 1999) as well as in individuals who rarely experience joy and pleasure (Vincent 1994; Messina *et al* 2003; Luby *et al* 2004). These findings have considerable implications for the welfare of animals that demonstrate high levels of these hormones. Learned helplessness in animals is perhaps the closest correlate to depressive states in humans and very high levels of GCs have been found in animals suffering from this condition (Gregory 2004).

Unlike other hormones associated with cardiovascular regulation, such as adrenaline and endorphins, cortisol does not seem to increase in many activities that would be deemed pleasurable as opposed to stressful (Few 1974; Hawkes 1992; Esch & Stefano 2004); for example, sexual excitement in humans has no influence on cortisol (Exton *et al* 1999) whereas levels decrease when we laugh (Berk *et al* 1988). Similarly what we would perceive to be pleasurable experiences for animals, such as environmental enrichment in capuchin monkeys (Boinski *et al* 1999) and the petting of dogs (Hennessey *et al* 1998) have been shown to reduce the GC response.

The fact that GCs are so closely linked to stressful events, rather than being consistently produced in all energy heightened states, is, perhaps, not surprising when we consider the action these hormones have on the body. GCs cause catabolic production of glucose by breaking down muscle protein, directing this energy resource towards the central nervous system and simultaneously releasing fatty acids for muscle use. This combination of actions is very energy demanding and results in a cost that the body is not going to pay unless absolutely necessary.

Are factors other than stress linked to rises in glucocorticoids?

Are glucocorticoid levels affected by species differences?

Corticoids differ from species to species, not only in the amount of circulating hormone but also in the type of GC, ie whether it is predominately corticosterone (eg rats), cortisol (eg man), or an equal mixture of both (eg pig). Furthermore, as basal levels of GCs are seen to vary hugely between species it precludes any direct species comparisons (Schatz & Palme 2001; von der Ohe & Servheen 2002; Meyer *et al* 2004). However, the fact that stressful events increase GC levels is universal and has now been demonstrated in every vertebrate genus (Klein 2000). The basal

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GC levels of a species need to be assessed before any measurement or inference of stress can be made and stand-alone time point samples cannot be compared between different species. This, however, is analogous to most physiological and behavioural parameters.

Are glucocorticoid levels affected by sex differences?

There have been relatively few studies that have investigated gender differences in GC levels. The available data however, suggest that over a range of species (eg monkey [Tilbrook *et al* 2000], alligator [Medler & Lance 1998], sheep [van Lier *et al* 2003], deer [Huber *et al* 2003] and tortoise [Lance *et al* 2001]) only minor differences exist in the basal levels of GC between males and females. Similarly, many clinical studies of the stress response have shown that the basal cortical levels of men and women do not differ significantly (eg Kirschbaum *et al* 1999) although women do tend to have slightly raised levels compared to men. However, these clinical studies have found significant sex differences in GCs when the body is challenged (eg Klein *et al* 2000). ACTH challenges in women evoke larger cortisol responses than in men (Silva *et al* 2002), and this phenomenon has also been demonstrated in sheep (van Lier *et al* 2003). This difference is probably linked to the positive feedback effect that oestrogens have on the HPA axis (Coe *et al* 1986). Oestrogens act positively on the anterior pituitary gland by both stimulating ACTH production directly and increasing the reactivity of the gland to CRH, thereby stimulating GC production (Kitay 1963).

Interestingly, when specific stressors are implemented, men and women are shown to offer differing responses based on the type of stressor. For example, women show a significantly greater cortisol response to social rejection stressors (Kirschbaum *et al* 1992; Stroud *et al* 2002) while men show larger responses to achievement challenges, such as mental arithmetic (Stroud *et al* 2002). The differential GC response to stress between men and women, therefore, seems to be related to the perception of the stressor rather than the stressor itself. Whether perception of stressors differs with gender in animals is yet to be determined but it would appear likely due to the differing social stressors that males and females are exposed to. For example, males might show a greater response to a conspecific challenge due to sexual competition pressure (see Schaffner & French 2004) whereas females may respond more to a predator's call due to their protective maternal instincts (see Blanchard *et al* 1998, Romero 2002). Studies have also shown that there are sex differences in the ratios of GC metabolites in the urine of women and men (Raven & Taylor 1996; Zimmer *et al* 2003) and in faecal samples of mice (Touma *et al* 2003; Touma *et al* 2004).

