Wildlife Welfare: The effect of park management factors on glucocorticoid receptor levels in the hippocampus and cortisol levels in hair of fallow deer (Dama dama) from wildlife parks in the UK and in the Netherlands as indicators for chronic stress

Research internship veterinary medicine Utrecht University
Dagmar Joostema, 3906132
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Supervisor: dr. H.M.B Lesscher

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Abstract

Human interference in the habitats of different wildlife animals has increased in the past decades, which has led to a decrease of the living area of these animal species. This has consequently created a public sense of responsibility for animal welfare. In the case of wildlife, it is important to be able to determine the welfare status of wildlife species. In that respect, chronic stress may be considered a potential indicator for animal welfare, because chronic stress is considered to result in ineffective adaptability to prevailing circumstances, and therefore to reflect a negative state of welfare. A way to measure the amount of chronic stress, is determining the amount of glucocorticoid receptor (GR) mRNA expression in the hippocampus. With chronic stress, due to downregulation of mRNA of GRs, there are fewer GRs available. This is thought to contribute to impaired negative feedback of the hypothalamus-pituitary-adrenal axis, resulting in accumulating levels of cortisol in the circulation. Moreover, GR downregulation has consistently shown to emerge in response to chronic stress across species. In more recent years, hair cortisol concentration (HCC) has been applied to measure the amount of glucocorticoids that have been accumulating over a longer period of time.

In this study, HCC and GR mRNA levels were analyzed to determine the impact of several wildlife park management factors on chronic stress in fallow deer. To this end, data on HCC and GR mRNA levels from brain and hair samples of fallow deer that were collected form parks in the UK were compared. Next to this, data for the Amsterdamse Waterleidingduinen (AWD) were extended. For the UK parks we found that pasture density, fallow density and the average temperature in May-June are important factors that predict if fallow deer experience chronic stress in their habitat. These effects were found to be significantly gender dependent ($p<0,05$).

Moreover, HCC was aimed to validate as an adequate indicator for chronic stress, in comparison with the amount of GR mRNA and MR mRNA. This was accomplished by looking for correlations between the data of HCC and the data of the level of GR and MR mRNA from different hippocampal regions, namely CA1, CA3 and the DG. No significant correlations were found ($p>0,05$).

Based on this study, we suggest that to maintain positive welfare in fallow deer, population density should be considered in management strategies of wildlife parks, especially when it concerns female deer.

1. Introduction

1.1 Fallow deer park management

Even though humans and animal wildlife live mostly separated, due to the growing density of the both human and wildlife populations and especially in densely populated countries as for example the Netherlands, they have increasingly come in close contact with each other (White & Ward, 2011). So much so, that restrictions to certain habitats often have to be made to decrease the amount of sometimes unpleasant interactions, such as road kills. The restrictions that humans put on the living area of the wild animals, have consequences for the animals. Putting fences around wildlife parks, creates a certain population density. One that could become high when there are for example no natural predators, and low when there are e.g. fast spreading diseases present. Park management factors can also have an influence on this population density, through e.g. culling or supplementary feeding when relative food availability is low.
Another way in which humans can interfere in wildlife, is through recreation, which can lead to disturbance of the animals. In the UK, there are currently around 200 to 300 deer parks. Most of these allow visitors, which creates an opportunity for the public to watch these free roaming animals themselves. The educational value of these parks is high. This high visitor pressure does however create situations in which routine activities of the deer have to be altered. Feeding time may be reduced, and individual deer may be chased by dogs that belong to the visitors (Bullock, 1993). In the Netherlands, fallow deer can be found in wildlife parks the Amsterdamse Waterleidingduinen (AWD) and the Hoge Veluwe. In these parks, recreational pressure has also increased over the past decades (de Boer, H. Y. & et al., 2013). One of the purposes of these parks, among others, is to teach the public about the wildlife living there and therefore it is part of the park management strategy.
Other management factors contribute to the preservation of wildlife and vegetation. Maintaining the size of populations can be achieved through e.g. culling or by introducing natural predator animals (Blumstein, 2010). Introduction of natural enemies, however, is often not desired by the public, as these wildlife parks are often open for visitors. Managing the vegetation of the parks can be achieved for example by introducing certain herbivores or by controlling the number of animals living in the park. Recently, an effect regarding the managing of population density can be seen in the AWD. Since introducing fallow deer in the AWD, the population has only been growing. So much so, that they began roaming the adjacent areas. At the end of 2012, a fence was placed around the AWD area to prevent the deer from roaming the nearby area. The deer couldn't escape to nearby crops anymore when food became scarce in the winter. The size of the population was too large, compared to the size of the now fenced habitat. In the following winters, a lot of these deer died of hunger or exhaustion (Niesen, 2016). Since this occurrence, many people protested and demanded for the deer to be supplied with extra food, to improve the welfare of these animals. The ultimate decision was to start culling late 2015.
Sometimes it is hard to combine animal welfare with management strategies, as welfare also encompasses the welfare of an individual rather than only focusing on the whole population (Paquet & Darimont, 2010). This results in different management strategies of various wildlife parks, as the priorities of stakeholders can vary the emphasis of the management factors. Because of the rapid decline of fallow deer in the AWD, it is interesting to put data regarding animal welfare from this park next to data from the UK, as the management strategies vary greatly.
Taken together, the management styles vary from region to region. For instance, the population densities are different, the composition of the population may vary, the culling regime may be

