The relation between environmental conditions, park management and chronic stress in Fallow Deer (Dama Dama)

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Abstract

Nowadays increased attention shows for the welfare of wildlife among the public, for instance the welfare implications for the housing of Fallow Deer in the Netherlands and the United Kingdom. Positive welfare is a state in which the animal has the freedom to adequately react to his living conditions and hence can reach a state which it perceives as positive. Under conditions of chronic stress, this adaptability may be compromised. Chronic stress results in impaired negative feedback of the hypothalamus-pituitary-adrenal axis, leading to prolonged and excessive release of cortisol in the circulation, followed by the accumulation of cortisol in hair over the course of weeks/months. Deer samples were obtained for fallow deer from the Amsterdamse Waterleidingduinen, the Netherlands and the different deer parks in the United Kingdom. In this study, brain samples these deer were sliced for the analysis of corticosteroid receptor levels in deer hippocampus, and an ELISA was conducted to measure the levels of cortisol in hair samples of these same deer.

The results from the AWD showed that the cortisol levels from the winter of 2015/2016 were significantly higher than from the winter of 2016/2017. that population density might be an important factor in chronic stress in fallow deer.

The data from the United Kingdom was analysed for possible correlations between the cortisol levels and the different park factors. Supporting the finding of the AWD, was the fact that for the UK data a trend towards significance was shown for the fallow deer density. When including also red deer, a significant correlation was found between the levels of cortisol and the total deer density. This can be explained by the experienced social stress and food availability. When comparing different cull winters, a significant difference was found, suggesting climate could also be an important factor. However, when considering the climate differences between the parks, only the average temperature in May and June was found to be significantly correlated with hair cortisol. Higher temperatures in May and June increases the germination and the growth of grass and therefore likely reflects increased food availability. Human disturbance showed a negative correlation with the levels of cortisol in hair, although this was only a statistical trend. This negative correlation might be explained by habituation of deer to the public in parks with high levels of human interference.

In conclusion, a substantial factor in the welfare of fallow deer are population density and the average temperature in May and June, which can be explained by experienced social stress and low food availability.

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Introduction

Fallow deer

The fallow deer (*Dama Dama*) is a native species to the western Eurasia, but also common among other countries. Belonging to the family of Cervidae, the fallow deer are present in The Netherlands and The United Kingdom, the provenance of our samples. Fallow deer are one of the most important ornamental park species, but can also provide substantial revenue from their venison (British Deer Society, 2017). Unfortunately, truly wild habitats no longer exist, as humans have had a comprehensive impact on the environment with the result of limiting natural habitats and therefore natural behaviour of wildlife (Sanderson et al., 2002). The deer adapted to the areas left to them, preferring grass pastures and mixed woodlands.
Nowadays, in the absence of a natural predator, culling is usually necessary to retain a healthy population and ecosystem. To protect the herd from starvation and health risks as a result of a large population, however the management strategies differ in the deer parks. The aim of this project is to determine which factors contribute to deer welfare. First the topic about public attention and accountability will be elaborated, then welfare and stress will be elucidated.

Public attention and accountability

Nowadays increased public attention shows for the welfare of wildlife (De Groot; KNMvD, 10 januari 2017; Staatsbosbeheer). In order to deliver solutions for the perceived welfare issues this subject finally gets the attention and funding it deserves.
As stated before, humans have had a comprehensive impact on the environment with the result of limiting natural behaviour of wildlife. This so-called Human Footprint represents the total of ecological effects caused by the human population (Sanderson et al., 2002). The effects include the transformation of land for (sub)urban development, agriculture, roads and power infrastructure. Ultimately enabling the greater consumption of resources by the increasing human population at the expense of nature. *Sanderson et al.* state that the global extent of the human footprint requires that humans are stewards of nature, whether we like it or not (Sanderson et al., 2002).
The history of animal welfare science was reviewed by *Broom et al.* stating that information regarding neurobiology became available for the public due to the media, which has been revolutionary in its impact on human attitudes (Broom, 2011). Studies of behaviour and how the brain controls behaviour show great similarities between people and a wide range of other species. A risk in the interpretation of animal behaviour and welfare is the over anthropomorphizing of animals by the public, with a result that people are less likely to see animals as an object, or as a being of no consequence. A development of major importance to the emerging concept of animal welfare. At the same time this requires more information about what welfare actually is and how welfare can be measured in an objective manner.
Altogether it is our moral duty to ensure the welfare of wildlife, such as the fallow deer, to develop new or assess current management strategies, however insight needs to be acquired regarding the current welfare of the deer. This raises the question: what exactly is welfare?

Welfare

Positive welfare is a state in which the animal has the freedom to adequately react to his living conditions and thereby can reach a state that it perceives as positive (Ohl & van der Staay, 2012). However, this capacity has its limits, for example when an individual is exposed to stress for a prolonged time because of illness, low food availability or high population density. As such, the prolonged exposure to such factors may negatively impact welfare, because they may ultimately render the animal unable to adequately react to his living conditions. A disturbance in the neuroendocrine responsivity to stress is a critical factor in this process, as will be explained below.

Originally animal welfare was assessed by means of observing the positive or negative emotional status of an individual, a process that is difficult to assess objectively (Yeates & Main, 2008).
In other words, the assessment of welfare requires a scientific, objective method to measure welfare.
Returning to the fundaments of the welfare definition: "the freedom to adequately react". The ability to adapt to challenging conditions is a sign of positive welfare, whereas not being able to adapt is a sign of negative welfare (Broom, 2011). When the conditions are beyond adequate adaptations capabilities the stress response can endure. Chronic stress can have deleterious effects and may impair the adaptive capacity of animals. These physiologic changes as a result of chronic stress are as such important candidates for the objective measurement of welfare, and therefore an indicator for negative welfare, as will also be explained in detail below.

Stress

To deal with stress, and therefore favoring survival, animals have evolved behavioral and physiological mechanisms. The stress response helps to restore homeostasis and thereby facilitates adaptation. The stress response is therefore a key reaction to any disturbance in the body or environmental conditions of any living organism (Mason, 1971). In fact, stress is not inherently bad, but rather an important coping mechanism.
Nevertheless, stress can have harmful effects also, the differences hinge on the duration, persistence, extent and reoccurrence of the secretion of the stress hormone, cortisol, in response to one or more stressor(s) (McEwen, De Kloet, & Rostene, 1986). If prolonged and severe, the exposure to environmental or internal stressors may exceed an animal's adaptive capacity. In order to adopt measures for chronic as indicators for welfare, it is essential to distinguish between acute and chronic stress, as I will explain in the text below.

Physiology of acute and chronic stress

- Acute stress
  In response to any disturbance in the homeostasis, the hypothalamic-pituitary-adrenal (HPA) system will be activated, see Figure 1. Centrally, during a stress response, neurons in the paraventricular nucleus of the hypothalamus, that express corticotrophin releasing hormone (CRH), will be triggered to release CRH. CRH in turn activates the pituitary to release adrenocorticotropic hormone, leading to the production of glucocorticoids (GCs) from the adrenal cortex (Blanchard, McKittrick, & Blanchard, 2001; Raone et al., 2007) , notably cortisol. These GCs generate major physiological changes, these include: the shift of energy towards the muscles, the increase of the cardiovascular tone, the stimulation of the immune system, inhibition of reproductive behavior and physiology, the decline in feeding and appetite, the increase of the cerebral perfusion and glucose utilization and final sharpened cognition (Sapolsky, 2000).
  To restore the balance after an acute stress response the HPA system includes a negative feedback loop, which is activated simultaneously during the stress response.

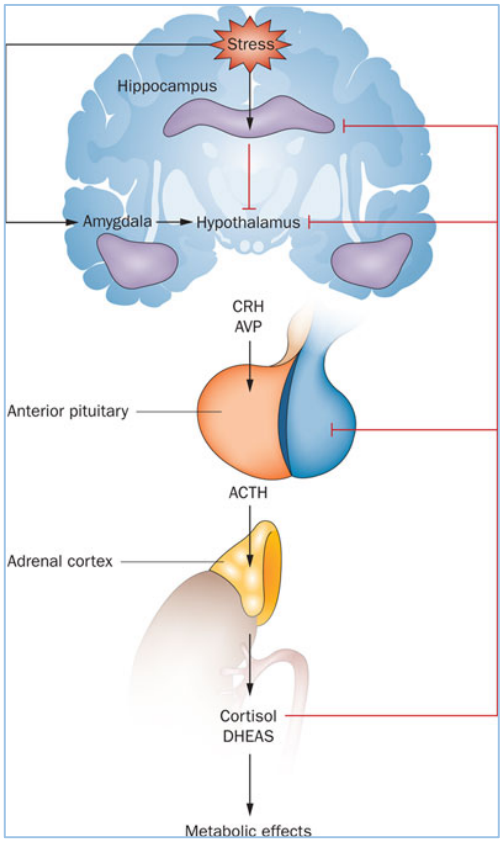

Figure 1 – Hypothalamic-pituitary-adrenal (HPA) axis (Papadopoulos & Cleare, 2012). The red line indicates the negative feedback.