As both sexes show increases in GCs to the same stressors it is possible to use these hormones as indicators of stressful events within mixed sex populations. However, gender differences in the respective magnitudes of the GC response to a given stressor should be borne in mind when analysing data; and separating the sexes will aid interpretation of the results.

Are glucocorticoids affected by oestrus cycle and reproduction?

By tradition many laboratory studies of endocrine stress responses have used only males to ensure that oestrus cycling could not be a confounding factor in the results. However several studies have found that the oestrus cycle seemingly has little affect on GC levels in a variety of species that includes tortoises (Ott *et al* 2000), sheep (Orihuela *et al* 2002) and hyenas (van Jaarsveld & Skinner 1992) under both basal and stressful conditions. Nevertheless, as already stated, there is a physiological link between oestrogens and GCs and in some cases the interaction between these hormones can have an effect on the resulting GC levels, particularly if oestrogen levels are high (Cavigelli *et al* 2003). Therefore, caution must be exercised when GCs are measured in cycling females even though it is, by no means, an impossible task.

Pregnant animals are an altogether different matter. Increases in the level of GCs are directly linked with increases in oestrogen levels during gestation, probably as a direct result of the positive effect of high levels of oestrogens on the HPA axis (eg Coe *et al* 1986; Stavisky *et al* 2001) and the release of CRH from the placenta (McLean & Smith 1999). This relationship is particularly robust, so much so that some studies have attempted to use GC levels as indicators of parturition (Hodges 1998; Sanson *et al* 2005). Thus GCs should never be used as indicators of stress during the latter stages of gestation.

The influence of the onset of mating and associated behaviours on GC levels in females is unclear. Although Schmil and Rissman (1999) found increases in GCs in conjunction with sexual receptiveness in female musk shrews, on the whole females show no changes in GC levels during or post mating, and in the majority of species there is also no effect prior to mating (eg Coe & Levine 1995; Strier *et al* 2003). In contrast to this the majority of the evidence in males suggests that the act of mating itself (eg Tamanini *et al* 1983; Howland *et al* 1985; Rabb *et al* 1989; Borg *et al* 1991; Colborn *et al* 1991; Levis *et al* 1995) and, in certain species, the onset of mating seasons (eg Strier *et al* 2003; Sands & Creel 2004) have positive effects on GC levels. These GC increases in males may be attributed to increases in aggression and conflict during mating periods and other changes in the endocrine system associated with the mating act. However, this theory is contradicted by one study in which no changes in GCs were found in the silver fox after mating although significant increases in testosterone and oestradiol were detected (Osadchuk 1996). Hence, the use of GCs as measures of external stressors in males during mating periods may also prove inaccurate.

Are glucocorticoid levels affected by diet?

Diet is easily controlled in laboratory animals and tends to be relatively consistent within, and often between, establishments. However, fluctuations are often seen in the diets of farm, zoo and especially wild animals, and, although it's possible to document these variations in zoo, laboratory and

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farm animals, it is seldom possible for wild animals. It is clearly important that the dietary effects on GCs are known and taken into account. A number of studies have investigated the effect that varying dietary content has on salivary and serum GC levels. These have sought to cover a wide range of nutritional factors and species of animal. These include: increased chromium ions in fish (Sahin *et al* 1997) and calf foods (Arthington *et al* 1997), increased lipid (Lochmann *et al* 2002) and nucleotide content (Leonardi *et al* 2003) in fish foods, increased dietary vitamin C in turtles (Zhou *et al* 2003), high-fibre diets in pigs (Rushen *et al* 1999), mineral content in the diets of hyenas (Dloniak *et al* 2004), and high fat diets in humans (Venkatraman *et al* 2001). Although all of these experimental manipulations involved considerable changes to the animals' dietary habits, not one of these studies revealed any significant effect on the levels of GCs. It would, therefore, appear that dietary changes that occur on a day-to-day basis do not have a major impact on the basal or stress levels of salivary or serum GCs.

