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Non-invasive techniques for analysing hormonal indicators of stress

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Summary. - The growing concern over animal welfare has led to an increased awareness of the need to monitor and reduce stress in laboratory, zoo, and farm animals. To do so, valid and reliable methods are necessary. In the present work, we discuss non-invasive techniques for analysing hormonal indicators of stress, particularly glucocorticoids. Specifically, we describe methods for analysing samples of saliva, urine, and faeces, the collection of which, unlike blood sampling, does not represent a source of stress and thus a potential source of bias. We also address species-specific responses to stress and inter-individual variability, the reliability of specific hormonal assays, and the use of indirect measures of circulating stress hormones.

Key words: animal welfare, glucocorticoids, catecholamines, saliva, urine, faeces.

Riassunto (*Tecniche non-invasive per analizzare gli ormoni indicatori di stress*). - L'importanza dell'uso di metodi affidabili per misurare il benessere negli animali (in laboratorio, zoo e fattorie) attraverso lo studio del loro stato di stress è ormai riconosciuta. I metodi che studiano particolari ormoni in campioni di saliva, urine e feci, si sono rivelati come particolarmente adatti a monitorare la difficoltà che un animale può incontrare nel gestire stimoli ambientali avversi. Questi metodi, infatti, usando campioni alternativi al sangue, non presuppongono il contatto con l'animale e, quindi, evitano l'ulteriore e inutile stress che è spesso legato al prelievo di un tale campione. Verranno presentati sia esempi di studi che hanno usato con successo queste tecniche, sia i problemi relativi alle tecniche stesse, e, più in generale, all'uso di ormoni per monitorare lo stress.

Parole chiave: benessere animale, glucocorticoidi, catecolamine, saliva, urine, feci.

Introduction

The behavioural or psychological responses of a body to environmental stimuli can, in some cases, be minimal and have no adverse effects, whereas in other cases, and particularly when such responses occur over a prolonged period of time, they may reduce, or be likely to reduce in the future, fitness, a condition that can be useful for defining stress [1]. Monitoring stress in animals in captivity, such as those in laboratories, farms, and zoos, is extremely important, especially in light of the growing awareness of the need to improve animal welfare and considering that stressed animals are more susceptible to disease, as demonstrated by studies that have proven the relationship among the nervous and endocrine systems and immune responses [2, 3].

There has also been growing awareness of the need to assess stress using valid and reliable methods [4]. For example, given that coping strategies differ according to the duration of the aversive stimuli, different sets of measurements must be used when evaluating short-term or long-term responses. Not only is it important to use appropriate indicators of stress, but the stress itself must be reduced, which, considering the impact of stress on behaviour and physiology, ultimately improves the quality and reliability of data. In the present work, we focus on the hormonal response, particularly, the glucocorticoid response, to stressful stimuli, and on non-invasive techniques for measuring this response.

Hormonal responses to stress

In mammals, environmental stimuli activate the adrenal axes: the sympathetic-adrenal medullary system (SAM) and the hypothalamic-pituitary-adrenal cortex system (HPA). Measuring the activity of both of

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these systems can be quite useful when attempting to assess how an animal copes with short-term problems, whereas for long-term problems it is more appropriate to measure only HPA activity. Hormones produced by the adrenal medulla (i.e. catecholamines) and cortex (i.e. glucocorticoids) provide readily available energy for emergency actions by increasing the metabolism of carbohydrates, proteins, and lipids, providing the body with more glucose (catecholamines) and more amino and fatty acids (glucocorticoids). The activation of the HPA axis increases the plasma levels of glucocorticoids, and the specific glucocorticoid produced depends on the species. Cortisol is predominantly produced in primates, dogs, cats, and most ungulates, whereas corticosterone is produced in rodents and chickens; in some species (e.g. pigs), both glucocorticoids are produced [1].

As mentioned, long-term stress results in immunosuppression [2, 3], and attempts have been made to assess stress by analysing the components of the immune system (e.g. [5]). One study has shown that, in captive juvenile rhesus monkeys (*Macaca mulatta*), the immune system can be negatively affected by social separation and reunion [6].

