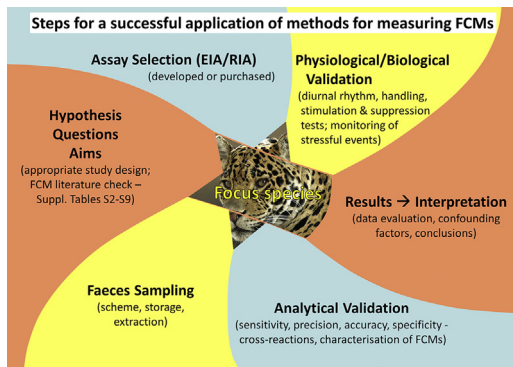Review

Non-invasive measurement of glucocorticoids: Advances and problems

Rupert Palme

Unit of Physiology, Pathophysiology and Experimental Endocrinology, Department of Biomedical Sciences, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria

GRAPHICAL ABSTRACT

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ABSTRACT

Glucocorticoids (GCs; i.e. cortisol/corticosterone) are a central component of the stress response and thus their measurement is frequently used to evaluate the impact of stressful situations. Their metabolites from faeces of various animal species are more and more taken as a non-invasive aid to assess GC release and thus adreno-cortical activity. The current literature review includes an extensive collection (1327 papers) and evaluation (see also Supplementary Tables) of the literature on faecal cortisol/corticosterone metabolite (FCM) analysis published to date. It aims at giving reference for researchers interested in implementing FCM analysis into their study or seeking to improve such methods by providing background knowledge on GC metabolism and excretion, conveying insights into methodological issues and stating caveats of FCM analysis and by highlighting prerequisites for and some examples of a successful application of such methods. Collecting faecal samples and analysing FCMs may appear simple and straightforward, but researchers have to select and apply methods correctly. They also need to be aware of the many pitfalls and potentially confounding factors and, last but not least, have to carefully interpret results. Applied properly, measurement of FCMs is a powerful non-invasive tool in a variety of research areas, such as (stress) biology, ethology, ecology, animal conservation and welfare, but also biomedicine.
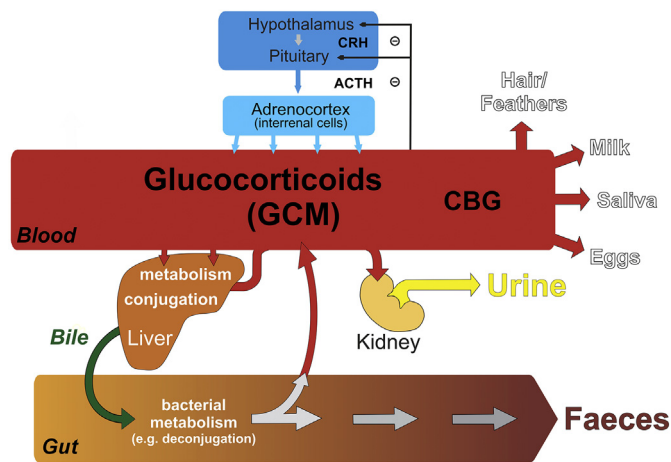
Fig. 1. Scheme of the secretion (HPA/HPI axis) and excretion of glucocorticoids (adapted from Möstl & Palme, 2002; for further details see Section 3.1). The different biological matrices for measuring GCs or their metabolites, such as blood, saliva, excreta (faeces and urine), milk and integumentary structures (e.g. hair and feathers) are indicated. CBG: corticosteroid binding globulin; GCM: glucocorticoid metabolites; CRH: corticotropin releasing hormone; ACTH: adrenocorticotrophic hormone; HPA axis: hypothalamus–pituitary–adrenal axis; HPI axis: hypothalamic–pituitary–interrenal axis (in fish).

1. Introduction

Organisms are frequently confronted with challenges or stressors. Such stimuli may be external or internal. When an animal experiences a stressful situation, a multitude of different responses is activated helping the organism to cope with the stressor. Among these are behavioural, physiological and neuroendocrine responses [1,2]. Because of these multifactorial responses, it is commonly accepted that different stress response parameters should be included when evaluating how organisms respond to stressful situations. Among these are measures evaluating the activity of the autonomic nervous system (catecholamines; heart rate variability [3]), or the endocrine system and the animal's behaviour [2,4]. The following literature review focuses on the endocrine stress system, and more specifically, the hypothalamic–pituitary–adrenal (HPA) axis and how its activity can be monitored non-invasively.

The endocrine system's role in modulating stress has been well known since the days of Selye [5]. Its main components, hormones and feedback loops (Fig. 1) have been extensively described and investigated (for examples see reviews [6–8]). The frontline hormones of the HPA (fish: hypothalamic–pituitary–interrenal) axis are the glucocorticoids (GCs), namely corticosterone (4-pregnene-11ß,21-diol-3,20-dione) and cortisol (4-pregnene-11ß,17α,21-triol-3,20-dione). Cortisol has an additional 17α-hydroxy group, which renders it slightly more hydrophilic than corticosterone. Birds, amphibians, reptiles and some rodents (Murinae and rabbits) secrete mainly corticosterone, whereas in fish and almost all other mammals secretion of cortisol predominates. Nevertheless even in cortisol-dominated mammals corticosterone is also found in the circulation [9]. As the two concentrations are not always correlated, it is speculated that corticosterone may exert some independent biological effects (negative feedback in the central nervous system [9]) or that its presence can be attributed to the fact that corticosterone is a direct precursor of the vital mineralocorticoid aldosterone.

GCs play a role in a variety of physiological processes. While they are typically considered "stress hormones", their primary role is basic energy regulation (acquisition, deposition and mobilization [10,11]). At high levels they orchestrate the changes associated with stress [11] to help an organism to cope with the stressful situation (for example, by making stored energy available). For that reason GCs may also be called

"anti-stress hormones". There are different concepts of what stress is and how it is defined (Classical, Allostasis and Reactive Scope Model, respectively – for more details see [12–14]) and thus different approaches to study it. Although measuring GC levels does not equate to measuring "stress", GCs are important mediators of the physiological stress response and thus their determination is frequently included as an important stress marker. Their baseline and response levels were found to be linked in an inverted U-shaped manner to survival, reproduction and fitness, and also to memory performance [11,15].

Traditionally, GCs have been determined from blood samples. However, as their collection by itself may be stressful and requiring special approval, alternative non-invasive sample materials have attracted much attention (see also Fig. 1). The pros and cons of various sample materials (blood, saliva, excreta, milk, hair/feathers and eggs) for assessing adrenocortical activity have been discussed before in detail (e.g. [6,7,16–18]). The present review focuses solely on the use of faecal samples.

2. Materials and methods (literature review)

The present literature review includes an extensive collection and evaluation of the published literature (1327 papers). Therefore, studies reporting faecal cortisol/corticosterone metabolite (FCM) data have been collected over the last 20 years. They were located in Clarivate Analytics' Web of Science (WOS; for a list of all WOS papers – 1250 as of the 20th November – see [19]) and in Elsevier's Scopus by using the search terms "cortisol or corticosterone or glucocorticoid" AND "faeces or feces or faecal or fecal". Additional papers were located via respective reference lists. With the help of ResearchGate further "in press" articles were found. Reviews, which only marginally deal with FCMs, and articles not written in English (which I could not fully evaluate) were not included in the collection. This was the case for 28 papers (listed separately at the end of the supplementary reference list).

My rating of the papers is focused only on FCM methodology, and most importantly whether biologically meaningful validations were conducted (indicated by a positive physiological or biological validation; see Sections 3.4.2 and 3.4.3). If such a validation has been successfully achieved for the described method (whether in this or another paper) the paper was rated "validated" ("1"), if not, it was rated "non-validated" ("0"). There were a few situations where I decided to use "0.5" (e.g. when two species were dealt with, and the method was only validated for one). Review papers and papers, which only deliver background information (e.g. radiometabolism studies), were not included in the number of non-validated studies. However, these papers were included in the total number of papers.

The following table (Table 1) provides the primary questions to be considered prior to a FCM study (see also the graphical abstract). In the following sections, those points will be dealt with in more detail.

3. Measurement of FCMs

3.1. Background knowledge (metabolism and excretion of FCMs)

GCs, secreted by the HPA axis and circulating in the plasma, are primarily metabolized by the liver, although many other organs

Table 1
Primary questions for the non-invasive monitoring of stress (HPA axis activity) using FCM (for details see respective sections).