Although dietary content does not markedly affect GC levels, the general nutritional state of the animal can have a considerable effect on the HPA axis. Clinical studies have demonstrated that people on restricted diets do have higher levels of GCs than normal (eg Hainer *et al* 2001; Anderson *et al* 2002). The same trend has been noted in animals when food resources are scarce (eg Abbott *et al* 2003; von der Ohe *et al* 2004). Variation in food availability could also be the underlying factor in studies that have found seasonal variation in GC levels (see below). Lack of sufficient food of an appropriate nature would be expected to increase GCs for two main reasons: First, starvation would be perceived as a large stressor from both a physiological and psychological standpoint and second, as already stated, GCs are needed to breakdown muscle protein to provide an energy supply once fat and carbohydrate stores have been depleted. Further possible effects of diet could be alterations to gut transit time, directly affecting the time lag between a stressful event and the presence of GCs in the faeces. However, as faecal GC metabolites are generally used as a measure of long term or chronic stress the fact that the lag time differs in the region of a few hours is generally not important (see later).

In summary, subtle changes in the day-to-day diet of both free-living and captive animals are unlikely to affect levels of GCs, but drastic changes in nutritional status leading to starvation will have a significant effect on GC levels.

Are GC levels affected by seasonality?

This is a difficult question to answer because seasonality has many diverse effects that are dependant on variables such as availability of food, weather clemency, presence of mating partners etc. Therefore in a study to investigate the effects of seasonality *per se* it may be impossible to tease out all the major factors that would have a direct effect on GCs, or indeed on any other welfare measure for that matter. For example, as already discussed, a severe lack of food can increase GC levels and such conditions are far more likely to show greater prevalence at certain times of the year compared to others.

Seasonal differences in GC basal levels have been demonstrated in animals with well-defined breeding seasons (eg Romero 2002) (see later). Other studies, however, have found no seasonal differences in basal GC levels across seasons. For example, over-wintering and autumn and spring migrations do not affect basal GC levels in sandpipers (O'Reilly & Wingfield 2003), although seasonal differences were found in the stress response of GCs when challenged.

When considering the effects of seasonality the real question is not whether or not seasonality affects GC levels, but whether different stressors are linked to different seasons. A few studies have investigated specific factors within different seasons rather than looking at seasonality as a whole. Increases in GC levels were most closely associated with low ambient temperatures and adverse weather conditions, such as snow (eg Romero 2000; Lanctot *et al* 2003). This would be expected since harsh environmental factors will reduce food availability, increase the metabolic stressors on the body and require catabolic production of energy resources; thereby necessitating an increase in GCs. In addition, different seasons in many species are likely to be associated with specific mating periods, which will result in altered effects on GC levels (eg Cooperman *et al* 2004) (see later).

Seasonality involves many factors that could be detrimental to welfare (Romero 2002) and, thus, measuring levels of GCs could be a useful tool for assessing the welfare of animals in differing seasons. However, if the welfare status of the animals in question is being assessed for factors extraneous to seasonality then it would be prudent to carry out such work where environmental stressors are likely to be few, for example, summer months with good food availability and ambient weather conditions.

Are GC levels affected by social status?

The establishment of social ranking within a group of animals may involve conditions that are stressful. Often determination of rank is decided through aggression and in such situations both the victor and the loser are likely to have experienced stress as a result (eg Abbot *et al* 2003; Schaffner & French 2004). Elevated GCs have been associated with both social subordination (eg Cavigelli *et al* 2003) and dominance (eg Correa *et al* 2003) especially in pack animals such as dogs (Creel 2001) and wolves (Sands & Creel 2004). Factors known to affect GC levels can also be modified due to social status. For example, in periods of low food availability nutritional deficits are far more likely to occur in subordinate animals and, as a result, GC levels would increase in these individuals. Muller and Wrangham (2004) concluded that the differences in GC levels in a group of chimpanzees were due to exposure to metabolic stress rather than social rank *per se*. In cases where the social ranking of the group is relatively stable, and hence there are few challenges by subordinates, the levels of GCs show no association with rank. This has been demonstrated

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in primates (eg Bercovitch & Clarke 1995; Stavisky *et al* 2001; Bercovitch & Ziegler 2002; Weingrill *et al* 2004; Bergman *et al* 2005) and in other social animals, such as Nile tilapia (Correa *et al* 2003) and the naked mole rat (Clarke & Faulkes 1998). In summary the majority of evidence suggests that social dominance is unlikely to have a significant effect on GC levels; any differences due to rank are more than likely to be relatively minor in comparison to a true stress response.

