Chronic stress in deer

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Abstract

Wildlife welfare is an emerging topic in our society, which requires methods for evaluating welfare. The welfare status of an animal is defined by the adaptability of that animal to different challenging conditions. Different aspects from different disciplines, such as the mental or physical state of an animal can influence the ability to adapt. Therefore, an interdisciplinary approach is needed to evaluate welfare. Chronic stress is one aspect that can lead to a modified mental or physical state and can therefore be measured to evaluate the welfare state of an animal. So far, it has been difficult to measure stress and to differentiate between acute and chronic stress. Stress can be measured using blood, excreta, or salivary samples. These samples mostly represent acute stress, because of the quick production and exiting of the body. Using hair to measure glucocorticoid (GC) levels is a new method that has not been validated yet, but is very promising, because the GCs can be accumulated due to the slow growth rate of hair. Several measurement techniques for measuring chronic stress have been thoroughly studied in some species, such as rats and dogs. This is, however, not the case for wildlife. This study is part of a larger project on chronic stress in deer and focusses on red and fallow deer from parks in respectively the Netherlands and the United Kingdom (UK), that apply different management strategies. The amount of glucocorticoid receptors (GRs) in the hippocampus were compared with GC levels in hair samples from the same animals. GC levels in hair were also compared between parks and between gender per park. The results showed that GC levels are significantly gender-dependent between the UK parks. It was interesting that the males in the UK park Belton had significant lower GC levels than those in Richmond, while for the females this was the other way around, which means that a factor in those parks leads to different GC reactions in males versus females. Beside these results, this study provides the promising result that deer that were culled for health reasons, have higher GC levels than deer that were culled for population management reasons. The findings of this study could be used to validate the use of hair samples for measuring cortisol and to create baselines for GC and GR levels in deer.

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1. Background

1.1 Welfare and the influence of society

Humans and animals have always had different relationships, varying from food/consumer to property/owner. According to Serpell (2004): 'Animals that are close phylogenetically to humans, or that are physically, behaviorally or cognitively similar to them, tend to evoke more positive affect than those that are phylogenetically distant or dissimilar. Those perceived as 'cute' or otherwise aesthetically appealing or admirable also tend to be preferred'. These different relationships lead to different views on our moral obligation towards animals. If a combination is made of several ethical approaches, that focus on both the conservation of nature and the welfare of an individual wild animal, it is accepted that humans have a moral obligation to all the 'sentient wild animals in whose lives we have interfered' (Beausoleil, Appleby, & Weary, 2014).

Humans often interfere with ecosystems, for example when building houses in rural areas, or when placing fences, which leads to fragmentation of living areas for wildlife. The interference has become especially prominent in the last 50 years, leading to the loss of biodiversity (Beausoleil et al., 2014; Millennium Ecosystem Assessment, 2005). According to the philosophical viewpoint anthropocentrism, humans are unique and have special rights, even if it means that animals will suffer (Paquet & Darimont, 2010). People who believe in anthropocentrism will only approve of conservation strategies if it benefits the human race (Beausoleil et al., 2014). If in the past no conservation strategies were applied, there would be even less biodiversity today (Millennium Ecosystem Assessment, 2005). The loss of biodiversity is important to many people, because of cultural values and because nature is important for humans and has therefore instrumental values (Beausoleil et al., 2014; Millennium Ecosystem Assessment, 2005).

Animal welfare and conservation strategies are becoming more interesting for society, due to the increasing awareness of the effects of the human interference in wildlife. Research of objective methods to evaluate welfare are, therefore, more required.

1.2 What is welfare and how can welfare be measured?

What is welfare

To evaluate animal welfare, we need to know how welfare can be best defined. For a long time, the five freedoms defined by the Brambell Committee were seen as the best way to determine the welfare state an individual experiences (Ohl & van der Staay, 2012). Ohl and Van der Staay (2012) posed that adaptability is a prerequisite for positive welfare and therefore modified the concept of welfare as follows:

"An individual is in a positive state when it has the freedom adequately to react to
• hunger, thirst or incorrect food;
• thermal and physical discomfort;
• injuries or diseases;
• fear and chronic stress, and thus,
• the freedom to display normal behavioural patterns that allow the animal to adapt to the demands of the prevailing environmental circumstances and enable it to reach a state that it perceives as positive".

Adaptability is thus an important aspect of welfare, because the ability of an individual to adapt will allow an animal to cope with environmental conditions and therefore will influence how an animal perceives a situation and its internal state. For example, when food availability is low, when the animal is suffering from an illness or when an animal lives in an area with a high population density, the animal might be exposed to stress. In the short-term animals can adapt to such environmental challenges. However, when these conditions surpass the adaptive abilities of the animal or persist for a prolonged period of time, the animal may no longer be able to adapt to these environmental

conditions, leading to a negative welfare state. Impairments in stress regulation, as a consequence of chronic stress, may be an important factor in impairing the adaptive abilities of an animal, as will be explained in the next section.

Stress responses help an animal to cope with events and changes in environment. Acute stressors such as a change in temperature or a sound, will trigger the hypothalamic-pituitary-adrenal axis (HPA-axis), which will help the individual to restore and maintain homeostasis (De Kloet, Joëls, & Holsboer, 2005; Sheriff, Dantzer, Delehanty, Palme, & Boonstra, 2011). The paraventricular nucleus of the hypothalamus secretes corticotrophin releasing hormone (CRH), which in turn triggers the release of adrenocorticotropic hormone (ACTH) from the pituitary. A high level of ACTH leads to the release of glucocorticoids (GCs; i.e. cortisol) from the adrenal cortex. GCs help to restore maintenance through impacting on different organ systems, thereby increasing energy resources and reducing immune function for example (Russell, Koren, Rieder, & Van Uum, 2012). At the same time, GCs bind to glucocorticoid receptors (GRs) in the hippocampus, which play an essential role in the negative feedback of the HPA-axis. According to Mizoguchi *et al.* (2003) the degree of increased GCs and the duration of increased GCs contribute to a change in the amount of GRs. When an animal is exposed to stress for a prolonged period of time, i.e. is confronted with chronic stress, this is known to result in impaired negative feedback as a consequence of reduced GRs in the hippocampus. As a consequence, GCs baseline levels are elevated for a prolonged period of time (De Kloet et al., 2005). However, some known cases do not show this increase in GC baseline levels (Mason & Mendl, 1993).

