Faecal cortisol metabolites as an indicator of adrenocortical activity in farmed silver foxes (*Vulpes vulpes*)

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Abstract

Measuring glucocorticoid metabolites in faeces has proven a useful, non-invasive method to monitor adrenocortical activity in several farm and wild species. Unlike plasma cortisol, whose sampling requires restraint and blood draws, faecal cortisol metabolites (FCM) may be particularly suitable for farmed silver foxes as these animals are sensitive to handling by humans. Prior to using FCM as a potential indicator of stress in silver foxes, however, a proper physiological and/or biological validation is required. Here, we determined FCM concentrations in 30 silver foxes (10 adult vixens, 10 juvenile females and 10 juvenile males) every alternate hour for 24 h after 1) an increase in cortisol induced by injection with synthetic ACTH (hereafter ACTH), and 2) a 2 min period of handling and restraint. Baseline FCM values, recorded every fourth hour for 24 h before the ACTH and handling treatments, served as controls. FCM values increased significantly following ACTH injection ($P=0.0001$) and handling ($P<0.0001$). The time to reach peak FCM concentrations after ACTH injection tended to differ between groups ($P=0.055$) averaging (± SE) 11.0 ± 1.04, 10.6 ± 1.30 and 7.8 ± 0.20 hours for vixens, juvenile females and juvenile males, respectively. After handling, peak FCM values were reached after 10.1 ± 0.55 hours with no significant differences between groups. Peak concentrations averaged 2143 ± 264 ng/g after the ACTH and 1008 ± 128 ng/g after handling, compared to 475 ± 48 ng/g for baseline levels. Peak FCM values tended to vary between individuals more in females than in males. Baseline FCM concentrations prior to handling were, unexpectedly, higher in more confident foxes ($P=0.004$), a finding perhaps indicating a potential preparative role of cortisol in silver foxes. There was also a negative trend between foxes' confidence and their times to reach peak FCM concentrations after handling ($P=0.062$), suggestive of a prolonged adrenocortical activation in more fearful individuals. Based on the rates that foxes produce faecal samples and the times to reach maximum FCM concentrations, we suggest a four hour delay to first faeces collection, before collecting samples every third hour the next 12 following hours to monitor elevations after an acute stressor. Our study confirms faecal cortisol metabolites as a valid indicator of adrenocortical activity in farmed silver foxes.

Keywords: silver fox, stress, validation, ACTH stimulation, handling, faecal glucocorticoid metabolites

1. Introduction

Silver foxes are black colour variants of the red fox (*Vulpes vulpes*) that are cage housed in outdoor barns for the commercial production of pelts. Fur production often attracts public debates centred on ethical concerns and claims that the animals' basic needs and welfare are not sufficiently maintained (Nimon and Broom, 2001; Norwegian Food Safety Authority, 2009). Scientific research on farmed fox behaviour and welfare has been conducted since 1946 (Pearson and Basset, 1946) focussing on several aspects of the housing environment (space, e.g. Korhonen et al., 2001; cage facilities, e.g. Jeppesen et al., 2000; human/animal relationship, e.g. Pedersen, 1994; social contact, e.g. Ahola et al., 2006), including methods for evaluating foxes' needs and motivations (e.g. Hovland et al., 2008; Koistinen et al., 2007). Parameters for assessing foxes' welfare state in different contexts include a variety of production and health related variables (e.g. litter size, growth rates [e.g. Bakken et al., 1994], immune status [Jeppesen and Pedersen, 1991]) together with several behavioural indicators (e.g. fearfulness, aggression, stereotypic behaviour [e.g. Ahola et al., 2000; 2006; Hovland and Bakken, 2010]) and physiological measures (e.g. stress-induced hyperthermia [Moe, 1996], adrenal size [Korhonen and Huuki, 2011], plasma cortisol [Moe and Bakken, 1996; Ahola et al., 2000]). Cortisol secretion has been a focus because glucocorticoids often increase during aversive conditions (e.g. Möstl and Palme, 2002). A disadvantage of assessing plasma cortisol, however, is that it requires repeated handling and immobilisation for blood collection, which stresses sensitive animals (Moe and Bakken, 1996), hence potentially interfering with the treatment effects. An alternative method that seems suitable for foxes is measuring faecal cortisol metabolites (FCM; Möstl and Palme, 2002; Palme, 2012). FCM reflect the glucocorticoid response over the previous few hours and are thus insensitive to very recent fluctuations caused by, for example, human approach (e.g. Palme, 2005). FCM can also be assessed without handling or direct contact, since in standard mesh-floored cages, droppings fall out of the cage for ready collection. FCM have previously been measured in farmed blue foxes (*Vulpes lagopus*), but the validity of the method was not assessed (Sanson et al., 2005). Prior to using FCM as a possible indicator of adrenocortical activity proper physiological and/or biological validation is crucial (Touma and Palme, 2005). A physiological validation is performed by inducing changes in circulating cortisol levels pharmacologically (typically by an ACTH challenge: Touma and Palme, 2005), and then assessing whether these are reflected in measured concentrations of FCM after a given time period. The delay between plasma and FCM peaks can also vary greatly between species (e.g., 4.2 h in mink [Malmkvist et al., 2011] and 22 ± 6 h in cats and 24 ± 4 h in dogs [Schatz and Palme, 2001]). Therefore, latency to reach peak FCM concentrations also needs to be empirically assessed as part of the validation. FCM can be measured by using enzyme immunoassays (EIA; Touma and Palme, 2005) or a radioimmunoassay (RIA; Young et al., 2004). Previously, cortisol immunoassays have proven useful for estimating FCM in dog faeces (Schatz and Palme, 2001) as well as in a variety of other carnivores (Young et al., 2004). Finally, a proper validation is also important because studies in several species have shown great individual variation and sex differences in both basal and ACTH-induced levels of faecal glucocorticoid metabolites (reviewed by Touma and Palme, 2005). Understanding how FCM excretion is affected by age, sex and individual identity is thus important when validating this approach in a new species. Lastly, it is also important to assess whether FCM actually change after a stressful experience, like, for instance, handling (e.g. physical restraint and immobilization [Bakken et al., 1999]) and whether variation in foxes' confidence towards humans affects FCM concentrations. The aim of our study was thus to evaluate the usefulness of FCM as an indicator of adrenocortical activity in farmed silver foxes through physiological and biological validations.

