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ORIGINAL PAPER

Non-invasive monitoring of adrenocortical activity in free-ranging fallow deer (*Dama dama* L.)

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Abstract Measurement of faecal glucocorticoid metabolites is increasingly used as a non-invasive tool to examine disturbances in various domestic and wild animals. Because measurements of faecal glucocorticoid metabolites has previously never been reported in fallow deer, we determined 11,17-dioxoandrostanes (11,17-DOA), a group of cortisol metabolites, in the faeces of four fallow deer yearlings after an adrenocorticotropic hormone (ACTH) challenge or control saline injection by an 11-oxoaetiocholanolone enzyme immunoassay (EIA), to validate a method. A 2.9- to 4.3-fold increase in measured cortisol metabolites in challenged animals after approximately 22 h demonstrated the suitability of this group-specific EIA to monitor adrenocortical activity in respective deer species. To determine faecal cortisol metabolites in fallow deer from a Mediterranean habitat, we collected samples during a 1-year study at Veliki Brijuni Island. The study confirmed seasonal pattern of cortisol release in fallow deer. Higher 11,17-DOA concentrations (median; min–max) were determined for November (99; 50–2,035), March (112; 25–315) and May (92; 40–196 ng/g faeces). Significantly lower concentrations were measured during July (30; 10–195 ng/g faeces). This study indicates that the analysis of faecal glucocorticoid metabolites is a valuable non-invasive technique for monitoring adrenocortical activity in fallow deer. This, together with information about the seasonal pattern of glucocorticoid excretion, could help to improve fallow deer management and welfare, especially in the case of farmed and park animals.

Keywords Fallow deer · Non-invasive · Stress · Enzyme immunoassay · Cortisol · Season

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Introduction

Animals are equipped to cope more or less efficiently with the constant changes in their dynamic environment through diverse behavioural and physiological reactions (Romero 2004). The modern wildlife and habitat management impose additional adverse situations to wildlife that range from simple harassment through the collection of forest products or recreational activities (Thiel et al. 2008) up to the real threats like hunting, habitat destruction and

fragmentation (Fahrig 1997; Huxel and Hastings 1999). Within these adverse situations, adrenal glands are the main organ involved that reacts via two axes, namely the sympatho-adreno-medullar and the hypothalamo-pituitary-adrenocortical (HPA) one. The activation of the HPA axis results in the release of glucocorticoids (cortisol or corticosterone, depending upon the species in question; Möstl and Palme 2002). These facts enabled long-term usage of glucocorticoids in stress evaluation. In contrast to a quantification of plasma glucocorticoids, where sampling by itself causes stress especially in wild animals, disturbing the final outcomes of the analysis, a non-invasive method using the indirect measurement via the glucocorticoid metabolites in the faeces of various species including deer has been launched during the late 1990s (Palme and Möstl 1997; Wasser et al. 2000; Dehnhardt et al. 2001; Millspaugh et al. 2001 and 2002; Creel et al. 2002; Huber et al. 2003a,b; Touma and Palme 2005; Taillon and Côté 2008; Christofoletti et al. 2010). They were developed on the basis of the research on glucocorticoid metabolism and excretion provided by Lindner (1972) and later by Palme et al. (1996, 2005). As pronounced species differences were found regarding the formed faecal glucocorticoid metabolites, a physiological validation of the methods used for their quantification is mandatory (Palme 2005; Touma and Palme 2005).

In this study, we present a validation of a method for measuring faecal cortisol metabolites (FCM) in fallow deer. In addition, adrenocortical activity in free-ranging fallow deer was evaluated during a 1-year study at the Brijuni National Park, Croatia.

Materials and methods

Method validation

We performed an adrenocorticotropic hormone (ACTH) challenge test in animals bred at the fallow deer farm Višnjica (eastern part of the Croatia, near the town of Slatina). Animals (four male yearlings, approximately 50 kg of body weight each) were part of a large herd consisting of approximately 200 individuals and could move freely between paddocks, yards and handling facility, on a total area of 30 ha. They were confined in separate paddocks (5×5 m) with solid wooden sides, which were placed within the handling facility, to prevent them from further visual stressors. They were acclimated for 2 days to their new environment. Three animals received $ACTH_{1\text{-}24}$ intramuscularly (approximately 0.30 mg/per animal (approximately 0.6 IU/kg of body weight); Sigma-Aldrich Co., Germany) diluted to a volume of 1.5 ml with saline. The remaining fourth yearling served as a control animal and received just 1.5 ml of saline. During the administration, animals were physically restrained. Faecal samples were collected 6, 12, 18 and 22 h after the injection. During the sampling procedure, animals were moved from one paddock to another by means of the sliding gates in order to minimise human induced disturbance. Immediately after sampling, faeces were frozen at −20°C in separate plastic bags, and the time of collection and individual number was noted. After the experiment was done, all animals were released. The experiment was conducted with the permission of the respective national authority (Kl.: 640-01/07-17/6; Ur. Br.: 61-01/139-07-10).

