A non-invasive method to assess the reproductive status of the European badger (*Meles meles*) from urinary sex-steroid metabolites

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ABSTRACT

Due to their unique reproductive physiology and behaviour, European badgers (*Meles meles*) are often used as a model to study mammalian reproduction. For reproductive endocrinology, circulating hormone levels are conventionally measured directly from blood samples. However, routine blood sampling is often not practical for wild animals and may induce stress affecting measurement accuracy. Non-invasive alternatives are thus of interest. Circulating hormones are metabolized through different routes, either by the kidneys, to be excreted through urine, or by the liver, to be excreted through faeces. These metabolites can thus be used as a proxy of hormone measurements, provided the species-specific metabolic characteristics are known. Here we tested the suitability of measuring urinary metabolites of circulating plasma sex-steroid hormones (testosterone in males and oestrogen in females) with enzyme immunoassays to assess the reproductive status of the European badger (*Meles meles*). Biological validation evidenced that urinary testosterone metabolite (UTM) and urinary total oestrogen metabolite (UEM) excretion patterns both corresponded with seasonal badger reproductive patterns on a population level, signaling correlation over a broad time frame. On an individual level, concurrent sampling of urine and plasma showed that male plasma testosterone and UTM levels correlated significantly across seasons, but no short term individual level correlation was evident for total oestrogen and UEM in females. Thus, in badgers, urinary sex-steroid metabolites can be used to reliably assess male reproductive status on the short term individual level, but only at broad term population level for females. Moreover, we demonstrate that importance of knowledge on biological patterns as a validation method.

Keywords: *biological validation of urinary hormone levels, European badgers, non-invasive monitoring of urinary hormone levels, reproductive status, sex-steroids, urinary testosterone and oestrone metabolites*

1. INTRODUCTION

Conventional hormone measurement from blood plasma quantify the immediate “active” hormone levels directly at the time of sampling and are thus considered to be the most accurate measurement of endocrine function when collected longitudinally (Heistermann et al. 1993; Graham 2004; Dehnhard et al. 2008; Heistermann 2010). However, routine blood sampling is often not practical and can be dangerous in wild animals (Monfort et al. 1997; Touma and Palme 2005) as it may induce stress, which in turn can trigger hormonal changes and/or affect assay accuracy due to cross-reactivity (e.g., with adrenaline metabolites: Dehnhard et al. 2008; Heistermann 2010). In terms of animal handling legislation, and the need to restrain/sedate unhabituated animals for invasive sampling, this approach typically requires licensing (e.g., UK Animals [Scientific Procedures] Act 1986), where ethical review standards apply the “3Rs” (i.e., Replacement, Reduction, and Refinement), and thus advocate the use of non-invasive techniques whenever practicable (Department of Health 2014; Heistermann 2010).

Sex steroids circulating in blood plasma are metabolized either by the kidneys and excreted in urine, or by the liver and ultimately excreted in faeces (Graham 2004; Touma and Palme 2005; Heistermann 2010) in either conjugated (as either glucuronides or sulphates, e.g. estrone conjugate E1C or pregnanediol-3-glucuronide, PdG) or unconjugated form (Schwarzenberger et al. 1996; Heistermann 2010). In addition, a small proportion of these unconjugated sex-steroids may be deposited in keratinised tissue (hair: Koren et al. 2002; claws: Mack and Fokidis 2017; Matas et al. 2016) or enter the saliva (Heistermann 2010). Sex-steroid measurements in these different mediums have thus been established as non-invasive proxies to estimate the levels of circulating hormone titres and reproductive parameters of a wide range of wild and captive species (Hindle et al. 1992; Monfort et al. 1997; Buesching et al. 1998; Graham 2004; Schwarzenberger 2007; Dehnhard et al. 2008); often easing sample collection (especially in wild animals) as they largely negate the need to handle and consequently stress the animal (Graham 2004; Heistermann 2010).

