Wildlife management factors and their effects on hair cortisol levels and glucocorticoid and mineralocorticoid levels in the hippocampus of fallow deer (*Dama dama)* as chronic stress and welfare indicators

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Abstract

A positive state of welfare for all animals including wild animals is becoming a more important field of interest for people. Welfare can be seen as a dynamic concept. According to this concept an animal has to be able to adapt itself to its surrounding and reach a state that it perceives as positive in order to be in a positive welfare state. Finding an adequate way to measure welfare in animals can be helpful in discussions concerning the human role in wildlife welfare. An adequate way to measure welfare might be by measuring chronic stress indicators. In this study we measured chronic stress in fallow deer by measuring hair cortisol levels and glucocorticoid receptor and mineralocorticoid receptor levels in the hippocampus. These results were combined with information on park management factors to determine which park management factors contributed to chronic stress and therefore impaired welfare. Fallow deer from eleven different parks in the UK and from the Amsterdamse Waterleidingduinen in the Netherlands have been used in this study. Population density has been identified as an important factor with an effect on chronic stress in fallow deer. These findings are more pronounced considering hair cortisol levels than glucocorticoid receptor levels. Based on these findings we may carefully suggest that management factors in nature parks should consider population density to reduce chronic stress and thereby improving the welfare status of fallow deer.

1. Introduction

Animal welfare is becoming a more important field of interest for people. We want our own animals as well as wild living animals to be in a positive state of welfare. Humans affect wildlife welfare by restriction of the living space of wild roaming animals by building roads and fences and removing forest area. Besides restriction humans also interfere with the living area of wild animals by visiting their living area leading to disturbance. Considering their interference with wildlife humans have a duty to care for wild animals in that they should prevent unnecessary suffering which may be consequential to human acts [1]. The growing attention for wildlife welfare also leads to large public and political discussions [2]. But what exactly is welfare? The concept of animal welfare has changed over time. Initially an animal was considered to be in a positive state of welfare when it had the ability to live according to the five freedoms of Brambell which were:

- An animal has to be free of hunger and thirst.
- An animal must be free of discomfort.
- An animal must be free of pain, injury and disease.
- An animal must be free of stress and anxiety.
- An animal has to be free to express normal behaviour.

This early concept of animal welfare was based on the exclusion of negative states. Later, this idea was adjusted to the dynamic concept of welfare. According to this concept, the animal has to be able to adapt itself to its surroundings and reach a state that it perceives as positive, for the animal to be in a positive welfare state [2,3].
Measuring welfare according to this concept is difficult. Finding an adequate way to measure animal welfare can be very helpful in the assessment of and discussions concerning wildlife welfare. Measuring chronic stress may be an interesting welfare indicator since the animals ability to adapt to its surrounding necessary to be in a positive state of welfare may be limited when an animal is exposed to chronic or severe stress due to for example illness low food availability or high population densities. In such cases an animal may experience chronic stress, which may result in a physiological state in which the animal is no longer able to respond to changes in its environment. Chronic stress can cause a prolonged suppression of the immune system, inhibition of the gastrointestinal function and inhibition of the growth, reproduction and thyroid function [4]. As a consequence, a chronic stressed animal is not able to adapt to the dynamics in its surrounding and may therefore be in a state of compromised welfare [1]. Measuring chronic stress could thus become an interesting welfare indicator, also for wild roaming animals and may be helpful in the discussion concerning the human role in wildlife welfare.

Park management

Humans affect wild roaming animals and their living area in different ways. First of all they affect the habitat of wildlife by restricting their living area. By building fences and roads humans create a restricted area, a nature park, for the animals to live in. This restriction may have consequences for the animals. The fences create a certain population density because the animals cannot move away from the area that they are living in. This population density can become low when there are for instance a lot of predators or there is a high virulence disease present in the nature park. However, the population density could also become very high. This will in turn have consequences, for instance for the food ability for the animals present. Relative food

availability can be influenced by park management factors as supplementary feeding or culling of animals to help control the population density. Another human factor affecting wildlife is human disturbance. Humans like to visit nature parks for recreation thereby possibly causing disturbance for the animals. In fact, wildlife parks have amongst others the purpose of teaching the public about wildlife.
Preservation of wildlife and vegetation variance in a nature park could be accomplished with certain management factors. Population density can be increased or decreased by respective introduction of new animals or culling of animals or by introduction of for instance predator animals. Vegetation variance can be sustained by introduction of certain herbivores or by controlling the population of these herbivores. These factors may have an effect on the animals present in the nature park [5]. Considering the fencing, human disturbance, supplementary feeding, introduction of predation but potentially also culling, these factors may affect wildlife. It is important to consider the impact of different management strategies. This will help understand the relative impact of specific interventions on the welfare of free-roaming deer and wildlife in general.

Stress response and chronic stress
Stress is a physiological response to a changing situation called a stressor. The stress response is a tool to restore the homeostasis in a living organism under a wide variety of conditions and is therefore essential for survival [6]. The stress response is mediated by the hypothalamic-pituitary-adrenal axis (HPA) and the locus coeruleus mediated norepinephrine-autonomic nervous system [7]. The HPA axis has an important role in the stress response. It consists of the hypothalamus, pituitary gland and adrenal glands. In response to stress the hypothalamus will secrete corticotropin releasing hormone (CRH) [8]. CRH will in turn stimulate the pituitary gland to secrete adrenocorticotropic hormone (ACTH). ACTH is then transported through the blood to the adrenal gland cortex, which will be stimulated to secrete stress hormones, glucocorticoids, the most important of which for the physiological stress response is cortisol in humans and higher animals and corticosterone in rodents [7]. The stress system is illustrated in figure 1 [7].

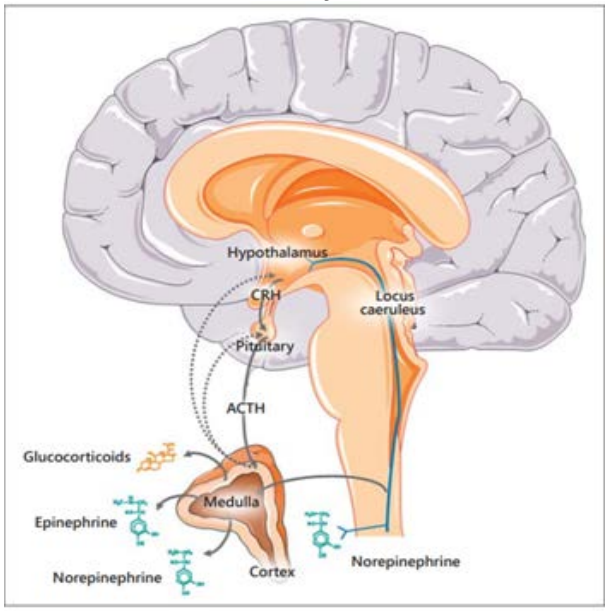

Figure 1. The stress system

Glucocorticoids are steroid hormones with effects in almost all organs and tissues [7]. These effects lead for example to an increase in blood glucose, heart rate, blood pressure and arousal while vegetative functions like feeding and reproduction will be inhibited [6]. The stress response is meant to be of short duration. This is for a large part accomplished by a negative feedback loop mediated by glucocorticoids. Glucocorticoids will bind to glucocorticoid receptors (GR) at the level of the hippocampus and hypothalamus. This in turn leads to downregulation of the stress response and ultimately a decline in circulating stress hormones.
However, in the event of chronic stress, the level of stress hormones will stay high due to the constant or repeated stimulation of the HPA axis and failure of the negative feedback loop. This failure is thought to be due to tolerance of the GR. As a consequence, the circulating glucocorticoids will have a prolonged effect on different organs and tissues. Circulating glucocorticoids have amongst others an effect on the innate and acquired immunity. For example, they suppress the secretion of proinflammatory cytokines and glucocorticoids influence the trafficking and functioning of accessory immune cells and leukocytes [4]. These effects cause a downregulation of the immune system and a consequential higher susceptibility for infections. Another effect of the prolonged higher levels of circulating glucocorticoids is the downregulation of the secretion of growth hormone (GH) and inhibition of the actions of insulin like growth factor I at its targets [7]. Furthermore, chronic stress also inhibits the thyroid hormone axis [4,7]. At the site of the hippocampus chronic stress leads to debranching and shortening of dendrites, at the site of the amygdala chronic stress will have the opposite effect leading to longer and more complex dendrites [9]. These dendritic changes will have an effect on hippocampal and amygdala-dependent learning and memory processes [9].
All these effects taken together may cause a chronic disturbance of the homeostasis of the animal. The situation causing the chronic stress has then exceeded the adaptive capacity of the animal therefore reflecting a state of compromised welfare.

The glucocorticoid and mineralocorticoid receptor in brain tissue
Two glucocorticoid sensitive receptors are located in brain tissue, the glucocorticoid receptor and the mineralocorticoid receptor (MR) [10]. These are nuclear receptors that act via transcriptional regulation of genes, their actions will develop slowly and have persistent effects [11] . Both receptors are widely abundant in the brain and their expression is especially high in the hippocampus [12] . The MR and GR are not evenly distributed throughout the hippocampus. The hippocampus can be divided in a CA1, CA2, CA3 and dental gyrus (DG) region, these regions are shown in figure 2. MR and GR levels differ throughout these regions.