☐ 1. What has already been published (including radiometabolism studies) about your animal species/group (see Supplementary Tables S2-S9)?
☐ 2. What are the aims of the study? Do you want to monitor effects of acute/chronic stressors (Section 3.3)?
☐ 3. How are faecal samples collected and stored (Section 3.2)?
☐ 4. How are FCM analysed (extraction and assay selection; Sections 3.2–3.4)?
☐ 5. What confounding factors need to be taken into account (Section 3.6)?

contribute to the process [20]. The resulting metabolites are excreted via bile or urine predominantly as conjugates (Fig. 1). In the intestine further metabolism of steroids excreted via the bile by bacterial enzymes may occur, and metabolites can also be partly reabsorbed (enterohepatic circulation). This metabolism can include 5α-reduction, 5β-reduction, hydroxylation, reduction of functional groups or, in the case of 17α-hydroxylated metabolites, side-chain cleavage by C-17,20-desmolase [21–23]. The GC metabolites will show up in the faeces after a time delay, which is species-specific and roughly corresponds to the gut passage time [24]. Understanding the passage time is an important part of the FCM validation process (see below).

So-called radiometabolism (or radioinfusion) studies have helped tremendously to elucidate the metabolism and excretory pathways of (stress) hormones. In these studies, a small amount of radiolabelled steroid hormone is injected into the animal (e.g. GC labelled with ^{3}H or ^{14}C) and all voided excreta collected afterwards (ideally until background values of radiation are reached). Among the first studies of that kind was a seminal study of Lindner [25], revealing an enterohepatic circulation of cortisol in sheep. Over the years 40 such studies (injecting radiolabelled cortisol or corticosterone) have been reported in various species (see [26] for a comprehensive list of the earlier studies, but also [27–30] and the Supplementary Tables of this review for more recent examples). Although limited by some constraints (e.g. special permissions needed to perform animal experiments and to handle radioactive materials; expensive compounds), radiometabolism studies helped to gain important background knowledge needed to develop non-invasive methods for evaluating adrenocortical activity [26,31]. They can be used to measure the time lag between the secretion of GCs into the blood and the excretion of their metabolites and they also give information about the proportional excretion via faeces and urine [26]. In most bird species, faeces and urine are excreted together in the form of droppings [32]. Therefore two peaks of radioactivity, reflecting urinary and faecal excretion of injected ^{3}H-corticosterone, can be found in birds [26,33]. This information may be especially relevant for choosing sample collection times for birds in case a selected assay predominantly picks up urinary or faecal metabolites, which would result in expected peak delay times of about or less than one or several hours, respectively [34–36].

Faecal samples derived from radiometabolism studies are ideal for optimising extraction procedures and for assay selection [26,37]. These samples allow for FCM characterisation and the investigation of steroid metabolite cross-reactivity with the antibody of an assay. The latter is achieved by conducting HPLC (High Performance Liquid Chromatography) immunograms. For this purpose, HPLC separations of the radiolabelled metabolites are performed (mainly using reverse phase systems). The eluent is fractionized and an aliquot of each fraction measured in the respective immunoassays. Samples derived from radiometabolism studies are most helpful here, as they enable an easy detection of the naturally occurring metabolites by additional measurement of the radioactivity in the fractions. A co-elution of radioactive and immuno-reactive peaks is a strong indication that the metabolites formed are recognized by the antibody used in the assay. HPLC immunograms proved that steroid hormones are heavily metabolized and thus only negligible amounts, if any, of the parent compounds are usually present in the faeces [26]. They also yield interesting information about pronounced species and sex differences in formed metabolites [26,38]. In some species (e.g., cats or North American red squirrels [39,40]) polar metabolites prevail (mostly resisting an enzymatic hydrolysis), whereas in others (ruminants) almost all metabolites are unconjugated [41]. In addition, such methods helped to elucidate a possible co-measurement of androgen metabolites with FCMs (for example in dogs and elephants [39,42]). However, HPLC separations require expensive equipment, special expertise and they are time consuming to perform. Thus, they are only recommended in certain circumstances, ideally in combination with radiometabolism studies. Given the complexities and variation of steroid metabolism and

excretion, providing validation that meaningful changes in plasma steroid hormones are appropriately reflected in the measured faecal metabolites of a given species is the most important aspect of the sound application of non-invasive methods (see 3.4.2. and 3.4.3.).

3.2. Collection, storage and extraction of faecal samples

Studies utilizing non-invasive methods need especially careful planning. Most important is the schedule for collection of samples (and their storage). Whatever mistakes are made here cannot be compensated for later. Corresponding with the aim of the study (e.g. evaluation of chronic or acute stressors) single samples might suffice or frequent serial sampling may be carefully taken into consideration to avoid missing the peak excretion sample. In both cases, faecal sample collection offers the advantage that it enables longitudinal studies where an animal can serve as its own control (thus facilitating better statistical power) and short episodic (ultradian) GC fluctuations are smoothened when measuring FCMs [26,43,44].

Several papers ($n = 74$) report experiments evaluating extraction procedures and/or sample storage conditions, some of which are totally dedicated to this topic (e.g. [45,46] and see also Supplementary Tables S2–S9). However, such experiments are only valid for the species and methods (sample processing/FCM analysis) used in the particular experiment (exception: radiometabolism studies; see Section 3.1) and of limited value, if the applied method has not been validated. Caution is advised if another species or different methods are used. For example, stability of FCMs has been tested in storage experiments (leaving fresh samples at room temperature for certain time periods) with ruminant faeces. Results indicated that FCM concentrations can increase, decrease or remain unchanged depending upon the analytical method used [37,47,48]. These studies demonstrate that, except for rapid freezing, storage conditions have unpredictable effects on FCM concentrations and thus, there is no reliable standard storage protocol that is transferable between projects. Instead, when samples cannot be frozen soon after defecation, the best practice is to include a small experiment testing the conditions of the performed real world experiment to be sure that measured FCMs remain unchanged (e.g. [45,49]).

In total, > 425,000 samples have been analysed in the publications included in this review (median: 180; min.: 8; max.: 10,163 samples per study). While there are many studies (~350) with a relatively low (100 or less) number of samples, only ~80 studies analysed 1000 or more samples. In the majority of published articles reporting respective details, methanol ($n = 749$) or ethanol ($n = 410$), mostly at a high percentage (80% or 90%), were used for extraction. Only a small number of studies used other solvents like water or buffer ($n = 25$), dichloromethane ($n = 25$), or diethylether ($n = 20$). Caution is advised here, because water (buffer) may be too polar in case of unconjugated FCMs [50], while ether is not polar enough for conjugated FCMs (e.g. birds) and both conditions will result in lower extraction efficiencies. The recommendation (see [37] for details) for extracting FCMs is to suspend mammalian faeces in a high percentage of a primary alcohol (80% aqueous methanol proved best for virtually all mammalian species tested; and 60% methanol is suggested for birds) and to transfer an aliquot of the supernatant (after centrifugation) into the immunoassay (after dilution with assay buffer). In rare situations (low amounts of metabolites, or sub-optimal storage conditions) more sophisticated procedures need to be applied [37]. For more detailed questions regarding collection, storage and extraction of faeces see the review by Palme et al. [37]. Two things may serve as a rule of thumb here: "Keep it (as) simple (as possible)" and "Whatever you do, be consistent across all samples".

3.3. FCM assays

Wudy et al. [51] in their review "The art of measuring steroids" note that in depth knowledge, as well as skill, are indispensable prerequisites

for successful steroid determination. This is especially true for FCM analysis. There are mainly two methods to measure steroid metabolites: Chromatographic methods (e.g. liquid chromatography – LC) in combination with mass spectrometry (MS), and immunoassays [51]. Several review papers have already compared both and discussed pros and cons when determining FCMs [51–53]. Briefly, while LC-MS is state of the art for measuring plasma steroids (due to its high specificity and the possibility to simultaneously quantify several compounds), the situation is different in the faeces. It is better to measure groups of metabolites there, and in contrast to immunoassays the chemical identity of the measured metabolites has to be known (which is hard to achieve due to a lack of available standards) in order to measure them with LC-MS. In addition, LC-MS is more expensive and not well suited for large scale analysis. Thus, to date immunoassays are almost exclusively utilized. The assays used are competitive immunoassays, where the steroids from the sample/standard compete with labelled steroids for limited binding places of the antibodies (for more details see [22]). Depending upon the label and thus the detection system used, they are classified as radioimmunoassay (RIA; tracer labelled with ^{3}H or ^{125}I) or enzyme immunoassay (EIA). On a few occasions, chemiluminescent assays were also used [53]. EIAs have mostly displaced RIAs in recent years (all together: 870 vs 370 papers, respectively), because they utilize less expensive equipment and no special permits/laboratories for handling and measuring radioactivity are needed for EIA analysis [22,53].