Choosing the most appropriate sample medium, however, depends on the research question (Graham 2004; Touma and Palme 2005), because each of the hormone measures represent an aggregate of hormone levels over a period of time (Graham 2004; Touma and Palme 2005). A short time-lag between the between plasma hormone levels and deposited/ excreted

hormones will give a closer estimate of the current circulating hormone levels but will also be more affected by transitional effects such as circadian variation and pulsatile secretion (Touma and Palme 2005; Heistermann 2010). A longer time-lag gives a more averaged estimate of circulating hormone levels over a period of time, and is thus more consistent, but may not be able to capture short-term effects or patterns that occur rapidly (Graham 2004; Touma and Palme 2005).

For many research questions, the most suitable non-invasive method is the analysis of urinary and/ or faecal sex-steroid levels (Heistermann 2010) that are essentially waste products and can be collected remotely upon observing when and where specific target wild individuals urinate or defecate (Kirkpatrick et al. 1988; Hutchings et al. 2001). In wild animals, faecal sample collection can be practically easier than urine collection (Graham 2004; Touma and Palme 2005; Heistermann 2010), but the time lag between circulating plasma hormones and faecal excretion is typically more than 12 hours (Palme et al. 1996; Graham 2004; Heistermann 2010), compared to only 2-8 hours in urine (Palme et al. 1996; Heistermann 2010). In carnivores, hunting success and diet affects scat deposition rates and content (Ray 1998), and irregular deposition can affect measured steroid levels (Kelly et al. 2012) possibly due to changes in gut microbiota populations (commonly abundant in faeces but less so in urine; Lubbs et al. 2009; Jain et al. 2019), which in turn can continue to metabolize (and thus affect) hormone levels in scat even after excretion (Kelly et al. 2012).

Using hormone levels in excreta requires species specific validation (e.g. gonadotrophin releasing hormone challenge, validation with known biological patterns/behaviour, correlation with plasma hormone levels, etc.; Swinbourne et al. 2017; Heistermann 2010) to ascertain that the patterns of circulating hormone levels is reflected in the hormone levels found in the excreta. This is because: i) The predominant metabolic pathway (urine vs. faeces) can differ significantly even between closely-related species (Schwarzenberger et al. 1996; Schwarzenberger 2007); ii) hormones are typically metabolized before being eliminated in excreta, the inherent time lag between plasma and faecal/ urinary hormone levels can vary considerably depending on species, individual, metabolism, water-/ food intake, and activity-level (Palme et al. 1996; Graham 2004; Touma and Palme 2005; Schwarzenberger 2007); and iii) excretions are discrete events (i.e., bowel movements/ emptying of bladder), hormone metabolites are pooled in these excretions over a (variable) period of time (Graham 2004; Touma and Palme 2005). Concentration can thus vary

according to circadian rhythm, activity levels, and fluid/ dietary fibre intake, necessitating careful assay calibration and sample quantification/ standardisation (Miller et al. 2004).

The European badger (*Meles meles*; henceforth “badger”) is one of the most intensively studied wild carnivores in the world and has been recognised as a suitable model species for many biological paradigms (Stopka and Johnson 2000), of which many rely on accurate classification of the focal animal’s reproductive status (Sugianto et al. 2018). Badgers are unusual amongst mammals in that they exhibit an atypical reproductive strategy incorporating delayed implantation (fertilized egg cells from mating throughout the year are in stasis and not implanted until the next spring) and superfoetation (new egg cells may be produced while previously fertilized eggs exist in stasis; Yamaguchi et al. 2006), further exacerbating the difficulty to determine reproductive status. Previous studies have investigated plasma sex-steroid profiles in the context of mammalian reproductive strategies (testosterone: Buesching et al. 2009; Woodroffe et al. 1997; Sugianto 2018; Sugianto et al. 2019a; 2020; progesterone and oestrogens: Mondain-Monval et al. 1980; Sugianto 2018; Sugianto et al. 2019a; 2020), and often linked external genitalia morphology (as proxy for reproductive status: Woodroffe et al. 1997, but see Sugianto et al. 2018) to behavioural parameters (Macdonald et al. 2015). Nevertheless, trapping, sedation and handling of badgers necessary for blood sample collection causes stress and the associated cortico-steroid hormone release can cross-react with sex-steroid assays, making results difficult to interpret (Dehnhard et al. 2008; Heistermann 2010). Although, from our previous studies on badgers (Sugianto 2018 and Buesching et al. 2009) we find that our current method and measurements correlate well with biological activities, invasive procedures in badgers is regulated by strict national and international laws (Bern convention; Convention on the Conservation of European Wildlife and Natural Habitats), which requires rigorous training and often extensive governmental licensing (UK Animals [Scientific Procedures] Act 1986). Surprisingly, thus far no non-invasive method to measure circulating sex-steroid hormone levels has been established for this species, often curtailing the possibility of a physiological component particularly to behavioural studies. Thus, here we aim to establish reproductive status of wild male and female badgers from non-invasive measurement of urinary sex-steroid metabolites.