Do GC levels increase with exercise?

It has been suggested that exercise is a variable that needs to be considered when using GCs to assess the welfare of free-running animals. As one of the main functions of GCs is to break down proteins to synthesise glucose for energy, it would seem logical for these hormones to be involved in some way with the energy expenditure associated with exercise. However, in experiments where the level of exercise has been documented, significant increases of GCs have only been seen to occur at extreme levels of exercise; mild and moderate exercise seemingly having no effect on GC levels (eg McCarthy *et al* 1992; Stupnicki & Obminski 1992; Duclos *et al* 1997; Del Corral *et al* 1999; Jacks *et al* 2002). Such studies indicate that if energy expenditure can be met by fat mobilisation and carbohydrate stores then there is no measurable increase in GCs. However, if these stores are depleted and catabolism is required, as is seen during prolonged and heavy exercise, then GC levels are increased (eg Maestu *et al* 2003; Jurimae *et al* 2004; Ratamess *et al* 2005). Extreme exercise is, in itself, a major physiological stressor and, in such circumstances, the GCs are, in effect, acting as stress markers. The fact that GCs are raised only in exercise when catabolism is necessary is demonstrated by the fact that high intensity exercise of short duration (eg Volek *et al* 1997) and long duration exercise of low intensity do not cause a significant increase in these hormones (Kraemer *et al* 1989; Monnazzi *et al* 2002).

Are the non-invasive methods of measuring GCs reliable?

Non-invasive measures of physiology and behaviour would seem the ideal choice for assessing welfare both from a practical and an ethical standpoint. Urinary GCs are used relatively infrequently in contrast to salivary and faecal measures, mainly due to the practical problems of collecting this type of sample and the fact that the diuresis of the sample needs to be taken into account. Hence this section of the paper concentrates on salivary and faecal samples and their associated benefits and problems.

Do salivary GC levels accurately reflect plasma levels?

A few studies have argued that salivary GCs are not consistent with the GC levels measured in the plasma (Dorn & Susman 1993; Anderson *et al* 1999; Wong *et al* 2004) and have advocated the use of blood sampling to obtain GC measures. However, apart from the obvious welfare issues, there are other problems associated with measuring plasma GC levels. Cortisol in the circulation exists principally bound to cortisol-binding proteins (transcortins); only approximately 10% of plasma cortisol is ever actually in the 'free' state and it is only whilst in this free state that cortisol has any biological activity (eg Vining *et al* 1983). Most ELISA (Enzyme-Linked Immunosorbent Assay) and RIA (radioimmunoassay) techniques measure both the free and the bound cortisol (eg Cooper *et al* 1989). Although the ratio of free to bound cortisol remains constant under most normal conditions, during times of stress the total cortisol concentration increases and, as the binding proteins become increasingly saturated, the proportion of the hormone in the free form increases disproportionately to the total concentration (Cook *et al* 1997). Therefore, when comparing basal levels of plasma cortisol to those of an animal under stress the true extent of the increase is not realised. In contrast salivary cortisol is directly and accurately correlated with the free fraction of cortisol (eg Riad-Fahmy *et al* 1982; Aardal & Holm 1995). Cortisol enters the saliva mainly by passive diffusion and, as the bound fraction of the hormone is unable to cross the blood-saliva barrier due to its size, only the free hormone enters the saliva. Antibodies have been produced that will only recognise and measure the free form of cortisol and these can be used effectively for plasma measurement (Lewis *et al* 2003). The use of these antibodies has confirmed that the level of the hormone in saliva is a direct reflection of the free cortisol in plasma (Le Roux *et al* 2002). Consequently, measurement of salivary cortisol by ELISA or RIA is a direct reflection of the free, ie biologically active, hormone in the circulation. The clinical use of salivary cortisol has been commonplace since the early 1980s and has gradually gained acceptance as both a reliable 'stress-marker' and a diagnostic test of HPA malfunction in humans (eg Guechot *et al* 1982). The level of salivary GCs has, therefore, been shown to be an accurate measure of circulating levels and the non-invasive methods by which it can be measured have obvious practical and welfare benefits (Morgan Jones 1996).