Every immunoassay relies on antibodies [22]. Many researchers use antibodies designed to measure cortisol or corticosterone (among these are all the commercially available immunoassay kits), originally used for blood samples (e.g. [54,55] for a comparison of several assays), but more recently applied to faecal samples. A second approach utilizes so-called group-specific antibodies [2,22]. Such EIAs are specifically designed to measure groups of metabolites. They detect a variety of faecal GC metabolites, usually yielding a much stronger signal [22]. Several group-specific EIAs have been developed and successfully validated for a variety of mammal and bird species [2,22]. Such assays can be easily recognized, because their standard (of course also, but less obviously, the immunogen and the label) is a steroid other than cortisol or corticosterone.

Among the group-specific EIAs developed by our group in Vienna (utilized in ~36% of all papers; a detailed laboratory protocol is made available upon request) are an 11-oxoaetiocholanolone EIA (measuring 11,17-dioxoandrostanes [50]), which was the first one of this kind and another 11-oxoaetiocholanolone EIA (immunogen and label coupled at C-17 [41]), which were used in 102 (86% validated) and 98 (89% validated) studies, respectively. A 5α-pregnane-3β,11β,21-triol-20-one EIA [38] was used in 112 published studies (97% validated; mainly applied to study rodents, but also other species such as silver foxes [56]), an 11ß-hydroxyaetiocholanolone EIA [57,58] used in 82 studies (91% validated) and a cortisone EIA [33] used in 42 studies (93% validated).

Coralie Munro (University of California Davis, USA) produced a cortisol EIA (antibody "R4866" [59]) and a corticosterone EIA ("CJM006" [60]), which have been used in 145 (57% validated) and 31 published studies (50% validated), respectively. However, the assay mostly utilized in all FCM studies and thus published in a large number of papers ($n = 262$; 65% validated) was a commercial ^{125}I-corticosterone RIA kit (MP Biomedicals, USA; former ICN Biomedicals), which proved suitable in a wide array of species (e.g. [61]). All other commercially available corticosterone or cortisol assay kits were used in 145 and 66 studies (15% and 27% validated), respectively. The advantage of commercial kits is that they are readily available and easy to handle (although relatively expensive). However, this may very well be the reason why a high percentage of studies using such assay kits lack a sound validation, because more researchers not familiar with FCM analysis may use them. Companies selling their kits could help by making information about successful (including a physiological or biological validation) applications of their methods available and by

highlighting the need for such validation when dealing with species not investigated to date.

As there is a multitude of different metabolites present in the faeces [26] and immunoassays have to rely on cross-reactivities of their antibodies (especially in the case of cortisol or corticosterone assays) to measure FCMs, measured concentrations are largely method dependent [22]. Thus, it is not possible to compare absolute concentrations across studies (see [31] for an example) and laboratories (unless exactly the same extraction/assay was used for the same species), and also not within studies, if sex differences in FCM are present (e.g. [49,62,63]). Thus, in such cases where higher FCM concentrations are found, they are not necessarily indicating a higher adrenocortical activity (or a more expressed stress reaction), but may have their basis in methodological issues (e.g. [49,63]). Likewise, it is not reasonable to compare absolute FCM concentrations or percentage increases after a stressor (or ACTH injection) between different species.

In order to accentuate a common misconception, I want to use a quotation from the highlights section of a recently published article [64], which states: "Corticosterone metabolite is the predominant GC in the faeces from blue whales." That statement is representative of several similar occurrences in the literature, but unfortunately incorrect for three reasons: GCs are heavily metabolized (usually no native hormones are present in faeces) resulting in a variety of different metabolites rather than a single metabolite being excreted in the faeces [22,26]. Those metabolites have no proven biological effects, and thus cannot be called GCs. Last but not least: As long as limited information on cross-reactions is given for the antibodies used and no chemical characterisation of the measured metabolites has been undertaken, metabolites picked up by a corticosterone assay are not necessarily "corticosterone metabolites", because cortisol metabolites may very well cross-react in a corticosterone assay as well (and of course vice versa). More sophisticated techniques (LC-MS) would be necessary to identify the formed metabolites in order to elucidate from which GC they are derived [51–53].

To conclude, assay selection for measuring FCMs is of great importance. Depending upon the aim of the study, different sample numbers are to be expected. When monitoring acute stressors more frequent sampling is necessary due to variable excretion delay times, as peak FCM samples might be otherwise missed. When evaluating chronic stressors, fewer samples will suffice. The number of samples is critical for choosing a method for FCM analysis. If there are a relatively low number of samples (up to a few hundred) buying a commercial assay kit is probably the best option. Ideally, one would chose a kit that has already been fully validated for the species of interest (check the literature provided in the Supplementary Tables S2–S9). If information about a successfully validated assay is lacking, special validation experiments need to be included in the study (see Section 3.4. for further details). Large sample numbers (beyond a thousand) make it attractive to set up an assay system by buying the key reagents (antibody and label of immunoassays) in bulk or even producing them (probably only an option for a few specialized laboratories). Whatever the number of samples, if someone lacks special laboratory facilities or is new in the field, finding a suited collaborator to outsource FCM analysis is strongly recommended (see Supplementary Tables S2–S9 and Section 3.4.4.).

3.4. Validation of methods to measure FCMs

In recent years the term "validated" has increasingly been used in papers dealing with FCM analysis. That may very well be the result of a series of review papers resulting from a workshop ("Analysis of hormones in droppings and egg yolk of birds" [65] held at the MPI Seewiesen (Germany), but also later reviews emphasising the importance of a sound validation of an applied method for FCM analysis [7,17,22,31,43,66]. However, just to label an assay validated is misleading, if the method has not been fully validated, and that includes not only an analytical validation, but most importantly also a

physiological and/or biological validation (for details see below).

3.4.1. Analytical validations

Analytical validations comprise measures of precision, sensitivity, specificity and accuracy. It is beyond the scope of this review to go into the details of those validations (but see [22,53]). To indicate precision, intra- and inter-assay coefficients of variation (CV) of low and high concentration pool (or standard) samples of the respective study need to be given. Sensitivity of the whole method is best expressed in ng/g (or nmol/kg) faeces. However, because FCMs tend to be present in very high concentrations, lack of sensitivity is hardly a problem. Cross-reactions of the antibody (also with 5α/5β-reduced steroids) should be included (or a relevant paper cited). Unfortunately, especially for commercial assays, most of the tested steroids (sex steroids) are not relevant, and cross-reactions of only a few (if any) FCMs are given (e.g. [54,55,67]). Moreover, in many cases the exact nature of the immunogen that was used for the antibody production is not given, thus making it even harder to estimate possible cross-reacting substances. Nonetheless, such assays also rely on their cross-reactions with FCMs, as native GCs are usually not excreted in the faeces. Serial dilutions of faecal extracts are performed to check whether the resulting values are in parallel with the standard curve. Thereby the presence of interfering substances can be excluded [2,22,60]. Parallelism is not a marker of the absence of other substances in the extract, which are cross-reacting with the antibody (specifity), but a proof of a dose–response relationship [22]. Because the parent stress hormones are usually not present in the faeces, but only their metabolites, adding cortisol/corticosterone to the extracts (or faeces) for testing the accuracy (or extraction efficiency) is highly artificial [31,37,60] and thus of rather limited value. It may only be useful for checking the precision of the person analysing the samples.

Several papers (see citations marked with a "^" in the Supplementary Tables S2–S9) also estimate which steroids are actually present in the faeces and picked up by the antibody (sometimes low cross-reacting steroids may give respectable readings in the assay if present in high concentrations; e.g. [68,69]. Such HPLC immunograms are especially useful when radiolabelled GCs were administered and thus the radioactive metabolites can be matched with the immunoreactivity of the assay [26,31,53]. For example, they provided evidence that native GCs are absent in the faeces and that even cortisol (or corticosterone) immunoassays work due to their cross-reactivity with other steroids. They also enabled the discovery that androgen metabolites were picked up by an EIA to measure FCMs in elephants [42]), and thus revealed that another EIA (both assays passed the ACTH stimulation test) was better suited for FCM analysis in this case.

To save time and assay ingredients, it is better to perform the physiological or biological validation (see below) first. The aim is to test whether increases (or decreases) in circulating GC levels are reflected in measured FCM concentrations. That is the most critical factor and there are only a few examples available where an assay had to be excluded because of a failure to accomplish the analytical validation. However, there are ample examples of assays, which passed an analytical validation, but were unable (or less suited) to pick up the biological signal in faeces (e.g. [70]). It is because of this short coming of analytical validations that studies which only (in most cases a check for parallelism) utilized an analytical validation (but no physiological or biological validation) were scored as "non-validated".

