Methods in Ecology and Evolution

British Ecological Society

Methods in Ecology and Evolution 2012, **3**, 757–765 doi: 10.1111/j.2041-210X.2012.00203.x

REVIEW

On the use of non-invasive hormone research in uncontrolled, natural environments: the problem with sex, diet, metabolic rate and the individual

Wolfgang Goymann

Max-Planck-Institut für Ornithologie, Abteilung für Verhaltensneurobiologie, D-82319 Seewiesen, Germany

Summary

1. Methods to measure metabolites of steroid hormones from faeces have become very popular in wildlife conservation and ecology, because they allow gathering physiological data without the necessity to capture the animals. However, this advantage comes at costs that are particularly relevant when studying free-living animals in their natural environments. Previous methodological reviews have stressed the importance of validations to prove that real metabolites of the hormone in question are measured, but the research community has largely ignored further caveats relating to sex, diet, metabolic rate and individual differences in hormone metabolite formation.

2. Often the sexes differ in how they metabolize hormones. As a consequence, one may not be able to compare hormone metabolite concentrations between males and females of one species.

3. Diet can alter the way hormones are metabolized, and different diets can change the amount of faecal bulk. Both phenomena can result in measurement artefacts that may seriously distort the estimation of hormone metabolite concentrations. As a consequence, comparisons of hormone metabolite concentrations, for example, between seasons or populations, may become problematic.

4. Changes in ambient temperature and food availability may trigger large fluctuations in metabolic rate of free-living animals. These fluctuations may then result in major distortions of faecal hormone metabolite concentrations without any change in bioactive hormone levels.

5. Bacteria metabolize hormones in the gut. Individual differences in bacterial composition can cause differences in how hormones are decomposed. Thus, individuals may differ with regard to what kind of hormone metabolites they form and with regard to the relative composition of these hormone metabolites. As only specific metabolites are measured, differences in metabolism may distort the results.

6. In summary, non-invasive hormone research measures various end products of a hormone after its clearance from the circulation and extensive modification by bacteria. Not only does this increase random variance, it may also generate systematic noise, which may seriously distort the signal (i.e. the hormonal status of the individual) in a non-random manner. Thus, we still need to learn much more about whether this widely used technique reliably measures the physiological status of animals in uncontrolled environments.

Key-words: bacterial metabolism, bird droppings, cortisol, diet, endocrinology, faeces, glucocorticoids, hormone metabolites, metabolic rate, sex differences, testosterone

Introduction

With some delay after their development (Taylor 1971; Czekala & Lasley 1977; Bercovitz *et al.* 1982; Möstl *et al.* 1984) methods for measuring steroid hormone metabolites from animal excrements have become very popular in studies of wildlife conservation and physiological ecology during the past decade. For field researchers, it is very attractive to be able to link the physiological status of wild animals to behaviour or other life-history traits without the necessity for capture, restraint and taking invasive samples. This is a clear advantage of this method compared with traditional measures of reproductive and stress hormones obtained from serum or plasma samples. A further advantage of non-invasive methods is that sampling does not

*Correspondence author. E-mail: goymann@orn.mpg.de

© 2012 The Authors. Methods in Ecology and Evolution © 2012 British Ecological Society

necessarily interfere with the natural behaviour of the animals. Hence, the boom of non-invasive hormone metabolite measurements in recent years comes as no surprise. Unfortunately, however, the advantages of non-invasive hormone research come at costs that are particularly relevant when studying animals in uncontrolled environments. But surprisingly, there is little debate about these caveats. The aim of this article is to point out the limitations and pitfalls that have not yet been addressed and continue to be neglected by the research community. Ecologists and conservation scientists need to be aware of these issues because they may affect the interpretation of hormone metabolite data obtained from wild, free-living animals. I will focus on hormone metabolite measurements from faeces (in mammals) and droppings (i.e. the mixture of urine and faeces in the cloaca of birds, reptiles or amphibians), but some of the issues are also relevant with respect to urinary analyses.

A number of published reviews and guidelines on non-invasive hormone research have sufficiently highlighted the importance of proper biological and physiological validations (e.g. Goymann 2005; Palme 2005; Touma & Palme 2005; Wielebnowski & Watters 2007; Scott *et al.* 2008; Sheriff, Krebs & Boonstra 2010; Sheriff *et al.* 2011). Hence, I will only briefly touch upon this highly relevant topic. The main focus of this contribution will be on further issues, namely effects of sex, diet, metabolic rate and individual differences in hormone metabolism on the measurement of hormone metabolite levels. These topics are of paramount importance particularly with respect to research in uncontrolled environments, that is, ecological- and conservation-oriented research on wild animals in their natural habitats. To my knowledge, the implications of these factors have hardly been touched upon.

Hormones vs. hormone metabolites – a crucial difference

Measuring plasma or serum hormone concentrations means to measure the actual hormone, that is, a signal molecule that is transported in the blood and binds to specific receptors in target tissues. There, the hormone mediates some kind of effect, such as altering the cell activity or leading to protein synthesis (e.g. Bentley 1998; Nelson 2011). In contrast, non-invasive measurements of steroid hormone metabolites do not capture the active signalling molecule. Instead, these methods measure various metabolic end products of the hormone after it has been cleared from the circulation and extensively modified by bacteria in the gut (see e.g. Taylor 1971; Goymann 2005; Klasing 2005; Palme *et al.* 2005; Hirschenhauser *et al.* in press). During this metabolic process, a specific hormone is typically broken down into various metabolites (Palme 2005), which are then measured in faeces or droppings. This fact is the source of many complications that need to be considered for a meaningful interpretation of hormone metabolite data.