This negative feedback involves the binding of GCs to glucocorticoid receptors (GRs) (MUNCK, GUYRE, & HOLBROOK, 1984). These receptors are located throughout the brain, though the negative feedback of the HPA system mostly involves the GRs in the hippocampus (Boyle et al., 2005; E. R. De Kloet, Vreugdenhil, Oitzl, & Joëls, 1998; Uno et al., 1994). The activated GRs facilitate the recovery from stress by inhibiting the activity of CRH neurons indirectly and thereby inhibiting and terminating the HPA axis activity (E. R. De Kloet et al., 1998; Reul & de Kloet, 1985). This is essential for the recovery from stress.

- Chronic stress
  Under conditions of chronic stress the presence of GCs in the circulation is prolonged and excessive (Calvano, 1984; Fuchs, Uno, & Flügge, 1995; Stoner, 1983). The excessive amounts of circulating GCs will in turn cause tolerance, involving a decline in the number of GRs in the hippocampus that will eventually result in an impaired negative feedback of the HPA system (Chiba et al., 2012; E. De Kloet & Reul, 1987; Maroulakou, Kitraki, & Stylianopoulou, 1992; SAPOLSKY, KREY, & McEWEN, 1984). As a consequence, there will be more GCs, resulting in a further derailed system, with consequences for brain and behavior, which will eventually hamper the individual's adaptive capacity (E. R. De Kloet et al., 1998; Raone et al., 2007). Taken together, brain GRs can be considered a reliable indicator for chronic stress, which is causal to impaired adaptive capacity and as such may also be considered a good indicator for negative welfare.

  However, measurement of GR levels can only be conducted in post-mortem material, restricting the assessment of the stress to the number of culled animals. Post-mortem samples are not in any case practical to assess welfare in the field. Moreover, this analysis can only be used to monitor wildlife populations when proactive culling is applied. Therefore, non-invasive methods need to be explored to obtain an actual status quo of the living herd. In situations of chronic stress, cortisol levels are permanently increased. This increase can be measured in blood, saliva, urine and feces (Sheriff, Dantzer, Delehanty, Palme, & Boonstra, 2011). The methods however are point measurements of the amount of cortisol present and are not by definition representative for chronic stress, but may also reflect an acute stress response. By contrast, cortisol accumulates in hair over the course of weeks/months. Therefore, cortisol in the fur of the deer could be a good (additional) indicator for chronic stress (Cone, 1996; Davenport, Tiefenbacher, Lutz, Novak, & Meyer, 2006; Erickson, Browne, & Lucki, 2017; Meyer, Novak, Hamel, & Rosenberg, 2014; Yu et al., 2015), although this has so far not been formally tested.

Measurement

In this study, a scientific, objective method to measure deer welfare is explored. In order to compare and assess the different management strategies regarding the housing of fallow deer. The deer welfare is assessed based on indicators for chronic stress that reflect negative welfare. To address this, we collected brain and hair samples from fallow deer from different parks in the UK and the Netherlands. We analyzed GR levels in deer hippocampus and cortisol in deer fur samples. Two ways of objectively measuring chronic stress are applied in this overall research.

- GR in hippocampus
  This potential method can only be conducted in post mortem samples, as pointed out before. The amount of GRs are determined in sub regions of the hippocampus. For the future, perhaps the amount of mineral corticoid receptors and corticotropin-releasing factor receptors are also relevant, but beyond the scope of this paper (Fuchs & Flügge, 1995; Ramot et al., 2017).
  However, the feasibility of the assessment of GR in the hippocampus is not in any case practical to assess welfare in the field. Moreover, this analysis can only be used to monitor wildlife populations when proactive culling is applied.
- Cortisol in hair
  As a non-invasive way to measure chronic stress, in which the amount of cortisol accumulated in the hair of the deer is determined, reflecting stress over the past weeks – months (Cone, 1996; Davenport et al., 2006; Erickson et al., 2017; Meyer et al., 2014; Yu et al., 2015). This method will be elaborated in this paper.

Hypotheses

We hypothesized that deer in parks with higher population density in comparison with deer in parks with lower population density, would have lower GR levels in the hippocampus, and higher cortisol levels accumulated in hair, reflecting chronic stress and lower welfare.

We hypothesized that deer in parks with higher human disturbance in comparison with deer in parks with lower human disturbance, would have lower GR levels in the hippocampus, and higher cortisol levels accumulated in hair, reflecting chronic stress and lower welfare.

We hypothesized that deer in parks with no supplementary feeding in comparison with deer in parks with supplementary feeding, would have lower GR levels in the hippocampus, and higher cortisol levels accumulated in hair, reflecting chronic stress and lower welfare.

We hypothesized that deer in parks with colder, more extreme winters in comparison with deer in parks with milder winters, would have lower GR levels in the hippocampus, and higher cortisol levels accumulated in hair, reflecting chronic stress and lower welfare.

Materials and methods

In this section the experimental details will be explained. This section consists out of different subsections, first the general tissue sampling, processing and analysing will be covered. Which is similar for the two datasets, as the samples were available from two different locations: the Amsterdamse Waterleidingduinen (AWD), The Netherlands, and different Deer Parks from the United Kingdom (UK). First the experimental details for the data set from The Netherlands will be discussed, followed by the dataset from the UK.

Tissue sampling and processing

The hair and brain samples were available per individual deer, enabling intra-individual correlation of brain GRs and hair GC levels. After being shot the deer were decapitated and the heads were then chilled and transported to either the University of Utrecht for the Dutch samples, or to the University of Glasgow for the UK samples. The deer heads were chilled at 4°C for 3 days before being processed. From the deer heads the fur samples were taken first, followed by the brain samples:

- Fur
  Approximately 500mg fur was shaved in front of the ears of the deer, which was stored in aluminum foil, in the freezer (-20°C, to kill any ticks).
- Frozen brain slices
  The deer heads were first cut along the midline. Subsequently both hemispheres were cut into 1 cm coronal slices using a 3D printed mold. The slices containing the hippocampus were stored in the –80°C freezer, awaiting further processing.

Hair Cortisol analysis

The protocol was adapted from a protocol from the Biomedical Primate Research Centre (BPRC), which was in turn adapted from a protocol by Davenport, 2006 (Davenport et al., 2006). Approximately 300mg hair was first washed in 30 mL 0,9% Saline solution, three times for 1 minute in the Multi Reax (Heidolph) at 2040 rpm. These steps are essential to make sure no blood contamination was present in the sample. These washes were followed by two washes with 15 mL isopropanol, to remove further contaminants such as sweat and sebum, gently mixing by hand for 1 minute per wash. To make sure the hairs were completely dry, the samples were placed in a stove at 37°C for at least 96h.
For maximal extraction of cortisol, the hairs were pulverized in a 2 mL Eppendorf tube at 30 Hz for 15 minutes with a Tissue Lyser II using 3 beads. After centrifuging for 3 minutes, the process was repeated for another 15 minutes. Subsequently 35 mg hair powder was dissolved in 1,5 mL methanol and incubated at room temperature for 24h on an end-over-end roller to extract the steroids. The tubes were centrifuged for 5 minutes at 14000 rpm, subsequently as much of the supernatant was extracted and centrifuged again. 1 mL of the steroid extract was then transferred in a new reaction vial (Eppendorf tube), after which the methanol was evaporated in a Speed Vac Concentrator at 42°C for 2.5 hours. The precipitate was re-dissolved in 60µL ready-to-use phosphate buffer (Salimetrics, USA) using the heating block at 50°C at 1400 rpm for 30 min, after which the samples were centrifuged at 14000 rpm for a few seconds. Quantification of the cortisol was then performed using a High Sensitivity Salivary Cortisol ELISA kit (Salimetrics, USA). The sample plates were read at 450 and 490 nm using a plate reader (Multiscan EK).
The absorbance results of the ELISA were transferred into concentrations (µg/dl) by means of a concentration curve and Graphpad Prism software. The cortisol concentrations (in pg/mg) were corrected for the use of 1 mL of the 1,5 methanol dissolved steroids during the procedure and the exact weight of the starting material.

The Amsterdamse Waterleidingduinen

With 3400 hectares (ha) this dune area is a nature reserve in the provinces of Holland, which is open for public. Housing the largest fallow deer population in the Netherlands (Waternet, 2015), this park is experiencing an extreme overpopulation (>3800 in 2016) (Waternet, May 2016). Resulting in a population density of approximately 1,1 deer per ha. Considering the overpopulation and impact on vegetation in this area, the decision was made to cull the fallow deer proactive, meaning healthy deer were culled to reduce population numbers (ANP Volkskrant, 2016). In order to balance fallow deer with other animals and plants in the area and reduce the nuisance for traffic and surroundings. The proactive culling takes place between November and March, to avoid disturbing the calving time and oestrous period. The deer receive no supplementary feeding during the winter times (Waternet, 2015).

The samples available from the AWD were from two consecutive years, therefore allowing a comparison between the two years for different population densities as the population diminishes every year as a result of the proactive culling.

The parks from The United Kingdom

As deer parks are abundant in the UK this is an appropriate study area for fallow deer. From the start of this overall research in 2013, information concerning the status quo of the deer and the involved parks themselves (Fig. 2) has gathered. This provides us the opportunity to analyse possible correlations between chronic stress and different management strategies or environmental conditions for the parks.
For every park a few standard factors were obtained, such as population density for fallow deer (FD) and the total ungulate density, human disturbance, supplementary feeding and climate. These factors will be elucidated individually per park below.
The human disturbance was determined in percentages (ha public access/total ha) by the parks and then scaled into low, medium or high (See Table 1). Climate information was obtained through the Met Office (website). We calculated the average temperatures from November-March, from January-February and May-June, the amount of days with snow and air frost, based on the information from the nearest Met Office climate station (Met Office, 2017).
For the individual fallow deer, information regarding their age, sex and cull date were also available.