pro-active or reactive, there may be supplementary feeding etc. But what is the impact of human disturbance and more specifically wildlife management on the welfare of free roaming species? This topic remains understudied, in particular in wildlife. Therefore, the overarching aim of this study is to identify factors that may affect wildlife welfare.

1.2 Defining animal welfare

As stated before, human interference in the habitats of wildlife animals has increased in the past decades (de Boer, Hanneke, van Breukelen, Hootsmans, & van Wieren, 2004), which has led to a decrease of the living area (Beausoleil et al., 2014). This has consequently created a public sense of responsibility for the welfare of wildlife animals (Broom, 2010; Stewart, Webster, Schaefer, Cook, & Scott, 2005). This poses a need for knowledge about animal welfare, about how animal welfare can be perceived and how it can objectively be measured. So how can we define animal welfare?

There are a number of ways to define animal welfare. One of the early definitions of animal welfare was based on the Brambell report (Brambell & Britain, 1965), which was ultimately developed into the five freedoms by the Farm Animal Welfare Council (FAWC) (FAWC, 1993). Today, we still call this the Five Freedoms of Brambell. This theory of animal welfare, is based on the exclusion of negative states. According to this theory, an animal is in a positive welfare state when it has freedom of hunger and thirst, freedom of discomfort and pain, freedom of injury and disease, freedom of stress and anxiety and the freedom to express natural behavior. There are, however, numerous natural occurring situations that animals sometimes choose to be in that may impose negative states such as injury, stress or discomfort. However, in general individuals can adapt to these situations to return to a state that they perceive as positive. This notion of adaptive capacities of animals to return to a state that it perceives as positive has led to the welfare concept described by Ohl and van der Staay (2012). They suggested to alter the original concept of welfare by including the animal's ability to adapt to environmental conditions and challenges. Such that the animal perceives its situation as positive. Despite this novel view on animal welfare, different approaches are still being utilized when assessing animal welfare. In some papers, an animal is deemed in a state of good welfare if it is e.g. free of disease and has a normal reproduction process (Fraser, 2008). Others adapt the notion that an animal has a positive state of welfare when the animal can exhibit their natural behavior, even if the animal is at higher risk at for example parasites or other diseases (Lund & Algers, 2003). It is, however, important to consistently use a clearly defined concept of animal welfare for precise scientific measurements, legal documents, and in public statements (Broom, 2010).

Regardless of the concept of welfare, most welfare assessments rely on behavioral observations and interpretations. This approach may not be feasible for all species, and are particularly challenging for wildlife species. To be able to say something about wildlife welfare, it is necessary to identify a feasible and objective measure for welfare. Multiple ways to study factors that can be indicated as parameters for welfare have been described. For animals that live in the wild, it is interesting to measure chronic stress as an indicator for animal welfare. Chronic stress can lead to suppression of the immune system, as well as contribute in inhibition of reproduction, wasting of muscles and suppression of growth (Koolhaas et al., 1999; Ashley 2011). This, among other things, then can lead to e.g. cardiovascular, gastric or even neurological pathologies. When animals are experiencing diseases that result from chronic stress, the ability to adapt itself to a positive state may be compromised, and thus, the welfare of the animal may be affected. Moreover, chronic stress in itself can decrease the adaptability of the animal (Ohl & van der Staay, 2012).