The effects of stress on female reproductive function have long been known (e.g. [7]). The levels of some reproductive hormones, such as testosterone, prolactin, luteinizing hormone (LH), FSH, and naturally occurring opioids (e.g. β-endorphin), are affected by exposure to conditions with which the animal has difficulty coping. The effect on testosterone is unpredictable and thus of little use in attempting to evaluate stress. The effect on prolactin, LH, and FSH depends on the stage of the ovulatory cycle. For example, in monkeys, restraint inhibits LH secretion during the follicular phase (rhesus macaques [8]) yet it does not affect a major event such as the mid-cycle LH surge (rhesus macaques [9]). Plasma opioid levels usually increase during difficult situations, even to the point of suppressing extreme pain, yet they are not useful for assessing short-term problems ([1]; see [10] for references on the role of endogenous opioid peptide in mediating stress-induced effects on gonadotrophin secretion). The hormones that are most involved in the body's response to difficult conditions are glucocorticoids, which have been shown to be good indicators of stress in a wide variety of captive mammals [1, 11-13].

Levels of stress hormones in plasma

Several studies have assessed stress based on plasma corticosteroid levels [14]. For example, it has been shown that, in dairy cows, repeated jugular puncture increases cortisol levels [15]. In primates, which are highly social animals, there is evidence that low social status (or social isolation) may influence cortisol profiles, as suggested by plasma glucocorticoid levels. In wild male baboons (*Papio cynocephalus*), a low social rank has been found to be associated with hypercortisolism and resistance in the feed-back system [16], and a subordinate social status induces chronic low-intensity social stress (i.e. victim of more aggression, less affiliative interactions, more time spent alone), which results in HPA hyperactivity. Stressful events typically experienced by a captive animal (e.g. poor housing conditions, surgery, anaesthesia, and restraint) have been shown to increase glucocorticoid levels in carnivores (e.g. leopard cat, *Felis bengalensis* [17]; dog [18, 19]; cat [20]; and silver fox, *Vulpes vulpes* [21]) and primates (e.g. brown capuchin, *Cebus apella* [22], [23]).

The effects of stress are also reflected in the plasma levels of reproductive hormones. In a study on marmosets (*Callithrix jacchus*), restraining the animals for collecting blood samples was found to have been associated with marked decreases in LH levels, yet only when followed by aggressive encounters and only in individuals with a low social rank; physical restraint or an aggressive encounter alone had no effect on the LH levels [24]. In wild baboons, physical restraint and anaesthesia have both been reported to result in an increase in plasma cortisol levels, followed by a decrease in plasma testosterone levels [25].

Non-invasive techniques for assessing stress

Animal welfare is a complex matter, and accurately assessing it entails evaluating a wide range of physiological, endocrinological, and behavioural parameters. Although the study of behaviour constitutes a non-invasive means of assessing stress, it can be greatly improved by the knowledge of the underlying hormonal response. In fact, stressful stimuli do not always induce behavioural changes, and in these cases stress must be evaluated based on physiological data (e.g. [26]). A conventional means of measuring stress hormones is the analysis of blood, yet the procedures for collecting blood samples (i.e. capture, handling, restraint, and venipuncture) represent in themselves a source of stress and can thus alter hormone levels, as shown, for example, in rhesus monkeys [27] and rats [28]. Furthermore, since the act of taking blood induces an increase in plasma glucocorticoids within about 2 minutes, the blood sample must be extracted rapidly [1]. It must also be considered that plasma glucocorticoid levels may not be a good indicator, particularly if used to assess long-term stress. In fact, the feed-back regulatory system works to reduce the levels of stress hormones: high glucocorticoid levels tend to decrease glucocorticoid production by acting on the pituitary

gland and inhibiting ACTH production. Usually, high plasma glucocorticoid levels last no longer than 90 minutes [29, 30].

As an alternative to analysing blood, in various animals stress has been evaluated by measuring glucocorticoid levels (and in some cases immunoglobulin levels) in saliva, urine, and faeces, samples of which can be collected using non-invasive stress-free methods. The analysis of these biological materials also allows both short-term stress (salivary and, in some cases, urinary hormones) and long-term stress (urinary and faecal hormones) to be more accurately monitored.