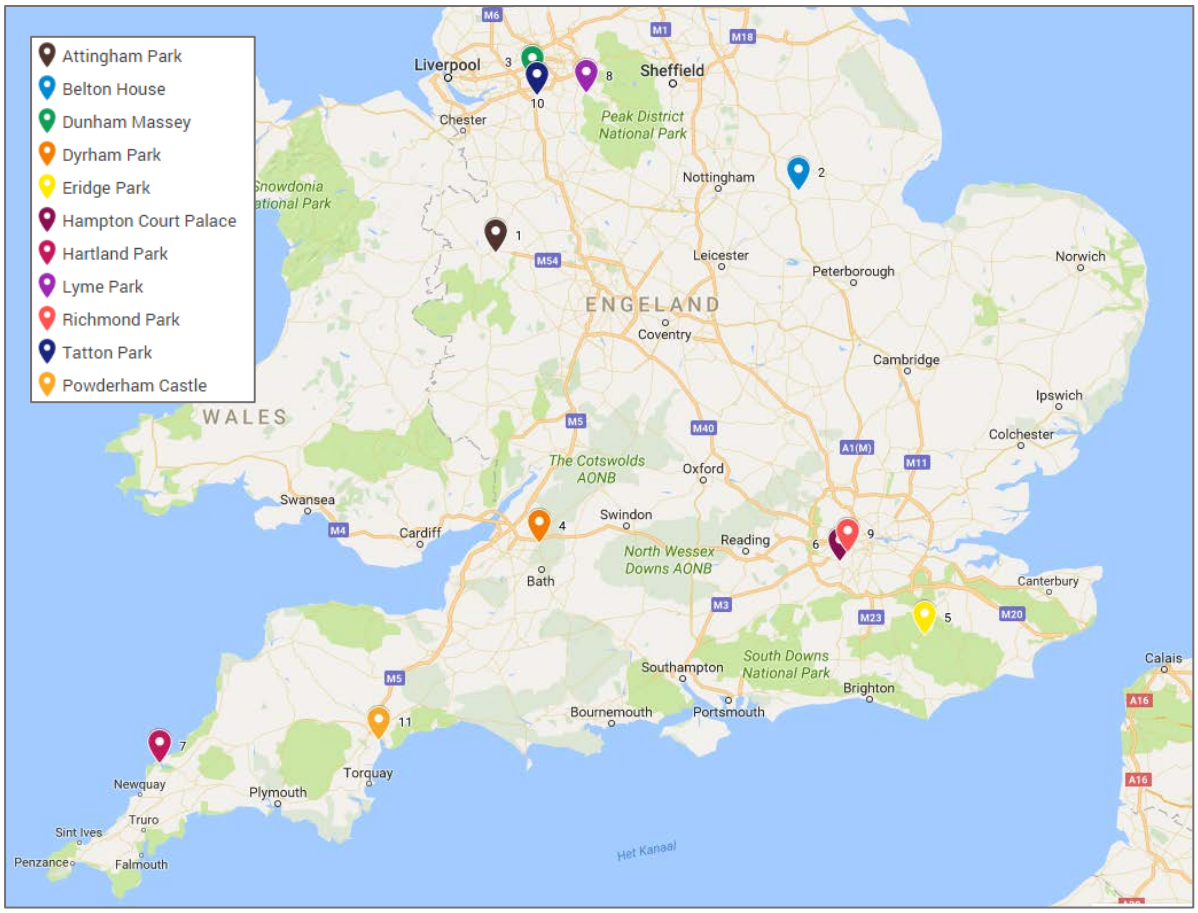

Fig. 2 – The locations of the deer parks in the United Kingdom. Numbered accordingly in the map.

Attingham Park (Attinghamd Park, 2017)
Surrounded by parkland this Mansion in the West Midlands of England covers 53 ha of land, with 43 ha of grass pasture. Approximately 196 fallow deer roam the land, resulting in a density of 3,7 deer per ha of land. During winter times the fallow deer are supplementary feed to help them through the colder weather. Only 5% of the pasture is open to public.

Belton House (National Trust, 2017)
This English country house in the East Midlands of England has a historic 303 ha deer park, divided in 295 ha of pasture and 8 ha of woodlands. Only fallow deer roam the park, approximately 700: from which 300 does, 200 bucks and 200 fawns. Resulting in a population density of 2,3 deer per ha. These deer receive no supplementary feeding during the winter.
The park is entirely open for public, so 100% public access.

Dunham Massey Park (Dunham Massey, 2017)
This great medieval deer park is 82 ha of mostly pasture-woodland from which 29 ha is grass pasture. Approximately 150 deer roam the park, resulting in 1,8 deer per ha. The animals receive supplementary feeding during the winter times from the park rangers. Only 46% of the park is open for public use.

Dyrham Park (Dyrham Park, 2017)
This late 17th century baroque mansion and deer park in South West England covers 95 ha, with 93 ha grass pasture and 2 ha woodland. Only approximately 178 fallow deer roam the park, from which 100 does, 38 bucks and 40 fawns. Resulting in a population density of 1,9 deer per ha. These deer are supplementary fed during winter time, depending on the weather conditions per day.
The public access of the park is 100%.

Eridge Park (Eridge Park, 2017)
This country estate resides in the South East of England and the total park covers 345 ha of which 174 ha is grass pasture. The remaining grounds are mostly woodland. The total number of fallow deer is approximately 700, from which 350 does, 145 bucks and 205 fawns. As a result the population density comes down to 2 deer per ha. During the winter the deer receive supplementary feeding. The whole park is open to visitors, resulting in 100% public access.

Hampton Court Palace (HCP) (Hampton Court Palace, 2017)
This Royal Palace in South East England is open to public as well as it 389 ha gardens, with around 287,9 ha grazing pasture. Containing 213 fallow deer, and therefore resulting in 0,5 deer per ha. No information was given about possible supplementary feeding. All of the grounds are open for the public (100% public access)

Hartland (Prideaux Place, 2017)
In South West England, this estate includes a deer park which covers 22 ha of grass pasture and contains 60 fallow deer. The population density comes down to 2,7 deer per ha. During the winter the deer are supplementary fed near the terrace tearoom of the mansion. In the actual deer park no visitors are allowed, so the public access is 0%.

Lyme Park (Lyme Park, 2017)
This estate consists of a mansion surrounded by 17,5 ha of pasture, of which 6,3 ha are grass pasture. Housing 46 fallow deer, which comes down to 2,6 fallow deer roam per ha. Red deer are also present in the park, however the number of red deer is currently unknown. In the winter supplementary feeding is provided. Only 80% of the pasture is open for the public.

Richmond Park (Richmond Park, 2017)
Belonging to the London's Royal Parks Richmond Park is the largest park with 1012 ha of land. From which 787 ha of grass pasture. Approximately 380 fallow deer roam the park, which are accompanied by 300 red deer. The 300 fallow deer comprise of 200 does, 100 bucks and 80 fawns. Resulting in 0,38 fallow deer per ha. The deer receive supplementary feeding during the winter times from December to April. Of the total grounds of the park only 80% is open for the public.

Tatton Park (Tatton Park, 2017)
This historic estate in North West England with approximately 430 ha of deer park, from which 340 ha of grass pasture and the remaining 90 ha woodland. Both fallow deer as red deer roam the park, accompanied by sheep in the summer. The total number of fallow deer is roughly 256, with 118 does, 28 bucks and 96 fawns. Resulting in a population density of 0,6 deer per ha. The deer receive supplementary feeding overwinter (December - April), which consists of carrots (1500kg), haylage (80 bales per year) and cattle nuts (50kg).
The coverage of the visitors in the park is about 70%.

Data Analyses

For the statistical analyses of the data SPSS 24 was used.
For the data from the AWD an independent t-test was preformed to assess possible statistical differences. Possible outliers were filtered using an outlier test for the data per year, followed by exclusion from further analysis.

For the samples from the UK the correlations between the park data (Table 1) and individual hair cortisol levels, average cortisol levels per park and standard deviation in cortisol levels per park were assessed (Spearman's SPSS).
In addition, an ANOVA was performed in order to assess a possible significant difference between the individual cortisol concentrations and the year/winter the deer were culled.

Table 1 – Overview of variables per deer park. * = Respectively the winters of 2013/2014, 2014/2015 and 2015/2016. X = Unknown.