2. Materials and methods

2.1. Animals and housing

Subjects were thirty silver foxes (*Vulpes vulpes*) from a commercial Norwegian line born and reared in the research farm at the Norwegian University of Life Sciences (NMBU). The animals included 10 adult females (2 – 4 years old, 7.32 kg ± 0.43 kg), 10 juvenile females (5-6 months old, 6.74 kg ± 0.63 kg) and 10 juvenile males (5-6 months old, 7.73 kg ± 0.46 kg). They were housed in an outdoor barn providing natural light and temperatures and kept singly in standard plastic coated wire mesh cages (1.2 m x 0.76 m x 1.06 m) with a wooden nest box (with wire roof), a wire mesh shelf (0.25 m x 1.06 m) and a gnawing object (a wooden stick). The foxes had *ad libitum* access to standard food paste for fur animals and to automated water drinking nipples. The experimental animals were housed in a row with neighbouring foxes of same sex and age. The study was completed between September 12th and October 8th 2011 and was approved by the Norwegian Animal Research Authority (ID 3651).

2.2. Experimental procedure and collection of faeces

To habituate to their new cages and neighbours, all animals were placed in their experimental cages 16 days before the start of the experiment. For proper collection of faeces without urine contamination, a wire mesh (1x1 inch) was mounted beneath the cages. Throughout the habituation period, a person dressed in a white coat cleaned this wire mesh under the cages every second day to habituate the foxes to the sampling procedure. All faeces were collected with a plastic spatula. Any hair and wooden splinters were then removed, before storage in plastic bags at -20°C. In cases of diarrhoea, collecting a complete faecal sample was impossible, but this constituted less than 0.1 % of the samples. Before the treatments (handling; ACTH injection) baseline FCM were evaluated by sampling all dropped faeces every 4th h for 24 h. Following treatments all dropped faeces were collected every 2nd h for another 24 h to establish more precisely at what time FCM levels peaked. The handling and ACTH test were conducted 7 days apart. We tested the effect of handling on FCM concentrations before testing the effect of ACTH injection to avoid a possible carryover effect from sensitization from repeated handling and from ACTH injection itself. Sensitization could potentially increase animals' baseline FCM concentrations (the control values) concealing a possible, and more subtle, effect of handling. As confirming a significant increase in FCM concentrations after ACTH injection is a premise for assessing the effect of a biological stressor (handling), the results are presented paralleling this rationale and not according to experimental test order. A time line for the experimental procedures is given in Table 1. To assess the best faeces sampling frequency to detect peak FCM values we recorded the number of times we collected a faecal sample out of all sampling attempts during the different 24 h periods.