1.3 Stress response and chronic stress

Animals continuously have to process external and internal floods of information (Scott Creel, Ben Dantzer, Wolfgang Goymann, & Dustin R. Rubenstein, 2013). While dealing with all this information, there will be several physiological responses that result in the restoration of homeostasis in the body. The homeostasis of an individual is determined by parameters that are essential of survival, such as pH, blood pressure and body temperature (McEwen, 2000). Stress is often defined as the biological response to stressors, as these stressors compromise the equilibrium or homeostasis (de Kloet, Joëls, & Holsboer, 2005; McEwen, 2000). The process in which the body tries to maintain in a state of homeostasis, is called allostasis, allostatic load is the price the body pays to this continuous stress burden (McEwen, 2000; Fotta, 2017). The immediate response of the body is to correct the destabilizing effect of the stressor. In vertebrates, the hypothalamic-pituitary-adrenal (HPA) axis, is one of the most prominent and best described physiological responses to stress (Nicolaides et al., 2014; Sanchez, Young, Plotsky, & Insel, 2000). The HPA-axis is activated in response to stress, which can be physical as well as psychological. The HPA-axis can be activated in multiple ways. One of them is through the amygdala, which is known for its role in detection of fear (Marin et al., 2011). The pathways that follows activation of the HPA-axis are however the same, regardless of what triggered the response. The main initiator of the HPA-axis is corticotropin-releasing factor (CRF), which is released from the paraventricular nuclei (PVN) of the hypothalamus. CRF is secreted into the hypophyseal portal system and binds to receptors in the anterior lobe of the pituitary (Nicolaides et al., 2014). Consequently, adrenocorticotropic hormone (ACTH) is released into the bloodstream, until it reaches the receptors of the adrenal glands. The end result of the activation of the HPA-axis, glucocorticoids, are secreted from here. Glucocorticoids are molecules derived from cholesterol (Nicolaides et al., 2014).

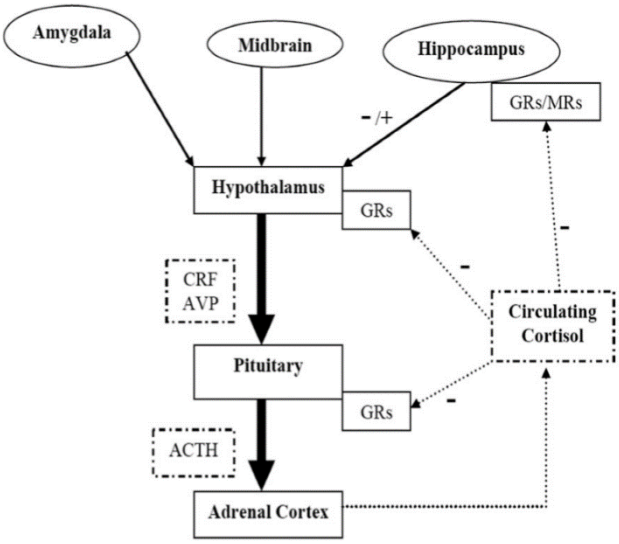


Figure 1 A schematic representation of the HPA-axis. Adopted from (Papadimitriou & Priftis, 2009)

Activation of the stress system leads to a series of behavioral and physical changes, that increases the chances of acute survival. Individuals will show an increased state of e.g. vigilance, arousal, improved cognition and focused attention (Nicolaides et al., 2014; Silverman et al., 2005). Because glucocorticoids have a liposolubility, it can pass the blood-brain-barrier and reach glucocorticoid receptors in various brain regions (Kudielka & Kirschbaum, 2005; Marin et al., 2011). There are two types of corticoid receptors. The mineralocorticoid receptors (MR), or type I receptors, take part in regulating the basal levels of glucocorticoids, whereas glucocorticoid receptors (GR), or type II receptors, focus on stopping the activation of the HPA-axis. This way, the GR contributes to restoration of homeostasis (Marin et al., 2011; Sanchez et al., 2000). The affinity of glucocorticoids on the MRs is about a tenfold higher than the affinity for GRs. Activation of the MRs occurs at basal levels of glucocorticoids (Frodl, 2012; de Kloet et al., 2005; de Kloet et al., 2016). The hippocampus can be divided in several