Whereas for nearly all species free cortisol has been detected and measured in saliva, the hormone metabolites excreted in urine and faeces may differ by species and be preferentially excreted in one or the other excreta. In fact, for several domestic and non-domestic species, radioactive corticosteroids have been administered to monitor: the route of excretion (faeces or urine); the time course of excretion (time lag); and the type of metabolites in urine and faeces. These and other variables (e.g., the correspondence between the hormone levels in saliva, urine, and the levels detected in plasma) are discussed in the following sections.

Saliva

The use of salivary cortisol levels as a means of evaluating stress was first validated in humans (see [31] for a review) and has since been validated in other mammals (dog, [4, 26]; rhesus monkey [32]; squirrel monkey [33, 34]; and tree shrew [35]). Salivary cortisol levels are particularly suitable for assessing plasma cortisol levels because they accurately reflect the level of the biologically active free fraction of the hormone in the plasma [36], although salivary cortisol levels are relatively low, since only 15% of the unbound cortisol plasma fraction transfers into saliva. Samples are easy to collect and store, and sampling can be performed frequently, which is particularly important as it allows acute stress to be monitored by measuring short-term changes in cortisol levels. Cortisol is a small and lipid-soluble molecule which easily transfers from the blood stream, through cells, into saliva. Moreover, since cortisol enters the saliva through passive diffusion, with no involvement of active transport mechanisms, the saliva flow rate does not affect salivary cortisol levels. This has been proven in humans [36] and indirect evidence has been obtained in dogs [4].

Some laboratories still collect saliva samples using some form of restraint (e.g. in dogs, saliva collected from cheek pouches by sucking through a collection tube [4, 26]) or through opportunistic methods (e.g. saliva collected by Q-tips moved over the gums in squirrel monkeys seated on a primate chair for a behavioural study [34]). However, methodological improvements have now made it possible to collect saliva samples from free-moving individuals (e.g. rhesus monkey infants [32] and adult male rhesus monkeys [33]) by training or allowing the animals to do the following: chew or suck, for a certain amount of time, on pre-treated cotton dental rolls, which in some cases are attached to a "pole apparatus" [26, 32, 35]; or lick a gauze pad, attached to a "screen apparatus" sprinkled with dry Kool-Aid and covered by a mesh [33]. Saliva is then retrieved by centrifugation. Samples are usually stored within one hour of collection at a temperature between -20 °C and -28 °C and then analysed using radio-immune assays (RIA). In Table 1, some examples of techniques used to collect, store, and analyse saliva samples are provided.

Some studies have compared hormone levels in saliva and plasma. In dogs, for example, following the chemical stimulation of the HPA axis by injecting ACTH, a significant correlation was found between plasma and salivary cortisol levels in samples collected at 0.25, 0.5, 1, 2, and 2.5 hours [26]. The use of salivary cortisol levels in dogs was also validated in a study that used a model of insulin-induced hypoglycaemia, which stimulates the HPA axis (and the SAM axis, see below) [4].

When measuring cortisol levels, the time pattern of production must be taken into account. Although in captive infant rhesus monkeys diurnal salivary cortisol levels have been shown to peak at around 2:00 p.m. [32], in mammals, the production of basal cortisol generally peaks at the beginning of daily activity, which can be explained by a circadian timing system that has evolved to help the organism predict daily environmental challenges, such as food seeking and consumption [38].

In socially inexperienced male tree shrews, the psychosocial stress induced by singly transferring the animals as intruders to another animal's cage for one hour a day for 28 days was shown to be reflected by salivary cortisol levels, which were measured before and during the exposure to stress and after recovery [35]. In the same animals, salivary cortisol levels collected before and after a cognitive task during non-stressful (control) and stressful conditions did not differ.