Table 1

Timeline for the experiment. Abbreviations: IN=animals placed in their experimental cages; HAB=habituation period; FS=faeces sampling; Handling=1 min handling and body weight recording; ACTH=ACTH-challenge test; BL=collection of faeces for measuring baseline FCM levels.

		HAB	**FS**	**FS**			**FS**	**FS**		
	IN		BL	Handling			BL	ACTH		Titbit test
Day	1	2-17	18	19	20	21 - 24	25	26	27	36 - 37

For the ACTH injection, each fox was captured and held with its front part inside the cage and injected intramuscularly with 1 ml Synacthen® (0.25 mg ml^{-1} tetracosactid, Defiante Pharmaceutica) in the upper thigh (*biceps femoris*) using a 2 ml syringe and 16 mm needle before being returned to the cage. The ACTH procedure lasted for approximately 1 min per fox and was completed for all subjects within 30 min (10:05-10:35 am). In the handling test, each fox was captured, taken out of the cage and then held for 1 minute. Subsequently, the fox was weighed before being returned to its cage. The Handling procedure lasted for approximately 2 min per fox and was completed for all subjects within 70 minutes (09:15-10:25 am). Three persons were present during both procedures. The animals were handled in consecutive housing order (adult vixens, juvenile males, and juvenile females) as this was the most efficient (time saving) way to handle the animals. This procedure was chosen to minimize the total handling time during both treatments. To assess the possibility that the latest handled animals (juvenile females) were more affected than the first ones (due to anticipatory stress [e.g. Sapolsky et al., 2000]), the effect of handling order within group (1 – 10) on latency to reach peak FCM concentrations was examined statistically.

2.3. Analysis of faecal cortisol metabolites (FCM)

The frozen faecal samples were thawed at 60°C in a drying cabinet for about 45 min and then homogenized inside the plastic bags. A 0.5 g portion of each sample was extracted with 5 ml of 80 % methanol by shaking with a hand vortex mixer for 1.5 - 2 minutes before centrifugation at 2500 *g* for 15 minutes (Palme et al., 2013). An aliquot of 0.5 ml of the supernatant was pipetted in 1.5 ml Eppendorf tubes that were placed in a heating block until the samples were dried up (2.5 – 4 hours). Dried down supernatants were sent to the Vetmeduni Vienna where they were redissolved in 0.5 80 % methanol and diluted (1:10) with assay buffer before EIA analysis. To determine the amounts of FCM the supernatants were first analysed with a cortisol enzyme immunoassay (EIA, Palme and Möstl, 1997). As this assay, and also an 11-oxoaetiocholanolone EIA described by Möstl et al. (2002), failed to produce expected FCM increases after ACTH injection and produced rather low values overall, we analysed the supernatant using a different EIA (5α-pregnane-3ß,11ß,21-triol-20-one EIA) as first described by Touma et al. (2003). The inter-assay coefficients of variation for a low and high concentration pool sample were 14.6 % and 10.3 %, respectively. The time to reach individual peak FCM concentrations after treatment was determined based on the highest FCM value measured after treatment. We estimated the minimum gut passage time to be about 3 h based on data from related fox species (4 h in Arctic fox [*Vulpes lagopus*] [Graae et al., 2004]; 2 h in Pampa fox [*Pseudalopex gymnocercus]* and 4 h in Crab-eating fox [*Cerdocyon thous*] [Varela and Bucher, 2006]; *pers. comm.* Øystein Ahlstrøm and Anders Skrede). Two animals had peak FCM values 0.5 and 2 h after treatment which is biologically unlikely based on the estimated minimum gut passage time; new peak values were therefore defined for these animals.

2.4. Assessment of confidence towards humans – the titbit test

As foxes' fear towards humans may reflect the nature and magnitude of their adrenocortical activity response during handling we examined whether confidence level, measured by a titbit test at the end of the experiment, affected their FCM concentrations before (baseline) and following handling. In the titbit test (Rekilä et al., 1997) foxes' tendency to accept a small food reward from the observer's hand is measured, reflecting its fearfulness towards the observer (Rekilä et al., 1997). During the test the observer stood in front of the cage offering a titbit (Frolic®, dog biscuit) through the wire-mesh wall. Both observers were dressed in a white plastic coat to resemble the white coat used during faeces collection. The test was performed on two separate but consecutive days, at the same time 2 h post feeding each day. The test duration was 30 sec and it was recorded whether the fox took the titbit or not. Just after finishing the first round a second round was completed. The average score based on a total of 4 tests was calculated for each

fox where 1 was the maximum score denoting that the fox accepted the tit bit every time it was offered. The relationship between confidence and the magnitude of the stress response (peak FCM concentrations) and its duration (time lag to reach peak FCM levels) were tested. Also, the relationship between confidence score and foxes' average and peak baseline FCM concentration was examined. More exploratory, fast responding individuals (sometimes described as having "proactive" behavioural strategies: Sih et al., 2004), typically have relatively low HPA axis reactivity (e.g. Koolhaas et al.,1997). We therefore predicted that less confident foxes would have higher baseline FCM levels; higher FCM concentrations after handling and shorter time to reach peak FCM concentrations.