We investigate whether urinary hormone metabolites (urinary testosterone metabolite and urinary total oestrogen metabolite, UTM and UEM respectively) correlate sufficiently with

reproductive status by i) testing whether urinary metabolites alone, in males and females, mirrored the seasonal reproductive activity patterns as biological validation on a seasonal time frame and population level, as well as ii) comparing urinary metabolites with circulating plasma hormone levels (testosterone in males; total oestrogen in females) from paired samples across seasons on to investigate any short term individual level correlation. Based on our results, we discuss the suitability of UTM and UEM measurements and emphasize the importance of known biological patterns as an alternative for validating measures.

2. MATERIALS AND METHODS

2.1 Badger trapping and sampling

Blood and urine samples were collected as part of an ongoing long-term research project from a high-density badger population in Wytham Woods, Oxfordshire, UK (51°46'26" N, 1°19'19" W; see Macdonald et al. 2015) between 2012-2017. Badgers were trapped three times annually in: (1) *spring* (May/June) towards the end of the main mating season and after females ceased lactation; (2) *summer* (August/September) during the second mating peak previously reported in other badger populations; and (3) *autumn* (November) during reproductive quiescence, following the methodology described in Sun et al. (2015). Traps were checked (6.30-8.00 am) and captured badgers were transported to a central field station to then be sedated (usually starting no later than 8 am) by intramuscular injection of 0.2 mg ketamine hydrochloride/ kg body weight (McLaren et al. 2005; Thornton et al. 2005; Sugianto et al. 2019b) with an induction time between 3-4 minutes. Processing of all captured badgers was completed before noon with a typical procedure (data collection and handling) of 10-15 minutes per badger (Sun et al. 2015), from sedation to completion. At first capture, typically as cubs, all badgers received a permanent unique tattoo allowing individual identification (ID) and accurate aging thereafter. If new individuals were first caught as adults, age was estimated using toothwear (Harris et al. 1992). The badgers caught during the period of this study were all adults with no recaptures of the same individual within the same season (any recaptures were from subsequent years). Autumn and summer samples were obtained in 2012 whilst spring samples were obtained in 2016-2017.

2.2 Plasma and urine samples

Paired blood and urine samples were collected from captured adult individuals (66 males and 61 females) under sedation. Blood samples (not exceeding 2 ml) were obtained by jugular venipuncture in K2-EDTA (di-potassium ethylene diamine tetraacetic acid) vacutainer tubes (Becton-Dickinson), and centrifuged at 10°C for 10 min at 2,500 rpm (1470g) within 30 minutes. Plasma was then transferred into Eppendorf tubes (1.5 ml) and frozen immediately at -20°C. Urine samples were collected in glass bottles with stainless steel layered caps (10 ml) by manual bladder palpation (wearing a fresh pair of latex gloves after each animal) and then transferred with a plastic pipette into Eppendorf tubes (1.5 ml) and frozen immediately at -20°C. All sampling times were standardised to account for circadian variation in hormonal profiles (Buesching et al. 2009).