How to measure chronic stress

Different techniques can be used to measure stress, both subjective and objective. Observing the behaviour of an assumed stressed animal is an example of a subjective technique. To avoid possible influences of subjectivity, objective techniques are required. Classically, the objective way to evaluate the stress status of an animal is to measure GC levels in blood, saliva, hair and excreta (Sheriff et al., 2011). GC levels in blood, saliva and excreta are considered to represent the acute released GC levels, ranging from minutes to hours after onset of the stressor (Russell et al., 2012; Stalder, 2012). Hence, they can be used for measuring acute stress. GC levels in hair however accumulate due to the slow hair growth and are therefore suitable to measure chronic stress (Russell et al., 2012; Stalder, 2012). However, systematic studies providing that hair GC levels indeed reflect chronic stress are lacking. Besides, the amount of GCs 'vary with diurnal rhythms and are released in a pulsatile or episodic manner', which is why, even when measuring the GC levels in hair samples, it is typically very difficult to dissociate acute and chronic stress when only measuring GC levels (Mason & Mendl, 1993; Russell et al., 2012).

As mentioned, GC levels can influence the GR levels. Therefore, GR levels can also be used to measure stress. Multiple studies have measured the amount of GRs in rats and tree shrews, influenced by chronic stress, of which only a few are selected here (Jöhren, 1994; Mizoguchi, Ishige, Aburada, & Tabira, 2003; Raone et al., 2007). Raone *et al.* (2007) described that rats which experienced chronic stress show a significant decrease in GRs in the hippocampus versus control rats that did not experience chronic stress. Jöhren *et al.* (1994) described the same results with tree shrews. There are, however, no known data of the baseline of GRs or of the amount of GRs influenced by chronic stress in wildlife, including deer.

In this project we aim to validate the measurement of GRs as an indicator of chronic stress in deer. At the same time, we will measure GCs accumulated in hair as a potential non-invasive measurement for chronic stress (Sheriff et al., 2011).

1.3 Difficulty of evaluating animal welfare

Different aspects from different disciplines, such as the mental or physical state of an animal can influence the ability to adapt. Therefore, the key to evaluating welfare is to use an integration of multiple types of measurements from different disciplines (Mason & Mendl, 1993; Sandøe & Simonsen, 1992) Welfare can be evaluated differently. For instance, welfare of domestic animals, wildlife, individuals or of populations. The difference between domesticated animals and wildlife is that the first are kept and cared for by humans and that the latter are free-ranging animals, which, in principle, do not come in close contact with humans. It is relatively easy to measure welfare for domestic animals, or animals that are held in captivity. By contrast, it is difficult to assess welfare for wild animals, due to the limitations in collecting samples from individuals (Beausoleil et al., 2014). There are some differences between evaluating the welfare of an individual and the welfare of a population. For the first, the experiences of an individual are evaluated and for the second the amount of affected individuals and the sum of individual experiences (Beausoleil et al., 2014). Conservation studies, so far, have mainly focused on populations and entire species, which means that the data collected mostly show fitness parameters and not the mental states of individuals or small groups (Beausoleil et al., 2014). Species, subspecies and individuals differ on how they experience a situation and on how they respond to it, because of their different subjective views (Ladewig, 2000; Mason & Mendl, 1993). Just as is the case for humans, what may be negative experiences for one animal, might not be so negative for another animal. Individuals differ in their adaptability capacities, which can influence the way they experience a challenging condition.

Besides the subjectivity of an individual, the subjectivity of the researcher is important, because it can influence the way results are being interpreted (Mason & Mendl, 1993). Science can give objective parameters that can serve as evidence for subjective experiences (Sandøe & Simonsen, 1992). For instance, the knowledge of baselines can decrease the influence of subjectivity. It is, however, not possible to know exactly whether an animal experiences a good welfare, because the thoughts and exact feelings of animals are unknown. Therefore, the influence of subjectivity can, even when incorporating science, not be completely left out in evaluating animal welfare.

Another difficulty in evaluating welfare is the possible influence of the duration of a negative experience. Experiencing a negative situation for a prolonged period of time, could lead to habituation (Mason & Mendl, 1993). Habituation can lead to responses that are suppressed, returned to normal, or increased due to sensitization, which makes it difficult to decide whether an animal experience a good welfare (Ladewig, 2000).

1.4 Wildlife welfare and wildlife management

Humans and free-ranging animals normally live separately. However, due to increasing wildlife populations and human interfering in ecosystems, they sometimes come in close contact with each other. Today, cases of wildlife involved traffic accidents are more common than before. For instance, in the year 1989, 1937 collisions with roe deer were recorded in the Netherlands (Groot Bruinderink & Hazebroek, 1996). This number increased to 2338 in 1993. This is, of course, not only due to the increase in wildlife populations, but also due to the increase of traffic (Putman, 2011). The close contact between wildlife and humans is one of the reasons why many people deem wildlife conservation necessary. It is, however, not easy to decide which wildlife management strategy should be applied, because there are several stakeholders, each with their own view on what is good for conservation and wildlife welfare. According to Riley *et al.* (2002) ‘A stakeholder is any person who will be affected by, or will affect, wildlife or wildlife management’, meaning that landowners, government, visitors and hunters are all examples of stakeholders. Approaches can be political, economic, social, cultural, recreational, or involve safety and health interests (Riley et al., 2002). A difference between the general public and professionals such as foresters, is that the latter usually has

more knowledge of how environmental changes can influence wildlife welfare. For instance, the general public often does not believe that the amount of deer in an area can be considered too high, while the foresters notice negative impacts on either deer, environment or both due to the overabundance of deer (Dandy, 2012). Some people may see predation as an indicator of a natural functioning ecosystem, while others interpret it as a factor that is negative for prey populations (Riley et al., 2002).