Although the use of saliva provides many benefits by allowing the assessment of short-term stressors that may be difficult with other non-invasive techniques (eg faecal samples), there are some caveats attached to the use of this technique. First, the influence of the circadian rhythm on the levels of these hormones needs to be taken into account. Where possible this can be overcome by limiting sampling to the middle portion of the day (ie the plateau phase) and/or by taking saliva samples as time-matched replicates and controls. Second, although GCs in the saliva are independent of salivary flow rate, the time lag that exists between the appearance of GCs in the saliva after they have been released into the bloodstream is subject to individual variation (eg McCracken & Poland 1989; Shinkai *et al* 1993). This should not lead to any problems when one source of potential stress is being investigated since repeated sampling can be employed. However, it may pose a problem if the animal is exposed to a chain of stressors

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and in such cases it may be necessary to introduce pilot studies to determine lag times for each animal.

Are faecal GC levels reliable?

Particular sources of error and variability have been linked with the measurement of faecal GC metabolites and these include dietary effects on the gastrointestinal system and possible time lag factors when collecting samples from the wild. It has been suggested that dietary differences may affect the pooling time of steroids and increase metabolite variability (von der Ohe & Servheen 2002). While we should be aware of this possibility it is unlikely to have a major impact on the reliability of faecal measures. A day–to–day change in dietary fibre may have a significant effect on gastrointestinal time but, as faecal measurements of GC metabolites tend to be part of long-term studies, a change in the time lag of a few hours is unlikely to impact on the accuracy of the final data.

Millspaugh and Washburn (2004) have suggested that differences in faecal collection and storage time could have a significant effect on the relative amounts of GC metabolites measured in the faeces. Khan *et al* (2004) found increases in GCs over time, whilst Terio *et al* (2002) report decreases. Rather than storage time *per se* it may be that the method of storage has an effect on the measurement of faecal GCs; in particular the presence of water in the sample enabling metabolic processes to occur. This is demonstrated by fluctuations in GC levels occurring when samples are desiccated in a solar oven (Terio *et al* 2002), or when they are kept on ice for extended periods as opposed to immersion in liquid nitrogen (Tempel & Gutierrez 2004). Nevertheless, one of the main advantages of using faecal steroid hormone metabolites is that they are relatively inert, stable compounds. Studies have shown that faecal measurements of GC metabolites are not affected by: (i) time of day of faecal deposit, (ii) number of deposits, (iii) time elapsed between collection and analysis or freezing, (iv) time elapsed between deposition and collection, (v) elution by different solvents, and (vi) storage duration — with GC levels shown to be unaffected after 40 days at ambient temperatures and 400 days when frozen (eg Lynch *et al* 2003; Beehner & Whitten 2004; Mashburn & Atkinson 2004).

There may be a number of reasons for the variability found in the literature. First, species differences can be important, for example avian faecal samples contaminated with caecal material can yield much higher GC levels and, in addition, variability may be higher in bird species since the faeces and urine are excreted together in a manner that changes with species (eg Tempel & Gutierrez 2004; Goymann 2005). Second, samples tend to have large variations in water content and hence they should be lypophilised where possible. In addition, if wet samples are used and stopped from metabolising with the use of ethanol, then the storage of these samples for differing periods can affect the amount of metabolites that are present and detected, leading to another source of variation (Hunt & Wasser 2003) Third, the faecal samples of some species may not be homogenous and in such cases a sample should be taken from a homogenate of the whole bolus (Palme 2005). Fourth, and probably most importantly, is the significant effect of type of antibody and assay. One confounding factor of GC assays is that the assay measures metabolites rather than the whole hormone. It is, therefore, conceivable that the recognition of smaller GC metabolites by the antibody/assay could result in increases in GCs over time rather than decreases. It is vitally important that the antibody used is known to have a high cross-reactivity with the major faecal metabolites. It is also essential that if samples are not to be purified and separated by High Performance Liquid Chromatography (HPLC) (Palme 2005), then cross reactivity with other steroids and their metabolites needs to be discounted. Ignoring these factors has shown to result in high variability between samples which, in turn, has lead to the measurement of faecal GC metabolites receiving a bad press. It is essential, therefore, that before embarking on any research involving the measurement of faecal GCs the most appropriate antibody and assay is carefully selected and/or validation studies are carried out (see Wasser *et al* 2000; Mostl *et al* 2005; Palme 2005; Touma & Palme 2005). If this is done then the technique is reliable and faecal GC metabolites have now been validated over a large number of species using both ACTH challenges (eg Mashburn & Atkinson 2004; Morrow *et al* 2002; Touma *et al* 2004; Young *et al* 2004) and radio-labelling studies (eg Graham & Brown 1997; Mohle *et al* 2002; Turner *et al* 2002; Rettenbacher *et al* 2004; see also review by Palme *et al* 2005).