2.5. Statistics

As the sampling interval differed following treatment (every 2nd hour) and baseline (every 4th hour), faecal samples were grouped into 4-hour intervals for statistical comparisons between baseline and treatment, in total six intervals labelled 'period'. When two samples from an animal were present within a certain time interval, the mean FCM concentration was calculated. Based on a Goodness-of-Fit Test the FCM variable did not fit the criterion for normal distribution. Therefore, the data were Box-Cox transformed so that the assumptions of normality were met. Treatment effects were examined separately for each treatment (ACTH injection vs. baseline and handling vs. baseline) and were tested with mixed models where 'treatment', 'period' (1-6) and 'group' (vixens, females and males) and all two-way interactions were included as fixed effects. 'Fox' nested in 'group' and the interaction with 'fox' and 'treatment' and 'fox' and 'period' were included as random effects. Average values for baseline FCM before treatment were calculated for each experimental animal based on the 6 sampling periods. For between-group comparisons of peak FCM concentrations, latency to reach peak FCM after treatment and confidence score were analysed with one-way ANOVA. As animals were handled in consecutive housing order (vixens, juvenile males and juvenile females) during both treatments we included 'handling order' (values from 1 to 10) to test for a possible effect of sensitisation on time to reach peak FCM concentrations. The ANOVA model included 'group' and 'handling order' and their interaction. Within group differences in peak FCM levels between the ACTH test and the handling test were examined with paired Student's *t*-tests for matched samples. The effect of foxes' confidence towards humans on their FCM values was tested with a model including 'group' and 'confidence score' (as a continuous variable) and their interaction. The coefficient of variation (SD/mean) was also calculated for the variables 'time to reach peak FCM concentration' and for 'peak FCM concentration' for both treatments within group, in order to summarise inter-individual variability. Mean ± SE values are given. JMP® 13.0 was used for all statistical analyses.

3. Results

3.1. The ACTH challenge test

FCM concentrations before (baseline) and after ACTH injection within groups are given in Fig. 1. There were significant effects of treatment ($F_{1,28.9}$=19.8, P=0.0001), period ($F_{5,123.6}$=26.4, P<0.0001), the period*treatment interaction ($F_{1,115.3}$=6.6, P<0.0001) and the group*period interaction ($F_{10,119.5}$=2.9, P=0.003). The results showed that the FCM levels were significantly increased compared to baseline levels, particularly during period 3 to 5. No significant effects were found for group ($F_{2,27.6}$=1.3, P=0.280) or for the group*treatment interaction ($F_{2,26.6}$=0.63, P=0.542). The time to reach peak FCM values after ACTH injection tended to differ between groups (F_2=3.2, P=0.055) and was 11.0 ± 1.04 h for adult vixens, 10.6 ± 1.30 h for juvenile females and 7.8 ± 0.20 h for juvenile males. The peak FCM concentrations did not differ significantly between groups (F_2=0.54, P=0.587) and averaged 2143 ± 264 ng/g. Twenty-eight of the foxes (93.3 %) reached the peak FCM concentration 6-14 h after handling. Coefficients of variation for the peak FCM concentrations after ACTH injection was 90.3 % for adult vixens, 68.2 % for juvenile females and

51.7 % for males. For the time to reach peak FCM concentration coefficient of variation was 30.0 % for adult vixens, 38.8 % for juvenile females and 8.1 % for males. No significant effect of handling order on time to reach peak FCM following ACTH injection was found (F_1=0.67, P=0.422).