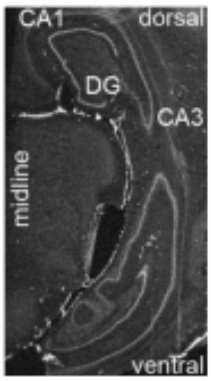

figure 2. Hippocampus subregions in deer brain

The MR is expressed in high levels in the CA2 region and at intermediate levels in the CA3, CA1 and DG regions of the hippocampus [9,10]. The GR is most abundant in the CA1, is expressed in intermediate levels in the DG region and in lowest levels in the CA3 of the hippocampus [9,13].
The glucocorticoid receptor in the brain plays an important role in the regulation of the stress response, particularly when endogenous levels of glucocorticoids are high as is the case during diurnal active phases and in response to stress. In the event of stress, the binding of glucocorticoids to the GR will result in downregulation of the stress response. Glucocorticoids and the GR form a negative feedback-loop for the stress response. The glucocorticoid receptor is closely related to the mineralocorticoid receptor (MR). The hippocampal MR has lost its aldosterone binding selectivity. This leads to binding of glucocorticoids to this non-selective mineralocorticoid receptor with high affinity. This affinity is approximately 10 times higher than the affinity of the GR and binding of glucocorticoids to the MR will therefore also occur under basal conditions [13,14]. Activation of the MR is necessary for the survival of granule cells in the DG [12]. Glucocorticoids can thus activate two different signalling pathways via MR and GR at the level of the hippocampus, depending on the levels of circulating glucocorticoids [15]. Co-activation of the MR and GR will consequently happen in the state of chronic stress.
In a chronic stressed condition down regulation of the MR and GR mRNA and consequently also of glucocorticoid receptor protein levels will occur, probably due to tolerance of the receptor as a result of the constant high levels of glucocorticoids [16,17]. The reduction in GR levels in brain tissue has been identified as in important indicator for chronic stress [9]. Studies on chronic stress and GR levels have been performed in rats, but also in a wide array of other species including birds and monkeys [18,19].

Hair cortisol levels
In the event of chronic stress cortisol levels will increase in blood, faeces and hair [20]. Blood cortisol levels are a representation of glucocorticoids secreted by circadian cycles, acute stress and chronic stress [20]. To conclude something on the circulating cortisol levels, basal levels and therefore multiple blood samples to distinguish baseline from potentially stress-induced levels are necessary. Collecting multiple blood samples of an animal requires handling by humans and in the case of wild animals also capturing. This may lead to acute stress in and of itself and therefore high cortisol levels [17]. These cortisol levels cannot be distinguished from the high baseline cortisol levels due to chronic stress.
Glucocorticoids are metabolized and excreted via the faeces there are no unmetabolized GCs present in faeces [21]. The structure and proportion of metabolized GCs in faeces can differ among species and also between males and females [21]. When measuring GCs in faeces it should be done as soon as possible after defecation to prevent bacterial or microbial degradation [20].
In the event of chronic stress cortisol will accumulate in the fur of the animal [22-24]. Cortisol is incorporated in the hair shaft in two ways. Free unbound cortisol is incorporated during the growth of the follicle [25,26]. The second way is via cortisol present in secreted sweat and sebum which can become incorporated in the hair shaft [27]. Therefore, hair samples offer a way to measure cortisol accumulated over the lifetime of the hair [28]. The advantage of measuring cortisol in fur instead of faeces or blood is that cortisol levels in hair are an indicator for the amount of cortisol present in the animal over a larger period in time. The cortisol in hair will be

accumulated over weeks or months rather than hours or days [20]. Hair cortisol analyses could be of interest in the long-run because, especially in the case of wildlife animal research, it is easier to collect hair samples than faecal or blood samples [20]. Hair cortisol has been suggested as an important indicator for chronic stress and is a very practical way to measure chronic stress especially in wildlife, it has been used in studies on different kinds of animals [22,23]. Previous studies have stated that hairs on different anatomical regions differ in growth rate [22]. Therefore, it is best to use hairs collected from the same anatomical region and within a similar time-window in the year.

Aim of the study

The aim of the overarching study is to determine which factors in park management may contribute to the welfare of fallow deer (*Dama dama)* in nature parks in the United Kingdom as well as fallow deer living in the Amsterdamse Waterleiding Duinen (AWD) and red deer (*Cervus elaphus)* living in the Oostvaardersplassen (OVP) and the Hoge Veluwe (HV). Information on park management, population and feeding density and human interference is available for each of these parks. For this study, we will use brain GR and hair cortisol as the critical parameters, with the secondary aim to validate hair cortisol as a measure for chronic stress, against GR that we consider as the golden standard given the consistent relation of GR levels with chronic stress in multiple species. The OVP deer form an interesting group since the policy in this park is to cull only the very weak and sick animals who are not likely to survive the upcoming weeks. Therefore, the deer live in an area with high population density and low food availability. These red deer will form a positive control for chronic stress because the culled animals have been in a prolonged bad condition. Initial analyses of deer from the UK revealed that population density might be an important factor to determine deer welfare (Lesscher et al.,unpublished findings), but more samples were needed to draw reliable conclusions regarding the park (management) factors and welfare. A study on hair cortisol levels in red deer in four different nature parks throughout Italy also revealed that population density is an important factor with an effect on chronic stress [29]. The fallow deer in the AWD form a particularly interesting group considering the initial evidence for population density as being a potential critical factor. The reason why this park is so interesting is that the population density in the AWD will be brought down over the next years by means of proactive culling, which started in 2015/2016. We have the opportunity to sample deer in a longitudinal approach since the start of the culling measures. This allows us to determine the impact of reduction of the population size on chronic stress measures in a longitudinal approach.

In this specific study the focus was on extension of the UK data and starting the AWD analyses. For that purpose, brain samples from the AWD deer were cut, cortisol analyses for AWD hair samples were performed and UK brain samples that have already been cut and processed for GR and MR measurements were analysed. This study forms a part of a bigger study which has been going on for several years.

Hypotheses

For the UK parks, we hypothesized that deer in parks with a higher population density and with higher human disturbance would show higher levels of chronic stress. This would be reflected in a lower number of GR's in the hippocampus and a higher level of cortisol in the hair.

For the AWD, we hypothesized that together with the reduction in population density there would be a gradual decline in chronic stress. This would be reflected in a rise in GR's in the hippocampus and a decline in hair cortisol levels.

2. Material and method

2.1 Tissue collection

In this study brain tissue and fur of fallow deer from eleven different parks throughout the UK and fur of fallow deer from the AWD was used. The UK parks were Attingham, Belton House, Dunham, Dyrham Park, Eridge, Hampton Court Palace, Lyme Park, Richmond Park, Prideaux, Tatton Park and Powderham Park. The UK deer were culled in 2014-2015. The AWD deer are culled in the period 2015 till 2018 and are divided in three groups 2015-2016, 2016-2017 and 2017-2018.
The heads of the culled deer were cooled and transported to the university. At the university fur samples were taken first. Approximately 500 mg of fur located between the eyes and ears of the deer was shaved. This region was standardized across the samples and is represented by the red circle in figure 3. The samples were wrapped in tin foil and provided with identification tags before storage in the dark at room temperature. The samples were stored until the cortisol analysis.

Figure 3. Shaving region on the head of the deer

After collection of the fur samples the heads were cut in a sagittal plane in order to collect both brain hemispheres. The brain region containing the hippocampus from both hemispheres was collected by slicing the brain in coronal slices of 1,5 cm using a 3D printed deer brain mold. The slices containing the hippocampus were provided with identification tags and stored until further slicing at -80 °C. Further slicing was performed with a Leica CM3050 S cryostat. The brain slices were sectioned in slices of 20 µm at a temperature of approximately – 20 °C. The slices were collected on Starfrost slides and stored at -40 °C until the in situ hybridization took place.

2.2 In situ hybridization (ISH) and analysis of hippocampus tissue

To navigate through the hippocampus and to ensure that a similar region of the hippocampus was analysed in all the deer the hippocampus was divided in four subregions that can be visually distinguished during slicing. The subregions were named A1, A2, A3 and A4 and they differ in their fusion of hippocampus areas. When slicing was performed from anterior to posterior the first region to be

encountered was the A1 region. At this region there was no fusion of the hippocampal parts visible, when continuing slicing the parts would start fusing this was the A1/A2 transition. When the two internal white hippocampal parts started fusing the A2/A3 region had been encountered. Complete fusion of the whole hippocampus would indicate the A4 subregion. The subregions used are shown in figure 4.