2.3 Sample extraction

Plasma (0.1 mL) was extracted with 2.5 mL petroleum ether. After freezing at -80°C, the organic phase was decanted, dried at 50°C under a stream of nitrogen, and dissolved in 1 mL 40% methanol. Analysis was conducted using 20 µl aliquots for oestrogen and testosterone. To 0.1 ml urine, 0.5 ml 0.05 M acetate buffer pH 4.8 was added including β-glucuronidase / arylsulfatase from *Helix pomatia* and incubated for 2 h at 37°C. For extraction, a mixture of 2.5 ml tert butylmethylether/ petroleum ether 30/70 % v/v was added, and samples were shaken for 20 min and frozen at -80°C. The organic phase was then decanted and dried under a 50°C stream of nitrogen before being dissolved in 1 mL 40% methanol.

2.4 Hormone analyses

Analyses of urinary total oestrogen metabolites (UEM) titres and plasma total oestrogen were carried out using a polyclonal antibody raised against 1,3,5(10)-estratrien-3-17β-diol-17-HS-BSA and 1,3,5(10)-estratrien-3-17β-diol-17-HS-peroxidase label in rabbits. Oestrogen cross-reactivities were as follows: 1,3,5(10)-estratrien-3,17-one (oestrone), 100%; 1,3,5(10)-estratrien-3,17b-diol (17β-E2), 100%; 1,3,5(10)-estratrien-3,17a-diol (17a-E2), 66%; 1,3,5(10)-estratrien-3,16a,17b-triol (oestriol), 1.5%; and 0.1% for 19-nortestosterone, P4 and testosterone. The intra-assay and inter-assay CVs for total oestrogen were 2.97% and 13.21% for low concentration samples and 3.46% and 13.20% for high concentration samples. Sensitivity of the assay was 0.32 pg/20 µL with a linear range of 0.32 – 20.02 pg/20 µL.

Urinary testosterone metabolite (UTM) and plasma testosterone analyses were conducted using antibodies immunized against 17 α -OH-testosterone-HS-BSA in rabbits (provided by Prof. Meyer, Weihestein, Germany) with testosterone-3-CMO-peroxidase used as enzyme conjugate. Antibody cross-reactivity with other steroids were: 4-androst-17 β -ol-3-one (testosterone: 100%), 5 α -androst-17 β -ol-3-one (dihydrotestosterone [DHT]: 13.6%), 5 α -androst-2-en-17 β -ol (1.5%), and < 0.1% for 5 α -androst-3 β -ol-17-one (epiandrosterone), 5 α -androst-3 α -ol-17-one (androsterone), 5 α -androst-2-en-17-one. The intra-assay and inter-assay CVs for testosterone were 4.37% and 19.68% for low concentration samples and 11.90% and 12.18% for high concentration samples. Sensitivity of the assay was 0.43 pg/20 μ L with a linear range of 0.43 - 24.14 pg/20 μ L.

Standardisation of urinary hormone levels with specific density

All urinary hormone levels were standardised according to the sample's specific density (Miller et al. 2004) to account for each badger's fluid intake. Aliquots of 50 μ L of urine were vortexed for approx. 2 minutes (until supernatant was clear) and measured using a KERN OPTICS digital refractometer ORD 1 PM. Hormone levels were corrected according to the following equation:

$$HC_{SD \text{ corrected}} = HC_{\text{Measured}} \times (SD_{\text{target}} - 1.0) / (SD_{\text{sample}} - 1.0)$$

where $HC_{SD \text{ corrected}}$ is the specific density corrected hormone concentration, HC_{Measured} is the hormone concentration measured directly from urine samples, SD_{target} is the population mean specific density (of all samples) and SD_{sample} is the specific density of each sample (Miller et al. 2004).

2.5 Statistical analyses

As a preliminary calibration, we first tested if there were seasonal differences in urine concentration using a linear model with specific density as response and season as predictor. Tests were conducted separately for both sexes. In cases where significance was found, a-priori tests were employed to further investigate the differences between seasons.

