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Review article

Non-invasive monitoring of physiological markers in primates

V. Behringer *, T. Deschner

Max Planck Institute for Evolutionary Anthropology, Department of Primatology, Deutscher Platz 6, D-04103 Leipzig, Germany

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ABSTRACT

The monitoring of endocrine markers that inform about an animal's physiological state has become an invaluable tool for studying the behavioral ecology of primates. While the collection of blood samples usually requires the animal to be caught and immobilized, non-invasively collected samples of saliva, urine, feces or hair can be obtained without any major disturbance of the subject of interest. Such samples enable repeated collection which is required for matching behavioral information over long time periods with detailed information on endocrine markers.

We start our review by giving an overview of endocrine and immune markers that have been successfully monitored in relation to topics of interest in primate behavioral ecology. These topics include reproductive, nutritional and health status, changes during ontogeny, social behavior such as rank relationships, aggression and cooperation as well as welfare and conservation issues. We continue by explaining which hormones can be measured in which matrices, and potential problems with measurements. We then describe different methods of hormone measurements and address their advantages and disadvantages. We finally emphasize the importance of thorough validation procedures when measuring a specific hormone in a new species or matrix.

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* Corresponding author.
E-mail address: verena_behringer@eva.mpg.de (V. Behringer).

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1. Introduction

Within the last decades, non-invasive monitoring of endocrine and other physiological markers has become an invaluable tool for researchers interested in the behavioral ecology of wild animals. For primatologists, the possibility of acquiring information about an animal's physiological state without darting and capturing the individual is essential as immobilization poses considerable risks to the individual's health, especially for arboreal primates, and additionally, the process could bias the occurrence of behaviors and interfere with habituation and long term behavioral observations (Cunningham et al., 2015). Furthermore, repeated darting and capture is also an ethical concern as it is considered to be undesirable and detrimental to the animal's well-being (Fedigan, 2010). Therefore, non-invasive monitoring of endocrine markers permits the examination of proximate mechanisms that influence individual variation in behavior and the consequential outcomes for the individual's fitness in wild populations in an ethically and scientifically appropriate way to answer ultimate questions about behavioral adaptations to ecological circumstances (Dufty et al., 2002; Klein, 2000; Wingfield et al., 1998).

In this review we will first give an overview of general areas of research relevant to primate field endocrinology, and discuss the hormones that have been measured so far to investigate questions related to reproductive and nutritional status, changes during ontogeny, social behavior such as rank relationships, aggression and cooperation as well as welfare and conservation issues. Thereafter, we will point out additional non-hormonal physiological parameters that have the potential to become important in the field of primate behavioral ecology. In the next part, we present a synopsis of available matrices that can be sampled non-invasively and that provide information about an individual's physiological state, and the hormones that can be measured in them. We then give a brief summary of the advantages and disadvantages of methods available to measure hormones in these matrices, and finally we highlight the importance of validation.

1.1. Applications of non-invasive monitoring of endocrine markers in primate behavioral ecology

1.1.1. Endocrine patterns in female reproduction

Historically, first efforts to measure endocrine parameters in non-invasive samples from primates were directed at measuring female reproductive steroid hormones in urine to characterize cycle patterns in zoo living individuals (Czekala et al., 1981; Hodges et al., 1979; Shideler et al., 1983). Several years later, the measurement of female reproductive steroid hormones in fecal extracts was successfully established (Wasser et al., 1988; Ziegler et al., 1989). The development of these methods was facilitated by the fact that steroids are generally rather stable chemical substances and are cleared from the body through the kidneys and the gut with only minor metabolic changes to their chemical structure (Lasley and Savage, 2007). Following the successful endocrinological assessment of female reproductive condition in captivity the methodology was then implemented in primate field studies (Andelman et al., 1985). For a detailed review on the historical development of non-invasive methods in primatology see Higham (2016).

The measurement of female sex steroids allows for monitoring of female reproductive functioning and fecundity. With a dense sampling regime, the day of ovulation can be pinpointed, a fertile window can be estimated and potentially fertile and infertile female periods in the menstrual cycle can be distinguished. Monitoring these changes in hormone levels allows for the investigation of male and female mating strategies and situation dependent mate choice (e.g., Engelhardt et al., 2004; Stumpf and Boesch, 2006, 2004). Furthermore, these hormonal changes can be put in context with changes in perineal swelling size (Heistermann, 1996; Thierry et al., 1996; Whitten and Russell, 1996).

The function of reproductive signals such as sexual swellings has been suggested to advertise or conceal the timing of ovulation to distinguish or confuse paternity, respectively (Nunn, 1999). Non-invasively measured changes in estrogen and progestin levels facilitate the investigation of the reliability of these signals as an indicator of imminent ovulation. While sexual swellings seem to be graded signals that indicate ovulation with a certain probability (Nunn, 1999), this probability seems to be rather variable across species. Sexual swellings indicate ovulation with a high degree of probability in Barbary macaques (Brauch et al., 2007), but only to a moderate degree in baboons (Higham et al., 2008), gibbons (Barelli et al., 2007), and chimpanzees (Deschner et al., 2003; Emery and Whitten, 2003). In primate species such as bonobos (Douglas et al., 2016; Reichert et al., 2002) or long tailed macaques (Engelhardt et al., 2005), sexual swellings are a poor indicator of the timing of ovulation.

The "Bruce effect", where pregnancies are terminated after females are exposed to unfamiliar males (Bruce, 1959) has been described for many female mammals in captivity, but evidence that this effect is also found in wild populations is indirect or anecdotal (Roberts et al., 2012). The non-invasive monitoring of fecal progesterone levels confirmed the termination of pregnancies after encounters with unfamiliar males in gelada baboons that would otherwise have gone unnoticed (Roberts et al., 2012). This study supports the notion that the Bruce effect is an adaptive female reproductive strategy in order to minimize investment in an offspring that would be at high risk of infanticide by unfamiliar males (Hrdy, 1979; Schwagmeyer, 1979).

Environmental and social parameters can affect the secretion of female sex hormones and thereby a female's fecundity. Female chimpanzees are more likely to have menstrual cycles and conceive more often during periods of high fruit availability (Emery Thompson and Wrangham, 2008), and home range quality correlates positively with ovarian hormone production, shorter birth intervals, and higher infant survivorship (Emery Thompson et al., 2007). Furthermore, phytohormones that mimic the effects of sex steroid hormones by binding to the appropriate receptors are suspected to influence reproductive function and behavior. In baboons and chimpanzees, the consumption of *Vitex* spp. fruits and plant parts is positively correlated with an elevated excretion of immuno-reactive progestogens (Emery Thompson et al., 2008; Higham et al., 2007; Lu et al., 2011), and the cessation of cyclic ovarian activity (Higham et al., 2007). In red colobus monkeys the consumption of estrogenic young leaves of *Millettiass dura* was positively correlated with both fecal estradiol and cortisol levels, as well as to higher rates of aggression and copulation, and lower rates of grooming (Wasserman et al., 2012).

In urine samples, but not in fecal extracts, other hormones that can be measured as indicators of female reproductive function are the gonadotropin hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In captivity, FSH and LH have been measured for monitoring the ovarian cycle and pregnancy in a variety of primate species, including humans and great apes as well as Old World- and New World monkeys (Czekala et al., 1981; He et al., 2001; Hodges et al., 1979; Lasley et al., 1980; Shimizu et al., 2003; Ziegler et al., 1987).