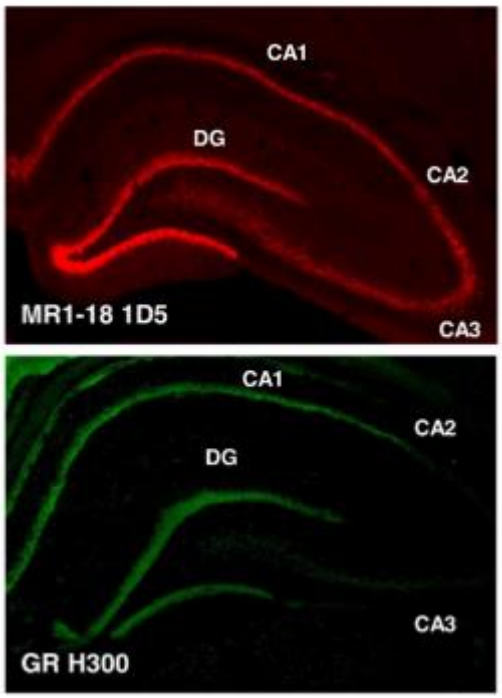


Figure 2 Distribution and quantification of MR and GR immunoreactive cells in the rat hippocampus.
Adopted from (Sarabdjitsingh et al., 2008)

subregions, the CA1, CA2, CA3 and Dentate Gyrus (DG) (Knierim, 2015). The expression of MR's and GR's differs in these regions, as illustrated in figure 2 (Sarabdjitsingh et al., 2008).
In the state of chronic stress, the hippocampus is an especially important structure in the stress response regulation, as it is a big contributor to the regulation of the negative feedback on the HPA-axis (Levone & et. al., 2015). If glucocorticoids bind to the GRs in the hippocampus and hypothalamus, the stress response will be down regulated so that the amount of circulating stress hormones will decline (de Kloet et al., 2005). When the levels of glucocorticoids are repeatedly high, as is the case in chronic stress, this will downregulate the amount of GR mRNA, as GRs are sensitive to elevated glucocorticoid levels (Mizoguchi et al., 2001; Sapolsky et al., 1990). This reduction of GRs in the hippocampus has been identified as an important indicator when an individual has experienced chronic stress (Vyas et al., 2002). Dendritic remodeling stands at the base of the changes seen in the hippocampus after a prolonged period of stress. A reversible shortening of the dendrites and debranching of the apical site of the dendrites takes place. (Conrad et al., 1996) This then causes an impairment of hippocampal-dependent learning, which can decrease the ability of the individual to adapt. As the negative feedback loop is disturbed, the level of glucocorticoids will stay elevated. As a result of this, glucocorticoids will have a prolonged effect on all different kinds of organs, with all its consequences, such a negative impact on cardiac health due to an excess of catecholamines which can lead to a decreased ability for an appropriate flight or fight response, which is crucial for survival (Koolhaas et al., 1999; Dickens, Delehanty, & Michael Romero, 2010).
In recent years, it has been described that the reaction to stress can be gender specific. Sexual dimorphism in the responsiveness of the HPA-axis has been well recorded in rodents, in which males show lower adrenocortical responses than females (Ferin, 2006). Further studies shall show if these correlations are also applicable to other specific animal species. In another study in humans, it has been reported that in women, the cerebral blood flow in the hippocampus is positively correlated with perceived stress during tasks, while in men it is negatively correlated (Dedovic et al., 2009). These results show that the way people or animals react to stressors can be gender dependent. Therefore, the possible difference between genders are going to be taken into account in this study.

1.5 Hair cortisol

Glucocorticoids are commonly used as biomarkers of stress. In humans, non-human primates and most large mammals cortisol is the predominant glucocorticoid, while corticosterone is the most important glucocorticoid in rodents (Russell et al., 2011). Cortisol and corticosterone can be found in blood, sweat, saliva, faeces, urine and hair (Moya, Schwartzkopf-Genswein, & Veira, 2013). The most commonly investigated samples used to review cortisol or corticosterone are urine, blood serum, urine and saliva samples (Russell et al., 2011). Blood serum samples are a great indicator for the circadian cycles, acute stress and chronic stress. For