In humans, a decrease in salivary IgA levels has been shown to be a reliable indication of stress in several situations, such as school examinations [39], childbirth [40], and intense physical stress [41], whereas relaxation has been shown to increase salivary IgA levels [42]. The measurement of salivary IgA levels has become an increasingly common means of

Table 1. - Examples of techniques used to collect, store, and analyse saliva samples

Species (housing at time of collection)	Collection method (duration)	Minimum quantity	Storage	Hormone	Assay	Reference
Tupaia (single caged)	Flavoured cotton roll (chewed at least 5 sec)	-	-28 °C	cortisol	in house immune assay	[35]
Infant rhesus monkey (single caged)	Flavoured cotton roll (for 10 min)	100 µl	-20 °C 1 day then - 80 °C	cortisol	RIA	[32]
Squirrel monkey (restrained)	Q-tip moved over gums (n/a)	-	-28 °C	cortisol	in house immune assay	[34]
Dog (restrained)	Cotton wool buds (chewed at least 30 sec)	0.5 ml	-20 °C	cortisol	RIA	[26]
Dog (restrained)	Collection tube, sucking from cheek pouches (n/a)	-	-20 °C	cortisol	RIA	[4]
Adult rhesus monkey (single caged)	Screen apparatus pole apparatus (10 min access)	0.4 ml	-20 °C	cortisol	RIA	[33]
Rat (restrained) *(*)*	Disc of filter paper under tongue (approx. 30 sec)	-	-20 °C	IgA	ELISA	[37]

RIA, radio-immune assay. *(*)* Rats were caged in same-sex groups and picked up for collection.

assessing stress in non-human species. For example, salivary IgA levels have been measured in rats by rocket immunoelectrophoresis [43] and ELISA [37]. In these studies, the specific procedure consisted of picking up the rat, showing it a piece of chocolate, placing a disc of filter paper under its tongue for about 30 seconds, storing the disc at -20 °C until analysis, and returning the rat to the cage and rewarding it with the chocolate. Salivary IgA was measured in duplicates obtained from pools, and although it was easily detectable with both assays, there was up to 15% variation between duplicates, which can most likely be attributed to the viscosity and heterogeneity of each sample [37]. Moreover, when measuring IgA in multiple samples from individual rats, the salivary IgA levels varied up to 28%. These problems were enhanced by the small size of the samples obtained. In dogs, marked decreases in salivary IgA levels have demonstrated just how demanding training is, and a negative correlation has been found between salivary cortisol and IgA levels [44] (see also, for humans, [45]).

Urine

Assays for measuring stress hormone metabolites in urine have been validated in sheep, cattle [46], and rats [47] and have been used to monitor stress in farm animals (e.g. cattle [48]), domestic and non-domestic felids [49], and dogs [4, 50]. In primates, there is a long history of monitoring stress with the non-invasive method of measuring both the adrenocortical response [51-54] and the adrenomedullary response [55]. Recently, the use of urine has been validated to assess acute stress by monitoring hypothalamic-pituitary-gonadal axis perturbance [56].

Since the specific metabolite excreted in urine differs by species [57], the choice of assay must take into consideration the species being studied. Moreover, urine hormone levels must be compared to the levels of creatinine. In fact, urine samples may differ in terms of volume and concentration. Creatinine, which is a product of the metabolism of muscular activity and thus depends on the extent of locomotion and the body mass, is excreted at a relatively constant rate when comparing animals living in the same environment. By expressing urinary hormone levels as the ratio of the hormone to creatinine (H/C), it is possible to control for differences in urine concentration (both among and within individuals) attributable to differences in body weight, urine output, and water consumption [1, 58]. In a study on stress response to general anaesthesia in chimpanzees [53], pooled urine samples collected over an approximately one-day period were used to measure cortisol concentrations: in this case, the samples were not normalised by creatinine, and cortisol content was expressed as micrograms per decilitre.

If urine samples are to accurately reflect the levels of stress-related hormones in the plasma, then a certain amount of time must be allowed to elapse between the stress-inducing event and sample collection. Many studies have assessed the delay of metabolite excretion in urine, by injecting either radioactive corticosterone or cortisol, depending on the species, and monitoring fluctuations in urine concentration. Given that the delay time is usually quite short (in most cases less than 5 hours), urine analysis can be used to measure the responses to both short-term events and chronic stressful conditions. Radioinfusion has also shown that after injection, steroids are rapidly conjugated in the plasma and then excreted into the bile and urine. The pathway of excretion can also differ by species. For example, in rats, an estimated 20% of the excreted metabolites have been reported to be found in urine and 80% in faeces [47], whereas in dogs, most cortisol metabolites have been shown to be mainly present in urine [57].