3.4.2. Physiological validations

Physiological validation experiments induce changes in circulating GCs in a standardized way, mostly by a pharmacological stimulation (or suppression) of HPA axis activity. Thus, state-of-the-art experiments include an adrenocorticotropic hormone (ACTH) challenge test [2,7,17,31,43,61,66] (see citations marked with "↑" in the Supplementary Tables S2–S9). The advantage of such a physiological validation is that the adrenals are directly stimulated by the injected ACTH,

and thus the other parts of the HPA axis and the central nervous system are circumvented (and thus their stimulation/activation is not a prerequisite). After a pre-treatment period, the pituitary hormone ACTH (mostly as a synthetic peptide analogue) is injected into the animal, causing the adrenal cortex to release GCs into the circulation. Frequently, tetracosactide (also known as tetracosactrin or cosyntropin) is used (e.g. Synacthen®, Novartis Pharma; Cortrosyn®, Amphastar Pharmaceuticals). It consists of the active N-terminal amino acids 1–24 of the ACTH molecule. Tetracosactide is available in a rapid-acting formulation (given by i.v., i.m. i.p. or s.c. injection) or a depot formulation (injected i.m.), and both are used (the latter causing a longer lasting increase). After administration, a peak increase in FCM levels occurs after a species-specific delay time [26,43]. If such an increase is detected by the assay, the technique has been successfully validated. In addition, information about the delay times (mean and variation) between stimulus and detection of faecal peak FCM levels is also determined by such experiments.

Normally a high ACTH dose is used [31,70] to see if its effect is reflected in the FCM levels. However, this may be problematic. The aim is not to see an effect of a huge dose of injected ACTH, but to elucidate the biological sensitivity of the applied FCM determination. Obviously, a more sensitive method will be able to trace even smaller changes in adrenocortical activity. Compare for example Touma et al. [62] with Abelson et al. [67]: In the latter, the authors state that a single ACTH dose of 2 mg/kg body weight was not sufficient to increase FCM levels in mice, which were measured by 4 different commercial corticosterone EIA kits. However, as demonstrated by Touma et al. [62] for the same species a much lower dose (0.2 mg/kg) lead to a significant increase in FCM concentrations when measured with the 5α-pregnane-3β,11β,21-triol-20-one EIA. Thus, this group-specific EIA is well suited for detecting even small increases in adrenocortical activity in mice, while the commercial assays tested are not.

Thus, it would be advantageous to determine the dose dependent response to ACTH by administering several different ACTH doses to individuals. However, there are only a few studies (e.g. [62,71]) where this has been carried out. In most cases when a higher dose was injected, this was done because the lower failed to give the expected results [72,73]. Palme and Wetscher [44] evaluated diurnal variations in FCM excretion in cattle and the biological sensitivity of an 11-oxoaetiocholanolone enzyme immunoassay by a dose response experiment over a wide range (including data from [71]) of intravenously injected synthetic ACTH amounts (0.016 to 3.0 mg; see Fig. 2 for an example). They found that baseline variations of FCMs were less pronounced than those of plasma cortisol. Even the lowest ACTH dose (0.016 mg) gave a clear signal in both sample types. However, dose of ACTH was only well correlated ($r = 0.819$; $p < 0.0001$) with the percent increase (above baseline) of FCMs, suggesting that their concentration is a better reflection of HPA axis responses than plasma cortisol values. It should be mentioned that routinely (also in the above two studies) only total GCs are measured in plasma, but evidence indicates that FCMs reflect circulating free GCs [29,74,75], which is the biologically active portion. Episodic variations in plasma GC are smoothened in FCM levels [26,44], which renders faeces better suited for assessing baseline or chronic HPA activity (especially when only a few samples can be collected). In addition, the group-specific 11-oxoaetiocholanolone EIA was characterized by a high biological sensitivity, which enables the detection of minor stressful events in cattle.

Another possibility for physiological validation is an administration of GCs (oral, dermal or by injection) to increase blood levels (e.g. [76–78]). However, attention has to be paid to ensure that GC concentrations are not increased far above physiological levels. In a recent study, McDonald et al. [76] administered food supplemented with an extremely high dose of cortisol (18 mg) to arctic foxes (about 3.6 kg body weights). Nevertheless, FCM levels were only moderately increased afterwards. Their conclusion that cortisol ingested via prey (note: amounts are two to three orders of magnitude lower) affects the
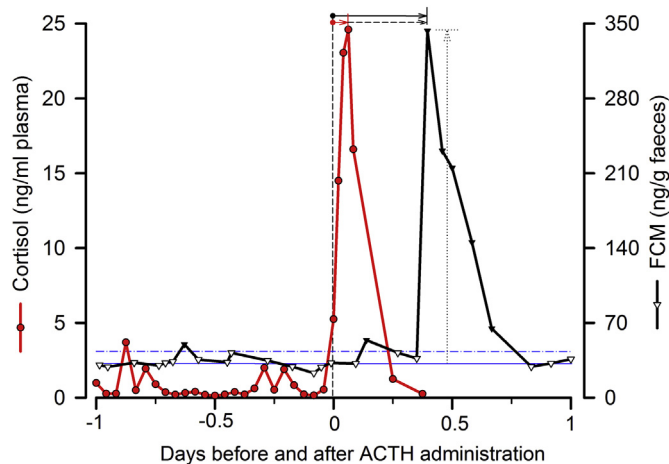
Fig. 2. Example of a time series (before and after ACTH administration) of blood and faecal samples collected in a cow receiving 125 μg ACTH (i.v.). Baseline (mean) FCM level (calculated by an iterative approach; details see 3.4.4) is given as blue line (dotted line: mean + 2 SD). FCM levels above that threshold are marked by filled triangles. Horizontal arrows indicate delay times between injection and plasma cortisol (red) or FCM (black; fully lined) peak, and between both peaks (black; dashed line). The vertical arrow denotes the increase of peak FCM levels above baseline. Please note the different scaling of both y-axes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

usefulness of FCM analysis is not supported by the presented data.

Dexamethasone suppression tests are applied to reduce FCM levels due to the negative feedback on the HPA axis activity, hence inhibiting GC release into the bloodstream. Thus, after the species-specific delay time, low levels of FCMs are expected. Such tests can help to discover interfering substances, such as steroids of gonadal origin, but also nonspecific interferences (e.g. by lipids or solvents used for extraction). Only a small number ($n = 23$) of papers included such a suppression test (see citations marked with "↓" in the Supplementary Tables S2–S9) where between 1 and 12 animals per sex were used (e.g. [27,62,79]). Interestingly, but in accordance with other studies using plasma samples, in none of three bird studies conducted so far [33,36,80] could a suppression of the adrenocortical activity be observed.

Some researchers use "handling" or "saline injection" as a control experiment in the context of physiological validation studies (e.g. [36,56,62,79–82]). In animals familiar with such procedures, no increase in FCMs happens [79,80]. In others (wild animals) such a procedure can cause a stress response and thus an increase in FCM levels indistinguishable from that of an ACTH injection ([82]; but see [83] for contrasting responses of two related species). This information is of interest, if the stressfulness of the handling/injection procedure needs evaluation, or the process might act as a sort of biological validation in wild animals (see below).

It is also advised (especially in rodents and other nocturnal species) to include an experiment checking for the presence of a diurnal rhythm in FCM excretion (e.g. [27,62,84]). If such a rhythm is not accounted for, increases or decreases during the day may be misinterpreted as resulting from stressful procedures or the opposite. This was nicely demonstrated in mice and rats, where the FCM peak of the ACTH challenge test (performed in the morning) preceded the peak of the normal diurnal rhythm only by a few hours [27,62].

3.4.3. Biological validations

Physiological validation experiments require special permission to conduct animal experimentation. With some species (e.g. endangered species) such experiments cannot easily be performed. In such cases (but also in others), a biological validation can be used. This consists of measuring FCMs before and after a stressful event that is strong enough

to elevate HPA activity for an extended period (many minutes to hours [43,85], which should yield a peak in FCM levels. This kind of validation demonstrates that the technique can detect biologically meaningful changes in adrenocortical activity. However, negative results are not conclusive, given that the event used may not have been stressful enough to elicit FCM peaks [70,79]. In addition, a complete suppression of HPA activity is not possible by environmental intervention only. In total 58 papers describe such a biological validation. In most cases capture, restraint, transportation, isolation, regrouping, physical examination, veterinary procedures or other anthropogenic disturbances were used as stressors (e.g. [81,86,87]). Caution is advised here to avoid circular reasoning. If a procedure is used to select an assay and to prove its biological validity, it should not be argued that the aim of the study was to evaluate the stressfulness of the very same procedure.