1.1.2. Social and ecological correlates of stress

Animals have to adapt their behavior, morphology, development, and their physiology in response to environmental cues to maintain homeostasis. The two most important physiological responses to stressors are the stimulation of the sympathetic nervous system that results in the release of catecholamines and the activation of the hypothalamic–pituitary–adrenal (HPA) axis that culminates in the secretion of glucocorticoids (GCs) that help to restore homeostasis (Reeder and Kramer, 2005; Sapolsky, 2000). An elevation in GC concentration increases the energy supply to tissues and thereby facilitates to fight or escape from life-threatening situations (Wingfield et al., 1998). After the individual has successfully coped with the stressor (behaviorally or physiologically), GC levels decrease. However, chronic activation of the HPA axis and

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thereby chronically elevated GC levels has detrimental consequences to immune function, cognitive ability, growth, reproduction, and finally survival (Boonstra, 2013; Sapolsky, 2000). Therefore, GC measurements are used to understand how environmental challenges such as predation risk, climate change, and habitat disturbance and social interactions with conspecifics affect the survival and reproductive success of free-living animals and thus impact populations (Boonstra, 2013; Creel et al., 2013; Fairhurst et al., 2013; Monclús et al., 2009). As darting and capture of animals is highly stressful to animals and results in elevated GC levels, study designs using urinary glucocorticoid metabolite (uGCM) and fecal glucocorticoid metabolite (fGCM) levels are highly valuable since sample collection does not in itself cause elevations in GCs (e.g., Möstl and Palme, 2002; Sheriff et al., 2011).

Several comprehensive reviews summarize behavioral and physiological aspects of the stress response in relation to an individual's sex and social status, coping mechanisms, metabolism and as an indicator of welfare in primates (Cavigelli and Caruso, 2015; Cheney and Seyfarth, 2009; Honess and Marin, 2006; Novak et al., 2013). Therefore, we will present only a few examples of questions that have been investigated by monitoring GCs in non-human primates, other examples as well as major problems with use of the term "stress" for cause and effect, are presented in this issue in Beehner and Bergman (2017).

Social rank and dominance interactions are powerful behavioral stressors that result in systematic individual differences in GC levels (Creel, 2001). In female ring-tailed lemurs, a species where females are dominant over males, a high dominance status correlated positively with fGCM levels (Cavigelli et al., 2003), while in free-ranging female chacma baboons fGCM levels were unrelated to rank (Weingrill et al., 2004). Similarly, in wild male chimpanzees, dominance rank was positively correlated with uGCM excretion in a stable dominance hierarchy (Muller and Wrangham, 2004) and the alpha males had significantly higher fGCM than all other males in a study of wild baboons in Amboseli, Kenya (Gesquiere et al., 2011). However, dominant individuals do not always exhibit higher GC levels than subordinates across the primate taxa. A meta-analysis of studies from wild and captive non-human primates identified two variables which significantly predicted higher GCM levels in subordinate individuals when they (1) were more often confronted with stressors, and (2) had fewer opportunities for social (including close kin) support (Abbott et al., 2003).

Anthropogenic disturbance and habitat destruction are some of the major concerns to the conservation of endangered primate species, because of their potentially negative impact on animal populations. Examples here for are presented in this issue in Emery Thompson (2016). Another factor that potentially interacts with habitat fragmentation is an increase in human disturbance. Although to date no studies have investigated this interaction, a study of wild Barbary macaques found that fGCM levels were positively related to rates of aggressive interactions between humans and monkeys, indicating a negative impact of tourist activity on animal stress levels (Maréchal et al., 2011).

Recently, cortisol measurements in hair of non-human primates have been increasingly used to assess chronically elevated cortisol levels during long-term stressful periods and high population densities (Carlitz et al., 2014; Davenport et al., 2006; Dettmer et al., 2014). Therefore, cortisol measurements in hair samples could become a valuable tool to assess long-term elevations in GCs in response to social and environmental stressors (for potential problems with this method see below).

1.1.3. Dominance, competition and androgens

The "Challenge Hypothesis" (Wingfield et al., 1990) proposes that seasonal changes in androgen levels are a function of mating system, male–male aggression, and paternal care. However, seasonal impacts on androgen levels and androgen responsiveness to male-male competition need to be controlled for (Goymann et al., 2007). It is assumed that during mating periods an increase in aggression levels is associated with a rise in testosterone levels which is beneficial for male reproductive competitiveness. Evidence for this assumption, however, is mixed across mammal and bird species (Hirschenhauser and Oliveira, 2006). As most research on the Challenge Hypothesis so far has focused on avian species where blood samples are habitually collected, the possibility to measure androgen levels in non-invasively collected samples allows the testing of the feasibility of the Challenge Hypothesis in a broader array of vertebrate species. This comparison will increase our understanding of the role of androgen level changes in mediating aggression, paternal care, and male-male competition in different social systems (Moore, 2007). More details and examples about the Challenge Hypotheses and how testosterone increases reproductive output but adversely impact survival are presented in this special issue in Muller (2016).

1.1.4. Physiological changes during ontogeny

A large body of literature exists on the developmental changes in primate hormone levels. These studies have almost exclusively relied on blood samples, for example in the genus *Macaca* (e.g., Kemnitz et al., 2000; Lane et al., 1997; Muehlenbein et al., 2003; Smail et al., 1982; Snipes et al., 1969), the genus *Papio* (e.g., Castracane et al., 1981; Copeland et al., 1982; Crawford et al., 1997), chimpanzees (e.g., Copeland et al., 1985; Kondo et al., 2000; Kraemer et al., 1982; Marson et al., 1993, 1991; Nadler et al., 1987; Winter et al., 1980), and of course in humans (e.g., August et al., 1972; Bribiescas et al., 2012; Ducharme et al., 1976; Elmlinger et al., 2005, 2002). The use of blood samples restricted the number of samples that can be obtained from a certain individual within a certain timeframe, and such sample collection is especially problematic for young individuals. Therefore, non-invasive samples provide an opportunity to obtain high resolution and longitudinal information on hormonal patterns across an individual's lifetime. The detailed knowledge of blood hormone levels during ontogeny that is already available serves as a basis for the physiological validation of non-invasive methods for several hormones and matrices (see below).

One early event during ontogeny in humans is adrenarche, the maturation of the adrenal cortex. The *zona reticularis* (ZR) of the adrenal cortex mainly produces dehydroepiandrosterone (DHEA), its sulfo-conjugate (DHEA-S), and androgens (e.g., Abbott and Bird, 2008; Conley et al., 2004; Dhom, 1973). Early after birth, the ZR shows an involution, a spontaneous back formation that is accompanied by a cessation of DHEA and DHEA-S excretion. This inactive state persists until shortly before the onset of puberty, when the ZR is re-established and DHEA and DHEA-S levels start to increase. While the human pattern of adrenarche has also been confirmed for chimpanzees and bonobos (Behringer et al., 2012b; Collins et al., 1981; Cutler et al., 1978; Watts and de Vries, 2009), the back- and reformation of the ZR and the related changes in DHEA and DHEA-S levels are present, but happen before birth in Old World primates (Abbott and Bird, 2008; Bernstein et al., 2012; Conley et al., 2012; Nguyen and Conley, 2008). In the few New World primate species investigated so far, the development of the ZR in females depends on their dominance rank and their gonadal development. In contrast, males lack a functional ZR (Pattison et al., 2009, 2005). Adrenarche related, ontogenetic changes in DHEA-S excretion can be detected in urine of humans (Remer et al., 2005), chimpanzees, and bonobos (Behringer et al., 2012b; Collins et al., 1981; Cutler et al., 1978; Watts and de Vries, 2009). While the adaptive significance of adrenarche in human and apes is still debated (e.g., Campbell, 2006; Nguyen and Conley, 2008; Bernstein et al., 2012), the availability of a non-invasive technique facilitates research in other non-human primate species to investigate the evolutionary origin of adrenarche.

In humans, after the first year of life, the hypothalamic–pituitary–gonadal (HPG) axis is suppressed for almost ten years. During this time, which is called the "juvenile pause", sex steroid and gonadotropin secretion is inhibited (Grumbach, 2002). With the reawakening of the HPG axis at the beginning of puberty, LH levels increase, triggering the maturation of the ovaries and testes, which then start to produce sex

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steroids (Aranoff and Bell, 2004; Forest, 1979). Non-invasive monitoring of sex steroids has confirmed a comparable pattern to the juvenile pause in humans for a range of non-human primate species. Analyses of fecal samples for sex steroids and their metabolites determined a juvenile pause in baboons (Beehner et al., 2009; Gesquiere et al., 2005), and in white-faced capuchin monkeys (Jack et al., 2014). In captive bonobos and chimpanzees, urinary testosterone levels are also indicative for a juvenile pause (Anestis, 2006; Behringer et al., 2014a). Together, these results indicate the general existence of a juvenile pause in primates.