In Table 2, some examples of techniques used to collect, store, and analyse urine samples are provided.

As opposed to saliva, urine is spontaneously voided, so that non-invasive collection is inevitably performed on an opportunistic basis. If necessary, however, urine samples may be collected as daytime urine, as opposed to nighttime urine. Early-morning voided samples are preferred to daytime samples because they are the result of night accumulation and are thus more concentrated. Nonetheless, in contrast to saliva, the quantity of urine in a single sample is usually sufficient for measuring multiple hormones. This is particularly useful when studying the impact of stressful events on the ovarian cycle, so that, in addition to the specific stress hormone studied, all reproductive hormones needed to monitor the ovarian cycle can be non-invasively measured (e.g. [56]).

Urine samples are usually collected from individually housed animals and retrieved from stainless steel pans placed underneath the cage. A stainless steel mesh prevents faeces and most food particles from contaminating the urine (e.g. [58]). However, other methods for collecting urine samples have been developed. For example, in one study,

Table 2. - Examples of techniques used to collect, store, and analyse urine samples

Species (housing at time of collection)	Collection method (duration)	Time lag	Storing	Hormone	Assay	Reference
Cat (single caged)	In the litter	9±3 h	-24 °C	[^{14}C] cortisol *(*)*	-	[57]
Dog (single caged)	A tray on a long stick during outside walking	3±1 h	-24 °C	[^{14}C] cortisol *(*)*	-	[57]
Dog (single caged)	-	-	- 20°C (added with formic acid)	cortisol	RIA	[4]
Dog (single caged)	-	-	-20°C (added with formic acid)	adrenaline noradrenaline	see ref.	[4]
Rat (single caged)	Plastic tube, bottom of the metabolic cage	3.2±1.9 h		[^{3}H] corticosterone *(*)*	RIA	[47]
Long-tailed macaque (single caged)	Metabolism pan, with urine collection box, filled with dry ice	-	-20 °C	free cortisol	RIA	[58]
Black tufted-ear marmoset (group living)	Trained to urinate in hand-held pans	-	-20 °C	cortisol	EIA	[59]
Gorilla (wild)	Habituated: collecting tube in the urine stream	-	-20 °C (within 24 h)	cortisol	RIA	[60]
Chimpanzee	Metabolic cage	-	-20 °C	cortisol	RIA	[53]
Rhesus macaque Long-tailed macaque (single caged)	Metabolic cage	-	-20 °C	FSH	see ref.	[56]

RIA: radio-immune assay. EIA: enzyme-immune assay. *(*)* Radio-infusion study to determine time lag and characterise excreted metabolites.

group-living marmosets were trained to move, in response to the light being switched on in the morning, from their sleeping boxes through corridors to single compartments, where they received a reward and micturated within minutes [61]. In another study, group-living female capuchins were trained to move from their home cages, through connecting doors, to adjacent experimental cages, one per animal; the animals were monitored through glass windows and were immediately released and rewarded after urinating [62].

As with salivary cortisol, several studies have shown that urinary hormone levels (e.g. FSH [56] and cortisol [58]) accurately reflect plasma hormone levels. To assess psychosocial stress in black tufted-ear marmosets (*Callithrix kuhli*), urinary cortisol was measured in animals exposed to different situations, specifically: sibling fights and the placement of daughters in different social settings (i.e. anovulatory daughters living in their family group; daughters commencing their ovulatory function living in their family group; and established breeding females living with their long-term pair-mate and in some cases offspring) [59]. Sibling fights were shown to stimulate high HPA activity, as revealed by increased urinary cortisol levels. However, no differences were observed when comparing anovulatory daughters, ovulating daughters, and breeding females in almost all stages of pregnancy, leading the authors to conclude that reproductive anomalies are not related to the activation of the HPA in this species, that is, the stress-induced activation of the HPA axis is not responsible for reproductive suppression. The urinary cortisol levels also provided evidence of certain social partners acting as buffers on HPA activity. Removing females from their family groups resulted in a dramatic increase in HPA activity when the animal was housed alone yet it produced no effect when housing the animal with a sister.