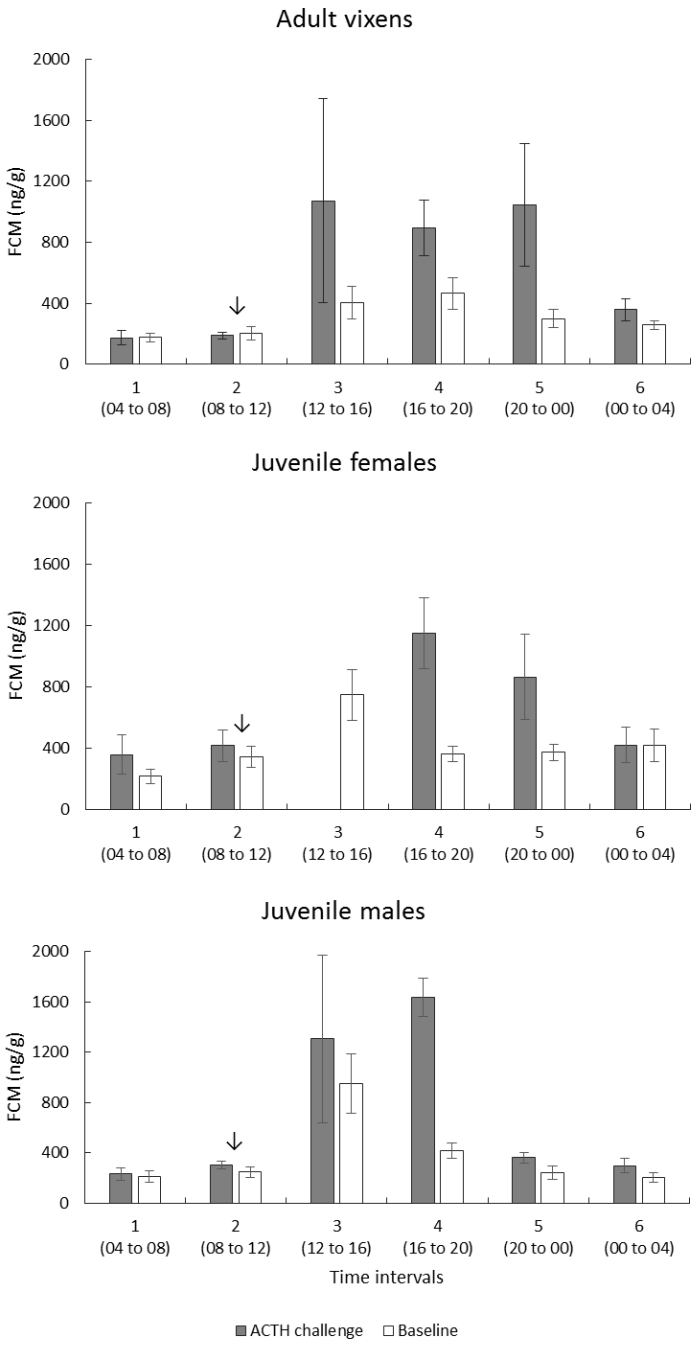

Fig. 1. Concentrations (mean ± SE) of faecal cortisol metabolites (FCM) in adult vixens, juvenile females and juvenile males following ACTH injection (gray bars) compared to prior baseline concentrations (white bars). The arrow signifies the time of the ACTH injection that took place between 10:05 and 10:35 am.

3.2. The handling test

FCM concentrations before (baseline) and after handling within groups are given in Fig. 2. There were significant effects of treatment ($F_{1,54.8}$=38.0, P<0.0001), period ($F_{5,118.1}$=21.2, P<0.0001), the period*treatment interaction ($F_{5,115.5}$=4.1, P=0.002) and the group*period interaction ($F_{10,118.9}$=2.2, P=0.020). FCM concentrations increased following the treatment, particularly during periods 3 to 5. No significant effects were found for group ($F_{2,29.4}$=0.59, P=0.558) or for the interaction group*treatment ($F_{2,25.4}$=0.34, P=0.717). The time to reach peak FCM values after handling was not significantly different between groups (overall mean 10.1 ± 0.55 h; F_2=1.7, P=0.202). Peak FCM concentrations after handling did not differ significantly between groups (F_2=2.0, P=0.161) and was on average 1008 ± 128 ng/g. This was significantly lower (approximately 53 % reduction) than the FCM concentrations reached after the ACTH challenge test (paired T-test; T_{29}=5.1, P<0.0001). There was no significant effect of group on the highest baseline FCM concentration (475 ± 48 ng/g; F_2=1.9, P=0.173), a value that was about half of the peak FCM concentration following handling. Twenty-five of the foxes (83.3 %) reached the peak FCM concentration within the period of 6-14 h after handling. Coefficients of variation for the peak FCM concentrations after handling was 105.0 % for adult vixens, 66.2 % for juvenile females and 36.6 % for males. For the time to reach peak FCM concentration coefficient of variation was 25.2 % for adult vixens, 32.8 % for juvenile females and 26.7 % for males. No significant effect of handling order on time to reach peak FCM after handling was found (F_1=0.15, P=0.706).