The onset of maturation is accompanied by major adjustments and transformations of the organism, including behavioral and morphological, but also physiological changes. Rising levels of circulating testosterone are an indirect marker for the reproductive maturation of the testes and ovaries (Sizonenko and Paunier, 1975). Testosterone levels obtained from non-invasively collected samples have been successfully used to pinpoint the onset of maturation in male primates. Increases in testosterone levels were detected in chimpanzee urine and fecal samples (Anestis, 2006; Behringer et al., 2014a; Seraphin et al., 2008), in Old World primates e.g., baboon fecal samples (Beehner et al., 2009; Gesquiere et al., 2005), and New World primates e.g., white-faced capuchin fecal samples (Jack et al., 2014). This increase in testosterone production is accompanied with an increase in testes size (Castracane et al., 1986; Crawford et al., 1997; Nieuwenhuijsen et al., 1987). Male orangutans exhibit two adult phenotypes, flanged and unflanged males. Unflanged males appear to be arrested in their development, and the impact of this status on androgen levels was successfully monitored in urine samples of wild and captive individuals. Unflanged males had significantly lower levels of testosterone than developing males (Maggioncalda et al., 1999; Marty et al., 2015).

In women, the existence of menarche facilitates the detection of the onset of sexual maturation compared to males. Menarche signifies ovarian maturation that is accompanied by an increase in the production of sex steroids, particularly estradiol and progesterone. Consequently, female reproductive maturation can be monitored as well by the non-invasive measurement of metabolites of these steroids in urine and fecal samples, as has been successfully shown in baboons (Gesquiere et al., 2005; Onyango et al., 2013). Alternatively, changes in testosterone levels can be used too to investigate the onset of maturation in females, as has been done by using urine samples of chimpanzees and bonobos (Behringer et al., 2014a).

Additional hormones showing consistent changes during development are growth hormone (GH) and thyroid hormones (THs), which are involved in growth processes. For these hormones, urine samples can be used to explore whether their production is correlated to expected differences in somatic growth patterns. For example, urinary GH levels were significantly elevated in developing orangutan males in comparison to arrested individuals (Maggioncalda et al., 2000). Interestingly, no difference was found in urinary thyroid-stimulating hormone (TSH) levels between the two orangutan phenotypes (Maggioncalda et al., 2000). Changes in urinary TH levels during development show a similar pattern in bonobos, chimpanzees, and humans. However, the decrease in adulthood in bonobos occurs ten years later than in chimpanzees, and five years later than in humans (Behringer et al., 2014b).

1.1.5. Endocrine correlates of nutritional status/balance

The importance of energy on life history processes, social behavior, and on fitness is reviewed in detail in this special issue in Emery Thompson (2016). In the mentioned review many hormones involved in metabolic regulation are presented with their function. One of such already validated markers is urinary c-peptide (UCP), to assess energy balance in primates (Deschner et al., 2008; Emery Thompson and Knott, 2008; Girard-Buttoz et al., 2011; Sherry and Ellison, 2007). UCP levels are not only an indicator of nutritional quality and quantity, but also respond to other metabolic challenges. For example, females have low UCP levels during lactation when energetic costs are high (Emery Thompson et al., 2012a). Similarly, illness and the activation of immune responses is an energetically demanding process, and UCP levels decreased in wild chimpanzees during times of illness (Emery Thompson et al., 2009). The evidence that social challenges and competition can increase energy demands for certain individuals is thus far ambiguous. Wild male bonobos had higher UCP levels when they were associating in bigger parties, but there was no correlation with mate guarding or mate competition (Surbeck et al., 2015). Also, mate guarding did not affect UCP levels in wild male long-tailed macaques (Girard-Buttoz et al., 2014).

In many primate species the collection of urine samples in the wild and/or in captivity is not possible. As c-peptide levels cannot be measured in fecal samples, there is the need for an alternative biomarker to monitor energy balance. Thyroid hormones (THs) play a pivotal role in the regulation of metabolic activity throughout all life stages in vertebrates in response to environmental and physiological challenges and are therefore a promising marker. Furthermore, THs are involved in the regulation of basal, or resting metabolic rate in euthermic animals (Speakman and Król, 2010), in adaptive thermogenesis in response to cold, and in the functioning of mechanisms that protect energy reserves in challenging situations like seasonal food shortages, fasting, and illness (Mullur et al., 2014). Two main THs are present in the blood of mammals, thyroxine (T4) and triiodothyronine (T3). T3 is more biologically active and potent than T4 and therefore, has greater biological and clinical importance (Burke and Eastman, 1974; Fisher and Polk, 1989; Tomasi, 1991). Increased levels of T3 and T4 are correlated with a rise in resting energy expenditure and the production of metabolic heat (Barboza et al., 2009; Norris, 2007). THs rapidly decrease in response to nutritional deficits, thereby lowering metabolic rate and allowing the body to conserve energy (Eales, 1988; Flier et al., 2000; van der Heyden et al., 1986). To date, most studies investigating TH levels have relied on blood samples, but THs can be reliably measured in urine and fecal samples (Behringer et al., 2014b; Gobush et al., 2014; Schaebs et al., 2016; Wasser et al., 2010). Recent studies in two primate species have reported that caloric restriction is accompanied by a decrease of T3 levels in the feces of howler monkeys (Wasser et al., 2010) and yellow-breasted capuchins (Schaebs et al., 2016). Therefore, THs are a promising marker of nutritional status that can be readily measured in fecal and urinary samples.

1.1.6. Endocrine correlates of cooperation and social bonds

Within the last years, the endocrinological mechanisms of sociality have received increasing attention. The peptide hormone oxytocin (OT), which is involved in the establishment and maintenance of social bonds (Crockford et al., 2014), has received most of this attention, but other hormones such as prolactin have been investigated as well, for more details see Ziegler and Crockford (2017) in this special issue.

Caregiving of mothers is associated with higher OT and arginine vasopressin (AVP) levels in rodents (Carter et al., 2009) and humans (Sanchez et al., 2009), and certain aspects of caregiving are also positively correlated to OT levels in parents and alloparents of cotton-top tamarins (Finkenwirth et al., 2016). Within a pair bond in tamarins, rates of affiliative behaviors are linked to OT levels (Snowdon et al., 2010) and pairs with strong social bonds have more synchronized longitudinal fluctuations of OT levels (Finkenwirth et al., 2015). The strength of social bonds outside of exclusive pair bonds is associated with OT levels as well. In chimpanzees, urinary OT levels are elevated after grooming with a bond partner as compared to a non-bond partner (Crockford et al., 2013). Additionally, OT levels are elevated in the giver and receiver after food sharing, independent from bond status (Wittig et al., 2014).

However, misconceptions persist regarding the relevance of peripheral OT measurements. Because peripheral OT does not cross the blood-brain barrier, the relevance of peripheral OT to behavioral correlates has been called into question (Higham, 2016). However, researchers using

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peripheral measures of OT in relation to affiliative behaviors have never claimed such a feed-back loop exists, but rather describe peripheral OT levels as an indicator of central levels due to coordinated release of OT into the brain and the periphery (Crockford et al., 2014; MacDonald and Feifel, 2015). Still, justified skepticism exists as to whether peripheral OT levels are representative of central levels as well as to general methods of measurement of peripheral OT (McCullough et al., 2013). Therefore, as with other measurements of endocrine parameters, a rigorous validation of collection, storage and assay procedures is necessary when applying OT measurements to new species and/or matrices. Particularly, the measurement of salivary OT levels awaits further rigorous validation (Horvat-Gordon et al., 2005).