In long-tailed macaques, Crockett *et al.* [58] monitored urinary cortisol levels in response to potential stressors commonly found in laboratories [e.g. changes in cage size, in housing level (upper *vs* lower cage), and in the housing room; tethering; sedation; surgery; and chronic catheterisation]. No significant differences in cortisol levels were observed when moving the animals to different sized cages every two weeks (a total of 5 cages sizes were used, with the smallest only large enough to allow the animal to sit, stand, and turn around), nor were there significant changes observed when changing the cage level or the housing room. Although previous studies have reported that these factors affect behaviour, Crockett *et al.* claim that their study is the first not to be influenced by confounding variables such as extraneous events, observation in an experimental cage (as opposed to the home cage), and the frequent presence of humans. Nonetheless, a significant increase in urinary cortisol levels, which is indicative of stress, was detected when the animals were exposed to surgery, sedation, and adaptation to tethering (jacket, cable, tether, and catheterisation).

FSH has been proven to be a reliable indicator of pituitary function (in humans [63]) and, as such, to reflect stress-induced alterations in hypothalamic-pituitary-gonadal activity. When animals are exposed to aversive stimuli, such as capture and restraint, at the luteal-follicular transition (when the synchrony between gonadotropins and follicles is crucial), urinary FSH reliably reflects the impact of these stimuli on pituitary activity, revealing secretion perturbations which may alter subsequent menstrual cycles (long-tailed macaques [56]). That alterations in urinary FSH secretion have been shown to occur not at the first but at the second exposure to capture and restraint suggests that the animals may not respond immediately to a stressful event and that they are instead sensitised to it, experiencing discomfort only after subsequent exposure.

Faeces

Assays for measuring stress hormone metabolites in faeces have been validated in domestic live-stock (in which cortisol metabolites were measured [13, 64]); in cats and dogs (cortisol metabolites [65, 57]); in wild carnivores such as cheetah (*Acinonyx jubatus*) [66], spotted hyena (*Crocuta crocuta*) [67], and African wild dogs (*Lycaon pictus*) [68]; and in primates [69, 70]). The assays have been used to monitor stress in, for example, primates (e.g. chimpanzee [53] and brown capuchin [22]). Measuring IgA levels has been validated as a means of monitoring stress in rats [37]. As with urine, the specific glucocorticoid metabolite excreted in faeces is species-specific and may need to be characterised using high-performance liquid chromatography (HPLC), followed by either enzyme-immune assay (EIA) or RIA (e.g. sheep [71]; rats [47]; and cats and dogs [57]; see also [72] for a review on faecal steroid analysis). When specific assays are not available, assays that cross-react with a broad range of cortisol metabolites can be used (e.g. sheep and cows [64]).

As with urine, many studies have monitored the time-lag of metabolite excretion in faeces. Since a large amount of time elapses between secretion in blood and excretion in faeces, faecal hormone levels are relatively unaffected by the collection of the faeces sample, making faeces the ideal excreta for measuring hormonal indicators of chronic stress. The time-lag of faecal steroid metabolite excretion is species-specific and is related to the amount of time needed for the

Table 3. - Examples of techniques used to collect, store and analyse faecal samples

Species (housing at time of collection)	Collection method	Time-lag	Storing	Hormone	Assay	Reference
Cat (single caged)	From the litter	22 ± 6 h	-24 °C	11,17-DOA (*)	EIA	[57]
Dog (single caged)	Walking the dog, collected from the ground	24 ± 4 h	-24 °C	cortisol equivalents	EIA	[57]
Rat (single caged)	Metabolic cage	16.7 ± 4.3 h	-24 °C	corticosterone metabolites	EIA	[47]
Brown capuchin (single caged)	Metabolic cage	-	-70 °C	cortisol	RIA	[22]
Chimpanzee (single caged)	Metabolic cage	48 h	-20 °C	cortisol	RIA	[53]

(*): 11,17-dioxoandrostanes; EIA: enzyme-immune assay. RIA: radio-immune assay.

cortisol metabolites to pass through the gut [46]; however, it is usually no less than 12-24 hours and tends to range from 24 to 48 hours in hindgut fermenters (e.g. primates [72]) (see Table 3).