3.4.4. Procedures for performing physiological or biological validations

In order to perform such physiological or biological validations properly, a FCM baseline has to be established by collecting several samples before the ACTH is injected, or the stressor occurs. Afterwards, ideally all voided faeces should be collected over a certain period of time that should extend well beyond the time window during which the FCM peak is expected for the given species. This depends upon the expected intestinal passage times [26], and will be 10–24 h in most species, but may also be shorter (e.g. birds), or longer (e.g. large carnivores). Researchers must be careful to avoid any disturbance during and before the sample collection period, as this might be reflected in additional FCM peaks, which will distort the true baseline. Baseline FCM concentrations are calculated in different ways. To avoid extreme values (potentially reflecting preceding stressful events) from exerting a strong influence, the median (instead of the mean) of all samples can be taken, or an iterative procedure is used [88]. In the latter approach, the baseline is calculated through an iterative process excluding points greater than a certain threshold (e.g. mean + 2 SD) until no points fall above it. Peaks are classified as levels above that threshold [70,79]. These are normally expressed as a percentage above the baseline, and assays yielding stronger signals are favoured, because such assays reflect higher biological sensitivities [70].

Validation experiments should be carried out on several ($n > 2$) animals and, when of interest, in both sexes. However, the fact that many species of interest are wildlife animals or even endangered species, where only a few individuals are accessible (e.g. housed in zoos) may pose serious limitations. Nevertheless all possible efforts should be undertaken to take advantage of necessary stressful events (transportation/veterinary investigations or treatments) and to collect samples from as many individuals as possible.

It is also best practice to test more than one immunoassay on validation samples to inform the selection of the best suited EIA (for some recent examples see [56,70,87,89–93] but see also citations marked with a "+" in the Supplementary Tables S2–S9). Besides the maximal increase (% above baseline) in FCM following the stressor, the robustness (ability of the assay to remain unaffected by small variations in the assay parameters) and availability (costs) of the assay, its potential for a co-measurement of metabolites of different origin (e.g. cross-reactivity with androgen metabolites), and also the stability of the measured metabolites (less prone to degradation) may be taken into consideration when choosing the assay. Contrary to some claims (e.g. by companies selling assay kits), but in accordance with many findings [22,58,70], there is no single assay which is suited for all species. This is best expressed by the title of a study in marsupials "One size does not fit all" [70].

There are unsuitable approaches to achieving such a physiological or biological validation, such as solely relying on diurnal [94] or seasonal [95] FCM variations (see confounding factors; Section 3.6), or mere correlational studies. Even if performed and sometimes recommended, no validation should be based on single faecal samples (per animal) and a correlation with another parameter (also measured

in a single sample; for a recent example see [96]. Correlations are always a questionable approach to prove the validity of a method, and the presence (or absence) of a correlation between two measures of stress does not prove much. Furthermore, the other parameter chosen to evaluate measured FCM levels is automatically set as the golden standard, which is questionable, especially in stress research. In most cases blood samples are used, but there are several reasons to doubt the suitability of measured plasma GC as the reference (e.g. presence of episodic GC fluctuations and a varying portion of biologically active, free GCs; e.g. [8,44]). In addition, this approach is even more questionable when the two measures reflect the endocrine status of a different time window (e.g. blood/saliva and faecal samples taken at the same time). Fig. 2 shows the results of an ACTH challenge test in a cow, where frequent serial blood and faecal samples were collected [44]. This should help to illustrate the problem with correlating single plasma and faecal samples. As expected, ACTH injection resulted in a sharp peak in plasma cortisol concentrations, which was well reflected by a FCM peak. However the FCM peak occurs with a certain delay time, which may vary to some degree (depending upon the defecation rate). Thus, it is clear that correlating GC (FCM) levels in plasma and faecal samples taken at the same time does not make sense, but matching (peak) samples need to be correlated. This however requires frequent sampling (all defecations), because otherwise the peak sample might be missed. If only single plasma/faecal samples are collected, the selected time point would strongly influence measured GC (FCM) levels, and thus yield totally different correlations. In addition, correlating levels of single samples does not permit the evaluation of the number one criterion for assay selection, which is to choose the assay with the biggest difference between baseline and stress-induced FCM concentrations.

Although some (e.g. [14,97]) focus on direct comparison between hormone (metabolite) measurements from blood and faeces (and highlighted possible problems with the latter), I think much might be gained by looking at both sample types as complementary (by carefully considering strength and weaknesses of both). Measured GCs (or their metabolites) reflect different things in blood and faeces: A snapshot in time and a more integrated measure of adrenocortical activity over a certain time period [31], which however reflects the endocrine status some time ago (depending upon the species-specific delay time in faecal excretion). Thus, when animals are captured to draw a blood sample, also faeces can be collected, and the measured GCs or their metabolites, will enable the researcher to gain insights into the immediate and the previous endocrine status of the respective individual (for an example see [98]). Or, as another possibility, several faecal samples are collected over a longer time from selected animals and complemented by single blood samples to help to elucidate confounding factors.

In the published literature, 279 papers reported a physiological (*n* = 186; 67%) or some sort of a biological (*n* = 93; 33%) validation experiment. In 18 cases (6.5%) only a single animal was used, and in another 36 papers (12.9%) only one sex (with 2 to 27 animals) was investigated. Unfortunately, utilized methods in roughly one third (37%) of all published papers (see Fig. 3; Table 2, but also the Supplementary Tables S2–S9) lack a sound physiological or biological validation in terms of demonstrating that an increase in adrenocortical activity is well reflected in measured FCM concentrations. As stated earlier [66], although the highest standards need to be kept, compromises cannot always be avoided (especially in field studies, or when dealing with endangered species). In such cases, these compromises (e.g. single animals used in validation studies) should be addressed frankly. Hence, to save time and money and to avoid some of the many pitfalls, it is advisable to check the available literature on the investigated species carefully and to use a method, which has already been successfully validated for the species under investigation. It also is recommended to get in contact with experienced researchers before the start of the experiments (see Supplementary Tables S2–S9). Help may be also found at the International Society for Wildlife Endocrinology

(ISWE), which aims to serve as a platform for people interested in non-invasive methods (http://www.iswe-endo.org). A major goal there is to improve access and distribution of resources [99]. Many researchers experienced with FCM analysis are also members of ISWE.

The percentage (~37%) of publications utilizing methods which were not validated for use in the investigated species is of serious concern and this shortcoming should be taken seriously. Especially meta-studies should only rely on validated papers in order not to confuse their findings. The use of non-validated (but also biologically insensitive) methods may be even more problematic in case of negative findings (no difference in FCM levels). Such results may turn out to be misleading, and even harmful, because procedures, situations or disturbances may be erroneously rated as not stressful (e.g. [100,101]).

Finally, it has to be mentioned here that there is a widespread misunderstanding (e.g. for a recent occurrence [102]) regarding FCM analysis: You cannot per se validate the measurement of FCM in a given species (or even worse animal taxon). Thus, validating one set of methods for the measurement of FCMs in a species does not validate all possible methods for the measurement of FCMs in that species. Likewise, but contrary to claims (or advertisements in case of commercial assay kits), it is also impossible to validate an assay for general use or for a certain sample matrix (e.g. faeces). If literature is available reporting that an ACTH stimulation (challenge) test provided evidence that the expected increase in FCM levels could be measured with a certain method (extraction and assay), then only this method in the specific species can be rated "validated". This is no indication whether other assays are also suitable. On the contrary, there is ample evidence that some assays may work in a given species, while others do not [58,70].

3.5. How to name the substances measured in the faeces

There are several appropriate abbreviations used frequently in papers: FCMs – faecal cortisol/corticosterone metabolites (some also use: immunoreactive cortisol/corticosterone metabolites); GCMs – glucocorticoid metabolites; FGMs or fGCMs – faecal glucocorticoid metabolites. "Faecal CORT" is double incorrect; first, although frequently used in rodent and bird literature, CORT (all capitals) implies an acronym (like ACTH for AdrenoCorticoTropic Hormone), which it clearly is not (see commentary by Raff [103]). And, secondly, there is usually no unmetabolized corticosterone (cortisol) present in the faeces. For the latter reason, also FC (faecal cortisol/corticosterone), or FGC (faecal glucocorticoid) should be avoided.