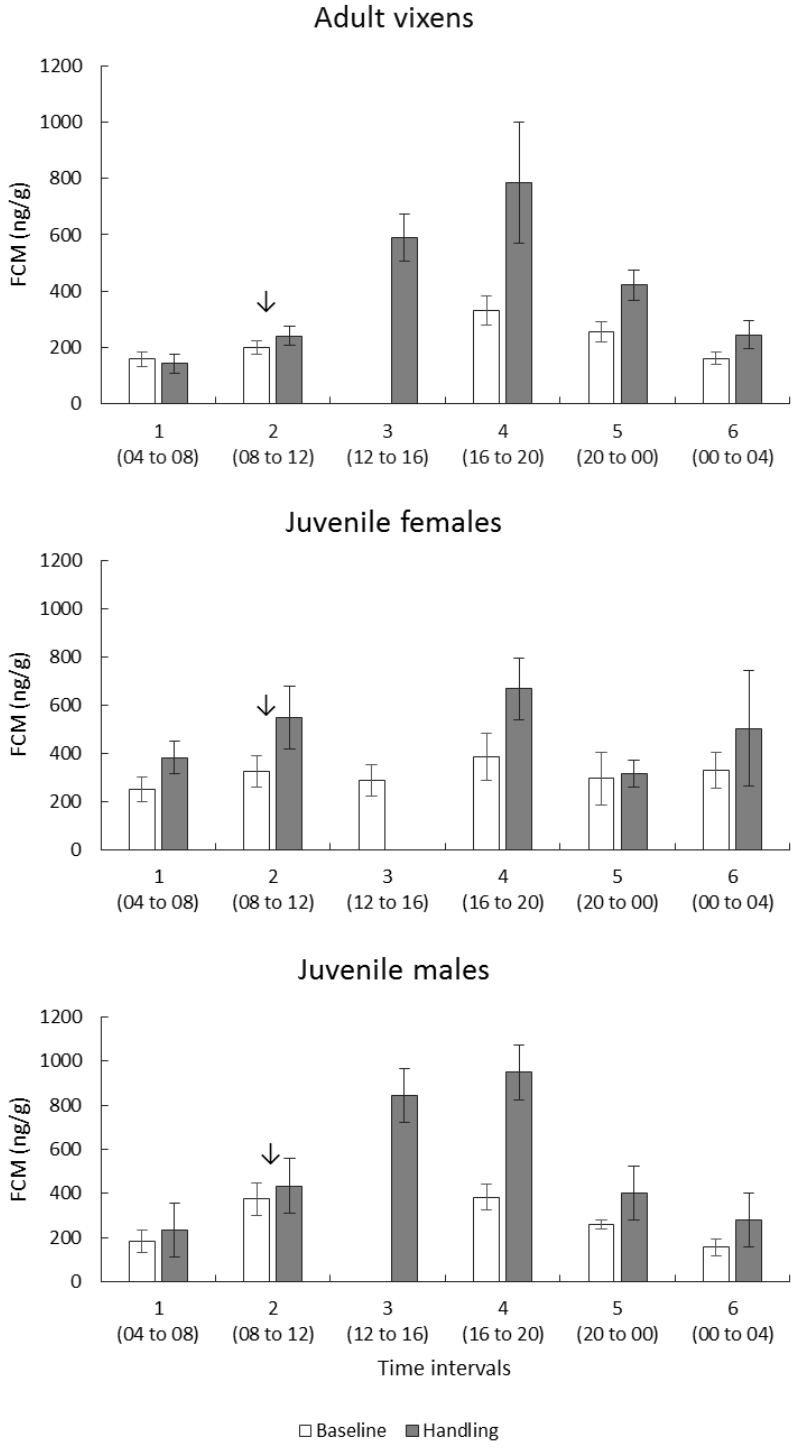

Fig. 2. Concentrations (mean ± SE) of faecal cortisol metabolites (FCM) in adult vixens, juvenile females and juvenile males following 1 min handling and immobilisation (gray bars) compared to prior baseline concentrations (white bars). The arrow signifies the time for the handling event that took place between 09:15–10:25 am.

3.3. The relationship between confidence scores and FCM concentrations before and after handling

There were no significant differences in confidence score between the groups (F_2=0.84, P=0.444) with an average score of 0.4 ± 0.07. Average baseline FCM values before handling were significantly related to confidence score, where higher confidence scores predicted higher levels of baseline FCM (F_1=10.2, P=0.004). After handling, there was no significant effect of confidence score ($F_{1,24}$=1.54, P=0.226) or the confidence score*group interaction ($F_{2,24}$=0.17, P=0.890) on peak FCM concentration. However, times to reach peak FCM concentration tended to negatively covary with confidence score ($F_{1,24}$=3.82, P=0.062).