Whether metabolites are excreted in a conjugated or unconjugated form has also been found to depend on the species. In most mammals, faeces contain more unconjugated steroids; this because steroids are usually de-conjugated during their transport in the intestine. However, in carnivores (dogs and cats [57]), it has been shown that the predominant metabolites are either conjugated or polar unconjugated (see also [65] for cats). The pathway of excretion can also differ by species. For example, cortisol metabolites are mainly excreted through the faeces in cats (about 86%) and through urine in dogs [57, 65].

No animal material is easier to collect than faeces. For animals that void urine by spraying it (e.g. felids), faecal samples represent a practical alternative. In other animals (e.g. horses and swine [46]), because faecal steroids may be unevenly distributed in the faecal balls, the sample may need to be mixed. As with urine, faecal samples can be preserved at -20 °C. In Table 3, some examples of techniques used to collect, store, and analyse faecal samples are provided.

Möstl *et al*. [64] collected faecal samples from cows used to teach non-invasive sampling procedures to university students and from cows used to teach invasive procedures. The control cows were living on the university farm. Samples were collected immediately after transport and later during classes. The stress related to transport, which was mainly attributable to being confined in a moving vehicle [73] and to novel housing conditions, was reflected by an increase in faecal cortisol metabolite concentrations. These concentrations remained elevated for about 5 days after transport, probably because the cows were brought to a new environment; in another study [48], in cows exposed to transport-induced stress, faecal cortisol metabolite excretion returned to normal within one day of transport, when the cows were already back in their home environment. In the study by Möstl *et al*., there were surprisingly no differences in cortisol metabolites between control cows and cows used to teach invasive procedures. The authors suggest that either not all kinds of stressful stimuli activate the HPA axis or the EIA used had not been able to detect that particular type of stress.

In non-human primates, undergoing general anaesthesia induces stress (see related references in [53]), which, however, is apparently due to the disorientation of undergoing or emerging from anaesthesia and not to the effect of the anaesthetic on the pituitary adrenal axis [74]. In chimpanzees, faecal cortisol levels have been shown to be a good indicator of even short-lasting stress such as anaesthesia. However, since the cortisol level in faeces is the result of the accumulation of cortisol over a prolonged period, it may not reflect the fluctuations in plasma cortisol levels, especially if these fluctuations are brief. Moreover, since the ratio of faecal cortisol levels to plasma cortisol levels can vary by individual faecal sample, a single faecal sample may not be representative. Nonetheless, in the study by Whitten *et al*. [53], faecal cortisol levels reflected 7 of the 8 events of anaesthesia, with a delay of 2 days, demonstrating the great potential of the use of faecal cortisol levels for measuring the stress induced by anaesthesia.

Although the importance of environmental enrichment for the wellbeing of captive animals has been widely recognised [75, 76], it has not been established what exactly constitutes enrichment for primates [77]. One study has used faecal cortisol levels, in addition to behavioural measures, to evaluate the effectiveness of environmental enrichment in decreasing abnormal behaviours in singly housed brown capuchins [22]. The environmental enrichment consisted of either adding two plastic toys to the animal's cage or providing access to a foraging box with food treats hidden in crushed alfalfa, or both. Both the behavioural measures and the faecal cortisol levels reflected the positive effects of environmental enrichment, although the behavioural measures were more sensitive, in that they also detected an increase in normal behaviours as the complexity of the enrichment progressed.

Additional considerations in assessing hormonal indicators of stress with non-invasive techniques

In this section, we provide examples of the potential problems encountered in assessing hormonal indicators of stress, such as species differences in the responses to stressful stimuli, the degree of reliability of the hormonal indicator, problems related to the indirect assessment of circulating stress hormones, and factors potentially affecting inter-individual variability even before exposure to stress.