3.6. Confounding factors that can influence FCM levels

As pointed out by almost all reviews, there are many potentially confounding factors, which need to be taken into consideration when doing FCM analysis (e.g.: [26,31,66,97,104] but see also Supplementary Table S1). While sometimes highlighted as a special problem with FCM analysis [14,97], the same factors may also influence plasma GC levels (or levels in any other sample matrix [4,105]). Some factors are particularly influential on FCM levels (e.g. diet; factors relevant for metabolism and excretion [32,97]), but this is the case with every sample material [7]. Again it is important to take into account and acknowledge potential confounding factors, and use a rigorous experimental design to ensure proper interpretation of results.

Most important among all factors is that the "individual" plays a crucial role in all aspects of stress research and individual differences have been observed in all parts of the HPA axis, and may be also related to GC metabolism and excretion [6,31,66,106,107]. These differences may be of genetic origin [108], but may be also acquired during life. For example, previous experiences [6] and even pre-natal stress [109] have been reported to exert an influence on GC levels later in life. Here, a special advantage of non-invasive methods using FCMs is that they enable longitudinal studies, whereby an animal can serve as its own
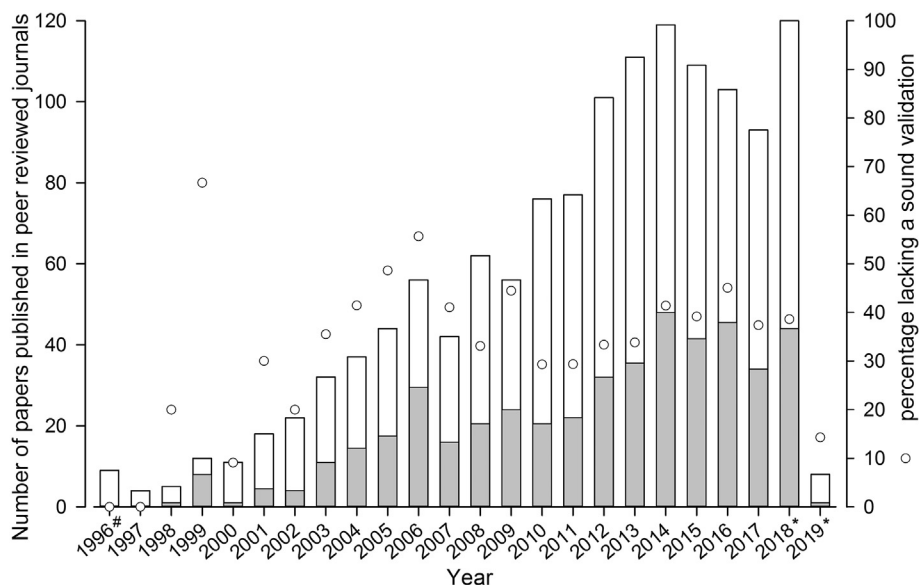
Fig. 3. Number of FCM papers published by year (grey bar: number of papers representing non-validated FCM studies, white bar: number of papers of validated studies plus reviews) and the percentage (○; right y-axis) of non-validated studies (the 55 reviews were excluded). If a method utilized to measure FCM was not physiologically or biologically validated, that paper was rated "non-validated" (see Section 2). # ≤1996; *as of the 20th November 2018.

control [17,66]. This increases the power of applied statistics [110], and especially in laboratory animals can reduce the number of animals needed for experimentation, thus contributing to fulfilling the 3R concept [111].

Sex (the term "gender" should be avoided in this context [112]) plays also an important role when studying GC and stress in animals. Sex differences regarding the central nervous system, the HPA axis, and last but not least also the metabolism and excretion of GCs have been reported. The latter were first described in detail in mice, partly with the help of a radiometabolism study [38,62]. The possible presence of sex differences in a given species should be evaluated within the physiological/biological validation. While such differences have been found in some species [29,38,49,113], they are absent in others [84,114]. When present, FCM levels of both sexes should not be pooled, but analysed separately (e.g. "sex" included as co-variable).

Individual and sex can be accounted for when collection of "anonymous" faeces is avoided. This is easy when dealing with domestic livestock or laboratory animals, but may be more problematic in wildlife studies. For the latter, when directly observing the defecating animal [115] is impossible, this problem can be overcome by a combination with genetic analyses [116,117]. In cases where more animals are housed together (zoos, private homes or social laboratory animals) indigestible markers can be fed to identify individual animal faeces [118], or special cages can be used, which allow for short term separation of individuals [119].

Reproductive state (which is of course connected with season in wildlife animals) influences GC and FCM levels. In many mammalian species, GC levels are elevated during pregnancy and increases in GCs trigger parturition [2], thus higher FCM concentrations during pregnancy and around parturition are found [120]. The developmental stage (e.g. infant, juvenile or adult), and thus age may also have an effect on GC or FCM levels [56,121]. Especially in social animal species, the social environment (group structure, degree of social integration or isolation, hierarchical rank) can exert an important influence on GC and FCM concentrations [35,122,123].

Seasonal variations in plasma GC and likewise in FCM levels have also been reported [124–126]. Most animals show a pronounced diurnal rhythm in basal HPA activity, with maximal levels (basal peak) corresponding to the onset of the circadian active period [8]. These changes are reflected in FCM levels (e.g. mice [62]). To account for this, a control group needs to be included in the experimental setup, or faeces should be collected at the same time of day. To be on the safe side, the latter is recommended when it is not possible to check the presence of such a rhythm in a given species. If this is not possible it is important to include "time of day" of the sample collection as a co-variable into the statistical analysis.

Diet (or changes in diet) may affect plasma GC levels to some extent [127], but more seriously FCM concentrations [31,66,97,128]. Diet and bacterial composition can potentially alter metabolism of GCs. Changes in diet can also influence faecal bulk and metabolic rate; both may affect FCM levels, and also defecation frequency. However, it is unclear if increased metabolic rate may be compensated by HPA feedback loops when plasma levels are decreased (e.g. [129]). Dietary effects may be more pronounced in birds, because their diet can change dramatically over the course of a year, and because they excrete urine and faeces together in the form of droppings [31,32].

In general, the more standardized the environment, the less problematic those confounding factors should be. Standardization can be expected to increase from wildlife animals to pet animals, domestic livestock, zoo animals and laboratory animals. In any case, special attention needs to be given to all those possibly confounding factors when performing studies using FCMs.

4. Applications

4.1. Overall comments

The absolute number of publications on FCMs has tremendously increased over the last 20 years. My paper collection comprises 1327 publications in 300 journals encompassing 362 different species. The top 10 journals (Fig. 4) covered almost 40% (top 77: 76%) of all publications. Among those "General and Comparative Endocrinology" has published the highest number of FCM papers and also has the lowest percentage of articles using non-validated FCM methods. Interestingly, two recently founded journals ("Conservation Physiology" and PLoS One) show strong increases in the number of publications of FCM studies and managed to get into the top 10. Roughly half of all journals published only a single FCM paper. Although approximately 3600 researchers have (co)authored FCM papers, most were probably not directly involved (about 2600 published exactly one paper, only 456 of them as first author). Thus the group of researchers who has been more frequently involved in FCM research is rather small. Only 32 authors have published at least 10 papers (including reviews) reporting studies using validated FCM methods.

Table 2 gives a summary (by phyla or order, respectively) of the number of species (papers) where a FCM analysis has been described,
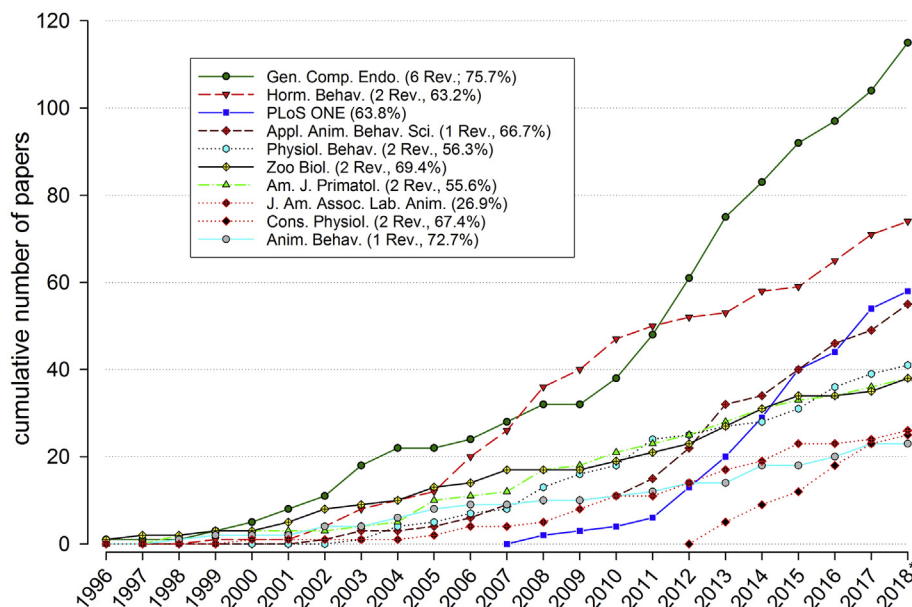
Fig. 4. Cumulative number of FCM papers published in the top 10 journals. The number of reviews and the percentage of papers from studies using validated FCM methodology (reviews excluded) are given in brackets. *as of the 20th November 2018.