Physiological and biological validations

A convenient, economic (and thus common) method to determine steroid hormone levels is via immunoassay. With this method, the hormone (antigen) in the sample is bound to a specific antibody against this antigen and detected either through enzymatic (enzyme-immunoassay) or radioactive labelling (radio-immunoassay). The concentration of the hormone is then determined using a calibrated standard curve of the same hormone (for details see, e.g. Chard 1995). In a medium such as blood plasma, this usually works quite well. Here, the steroid hormone in question occurs in relatively high concentrations in its biologically active form. Potential cross-reactions of the specific antibody with structurally similar antigens are negligible, because these cross-reacting substances (for example, structurally similar metabolites of the steroid hormone) occur only in low concentrations compared with the active hormone. In contrast, excrements from animals are composed of myriads of substances including hormone metabolites. In faeces or droppings, the metabolites of steroid hormones occur at much higher concentrations than the original and biologically active steroid (e.g. Palme *et al.* 2005; Touma & Palme 2005). In fact, the original steroid is typically completely metabolized so that it is no longer present in faeces or droppings at all (e.g. Graham & Brown 1996; Palme *et al.* 1996; Wasser *et al.* 1996; Goymann, Möstl & Gwinner 2002a,b; Möhle *et al.* 2002; Baltic *et al.* 2005; Thiel, Jenni-Eiermann & Palme 2005; Dantzer *et al.* 2011; Hirschenhauser *et al.* in press). As long as the assays' antibody cross-reacts only with substances that are true metabolites of the original hormone, the problem is a minor one. However, if the antibody cross-reacts with metabolites of structurally similar but functionally different hormones, this can have major and distorting impacts on the results of the study. Thus, a meaningful immunoassay for hormone metabolites relies on substantial cross-reactivity of the assays' antibody with one or several main metabolites of the steroid in question, while at the same time, the antibody should not react with metabolites of other steroids. As a consequence, assays to measure hormone metabolites require a careful physiological or biological validation to demonstrate the assay system's capability to detect relevant metabolites only. This topic has been extensively dealt with in the literature (Goymann 2005; Möstl, Rettenbacher & Palme 2005; Palme 2005; Touma & Palme 2005; Wielebnowski & Watters 2007; Scott *et al.* 2008; Sheriff, Krebs & Boonstra 2010; Sheriff *et al.* 2011), but unfortunately, papers without these essential validations continue to get published even in high-quality journals (e.g. Langmore, Cockrem & Candy 2002; Lucas *et al.* 2006; Sorato & Kotrschal 2006; Stöwe *et al.* 2008; Arlet *et al.* 2011; Selva *et al.* 2011; Ezenwa, Ekernas & Creel 2012). Many authors, editors and referees do not seem to be sufficiently aware of this essential validation requirement, which might result in the publication of seriously flawed data.

Sex differences and hormone metabolite excretion

A number of studies have reported sex differences in the composition of hormone metabolites, that is, females and males metabolize the same hormone differently and excrete different metabolites of this hormone. For example, females and males

excrete different kinds of corticosterone metabolites in mice (*Mus musculus*; Touma *et al.* 2003), domestic chicken (*Gallus gallus*; Rettenbacher *et al.* 2004), black grouse (*Tetrao tetrix*; Baltic *et al.* 2005), Japanese quail (*Coturnix japonica*; Hirschenhauser *et al.* in press) and European stonechats (*Saxicola torquatus*; Goymann, Möstl & Gwinner 2002a). Sex differences also exist in the excretion of androgen metabolites. For example, male and female European stonechats metabolize testosterone differently; as a result, testosterone metabolite measurements from droppings reflect testosterone production in males, but not in females of this species (Goymann, Möstl & Gwinner 2002b; Goymann 2005). Recently, Preis *et al.* (2011) very elegantly demonstrated that an immunoassay to measure testosterone metabolites in the urine of chimpanzees (*Pan troglodytes*) does indeed detect testosterone metabolites of gonadal origin in males. In female chimpanzees, however, the same immunoassay detects androgen metabolites of adrenocortical origin. Thus, the information gained from androgen metabolites in urine of male chimpanzees is related to reproduction, whereas the information gathered from androgen metabolites of female chimpanzees may rather be related to stress.

Surprisingly, the fact that the sexes differ in how they metabolize hormones has resulted in little debate (but see Millspaugh & Washburn 2004; Goymann 2005), even though the consequences are far-reaching. First of all, sex differences in the hormone metabolism suggest that biological and physiological validations of hormone metabolite measurements may need to be performed for each sex separately. Secondly, if there are sex differences in how hormones are metabolized and if a particular immunoassay picks up different metabolites in females and males (or only metabolites of a particular hormone in one sex), then a quantitative comparison of absolute hormone metabolite concentrations between the sexes does not make sense. Such comparisons merely reflect the magnitude of cross-reactivity of the antibody with particular and sex-specific hormone metabolites, but not an actual difference in hormone levels between the sexes (Fig. 1). Thus, these data neither carry any meaningful biological information regarding the physiological ecology of animals nor would they be relevant for questions concerning conservation.

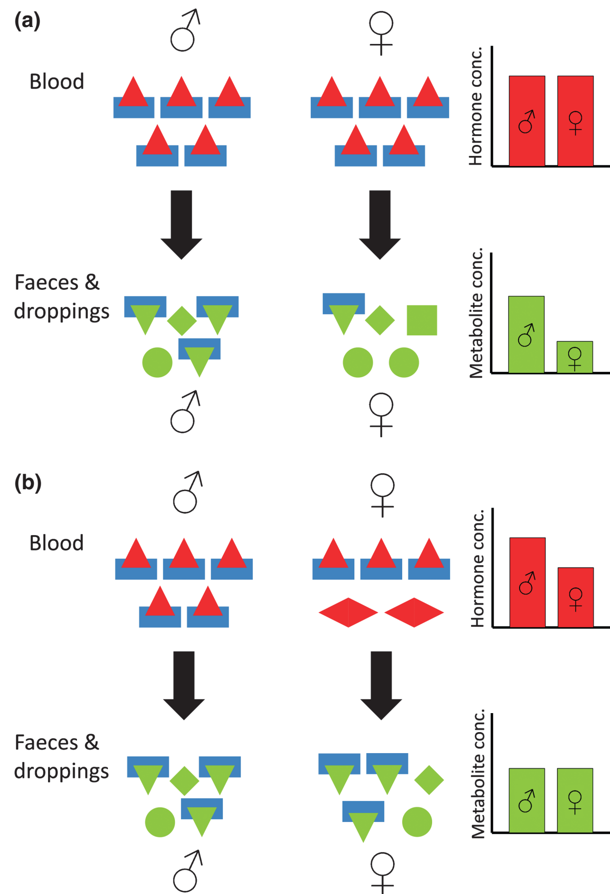

Fig. 1. Potential differences between the sexes in hormone metabolite formation. The scheme represents an immunoassays' antibody reaction with hormones in blood or hormone metabolites in faeces or droppings. A specific antibody (blue square) detects the native steroid hormone (red triangles) in blood plasma from females and males. (a) In the first scenario, the concentration of the respective steroid hormone is similar in the plasma of females and males. Hence, the immunoassay correctly measures that there are no sex differences (bar graph with red bars). In faeces or droppings, the native hormone is metabolized to various compounds in a sex-specific manner (green symbols). However, only some of these metabolic compounds react with the antibody of the immunoassay. Females and males differ in how they metabolize the original hormone. Because the antibody picks up only some of these metabolites, the concentration of hormone metabolites differs between the sexes (bar graph with green bars). This is correct with respect to the particular metabolites measured, but does not carry biologically meaningful information with respect to sex differences in hormone concentrations. (b) In the second scenario, the concentration of the respective steroid hormone differs in the plasma of females and males (red triangles). Hence, the immunoassay correctly measures an existing sex difference (bar graph with red bars). In addition, females secrete a second but structurally similar hormone (red trapezoids) not detected by the antibody. In faeces or droppings, the two hormones are metabolized to various compounds. Some of the metabolites of the other female-specific hormone cross-react with the antibody (green symbols). As a result, there are no differences in hormone metabolite concentrations (bar graph with green bars). Again, this is correct with respect to the particular metabolites measured, but does not carry biologically meaningful information with respect to existing sex differences in target hormone concentrations.