Prolactin is related to the regulation of parental behavior in females and males. Maternal behavior is positively correlated to care directed to infants in squirrel monkeys (Soltis et al., 2005). Experimental reduction of prolactin secretion leads to a decrease in care-giving behavior in both female and male marmosets (Roberts et al., 2001). In New World primates, variation in prolactin excretion in males across species however seems to be a function of experience, age and care-giving behaviors (Schradin et al., 2003). In male marmosets experimentally reduced prolactin levels do not affect care-giving behavior in experienced males (Ziegler et al., 2009). Prenatal prolactin levels increase in experienced fathers in cotton-top tamarins (Ziegler et al., 2004; Ziegler and Snowdon, 2000). Similar patterns have been reported for humans (Delahunty et al., 2007; Fleming et al., 2002).

Unfortunately, large amounts of urine are necessary to measure prolactin with immunoassays (Schradin and Anzenberger, 2004; Ziegler et al., 1996b), and therefore obtaining enough urine from free ranging animals might be a constraining factor for the use of this hormone in field studies. More information about neuroendocrine control in non-human primate sociality is reviewed in this special issue in Ziegler and Crockford (2017).

1.1.7. Additional physiological markers

A well-functioning immune system is important for resistance to infections and ultimately for survival. Therefore, the monitoring of health status and immune function in combination with endocrinological parameters is of importance in wild and captive non-human primates to better understand the progression of diseases, and the impact of health status on fitness (French et al., 2009). So far, the immune response has been less studied in wild and captive animals because of the lack of non-invasive techniques. Traditionally, the health status of non-human primates has been non-invasively monitored by visual inspection (Archie et al., 2012), or by quantification of parasitic load (Gillespie et al., 2005). In wild chimpanzees, total parasite richness in fecal samples was correlated with fecal cortisol and testosterone levels (Muehlenbein, 2006). Another method to determine health status is urinalyses. Urinalyses is mainly performed with a dipstick to diagnose urologic conditions or diseases of the kidneys by measuring for example bilirubin, urobilinogen, glucose, haemoglobin, ketones, and nitrites (Simmerville et al., 2005). However, in some species such as wild chimpanzees, dipstick analyses alone have turned out to be too unreliable for assessing health status, as the semi-quantitative assessment of urinary proteins was not associated with obvious external signs of illness (Kaur and Huffman, 2004; Leendertz et al., 2010). Very recently, proteins such as haptoglobin, neopterin, and c-reactive protein in urine and fecal samples of monkeys were evaluated as non-invasive markers of immune activation and inflammation (Higham et al., 2015). Urinary neopterin was significantly correlated with serum neopterin, and was also highly responsive to infections (Higham et al., 2015). Furthermore, urinary neopterin was tested for the collection and storage in the field, and found to be stable after contaminated with dirt, after thawing and freezing cycles, and at room temperature (Heistermann and Higham, 2015).

1.2. Measuring hormones in non-invasive matrices

The choice of which non-invasive matrix to collect for hormonal measurements, depends not only on its convenience of collection, but also on other considerations that are related to the research question. When relating changes in hormone concentration from non-invasive samples to behavioral or environmental stimuli, the time lag between the secretion and the excretion of the hormone in the matrix of choice (Fig. 1) is an important factor to consider (Palme et al., 2005; Whitten et al., 1998).

Changes in hormone production are detected within seconds to minutes in cerebrospinal fluid, blood, and saliva. Thus, among these matrices, only saliva is suitable to non-invasively detect short-term changes in hormone production, as salivary hormone concentration represents a snapshot in time (Anestis, 2010). For example, the equilibrium between cortisol levels in blood and saliva is reached in less than 5 min (Landon et al., 1984; Riad-Fahmy et al., 1982). However, many hormones like testosterone, LH, and FSH, are not released at a steady rate, but in pulses (Veldhuis et al., 1987). Therefore, hormone levels obtained from these matrices can vary considerably within a few minutes, and this noise might obscure any effects one intends to measure. In contrast, in urine and fecal samples the hormone concentration reflects the cumulative secretion and elimination from the blood stream over hours or even days. The time lag of hormone/metabolites excretion in feces is species-specific and mainly a function of gut passage times (e.g., Palme et al., 1996; Schwarzenberger et al., 1996; Whitten et al., 1998). In primates, gut passage time varies from hours (e.g., marmosets and capuchins) to days (e.g., chimpanzees) (Bahr et al., 2000; Murray et al., 2013; Wheeler et al., 2013).

In cases where samples cannot be regularly collected, measuring hormone levels in hair samples has the potential to provide a long record of an animal's hormone level. The slow growth of hair means that the time scale over which one can measure the animal's hormone levels is typically measured in weeks and months (Gao et al., 2015; Sheriff et al., 2011; Stalder and Kirschbaum, 2012). Therefore, this method is useful for tracking long-term changes in hormone levels associated with seasonality or reproductive status, but not for investigating short-term events.

1.2.1. Saliva

Because the focus of this special issue is on field endocrinology and saliva is still difficult to collect from wild animals, the review on hormonal measures in saliva is condensed, but nevertheless, the specific problems pertaining with salivary hormone measures are noted.

Saliva collection is a non-invasive sampling method in humans; but in animals it necessitates either the restraint or the cooperation of the subject. Saliva collection has the added advantage that it can be repeated multiple times over short intervals, and it avoids numerous ethical issues related to serum sampling. At least for humans, it is considered as a relatively stress free procedure that can be performed even at remote sites by untrained persons (Dabbs, 1990; Gröschl, 2008; Hofman, 2001; Lipson and Ellison, 1989; Quissell, 1993; Riad-Fahmy et al., 1982).

Saliva is an aqueous fluid that contains many analytes of interest, e.g., steroids, peptides, and amines (Gröschl, 2008; Hofman, 2001; Lima et al., 2010). In humans, salivary steroid hormones have been measured, for example to determine adrenal activity and gonadal function (Lipson and Ellison, 1996; Riad-Fahmy et al., 1982). In captive non-human primates, steroid hormones, such as cortisol or testosterone have been measured in salivary samples of lemurs (von Engelhard et al., 2000), New World primates (Espinosa Gómez et al., 2015; Fuchs et al., 1997), Old World primates (Arslan et al., 1984; Boyce et al., 1995; Lutz et al., 2000; O'Brien et al., 2008; Pearson et al., 2008), and Great Apes (Behringer et al., 2014c, 2013, 2012a, 2009; Elder and Menzel, 2001; Heintz et al., 2011; Hohmann et al., 2009; Kuhar et al., 2005; Kutsukake et al., 2009; Wobber et al., 2013, 2010). However, saliva collection under field conditions is challenging. Pioneering work allowed

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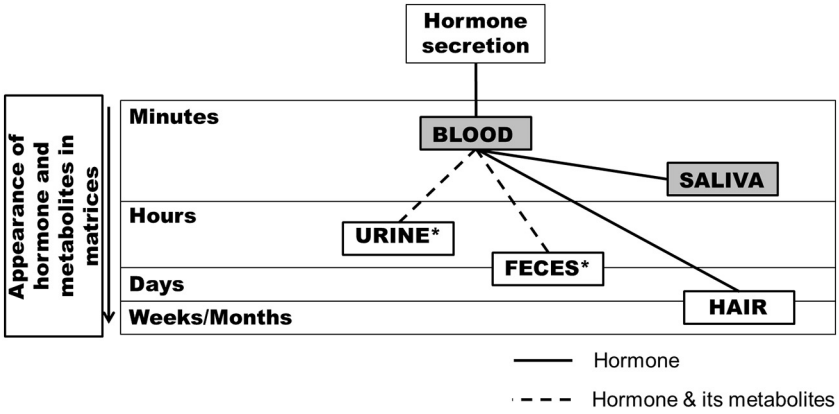

Fig. 1. General overview of the time lag between hormone secretion and the appearance of the hormones and their metabolites in different matrices. *time lag is highly variable by species.

for saliva collection in free ranging rhesus macaques at Cayo Santiago by providing ropes with oral swabs sewn on the end on which the animals chewed (Higham et al., 2010), and in free-ranging mountain gorillas by recovering saliva from discarded forest food parts (Smiley et al., 2010).