3.4. Optimal faeces sampling interval - the frequency of defecations

The number of faeces samples collected during the two 24 h baseline periods, after handling and following ACTH injection, is given in Table 2.

Table 2

The number (mean ± SE) of faecal samples collected during the two 24 h baseline periods, following ACTH injection and after handling. The percentage of times faeces were present out of all possible sampling intervals (N=12) is given in brackets.

Sampling frequency	Adult vixens	Juvenile females	Juvenile males
Baseline every 4th h	9.8 ± 0.33 (82 %)	10.9 ± 0.23 (91 %)	10.3 ± 0.26 (86 %)
ACTH every 2nd h	6.8 ± 0.55 (57 %)	7.3 ± 0.43 (61 %)	7.3 ± 0.30 (61 %)
Handling every 2nd h	5.6 ± 0.45 (47 %)	6.0 ± 0.56 (50 %)	6.8 ± 0.42 (57 %)

4. Discussion

The aim of this study was to evaluate the usefulness of FCM as an indicator of adrenocortical activity in silver foxes, with the ultimate aim of using FCM in future welfare assessment studies. We tested whether increasing levels of circulating cortisol, brought about by an ACTH injection and a stressful experience like handling and immobilisation were well reflected in levels of faecal cortisol metabolites (FCM). For comparison, baseline FCM levels were measured in faecal samples from the unstressed individuals taken 24 h prior to the experimental treatment.

The results showed that both the ACTH challenge test and the handling procedure increased FCM concentration compared to baseline levels in adult vixens, juvenile females and juvenile males. This finding confirms FCM as a valid parameter for measuring adrenocortical activity in farmed silver foxes. Glucocorticoid metabolism can be affected by various factors like e.g. individual differences, sex and age (Palme, 2005; 2012). In our study, there was no clear difference in the magnitude of peak FCM concentrations between the different groups, but the time to reach peak values after ACTH challenge tended to differ. Juvenile males reached maximum concentrations about 2.5-3 h earlier than adult vixens and juvenile females. The time lag to reach peak concentrations after handling and restraint did not differ significantly between the groups and was on average 10.1 h. We noticed that variation in defecation pattern was most pronounced during period 3, particularly for the baseline sampling period prior to and after the handling treatment, as only a few fecal samples were collected in this period. Most likely, this was related to a temporary and random variation in defecation pattern. Individual variation in peak FCM values was overall, less pronounced in males than females. That males showed less inter-subject variation in excreted glucocortiod metabolites parallels results found in a FCM validation study on rats by Lepschy et al. (2007),

and suggests that using within-subject designs and/or large sample sizes should be a more important consideration in future research using female foxes than males.

Peak FCM concentrations after ACTH injection were twice the levels after handling, which again was about double the highest baseline FCM concentration measured during the 24 h before handling. These values illustrate that the ACTH challenge induces a strong activation of the adrenocortical system while the experience of handling and 1-minute restraint elicit an intermediate response. Handling has previously been shown to activate the HPA axis in silver foxes, wherein plasma cortisol levels following repeated handling, at 5-min intervals, doubled after 1 h compared to initial levels (Moe and Bakken, 1997). Interestingly, and against our predictions, the foxes' confidence scores showed a negative relationship with the time to reach peak FCM values after handling. This might suggest that foxes that were more fearful had a prolonged adrenocortical activation after handling compared to individuals that were more confident. Our analysis also showed that average baseline FCM levels positively correlated with confidence scores. Thus, surprisingly, foxes that were more confident had higher baseline FCM values. Based on data from many other species, we had anticipated the opposite pattern (cf. e.g. Sih et al., 2004, Koolhaas et al., 1997), particularly since previous studies have shown that domesticated (and less fearful) foxes have lowered plasma cortisol (e.g. Gulevich et al., 2004). Applying the concepts of active and passive coping styles as an alternative explanatory basis for the link between FCM and confidence is also relevant. Here, a likely term for the behavioural style of confident foxes (that actively approached the observer) would be proactive, whereas the style best fitting the unresponsive and more fearful foxes would be reactive. However, as a proactive copying style is linked to increased activation of the sympathetic-adrenal-medullary axis followed by an elevated catecholamine secretion rather than increased glucocorticoid secretion (e.g. Koolhaas et al., 1999) this rationale is still in contrast to our findings showing elevated baseline FCM in the confident and more proactive foxes. Sapolsky et al. (2000) discuss the concept of behavioural preparedness as a part of a more proactive strategy, suggesting that high baseline cortisol levels could reflect an individual's preparedness to respond efficiently to a future stressor and that glucocorticoid release may have a 'preparative' function, 'adapting the organism for responding to the next stressor' (Sapolsky et al., 2000). Whether cortisol may have a preparative function in relation to coping with various stressors, like e.g. handling in silver foxes, is a potential topic for future investigation. Anyway, our finding somewhat parallels data from a study of kennel housed dogs where fast learning individuals (termed more proactive dogs by the authors), showed lower levels of fearful behavior but elevated cortisol/creatinine concentrations (Blackwell et al., 2010).