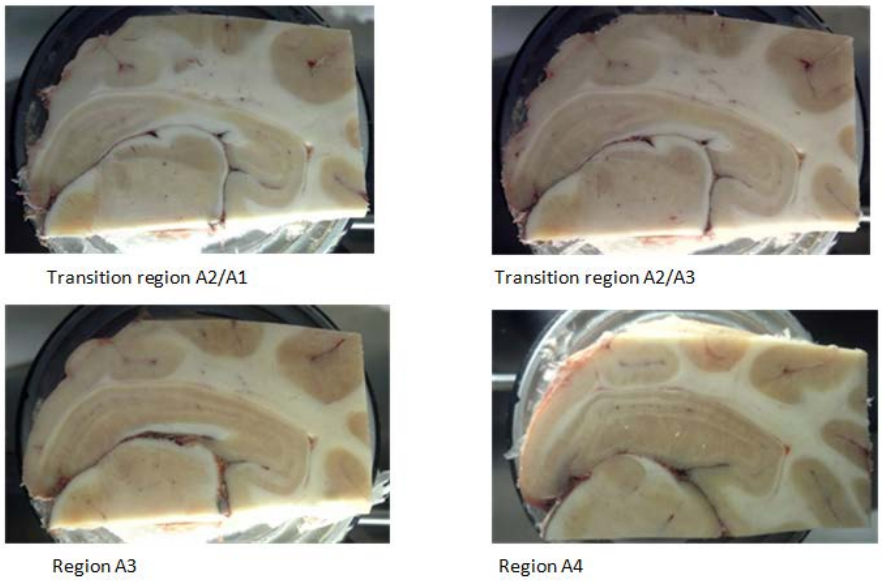

figure 4. The subdivision in hippocampus regions to help navigate through the hippocampus

Each individual slide was scored on the visibility and integrity of the DG, CA3 and CA1 region of the hippocampus. The regions were scored – when the region could not be analysed due to an impairment unless that slide was the only possibility. The region was scored -/+ if there was a small impairment in the region. If the region was intact it was scored +. The four best consecutive slides located as close as possible to the A1/A2 region transition were used for the ISH. Two of these slides were used for the MR analysis the remaining two slides were used for the GR analysis. These slides were selected for the left and right hemisphere resulting in 4 slides per animal per receptor. The first, last and A1/A2 and A2/A3 transition slides were coloured using a Nissl colouring this provides the nervous system cells with a purple colour.
Radioactive ISH was performed according to a standard operating protocol included as appendix at the end of this paper. In previous studies a deer-specific in situ hybridization probe has been developed for the GR and MR. The radioactive slides were developed on a light-sensitive film.
After development of this film the slides were scanned and quantified using imageJ software. The CA1, CA3 and DG regions were individually quantified by using the ROI manager function of the software. Using the marker values of each film and a polynomial regression function calculated by http://www.xuru.org/rt/pr.asp the results given by image J software were transformed into Becquerel values. The mean of the 4 measurements of one subregion form both hemispheres and two slides per animal was calculated and used for the data analysis.
The quantification of the films was done by two students to compare the values.

2.3 Cortisol analysis in hair

The cortisol analysis was done following a standard operating protocol which is included as appendix at the end of this paper. The hairs were washed once with 20 mL PBS in a 50 mL tube by gently mixing by hand. Then the hairs were washed twice with 20 mL isopropanol in a 50 mL tube by hand for 3 minutes per wash at room temperature. New isopropanol was used for the second wash. After the second wash the hairs were dried in a stove at 37 °C for 96 hours until the hairs were thoroughly dry. The washing procedure was necessary to eliminate contaminating cortisol originating from sweat or saliva. A study in polar bears and grizzly bears showed that hairs washed with isopropanol showed a higher cortisol level than hairs washed with methanol [30]. After the washing procedure 60-70 mg of hair was pulverized using a tissue-lyser II (Quiagen) and three 5 mm steel balls. 50 mg hair powder was incubated in methanol to extract the steroids. After centrifugation the samples were dried in a stove. Using a High Sensitivity Salivary Cortisol ELISA kit (Salimetrics) the pg cortisol / mg hair was measured and used in further data analysis.

2.4 Data analysis

All statistical analyses were performed using SPSS 20 for windows.
For the UK data, a linear mixed model was used to analyse the relation of the outcome variables (hair cortisol, GR mRNA and MR mRNA) with population density (fallow deer density, female fallow deer density, male fallow deer density), human disturbance, supplementary feeding, altitude and average temperature in November – March. These variables were entered as covariates in the model, along with park as random factor. These variables were tested for significance, and variables that were not significant were deleted from the model using a backward strategy [31]. Data are presented as mean ± SEM; significance was accepted at $P < 0.05$.
The analysis on cortisol in hair for the AWD deer was performed using a univariate ANOVA with pg cortisol/mg hair as the dependent variable and year and gender as the between-subjects factors. These variables were tested for significance. Where relevant, post hoc pairwise comparisons were performed. Data are presented as mean ± SEM; significance was accepted at $P < 0.05$.

3. Results

3.1. UK deer

Comparison of the cortisol data revealed a significant effect of fallow deer population, female fallow deer population and male fallow deer population on hair cortisol levels ($P < 0.01$). Moreover, human disturbance and supplementary feeding also contributed to the variance in hair cortisol ($P < 0.01$). As is shown in table 1. Scatterplots are shown in figure 5 for the significant variables contributing to the variance in cortisol. No significant correlations were found considering these scatterplots. When analysing the male and female population separately a significant non parametric correlation (P<0,01) was found for fallow deer density in the female population, this correlation was not significant in the male population. Scatterplots of these correlations are shown in figure 6. The effects of fallow density, male fallow density, female fallow density and supplementary feeding are sex dependent as is evident from a significant interaction (P<0,01) of these factors and gender. Separate analyses per sex confirmed the significant effect of fallow density in the female population (P<0,01) and in the male population(P<0,05), and female fallow density in the female population (P<0,01) and in the male population(P<0,05). As is shown in table 2.a. for the male population and in table 2.b. for the female population.

Estimates of Fixed Effects[a]

Parameter	Estimate	Std. Error	df	t	Sig.
Intercept	-34,494592	16,801607	82,000	-2,053	,043
fallow_dens	30,495035	9,647819	82	3,161	,002
male_fallow_dens	-22,759579	8,289082	82	-2,746	,007
female_fallow_dens	-39,145196	13,928645	82,000	-2,810	,006
human_dis	7,165057	2,351852	82	3,047	,003
supp_feed	12,543071	4,652726	82,000	2,696	,009

a. Dependent Variable: cort_pg/mg.

Table 1. the significant variables contributing to the variance in cortisol levels

Estimates of Fixed Effects[a,b]

Parameter	Estimate	Std. Error	df	t	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	28.395716	8.149712	17.000	3.484	.003	11.201327	45.590106
fallow_dens	-41.716491	18.797774	17.000	-2.219	.040	-81.376327	-2.056655
male_fallow_dens	51.206700	34.873089	17	1.468	.160	-22.369086	124.782486
female_fallow_dens	51.548062	20.368331	17	2.531	.022	8.574640	94.521485
human_dis	-1.540286	2.427346	17	-.635	.534	-6.661538	3.580966
supp_feed	0[c]	0	.	.	.	.	.

Table 2.a. Variables contributing to the variance in cortisol levels in the male population

Estimates of Fixed Effects[a,b]

Parameter	Estimate	Std. Error	df	t	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Intercept	-11.566936	40.674778	43	-.284	.777	-93.595443	70.461571
fallow_dens	51.782010	13.817998	43.000	3.747	.001	23.915360	79.648659
male_fallow_dens	-72.054904	46.542807	43.000	-1.548	.129	-165.917420	21.807612
female_fallow_dens	-56.932591	15.149335	43.000	-3.758	.001	-87.484136	-26.381045
human_dis	5.324219	6.065257	43.000	.878	.385	-6.907537	17.555974
supp_feed	1.595819	10.706544	43.000	.149	.882	-19.995985	23.187622

Table 2.b. Variables contributing to the variance in hair cortisol levels in the female population