Diet and hormone metabolite excretion

Only few studies have investigated the potential impact of diet on hormone metabolites in excrements. In dairy cattle, changing the amount of dry matter in the diet did not affect faecal progesterone metabolite concentrations (Rabiee *et al.* 2002), but may have affected glucocorticoid metabolites (Morrow *et al.* 2002). In humans, a vegetarian diet high in fibres and low in fats increased oestrogen metabolite excretion (Goldin *et al.* 1981, 1982; Gorbach & Goldin 1987; Pusateri *et al.* 1990). The authors ascribed this effect to differences in the composition of bacteria in the gut of non-vegetarians and vegetarians, which may have resulted in a lower reabsorption of oestrogen conjugates and thus a higher excretion of oestrogen metabolites in people with a vegetarian diet (Pusateri *et al.* 1990). Similar to humans, an increase in dietary fibre increased testosterone and

corticosterone metabolite concentrations in faeces of red squirrels (*Tamiasciurus hudsonicus*; Dantzer *et al.* 2011).

In contrast to these results, an increase in dietary fibre led to a decrease in the amount of excreted progesterone metabolites per unit of faecal mass in baboons (*Papio cynocephalus*; Wasser *et al.* 1993), and experimentally adding fibres to food decreased corticosterone and testosterone metabolite concentrations in droppings of European stonechats (Goymann 2005). In baboons and stonechats, the high-fibre diet probably led to an increase in gut passage time and, more importantly, to an increase in excrement mass (because of the indigestible fibres). This means that the same amount of hormone metabolites per unit of time was placed into a larger amount of faeces or droppings per unit of time. This resulted in a lower concentration of hormone metabolites, as the concentration is typically expressed with reference to faecal or dropping mass (particularly in studies of free-living animals; see also Fig. 2). For stonechats, this was tested by calculating the hourly excretion rate of hormone metabolites. This measure is independent of excrement mass, and in fact, when this measure was applied, there was no difference in the excretion rate of corticosterone and testosterone metabolites between the high-fibre and the low-fibre group (Goymann 2005). In conclusion, there is some correlational and experimental evidence that changes in dietary composition content may influence the measurement of hormone metabolite levels.

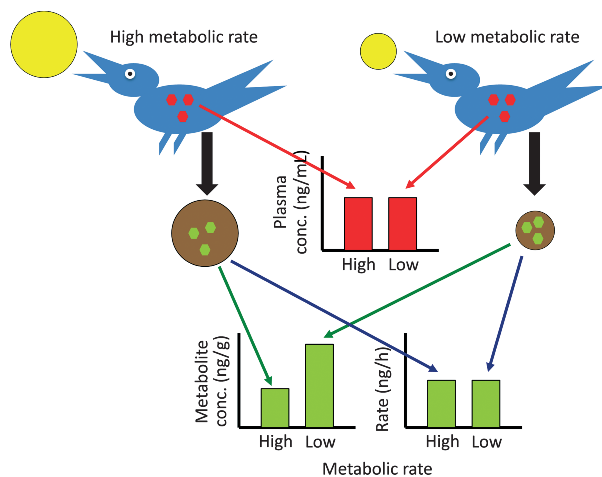

Fig. 2. Schematic representation of the influence of metabolic rate on hormone metabolite measurements. The left bird has a high metabolic rate and consumes a large amount of food (large yellow circle). The right bird has a low metabolic rate and consumes a small amount of food (small yellow circle). Both birds do not differ with respect to plasma hormone concentration (red symbols and central bar graph) and the amount of hormone metabolite production (green symbols inside the brown 'droppings', and lower right bar graph). However, because of its higher metabolic rate and food intake, the left bird puts the same amount of hormone metabolites into a larger dropping (brown circles). Because the concentration of hormone metabolites is expressed with respect to dropping mass as a reference, the concentration of hormone metabolites is lower in the bird with the higher metabolic rate than the bird with the lower metabolic rate (lower left bar graph). Thus, in this case, the concentration measurement of hormone metabolites underestimates the true hormone concentration of birds with a higher metabolic rate and overestimates the hormone concentration of birds with a lower metabolic rate. Such situations may be very common. For example, the metabolic rate of warm-blooded mammals and birds may increase substantially when ambient temperatures drop. Alternatively, during periods of low food availability, some organisms gain most of their energy from body reserves – mainly fat tissue – while feeding less. This means they drastically reduce food and water intake with subsequent changes in faeces and urine production.

Free-living animals often show large seasonal differences in the type and quantity of foods they consume. For example, many otherwise insectivorous birds rely on nectar and pollen in spring (Schwilch *et al.* 2001; Cecere *et al.* 2011) and berries or fruits in autumn (Bairlein 2002; McWilliams & Karasov 2005). Even dairy cows ingest different kinds of food in spring and autumn, which seems to affect gut passage time (Morrow *et al.* 2002). Also, the size and composition of the digestive tract may change during the year, especially in long-distance migrants (Gasaway 1976; Hume & Biebach 1996; Piersma & Lindström 1997; Bairlein 1999; Piersma & Drent 2003; Bauchinger, Wohlmann & Biebach 2005; McWilliams & Karasov 2005). Capercaillies (*Tetrao urogallus*) living in pine forests expressed higher levels of glucocorticoid metabolite levels than those living in spruce forests, suggesting that the different diets may have affected hormone metabolism (Thiel *et al.* 2011). A striking example in mammals comes from a study on Alaskan brown bears (*Ursus arctos horribilis*). These bears feed on a range of diets from pure grass, berries or meat to a mixed diet including all of these items. The best predictor of faecal glucocorticoid metabolite concentrations of brown bears was diet (von der Ohe *et al.* 2004), suggesting that the type of diet determined hormone metabolite formation. Thus, the gut of a brown bear feeding mainly on berries formed different metabolites of cortisol, the major glucocorticoid, than the gut of a brown bear feeding on meat. As the antibodies of immunoassays cross-react only with some of these metabolites, it may be difficult or even impossible to compare berry-eating bears with meat-eating bears. The two types of bears may not differ with respect to their levels of plasma cortisol, but if the immunoassays' antibody shows a higher cross-reactivity with cortisol metabolites formed in the guts of meat-eating bears than with cortisol metabolites formed in the guts of berry-eating bears, this will result in significant differences in glucocorticoid metabolite concentrations. These differences, however, have no meaning with regard to the physiological status of the brown bear. They are nothing but artefacts of the hormone metabolite measurement without any meaningful relationship to circulating hormone levels. The same study on brown bears also highlighted the potential importance of absorption and excretion of steroids directly ingested with the diet. Faecal glucocorticoid metabolite levels of meat-eating brown bears were highest during the time of salmon spawning, that is, the time when glucocorticoid levels of salmons were maximal (von der Ohe *et al.* 2004).