Table 2
Overview of investigated species (by number) of Mammalia, Aves and others – given in total (first line), but also split up by order or phylum. The number of papers and the percentage of papers from "non-validated" studies (%n-v) are also given.

Phylum	Order	Species (no.)	Papers (no.)[a]	%n-v
Sum		363	1345	37%
Mammalia		**274**	**1137**	**37%**
	Primates	67	254	43%
	Glires (Rodentia/Lagomorpha)	63	362	45%
	Artiodactyla	51	203	34%
	Perissodactyla/Paenungulata	13	105	19%
	Carnivora	52	167	29%
	Marsupialia/Xenartha/Chiroptera	28	46	33%
Aves		**69**	**187**	**28%**
	Anseriformes	9	31	16%
	Charadriformes	6	7	14%
	Galliformes	6	49	24%
	Passeriformes	17	34	26%
	Pelecaniformes	3	14	71%
	Psittaciformes	8	14	43%
	Sphenisciformes	3	7	0%
	Others	17	31	29%
Others		**20**	**21**	**81%**
	Reptilia	12	12	75%
	Amphibia	1	1	100%
	Fish	6	7	86%
	Mollusca	1	1	100%

[a] Notice that in the case a paper investigated more than one species, it is counted for each species separately.

and the percentage of non-validated studies. More details about the different groups and the investigated species can be found in the Supplementary Tables S2–S9. The highest numbers of species were investigated in "Primates" and "Glires", but in both groups also the highest percentage of non-validated methods was utilized. This may be caused by researchers who have easier access to (commercial) assays, but lack a special background in FCM analysis. Another explanation may be the high number of field studies with species involved, which

are less accessible to validation experiments. This may very well also be the explanation for a low percentage of validated methods in other groups (e.g. [130]).

4.2. Selected examples

There has been a plethora of studies in the past several years using FCMs (Fig. 3). The broad range of journals emphasizes the wide spectrum of research fields (see subheadings below – although there is some overlap between them) where such non-invasive methods have been applied (see Supplementary reference list). In the following a few examples (grouped according to their main focus) are chosen to highlight the usefulness of such methods, but also to point at different methodological issues discussed above.

4.2.1. Stress biology

An interesting longitudinal study in field endocrinology (combined with behavioural observations) was performed by Ganswindt et al. [115]. They followed wild-living African elephants (*Loxodonta africana*) and elucidated endocrine correlates of musth and the impact of ecological and social factors on its occurrence. Musth was associated with higher levels of faecal androgen metabolites, which were already elevated before the onset of physical musth signs, suggesting changes in androgen levels represent the initial stimulus. Interestingly, musth was accompanied with lower FCM concentrations in wild-living African elephants. Needless to say that such wildlife studies would not be possible without non-invasive methods.

Over 11 years Markham et al. [131] investigated the optimal group size in a highly social animal, the wild baboon (*Papio cynocephalus*). They analysed > 10,000 faecal samples from 157 adult females, making it the study with the highest sample number. Interestingly, they observed a U-shaped relationship between group size and home range area, average daily distance travelled and FCM levels. They postulated that large groups are constrained by within-group competition, whereas small groups face higher levels of between-group competition and predator pressure.

An experimental field study, performed by Dantzer et al. [132], demonstrated that social cues reflecting population density were sufficient to elicit increased offspring growth through an adaptive hormone-mediated maternal effect. Female red squirrels (*Tamiasciurus*

hudsonicus) in densely populated areas had faster-growing pups, which more likely survived their first winter. The researchers manipulated perceived density (by playbacks of squirrels' territorial cries) and fed GC to pregnant females. Increased FCM levels and heavier pups demonstrated in both cases that elevated maternal glucocorticoid levels were the mediator of increased offspring growth.

Spaan et al. [133] investigated the stress response in African buffalo (*Syncerus caffer*) subjected to an immobilization. They performed an ACTH challenge test and a saline injection in the same individuals (1 week apart). An untreated group served as controls. Serial plasma and faecal samples were collected. Two commercial assays, a ^{125}I-corticosterone RIA (MP Biomedicals) and a cortisol EIA, were used to measure FCMs. Following ACTH injection, increases in plasma cortisol and FCM levels were detected with both assays (more expressed with the RIA), proving their suitability for FCM analysis in buffalo. Interestingly, FCM results revealed that adults, but not sub-adults mounted a stress response to immobilization. This example underscores that caution is advised when using biological validations, as not every type of stressor may result in FCM increases, and underlines the possibility of age dependent differences in HPA activity.

4.2.2. Ethology

A study [35] combining behavioural and endocrine data investigated adrenocortical responses of ravens (*Corvus corax*) during separation. Strong social integration was related to low FCM levels when the individuals were within the group and high levels during separations. In contrast, poorly socially integrated ravens exhibited the opposite pattern, indicating that the birds' adrenocortical activity can be modulated by their social integration.

Dantzer et al. [134] investigated the influence of stress hormones and aggression on cooperative behaviour in subordinate, wild meerkats (*Suricata suricatta*). Provisioning individuals with exogenous glucocorticoids elevated plasma GC and FCM levels. Interestingly, the effects of manipulating GC concentrations on cooperative behaviour varied between cooperative activities as well as between the sexes.

4.2.3. Ecology

Invasive alien species are a serious ecological problem world-wide and represent a threat to native species. Eurasian red squirrels (*Sciurus vulgaris*) have gone extinct from many European sites after introducing American invasive grey squirrels (*Sciurus carolinensis*). Santicchia et al. [135] found that native red squirrels where they co-occurred with invasive grey squirrels had FCM concentrations that were three times higher than those in sites without the invasive species. Moreover, in a longitudinal study, stress hormones in red squirrels increased after colonisation by grey squirrels. When they experimentally reduced the abundance of the invasive grey squirrels, FCM concentrations in co-occurring red squirrels decreased significantly.

Another recent study [136] investigated the contribution of environmental stressors and life history stage to the allostatic load in a migratory population of zebras (*Equus quagga*) in the Serengeti ecosystem. They included an ACTH challenge to successfully validate a group-specific EIA for measuring FCMs. Higher FCM levels were found in animals in large aggregations and in band stallions (compared to bachelor males). They concluded that migratory zebra have elevated allostatic loads in large aggregations that probably result from their combined responses to increased feeding competition, predation pressure and various social stressors.

4.2.4. Animal conservation

Research also revealed the potential of non-invasive methods to help to inform management plans to conserve highly endangered species, because they allow more frequent and aggregate measures of physiology which produce minimal disturbance to the animals. Thiel et al. [63] investigated the impact of human outdoor recreational activities on capercaillie (*Tetrao urogallus*). Ski tourism affected both

habitat use (although only at a small spatial scale within home ranges) and endocrine status. FCM levels of individuals in areas with low recreation intensity were significantly lower than those in areas with moderate or high recreation intensity during the entire study period (three winters). In order to protect capercaillie populations, managers should keep forests inhabited by capercaillie free from tourism infrastructure, or at least retain undisturbed forest patches within skiing areas [63]. Similar FCM results were found in mountain hares (*Lepus timidus*) located in areas differently affected by winter tourism [137].

There are a number of different studies utilizing FCMs, which demonstrate that anthropogenic disturbances also increase adrenocortical activity in non-endangered animals (e.g. [138,139]). Most recent, Zbyryt et al. [93] found that stress in wild ungulate (red deer and roe deer) populations was lower and less variable in areas utilized by large carnivores (wolf and lynx) than in carnivore-free areas where human-related factors predominated. On the other hand, Rodrigues [140] found that spider monkeys (*Ateles geoffroyi*) could successfully cope with mildly disturbed habitats, and food provided by humans was a valuable resource. And for some species urban habitats may even be relatively benign. For example, Lyons et al. [141] discovered that urban chipmunks had lower FCM concentrations and were in better body condition than those from natural habitats.