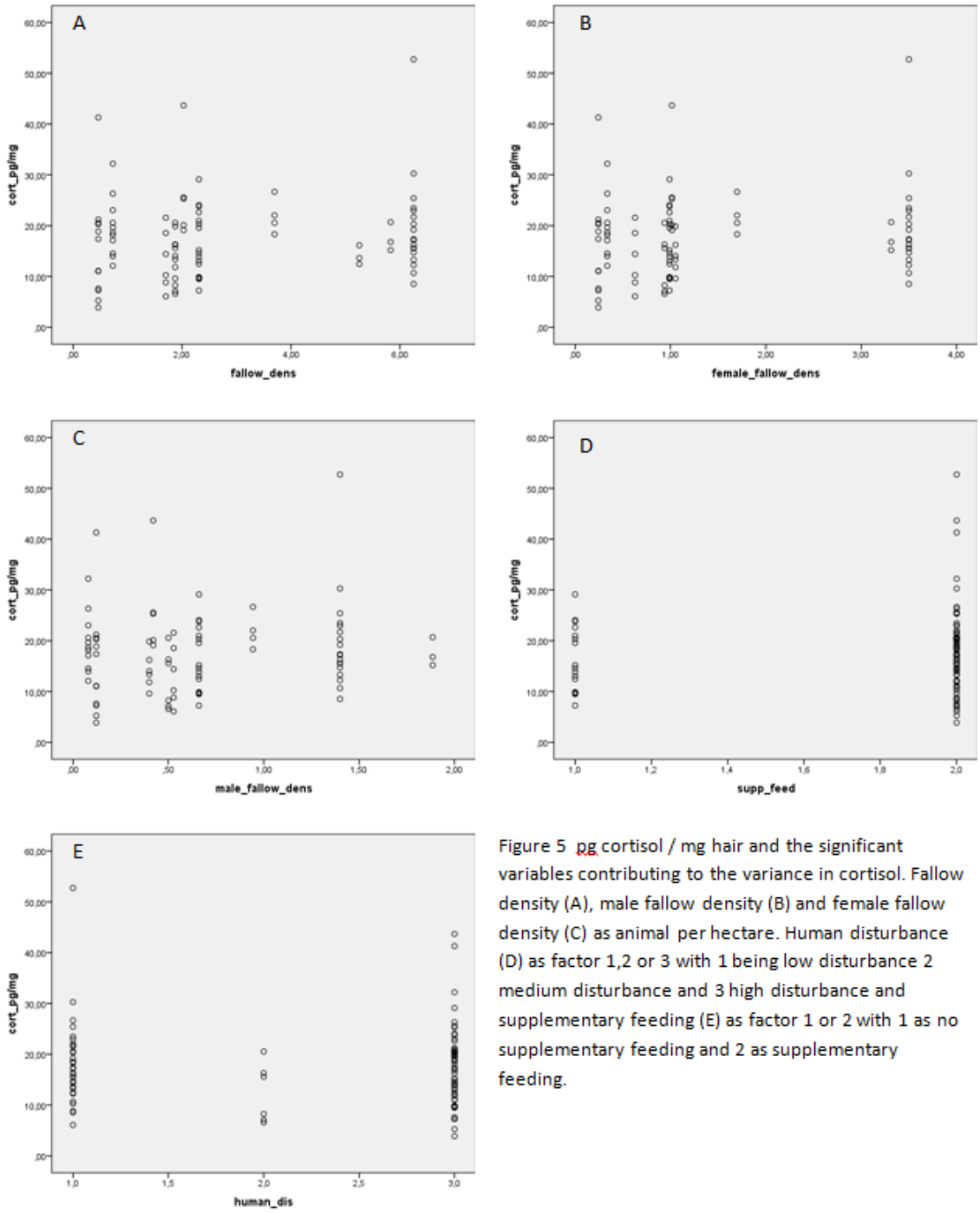

Figure 5 pg cortisol / mg hair and the significant variables contributing to the variance in cortisol. Fallow density (A), male fallow density (B) and female fallow density (C) as animal per hectare. Human disturbance (D) as factor 1,2 or 3 with 1 being low disturbance 2 medium disturbance and 3 high disturbance and supplementary feeding (E) as factor 1 or 2 with 1 as no supplementary feeding and 2 as supplementary feeding.

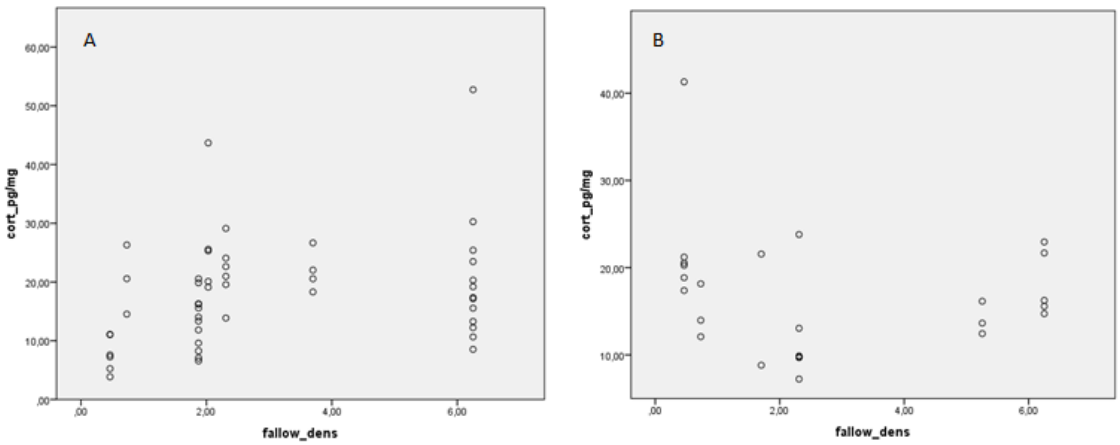

Figure 6. fallow density and the effect on pg cortisol/ mg hair in the female population (A) and male population (B)

Comparison of the GR DG date revealed a significant effect of male fallow density on GR DG levels p<0,05 this is shown in figure 7. A scatterplot showing male fallow density and GR DG levels is shown in figure 8.

Estimates of Fixed Effects[a]

Parameter	Estimate	Std. Error	df	t	Sig.
Intercept	122,236583	280,505607	33,000	,436	,666
male_fallow_dens	334,319290	160,821526	33,000	2,079	,045
female_fallow_dens	14,711781	223,660377	33,000	,066	,948
fallow_dens	-84,434936	158,679438	33	-,532	,598
human_dis	7,173086	40,338534	33	,178	,860
supp_feed	20,405943	74,703435	33	,273	,786

a. Dependent Variable: GR_DG.

figure 7. Male fallow density as the only significant variable contributing to the variance in GR DG levels.

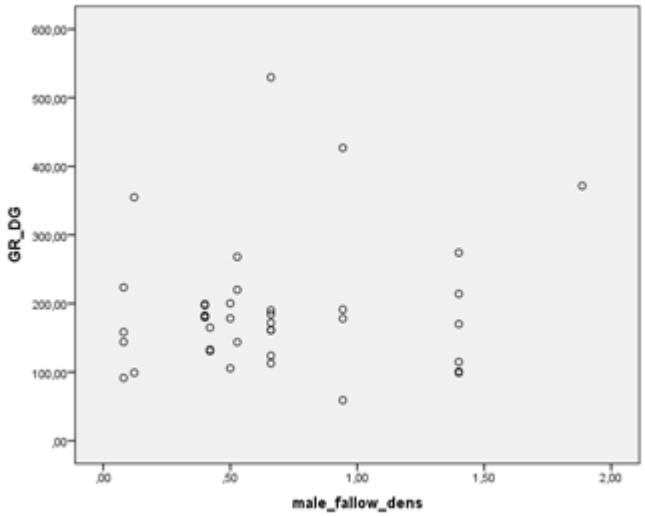

figure 8. Scatterplot of male fallow density and GR DG level.

3.2. AWD deer

Cortisol analysis of cortisol in hair from AWD deer showed an overall effect of year $F_{(2,55)year} = 3,8$ $p<0,05$. This effect is dependent on gender, as is evident from a significant year x gender interaction $F_{(2,55)\ year\ x\ gender} = 6,7$ $p<0,01$. No effect of gender was found $F_{(1,55)gender} = 1,3$ not significant.
Post hoc pairwise comparisons showed that cortisol was highest in the year 2015/2016 and gradually declined to 2016-2017 and 2017-2018 this is shown in figure 9. A post hoc pairwise comparison for year and gender combined showed that female hair cortisol was highest in 2015-2016 and gradually declined to 2016-2017 and 2017-2018 this is shown in figure 10. Male deer cortisol declined to 2016-2017 compared to 2015-2016 but it increased to 2017-2018 compared to 2015-2016 and 2016-2017 as is shown in figure 11.

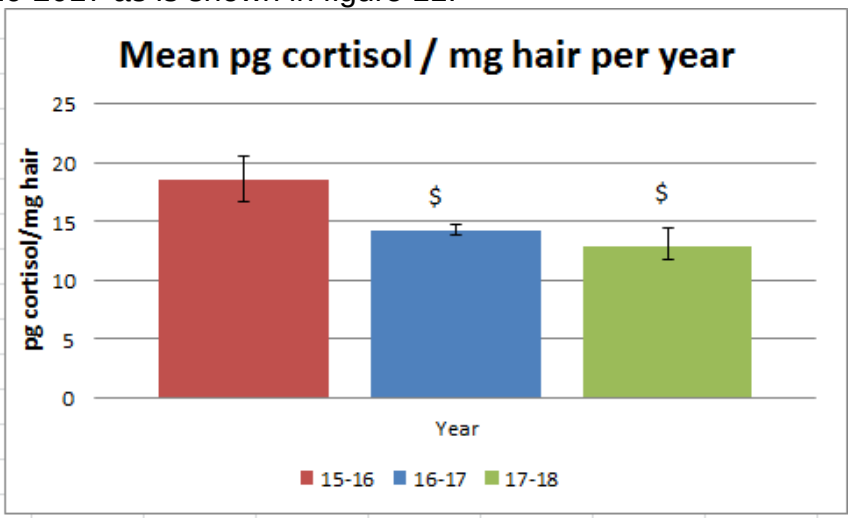

figure 9. Mean pg cortisol/ mg hair per year. $= p<0,05 compared to 2015/2016

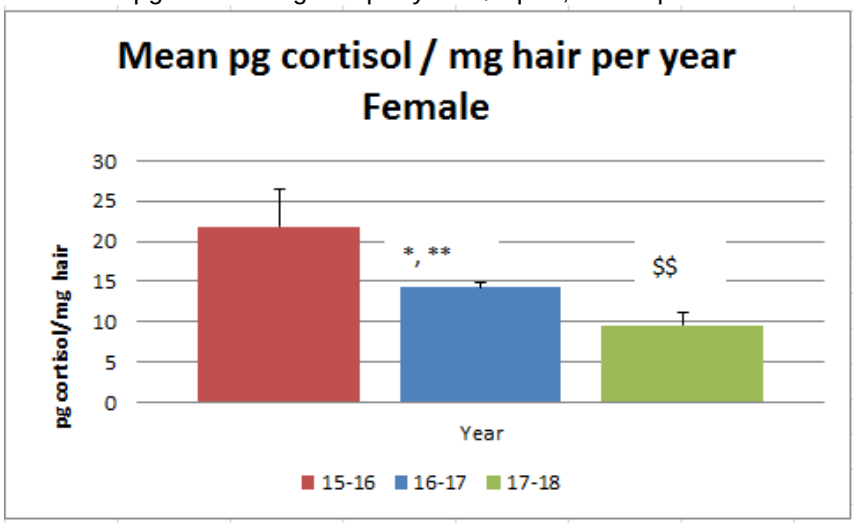

figure 10. The effect of year on mean female cortisol levels. *= p<0,05 compared to 2015-2016,**= p<0,01 compared to 2017-2018, $$ = p<0,01 compared to 2015-2016