The viscosity of freshly collected saliva impedes precise pipetting. After one freeze-thaw cycle, proteins and mucopolysaccharides precipitate, resulting in a less viscous saliva matrix (Read et al., 1990; Riad-Fahmy et al., 1982). However, the number of freeze-thaw cycles can also effect the concentration of the hormone. Salivary cortisol decreased significantly after five freezing-thawing cycles while progesterone was not affected by the procedure (Gröschl, 2001).

An exceptionally problematic source of sample contamination for saliva collection is the absorbing collection material. Some sampling materials contain interfering substances that introduce a significant, but unsystematic error after extraction. In the case of cotton rolls, these substances could be plant hormones that cross-react with the antibodies used in certain immunoassays (Dabbs, 1991; Granger et al., 1999). Additionally, some of the hormones of interest could adhere to the cotton fibers and are thus, removed from the measured sample (Shirtcliff et al., 2001). Specifically, the use of cotton-based absorbent materials has profound effects on the results of immunoassays for salivary dehydroepiandrosterone (DHEA), testosterone, and progesterone. Levels for these hormones were artificially high when collected with cotton, whereas results for secretory immunoglobulin A were artificially low (Dabbs, 1991; Granger et al., 1999; Krüger et al., 1996; Shirtcliff et al., 2001). Details regarding the "troubles" with salivary testosterone are described in detail by Granger et al. (2004). The same problems that are reported when using cotton rolls for human salivary testosterone measurements apply to their use in other species. In chimpanzees, immunoassay testosterone results were artificially high when saliva was collected with cotton based material, but after the cotton was cleaned with a procedure that involves several steps prior to saliva collection, testosterone concentrations could be accurately measured (Kutsukake et al., 2009). An alternative cleaning procedure for cotton based material is presented in Laudenslager et al. (2006).

Sample impurities, preservatives or salivary pH can interfere with assay performance. A possible solution is to extract steroids from saliva with diethyl-ether (Arslan et al., 1984; Dabbs, 1990; Hohmann et al., 2009). This also provides the possibility to concentrate samples, but it should be validated if matrix effects resulting from the simultaneous concentration of other saliva compounds influence correct hormone measurements.

Particular care should be taken when salivary flow rate is artificially stimulated to obtain higher sample volumes. While some steroid hormone concentrations were unaffected by changes in salivary flow rate (Lipson and Ellison, 1989; Riad-Fahmy et al., 1982), substances used to stimulate saliva flow rate may contain substances that cause interference with the assay. For example, non-steroid substances can affect assay results in progesterone (Lipson and Ellison, 1989), cortisol (Schwartz et al., 1998), and DHEA levels (Gallagher et al., 2006). It is possible that the stimulating substances produce changes in salivary acidity beyond the buffering capacity of the immunoassay, which may compromise the immunoassay binding reaction, resulting in artificially high values (Schwartz et al., 1998). Furthermore, salivary flow rate stimulation might alter the concentration of salivary proteins, thereby increasing matrix effects (Lipson and Ellison, 1989). For these reasons, it is recommended to collect saliva whenever possible without using oral stimulants, or to first check for potential interferences (Schwartz et al., 1998). Additional interferences can be caused by substances consumed prior to saliva collection, such as breast milk or food products containing phytoestrogens. Compounds of these substances can cross-react with the antibody of the immunoassay and cause erroneous hormone measurements (Laudenslager et al., 2006; Magnano et al., 1989). Therefore, smoking, drinking, and eating should be avoided around the time of salivary sample collection (Aardal and Holm, 1995).

Lastly, minor contamination of saliva with blood by e.g., shedding teeth or injury can drastically affect the quantitative estimates of salivary hormone levels. Most steroid hormones have tremendously higher concentrations in blood than in saliva, and even minor contamination can result in falsely high values (Quissell, 1993; Schwartz and Douglas, 2004). To control for blood contamination of saliva samples, they can be tested with microcuvettes for haemoglobin determination (Aardal and Holm, 1995).

1.2.2. Urine and feces

Hormones are extensively metabolized by the liver, and afterwards excreted via the kidneys into the urine, or via the bile ducts into the gut (Macdonald et al., 1983; Palme, 2005; Palme et al., 2005). Metabolic products can be conjugated to various degrees to glucuronides and sulfates (Bahr et al., 2000; Hauser et al., 2011). Therefore, in both matrices the primary hormones are only available in relatively small proportions, and excretion pathways can be variable depending on species and hormone (Bahr et al., 2000).

1.2.3. Urine

Urine contains soluble steroids and their metabolites, peptides, and some neurotransmitter metabolites (Carlstead et al., 1992; Jewgenow et al., 2009; Möstl and Palme, 2002). In the urine of primates, peptide hormones like c-peptide (Deschner et al., 2008; Emery Thompson and Knott, 2008; Grueter et al., 2014), oxytocin (Crockford et al., 2013; Finkenwirth et al., 2016, 2015; Wittig et al., 2014), and thyroid hormones (Behringer et al., 2014b; Wasser et al., 2010) can also be measured. Furthermore, additional non-hormonal substances such as pteridines for the monitoring of immune responses have been validated in primate urine (Heistermann and Higham, 2015; Higham et al., 2015).

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In captive animals, urine samples can be collected from the cage floor or from drip pans in metabolic cages (Anzenberger and Gossweiler, 1993; Whitten et al., 1998). For some studies animals were trained to cooperate for urine collection (Beerda et al., 1996; Bloomsmith et al., 2015; Laule et al., 1996). In the wild, diverse methods exist to collect urine samples (Danish et al., 2015; Whitten et al., 1998). Urine can be collected from a trap, the vegetation, on plastic sheets, or from the ground (Emery Thompson and Knott, 2008; Robbins and Czekala, 1997; Surbeck et al., 2015).

Urinary steroid hormones are generally assumed to be stable for 24 h at room temperature, and for 72 h at 4 °C (Grant and Beastall, 1983). However, the stability of steroid hormones should be considered with care in endocrinological studies, because the speed of bacterial degradation can vary depending on the metabolized form, e.g., conjugation of the respective steroid hormone. Urine samples should be stored frozen; alternatively urinary steroid preservation is also possible on filter paper (Knott, 1997). While some hormones are robust to multiple freezing and thawing cycles, the procedure should be avoided by dividing the sample into aliquots before freezing or validated for the hormone in question (Heistermann, 2010). Before analysis, urine samples should be centrifuged to remove particulate matter (Heistermann, 2010).

Hormones in urine are excreted predominantly in a conjugated (water-soluble) form. For some hormones, measurement techniques and assays are available to assess the concentrations of the conjugated and unconjugated hormone separately. Other hormones can only be measured after deconjugation, which can be achieved by solvolysis or hydrolysis procedures (Hauser et al., 2008a; Preis et al., 2011). If enzymatic hydrolysis is applied to urine samples using enzyme extracts from *Helix pomatia* juice caution should be taken, because these contain enzymes which can result in hormone transformation (Hauser et al., 2008b). However, the efficiencies of deconjugation are dependent on conjugation patterns and therefore can vary across species and even from sample to sample. Therefore, validation tests of deconjugation methods are necessary (see validation part below).