In summary, comparisons of hormone metabolite concentrations between seasons when the type of food available and/or the amount of food ingested differs or comparisons between populations with access to different types and quality of food can be problematic: any differences in hormone

metabolite concentrations may not reflect true differences in bioactive hormone levels, but may represent artefacts of diet. The potential influence of type and quality of food on hormone metabolite concentrations is not only relevant for studies in physiological ecology, but may also impact conservation related research, for example, when comparing hormone metabolite profiles of animals in undisturbed and disturbed habitats (e.g. Wasser *et al*. 1997; Lucas *et al*. 2006) that may differ in the kind and quality of available food.

Metabolic rate, food intake and hormone metabolite excretion

Warm-blooded animals that live in harsh and highly seasonal environments face the problem that the energetic costs for maintaining a high body temperature are higher, and food availability is lower in winter compared with that in summer. Thus, animals living in seasonal environments undergo major changes in physiology and metabolic rate during their life cycle (McNab 2002). Many small mammals cope with this challenge by daily torpor or hibernation. In birds and mammals that maintain a high body temperature also during winter, daily and seasonal fluctuations in ambient temperature may have a large effect on energy metabolism (Dawson, Marsh & Yacoe 1983; McNab 2002). For example, upon cessation of herbage growth during winter, deer and other large herbivores are confronted with a nutritional bottleneck and, as a consequence, reduce food intake, body temperature and metabolic rate (e.g. Milne *et al*. 1978; Freudenberger *et al*. 1993; Arnold *et al*. 2004; Arnold, Ruf & Kuntz 2006; Kuntz *et al*. 2006). Such differences in food intake and metabolism may have large effects on hormone metabolite measurements, but have received astonishingly little attention. And again, to know about such effects would be particularly useful for all studies conducted in 'uncontrolled' natural environments, where fluctuations in food intake and metabolic rates are the norm rather than the exception (but even under constant temperature conditions in the laboratory animals still exhibit seasonal changes in metabolism and food intake; see for example Goymann & Trappschuh 2011). I am aware of just one study addressing this topic: In this experiment, European stonechats were maintained at an ambient temperature of either +5 °C or +22 °C (Goymann *et al*. 2006). This difference in ambient temperature corresponded to a change in resting metabolic rate of more than 30% in this species (Tieleman 2007). At +5 °C, the food intake of stonechats was about twice as high compared with an ambient temperature of +22 °C. As a consequence, also the amount of excreted droppings changed: at +5 °C, the dry weight of droppings almost doubled compared with the +22 °C condition. As faecal or dropping dry weight is used as the standard reference to calculate hormone metabolite concentrations (e.g. in nanogram hormone metabolite per milligram of droppings or faeces), the change in dropping mass had a large effect on hormone metabolite concentration (see Fig. 2 for a schematic illustration of the effect). While the plasma levels of testosterone and corticosterone did not differ between stonechats maintained at +5 °C or +22 °C, there was a huge difference in hormone metabolites: the concentrations of testosterone and corticosterone metabolites were less than half as high at +5 °C compared with +22 °C. This effect was entirely driven by the change in dropping dry mass. If the excretion rate of hormone metabolites was considered (by replacing dropping dry mass with time as a reference for the amount of hormone metabolites excreted), the difference between treatments disappeared (Goymann *et al*. 2006). Hence, the change in metabolic rate caused by the manipulation of ambient temperature did not alter hormone concentrations or the rate of hormone metabolite excretion. However, the concentration of hormone metabolites could not be used as a reliable proxy for circulating hormone levels, because the change in metabolic rate with a subsequent change in food intake and dropping production rendered dropping dry weight an invalid reference for the estimation of hormone metabolite concentration in stonechats. Other studies in different species also concluded that the rate of excretion may be more reliable than the concentration (e.g. Royo *et al*. 2004; Cavigelli *et al*. 2005; Carlsson *et al*. 2007; but see Lepschy, Touma & Palme 2010), but it is unknown how influential metabolic rate is in distorting hormone metabolite patterns in species larger than stonechats.

What do these results imply for studies conducted in uncontrolled natural environments? In a northern temperate climate, a difference in ambient temperature between +5 °C and +22 °C corresponds to the difference between a warm and a cold day during spring or fall, or the difference between night and day within these seasons. Hence, such differences in ambient temperature are quite common, but could cause major differences in hormone metabolite concentrations without any change in bioactive hormone levels. Thus, these huge differences in hormone metabolite concentrations would not carry any meaningful information with respect to the hormonal status of the individuals compared. If an animal lowers its energy expenditure when the ambient temperature drops, the effects on hormone metabolite levels may become even more complicated.

Measuring the excretion rate of hormone metabolites instead of the concentration is a good solution for the laboratory, but quite difficult in the field. Hence, field investigators will have to rely on concentration measurements, but should be aware that environmental variables such as ambient temperature or internal variables that change the metabolic state of the study organism could impact their results. Thus, scientists may be well advised to consider alternative explanations when they find differences in hormone metabolite concentrations, for example, between seasons, according to time of day or depending on weather conditions. The effect of ambient temperature on metabolic rate is probably more severe in smaller than in larger birds or mammals. Reptiles and amphibians may be even more problematic because of potentially larger fluctuations in core body temperature and very irregular feeding events. Also, any other factors that may systematically affect metabolic rate, food intake or excrement output need to be considered. For example, most vertebrates cycle through a number of life-history stages that are associated with particularly high or low metabolic rates, such as breeding, moult and

migration in birds, or pregnancy, lactation and hibernation in mammals. Thus, any of these life-history stages could lead to differences in hormone metabolite concentrations, without these differences necessarily corresponding to changes in circulating hormone levels.