To establish whether urinary hormone levels exhibited similar variation to known biological patterns, we compared urinary hormone levels to known seasonal reproductive patterns (Sugianto et al. 2018) that were also confirmed here by plasma hormone levels. We used general linear models with urinary hormone levels (testosterone ng/ml for males; total

oestrogen pg/mg for females) as the response variable and season (spring, summer, autumn) as the predictor. Significant results were subjected to post-hoc tests to ascertain specific inter-seasonal changes. All samples used in the analyses were independent (i.e., no individual contributed more than one sample to each dataset). Patterns of residuals, normality, and mean variance relationship for each model were checked using diagnostic plots in R.

To test for correlation between plasma and urinary sex-steroid levels on an individual level, a general linear model was conducted using urine hormone levels (UTM ng/ml for males; UEM pg/ml for females) as response, plasma hormone levels (testosterone ng/ml for males; total oestrogen pg/ml for females) as predictor, season (spring, summer, autumn) as covariate, and including the interaction-term between plasma hormone levels with season to investigate whether potential correlations were season-specific. For males, plasma testosterone was log10 transformed for best model fit. The following models were analysed: Males= $\text{lm}(\text{Urine testosterone (ng/ml)} \sim \text{log10(Plasma testosterone, ng/ml)} + \text{Season} + \text{log10(Plasma testosterone)*Season}$; Females= $\text{lm}(\text{Urine total oestrogen (pg/ml)} \sim \text{Plasma total oestrogen (ng/ml)} + \text{Season} + \text{Plasma total oestrogen*Season})$. As mentioned previously, all samples obtained were independent (a very small number of recaptures recorded, but never in the same season, thus treated as independent). All statistical analyses were performed using RStudio (0.99.896; RStudio Team 2015) and R (R-3.2.4; R Core Team 2016).

3. RESULTS

3.1 Seasonal variation in specific density of badger urine

Urine specific density did not vary significantly between seasons in either sex (males: $F_{2,70}=1.409$, $p=0.251$; females: $F_{2,79}=0.165$, $p=0.848$; Fig. 1).

Figure 1. Specific density of seasonal urine samples for males (a) and females (b)

3.2 Testosterone

3.2.1 Seasonal patterns in urine and plasma testosterone profiles on a population level

Urinary testosterone metabolite (UTM) levels in urine showed similar seasonal patterns to plasma testosterone, where urine levels varied significantly with season ($F_{2,63}=15.43$, $p<0.001$). Levels were significantly higher in spring (urine= 1.34 ± 0.81 ; plasma= 1.63 ± 2.03 ; $n=30$; post-hoc test, spring vs summer+autumn: Estimate= 0.861, SE= 0.169, $t=5.108$,

$p < 0.001$), lower in summer (urine= 0.68 ± 0.60 ; plasma= 0.49 ± 0.69 ; $n = 19$), and lowest in autumn (urine= 0.26 ± 0.4 ; plasma= 0.28 ± 0.49 ; $n = 17$; Fig. 2), although the latter two seasonal differences were not significant (Estimate= 0.384, SE= 0.266, $t = 1.444$, $p = 0.154$; post-hoc test).

Figure 2. Urinary testosterone metabolite levels (ng/ml) in urine (a) and plasma testosterone (b) during spring, summer, and autumn.

3.2.2 Individual plasma and urine testosterone correlation

In males, UTM levels correlated positively with paired plasma levels ($F_{1,59} = 27.71$, $p < 0.001$, Fig. 3, $r_{\text{spring}} = 0.25$, $r_{\text{summer}} = 0.28$, $r_{\text{autumn}} = 0.77$) in all seasons (plasma testosterone-season interaction term was non-significant: $F_{2,59} = 0.4893$, $p = 0.616$), when season was accounted for ($F_{2,59} = 6.07$, $p = 0.004$).

Figure 3. Correlation between urinary testosterone metabolite levels (ng/ml) in urine and plasma testosterone during spring, summer, autumn.

In each individual, UTM titres were similar to plasma testosterone titres, and individual hormone levels followed the seasonal distribution curve, corroborating the population-level correlation. However, plasma levels tended to exhibit larger variation than urine profiles, causing slight within-individual asynchrony between the two hormone measures in some individuals (e.g. as seen in badger ID 1488 and 1659 in spring; Fig. 4a) although not as much as to break the correlation as whole.