reviewing long term stress, this would require multiple blood samples to distinguish basal cortisol levels from stress induced levels. When it comes to researching the adaptive capacities of animals, it is more relevant to measure basal levels of glucocorticoids in combination with responses on several and repeated applied stressors. For wildlife, conducting such research is mostly not possible. Collecting blood samples from wild animals is invasive. It also requires specific knowledge about the natural history of the animal, as well as knowledge of the HPA-axis and of stress to be able to make sense of the samples (Sheriff, Dantzer, Delehanty, Palme, & Boonstra, 2011). This would also require capturing the animals multiple times, which would be a stressful operation in itself (Moya et al., 2013).
More recently, the concentration of hair cortisol has been shown to be a useful measure of long term stress (Yamanashi et al., 2016). It is not subject to circadian fluctuations during the day, and it can spare the animal in certain test formats from the momentary stress of being captured for repeated blood sampling. Previous studies have suggested that blood-borne substances, for example cortisol, enter the hairs through passive diffusion from capillaries into growing cells of the hair follicle (Moya et al., 2013). These substances will thus be deposited into the hair shaft. Another way for the cortisol to enter the hair is via cortisol present in secreted sweat and sebum which then can become incorporated in the hair shaft. These mechanisms are however not yet fully understood.
Hairs are considered to have the longest record of GC exposure of an animal (Sheriff et al., 2011). The slow growth of hair means that the time scale in which the hairs are incorporating cortisol is weeks to months, instead of hours to days. This makes hair cortisol concentration (HCC) a useful measure for examining long term stress (Sheriff et al., 2011). For wildlife, this is a practical and reliable way to measure the long term cortisol concentration as a parameter for chronic stress. Something to consider when analyzing HCC, is that there are multiple ELISA kits commercially available, but not all kits are evenly sensitive and therefore not evenly accurate. To correctly reflect the amount of hair cortisol, an appropriate kit has to be chosen (Kroshko et al., 2017).

1.6 Aim of the study

The aim of this study, was to determine which factors in wildlife park management influence chronic stress levels of fallow deer. For this purpose, this study contains two lines of research. Firstly, culled fallow deer from several parks from the UK were collected to investigate multiple management factors that could be of importance regarding chronic stress.
Secondly, fallow deer from the AWD that were culled from 2015 and onwards were obtained, to research if a decline in population density through, in this case, culling could have an effect on the amount of stress in this population.
For this study, we collected brain samples and fur samples, that originated from fallow deer from these areas who were send in after culling. To determine chronic stress levels in these animals, we measured the levels of GR mRNA expression in the hippocampus , and the levels of cortisol in hair samples.
A secondary aim of this study was to evaluate whether HCC is an accurate indicator for chronic stress, in comparison to GR mRNA levels in the hippocampus, as a more established indicator for chronic stress in different species. (Boleij et al., 2014; Dickens et al., 2009; Gomez et al., 1996; Patel et al., 2008; Sterleman et al. 2008). In this analysis, we also took MR mRNA into account, as a decrease in GR mRNA expression is often accompanied by a decrease in MR mRNA expression (Boleij et al., 2014).

This study is part of a bigger study which has been going on for several years. Initial analyses of deer from the UK revealed that population density is an important factor to determine chronic

stress in deer, as a proxy for animal welfare (Lesscher et al., unpublished findings), but the sample size was still low and more samples needed to be analyzed to draw reliable conclusions regarding the management factors and animal welfare. The fallow deer in the AWD formed a particularly interesting group considering the initial evidence for population density being a critical factor, and the fact that the population density in the AWD would be declining by culling over several years. This has made it possible to investigate the impact of the reduction of the population density on the chronic stress parameters. The samples are taken at the same time each year, thereby excluding the impact of seasonal changes in environmental conditions such as breeding and rutting.

The aim of this specific study was to extend the analysis of the UK data and to extend the AWD data. As part of my internship, I processed brain samples from the AWD from the previous winter (2017/2018). The samples were cut, processed and stored for in situ hybridization. In addition, the most up to date file with HCC and brain GR mRNA levels from UK samples was used for statistical analysis. The results of this analysis are described in the results section of this report.

1.7 Hypotheses

The hypothesis is that the amount of stress in deer living in UK parks with higher population density will be elevated compared to deer in the UK parks with lower population density. This would be reflected by a lower expression of GR mRNA in the hippocampus and a higher level of HCC in deer from parks with a higher population density.

2. Material & Methods

2.1 Tissue collection and preparation

Brain tissue and hair samples were collected from fallow deer that came from parks in the United Kingdom and the Netherlands. For this study, the UK parks that participated are Attingham, Belton House, Dunham, Dyrham Park, Eridge, Hampton Court Palace, Lyme Park, Richmond Park, Prideaux, Tatton Park and Powderham Park. The park from the Netherlands that participated in this study was the Amsterdamse Waterleidingduinen (AWD). The deer from this park were culled in the period of 2014 up to and including 2017. The deer from the UK parks were culled in the period of 2014 up to and including 2016. The fallow deer were culled in the same period every year, from November to March.