It is necessary to adjust hormone measurements for the concentration of the urine sample, because urine concentration varies with fluid intake and with the time since last urination (Miller et al., 2004). An adjustment of urinary hormone concentration can be achieved by correction for urinary creatinine levels or specific gravity (SG) of the urine sample. Although widely used, creatinine as a correction factor can be problematic depending on the study design. Creatinine values show inter- and intra- subject variability that can depend on sex, age, activity, and diet (Carrieri et al., 2000; Emery Thompson et al., 2012b; Miller et al., 2004). Because creatinine is a breakdown product of muscle metabolism, urinary creatinine can vary in relation to individual muscle mass. Therefore, in studies that focus on comparisons across animals with considerable variation in muscle mass, e.g. males vs. females or adults vs. juveniles, correction of urine concentration by creatinine can lead to misleading results and should be avoided (Alessio et al., 1985; Emery Thompson et al., 2012b). Also, chimpanzees younger than four years of age were found to produce small quantities of highly dilute urine with low creatinine levels, and the creatinine is probably skewed by enhanced protein intake during nursing (Emery Thompson et al., 2016). The correction for urine concentration by using SG has three advantages. Firstly, it can be carried out easily already in the field with a handheld refractometer. Secondly, only a small amount of urine is necessary for measurement (20–30 μl) and thirdly, in contrast to creatinine, SG is not vulnerable to degradation (Anestis, 2010; Hodges and Heistermann, 2011). In addition to using creatinine and SG measures for correction of urine concentration, they can also be used to estimate muscle mass in adult and lean body mass in juvenile chimpanzees (Carrieri et al., 2000; Emery Thompson et al., 2016, 2012b).

As in saliva, the circadian pattern of some hormones can also be observed in urine samples (Heistermann, 2010). For example, urinary cortisol and its metabolites are elevated in early morning samples collected after the animals wake up and then levels gradually decrease throughout the day in marmosets (Smith and French, 1997), chimpanzees (Muller and Lipson, 2003), and spider monkeys (Davis et al., 2005). The same circadian pattern was found for urinary testosterone and its metabolites in chimpanzees (Muller and Lipson, 2003). Therefore, sampling time needs to be kept constant or controlled for in the statistical analysis.

In urine samples a limitation of measuring c-peptide is its susceptibility to degradation. UCP degradation depends on processing, storage, and transport. Furthermore, contamination of urine with feces was found to reduce measured UCP levels, whereas the contamination with dirt did not affect measured UCP levels (Higham et al., 2011).

1.2.4. Feces

Endocrinological monitoring based on fecal samples is particularly valuable when the research focus is on long-term patterns, like chronic stress, seasonal patterns, or reproductive status (Touma and Palme, 2005; Wielebnowski and Watters, 2007).

Until recently, measurements in fecal samples of non-human primates have focused exclusively on steroid hormones (Heistermann, 1996; Lasley and Kirkpatrick, 1991; Möhle et al., 2002; Schwarzenberger et al., 1996; Weltring et al., 2012; Ziegler et al., 1989). Except for the successful validation of TH measurements (Schaebs et al., 2015; Wasser et al., 2010), the measurement of other hormones is precluded by the fact that they are either not excreted into feces or as heavily degraded compounds which are not detectable by the available measurement techniques.

Ideally, after defecation the fecal sample should be immediately collected and frozen, because hormones in feces are prone to bacterial metabolism (Hodges and Heistermann, 2011; Millspaugh and Washburn, 2004; Palme, 2005). Sample degradation can lead to changes in the relative amount of cross-reacting metabolites (Khan et al., 2002; Price and Newman, 1991). Depending on the assay used, degradation may cause fluctuations in hormone levels with increasing age of the sample (Palme et al., 2013). When freezing is not possible, for example under field conditions, hormonal degradation can be prevented by removing water from the samples. This can be achieved by drying or by adding of hygroscopic chemicals such as alcohol (Palme, 2005). Preservation in alcohol has the advantage that samples can be stored at ambient temperature. However, a slight degree of hormone degradation can still persist and alcohol evaporation, spillage and leakage can create erroneous concentrations (Hodges and Heistermann, 2011). Improved preservation of fecal samples in alcohol at room temperature can be achieved by adding sodium azide or other acids, but these can be highly toxic and dangerous for the environment (Whitten et al., 1998). To avoid the problems associated with sample storage and transport in field settings, extracting fecal samples already in the field has become increasingly common in the last years (Beehner and Whitten, 2004; Kalbitzer and Heistermann, 2013; Whitten et al., 1998; Ziegler and Wittwer, 2005). In addition, the extraction process separates the hormones and their metabolites from the potentially pathogenic fecal matrix (Ziegler and Wittwer, 2005). Commonly, a certain amount of fecal sample is mixed with a certain amount of 80% methanol or 80–90% ethanol. The hormones in the suspension can be separated through centrifugation from the fecal matter and the supernatant is stored separately (Wielebnowski and Watters, 2007). The efficiency of such an extraction depends on the polarity of the hormone or metabolite of interest (Möstl et al., 2005; Palme et al., 2013). Even in fecal samples, some hormones can be conjugated and therefore might require additional enzymatic hydrolysis and/or solvolysis before analysis (Ziegler et al., 1996a).

A test with steroid hormones in baboon fecal samples revealed that if samples have to be stored for up to 24 weeks at room temperature the preferred method is the storage of liquid extracts in a dark and cool place. For longer periods, solid phase extraction (SPE) cartridges should be applied (Kalbitzer and Heistermann, 2013). Permit guidelines mandated by country-specific regulations when samples have to cross

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borders may also affect the choice of sample storage and transport vessel (Wielebnowski and Watters, 2007).

Hormones are unevenly distributed throughout a fecal sample necessitating a thorough homogenisation before extraction (Millspaugh and Washburn, 2004; Palme, 2005). Furthermore, larger pieces of non-fecal material, like big seeds and parts of insects, should be discarded during sample processing (Hodges and Heistermann, 2011).

Contamination is also a problem when working with fecal samples, and contamination of fecal material with urine needs to be avoided. Depending on the main excretory pathway of the species the hormone or metabolite concentration may be higher in urine, which can lead to an overestimation of fecal hormone concentrations if the method for hormone measurement is also able to detect urinary metabolites of the hormone of interest (Bahr et al., 2000; Whitten et al., 1998).

In contrast to saliva and urine samples, diurnal variation of hormone production is only an issue in fecal samples when the species of interest has a fast gut passage rate (Wielebnowski and Watters, 2007). For example, cortisol and progesterone showed diurnal changes in female common marmosets, with significantly elevated cortisol levels in the afternoon (Sousa and Ziegler, 1998). Other factors can contribute to inter- and intra-individual differences that have to be taken into account. Individual differences in gut bacterial composition can cause differences in how fecal hormones are metabolized. Thus, individuals may differ with regard to what kind of hormone metabolites they form and to the relative proportions of these hormone metabolites they possess. Assay methods that are sensitive to only one specific metabolite might therefore create differences in fecal steroid concentrations between individuals that are not representative of serum levels (Goymann, 2012; Habumuremyi et al., 2014; Palme, 2005).

Additionally, variation in diet composition can influence hormone metabolism, hampering the comparison of hormone metabolite levels across seasons. For example, in women it was found that an increase in dietary fiber resulted in a shorter gut transit time, although both, fecal bulk and fecal estrogen concentration increased (Goymann, 2012, 2005; Millspaugh and Washburn, 2004). In baboons, fecal progestogen was relatively independent of the amount of dietary fiber consumed by the individuals (Wasser et al., 1993).

1.2.5. Hair

The measurement of steroid hormones in hair is still in its infancy. Until now, most research has been performed on humans in medical studies and only a few investigations relate hormone measurements derived from hair with behavior or the environment. The majority of studies have focused on cortisol, but in human and rhesus monkey hair, testosterone, estradiol, progesterone, cortisone, and DHEA have been measured as well (Chen et al., 2013; Gao et al., 2015, 2013; Grass et al., 2016; Kapoor et al., 2014; Yang et al., 1998).