Bacterial degradation and hormone metabolite excretion

The metabolites of steroid hormones that are measured in faeces or droppings are the products of extensive modification by bacteria in the gut (e.g. Taylor 1971; Klasing 2005; Palme 2005; Palme *et al*. 2005; Thiel, Jenni-Eiermann & Palme 2005). Modification by bacteria has also been made responsible for changes in hormone metabolite concentrations during storage of faecal samples (Khan *et al*. 2002; Hunt & Wasser 2003; Lexen *et al*. 2008). As a consequence, one should expect that the composition of bacteria should influence the type and quantity of hormone metabolites. Thus, the kind of hormone metabolites and the relative composition of these hormone metabolites may differ between individuals depending on the suite of gut bacteria (Fig. 3). Further, the composition of gut bacteria may change between seasons, for example, with a switch in diets. To my knowledge, few studies in non-invasive hormone research have addressed the question of whether individuals differ in how they metabolize steroid hormones. Furthermore, none of these few studies discussed the potential implications and consequences of such differences for the reliability of non-invasive hormone metabolite data, which could be huge (Fig. 3). The typical approach for estimating variability in hormone metabolism is to inject animals with a radiolabelled hormone, find the metabolites of this radiolabelled hormone in the faeces or droppings and then identify the cross-reactivity of the assays' antibody with these radiolabelled metabolites (e.g. Goymann 2005). Touma *et al*. (2003) injected 24 mice with radiolabelled corticosterone. The authors report '*some individual differences concerning the relative proportions of excreted metabolites*' (p. 273), but do not further specify these differences. In laboratory rats, Bamberg, Palme & Meingassner (2001) report '*high individual variability of the relative amounts of the metabolites present*' (p. 310), and also, Rettenbacher *et al*. (2004) state that individual chicken differed with respect to the quantity of 15 identified metabolites of corticosterone. Given that these studies refer to laboratory or farm animals fed on standard diets, one may expect that potential individual differences in the kind of metabolites excreted could be much more pronounced in free-living animals with more variable diets. The bacterial composition in the gut drives some of the differences in metabolite formation that have been observed between humans with different diets (Pusateri *et al*. 1990) and are probably also responsible for some of the differences between the sexes. Differences in bacterial metabolism could also explain some of the oddities observed in hormone metabolite levels as described by some authors. For example, in a study by Dloniak *et al*. (2004), one particular female spotted hyena consistently had more than 10-fold higher levels

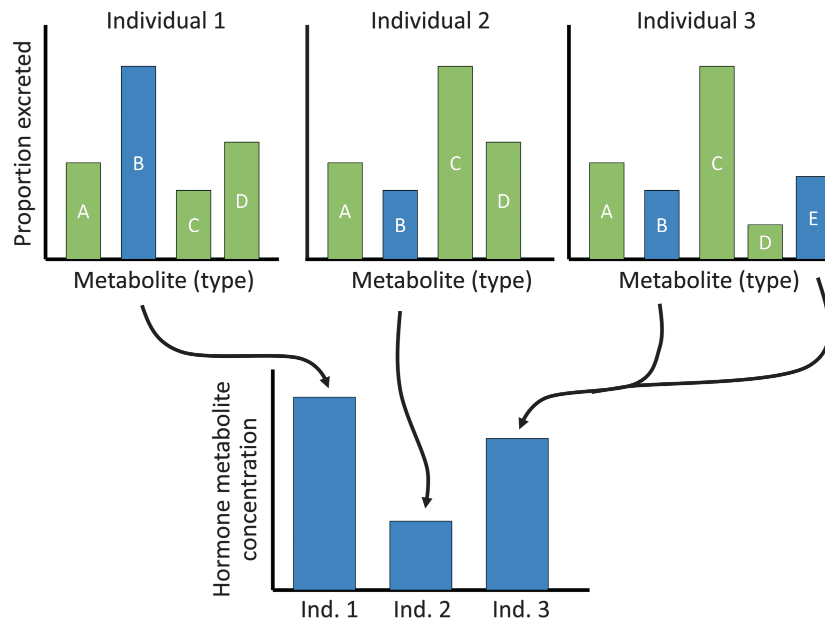

Fig. 3. Schematic representation of individual differences in hormone metabolism because of differences in gut bacterial composition. Individual 1 and individual 2 excrete the same kind of four different metabolites (A–D) of a steroid hormone; individual 3 excretes an additional metabolite (E). The total amount of excreted hormone metabolites is identical between all three individuals (blue and green bars in the upper 3 graphs), but the relative amounts of hormone metabolites differ. Individual 1 excretes a high proportion of metabolite B and a low proportion of metabolite C. In contrast, individual 2 excretes a low proportion of B and a high proportion of C. Individual 3 excretes a lower proportion of D than the other two individuals, compensated by a higher excretion of E. The assay is only capable of detecting metabolites B and E (blue bars). As a consequence, the assay measures high concentrations in individual 1, intermediate concentrations in individual 3 and low concentrations in individual 2. This result is an incorrect estimate of the physiological status of the three individuals, because all of them excrete the same total amount of hormone metabolites.

of testosterone metabolites than other individuals (even males that have much higher levels of plasma testosterone than females; Goymann, East & Hofer 2001), but it is rather unlikely that these differences would correspond with real differences in plasma hormone levels.

In summary, further studies are needed to find out how large the impact of individual differences in steroid hormone metabolism is on the accuracy and comparability of hormone metabolite measurements. In the worst case, metabolite formation may differ between individual to such an extent that a comparison of immunoreactive hormone metabolite concentrations may not be valid. While this is particularly problematic in comparisons between individuals, it could also be worrying on a population level: if individuals that differ in how they degrade hormones are unequally distributed between samples, this may systematically distort the results. We need studies that systematically compare the metabolism of steroid hormones in various individuals of the same species and sex and under different food conditions. Further, individual differences in hormone metabolism should be correlated with differences in the composition of gut bacteria.

Conclusion

Owing to their non-invasiveness, studies of hormone metabolite levels have become very popular in ecology and conservation research. This huge advantage compared with traditional measurements of hormones from serum or plasma comes with some caveats, however. Hormone metabolites in urine, faeces or droppings represent the end point of hormone metabolism and – in contrast to plasma – do not measure the actual signalling molecule. External and internal variables unrelated to the endocrine status of the individual can influence the composition and concentration of these metabolites. Despite the fact that measuring hormone metabolites has developed into a standard technique, we need to learn much more especially with respect to using this method in uncontrolled environments. Urging questions include the ones raised here (sex, diet, metabolic rate and bacterial degradation), but also others. For example, defaecation rates may change between seasons, between different times of the day or during stressful events. These factors could distort hormone metabolite measurements (see Goymann & Trappschuh (2011) for a detailed discussion of time of day and season). Further, circulating hormone levels may show diel rhythms. As the excretion of faeces or droppings may also vary with time of day, the two effects may either reinforce each other or cancel each other out. Further, at least in birds, it has been shown that hormone metabolite levels vary from dropping to dropping, suggesting that hormone metabolite levels should be based on several droppings (Klasing 2005; Scheiber, Kralj & Kotrschal 2005).