Figure 4. Testosterone levels (ng/ml) in plasma and urinary testosterone metabolite of each individual from spring (a), summer (b), and autumn (c).

3.3 Total oestrogen

3.3.1 Seasonal patterns in urine and plasma total oestrogen levels on a population level

Urinary oestrogen metabolite (UEM) levels varied significantly with season, mirroring plasma total oestrogen levels ($F_{2,58} = 3.97$, $p = 0.024$, Fig. 5), and being highest in spring (urine= 498.88 ± 230.36 ; plasma= 41.53 ± 18.15 pg/ml; $n = 34$: spring vs summer+autumn: Estimate= 131.44, SE= 49.56, $t = 2.652$, $p = 0.010$; post-hoc test), but with no significant difference between summer (urine= 401.58 ± 128.47 ; plasma= 28.29 ± 17.17 ; $n = 14$) and

autumn (urine= 330.68 ± 122.82 ; plasma= 34.13 ± 17.22 ; n= 12: Estimate= 65.41, SE= 77.84, t= 0.84, p= 0.404; post-hoc test).

Figure 5. Urinary total oestrogen metabolite levels (pg/ml) in urine (a) and plasma total oestrogen (b) during spring, summer, and autumn.

3.3.2 Individual plasma and urine total oestrogen correlation

Over the complete paired dataset, UEM levels did not significantly correlate with plasma total oestrogen levels ($F_{1,55} = 0.335$, $p = 0.565$, Fig.6, $r_{\text{spring}} = 0.051$, $r_{\text{summer}} = 0.052$, $r_{\text{autumn}} = 0.051$) when controlling for season ($F_{2,55} = 3.615$, $p = 0.034$), and the plasma total oestrogen-season interaction term was not significant ($F_{2,55} = 0.026$, $p = 0.975$).

Figure 6. Correlation between total oestrogen levels (pg/ml) in urine and plasma during spring, summer, autumn.

Unlike UTM, UEM levels were substantially higher than total plasma oestrogen levels, with UEM exhibiting considerably higher inter-individual variation (Fig. 7) than UTM (Fig. 2,3,7), resulting in highly asynchronous patterns that did not correlate within individuals.

Figure 7. Total oestrogen levels (pg/ml) in plasma and urinary total oestrogen metabolites of each individual from spring (a), summer (b), and autumn (c).

4. DISCUSSION

Urinary sex-steroid excretion patterns corresponded well with established seasonally-mediated reproductive events: In males, urinary testosterone metabolite (UTM) levels were highest at the end of the spring mating season, when male badgers are known to have elevated plasma testosterone levels (Buesching et al. 2009), and larger descended testes (Sugianto et al. 2018). Low UTM levels in autumn correspond with reproductive quiescence in badgers, when males have low plasma testosterone levels (Buesching et al. 2009) and smaller ascended testes (Sugianto et al. 2018). On an individual level, plasma testosterone and UTM levels also correlated significantly across all seasons, evidencing that UTM in urine accurately reflect changes in circulating testosterone titres and may therefore provide a reliable index of male reproductive condition in badgers up to the individual level.

In female badgers, urinary sex-steroid metabolites also exhibited significant seasonal variation at the population level, reflecting badger reproductive stages. UEM levels were highest during the spring mating season, coinciding with elevated plasma levels, when females typically exhibit a distinctly swollen, pink and everted vulva (Sugianto et al. 2018), and overall levels were lower outside of the mating season in summer and autumn, when there is no, or little, vulval swelling (see Sugianto et al. 2018). These urinary profiles thus corroborate previous findings that, at high badger population density (such as at this study site), plasma oestradiol levels show a single peak in spring, consistent with a single mating peak (see Sugianto et al. 2018; Sugianto et al. 2019a).