Park	Attingham Park	Belton House	Dunham Massey	Dyrham Park	Eridge Park	HCP	Hartland	Lyme Park	Richmond Park	Tatton Park	Powderham Park
Amount of Samples	4	17	6	6	5	6	18	3	12	11	3
Amount Female	4	X	6	0	5	X	5	X	6	X	0
Amount Male	0	X	0	6	0	X	13	X	6	X	3
FD Density (FD/ha)	3,7	2,31	1,88	1,87	2,03	0,55	2,73	5,83	0,38	0,73	5,25
Total Density (Deer/ha)	3,7	2,31	1,88	1,87	2,03	0,55	2,73	28,8	1,05	2,55	*
Human Disturbance	Low	Medium	High	High	Medium -High	High	Low	Low	High	High	Low
Supplementary Feeding	Yes	No	Yes	Yes	Yes	X	Yes	Yes	Yes	Yes	X
AVG Temp Nov-Mar (˚C) (Met Office, 2017)	8,74	8,06	8,1	8,96	8,86	9,42	9,62	8,1	9,66	8,1	9,9
AVG Temp Jan-Feb (˚C) (Met Office, 2017)	7,6	6,95	7,05	7,85	7,55	8,2	8,65	7,05	8,45	7,05	8,8
AVG Temp May-June (˚C) (Met Office, 2017)	17,95	17,55	17,15	18,1	17,8	18,95	16	17,15	19,5	17,15	18,3
Days of Snow (Met Office, 2017)	2,77	1,02	2,02	1,89	X	X	0,29	X	1,31	2,02	2,77
Days of Airfrost (Met Office, 2017)	51,8	48,1	44,8	34,9	39,9	33,3	13,7	44,8	44,8	44,8	38,7
Winters # Available*	1	1, 2, 3	1, 2	3	1	3	1, 2	3	1, 2	2, 3	3

Results

The cortisol data were analysed separately and will be described accordingly.

The Amsterdamse Waterleidingduinen, the Netherlands

The individual cortisol concentrations for the fallow deer in the Netherlands, the AWD, are shown in Figure 3. For the winter of 2015/2016 11 samples were available, from which one outlier was excluded, namely AWD #5. For the consecutive winter of 2016/2017 25 samples were available.

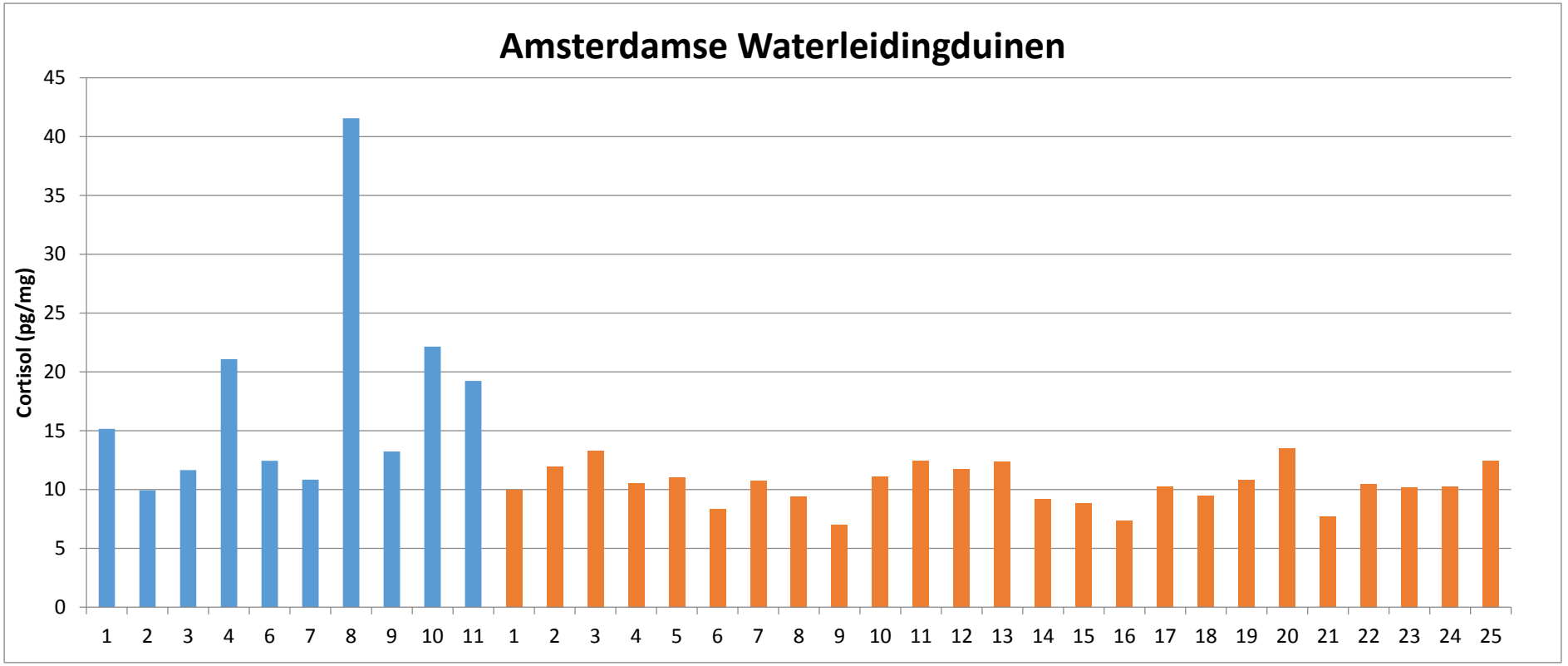

Fig. 3 – The individual measured concentrations of cortisol in pg/mg for the fallow deer of the AWD. The cortisol data for the samples taken in the winter of 2015/2016 are shown in blue, those of the samples taken in the winter of 2016/2017 are shown in orange.

For the comparison of the cortisol levels for deer in the AWD in the two consecutive years, the average cortisol concentrations were calculated per year. Figure 4 shows that the average cortisol concentration (pg/mg) for the winter samples of 2015/2016 (17,7 +/- 9,4) was significantly higher than for the samples taken in the winter of 2016/2017 (10,4 +/- 1,89, $t(35)=3,80$, $p<0,001$).

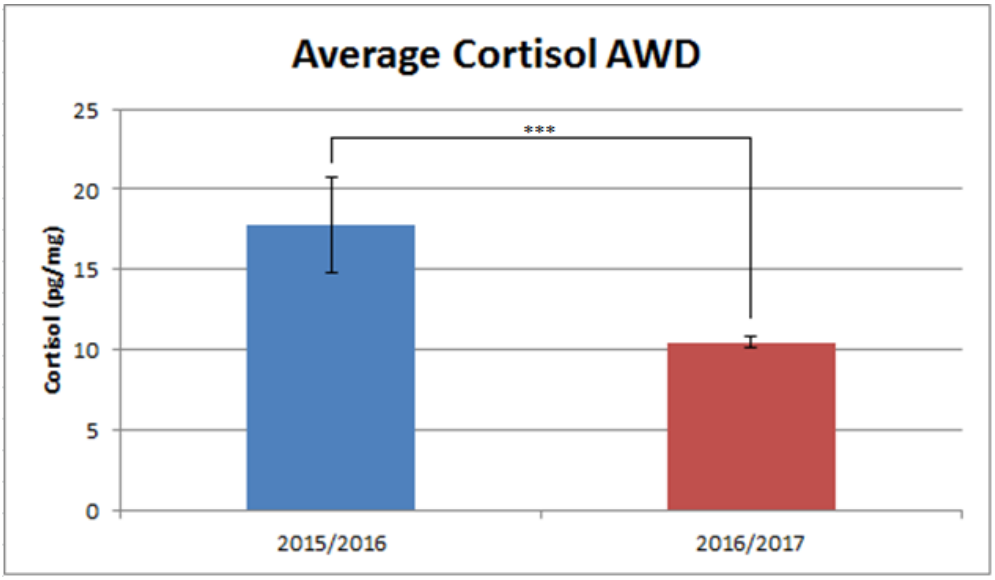

Fig. 4 – The average cortisol concentration of fallow deer from the AWD from the winter of 2015/2016 and 2016/2017. Displayed are the average cortisol concentrations in hair (pg/mg) from fallow deer in the AWD and the Standard Error of the Mean (SEM) for the two consecutive winters. $p < 0,001$

The United Kingdom

Figure 5 shows the individual cortisol concentrations (pg/mg) for the fallow deer in the UK. From these data 2 outliers (based on the data within respective parks) were excluded from the analyses, namely Hartland 161 and Richmond 5 (winter 2013/2014).