There are considerable complications and uncertainties with hair hormone measures that need to be taken into account when using this method. Hair cortisol concentrations in a number of animal species vary in relation to the hair's position on the body, highlighting the importance for the identification of a standardized location for hair sampling (Carlitz et al., 2015; Terwissen et al., 2013; Yamanashi et al., 2013). This renders random hair collection from traps or nests highly problematic. One explanation for the variation could be that different body parts are more or less exposed to sun light, because sun light exposure decrease hair cortisol and cortisone levels, but increase hair DHEA and progesterone levels (Grass et al., 2016). In comparison to other matrices, hair samples can be stored very easily at ambient temperatures in an envelope without risking hormonal degradation (Accorsi et al., 2008). Most protocols for hormone measurement in hair include one or more washing steps prior to analysis to prevent contamination from external sources (e.g., sweat). Such washing protocols include as washing solutions methanol, chloroform, methylene chloride, or isopropanol (Chen et al., 2013; Gao et al., 2015). A protocol for hormone extraction for LC-MS measurement from hair samples is described in detail in Gao et al. (2015). For this step, methanol is considered the preferential extract (Cooper et al., 2012).

Hair can be easily contaminated with hormones from external sources (Musshoff and Madea, 2007). In humans, the principal sources for contamination are sweat and sebum, while in numerous animal species secretions from scent glands can be incorporated into certain areas of the fur (Ferrero and Liberles, 2010). These secretions, as well as salivary GCs applied to the fur by licking, could contribute to variation in hair GC levels (Sheriff et al., 2011). Furthermore, hormone concentrations depend on the hair growth rate. In humans, hair growth rate depends among other factors on the anatomical region, age, gender, and ethnicity, which are nontrivial influences on the interpretation of hormone concentrations in hair (Taylor et al., 2015; Wennig, 2000). Hair hormone concentrations also seem to vary significantly with hair color between and within individuals, as described in dogs (Bennett and Hayssen, 2010), and chimpanzees (Yamanashi et al., 2013). Finally, local cortisol production in hair bulbs contributes to hair cortisol concentration thereby rendering the representativeness of hair hormone levels highly questionable, as shown in the hair of guinea pigs (Keckeis et al., 2012) and sheep (Stubsjøen et al., 2015). For more information on potential cofounding effects to hair hormone levels particularly in humans see Wennig (2000).

1.3. Methods of measurement

Hormones in non-invasively collected samples from animals are predominantly measured with immunoassays – radio- or enzyme immunoassays (RIA or EIA). In humans, the use of liquid chromatography mass spectrometry (LC-MS) methods has recently gained importance for measuring hormones in matrices other than blood, particularly in the fields of medicine, and doping analysis (Taylor et al., 2015).

1.3.1. Immunoassays (RIA/EIA)

The principal method to determine hormones in various matrices are immunological procedures using hormone- or group-specific antibodies (Heistermann et al., 2006; Holme and Peck, 1998; Katoch, 2011). Most often, radioimmunoassays or enzyme immunoassays, also known as ELISA (**E**nzyme **L**inked **I**mmuno **S**orbent **A**ssay), are used. Competitive immunoassays quantify hormones based on the binding specificity and selectivity of their antibody. The principle of a competitive immunoassay is the competition between an added, labeled antigen of a known concentration and an unlabeled antigen (i.e., hormone in the sample) for binding to the specific antibody. The advantages of RIA over EIA include higher measurement precision (reproducibility of replicate determinations, either within an assay batch or between batches, laboratories, or methods (Price and Newman, 1991)) and robustness, however a licensed radioactive laboratory is necessary, gamma and beta scintillation detection equipment as well as radiolabeled compounds are comparatively expensive and not always available e.g., for steroid metabolites. Furthermore the regulations for the disposal of radioactive waste have become more and more rigorous and expensive (Brown et al., 2004). For analysis of steroids, competitive enzyme immunoassays have been widely used because they are relatively simple to run, analytically sensitive, and a great number of commercial and in-house assays exist and have been validated for the use in a variety of matrices and species (e.g., Gröschl, 2008; Heistermann et al., 2006). A detailed illustrated description of immunoassay performance is given in Möstl et al. (2005). However, immunoassays do not always have the analytical specificity required for exclusive binding to the hormones of interest in the presence of structurally similar compounds (Cox et al., 2004). Most antibodies have varying degrees of cross-reactivity with the metabolites of the hormone in question or with hormones of a similar structure. Therefore, results produced with such assays would include measures of the hormone of interest as well as in parts the measurements of all the metabolites which the antibody of the assay cross-reacts with. Antibody cross-reactivity in assays

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can be advantageous as well as disadvantageous for the measurement of hormone levels in non-invasive sample matrices. In a situation where most of the hormone of interest is metabolized and the assay mainly picks up signals from these metabolites due to its cross-reactivity, the assay results might still be biologically meaningful, even in the total absence of the native hormone. In contrast, if the assay cross-reacts mainly with metabolites originating from different hormonal pathways that are not related to the native hormone, then assay measurements will be erroneous and maybe not meaningful (Möstl et al., 2005; Palme et al., 2013, 2005). For example, when fecal testosterone is measured in females, it is possible that the testosterone levels might originate from a different metabolic pathway, either from androgens (other than testosterone) coming from the adrenals or out of the progesterone pathway. If this is the case, the measured fecal testosterone levels would not represent biologically relevant information, independent of the method used to measure the hormone or metabolites. It is therefore important to test if for a specific study design an assay with a group- specific antibody is superior over an assay with a specific antibody. The group- specific antibody is directed against a group of metabolites whereas the specific one is directed against the native hormone. Therefore, several assays with different specificities should be tested for any given species and matrixes to evaluate which antibody is best to detect a hormone/metabolite level change (Heistermann et al., 2006). For example, different assays with antibodies produced against different hormones/metabolites can be tested with the same sample set, and the assay with the highest reactivity is then used. Because this assay allows for the detection of smaller hormone/metabolite level changes (e.g., Fanson et al., 2015; Möstl et al., 2005; Sheriff et al., 2011). For further explanation see Higham (2016).

The variation in cross-reactivity of antibodies with different compounds used among assays also makes a comparison of measured values across studies almost impossible. Different extraction or sample preparation protocols further contribute to the problem of comparability across laboratories (Millspaugh and Washburn, 2004; Palme et al., 2013). These aspects of immunoassay characteristics necessitate a rigorous analytical validation of the method used with respect to its sensitivity (defined as the smallest value that can be reliably discriminated from zero values with a 95% probability) as well as specificity, because matrix influences that are much more complex in non-invasive than in plasma samples can play a substantial role (Möstl et al., 2005).

1.3.2. LC-MS

Modern liquid chromatography (LC), coupled with mass spectrometric (MS) detection, has tremendously improved the analytical specificity and sensitivity of hormone measurements in biological samples (Cross and Hornshaw, 2016). LC-MS combines the separation capabilities of a high performance liquid chromatography (HPLC) system with those of a conjoined mass spectrometer (Murtagh et al., 2013). In contrast to immunoassays, LC-MS specificity does not depend on an antibody-antigen binding but rather on the separation of substances based on their chemical and physical properties such as polarity, mass and charge of the substance of interest. Apart from its high specificity, an additional advantage of LC-MS is the possibility for the simultaneous measurement of a large number of hormones and their metabolites (Gröschl, 2008). For detailed information about the functionality of this method and other advantages see Ackermans and Endert (2014); Couchman et al. (2011); Keevil (2013); Murtagh et al. (2013); and Soldin and Soldin (2009).

For endocrine measurements of non-invasive samples from animals, various LC-MS methods have been developed to quantify steroid hormones in fecal samples of domestic animal species (De Clercq et al., 2014), New World primates (Weltring et al., 2012), Old World primates (Gesquiere et al., 2014), and in urine of Great Apes (Hauser et al., 2008a). LC-MS also allows the measurement of peptide hormones in non-invasively collected samples. In human urine, c-peptide (Stoyanov et al., 2013) and gonadotrophin-releasing hormone (Thomas et al., 2008) have been measured. Furthermore, additional non-hormonal substances such as neopterin, which is used to measure immune responses, can be quantified with LC-MS in human urine (Yang et al., 2015).