In contrast to male testosterone patterns, however, in females circulating total oestrogen levels did not correlate significantly with UEM on an individual level. This may be caused by the pulsative nature of circulating hormone secretion (Veldhuis 2008), which can fluctuate due to multiple factors other than reproduction (e.g. circadian rhythm: Irvine et al. 1994; stress, social encounters, health status: Adkins-Regan 2005). Sex-steroid secretion into the bloodstream is rapid and episodic (Veldhuis 2008), whereas urinary metabolites are filtered via the kidneys continuously, and therefore levels accumulate in the urine over time (rather than ‘snap shot’ values; Knox et al. 1992), often resulting in a considerable secretion-excretion time-lag (testosterone: sheep [*Ovis aries*] = 3-4 hours; ponies [*Equus ferus caballus*] = 3-5 hours; cortisol: chimpanzee [*P. troglodytes*], long-tailed macaque [*M. fascicularis*], and common marmoset [*C. jacchus*] = 2-6 hours; Bahr et al. 2000). In males, this pulsative fluctuation only caused asynchrony between UTM and plasma testosterone levels in a few sampled individuals, but did not compromise the significance of the correlation. This may be explained by the fact that testosterone and oestrogen are metabolized differently (Palme et al. 1996), and thus the time-lag differs between male and female reproductive hormones. Radio-metabolism studies, where a radioactive component is administered into the blood stream and excreta are collected over the following time period (Palme et al. 1996; Schwarzenberger et al. 1996), could help elucidate the hormone-specific time-lag between plasma and urinary sex-steroid profiles in European badgers (Keckeis et al. 2012; Bahr et al. 2000). Inherent health-risks and logistical problems, however, typically prevent this approach being applied to free-ranging populations (Graham 2004). Alternatively, validation could be achieved in captivity by repeat sampling blood and urine from the same individuals over several days to document the post-oestrus decrease in plasma

oestrogens and delayed decline in urinary oestrogen metabolites as previously done in Knox et al. (1992).

Another potential reason for non-correlation on the individual level could be that the assay antibody was dissimilarly sensitive to circulating total oestrogen and UEM in badgers (cross-reaction : oestradiol [17β -E2: 100%; 17 α -E2: 66%], oestrone [100%], and oestriol [1.5%]), causing low levels of detection in plasma (but not in urine), and was thus not capturing exact individual values, although still indicating relative seasonal proportionality (see Fig. 6). Consequently only conjugated oestradiol may be present in blood/ plasma, which this assay would not be able to detect (e.g., in African wild dogs, *Lycaon pictus* the active circulating oestradiol is conjugated oestradiol glucuronide: Monfort et al. 1997; similarly in African elephants, *Loxodonta africana*: Hodges et al. 1983; and Asian elephants, *Elephas maximus*: Czekala et al. 1992). Future studies could investigate and address this limitation by using assays with high cross-reactivity against heavily metabolised oestrogen (Graham et al. 2001; Mostl et al. 2005), or employ a biochemical approach, where the chemical structure of the plasma oestrogen metabolite is identified and a specific antibody (GnRH challenge) is raised against it (Hindle et al. 1992). Alternatively, the use of different hormones such as progestogens (Heistermann 2010) may give better results for monitoring female reproductive status.

A range of studies on badger urination behaviour (White et al. 1993; Hutchings and Harris 1999; 2001; Roper 2010; Buesching and Macdonald 2004) have found that badgers display consistent urinating patterns, allowing for potential development of non-invasive urinary sample collection. A similar approach has already been established in other wild species research (Kirkpatrick et al. 1988; Downs and Perrin 1991; Jordan et al. 2010). In the case of the European badger, with our method we are able to determine reproductive activity on a population level for both sexes, and to the individual level in males specifically. We conclude that validation of non-invasive measures can be done using a range of methods which may not necessarily require direct comparison with invasive measures, for example using known biological patterns/behaviours. Once the relationship between steroid hormone metabolite concentrations and reproductive events has been characterized for a species, non-invasive endocrine monitoring could then be used as a tool to enhance the understanding and management of the species in captivity and in the wild (Graham 2004).

Declaration of interests: None

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