When faecal measurement of GC metabolites is carried out by a suitable and validated technique it can be an incredibly useful method with very few limiting factors. Faecal sampling is relatively simple and can usually be conducted without disturbing the subject and without interfering with other welfare measures running in parallel, for example behavioural assessment. As samples can be collected at leisure rather than at the specific time they are deposited, this technique has grown in favour particularly with zoo practitioners (eg Talling *et al* 2002; Mashburn & Atkinson 2004; Shepherdson *et al* 2004) and wildlife scientists (eg Armitage 1991; Teskey-Gerstl *et al* 2000; von der Ohe *et al* 2004; Gusset 2005).

Conclusions

The nature of the potential stressor needs to be taken into account when deciding whether GCs should be measured in saliva or faecal samples. In cases of chronic stress faecal samples are normally more reliable as they negate possible circadian effects and short-term stressors that could affect the overall profile. On the other hand short-term or immediate stressors are best investigated using salivary samples since these can be time linked more reliably to the stressor than faecal samples due to the variability in gut transit time. However, it is often the case that the type of sampling will be dictated by the species and/or the conditions in which the animals are kept. For example it is difficult to obtain saliva samples from a small animal like a mouse or from a dangerous animal like a tiger, and it is

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difficult to gain faecal samples from animals like pigs that are group housed under normal farming conditions.

One of the main advantages of using non-invasive measures of GCs is the fact that carrying-out repeated sampling need not be to the detriment of the animal, and, in the case of faecal sampling, with very little, if any, disturbance to their normal routine. A single stand-alone measure of GC is not an accurate method to assess stress or welfare. Sudden, ephemeral increases in these hormones could be attributed to a number of factors many of which would have very little impact on the welfare of the animal. The use of non-invasive technology, particularly faecal measures, not only allows many samples to be taken without fear of compromising the animals' welfare but also yields as accurate a representation of the animals' physiological status as possible.

The aim of this paper was to briefly discuss the factors most often credited with rendering GC measures unreliable. Some of these factors, such as starvation and exhaustion only have significant effects in the type of extreme circumstances that would generally be accepted as stressors in themselves. While others, such as species and/or gender, also affect most other welfare measures, including behavioural monitoring. Many factors influence behavioural and physiological welfare parameters and it is the responsibility of the investigator to minimise and be aware of such effects, as opposed to ignoring certain methods and/or disregarding results obtained through their use. In order to utilise any welfare measure the more information available on the lifestyle and experience of the individual animal the more accurate the measure is likely to be. However, even if a comprehensive data set is unavailable for a given animal concerning details such as social rank, it does not necessarily follow that welfare measures cannot be useful or accurate, as long as care is taken over the interpretation of any data gained. For example, in a recent study we investigated feather plucking in parrots (Owen & Lane 2006), behaviour usually ascribed to a stress response when all other physical factors (eg mites) have been ruled out. Faecal GC metabolites were compared between pet parrots (residing in their own homes) that did and did not self-feather pluck. There were significantly higher levels of faecal GC metabolites in the feather plucking parrots despite the fact that in this study diet, gender, housing, reproductive status and general lifestyle could not be controlled.

In conclusion, non-invasive GC measurement, especially when used in conjunction with other parameters such as behaviour, can give an accurate and important insight into the welfare status of an individual or a group of animals without the procedure itself causing any kind of distress or detrimental effects. It is clear that the use of non-invasive GCs should have a major place in animal welfare science.

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