1-year monitoring and enzyme immunoassay

To evaluate the influence of the season on FCM, we collected fallow deer faecal samples (n=134) on the island of Veliki Brijun during March (n=30), May (n=42), July (n=30) and November (n=32). With this distribution of sample collections, following seasons were covered: spring, late spring, summer and winter. With respect on studies performed in other deer species, this sampling pattern gave us the possibility to compare the periods were higher (spring and winter) and lower glucocorticoid release (late spring and summer) were expected. Furthermore, this sampling distribution included two periods with low or no touristic activity and two periods with peak human activity (touristic season at Brijuni islands starts at May and ends at the beginning of October). Brijuni islands (a group of 14 islands of different size) are categorised as a National Park, placed alongside the north Adriatic coast. The climate of the Brijuni islands is a typical Mediterranean, representing the original life conditions for fallow deer. However, fallow deer are not the original members of the Brijuni fauna, but were introduced by humans between the years 1902 and 1908, along with the axis deer (*Axis axis*) and the European mouflon (*Ovis aries musimon*). As sex differences in FCM were not observed in other deer species (Bubenik et al. 1998; Huber et al. 2003a), and due to the open habitat, samples were collected on an anonymous base. Fallow deer were allowed to move freely along the island, and were offered supplemental feed only during the winter season. During the sampling, animals were monitored from the distance, and defecation was noted on a separate map of the observed area. After the herd moved to another grazing area, the samples were collected in accordance to the aforementioned map, labelled and immediately placed into an ice chest while on the field and later frozen at -20°C. Only the fresh samples, based on their external appearance (Huber et al. 2003b), were considered adequate and collected. This protocol was followed since bacterial activity in faeces may be a cause of further FCM degradation (Touma and Palme 2005; Lexen et al. 2008).

Furthermore, samples were not collected during rain periods as rainfalls were reported to decrease levels of metabolites in exposed faeces (Rehnus et al. 2009). After thawing, samples were homogenised according to the standard protocol (Palme 2005). In brief, we extracted 0.5 g of faeces with 5 ml of 80% methanol. After 30 min shaking on a multivortex and centrifugation (2.500*g*, 15 min), we determined the amounts of cortisol metabolites (11,17-dioxoandrostanes) in the supernatant by a group specific 11-oxoaetiocholanolone EIA. Details of the EIA used, including cross-reactions, are described by Palme and Möstl (1997). Interassay coefficients of variation were 10.4% and 11.9% for a low and high concentration pool, respectively. Obtained results were evaluated using SigmaStat 3.1 (Systat Software, Erkrath, Germany). As 11,17-DOA values obtained during March and June were not normally distributed (Lilliefors test), different months were compared using a one-way ANOVA on ranks test, followed by a Dunn's test to isolate the groups that differ significantly.

Results

Results of the physiological validation of the EIA used for measurement of FCM in the fallow deer are presented in Fig. 1. Concentrations of 11,17-DOA in the samples collected after 6 h, which, on the basis of the literature (for example see Millspaugh et al. 2002; Huber et al. 2003b), were regarded still baseline, ranged from 88 to 262 ng/g faeces in the studied animals. Highest concentrations (representing a 2.9- to 4.3-fold increase) were found 18 h (animal no. 1) or 22 h (other two animals) after the ACTH challenge. The values in the control animal showed only a small increase (144%; Fig. 2).

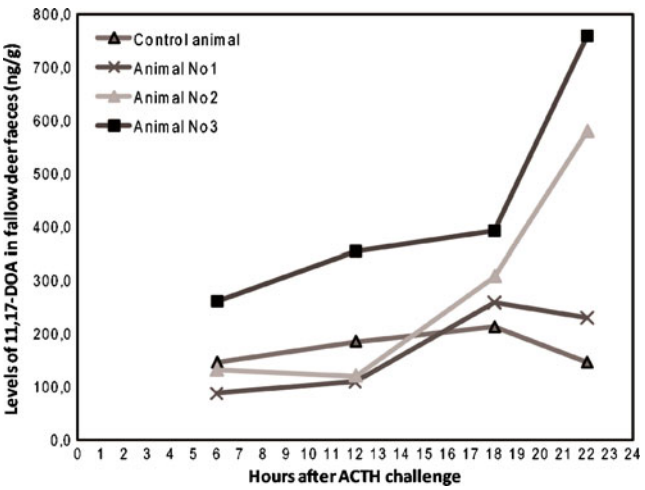

Fig. 1 Comparison of 11,17-DOA levels between ACTH challenged (*cross*: animal no. 1; *full triangle*: animal no. 2; *square*: animal no. 3) and control, saline-injected (*open triangle*) fallow deer yearlings