It is likely that in the future many of the problems described above for immunoassays will be resolved by the use of LC-MS. In fact, LC-MS has already been proposed as mandatory for reporting steroid levels in humans in some journals (Handelsman and Wartofsky, 2013). This proposition has been met with some resistance, with other researchers concluding that irrespective of the specific technique, all methods need proper validation and reporting of quality criteria (Wierman et al., 2014). Therefore, in the near future EIA and RIA will likely remain the preferred methods in most laboratories, but we expect that LC-MS will become a more and more important method for assay validation (Behringer et al., 2012b; Gesquiere et al., 2014; Habumuremyi et al., 2014; Kutsukake et al., 2009; Preis et al., 2011).

The high specificity of LC-MS is especially beneficial when measuring steroids and their metabolites in non-invasively collected matrices such as urine and feces. The advantage of LC-MS is that one can avoid cross-reactivity that might come from metabolites that originate from a different metabolism pathway (e.g., adrenal versus gonad glands) (Preis et al., 2011). However, high specificity in measurements may likewise lead to erroneous results if, for example, a particular hormone e.g. cortisol, can be metabolized into a variety of different metabolites with the metabolic pathways depending mainly on the bacterial gut flora. Indications of such an effect were found by Habumuremyi et al. (2014), who reported much wider differences in inter-individual female fecal pregnandiol levels when measured by LC-MS than when measured by EIA. An EIA that cross-reacts with a wide number of metabolites originating from the same pathway might reduce this problem. Alternatively, levels of metabolites stemming from the same pathway can be summed in order to provide a corrected excretion estimate as see, for example, Wittig et al. (2016, 2015).

Unfortunately, sample extraction for LC-MS measurement can be elaborate and time consuming. Furthermore, the instrument itself is very costly, needs regular expensive maintenance, and can only be run by trained and experienced people (Taylor et al., 2015).

1.4. Validation

A number of published reviews and guidelines on non-invasive hormone research have repeatedly highlighted the importance of proper chemical and physiological validations (e.g., Buchanan and Goldsmith, 2004; Goymann, 2005; Palme, 2005; Touma and Palme, 2005; Wielebnowski and Watters, 2007). Still, researchers attempting to use endocrine measurements in addition to behavioral observations and environmental variables might not fully appreciate the importance of assay validation procedures (Buchanan and Goldsmith, 2004). Although commercially available assay kits provide some data on immunoassay validation, those results are relevant only for the species and matrices tested by the commercial supplier. Therefore, it is a necessity to validate assays for the use in another species or in different matrices (Buchanan and Goldsmith, 2004; Goymann, 2005; Palme, 2005).

In the 1980s, endocrinological journals customarily required authors to publish the procedures for adequate assay validations. The validation of an assay for urinary growth hormone in humans is an example of this practice (Hourd and Edwards, 1989). Such validation experiments are essential in establishing the quantitation limits of a method, allow for estimating confounding effects, and thereby determining the limits within the results can be interpreted. Importantly, a validation shows the suitability of a certain assay for a given matrix in a given species.

1.4.1. Chemical validation procedures

For the chemical validation a parallelism experiment is necessary to ensure that the assay is capable of reliably measuring a certain hormone in samples of varying matrix concentrations. It involves running a series

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of dilutions using a pool combined from several different samples. The displacement curve of such a dilution series is then compared against the standard curve, which is produced using a synthetic form of the hormone dissolved in assays buffer. If the displacement curve of the sample parallels the standard curve, the sample solution is immunologically similar to the standard and can be measured proportionately. An illustrated example for a urinary testosterone curve is presented in Al-Dujaili (2006). Furthermore, the specificity, i.e., the cross-reactivity with other antigens, of an assay should be routinely tested and presented, since these parameters are essential for the assessment of accuracy (Remer et al., 1994). Accuracy evaluates the degree to which the measured concentration with the assay corresponds to the true concentration of a substance in a sample. It tests for potential interference caused by substances contained within the sample that are independent of specific antigen-antibody binding. An example for such a procedure is explained in detail in Brown et al. (2004).

To assess which metabolites and hormones other than the target hormones are bound with the antibody of a certain assay, samples are loaded on a reverse-phase high performance liquid chromatography (RP-HPLC), fractionated, and fractions are measured with the assay and compared to standard measurements of these compounds (Heistermann et al., 2006; Wheeler et al., 2013). Ideally such HPLC separations are performed with samples from a radiometabolism experiment, where all naturally formed metabolites are radiolabeled and their immuno-reactivity can be evaluated (Bahr et al., 2000; Möhle et al., 2002).

Extraction efficiency gives you an estimate of the proportion of the compound of interest in the sample which is finally recovered after the whole extraction procedure (Palme et al., 2013). Additionally, these tests can also be used to determine how consistent the extraction process is between samples. It is essential that the variability across several samples as well as the average efficiency are determined, as samples containing higher or lower hormone levels may not show equal extraction efficiencies (Buchanan and Goldsmith, 2004). Examples for the experimental set up and presentation of results can be found in Al-Dujaili (2006) for humans and in Behringer et al. (2014b, 2012b) for non-human primates.

Furthermore, stability experiments need to be carried out. Whenever possible, sample freezing-thawing cycles should be avoided. If this cannot be prevented, it is necessary to experimentally assess the effects of these cycles in a certain matrix on hormone concentrations (Wielebnowski and Watters, 2007). Examples for this procedure in bonobo urine are shown for example for DHEA-S (Behringer et al., 2012b) and for c-peptide (Deschner et al., 2008).

Assay measurements need to be compared to the linear range of the standard curve. Values for which the binding is less than 20% or greater than 80% of maximum binding should be excluded, because most standard curves are linear between 20 and 80% binding, and only for measurements within this range the quantity response is linear (Brown et al., 2004). Validation and standardization of an assay is only the first step towards establishing a reliable endocrine monitoring program. Further steps include the measurement of quality control samples (QC). As parameters of precision control usually two to three QC are run on each assay with concentrations around ideally 30%, 50%, and 70% binding of the assay. Ideally, a large pool sample for each QC is prepared along with the biological material and then aliquots are prepared to avoid repeated thawing (Brown et al., 2004; Möstl et al., 2005). Assay coefficients of variation (CV) are precision parameters, classically determined by pool samples (with high and low concentration), which are measured several times either within an assay (intra-assay CV) or across different assays (inter-assay CV) (Möstl et al., 2005). An intra-assay CV is most accurately determined by calculating the variation in assaying multiple replicates of one sample throughout one assay. The Inter-assay CV is determined by calculating the variation in values for QC run in every assay (Brown et al., 2004).

1.4.2. Physiological validation

It is essential to demonstrate that the values measured by an assay accurately reflect the physiological events of interest, in other words, that assay results provide physiologically relevant information. In this regard the golden standard of a physiological validation is to challenge the release of the hormone of interest. For the validation of the measurement of GCs, an adrenocorticotropic hormone (ACTH) challenge is performed by administering ACTH to stimulate cortisol release (e.g., Heistermann et al., 2006; Wasser et al., 2000; Whitten et al., 1998). This approach also clarifies the excretory lag-time between stimulation of an endocrine gland and the appearance of its hormonal metabolites in the matrix (Brown et al., 2004). In cases when such an invasive experiment is impossible, e.g., if the species has a high protection status or if it is not kept in captivity, a validation can be achieved by correlating non-invasive hormone measurements to certain states, events or other circumstances that are known to be correlated with the hormone in question. For example, urinary cycle profiles of sex steroids can be compared to external signs of reproductive status like swellings. Potentially stressful events, like parturition (Murray et al., 2013) injury, or anesthesia (Wheeler et al., 2013) can be used to validate glucocorticoid and alpha-amylase assays. Furthermore, the levels of hormones that are known to differ between immature and adult individuals can be compared (Gobush et al., 2014).

2. Conclusion

Within recent years, methods for the non-invasive measurement of hormones and their metabolites have been developed for an increasing number of hormones and validated for an increasing number of primate species and matrices. However, when applying hormone measurements to a new species or matrix it is essential to carry out rigorous validation prior to measurement, regardless of which assay method is used. Furthermore, existing problems with hormone measurements in different matrices need to be considered and the appropriate matrix chosen in accordance with the specific aims of the study.

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