It is particularly important to realize that the factors discussed here do not just increase the random noise of hormone metabolite measurements. Random noise would only increase the variance (= noise) around the signal of hormone metabolite levels (signal in this context means that differences in hormone metabolite levels correspond to real differences in circulating hormone concentrations). Rather, the factors discussed here can generate systematic error that would seriously distort the potential signal of hormone metabolite levels in a non-random manner. For example, if a seasonal change in ambient temperature causes a change in metabolic rate, this can systematically alter hormone excretion patterns. As a consequence, comparisons between seasons may no longer be meaningful (Goymann *et al.* 2006). In conclusion, in cases where this is possible, it may be well worth the additional effort necessary to catch an animal and take a blood sample for measuring the actual hormone (the active signalling molecule with a biological function). There is far less potential for systematic error in this measure compared with struggling with the delicacies of hormone metabolites in faeces or droppings. In cases where it is not possible to measure hormones from serum or plasma, extreme caution is required in the interpretation of hormone metabolite data.

Acknowledgements

I thank the Max-Planck Gesellschaft for funding, Barbara Helm, Katharina Hirschenhauser and Lisa Gill for critically evaluating earlier drafts of this manuscript and two anonymous referees for their helpful and constructive suggestions to improve the manuscript. The author declares no conflict of interest.

References

Arlet, M.E., Kaasik, A., Molleman, F., Isbell, L., Carey, J.R. & Mänd, R. (2011) Social factors increase fecal testosterone levels in wild male gray-cheeked mangabeys (*Lophocebus albigena*). *Hormones and Behavior*, **59**, 605–611.

Arnold, W., Ruf, T. & Kuntz, R. (2006) Seasonal adjustment of energy budget in a large wild mammal, the Przewalski horse (*Equus ferus przewalskii*). II. Energy expenditure. *Journal of Experimental Biology*, **209**, 4566–4573.

Arnold, W., Ruf, T., Reimoser, S., Tataruch, F., Onderscheka, K. & Schober, F. (2004) Nocturnal hypometabolism as an overwintering strategy of red deer (*Cervus elaphus*). *American Journal of Physiology – Regulatory and Integrative Comparative Physiology*, **286**, R174–R181.

Bairlein, F. (1999) Energy and nutrient utilisation efficiencies in birds: a review. *Proceedings of the 22 International Ornithological Congress, Durban* (eds N.J. Adams & R.H. Slotow), pp. 2221–2246. BirdLife South Africa, Johannesburg.

Bairlein, F. (2002) How to get fat: nutritional mechanisms of seasonal fat accumulation in migratory songbirds. *Naturwissenschaften*, **89**, 1–10.

Baltic, M., Jenni-Eiermann, S., Arlettaz, R. & Palme, R. (2005) A non-invasive technique to evaluate human-generated stress in the Black Grouse. *Annals of the New York Academy of Sciences*, **1046**, 81–95.

Bamberg, E., Palme, R. & Meingassner, J.G. (2001) Excretion of corticosteroid metabolites in urine and faeces of rats. *Laboratory Animals*, **35**, 307–314.

Bauchinger, U., Wohlmann, A. & Biebach, H. (2005) Flexible remodeling of organ size during spring migration of the garden warbler (*Sylvia borin*). *Zoology*, **108**, 97–106.

Bentley, P.J. (1998) *Comparative Vertebrate Endocrinology*, 3 edn. Cambridge University Press, Cambridge.

Bercovitz, A.B., Collins, J., Price, P. & Tuttle, D. (1982) Noninvasive assessment of seasonal hormone profile in captive bald eagles (*Haliaeetus leucocephalus*). *Zoo Biology*, **1**, 111–117.

Carlsson, H.-E., Lyberg, K., Royo, F. & Hau, J. (2007) Quantification of stress sensitive markers in single fecal samples do not accurately predict excretion of these in the pig. *Research in Veterinary Science*, **82**, 423–428.

Cavigelli, S.A., Monfort, S.L., Whitney, T.K., Mechref, Y.S., Novotny, M. & McClintock, M.K. (2005) Frequent serial fecal corticoid measures from rats reflect circadian and ovarian corticosterone rhythms. *Journal of Endocrinology*, **184**, 153–163.

Cecere, J.G., Cornara, L., Mezzetta, S., Ferri, A., Spina, F. & Boitani, L. (2011) Pollen couriers across the Mediterranean: the case of migrating warblers. *Ardea*, **99**, 33–42.

Chard, T. (1995) *An Introduction to Radioimmunoassay and Related Techniques*, 5th edn. Elsevier, Amsterdam.

Czekala, N.M. & Lasley, B.L. (1977) A technical note on sex determination in monomorphic birds using fecal steroid analysis. *International Zoo Yearbook*, **17**, 209–211.

Dantzer, B., McAdam, A.G., Palme, R., Boutin, S. & Boonstra, R. (2011) How does diet affect fecal steroid hormone metabolite concentrations? An experimental examination in red squirrels. *General and Comparative Endocrinology*, **174**, 124–131.

Dawson, W.R., Marsh, R.L. & Yacoe, M.E. (1983) Metabolic adjustments of small passerine birds for migration and cold. *American Journal of Physiology – Regulatory Integrative and Comparative Physiology*, **245**, R755–R767.

Dloniak, S.M., French, J.A., Place, N.J., Weldele, M.L., Glickman, S.E. & Holekamp, K.E. (2004) Non-invasive monitoring of fecal androgens in spotted hyenas (*Crocuta crocuta*). *General and Comparative Endocrinology*, **135**, 51–61.

Ezenwa, V.O., Ekernas, L.S. & Creel, S. (2012) Unravelling complex associations between testosterone and parasite infection in the wild. *Functional Ecology*, **26**, 123–133.

Freudenberger, D.O., Toyakawa, K., Barry, T.N., Ball, A.J. & Suttie, J.M. (1993) Seasonality in digestion and rumen metabolism in red deer (*Cervus elaphus*) fed on a forage diet. *British Journal of Nutrition*, **71**, 489–499.

Gasaway, W.C. (1976) Seasonal variation in diet, volatile fatty acid production and size of the cecum of rock ptarmigan. *Comparative Biochemistry and Physiology Part A: Physiology*, **53**, 109–114.

Goldin, B.R., Adlercreutz, H., Dwyer, J.T., Swenson, L., Warram, J.H. & Gorbach, S.L. (1981) Effect of diet on excretion of estrogens in pre- and postmenopausal women. *Cancer Research*, **41**, 3771–3773.