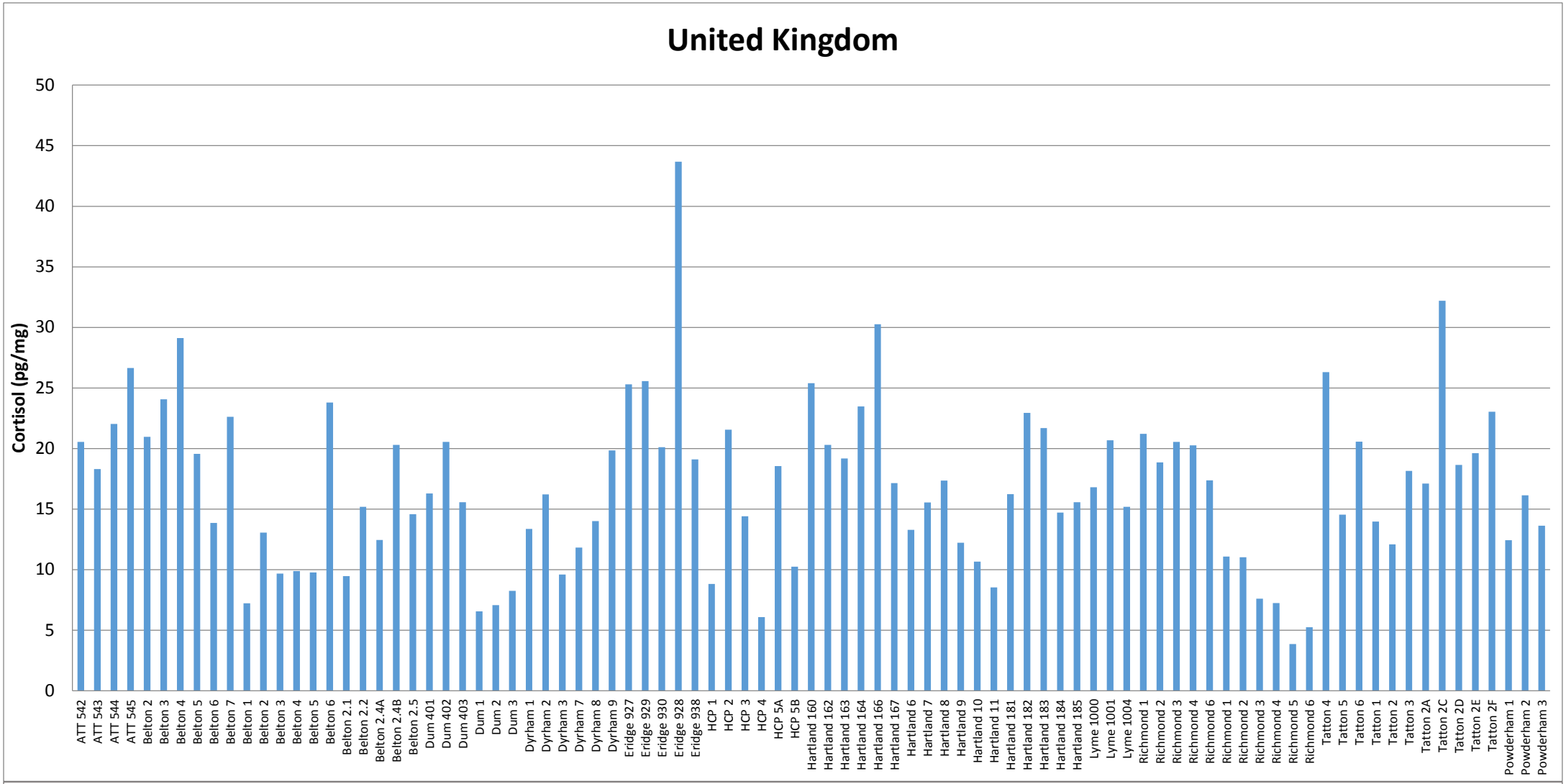

Fig. 5 – The individual cortisol levels in hair in pg/mg for the fallow deer of the UK parks. Abbreviations: ATT = Attingham, Dum = Dunham Massey, HCP = Hampton Court Palace.

In Figure 6 the average levels of cortisol per park are shown.

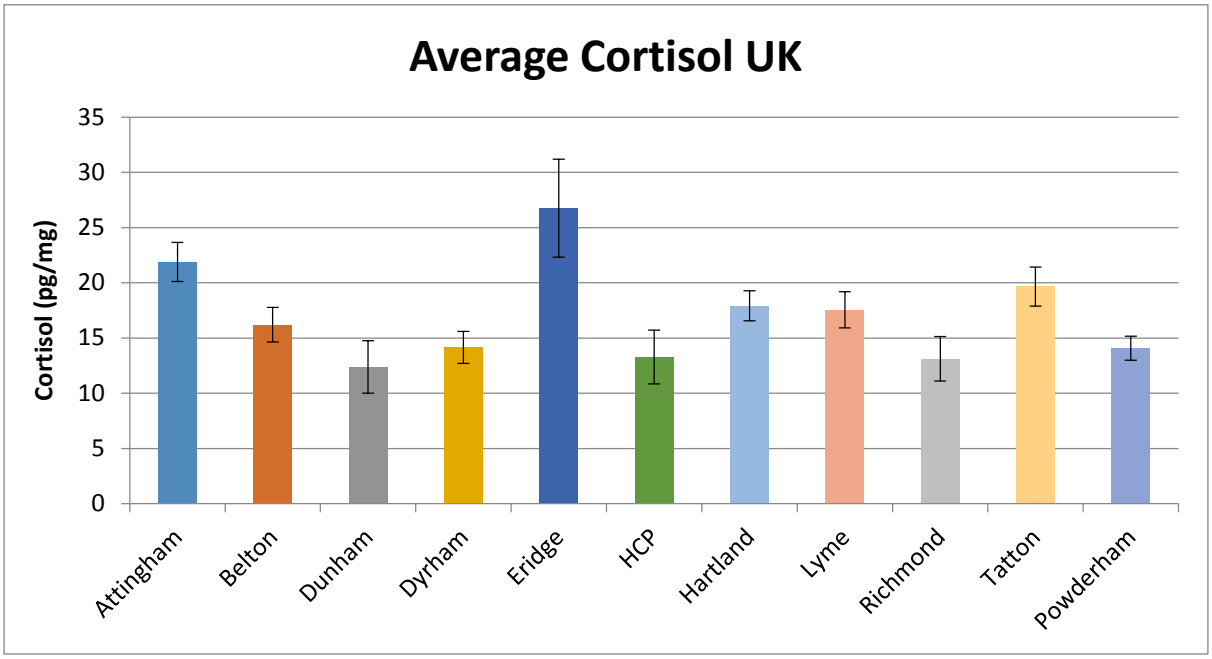

Fig. 6 – The average amount of cortisol (pg/mg) per deer park in the United Kingdom. Displayed the average concentrations of cortisol in the hair of the fallow deer ±SE.

To further compare the cortisol levels for the different parks and the impact of factors that differ between the parks on cortisol levels, the individual levels of cortisol in the deer were considered as well as the average cortisol levels per park and the standard deviation in cortisol concentrations per park. The results of the analyzed correlations regarding the different park factors are shown below and will be discussed further on in the paper.

Population Density

The population density as noted in Table 1 was plotted against the amount of cortisol per individual deer. Only a statistical trend towards significance (r_s=0,129, p=0,071) was found, see Figure 7. The correlation between fallow deer density and the average cortisol per park was non-significant. However there was a significant (r_s=-0,664, p=0,026) correlation between the fallow deer density and the standard deviation of the amount of cortisol in hair for deer in the individual parks.

Lyme Park, Richmond Park and Tatton Park also house red deer. When including other species of deer into the equation, the overall ungulate population density for these parks is therefore higher than the fallow deer density. For Powderham Park no total deer density was known, and for that reason this park was left out of the total deer density correlation analyses. As can be seen in Figure 8, there is a significant correlation between total population density and cortisol concentration (r_s=0,321, p=0,003). In line with these findings, the correlation between the total population density and the average cortisol concentration per park was also significant (r_s=0,648, p=0,043). Moreover, there was a strong trend towards a significant correlation of the total density with the standard deviation of cortisol per park (r_s=-0,624, p=0,054).

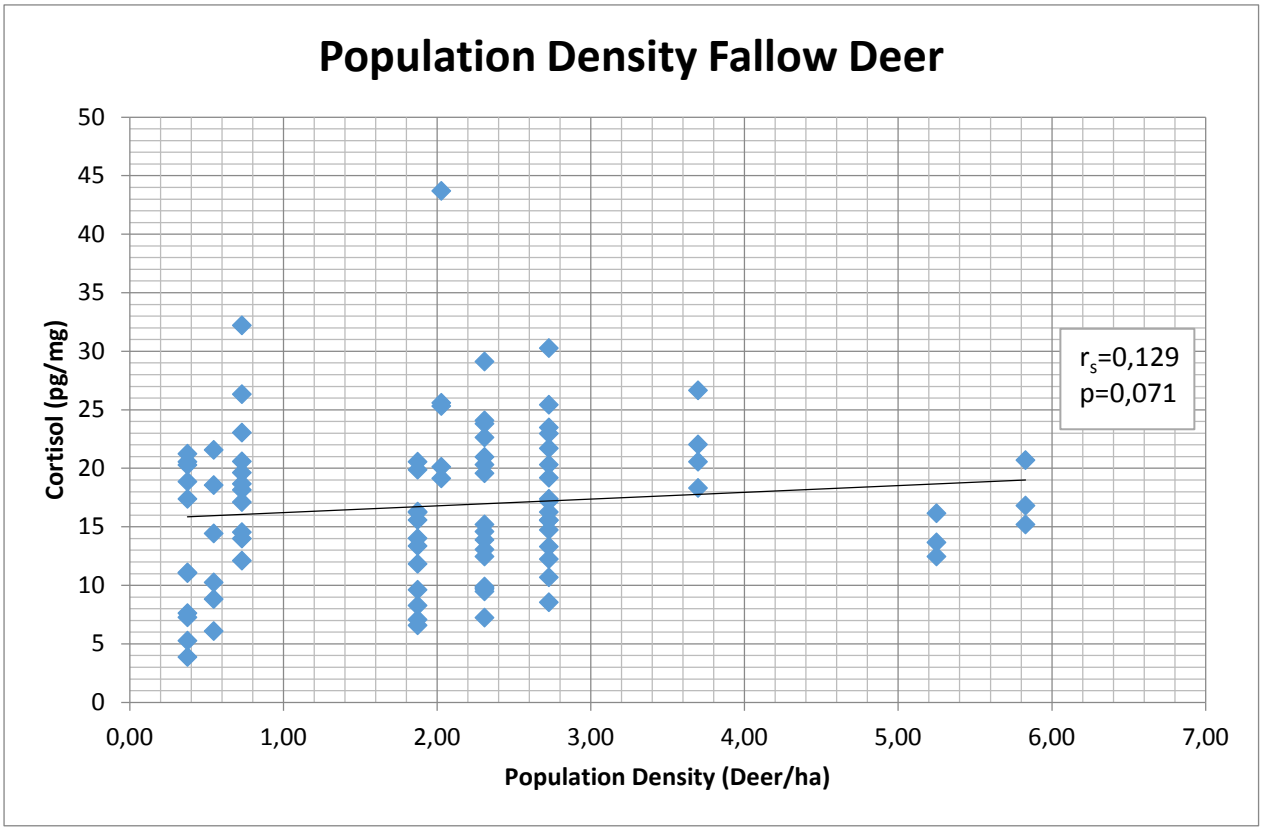

Fig. 7 –A strong trend towards correlation between population density and the amount of hair cortisol in fallow deer across the UK. r_s=0,129, p=0,071.