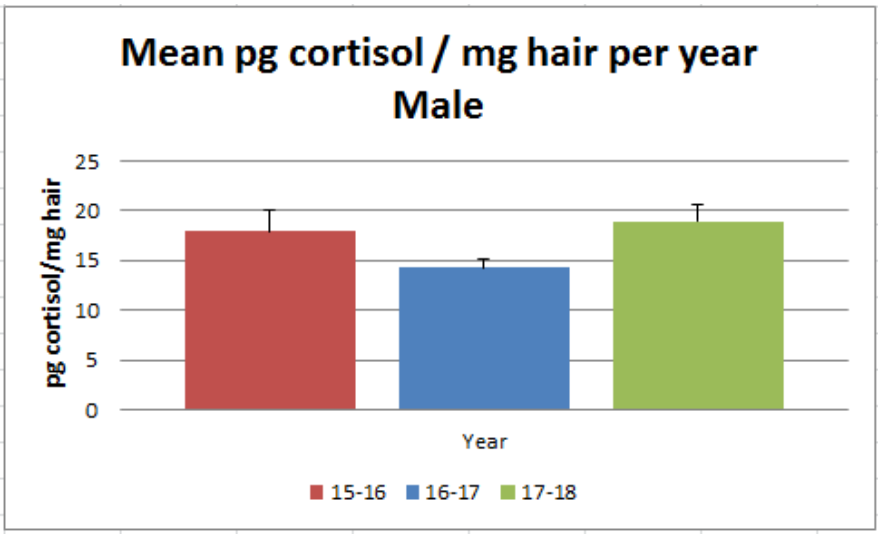

figure 11. The effect of year on male cortisol levels

4. Discussion

The aim of this study was to determine which park management factors have an effect on chronic stress and therefore on welfare in fallow deer in different parks throughout the UK and in the AWD. For both the UK deer and the AWD deer an effect of population density on chronic stress has been found.

4.1. UK deer

For the UK data an effect was found for the total fallow deer density and the female and male fallow density on hair cortisol levels. The effect of density cannot be seen in the scatter plots of figure 5 but is significant according to the statistical analysis.
The effects on hair cortisol levels are sex dependent. When analysing the female and male fallow deer population separately an effect was found for fallow density and female fallow density in both genders. The effect of both factors is more pronounced in female hair cortisol levels. This might be due to unequal sample sizes, we analysed 49 females and 24 males. The significant effects suggests that density has an effect on the variance of hair cortisol levels and therefore density is an important factor regarding the presence of stress in fallow deer. This finding is in line with a study done on hair cortisol levels in red deer in Italy [29]. In this study hair cortisol levels of red deer from four different parks with different population densities in Italy were analysed and showed higher cortisol levels in deer living in parks with higher population density [29]. However, more samples are needed and an equal distribution in male and female samples is needed to draw more reliable conclusions. In addition, our study identified human disturbance as a factor that explains the variation in hair cortisol levels in the UK deer as well. This effect was not seen in the separate gender analysis. This might suggest that the amount of human disturbance is an important factor for the degree of chronic stress experienced by fallow deer as well. An effect of human disturbance on ungulate flight responses has already been found in previous studies [32] .
Regarding the GR and MR levels we only observed a significant effect of male fallow density on GR DG levels. This might suggest that the GR in the DG region may be most sensitive to stress caused by male population density. This is in line with studies on other animals were the GR in the DG region was also identified as most sensitive to stress [10,33] . The lack of effect of density in other hippocampus regions and in MR levels could possibly be explained by missing data and therefore too little data to perform a statistical analysis with sufficient power. Although these findings appear to be in line with the cortisol data, the effect of population density on GR were much more subtle than those observed for hair cortisol. This may also have to do with the limited sample size for the GR and MR analyses. GR and MR data was complete for only 37 animals while the cortisol data was complete for 91 animals. The missing data are due to loss of tissue by incorrect dissection of the brain. The dissection procedures were adjusted to prevent future tissue loss that unfortunately occurred in the initial stages of sampling in this project. Another explanation for the lack of an effect or at least effects that were much less clear on GR and MR levels could be that GR and MR may be less sensitive to changes in response to chronic stress as compared to cortisol in hair. Perhaps the stressors the fallow deer are exposed to are sufficient to induce repeated acute stress responses, which do lead to accumulation of cortisol, but not sufficient enough to induce excessive levels of cortisol that result in tolerance of the GR. This would be in line with findings in a

study on chronic mild stress in rats where no changes in GR levels in the rat hippocampus after a period of chronic mild stress had been found [34].

4.2 AWD deer

For the AWD data an overall effect of year on hair cortisol levels was observed and this effect was dependent on gender. An overall decline of cortisol levels was apparent over the years that samples were processed this far. However, when looking at males and females independently the decline in hair cortisol was only apparent in the female population. The population density of fallow deer in the AWD is also declined due to the start of culling of random deer in 2015. The effect of year on hair cortisol levels might therefore indicate that this is because of the decline in population density. The effect of population density on cortisol levels would be in line with the UK data showing that fallow deer density is an important factor in the development of chronic stress. However, we cannot rule out at this stage that other factors may have contributed to the decline in hair cortisol, such as climate differences over the years that we have not considered yet, or subtle changes in park management, number of visitors etc. that may also explain the changes that we have observed. Nevertheless, since an effect of population density on chronic stress has been identified in the UK fallow deer and Italian red deer as well it is likely that this is also the case for the AWD deer. There is a difference in decline of hair cortisol levels between the male and female fallow deer. In the female deer there is a significant decline between all the years. In the male deer population there is a non-significant difference in hair cortisol over the years. A difference in hair cortisol levels between the male and female population has not been found in a study on hair cortisol levels in red deer in Italy [29]. The differences between the genders might be due to uneven sample sizes. In 2015-2016 there were 2 females and 9 males, in 2016-2017 there were 19 females and 6 males in 2017-2018 there were 13 females and 7 males. With only two female samples in 2015-2016 it is hard to make a male/ female comparison for this year as well as a female/female comparison for 2015-2016 and 2016-2017. Bigger and equal sample sizes throughout the years would make the comparison more reliable.
Data on the GR and MR levels of the AWD deer will be available as well in time. It would be very interesting to see if there are any changes in GR and MR levels over time as well. Since both measures are available for each deer a correlation between hair cortisol and GR levels may be determined in time.

4.3 Hair cortisol levels

Hair cortisol levels appear to be an interesting factor when looking at chronic stress in wildlife. The samples are easy to collect in wild roaming animals and are a reliable way to measure cortisol accumulated over a longer period of time. The hairs can be collected in a non-invasive way using barbed wire the disadvantage of this way of collecting is that the hairs will not be originated from the same anatomical location. To ensure that the hairs originate from the same location a more invasive way of capturing and collecting the hair samples must be done. The stress due to handling and human presence will not have an effect on the cortisol levels in the hair since these levels have accumulated over a longer period of time and are not a result of

acute stress [35]. This makes hair cortisol very useful when investigating stress levels in wild animals. This method could be used in different animals in other nature parks as well to determine stress levels. Further validation in different animal species is necessary due to differences in hair structure and porosity among other factors as difference in hair colour and gender [26].

4.4 Park management factors

In both the UK and AWD fallow deer density appears to be an important factor causing stress, since chronic stress decreases the adaptive capacity of animals to their surrounding it might affect the welfare status. Management measures to reduce population sizes and decline fallow deer density could therefore contribute to a positive state of welfare for the remaining animals. These findings can be helpful in current discussions regarding the human role in wildlife welfare at for instance the OVP . Based on our findings, it is tempting to state that the human role in fallow deer welfare should include the prevention of unnecessary suffering by reducing population density. However, more research is necessary to confirm our findings, in other areas and with larger sample sizes.

5. Conclusions

There is an increasing interest in wildlife welfare. This study aimed to determine which park management factors have an effect on chronic stress and therefore welfare in fallow deer in UK parks and the AWD. The main finding of this study is that fallow deer density is an important factors that determines chronic stress in fallow deer. These findings are more pronounced when considering hair cortisol than brain GR. Because hair samples are relatively easy to collect measuring chronic stress through hair cortisol level analysis is becoming a very interesting approach to study stress in wildlife on a broader scale. According to our findings there might be a gender dependent difference in chronic stress in fallow deer. These findings further suggest that fallow deer density may impact the welfare status of the fallow deer. Based on these findings, we may carefully suggest that management factors in nature parks should consider population density , with the ultimate aim to prevent unnecessary suffering and compromised welfare in the fallow deer.