Goldin, B.R., Adlercreutz, H., Gorbach, S.L., Warram, J.H., Dwyer, J.T., Swenson, L. & Woods, M.N. (1982) Estrogen excretion patterns and plasma levels in vegetarian and omnivorous women. *New England Journal of Medicine*, **307**, 1542–1547.

Gorbach, S.L. & Goldin, B.R. (1987) Diet and the excretion and enterohepatic cycling of estrogens. *Preventive Medicine*, **16**, 525–531.

Goymann, W. (2005) Non-invasive monitoring of hormones in bird droppings: biological validations, sampling, extraction, sex differences, and the influence of diet on hormone metabolite levels. *Annals of the New York Academy of Sciences*, **1046**, 35–53.

Goymann, W., East, M.L. & Hofer, H. (2001) Androgens and the role of female "hyperaggressiveness" in spotted hyenas (*Crocuta crocuta*). *Hormones and Behavior*, **39**, 83–92.

Goymann, W., Möstl, E. & Gwinner, E. (2002a) Corticosterone metabolites can be measured noninvasively in excreta of European stonechats (*Saxicola torquata rubicola*). *The Auk*, **119**, 1167–1173.

Goymann, W., Möstl, E. & Gwinner, E. (2002b) Non-invasive methods to measure androgen metabolites in excrements of European stonechats, *Saxicola torquata rubicola*. *General and Comparative Endocrinology*, **129**, 80–87.

Goymann, W. & Trappschuh, M. (2011) Seasonal and diel variation of hormone metabolites in European stonechats: on the importance of high signal-to-noise ratios in noninvasive hormone studies. *Journal of Biological Rhythms*, **26**, 44–54.

Goymann, W., Trappschuh, M., Jensen, W. & Schwabl, I. (2006) Low ambient temperature increases food intake and dropping production, leading to incorrect estimates of hormone metabolite concentrations in European stonechats. *Hormones and Behavior*, **49**, 644–653.

Graham, L.H. & Brown, J.L. (1996) Cortisol metabolism in the domestic cat and implications for non-invasive monitoring of adrenocortical function in endangered felids. *Zoo Biology*, **15**, 71–82.

Hirschenhauser, K., Spreitzer, K., Lepschy, M., Kotrschal, K. & Möstl, E. (in press) Excreted corticosterone metabolites differ between two galliform species, Japanese quail and chicken, between sexes and between urine and faecal parts of droppings. *Journal of Ornithology*.

Hume, I.D. & Biebach, H. (1996) Digestive tract function in the long-distance migratory garden warbler, *Sylvia borin*. *Journal of Comparative Physiology B*, **166**, 388–395.

Hunt, K.E. & Wasser, S.K. (2003) Effect of long-term preservation methods on fecal glucocorticoid concentrations of grizzly bear and African elephant. *Physiological & Biochemical Zoology*, **76**, 918–928.

Khan, M.Z., Altmann, J., Isani, S.S. & Yu, J. (2002) A matter of time: evaluating the storage of fecal samples for steroid analysis. *General and Comparative Endocrinology*, **128**, 57–64.

Klasing, K.C. (2005) Potential impact of nutritional strategy on non-invasive measurements of hormones in birds. *Annals of the New York Academy of Sciences*, **1046**, 5–16.

Kuntz, R., Kubalek, C., Ruf, T., Tataruch, F. & Arnold, W. (2006) Seasonal adjustment of energy budget in a large wild mammal, the Przewalski horse (Equus ferus przewalskii) I. Energy intake. *Journal of Experimental Biology*, **209**, 4557–4565.

Langmore, N.E., Cockrem, J.F. & Candy, E.J. (2002) Competition for male reproductive investment elevates testosterone levels in female dunnocks, *Prunella modularis*. *Proceedings of the Royal Society B*, **269**, 2473–2478.

Lepschy, M., Touma, C. & Palme, R. (2010) Faecal glucocorticoid metabolites: how to express yourself – comparison of absolute amounts versus concentrations in samples from a study in laboratory rats. *Laboratory Animals*, **44**, 192–198.

Lexen, E., El-Bahr, S.M., Sommerfeld-Stur, I., Palme, R. & Möstl, E. (2008) Monitoring the adrenocortical response to disturbances in sheep by measuring glucocorticoid metabolites in the faeces. *Wiener Tierärztliche Monatsschrift*, **95**, 64–71.

Lucas, J.R., Freeberg, T.M., Egbert, J. & Schwabl, H. (2006) Fecal corticosterone, body mass, and caching rates of Carolina chickadees (*Poecile carolinensis*) from disturbed and undisturbed sites. *Hormones and Behavior*, **49**, 634–643.

McNab, B.K. (2002) *The Pysiological Ecology of Vertebrates: A View From Energetics*. Cornell University Press, Ithaca, New York.

McWilliams, S.R. & Karasov, W.H. (2005) Migration takes guts. *Birds of Two Worlds* (eds P. Marra & N. Greenberg), pp. 67–78. Smithsonian Institution Press, Washington.

Millspaugh, J.J. & Washburn, B.E. (2004) Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. *General and Comparative Endocrinology*, **138**, 189–199.

Milne, J.A., Macrae, J.C., Spence, A.M. & Wilson, S. (1978) A comparison of the voluntary intake and digestion of a range of forages at different times of the year by the sheep and the red deer (*Cervus elaphus*). *British Journal of Nutrition*, **40**, 347–357.

Möhle, U., Heistermann, M., Palme, R. & Hodges, J.K. (2002) Characterization of urinary and fecal metabolites of testosterone and their measurement for assessing gonadal endocrine function in male nonhuman primates. *General and Comparative Endocrinology*, **129**, 135–145.

Morrow, C.J., Kolver, E.S., Verkerk, G.A. & Matthews, L.R. (2002) Fecal glucocorticoid metabolites as a measure of adrenal activity in dairy cattle. *General and Comparative Endocrinology*, **126**, 229–241.

Möstl, E., Rettenbacher, S. & Palme, R. (2005) Measurement of corticosterone metabolites in birds' droppings: an analytical approach. *Annals of the New York Academy of Sciences*, **1046**, 17–34.

Möstl, E., Choi, H.S., Wurm, W., Ismail, N. & Bamberg, E. (1984) Pregnancy diagnosis in cows and heifers by determination of oestradiol-17a in faeces. *British Veterinary Journal*, **140**, 287–291.

Nelson, R.J. (2011) *An Introduction to Behavioral Endocrinology*, 4th edn. Sinauer Associates, Sunderland, MA.

von der Ohe, C.G., Wasser, S.K., Hunt, K.E. & Servheen, C. (2004) Factors associated with fecal glucocorticoids in Alaskan brown bears (*Ursus arctos horribilis*). *Physiological and Biochemical Zoology*, **77**, 313–320.