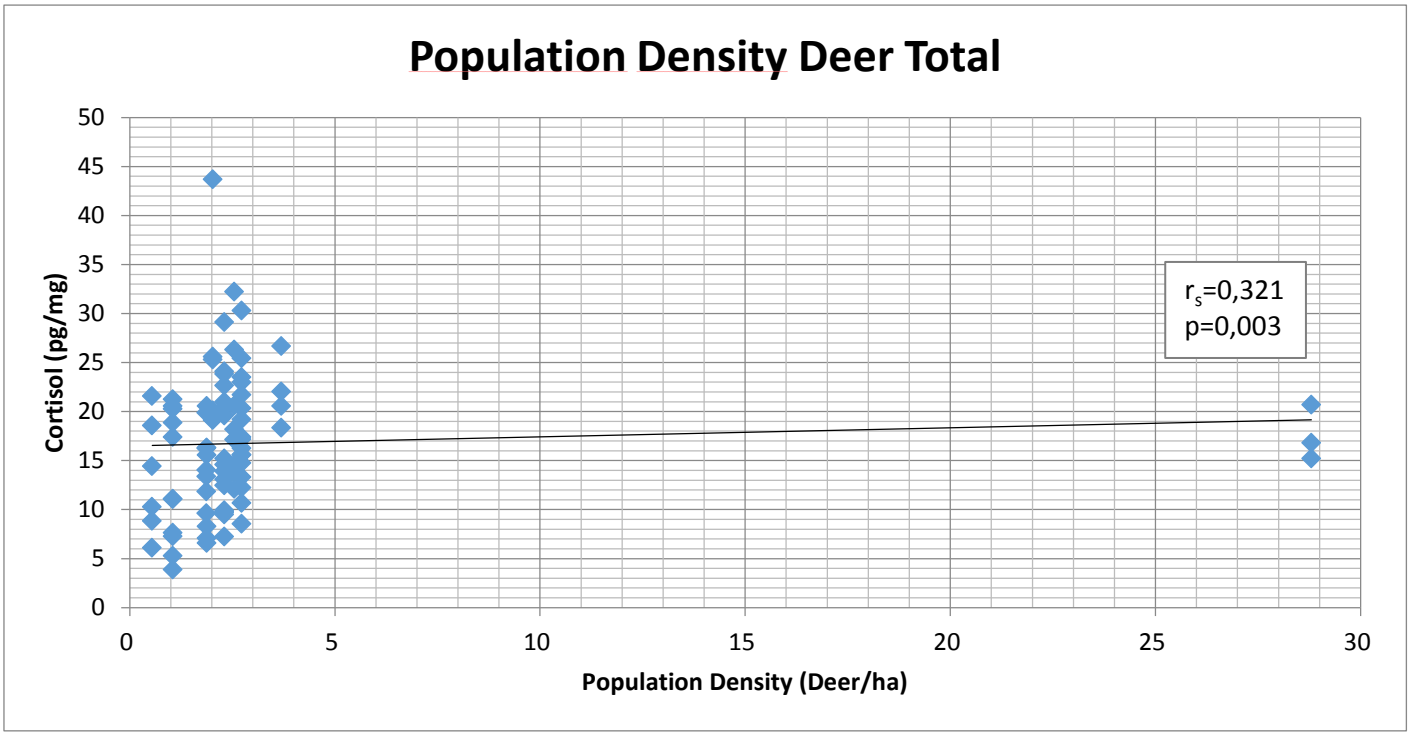

Fig. 8 –A correlation between the total deer population density and the amount of hair cortisol in fallow deer across the UK. r_s=0,321, p=0,003.

Year

The samples were collected over 3 consecutive years, i.e. the winter of 2013/2014, 2014/2015 and lastly 2015/2016. Year was found to be of significant influence on the concentration of cortisol levels in hair ($F_{(2,88)year}$=20,8, p<0,01), as shown in Figure 9. Posthoc multiple comparison revealed that the cortisol levels for 2013/2014 were significantly higher compared to the winter of 2014/2015 and the winter of 2015/2016 (p<0,001). These results suggest that possibly the climate prior to culling affected the amount of cortisol present, as the other factors of the different deer park remain stable over the different years.

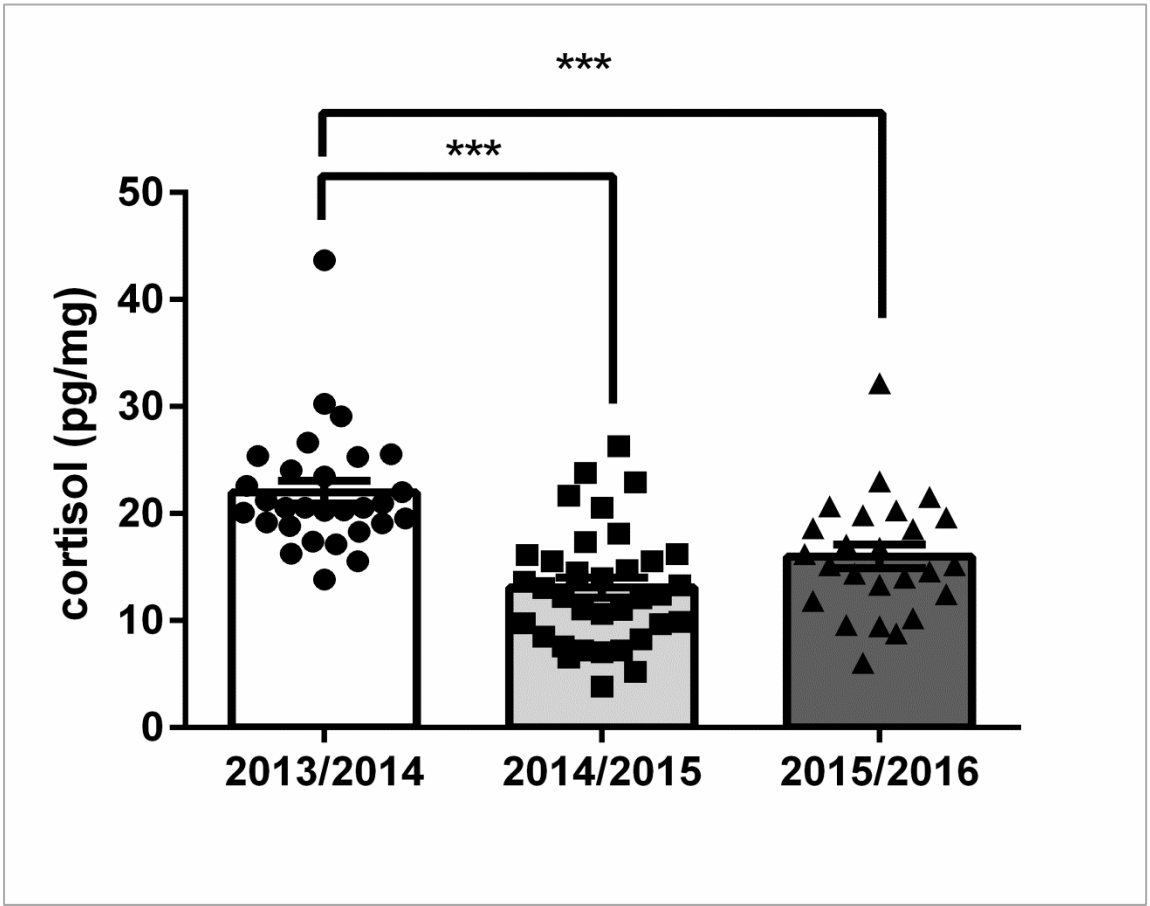

Fig. 9 – The relation between cortisol concentrations and the winter the deer were culled. Winter 1 = 2013/2014, 2 = 2014/2015 and 3 = 2015/2016. $F_{(2,88)year}$=20,8, p<0,001

Several potential correlations were explored between the amount of cortisol and climate factors, such as the average temperatures from November-March, January-February and May-June, the amount of days with snow and air frost (Table 1, Met Office(Met Office, 2017)).
The correlation between the amount of cortisol and the average temperature in May-June, as shown in figure 10, was found to be significant (p=0,005). However, other climate factors did not correlate with hair cortisol levels.