As explained, welfare is based on the adaptability of an animal to different types of situations it encounters in its environment. Some stakeholders do not take into account the important linkage between the welfare of a species (i.e. the animal welfare) and the welfare of an environment (Paquet & Darimont, 2010). Doing so will lead to a shift in the ecological system and to the local/regional (extirpation) or global extinction of species (Paquet & Darimont, 2010). A large deer population could, for instance, lead to a decrease in butterfly population or to a decrease of vegetation (Putman, 2011). On the other hand, for an animal to experience a good welfare, the environment must meet up with the ecological needs that the animals have. For instance, deer have the ecological need to have access to enough food, to sufficient space and to shelter opportunities.

Apart from the discussion about the application of wildlife management at all, an important next step will be to decide what kind of management strategy should be applied. Management strategies that are applied differ from doing nothing and let nature run its course, to culling some individuals to maintain a certain population size and to create a perfect balance between animals and vegetation. As mentioned, stakeholders have different considerations and use different approaches to wildlife management, which makes it difficult to form one strategy that will satisfy every single group of stakeholders. Some stakeholders, like welfare conservationists, are focused on habitats and populations, while others focus more on individuals (Paquet & Darimont, 2010).

As mentioned by Paquet & Darimont (2010): 'the conservation of species and populations often trumps all other values, including the welfare of individuals', which makes it difficult to combine conservation and animal welfare. According to the utilitarian approach of Singer, every individual counts and the best conservation strategy is the one with 'the highest ratio of benefit to cost', for all individuals involved (Beausoleil et al., 2014). Also, 'welfare impacts have to be balanced against conservation goals' (Beausoleil et al., 2014). This could mean that culling, placing fences or even doing nothing might be the best wildlife management strategy.

In different parks in both the Netherlands and the United Kingdom (UK), as in other countries, different managements strategies are applied. The Oostvaardersplassen (OVP) in the Netherlands chose for a natural approach and no interference. However, because individual deer should be free of suffering, the OVP does cull animals, but only those that are sick or severely wounded that will most likely not survive over the next couple of weeks, i.e. reactive culling. By contrast, other parks apply proactive culling. This strategy is applied, for example, in the Hoge Veluwe Nationale Park (EHV) in the Netherlands, where the population is kept at a steady level to ensure an optimal balance in the ecosystem and the wildlife's ecological needs, by culling healthy animals. According to Beausoleil (2014) this strategy: 'aims to reduce a pest population to an initial low abundance and suppress population growth using regular maintenance control'. This means that some individuals are being sacrificed for the population or species at large (Paquet & Darimont, 2010). It depends on the qualities of the ecosystem and on the beliefs of the stakeholders, what the best possible strategy is for wildlife welfare in that particular ecosystem.

1.5 Aim and approach of this study

This study is part of a larger project which develops methods and indicators to monitor the welfare of deer populations in parks with different management strategies. The first aim of this study is to measure the difference in chronic stress between red deer and fallow deer populations from parks that

apply different wildlife management strategies and are characterized by different environmental conditions. For that purpose we will analyse GRs in the hippocampus by performing in situ hybridisation with radioactive probes and GCs in hair from the same animals using an Enzyme-Linked immune Sorbent Assay (ELISA). The second aim is to use GR levels as a golden standard to validate hair GC levels as a measure for chronic stress. For this study, we had hair and brain samples available for analysis from red deer in the Netherlands. In addition to these samples, we had hair samples from fallow deer from different parks across the UK, where population densities, human interference (visitors, traffic crossing and surrounding the park), climate and the presence of other species differ.

The hypothesis is that deer that were exposed to chronic stress will have lower GR levels in the hippocampus and higher GC levels in the hair samples. This will be particularly clear for animals that were culled for health reasons when compared to animals that were culled for population management reasons, but were otherwise healthy.

2. Materials and methods

For this study, GRs in the hippocampus and GCs in hair are measured. The practical part of this study was divided into two experiments. In experiment 1 the GR levels of 14 deer from parks in the Netherlands were measured using an in situ hybridisation. Experiment 2 measured the GC levels in hair from 80 deer from parks both in the Netherlands and in the UK.

2.1 Materials

Experiment 1 (brain samples)

- Ten brains of deer from EHV (eight female and two male)
- Four brains of deer from OVP (all female)
- Cryostat (Leica CM 3050 S)
- Frozen Tissue Medium
- Microscope (Olympus BX51)

Experiment 2 (hair samples)

- Hair samples of 80 deer from two parks in the Netherlands and from eight parks in the UK
- Methanol
- Ethanol
- Saline
- Aluminium foil
- 50 ml tube
- Stove at 40°C and 37°C
- Sure cap Eppendorf centrifuge tubes (2 ml)
- Beads
- Tissue Lyser II
- Centrifuge
- Scale for weighing 35 mg
- Combitip 25ml Eppendorf
- Repeater-pipet
- Rotator wheel or shaker
- Reaction vial (1.5 ml)
- Vortex
- Speedvac
- Expanded Range High Sensitivity Salivary Cortisol Enzyme Immunoassay Kit (Salimetrics)
- ELISA reader (SoftMax Pro)

2.2 Methods

Experiment 1 (brain samples)

A total of 14 brains were available for this experiment, ten brains from the EHV and four from the OVP. Prior to this experiment, each brain was cut in half at the midline and then dissected in slices of 1 centimetre thickness using a specially designed mold. The most anterior slices were named left or right 1. The slices most posterior were named left or right 2 or 3. All the slices were stored at -80ºC until sectioning. The GR mRNA levels were measured in the sub regions dentate gyros (DG) and cornu ammonis one and three (CA1 and CA3) of the hippocampus, as was done in prior studies that showed a decrease in GR levels by chronically stressed animals (Jöhren, 1994; Mizoguchi et al., 2003; Raone et al., 2007). Hence, before and during sectioning the hippocampus was the focus point. The hippocampus was divided in three coronal regions which were, from anterior through posterior, called

A1, A2 and A3. From each slice 20 µm sections were made with the use of a cryostat. The 20 µm sections from the coronal region A2 were mounted on coated microscope glasses for subsequent staining and in situ hybridization was performed to determine GR mRNA levels. The coated microscope glasses were stored at -30ºC. To confirm the exact anatomical location of the sections, Nissl staining was performed per brain slice on the first and the last sections and on the sections with the transitions between the coronal regions (A2/A1 and A3/A2).