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7. Appendix

ISH with radioactive probes

Most recent adjustment made by: J.Hendriks **Date: 11-11-2014**
S.Kirchhoff **Date: 30-10-2012**

1. Introduction

In situ hybridization (ISH) is a type of hybridization that uses a labeled complementary DNA or RNA strand (i.e., probe) to localize a specific DNA or RNA sequence in a portion or section of tissue (in situ), or, if the tissue is small enough (e.g. plant seeds, Drosphila embryos), in the entire tissue (whole mount ISH). DNA ISH can be used to determine the structure of chromosomes. RNA ISH is used to measure and localize mRNAs and other transcripts within tissue sections or whole mounts.

For hybridization, sample cells and tissues are usually treated to fix the target transcripts in place and to increase access of the probe. As noted above, the probe is either a labeled complementary DNA or, now most commonly, a complementary RNA (riboprobe). The probe hybridizes to the target sequence at elevated temperature and then the excess probe is washed away (after prior hydrolyses using RNAse in the case of un-hybridized, excess RNA probe). Parameters such as temperature, salt and/or detergent concentration can be manipulated to vary the stringency of this washing step in order to remove any non-identical interaction (i.e. only exact sequence matches will remain bound). Then, the probe that was labeled with either radio-, fluorescent- or antigen-labeled bases (e.g., digoxigenin) is localized and quantified in the tissue using either autoradiography, fluorescence microscopy or immunohistochemistry, respectively. ISH can also use two or more probes, labeled with radioactivity or the other non-radioactive labels, to simultaneously detect two or more transcripts. This protocol presented here is specific for radioactive ISH followed by autoradiography.

2. Chemicals

Chemicals	Supplier	Product Nr.	Location
10x transcription buffer	Roche	11130900	Lab 383, Fridge #7
ATP (100 mM, 25 µmol)	Fermentas	#R0441	Lab 383, Fridge #7
CTP (100 mM, 25 µmol)	Fermentas	#R0451	Lab 383, Fridge #7
GTP (100 mM, 25 µmol)	Fermentas	#R0461	Lab 383, Fridge #7
UTP (100 mM, 25 µmol)	Fermentas	#R0471	Lab 383, Fridge #7
DTT	Sigma	D0632	Lab 383, Fridge #8
RNAse inhibitor (20 U/µl)	Roche	M14764	Lab 383, Fridge #7
DEPC	Calbiochem la Jolla, CA	298711	Lab 385, Fridge #4
^{33}P UTP (100 µl) enough for 20 labeling reactions	Perkin Elmer	NEG607H001MC	RA Lab
^{33}P UTP (25 µl) enough for 5 labeling reactions	Perkin Emer	NEG607H250UC	RA Lab
T7 RNA polymerase	Fermentas	#EP0111	Lab 383, Fridge #7
T3 RNA polymerase	Fermentas	#EP0101	Lab 383, Fridge #7
DNAse (RNAse free 40 U/µl)	Fermentas	#EN0521	Lab 383, Fridge #7
tRNA	Roche	10109525001	Lab 383, Fridge #7
Sodium acetate	Sigma	11OH-072015	Lab 383, Chemical cabinet #1
Ethanol 100%	Merck	1.00983.2500	Lab 383, fume cupboard
Paraformaldehyde (PFA)	Merck	PROL28793.292	Lab 385, Fridge #4
Acetate anhydride (pure)	Merck	1.00042.1000 1.00041.1000	Lab 383, fume cupboard #4
HCl (37.5%)	Merck	1.00317.1000	Lab 383, fume cupboard #6
Triethanolamine (TEA)	Merck	1.08379.0250	Lab 383, fume cupboard #4
Formamide	Roche	11814320001	Lab 383, Fridge #6
Dextran sulfate	Sigma	D-8906	Lab 383, Chemical cabinet #1
tRNA Bakers yeast	Roche	10954821	Lab 383, Fridge #7
Sonificated Salmon Sperm DNA	Stratagene	201190	Lab 383, Fridge #8
Ultima gold scintillation Cocktail	Perkin Elmer Precisely	6013119	RA Lab
Ficoll PM 400	GE Healthecare Diegem	17030050	Lab 383, Chemical cabinet #1
Polyvinylpyrrolidone	Sigma	P5288	Lab 383, Chemical cabinet #2
BSA-fraction V	Merck	126609-5GM	Lab 056, cold room

Acetic acid	Merck	1.0063.1000	Lab 63, fume cupboard
NaCl	Merck	1.04873.1000	Lab 63, chemical cabinet
KCl	Sigma	P9541	Lab 63, chemical cabinet #2
di-sodium hydrogen phosphate dihydrate	Merck	1.06580.1000	Lab 63, chemical cabinet

$Na_2HPO_4.2H_2O$			
Sodium citrate ($C_6H_5Na_3O_7.2H_2O$)	Backer J.T. Deventer	0280	Lab 63, chemical cabinet
RNAse A	Roche	10109142001	Lab 383, fridge #8
Acetic anhydride (pure)	Merck	1.00042.1000 1.00041.1000	Lab 383, fume cupboard #4
NaOH	Merck	6498.1000	Lab 63, Chemical cabinet #1
pH calibration buffers pH 7	Radiometer Copenhagen	943-112	Lab 385, cabinet above pH-measurement system
pH calibration buffer pH 4.1	Radiometer Copenhagen	943-111	Lab 385, cabinet above pH measurement system
pH calibration buffer pH 10.0	Radiometer Copenhagen	S 1336	Lab 385, cabinet above pH measurement system
Potassium dihydrogen Phosphate (KH_2PO_4)	Merck	1.04873.1000	Lab 63, Chemical cabinet #1

3. Materials and equipment

3.1 Materials

All materials marked must be autoclaved or baked at >200 ℃, to breakdown RNase.*

Material	Supplier	Article number
Beaker *(300 ml, 2x; 500 ml)	VWR	
Stirring flea (3x)	VWR	
Pipettes and tips 2.5 µl, 10 µl, 200 µl, 1000 µl, 10 ml	Finn/ Omnilabo	-
Eppendorf cups 1,5 ml	Eppendorf/Omnilabo	801161
Bottles* incl. Scraw cap (0.250, 0.5, 1 L)	VWR	
Measuring cylinder* (25 ml, 100 ml)	VWR	
Staining trays incl. racks	VWR	
Sterile tube (50 ml)	VWR	
Timer (2x)		
Polyethylene (PE) vials	Perkin elmer	
Forceps*, flat		
Films	Thermo scientific	Lot: NE1519097
Glue spray	Bison	Barcode: 8710439015118
Eppendorf tubes (RNAse free) (1.5 ml)	Eppendorf	Lot: A145172Q
Carbon-14 standard for autoradiography (RA lab)	Scopus Research	120308

3.2 Equipment

Material	Supplier	Article number
Water bath or stove (37 °C)	Lab companion	BW-05G
Water bath (65 °C)	Lab companion	BS-11
Hybridization stove (55 °C) Set point 56°C	Appligene	Notatia system detached
Stove (>200 °C)	Dépex b.v. De Bilt	Memmert ULE 500
Heating block (>90 °C)	Beun DeRonde	Lab line multi-blok heater 2000-ICE
Vortex	Scientific Industries	Vortex genie 2
Magnetic stirring device incl. heater (3x)	Tamson	IKA MAG RCT
pH-measurement system	Beun DeRonde	Consort C231
β- counter	Packard	Tricarb 1900CA

4. Solutions

DEPC will increase the pH of the stock solutions! Therefore check the pH of solution made with DEPC!

DEPC water	1 ml DEPC/litre Keep stirring for 2 h or O/N Autoclave (For 20 slides ± 3 liters needed)
10x PBS (1000 ml) In DEPC	80 gr. NaCl, 2.44 gr. KH_2PO_4, 17.8 gr. $Na_2HPO_4.2H_2O$, 2.0 gr. KCl Fill up till 900 ml with Milli Q and set pH: 7.5 (met NaOH-juli2014) End volume of 1000 ml Add 1 ml of DEPC Keep stirring for 2h or O/N Autoclave
20x SSC (1000 ml) (3M NaCl/300mM sodiumcitraat) In DEPC	NaCl 175.3 gr. Sodium citraat dihydraat 88.2 gr. Fill up to 900 ml with Milli Q and set pH:7.0 End volume of 1000 ml Add 1 ml of DEPC Keep stirring for 2h or O/N Autoclave
50x Denhardt's solution (50 ml)	0.5 gr. Ficoll-400; 0.5 gr. Polyvinylpyrrolidone; 0.5 gr. BSA-fraction V Dissolve in 50 ml DEPC (in 50 ml tube), by shaking for 10 minutes at 37 °C. Filtrate over 0.45 µm filter and fill out in eppendorf tubes (50x 1ml) Store at -20 °C