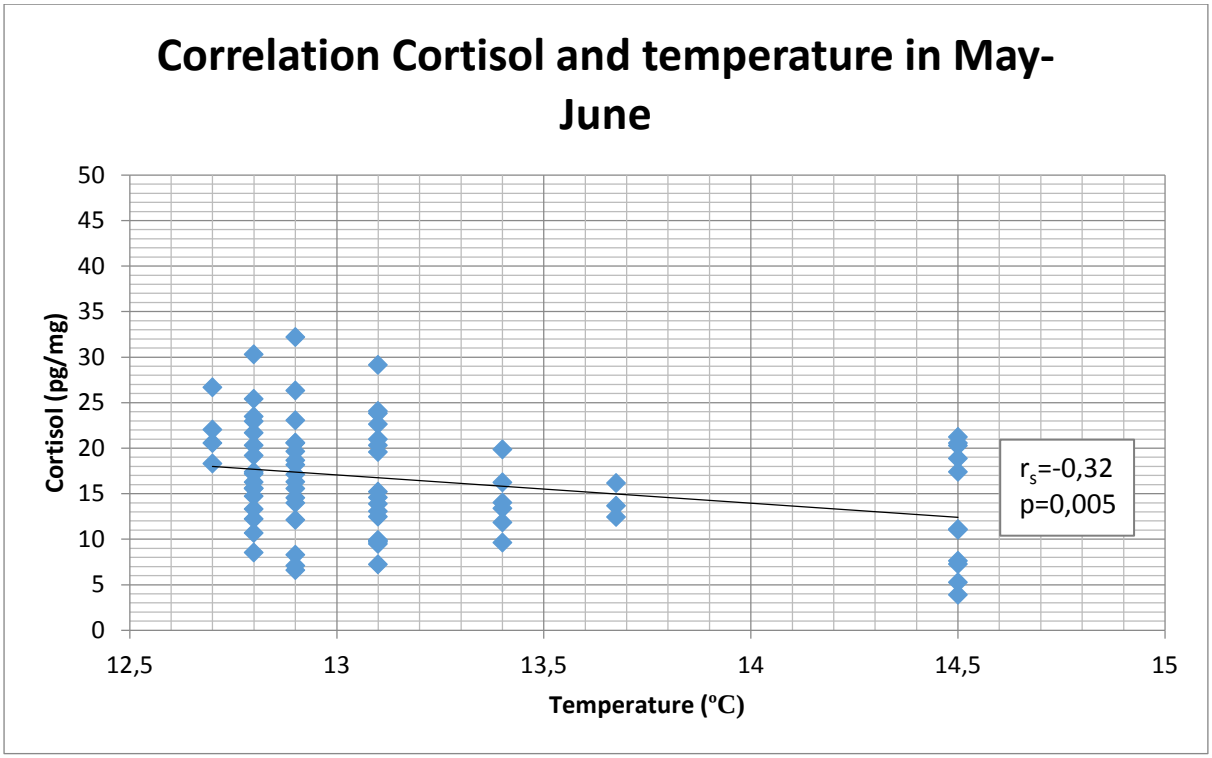

Fig. 10 – The correlation between the amount of cortisol and the average temperature in May and June. R=-0,32, p=0,005

Human Disturbance

This factor was taken into account as the deer may experience additional stress when (more) public can access their living area. However the correlation of human interference with the individual hair cortisol concentrations only reached a trend towards significance (r_s=-0,201, p=0,059) (Fig. 11). There was no significant correlation for human disturbance with the average cortisol levels per park or the standard deviation per park.

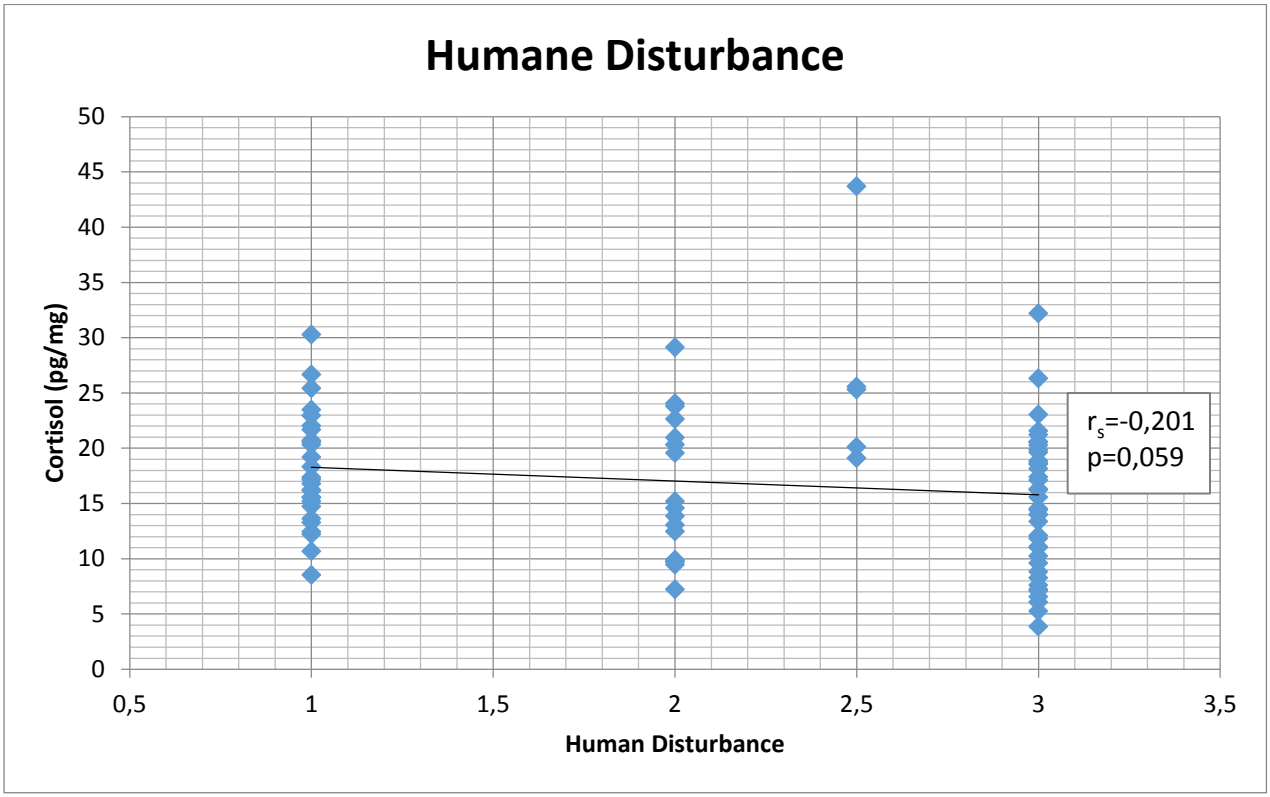

Fig. 11 – The correlation between the amount of cortisol (pg/mg) and the amount of human disturbance. The human disturbance was categorized as follows: 1 = low, 2 = medium, 3 = high. R=-0,201, p=0,059

Supplementary feeding

The data available for supplementary feeding were categorized into if the deer either received supplementary food during the winter times or not. There were no significant correlations between supplementary feeding and the levels of cortisol accumulated in hair.

Discussion

The main objective of this study was to determine which park management and environmental factors contribute to chronic stress in wild roaming fallow deer. For that purpose, we preformed correlation analyses for the different factors with the amount of cortisol present in the hair of the fallow deer. Two datasets were available, i.e. data from the AWD in the Netherlands and data from different deer parks in the UK. Our results show that population density in the deer park and the average temperature in May-June contribute to the welfare of fallow deer. These impact of these factors on welfare may be explained by differences in the extent of social stress and food availability (Côté, 2000; Degoutte et al., 2006; Hall, 1983; Jordan & Haferkamp, 1989; Krieger, Crowley, O'Donohue, & Jacobowitz, 1980; Ozoga, 1972; Townsend & Bailey, 1981; Went, 1953)

The AWD is experiencing extreme fallow deer overpopulation. The policy to proactively cull fallow deer was established last year with the goal to reduce the population numbers from ±3800 fallow deer to 800 in 5 years. Therefore the population density declines every year. We observed significantly higher average cortisol levels in deer that were culled in the winter of 2015/2016 in comparison with the winter of 2016/2017. Although the decline in fallow deer from last year to the current year is relatively small, these initial findings suggest that, as we hypothesized, a high population density may provoke chronic stress (Sibly, 1999). However more factors may be responsible for the apparent difference in chronic stress, reflected by the amount of cortisol in hair, in these fallow deer. For instance no data was yet collected regarding the climate factors during the two distinct winters/years. Whereas the weather conditions may influence the amount of food available (Jordan & Haferkamp, 1989; Went, 1953). Furthermore, the influence of the proactive hunting should not be underestimated. As this might be dissimilar for the two years, and the following years, as the hunters gain more experience for example. Additionally the amount of visitors may contribute to the amount of stress. This number may differ per year and should therefore be taken into account. Further research on this subject is essential to further substantiate our hypothesis. If a strong decline in cortisol is established in the following years, this will support the hypothesis that population density is an important factor in chronic stress for fallow deer.

Also for the UK data we observed a strong trend towards a significant correlation of fallow deer density with the individual hair cortisol levels. This indicates that population density might be of importance in the development of chronic stress, therefore giving the grounds for further exploration. In this analysis the presence of other ungulates was not taken into account. In different parks, including Lyme park, Tatton Park and Richmond park red deer (*Cervus Elaphus*) also roam the grounds. This means that in these parks the amount of total deer per hectare is in fact higher, and possibly influencing the amount of stress experienced. Therefore, we also compared the total deer density with the individual levels of cortisol in hair, which revealed a significant correlation. These findings suggest that (1) all deer in a park should be taken into account and (2) deer density is an important factor for chronic stress in wild roaming deer, and therefore for deer welfare.