Palme, R. (2005) Measuring fecal steroids: guidelines for a practical application. *Annals of the New York Academy of Sciences*, **1046**, 75–80.

Palme, R., Fischer, P., Schildorfer, H. & Ismail, M.N. (1996) Excretion of infused 14C steroid hormones via feces and urine in domestic livestock. *Animal Reproduction Science*, **43**, 43–63.

Palme, R., Rettenbacher, S., Touma, C., El-Bahr, S.M. & Möstl, E. (2005) Stress hormones in mammals and birds. Comparative aspects regarding metabolism, excretion, and non-invasive measurement in fecal samples. *Annals of the New York Academy of Sciences*, **1040**, 162–171.

Piersma, T. & Drent, J. (2003) Phenotypic flexibility and the evolution of organismal design. *Trends in Ecology & Evolution*, **18**, 228–233.

Piersma, T. & Lindström, Å. (1997) Rapid reversible changes in organ size as a component of adaptive behaviour. *Trends in Ecology & Evolution*, **12**, 134–138.

Preis, A., Mugisha, L., Hauser, B., Weltring, A. & Deschner, T. (2011) Androgen and androgen metabolite levels in serum and urine of East African chimpanzees (Pan troglodytes schweinfurthii): comparison of EIA and LC–MS analyses. *General and Comparative Endocrinology*, **174**, 335–343.

Pusateri, D., Roth, W., Ross, J. & Shultz, T. (1990) Dietary and hormonal evaluation of men at different risks for prostate cancer: plasma and fecal hormone-nutrient interrelationships. *The American Journal of Clinical Nutrition*, **51**, 371–377.

Rabiee, A.R., Dalley, D., Bormana, J.M., Macmillan, K.L. & Schwarzenberger, F. (2002) Progesterone clearance rate in lactating dairy cows with two levels of dry matter and metabolisable energy intakes. *Animal Reproduction Science*, **72**, 11–25.

Rettenbacher, S., Möstl, E., Hackl, R., Ghareeb, K. & Palme, R. (2004) Measurement of corticosterone metabolites in chicken droppings. *British Poultry Science*, **45**, 704–711.

Royo, F., Bjork, N., Carlsson, H., Mayo, S. & Hau, J. (2004) Impact of chronic catheterization and automated blood sampling (Accusampler) on serum corticosterone and fecal immunoreactive corticosterone metabolites and immunoglobulin A in male rats. *Journal of Endocrinology*, **180**, 145–153.

Scheiber, I., Kralj, S. & Kotrschal, K. (2005) Sampling effort/frequency necessary to infer individual acute stress responses from fecal analysis in Greylag Geese (*Anser anser*). *Annals of the New York Academy of Sciences*, **1046**, 154–167.

Schwilch, R., Mantovani, R., Spina, F. & Jenni, L. (2001) Nectar consumption of warblers after long-distance flights during spring migration. *Ibis*, **143**, 24–32.

Scott, A.P., Hirschenhauser, K., Bender, N., Oliveira, R., Earley, R.L., Sebire, M., Ellis, T., Pavlidis, M., Hubbard, P.C., Huertas, M. & Canario, A. (2008) Non-invasive measurement of steroids in fish-holding water: important considerations when applying the procedure to behaviour studies. *Behaviour*, **145**, 1307–1328.

Selva, N., Cortés-Avizanda, A., Lemus, J.A., Blanco, G., Mueller, T., Heinrich, B. & Donázar, J.A. (2011) Stress associated with group living in a long-lived bird. *Biology Letters*, **7**, 608–610.

Sheriff, M.J., Krebs, C.J. & Boonstra, R. (2010) Assessing stress in animal populations: do fecal and plasma glucocorticoids tell the same story? *General and Comparative Endocrinology*, **166**, 614–619.

Sheriff, M.J., Dantzer, B., Delehanty, B., Palme, R. & Boonstra, R. (2011) Measuring stress in wildlife: techniques for quantifying glucocorticoids. *Oecologia*, **166**, 869–887.

Sorato, E. & Kotrschal, K. (2006) Hormonal and behavioural symmetries between the sexes in the Northern bald ibis. *General and Comparative Endocrinology*, **146**, 265–274.

Stöwe, M., Bugnyar, T., Schloegl, C., Heinrich, B., Kotrschal, K. & Möstl, E. (2008) Corticosterone excretion patterns and affiliative behavior over development in ravens (*Corvus corax*). *Hormones and Behavior*, **53**, 208–216.

Taylor, W. (1971) The excretion of steroid hormone metabolites in bile and feces. *Vitamins and Hormones*, **29**, 201–285.

Thiel, D., Jenni-Eiermann, S. & Palme, R. (2005) Measuring corticosterone metabolites in droppings of Capercaillies (*Tetrao urogallus*). *Annals of the New York Academy of Sciences*, **1046**, 96–108.

Thiel, D., Jenni-Eiermann, S., Palme, R. & Jenni, L. (2011) Winter tourism increases stress hormone levels in the Capercaillie Tetrao urogallus. *Ibis*, **153**, 122–133.

Tieleman, B.I. (2007) Differences in the physiological responses to temperature among stonechats from three populations reared in a common environment. *Comparative Biochemistry and Physiology – Part A: Molecular & Integrative Physiology*, **146**, 194–199.

Touma, C. & Palme, R. (2005) Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of a biological validation. *Annals of the New York Academy of Sciences*, **1046**, 54–74.

Touma, C., Sachser, N., Möstl, E. & Palme, R. (2003) Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. *General and Comparative Endocrinology*, **130**, 267–278.

Wasser, S.K., Thomas, R., Nair, P.P., Guidry, C., Southers, J., Lucas, J., Wildt, D.E. & Monfort, S.L. (1993) Effects of dietary fibre on faecal steroid measurements in baboons (Papio cynocephalus cynocephalus). *Journal of Reproduction & Fertility*, **97**, 569–574.

Wasser, S.K., Papageorge, S., Foley, C. & Brown, J.L. (1996) Excretory fate of estradiol and progesterone in the African elephant (*Loxodonta africana*) and patterns of fecal steroid concentrations throughout the estrous cycle. *General and Comparative Endocrinology*, **102**, 255–262.

Wasser, S.K., Bevis, K., King, G. & Hanson, E. (1997) Noninvasive physiological measures of disturbance in the northern spotted owl. *Conservation Biology*, **11**, 1019–1022.

Wielebnowski, N. & Watters, J. (2007) Applying fecal endocrine monitoring to conservation and behavior studies of wild mammals: important considerations and preliminary tests. *Israel Journal of Ecology & Evolution*, **53**, 439–460.

Received 20 January 2012; accepted 6 March 2012
Handling Editor: Robert Freckleton