In a variety of species, high HPA activity, revealed by basal plasma glucocorticoid levels and variables such as high adrenal weight or adrenal response to stressful stimuli, has been shown to be associated with a low social status, that is, a chronic condition of psychosocial stress (e.g. baboons [16, 78]). However, in an increasing number of species, no such association has been detected (e.g. gorilla [60]) and an opposite association has instead been found (see [79] for related references). Saltzman *et al*. [79] have demonstrated that, when using glucocorticoid levels as indicators of rank-related stress, it is necessary to have taxon-specific knowledge of the behavioural and physiological variables related to social rank and associated with HPA activity. In marmosets, for example, the authors compared cortisol levels and reproductive hormone levels in cycling, ovariectomised, and anovulatory subordinate females: the results showed that the low cortisol levels in the non-cycling subordinate females were the result of a combination of factors that suppress HPA activity (i.e. hypoestrogenemia, social subordination, and suppression of circulating LH levels).

When using glucocorticoid levels, it must be considered that apparently not all stressors induce increases (e.g. [64]). Hau *et al*. [37] has criticised the use of classical glucocorticoids as indicators of stress for several reasons, including the significant intra- and inter-individual variation and feed-back inhibition. Inter-individual variability has been assessed, for example, in cats, dogs [57], and chimpanzees [53], and significant differences in individual baseline faecal cortisol levels have been found. Baseline measurements should thus be taken, so that each individual can serve as its own control (e.g. [46]). In baboons, Sapolsky [25, 80] found considerable inter-individual differences, which were related to individual social status. Using the dexamethasone suppression test (dexamethasone decreases cortisol levels by suppressing ACTH production), the author found that the higher cortisol levels in low-ranking animals had resulted from a dysfunction in the feedback regulation system between the hypothalamus/pituitary, and glucocorticoid levels [80]. In fact, whereas an increase in cortisol levels was induced by the injection of ACTH in both dominant and subordinate individuals, the subordinate individuals did not react to dexamethasone, as if their feed-back system was less effective than that of dominant individuals. Sapolsky [25] also found that the dominant baboons had a lower testicular sensitivity to the suppressive effects of high cortisol levels. In fact, the LH-induced decrease in testosterone secretion, which is caused by the stress of immobilisation, is preceded by increases in cortisol levels. Sapolsky speculates that resistant males may have been characterised by either a decreased cortisol receptor concentration or a different testicular sensitivity to LH that is linked to the action of glucocorticoids. Overall, what appears to be of extreme importance is knowing what represents a challenge to the HPA system and how to interpret results.

Potential problems may be specific to the type of excreta being analysed. For example, the acidity of some of the flavourings used to collect saliva samples could interfere with the cortisol assay (e.g. [81]), and although the cotton material does not affect the results of salivary cortisol assays [82], its use can result in artificially high levels of reproductive steroids and artificially low levels of IgA. Regarding the use of faeces, Möstl *et al*. [64] found more than 20 cortisol metabolites in faecal samples collected from sheep, which could complicate the standardisation of measurements among different laboratories, which may use different immunoassays and extraction procedures. Wasser *et al*. [83] report that there is a direct relationship between the amount of dietary fibres consumed and the delay in steroid excretion in faeces. However, these authors suggest that the variability due to different diets can be controlled by expressing hormonal levels per dry weight. They also stress the fact that faecal glucocorticoids are often more heavily metabolised than other faecal steroids

and are thus less likely to cross-react with the assays for measuring the levels of the given hormone in plasma. Finally, Möstl *et al*. [64] warn against bacteria, which can further the metabolism of cortisol and thus alter metabolite concentrations (see also [13]).

Conclusions

Overall, assessing stress is rather complex, in that it entails objectively quantifying stress, distinguishing between causes and effects and between psychological and physiological stress, understanding how a given species adapts to a stressful situation and how its living environment affects the reproductive and endocrinological measures of stress, and assessing inter-individual variability in response to stressful stimuli. Non-invasive techniques for assessing hormonal stress indicators have greatly improved, and the analysis of saliva, urine, and faeces seems to be the best alternative, in that these biological materials can be collected without subjecting animals to additional stress, which can bias the results. Moreover, non-invasive techniques allow physiological imbalances due to stress to be rapidly assessed, before the appearance of other markers of stress, such as weight loss, poor health, and infertility. What remains is to further validate these techniques and to standardise laboratory procedures and the interpretation of data.

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