Hybridization buffer (500 ml)	100% formamide 20x SSC 50x Denhardts 12.5 mg/ml tRNA bakers yeast 10 mg/mlSonificated Salmon Sperm DNA (ssDNA)	250 ml 125 ml 50 ml 10 ml 25 ml
RNAse A Stock	Stock solution 500 units/ml (100x) (50-60U/mg) Dissolve RNAse A powder in Tris buffer: For 50 ml Tris Buffer: Boil for 15 minutes at 100 °C Cool down slowly Store at -20 °C	 60.6 mg Tris, 43.8 mg NaCl in MilliQ pH 7.5
1M Tris pH 8	121,14 g / liter MilliQ Set to pH 8,0	
5 M NaCl	292,2 g/liter MilliQ	
1M DTT	Dissolve 154.25 mg in 1 ml Milli Q Store at -20 °C	
tRNA 10 mg/ml	Dissolve 10 mg in 1 ml DEPC-H_2O Store at -20 °C	
3M SodiumAcetate	Dissolve 24.6 gr in 70 ml Milli Q Set pH to 5.2 with Acetic Acid Fill up till 100 ml with Milli Q Autoclave	

50% ethanol	Dilute 125 ml 100% ethanol using Milli Q (RNAse free) End volume of 250 ml
70% ethanol	Dilute 175 ml 100% ethanol using Milli Q (RNAse free) End volume of 250 ml
80% ethanol	Dilute 200 ml 100% using Milli Q (RNAse free) End volume of 250 ml
10M NaOH (100 ml)	Mw: 40.00 g/mol Dissolve 40 gr. of NaOH in 100 ml Milli Q

5. Procedures

Probe synthesis

The probe is synthesized by in vitro transcription using radioactively labeled UTP. Linearized plasmide DNA or PCR product that contain a promoter (generally either T7 or T3) is used as a template.

RNAse free! Use DEPC-H_2O and baked end/or autoclaved glasswork!

Prepare fresh:

- 100 mM DTT
  - Dilute from stock solution 1M DTT with autoclaved Milli Q water
- 10 mM NTP's (ATP, CTP, GTP, UTP)
  - Dilute from stock solution 100 mM with autoclaved Milli Q
- 10 mg/ml tRNA
  - Dissolve 10 mg in 1 ml DEPC-H_2O

Protocol:
Voorwaarden PCR product: 260/280 1.8
230/260 minimaal 1.6
Indien onzuiver: lage inbouw 33P

- Labeling reaction pipetting scheme

Needed	**Concentration**
2.0 µl	10x transcription buffer
1.0 µl	10 mM ATP
1.0 µl	10 mM CTP
1.0 µl	10 mM GTP
2.0 µl	100 mM DTT
1.0 µl	100 µM UTP
0.8 µl	RNAse inhibitor
x µl	Plasmid (1µg) /PCR insert (200 ng/µl)
x µl	dd H_2O
14.25 µl	Total volume
Add in RA lab	
5.0 µl	^{33}P UTP
0.75 µl	T7 or T3 or SP6 polymerase
20.0 µl	Grand volume

- Incubate at 37 °C for 60' – 90'
- Add 0.75 µl DNAse (RNAse free, 40 U/µl)
- Incubate at 37 °C for 30'
- Add the following and mix by vortexing

80.0 µl	ddH_2O
1.0 µl	tRNA (10 mg/ml)
10.0 µl	3M NaAc (pH=5.2)
270 µl	Absolute ethanol

- Freeze at -80 °C for 15'- 30' or O/N at -20°C
- Centrifuge at maximum speed (130000 rpm) for 20' at 4°C
- Collect supernatant in tubes (SN1)
- Add 500 µl 70-80% ethanol and centrifuge for 5-10' at maximal speed at 4°C
- Collect the supernatant in tubes (SN2)
- Air-dry the pellet
- Dissolve pellet at 49 µl DEPC and 1 µl 1M DTT. Store at -20 °C until use

- Measure the efficiency *in vitro* transcription:
  - Pipette 1 µl of labelled riboprobe in scintillation tube
  - Pipette 7.6 µl of SN1 in scintillation tube (in general 1/50 of total volume)
  - Add 3 ml Ultima gold scintillation fluid. Shake solution thoroughly
  - Measure in β-counter

Important:
- The amount of DNA must not exceed 5.5 µl, because otherwise the total volume after addition of ^{33}P UTP and polymerase is too large for the transcription buffer
- The amount of absolute ethanol should be around 2.5x the volume of the total labeling reaction
- Cpm in labeled probe should be around 80% of total cpm (cpm probe + cpm supernatant)
- One labeling reaction is sufficient for ISH with 25 glass slides (2-4 mio cpm/glass slide).

Day 1: Prehybridization and hybridization

Work RNAse free! Use DEPC-H_2O and baked end/or autoclaved glasswork!

Prepare fresh:
One staining tray can contain 19 slides!
Solutions for 250 ml (= 1 tray)

- 4% Paraformaldehyde (PFA):
  - Dissolve 10 gram of PFA in 225 ml DEPC and 25 ml 10x PBS at 60-65 °C using a heating plate and magnetic stirrer (do not heat excessively as PFA breaks down!).
  - Weigh the PFA under a fume hood!!
  - If the PFA doesn't dissolve at 60-65°C, add a few drops of NaOH
  - Set the pH to 7.4
- 1x PBS:
  - Dilute from 10x PBS using DEPC. Make 500 ml.

- Triethanolamine (TEA):
  - 3.3 ml triethanolamine
  - 0,438 ml HCl (36%, fuming)
  - 250 ml MQ.
  - 0.625 ml Acetic anhydride (drop wise adding)

- 2x SSC
  - Dilute from 20x SSC using DEPC

- Alcohol series:
  - 50%, 80%, 100%.
  - Dilute 100% ethanol with DEPC water, to 50% and 80%

Preparations
- Heat the incubation oven to 55 °C
- Make sure the heating block is 85 °C

Protocol
- Take slides out of the -80°C freezer and let them defrost for ± 30 minutes
- Fixate the slides in 4% PFA for 30'- 60'
- Wash the slides 2x 5' with 1x PBS
- Acetate the slides in TEA for 10', add the acetic anhydride dropwise over the slides
- Rinse slides 10'with 2x SSC

Dehydrate:
- 1' 50% ethanol
- 1' 80% ethanol
- 1' 100% ethanol
- 1' 100% ethanol
- Air dry the slides and store at room temperature

Hybridization

Table 1 Hybridization mix

For 15 ml	For 50 ml		Final concentration
7.5 ml	25 ml	100% deionized formamide	50%
3.75 ml	12.5 ml	20x SSC	5x
1.5 ml	5 ml	50x Denhardt's	5x
0.3 ml	1 ml	12.5 mg/ml tRNA bakers yeast	250 µg/ml
0.75 ml	2.5 ml	10 mg/ml Sonificated Salmon Sperm DNA	500 µg/ml
1.2 ml	4 ml	H_2O	

- The hybridization mix should contain 2-4 million cpm of probe/120 µl. Heat the hybridization mix containing the probe for 10' at 85 °C
- Pipette 120 µl probe per glass slide, put coverslips on top
- Place the glass slides in a dark box with on the bottom some wet tissues
- Incubate O/N at 55 °C

Preparations for the next day:

- Preheat the solutions needed for the second day
- Put the oven or waterbath on at 37 °C
- Put the waterbath on at 65 °C

Day 2: Posthybridization

The use of DEPC is not necessary.

Preparations:

- RNAse solution for 250ml:
  - 500 µl of 10mg/ml RNAse, add just before use of the solution!
  - 25 ml 5M NaCl
  - 2.5 ml 1M Tris pH 8
  - 222.5 ml H_2O
  - Preheat the RNAse solution, so this is already around 37 °C.
  - 500 µl of 10 mg/ml RNAse, add just before use of the solution!
- 2x SSC
  - Dilute from 20x SSC, make at least 300 ml
- 1x SSC
  - Dilute from 20x SSC, make at least 300 ml
  - Preheat to 65 °C
- 0.2x SSC
  - Dilute from 20x SSC, make at least 300 ml
  - Preheat to 65 °C
- 0.1x SSC
  - Dilute from 20x SSC, make at least 600 ml
  - Preheat to 65 °C
- 0.5x SSC
  - Dilute from 20x SSC, make at least 300 ml
  - Preheat 65 °C
- Alcohol series

- Dilute 100% ethanol (technical) with Milli Q water, to 80% and 50%

Protocol:

- Remove the coverslips in 2x SSC
- Incubate the slides in RNAse solution at 37 °C for 15‘

Wash steps:

- 5’ with 1x SSC at 65 °C
- 10’ with 0.5x SSC at 65 °C (the rest of the washes can be regarded as non-radioactive)
- 15’ with 0.2x SSC at 65 °C
- 15’ with 0.1x SSC at 65 °C
- 5’ with 0.1x SSC at RT

Dehydrate:

- 1’ 50% ethanol
- 1’ 80% ethanol
- 1’ 100% ethanol
- 1’ 100% ethanol

- Air dry the slides
- Clean the autoradiography cassettes with ethanol and put filterpaper in the cassette
- Spray the filter paper with glue and paste the slides on the filterpaper and put it in the cassette
- In a dark room put film on top of the slides and expose the slides for at least 2 weeks