Statistical analysis of experiment 1
Due to the limited time available for this study, the in situ hybridization could not yet be performed. Therefore, no statistical analyses were performed for experiment 1.

Experiment 2 (hair samples)
This experiment was performed using hair samples of 80 deer from two parks in the Netherlands and from eight parks in the UK. The samples of the UK were collected from the following parks: Attingham Park (ATTCO1), Prideaux Park (Hartland), Richmond Park, Belton Park, Dunham Massey Park (DUM), Eridge Park, Powderham Castle and Tatton Park. Table 1 shows the exact numbers of deer per park and per gender.

Table 1: Number of deer per park and per gender

Land	Park	Total	Females	Males
NL	EHV	10	8	2
	OVP	4	4	-
UK	ATTCO1	4	4	-
	Belton	12	6	6
	DUM	6	6	-
	Eridge	5	5	-
	Hartland	18	13	5
	Powderham	3	-	3
	Richmond	12	6	6
	Tatton	6	3	3
	Total	**80**	**55**	**25**

The aim of this experiment was to determine the amount of glucocorticoids in hair with the use of an ELISA. To do this, a standardized protocol for cortisol/corticosterone analysis from hair (see appendix 2) was followed with some alterations. It became clear from previous experiments that the hair samples were still dirty and sticky after washing with isopropanol, which probably prevented optimal pulverization and extraction. Therefore, in this experiment, the hair samples were washed three times with saline prior to washing the hair twice with isopropanol, before being finely pulverized by beads.

Isopropanol was used to eliminate any contamination (Russell et al., 2012). After the hair samples were pulverized, 35 mg of the samples was weighed and added to methanol for extraction and placed on a slow rotator overnight (Gow, Thomson, Rieder, Van Uum, & Koren, 2010). Instead of 1.5 ml methanol, 1.2 ml methanol was added to the hair samples, because 1.5 ml did not fit in the Eppendorf tubes. Because of the small tubes, only 0.8 ml of the extract was placed in a new reaction vial, instead of 1 ml. In this experiment, a Speedvac was used instead of a shaking heating block, because the samples dried faster using a Speedvac, saving several hours compared to the shaking heating block. After the hair samples were dissolved in 80 µl assay diluent from the ELISA kit and were stored overnight, the commercially available salivary ELISA kit (Salimetrics) was used for analysing (protocol of that ELISA kit can be downloaded on this site: https://www.salimetrics.com/assay-kit/cortisol-salivary-elisa-eia-kit). Because of the large number of samples, three separate ELISA's were performed (appendix 3).

Statistical analysis of experiment 2

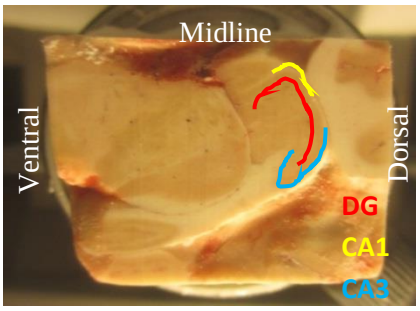
Three samples were classified as significant outliers using http://graphpad.com/quickcalcs/Grubbs1.cfm and were not included in the statistics: Hartland 161 (#6), Richmond 5 (#16) and EHV 503 (#33). Besides being an outlier, only 10 mg hair was available from sample Richmond 5, instead of the needed 35 mg. Sample 34 and 38 were labeled to be both derived from deer EHV 507. Therefore, the average of these two results was used in statistics as the outcome of sample EHV 507. In the end, the results of 76 samples were used for statistical analysis.

Statistical analyses were performed using SPSS (IBM SPSS Statistics 24) and graphs were prepared using GraphPad (Prism 6.05). GC data from 76 samples from different parks in the Netherlands and the UK were analyzed. The data from the red deer (eight samples from EHV and four samples from OVP) and the data from the fallow deer (64 samples from different UK parks) were analyzed separately using one-way ANOVA. The dependent variable was cortisol (pg/mg hair) and significance was assessed at $P < 0.05$. First separate comparisons were made between the averages of the parks in the Netherlands and between the averages of the parks in the UK. Secondly, separate analyses per gender were performed for the UK parks and Tukey's t-test was used to perform post-hoc comparisons. It should be mentioned that for this study, female and male samples were available from respectively seven and five parks from the eight UK parks in total that delivered samples. Hence, gender-dependent differences could not be performed on all parks. The separate analyses per gender could also not be performed for the parks in the Netherlands, because no samples were available from male deer from the OVP.

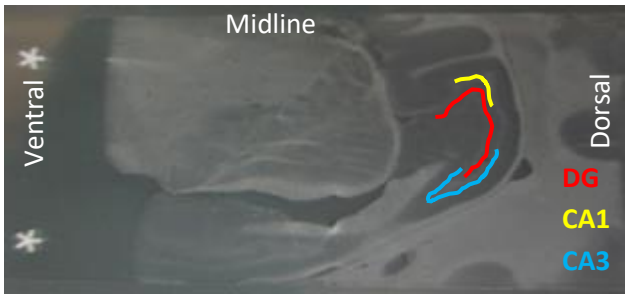
3. Results

Experiment 1(brain samples)

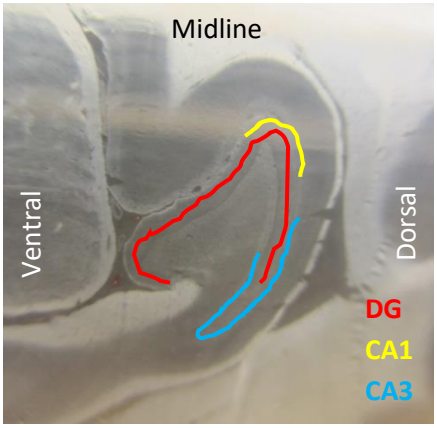
GR data are not known due to not performing the in situ hybridization. Below are photos of one slice and of two sections.