The deer were culled in different years. Much similar to the AWD data, cortisol levels were significantly different across the years. Climate may be an important factor in this. However, we do not (yet) have detailed information for the different parks for the individual years.
Moreover, as the information is originating from Met Office Stations across the UK and not from the parks themselves, and some parks were close to the same Met Office Station, the climate data does not differ per park. It is important to realize that the actual climate factors may not be equal for these parks. As such, the use of Met Office data is a limitation in data resources. Furthermore, the information available was an average of 15 years, rather than per year. That information would be

essential to determine whether climate differences across years contributes to the differences in hair cortisol between the years in which the samples were taken.
Nevertheless, in contrast to what I expected, the average temperature in May and June was found to be significantly correlating with the individual amount of cortisol, not the hypothesized average winter temperature, days of frost or snow. Suggesting that higher temperatures during May and June result in lower cortisol levels in fallow deer.

The significant correlations with the population density as well as the average temperature in May and June suggest that these two factors are important for the development of chronic stress in fallow deer. A high population density may be associated with social stress, due to the enforcement of a hierarchy in the herd of possibly the rutting season (Colborn, Thompson, Roth, Capehart, & White, 1991; Côté, 2000; Hall, 1983; Ozoga, 1972; Townsend & Bailey, 1981). And population density as well as the average temperature in May and June might both be determined by the food availability per fallow deer (Degoutte et al., 2006; Jordan & Haferkamp, 1989; Krieger et al., 1980; Ozoga, 1972; Went, 1953). As more ungulates graze the same amount of grass pasture, less grass is available for the individual. And restriction of food is known to increase the cortisol levels, and therefore the amount of stress experienced (Degoutte et al., 2006; Krieger et al., 1980).
The average temperature in May and June correlates with the growth of the grass in the summer of the UK. For as the germination of grass seeds positively correlates with the environmental temperature (Jordan & Haferkamp, 1989), and plant growth is depending on the environmental temperature as higher temperatures can stimulate the metabolism of the plants (Went, 1953). Hence it can be stated that the food availability may be higher after warmer temperatures in May and June. This could in turn explain the lower cortisol levels, reflecting lower chronic stress and higher welfare, in parks where the temperature in May and June is on average higher.

Additionally to these findings we found a statistical trend towards significance for human disturbance. A negative correlation, in which a higher human disturbance meant lower cortisol levels. I expected an inverted result, that higher human disturbance would generate a higher stress response. Though the results indicate otherwise. This negative correlation might be explained by the habituation of the animals to the public, because the human disturbance is more predictable and consistent for these animals (Recarte, Vincent, & Hewison, 1998).
Although for the human disturbance we only looked at the amount of hectares of the park accessible for the public. Passing the possible effect of other forms of human disturbance such as the deer hunting. The rules regarding the deer hunting will differ per park, and may therefore be an additional factor in human disturbance. The hunting should be taken into account when dividing the different deer parks in categories of human disturbance as it may provoke an stress response in the herd. The effect dependent on the skills of the hunter, the number of culled deer and the duration of the culling period, and therefore a potential subject for further research.

Data on supplementary feeding was available, however this data was extremely divergent and therefore difficult to compare. For that reason the decision was made to categorize the data into supplementary feeding during the winter times yes or no. No significant correlation was found with the levels of cortisol, possibly due to the lack of distinctiveness.

These outcomes are promising for further research regarding the welfare of fallow deer. Nevertheless more factors ought to be explored on this subject. I was not able to correlate the sex of the individual deer with the levels of cortisol, for the simple reason that the data set was incomplete.
Ideally the data received from the UK parks would be rated, classified and documented by the same expert, to limit the variation in the data and to ensure the completeness of the data set. Especially

referring to the classification of the supplementary feeding, considering that food availability might be an important factor in deer welfare.
For the samples of the AWD it would have been interesting to analyze possible correlations between the levels of cortisol and the climate factors, as for the UK parks. Unfortunately due to the lack of time, I was not able to obtain the information for the different years. Ideally the AWD data would have been integrated into the UK data set, to possibly strengthen the correlations.
Within my research I only looked at possible correlations between the cortisol levels and the park factors. When looking at the average cortisol levels per deer park in the UK, it's noticeable that Erigde park distinguishes itself from the other parks. When evaluating the park factors as mentioned in table 1, no apparent factors stand out. However in this research we did not compare the parks to each other directly.

Additional research regarding the population density may be necessary to expand the knowledge on the subject. Including for example red deer (*Cervus Elaphus*) and roe deer (*Capreolus Capreolus*), but also other ungulates, in the population density correlation with the levels of cortisol in fallow deer. As many parks keep more than one species in their pastures, the question raises: How do these interspecies enclosures influence the welfare of these animals?

In conclusion we can state that a substantial factor in the welfare in fallow deer are population density and the average temperature in May and June, which can potential be explained by experienced social stress and low food availability. Whereas these factors might compromise the adaptation of the fallow deer and prolonged exposure may negatively impact their welfare. Both correlations with the population density and the average temperature in May and June can be related to food availability and therefore we can state that this is an essential factor in chronic stress in fallow deer.

This overall research may deliver the solution, at least a part of it, for the perceived welfare issues of wildlife by the public. For we can partly provide scientific substantiation for the causes of chronic stress in fallow deer. And therefore alter management strategies in the deer parks in which deer were found to have increased cortisol concentrations, to reassure wildlife welfare.

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Appendix I

SOP Cortisol/corticosterone analysis from hair

Most recent adjustment made by: T. Helwig Date: 01.05.2017

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 assessments 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 color, 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
MeOH			
isopropanol			
High Sensitivity Salivary Cortisol ELISA kit	Salimetrics	1-3002	Suffolk, UK
Corticosterone RIA kit I125	MP Biomedicals,	07120102	Eschwege, Germany

3. Materials

- aluminium foil
- 50 mL tube
- stove at 37°C
- sure cap Eppendorf centrifuge tubes (2 mL)
- beads (Lab services BV Biospec products, 3.2 mm no 11079132).
- Tissue Lyser II (Cat. No. 85300, Quiagen)
- Centrifuge
- Scale for weighing 35 mg
- combitip 25mL Eppendorf
- repeater-pipet
- rotator wheel or shaker
- reaction vial (1.5 mL)
- vortex
- heating block
- Dilution solutions provided with the respective kits

4. Procedures

During whole protocol: keep samples in the dark! And wear gloves.

1. Shave approximately 500 mg hair from in front of the ears
2. Store in aluminium foil, in the dark, in the freezer to kill of any remaining ticks
3. Wash the hair three times with 30 mL physiologic salt water by using the multi reax (Heidolph) at max speed (2040 rpm) for 1 minute, decant the water and repeat procedure 2 more times.
4. Wash the hair twice with 20 mL isopropanol in a 50 mL tube by gently mixing (by hand) at room temperature for 3 minutes per wash, decant the isopropanol and add new isopropanol for the second wash
5. Dry the hairs for at least 96h in a stove at 37°C (has to be thoroughly dry) in a petri dish covered with anti-static paper.
6. Remove the hair from the stove and cut it with a scissor into small pieces on the aluminium foil. Use a forceps to hold the hair to minimize hair loss.
7. Place 60-70 mg washed hair in sure cap Eppendorf centrifuge tubes (2 mL - in 1,5 ml the balls cannot grind at the bottom of the tube) with 3 beads (Lab services BV Biospec products, 3.2 mm no 11079132).
8. 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, 2200) and repeat for another 15 minutes. If necessary repeat until ground to powder.
9. Weigh in 35 mg (+/- 5mg) hair powder into a clean 2 mL sure close Eppendorf tube by using a accurate scale. Note the exact weights. To reduce variability, try to use similar amounts of hair powder for each sample.

To reduce variability: All samples together from this point onwards.

10. 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 for 24h on an end-over-end roller to extract the steroids.
11. Centrifuge (5 min, room temperature, 14000rpm) and place as much as possible of the extract in a clean reaction vial (1.5 mL), for example pipet two times 650 µl . Centrifuge again (5 min, room temperature, 14000rpm) and place 1 mL of the extract in a new reaction vial (1.5 mL). Use reverse pipetting for this step.
12. Dry the methanol from the tubes in a Speed Vac Concentrator (CentriVsp Concentrator Labconco) at at 42 °C for 2.5 hours. The lids of the vials need to be open.
13. Dissolve the dried extracts in 60µl phosphate buffer that was provided in the essay kit (High Sensitivity Salivary Cortisol ELISA kit (Salimetrics) using the heating block at 50°C at 1400 rpm for 30 min (lids closed),
14. Centrifuge (3 min, 4°C, 14000)
15. Measure cortisol with using High Sensitivity Salivary Cortisol ELISA kit (Salimetrics) kit using dissolved extracts without further dilutions and following the instructions for the authors and read at 450 and 490 nm on a plate reader (Multiscan EK). Analysis was performed in duplicate. Corticosterone extracts were dissolved 1:4 in diluent included in the RIA kit. Analysis was performed in duplicate.
16. Calculate the ng 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 DEVIDED by 200 to get the absolute ng cort/ hair.

Reference

Matthew D. Davenport, Stefan Tiefenbacher, Corrine K. Lutz, Melinda A. Novak, Jerrold S. Meyer, 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,