From a conservation perspective, management of animal populations kept in captivity (ex situ) or in their natural habitat (in situ) is important. Identifying the ecological and anthropogenic processes that act as sources of physiological stress in wildlife is of prime importance to implementing appropriate management and conservation strategies [142]. By using FCMs as an indicator of stress, Santos et al. [143] examined various factors influencing a wild red deer (*Cervus elaphus*) population in the Mediterranean ecosystems of south-western Europe. They found that factors related to hunting management were the main drivers of FCM variation in red deer, followed by those related to environmental conditions and individual traits. Their findings provided an integrated perspective of how multiple factors impact on stress physiology in large wild herbivores and highlighted the importance of considering management practices, as well as spatio-temporal variation, when assessing stress-inducing factors in wild populations.

In an *ex situ* all-male colony of large flying foxes, Freeman et al. [144] investigated seasonal dynamics of agonistic behaviour and hormones. The authors performed a biological validation, which revealed that GC metabolites could be reliably measured in urine and faeces. Three agonistic behaviours were identified as early indicators to signal the onset of the breeding season. They could be used to implement *ex situ* management changes to reduce the incidence of agonism between individuals.

However, when dealing with conservation efforts, a study [145] in captive African lesser bushbabies (*Galago moholi*) utilizing FCMs indicated that caution is advised when comparing individuals held in captivity with free-ranging ones, because captivity itself or differences in diet between the populations may have an effect on detected FCM levels (or adrenocortical activity) of a species.

4.2.5. Animal welfare

For domestic livestock routine or management procedures have to be frequently performed. FCM levels can be used to reflect experienced stress. This helps to select less stressful procedures, and thus to improve animal welfare. The following two examples also demonstrate different sampling regimes needed to monitor short term (acute) or longer lasting stressors. In a large-scale on-farm study in cows ($n = 207$) two different devices for restraint during functional claw trimming were compared [146]. Frequent faeces collection was necessary to monitor this acute stressor (lasting for approximately 10 to 20 min). 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 [146]. In another experiment evaluating management procedures for the introduction of pregnant, young females into the herd of adult dairy goats, social behaviour and adrenocortical activity (via FCMs) were evaluated [147]. Here the establishment of a new dominance relationship was expected to act as a stressor over a longer period (days), thus less frequent sampling was performed. Actually, as the first sample after the introduction was collected after 3 days, acute effects were not captured. Results indicated that young goats experience less social stress when being introduced shortly after parturition and with fawn kids still present rather than during the "dry period" before parturition [147].

Although a consensus endocrine profile for chronically stressed animals does not exist [148], most researchers expect increased levels of GCs (FCMs). Interestingly, a recent study in horses [149] found that animals whose welfare was clearly compromised (as indicated by an unusual ears backward position, presence of vertebral problems or haematological anomalies) had lower levels of both FCM and plasma cortisol. This underlines the necessity to evaluate several stress response parameters for a correct interpretation of data. In another study [150] lower FCM levels were correlated with skin and oral lesions in rhinos (*Diceros bicornis*). Both studies suggest that welfare and health issues may be correlated in a U-shaped way with GC concentrations and that an animal's incapability to mount an adequate stress response has negative consequences.

4.2.6. Biomedicine

There is broad application of non-invasive methods in biomedicine, because they enable longitudinal studies and avoid the negative effects of blood sample collection. Animal models are widely used to elucidate pathogenesis and possible treatment of human diseases. Such a model for affective disorders has been established in mice, which were selected for extremes in stress reactivity [151]. Animals of both extremes showed behavioural, endocrine and sleep changes resembling major endophenotypes of depression. Hereby, FCM levels in frequently collected faecal samples revealed the typical changes in the diurnal rhythm of adrenocortical activity found in affective disorders [152]. As in this example, assessing adrenocortical activity may be of importance for the model itself, or regarding animal care welfare issues, which are of growing concern (see Section 5.1.). In both cases, FCM analysis is an ideal non-invasive method.

5. Future directions of research

There are two main directions of further research, one connected with a broader application of such non-invasive methods and another with a further refinement of them (including topics related to the metabolism and excretion of GCs, and possible biological actions of FCMs).

5.1. Broader application of FCM analysis

As outlined above (Section 4.2.) FCM analysis has proven useful in a variety of research areas. Here, some further areas for broader application are highlighted. Studies in free-ranging animals are often limited by "anonymous faeces" collection. The advent of cheaper methods to generate genetic data will be helpful to overcome this limitation in the future and will open up new possibilities in wildlife research [116,117]. Among others, especially in ecological research, questions related to cross-species variation or individual plasticity across different environments [153], and how life history relates to modulation of immunity and stress reactivity (e.g. [154,155]), could benefit from an application of FCM methods.

Animal welfare issues, especially in biomedical research (laboratory rodents), are of great concern. Here, measurement of FCMs could be further incorporated into evidence-based severity assessments of experimental procedures (possibly causing pain and distress; e.g. [156–158]). With respect to biomedicine there is an urgent need to

increase repeatability and replicability in laboratory experiments, and a greater need to examine individual-level variation, especially in the context of understanding human responses to disease. All those areas could benefit from including FCM analysis.

5.2. Further investigations into GC metabolism and the biological activity of FCMs

Laboratory animals are kept in highly standardized environments, where many confounding factors are kept constant. However, pharmacological substances (e.g. anaesthetic drugs) may exert an influence on excreted FCMs. And the same may be true in animal welfare questions within the field of veterinary medicine, when such drugs are applied for (e.g. pain) treatment of the animals. As many of those substances can affect liver enzymes [cytochrome P450 (CYP) enzymes] also involved in the metabolism of steroids [159], they may change the proportions of glucocorticoid metabolites, thus leading to misleading different assay values. To date the influence of substances affecting liver enzymes, and thus most likely GC metabolism, on FCM levels has not been investigated, but needs special attention. Likewise, the influence of diet (quality and amount), but also other factors such as corticosteroid binding protein (CBG) on metabolism and excretion of metabolites still needs more detailed investigations, probably best in combination with radiometabolism studies and steroid profiling with LC-MS.

Pronounced species differences regarding the metabolism and excretion of glucocorticoids have been found [22,26,43]. However, little is known about their basis. Thus more investigations elucidating the sources and reasons should be favoured. This is particularly relevant, because FCMs may not only be metabolic end products, but also have some biological activity [160]. For example, reduced steroid metabolites (e.g. dihydrotestosterone) are known to exert strong hormonal effects. Similarly, reduced FCM (which can be reabsorbed from the intestinal tract) may be the mediator of some biological effects within the gut−brain axis [161,162]. In addition, a mammalian cortisol metabolite shows androgenic activity in fish and could thus function as an endocrine disruptor [163]. Faeces deposited in feedlots release FCMs into the water and thereby may affect fish populations. Furthermore, 11-oxygenated C19 steroids (some of which are also among those described as FCMs) have been found to activate the human androgen receptor, thus possibly playing a role in androgen related diseases [164]. Taken together studies investigating intra- and inter-species biological effects of FCMs could open up interesting research questions.

6. Conclusion

Besides giving a comprehensive overview of the published literature on FCM (see also Supplementary Tables S2–S9), this review should be understood as an urgent plea for sound validation of applied methods, especially providing proof that the measured FCM reflect adrenocortical activity in the investigated species. Much harm and frustration with non-invasive methods derives from a lack of background knowledge and an application of non-validated methods. Unfortunately, the latter has remained almost unchanged over the years (~37% of the published articles utilized methods, which were not validated for use in the investigated species). Thus, a main aim of this review is to alert readers, authors and of course editors and reviewers to take this shortcoming seriously. Otherwise, as a consequence, many results, especially negative findings (no difference in FCM levels), may turn out to be misleading, if not harmful. Particular attention should be given to methodological shortcomings in meta-analyses, and papers using non-validated methods excluded. Collecting faecal samples and analysing FCM may appear simple and straightforward. However, researchers must properly collect and handle samples and avoid or take into account sources of confounding variation. In addition, interpretation of results might be incorrect without background knowledge of how FCM vary through time (delay times of excretion; diurnal rhythms). Applied

properly, FCM analysis as a non-invasive method for assessing HPA axis status has great potential and has proven useful in a variety of research areas, such as (stress) biology, ethology, ecology, animal conservation and welfare, but also biomedicine.

Declaration of interest

The author declares no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.physbeh.2018.11.021.

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