Picture 1: One slice with region A1



Picture 2a: One section with transition A2/A1



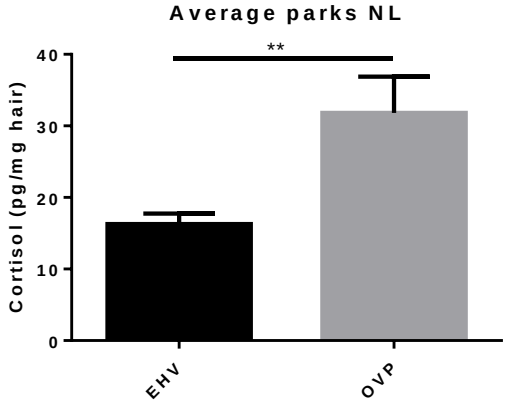
Picture 2b: One section with region A2

Experiment 2 (hair samples)

As mentioned, the samples from the park in the Netherlands were analysed separately from the samples from the UK parks.

Red deer (NL):

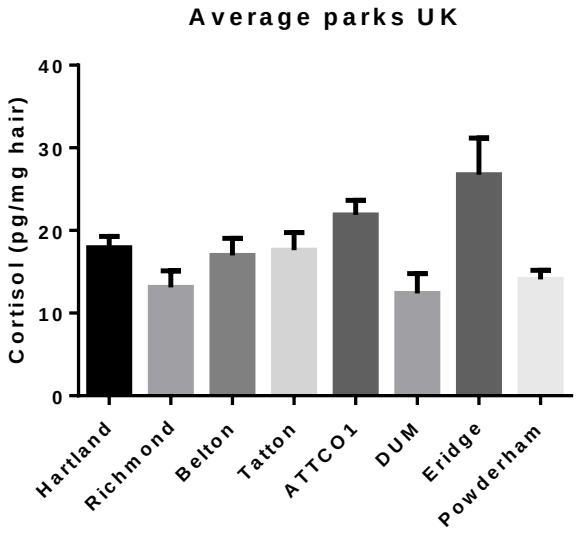
Graph 1 shows the average GC levels in hair from deer of the two parks in the Netherlands. An increase in average GC level can be seen between the EHV and the OVP, with the OVP having the higher average GC level compared to the EHV ($p < 0.01$).



Graph 1: Average per park (NL)

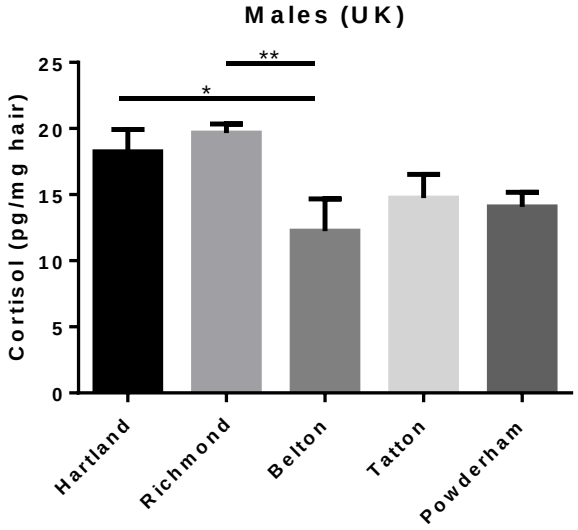
Fallow deer (UK):

The average GC levels per UK park are displayed in graph 2, which shows a large degree of variation between the parks. Statistical analysis confirmed that there is a significant difference between the UK parks ($F_{park\ (7,52)} = 3.8$, $p < 0.01$). Noticeable is that Eridge has the highest average GC level. Pairwise comparison revealed that the average GC level of Eridge was significant with the average GC levels of all the other UK parks, except with ATTCO1. Furthermore, the difference between UK parks was gender-dependent ($F_{park\ x\ gender\ (3,52)} = 7.9$, $p < 0.001$). Next, separate analyses per gender were performed.



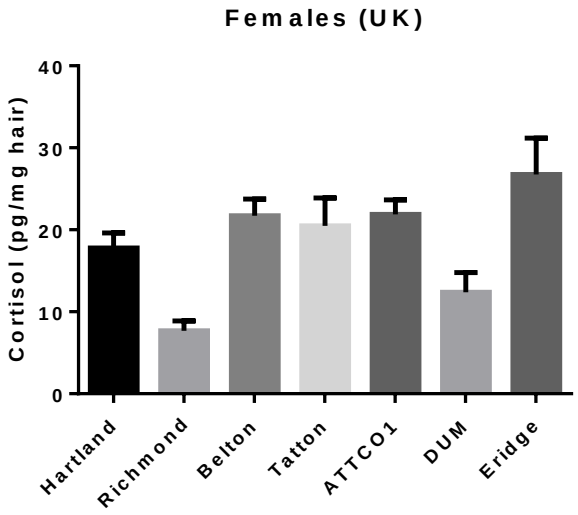
Graph 2: Average per park (UK)

The separate analyses for the males revealed an overall effect of average GC levels between the UK parks ($F_{park\ (4,17)} = 3.0$, $p<0.05$). Graph 3 shows the average GC levels of males between UK parks. Noticeable is that the GC levels from Belton are lower than the GC levels from Hartland and Richmond. Pairwise comparisons revealed that the male-dependent is significant between Richmond and Belton ($p<0.01$) and between Hartland and Belton ($p<0.05$).