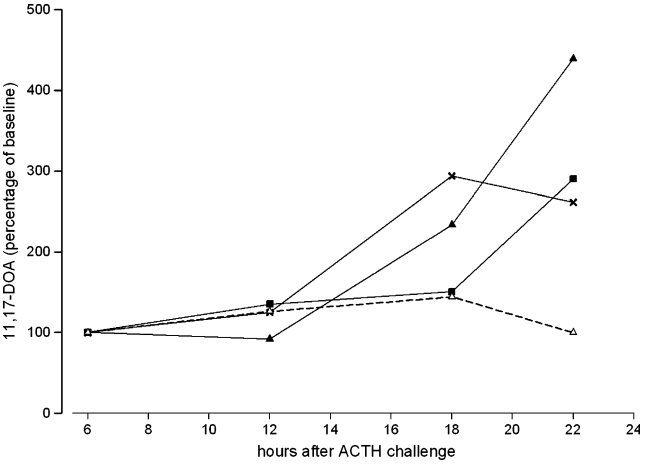

Fig. 2 Comparison of 11,17-DOA levels between ACTH challenged (*cross*: animal no. 1; *full triangle*: animal no. 2; *square*: animal no. 3) and control, saline-injected (*open triangle*) fallow deer yearlings, expressed in percentages

The results of the 1-year study at Veliki Brijuni Island are given in Fig. 3. Higher 11,17-DOA concentrations (median; min–max) were determined for November (99; 50–2,035), March (112; 25–315) and May (92; 40–196 ng/g faeces). Significantly lower ($p<0.05$) concentrations were determined during July (30; 10–195 ng/g faeces).

Discussion

Measurement of faecal glucocorticoid metabolites has been successfully validated and applied in an increasing number of ruminants, including several deer species (Palme and

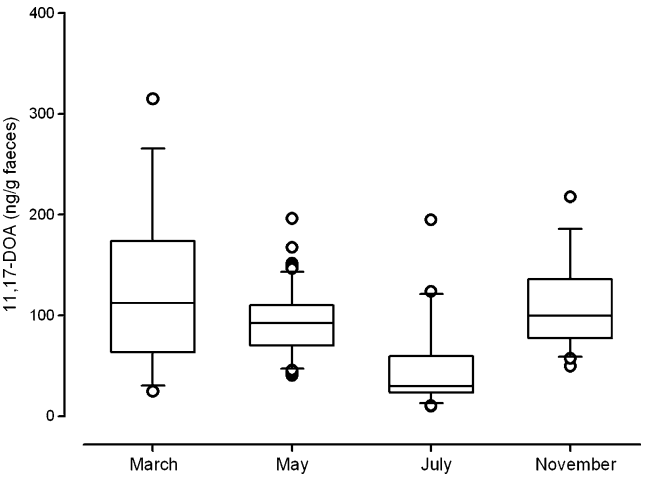

Fig. 3 *Boxplots* of concentrations (nanogrammes per gramme faeces) of faecal cortisol metabolites (11,17–DOA) in fallow deer during different seasons of the year. Only values in July differed significantly ($p<0.05$) from the others. Data are given as *box-whisker plots* showing medians (*lines in the boxes*), 25% and 75% quartiles (*boxes*), 10% and 90% ranges (*whiskers*) and outliners (*dots*). March represents spring, May–late spring, July–summer and November–winter period