Our data now add to several prior studies showing the usefulness of FCM as an indicator of adrenocortical activation and compromised welfare in carnivore species, including e.g. farmed mink (*Mustela vison*) (Malmkvist et al., 2011; Diez-Leon et al., 2013), cats and dogs (Schatz and Palme, 2001) and wolves (*Canis lupus*) (Molnar et al., 2015). However, only a few studies had focused on FCM as a potential non-invasive method for measuring stress in fox species. In farmed blue foxes (*Vulpes lagopus*), Sanson et al. (2005) measured FCM concentrations while monitoring vixens' hormonal status during pregnancy and parturition, and found that FCM concentrations doubled around whelping compared to values 2 days prior to and 3 days after delivery. As part of a FCM validation study in crab-eating foxes, Paz et al. (2014) found a 10-45 fold increase in FCMs after ACTH injections compared to baseline values. Later, the authors recorded FCM to examine welfare related effects of rehousing and reproduction in these foxes (Paz et al., 2015). Our data are the first to look for similar effects in silver foxes, and will now be used in fox welfare research.

Young et al. (2004) quantified faecal glucocorticoid metabolites in different carnivores with EIA and RIA procedures, and found both techniques suitable. Also Vasconcellos et al. (2011) confirmed both a cortisol EIA and RIA as suited to measure FCM in maned wolves (*Chrysocyon brachyurus*). Interestingly, we did not find a cortisol EIA to be suited for measuring FCM in silver foxes, as values were low, and an ACTH increase was missing. Instead, a 5αa-pregnane-3ß, 11ß,21-triol-20-one EIA (Touma et al., 2003) produced

expected increases after ACTH injection. This again highlights the need to validate an assay for each new species under investigation (Palme, 2012).

As 24 h faeces sampling is laborious and repeated visits represent some level of disturbance to the animals, finding an optimal sampling interval that is short enough to ensure that peak concentrations can be detected, but also long enough to reduce the frequency of 'empty' visits, would be useful. Preferentially, faeces should be collected as soon as possible after defecation, as bacterial degeneration of metabolites will escalate by both increasing time and temperature (Palme 2005; 2012). Our data showed a 'hits percent' (the number of times we collected a faecal sample out of all sampling attempts) of approximately 51 % after handling and 60 % after ACTH injection where sampling interval was 2 h. For the baseline samplings before treatment with 4 h sampling intervals the 'hits percent' was 82-91 %. Thus, we suggest that a sampling interval of 3 h would be advisable to reduce the number of empty visits but sufficient to detect peak concentrations after treatment. The time to reach peak FCM concentrations was on average 10.1 h after handling and varied between 7.8 to 11 h after ACTH injection, where about 83 % and 93 % of the foxes reached a peak within 6-14 h, respectively. Based on this, a delay of 4 h for the first sampling after an acute, biological stressor and then every 3rd h for 12 h would likely be sufficient to obtain most of the foxes' peak FCM concentrations.

Our findings of a positive correlation between confidence score in the titbit-test and baseline peak FCM concentrations raises two potential inquiries for future studies: 1) How do glucocorticoids affect behavioural stress responses? and 2) What kind of motivations underlie foxes' approach and acceptance of the food reward in the titbit test? The first issue is related to the potential preparative function of glucocorticoids and the second question to the differing physiological impacts of activating opposite motivational systems, as in principle, both reduced fear/increased confidence and increased boldness and/or aggressiveness could motivate such a response.

5. Conclusion

Our study demonstrates faecal cortisol metabolites as a valid parameter of adrenocortical activity in silver foxes. Their measurement can therefore be applied as a non-invasive method to evaluate stress. The magnitude of FCM concentrations and the time to reach peak values tended to vary more among adult vixens and juvenile females compared to that of juvenile males. Based on our findings, we suggest a delay of 4 h before the first sample is collected after a stressful event and then a sampling interval of three hours for the following 12 h.

Conflict of interest statement

The authors declare that there is no actual or potential conflict of interest.

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