Graph 3: Average males per park (UK)

Separate analyses for the females revealed an overall effect of average GC levels between UK parks ($F_{park\ (6,35)} = 6.2$, $p<0.001$). Graph 4 represents the average GC levels of females per UK park. Interestingly, the average GC level of Belton is higher than the average GC level of Richmond, while for the males, Belton was lower than Richmond. The pairwise comparisons revealed significant differences by nine comparisons which are represented in table 2. This means that the average female GC levels differ significantly between nine parks. Richmond differs significantly with all the other UK parks, except with DUM.



Graph 4: Average females per park (UK)

Table 2: Significant female gender-dependent differences

Couples	**P**
ATTCO1 x DUM	$p < 0.05$
ATTCO1 x Richmond	$p < 0.001$
Belton x DUM	$p < 0.01$
DUM x Eridge	$p < 0.001$
Hartland x Eridge	$p < 0.01$
Hartland x Richmond	$p < 0.01$
Richmond x Belton	$p < 0.001$
Richmond x Eridge	$p < 0.001$
Richmond x Tatton	$p < 0.01$

4. Discussion

The aim of this study was to measure GC levels in hair samples from red and fallow deer from respectively the Netherlands and the United Kingdom and also to measure GR levels in brains from red deer. We hypothesized that deer that encountered chronic stress, i.e. deer that were culled for health reasons or deer that were exposed to environmental challenges, would have higher GC levels and lower GR levels compared to healthy deer that were culled due to population management reasons or deer that live in less challenging conditions. Due to limited time for this project we were not yet able to perform the in situ hybridization. However, the hair GC data revealed clear differences between deer from different areas both within the UK and the Netherlands, suggesting there may indeed be differences in chronic stress and therefore also welfare between the different populations. These results are particularly promising, because they reflect relatively small samples sizes. The deer in the parks in the Netherlands are a different subspecies than the deer in the UK parks. Due to possible subspecies influences on GC levels, it was not possible to compare those parks, which is why separate analyses were performed per country.

Firstly, the parks in the Netherlands were compared with each other. The data revealed a higher average GC level in the OVP compared to the EHV. The deer from the OVP were believed to have experienced severe chronic stress due to illness or injuries. On the other hand, the deer from the EHV were believed to have less chronic stress than the OVP, due to being culled for population management reasons. The deer in the OVP should, therefore, have higher GC levels than the deer in the EHV. The data confirm this presumption. It is, however, not possible to draw conclusions about the entire deer population of the OVP, because only four unhealthy deer were analysed. Besides, the GC levels of the healthy deer were not included. It could be possible that the deer in the OVP have naturally high GC levels compared to the deer in the EHV. An additional consideration is that all the hair samples from the OVP came from females. It is, therefore, not possible to draw conclusions about the GC levels in male deer. If possible, further research should include more unhealthy deer and also healthy deer from the OVP, so baselines could be made. Furthermore, also samples from male deer should be analysed. Chronic stress is seen as a factor that can influence the welfare status of an animal.

Secondly, the UK parks were compared with each other. The data show a large degree of variation between the parks. For instance, the average GC level of Eridge was significantly higher than those of the other parks, except for ATTCO1. Environmental factors in the UK parks could lead to these differences. Parks, for instance, could differ in size of grazing area for deer, or in total number of deer. The differences between parks appeared to be gender-dependent. Some parks only delivered samples from males or females, while others delivered samples of both genders. Therefore, it is difficult to draw conclusions about the entire deer populations of the UK parks. Besides the skewed male female ratio, the GC levels were not consistent for males and females. The males in Belton have higher GC levels than in Richmond, while the females in Belton have lower GC levels than in Richmond. This shows that an environmental factor in those two parks influences the way males and females react to stress. It would be interesting, for future research, to compare the park details with the GC levels, to see which environmental factors influence the differences between parks and between gender.

With the help of the GR levels that will be known when the in situ hybridisation is performed, the method of measuring GC levels in hair can be validated as a good way to measure chronic stress. Using hair samples to measure cortisol levels and to evaluate welfare is a relatively new method and has yet to be validated (Sheriff et al., 2011). When the method of using hair samples is validated, this non-invasive method could be used for future chronic stress research. Although using hair samples is seen as a non-invasive method to measure chronic stress levels, it is not an easy method concerning wildlife. Our hair samples were collected from the same deer of which we also received the brains. We

were able to compare a non-invasive method with an invasive method. Due to the novelty of this method, no cortisol baseline levels are known at this moment and there are some limitations. A limitation for using hair samples could be hair colour. Some studies conclude that hair colour has no influence on cortisol levels, while other studies concluded the opposite (Gow et al., 2010; Russell et al., 2012). Moreover, studies do not agree on the cortisol incorporation in hair. It might be possible that peripheral HPA axis activity influences the cortisol levels in hair and that those levels, therefore, do not correlate to the systemic cortisol concentrations, or to the GR levels in the hippocampus (Russell et al., 2012). However, when GR levels of the in situ hybridisation are compared to the GC levels of the hair samples, this will no longer be a limitation.

More factors that can influence GR levels are (sub)species, age and gender (Gow et al., 2010; Russell et al., 2012). Species and subspecies differ in how they react to situations. For example, bulls that were injected with ACTH for several weeks, showed decreased GC responses, while pigs showed increased responses (Mason & Mendl, 1993). We had samples from different subspecies (red and fallow deer) available for this study. Due to possible subspecies differences, we were not able to compare the samples from red deer with the fallow deer. The influence of age could differ between species. For example, the adrenal glands of pigs with increasing age appear to response less to an injection of ACTH, leading to a smaller increase in GC levels as a pig gets older (Mason & Mendl, 1993). However, Pavitt *et al.* (2015) found that GC levels in red deer stags increased linearly with age. This might be due to desensitization of the HPA axis feedback loop (Pavitt, Walling, Möstl, Pemberton, & Kruuk, 2015). As a result GC baseline levels may vary dependent on the age. Increasing sample sizes over the course of 3-4 years will allow to control for age as a potential factor of influence. Moreover, performing research over a longer period will eliminate possible climate influences.