Möstl 1997; Palme et al. 1999; Wasser et al. 2000; Dehnhardt et al. 2001; Millspaugh et al. 2001, 2002; Creel et al. 2002; Huber et al. 2003a,b; Pesenhofer et al. 2006; Taillon and Côté 2008; Christofoletti et al. 2010). However, it has, to our knowledge, never been reported in fallow deer, so far. As species differences regarding the metabolism and excretion of glucocorticoids exist (Palme et al. 2005), it is necessary to physiologically validate such non-invasive methods for each species (Palme 2005; Touma and Palme 2005). Therefore, we performed a small validation experiment in fallow deer first. In order to avoid further disturbances of the animals used in the experiment, we started the collection 6 h after the ACTH injection and ended the experiment after 22 h. Based on the literature, we expected still baseline values in our first samples (for example see Millspaugh et al. 2002; Huber et al. 2003b). Highest concentrations 18 or 22 h after the challenge test are in accordance with reported delay times of peak FCM values in red deer ranging between 18 to 22 h (Huber et al. 2003b; Wasser et al. 2000). In contrast, in white-tailed deer, a larger variation (10 to 24 h) was reported (Millspaugh et al. 2002), and in brown brocket deer, they were somewhat longer (24 to 28 h; Christofoletti et al. 2010). Although we found expressed increases in ACTH-treated animals, the injection itself did only cause a small increase, suggesting that this procedure caused an only minor stress response. However, caution is advised with this interpretation, as we used only a single animal and large inter-individual variation in such reactions has been observed in red deer (Huber et al. 2003b). The most frequently given explanation for observed variations in baseline and response FCM values is in individual differences. They were found in almost all studies performed (Palme et al. 1999; Touma and Palme 2005) and were expected in this one. Taken together, the ACTH challenge test proved the physiological relevance of an FCM analysis to monitor adrenocortical activity in fallow deer.

Our second experiment revealed seasonal changes in FCM excretion in fallow deer. Similar pattern of adrenocortical activity were observed in other deer species that originate from temperate climate (Bubenik et al. 1983; Huber et al. 2003a). It is believed that this change in adrenal activity represents an adaptation to the environment when relatively low sources of natural feed are available. The increased production of the glucocorticoids in that time is associated with the shift from an anabolic to a catabolic state, engaged to satisfy energetic demands from own reserves rather than from feedstuffs. This statement is further confirmed by Pereira et al. (2006) who found elevated levels of FCM in Pampas' deer stags during the winter in Brazil. In the case of Brazilian climate, the most important difference between winter and summer period is not that much in temperature but in average precipitation. The dry winter season imposes deer to activate catabolism rather than to rely on natural feed. Large variations in FCM values can be attributed to individual differences, hierarchy, perception of the external stimuli, etc. In addition, we did not find differences in glucocorticoid production between the touristic and non-touristic season of the year. Even more, decreasing FCM values in May and lowest observed values in July (both in tourist season) exclude any significant influence of the human presence or vehicles as stressors on the Brijuni islands. However, it is important to mention that the park area is large enough to permit avoidance of the adverse situations and that vehicles of local services, electro-mobiles and bikes use asphalt roads and thus predictable ways of moving. This is a prerequisite for animals to adapt to such disturbances. Thus, the results obtained in this study represent baseline reference values of cortisol metabolites in fallow deer from an open Mediterranean habitat.

Besides pulsatile, diurnal and seasonal rhythms the release of glucocorticoids depends upon numerous factors (Mormède et al. 2007). Although caution is advised since glucocorticoids are not sensitive to every type of stressor, they are measured as a parameter for evaluating stress (Möstl and Palme 2002). Knowing the baseline values of FCM for a specific population could help us to separate the influence of regular daily activities (like feeding, exploring the habitat, courtship, mating, etc.) from those of other origin (mainly imposed by humans) on glucocorticoid levels. Despite some known disadvantages, glucocorticoid levels could, especially if combined with other parameters (e.g., reproductive success or life expectancy; Müller et al. 2010), provide answers on the aversiveness of certain events in the animal's environment (Mormède et al. 2007).

As suggested previously (Huber et al. 2003b), measurement of faecal glucocorticoid metabolites can provide at least three advantages if compared with other methods. These are: the non-invasive sampling method, an integrated measure of circulating cortisol concentration in blood (high cortisol level in plasma last no longer than 90 min) and the fact that it enables long-term longitudinal studies with examination even on a retrospective basis. Beside the severe stressors associated with capture or hunting, many of the stressors that can be found commonly in farmed or park deer are less severe, although, if persisting for longer time, their effects are equally as important (Griffin and Thomson 1998). Among them, both physical and psychological stressors, like transport, physical restraint, velvetting, overcrowding, poor nutrition, extremes in climate, infection, separation at weaning, establishment of hierarchies, breeding, touristic and recreational activities, whose effects are less noticeable, reduce the deer welfare. From the management point of view, it is important to detect not only the extreme but also the less severe stressors in order

to avoid or minimise its effect on animals. Understanding of seasonal pattern of glucocorticoid excretion and its comparison with metabolite levels after human induced disturbances (e.g., management techniques, handling, transportation, tourism, etc.) could provide answers on the level of stress experienced by the animals. Thus, measuring FCM could help to improve animal welfare during husbandry and wildlife management. This is of particular importance for farmed and park fallow deer.

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