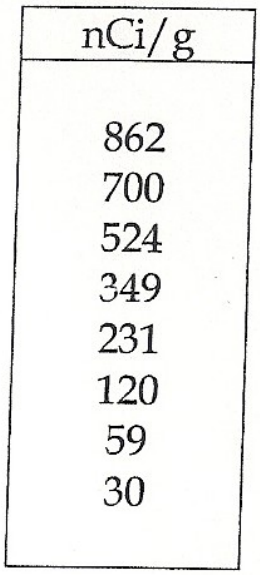
Important:

- Take controls when making use of new probes, to check the expression of probe on the film
- Take negative controls along with the slides for the In situ hybridization, this should be the labeled sense probe
- Take along a marker which contains Carbon-14 (attachment 1), so the amount of darkness on the film can be measured
- Don’t paste the slides too close to each other, but with some space between

Attachments

Attachment 1:

Figure of the Carbon-14 marker



Subject. Cortisol/corticosterone analysis from hair

Most recent adjustment made by: K.Hooijer Date: 25-04-2018

1. Introduction

Cortisol was shown to be incorporated into hair and correlate with circulating cortisol levels. Cortisol extraction from hair can be used as a measure for the relative amount of circulating cortisol over the time of the growth of the hair. By this means long term assesments of relative stress hormone levels can be made analyzing cortisol from hair. The technique was used in a lot of different species and can also be used analyzing corticosterone from bird feathers. A lot of reviews describe the possible influence of fur colour (dogs), hair lengths, and influences of washing.
Protocol adapted from a protocol from the BPRC adapted from a protocol by Davenport, 2006).

2. Chemicals

Chemicals	Supplier	Product Nr.	Location	Waste disposal
MeOH 				Non-Halogenated Waste
1x PBS				Drain
70% ethanol				
Isopropanol 				Non-Halogenated Waste
High Sensitivity Salivary Cortisol ELISA kit	Salimetrics	1-3002	Suffolk, UK	
Corticosterone RIA kit I125	MP Biomedicals,	07120102	Eschwege, Germany	

3. Materials

Accurate scale for minimum amounts (mg)
Aluminium foil + tape (for the mark)
Beads (Lab Services BV Biospec Products, 3.2 mm no 11079132)
Centrifuge
Combi-tip 25 mL Eppendorf
End-over-end roller
Eppendorf centrifuge tubes 2 mL(!!!), sure cap
Gloves
Instruments: Forceps, scissor and a scoop
Petri dishes
Pipet 1mL + tips and 200µL tips
Reaction vials 1,5mL, sure cap
Repeater-pipet
Sartstedt Micro tube with cap 2ml (ref 72.694)
Speed Vac Concentrator, Labconco
Stove at 37 ºC
Thermomixer, Eppendorf
Tissue Lyser II (Cat. No. 85300, Quiagen)
Tubes (50 mL)

4. Solutions

Dilution solutions provided with the respective kits

5. Procedures

<u>During whole protocol: keep samples in the dark! & wear gloves</u>

1. Shave at least 500 mg hair from the back of the neck
2. Store in aluminium foil, in the dark, at room temperature
3. Put some hair sample (approx. 50% of the tube) in a 50mL tube

Mark the tube, twice: one on top and one on the side (because work with –ol's), repeat this marking for all the tubes or reaction fails, during this protocol.

4. Wash the hair once in 20mL 1x PBS by roughly shaking (by hand) at room temperature for 2 minutes and decant the PBS, *at this step; most blood will be removed from the hair.*
   After the PBS wash, wash the hair twice with 20 mL isopropanol by gently mixing for 3 minutes per wash, decant the isopropanol and add new isopropanol for the second wash
5. After the wash, cover a petri dish with aluminium foil (with a mark!). Empty the tube above the dish.
6. Dry the hairs for 96h in a stove at 37°C (has to be thoroughly dry)
7. Remove the hair from the stove. If continue: Step 8.
   For storage: Fold the aluminium foil and make sure the mark (*the deer number)* is visible. Store in the dark at room temperature
8. Cut the hair with a scissor into pieces (as small as possible) on aluminium foil (approx. 80-90mg). Use a forceps to hold the hairs to minimize hair loss.
9. Put 3 beads(Lab services BV Biospec products, 3.2 mm no 11079132) into an marked Eppendorf tube of 2 mL(2 mL!!!- in 1,5 ml the balls cannot grind at the bottom of the tube). Weigh this on an accurate scale. Grab the hair with the forceps and put the hair in the Eppendorf tube. Weigh again and calculate hair weight. Place 60-70mg washed hair in sure cap Eppendorf centrifuge tubes.
   Clean the scissor and forceps with 70% ethanol, let it dry and collect the next sample.
10. Grind the hair samples with a Tissue Lyser II (Cat. No. 85300, 100–120/220–240 V, 50/60Hz, Quiagen) at 30 Hz during 15 minutes. Centrifuge (3 min, room temperature, 14000 rpm) and repeat for another 15 minutes. Centrifuge (Short spin). Ensure afterwards no clots of hairs are still present in the tubes. If necessary repeat until ground to powder.
11. Weigh in 35±2 mg hair (mink, deer) powder into a clean 2 mL Sarstedt tube with cap. (for rat and monkey (Rhesus and Java) use 50 mg hair). Note the exact weights.
    Clean the spatula with ethanol 70%, let it dry and weigh the next sample.
12. Add 1.5 ml methanol (using a combitip 25mL Eppendorf, on a repeater-pipet with a 200µl pipet-tip attached) and incubate the tubes at room temperature overnight on an end-over-end roller to extract the steroids.
    Make sure that the Sartstedt tubes are completely closed! Put 3 or 4 Sartstedt tubes in one 50mL tube.
    End-over-end roller present at third floor at room 384 in Nieuw Gildestein building Utrecht
13. Centrifuge (5 min, room temperature, 14000rpm) and pipet twice 650µL of the extract (1 mL pipet) into a clean reaction vial (1.5 mL). Centrifuge again (5 min, room temperature, 14000rpm) and use reverse pipetting to put exact 1 mL of the extract in a new reaction vial (1.5 mL).
14. Dry the methanol from the tubes in a Speed Vac Concentrator (CentriVap Concentrator Labconco) at 42ºC for 2.5 hours. The lids of the tubes have to be open.
    Speed Vac Concentrator present at second floor at room 272 in radioactive laboratory in Nieuw Gildestein building Utrecht

15. Dissolve the dried extracts of ferret and deer in 60µl phosphate buffer(assay buffer) that is provided in the essay kit (High Sensitivity Salivary Cortisol ELISA kit (Salimetrics) using the thermomixer at 50 ºC at 1400 rpm for 30 minutes with the lids of the tubes closed.
    (The rat extract were dissolved in 1 mL Diluent included in the corticosterone RIA kit MP Biomedicals, Germany).
16. Centrifuge the samples at room temperature for a short spin till 14000 rpm. Measure the cortisol with using the previous described ELISA kit by following the accessory protocol.
    Analysis are in duplicate.
    Plate mixer (DPC Micromix 5) present at laboratory of the Department of Animal in Science and Society, ground floor in Nieuw Gildestein building Utrecht; ELISA reader (Softmax Pro 5, Versa max microplate reader) present at second floor at room 236 in Nieuw Gildestein building Utrecht
    (Rat extracts were diluted 1:4 in diluent included in the RIA kit.)
17. Calculate the pg cortisol/ mg hair used (ng corticosterone/mg hair). (As the RIA from MP Biomedical is for plasma and all plasma samples are diluted 1:200 and the kit corrects for that, the results of the RIA analysis have to be DIVIDED by 200 to get the absolute pg cort/ hair).
    In EXCEL calculate OD 450nm (optical density) - OD 490nm and organize the data so the sample ID corresponds to the OD.
    Subtract the average OD NSBW (non-specific-binding-wells) from all the OD-values.
    Calculate log-values of the cortisol-standards' concentrations
    Open the program 'graph pad' -> use tutorial ELISA or RIA, follow this tutorial and, and put those log-values as known x-values in the program.
    The y-values are the OD's. Let the program calculate the X-values, this will be the log of the µg/dL, copy those to your EXCEL file
    Calculate the inverse log so the unknowns samples are now in µg/dL.
    <u>Compare the kit controls</u>, if it's in the range, continue, if not: re-do the whole protocol.
    The result (µg/dL) are multiplied by the amount of phosphate buffer (step 15) divided by 100 to convert units to [ng/µL]
    Multiply this by 1.5 (to control for loss; step12: 1.5mL -> 1mL (step 13)). So the outcome will be the total cort (ng) extracted
    Divide total cort [ng] by hair weight [mg] (step 11)
    Multiply this by 1000 to convert units to pg cortisol/ mg hair.
18. Clean the beads for re-use.
    After removing the beads from the tubes wash them by the following steps:
    1)Wash the dirty beads in a petri dish with tap water until they are visibly clean
    2)Put the beads in a 50 mL tube and add methanol
    3)Incubate this tube on a gentle mixer overnight
    4)Decant methanol and add pure ethanol
    5)Dry the beads and put them again in a clean petri dish

6. Reference

Davenport, M. D., Tiefenbacher, S., Lutz, C. K., Novak, M. A., & Meyer, J. S. (2006). Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. *General and Comparative Endocrinology*. Volume 147, Issue 3, July 2006, Pages 255-261, ISSN 0016-6480