This study shows promising results that, by measuring GC levels in hair samples, healthy deer that were culled for population management reasons most likely experienced less chronic stress than deer that were culled for health reasons and, because of that, would seem to have a better welfare status. Moreover, differences in GC levels were found between UK parks and between genders per park, which may be related to climate differences or other environmental or management factors that are important for animal welfare. The results of this study can contribute to improve the welfare of deer and other wildlife.

5. Acknowledgements

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6. Literature

Beausoleil, N. J., Appleby, M. C., & Weary, D. M. (2014). Balancing the need for conservation and the welfare of individual animals. (pp. 124). Wallingford: CABI.

Dandy, N. (2012). Exploring beliefs behind support for and opposition to wildlife management methods: A qualitative study. *European Journal of Wildlife Research, 58*(4), 695. doi:10.1007/s10344-012-0619-1

De Kloet, E. R., Joëls, M., & Holsboer, F. (2005). Stress and the brain: From adaptation to disease. *Nature Reviews Neuroscience, 6*(6), 463-475. doi:10.1038/nrn1683

Gow, R., Thomson, S., Rieder, M., Van Uum, S., & Koren, G. (2010). An assessment of cortisol analysis in hair and its clinical applications. *Forensic Science International, 196*(1), 32-37.

Groot Bruinderink, G. W. T. A., & Hazebroek, E. (1996). Ungulate traffic collisions in europe. *Conservation Biology, 10*(4), 1059-1067. doi:10.1046/j.1523-1739.1996.10041059.x

Jöhren, O. (1994). Hippocampal glucocorticoid receptor expression in the tree shrew: Regulation by psychosocial conflict. *Cellular and Molecular Neurobiology, 14*(3), 281. doi:10.1007/BF02088326

Ladewig, J. (2000). Chronic intermittent stress: A model for the study of long-term stressors. *The Biology of Animal Stress, ,* 159-169.

Mason, G., & Mendl, M. (1993). Why is there no simple way of measuring animal welfare? *Animal Welfare, 2*(4), 301-319.

Millennium Ecosystem Assessment. (2005). Ecosystems and human well-being: Biodiversity synthesis. *Washington, DC,*

Mizoguchi, K., Ishige, A., Aburada, M., & Tabira, T. (2003). Chronic stress attenuates glucocorticoid negative feedback: Involvement of the prefrontal cortex and hippocampus. *Neuroscience, 119*(3), 887-897. doi:http://dx.doi.org/10.1016/S0306-4522(03)00105-2

Ohl, F., & van der Staay, F. J. (2012). Animal welfare: At the interface between science and society. *The Veterinary Journal, 192*(1), 13-19. doi:http://dx.doi.org/10.1016/j.tvjl.2011.05.019

Paquet, P. C., & Darimont, C. T. (2010). Wildlife conservation and animal welfare: Two sides of the same coin? *Animal Welfare, 19*(2), 177-190.

Pavitt, A. T., Walling, C. A., Möstl, E., Pemberton, J. M., & Kruuk, L. E. B. (2015). Cortisol but not testosterone is repeatable and varies with reproductive effort in wild red deer stags. *General and Comparative Endocrinology, 222*, 62-68. doi:http://dx.doi.org/10.1016/j.ygcen.2015.07.009

Putman, R. (2011). Identifying threshold densities for wild deer in the UK above which negative impacts may occur. *Mammal Review, 41*(3), 175. doi:10.1111/j.1365-2907.2010.00173.x

Raone, A., Cassanelli, A., Scheggi, S., Rauggi, R., Danielli, B., & De Montis, M. G. (2007). Hypothalamus–pituitary–adrenal modifications consequent to chronic stress exposure in an experimental model of depression in rats. *Neuroscience, 146*(4), 1734-1742. doi:http://dx.doi.org/10.1016/j.neuroscience.2007.03.027

Riley, S. J., Decker, D. J., Carpenter, L. H., Organ, J. F., Siemer, W. F., Mattfeld, G. F., & Parsons, G. (2002). The essence of wildlife management. *Wildlife Society Bulletin, ,* 585-593.

Russell, E., Koren, G., Rieder, M., & Van Uum, S. (2012). Hair cortisol as a biological marker of chronic stress: Current status, future directions and unanswered questions. *Psychoneuroendocrinology, 37*(5), 589-601.

Sandøe, P., & Simonsen, H. B. (1992). Assessing animal welfare: Where does science end and philosophy begin? *Animal Welfare, 1*(4), 257-267.

Sheriff, M. J., Dantzer, B., Delehanty, B., Palme, R., & Boonstra, R. (2011). Measuring stress in wildlife: Techniques for quantifying glucocorticoids. *Oecologia*, *166*(4), 869-887.

Stalder, T. (2012). Intraindividual stability of hair cortisol concentrations. *Psychoneuroendocrinology*, *37*(5), 602. doi:10.1016/j.psyneuen.2011.08.007

7. Appendices

7.1 Appendix 1 - Pictures of standardized ROI with transitions for experiment 1

Indeling hippocampus (van het damhert) in verschillende gebieden

Most recent adjustment made by: Judith Hendriks Date: 08/07/2015

Deze indeling hebben we gebruikt voor de hippocampus tijdens het snijden op de cryostaat.

Hierdoor kunnen we voor het experiment een selectie van de coupes maken, die in ongeveer hetzelfde gebied liggen. Daarnaast worden ook de Nissl glaasjes gebruikt voor een zo'n uniforme selectie.

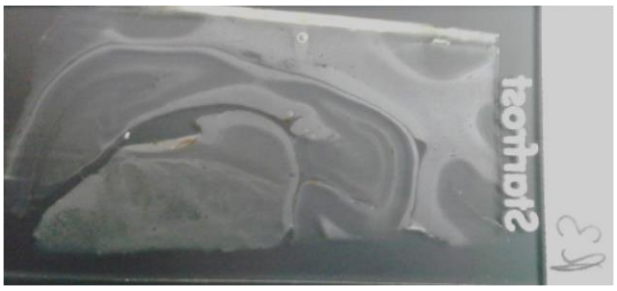
Posterior → Anterior

A4

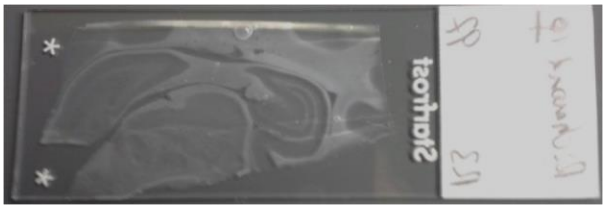
A3

A3/A2 (ROI-region of interest)

A2/A1 (ROI)



Gestopt (ROI)



Het moment van overgang A3/A2 en A2/A1 wordt bepaald volgens onderstaande foto's.

A3/A2

2 gebieden gaan los van elkaar

A2/A1

2 witte gebieden gaan dicht

7.2 **Appendix 2 -** SOP Cortisol/corticosterone analysis in hair samples

Subject. Cortisol/corticosterone analysis from hair

Most recent adjustment made by: A. de Rooij Date: 09.08.2016

1. Introduction

Cortisol was shown to be incorporated into hair and correlate with circulating cortisol levels. Cortisol extraction from hair can be used to measure the relative amount of accumulating cortisol over the time of the growth of the hair. The release of cortisol over a long term can be assessed through the analysis of cortisol extracted from hair. This 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**
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 40°C and 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
Speedvac

4. Solutions

Dilution solutions provided with the respective kits

5. Procedures

During whole protocol: keep samples in the dark!

1. Shave approximately 500 mg hair from the back of the neck
2. Store in aluminium foil, in the dark, at room temperature
3. Wash the hair three times with 30 ml saline in a 50 ml tubes by mixing at room temperature with the Multi reax for 1 min (maximum speed) per wash, decant the saline and add new saline for the second wash and repeat for the third wash. Dry off the hair samples using Klimtech tissues.
4. Wash the hair twice with 15 mL isopropanol in a 50 mL tube by gently mixing (by hand) at room temperature for 1 minutes per wash, decant the isopropanol and add new isopropanol for the second wash.
5. Dry the hairs at 40°C for a few hours and then at 37°C for 2 nights (has to be thoroughly dry)
6. Weigh in 2 times 30 mg washed hair in 2 separate 2 ml sure cap Eppendorf centrifuge tubes with 3 beads (Lab services BV Biospec products, 3.2 mm no 11079132).
7. 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 (1 min, room temperature, 14000rpm) and repeat for another 15 minutes. If necessary repeat until ground to powder.
8. Weigh in 35 mg hair (mink, deer) powder into a clean 2 mL sure close Eppendorf tube. (for rat and monkey (Rhesus and Java) use 50 mg hair). Note the exact weights.
9. Add 1.2 ml methanol (using a combitip 12.5 mL Eppendorf, on a repeater-pipet with a 200µl pipet-tip attached) and incubate the tubes at room temperature for 24h on a rotator wheel to extract the steroids.
10. Centrifuge (10 min, room temperature, 14000rpm) and place as much as possible of the extract in a clean reaction vial (1.5 mL). If necessary centrifuge again (5 min, room temperature, 14000rpm) and place 0.8 mL of the extract in a new reaction vial (1.5 mL). Use reverse pipetting for this step .
11. Dry at 42 °C in Speedvac (programme 1 for 2.5h; trap erin).
12. Optional: store dark at RT if necessary.
13. The dried extracts of ferret and deer were dissolved in 80 µl assay diluent 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 5 min (lids closed), monkey samples had to be dissolved in 400µl buffer. After that the samples were stored overnight on slow rotation at 4°C.
    The rat extract was dissolved in 1 mL Diluent included in the corticosterone RIA kit MP Biomedicals, Germany).
14. Centrifuge (3 min, 4°C, 14000rpm)
15. Store at 4°C for up to 48h (if longer is necessary: -20°C)
16. 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.
17. 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.

6. 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,

7.3 **Appendix 3 -** ELISA plate layouts

ELISA 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	3 Std	3 Std	NSB	NSB	6	6	14	14	22	22	30	30
B	1 Std	1 Std	ctr hi	ctr hi	7	7	15	15	23	23	31	31
C	0.333 Std	0.333 Std	ctr lo	ctr lo	8	8	16	16	24	24	32	32
D	0.111 Std	0.111 Std	1	1	9	9	17	17	25	25	33	33
E	0.037 Std	0.037 Std	2	2	10	10	18	18	26	26	34	34
F	0.012 Std	0.012 Std	3	3	11	11	19	19	27	27	35	35
G	0.006 Std	0.006 Std	4	4	12	12	20	20	28	28	36	36
H	Zero	Zero	5	5	13	13	21	21	29	29	37	37

ELISA 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	3 Std	3 Std	NSB	NSB	51	51	60	60	68	68	76	76
B	1 Std	1 Std	ctr hi	ctr hi	52	52	61	61	69	69	77	77
C	0.333 Std	0.333 Std	ctr lo	ctr lo	53	53	62	62	70	70	78	78
D	0.111 Std	0.111 Std	38	38	54	54	63	63	71	71	79	79
E	0.037 Std	0.037 Std	39	39	56	56	64	64	72	72	80	80
F	0.012 Std	0.012 Std	40	40	57	57	65	65	73	73	81	81
G	0.006 Std	0.006 Std	41	41	58	58	66	66	74	74	82	82
H	Zero	Zero	50	50	59	59	67	67	75	75	83	83

ELISA 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	3 Std	3 Std	NSB	NSB	55	55		22 (1:1)				
B	1 Std	1 Std	ctr hi	ctr hi				23				
C	0.333 Std	0.333 Std	ctr lo	ctr lo				24				
D	0.111 Std	0.111 Std	84	84				25				
E	0.037 Std	0.037 Std	85	85				26				
F	0.012 Std	0.012 Std	86	86				27				
G	0.006 Std	0.006 Std	87	87				28				
H	Zero	Zero	88	88				29				