The heads of the culled fallow deer were cooled at 4 degrees Celsius and sent to the Faculty of veterinary medicine of Utrecht University. Here, approximately 500 mg of fur, which is equivalent to approximately 2 cm^2, was shaved with an electric razor from the space between the eyes and the forehead. This was standardized for all the samples to facilitate comparison between samples. After shaving the hair, the sample was wrapped in tin foil. If the collected hair was wet, it was set aside in a drawer, to allow it to dry without the influence of light.
For brain dissection, the head was cut medially in the sagittal plane, and both brain hemispheres were dissected from the head. The hemispheres were cut in sections of approximately 1,5 cm thickness in the coronal plane with help of a pre-made 3D printed mold. The optic nerve was placed on a cutting site and was used for orientation. The part that contained the hippocampus, which is in the slice posterior to the optic chiasm, was selected and transferred to a Ziploc plastic bag. The slices were placed in the Ziploc plastic bag in a way that made sure it was clear which side was anterior and which was posterior. Then, they were frozen at a temperature of

minus 80 degrees Celsius. These parts were stored until further cutting and slicing was performed.
To make sure similar regions of the hippocampus were used for analysis, areas of importance were marked, ranging from A1, being most anterior until A4, being most posterior. The region of interest (ROI) is the region that ranges from the A3/A2 transition until the A2/A1 transition. These areas were visually distinguishable during slicing. In table 1 an overview of the recognition of these transition locations can be found.

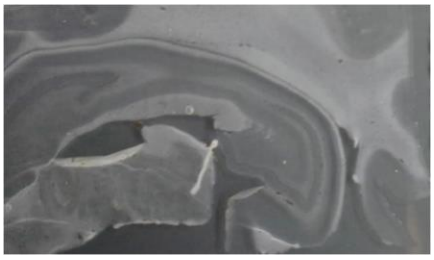
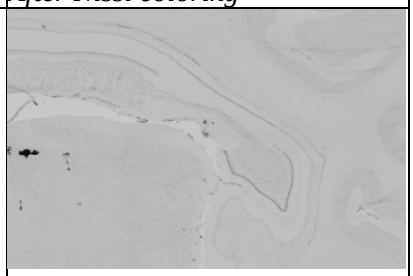
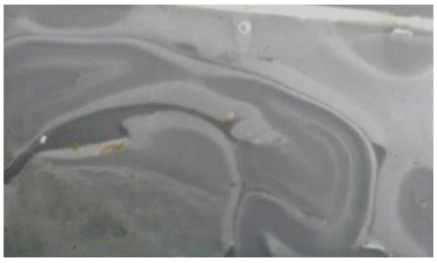
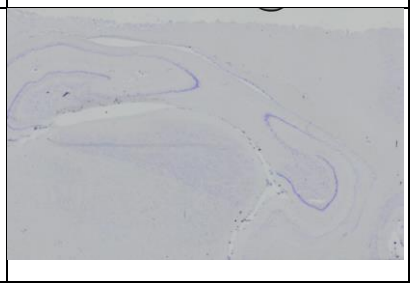
Transition area	*On slide*	*After Nissl coloring*
A3/A2		
A2/A1		

Table 1 Overview of the recognition of the transition regions when on the slide (L) and after Nissl coloring (R).

Slicing was performed from posterior to anterior, unless the brain section was unfit for a posterior to anterior slicing. The brain samples were sliced using a Leica CM3050 S cryostat, set at a temperature of minus 20 degrees Celsius, with a preset thickness of 20 μm. The slices were mounted on a Starfrost slide, and the CA1, CA3 and dentate gyrus (DG) regions were individually rated on integrity, visibility and usability directly after. The region was scored + when this region was suitable for analysis, +/- when the region was questionable, and – when the region was unsuitable for analysis.
To get a clear indication of the placement of all the slices in the brain, a few slides were kept apart for a Nissl coloring, which stains nucleic acids. These slides were selected as followed: the first slide, a slide on the A3/A2 transition, a slide on the A2/A1 transition and the last, or one of the last, slides. This was done for a better orientation of the hippocampus. The full protocol for the Nissl coloring can be found in the appendix.
The remaining slides were stored at minus 30 degrees Celsius until the in situ hybridization and further research was conducted.

2.2 In situ RNA hybridization and analysis hippocampus tissue

The complete protocol for the ISH can be found in the appendix. The ISH was performed before this specific study, the results were already available for statistical analysis. For the ISH a deer-specific RNA probe for the MR mRNA and GR mRNA, which was developed in previous studies, was used. Briefly, for the in situ hybridization (ISH), the four best consecutive slides from the A2 region closest to the A2/A1 transition from both the left and right hemisphere were selected. Thus, a total of 8 slides per animal were chosen. From each hemisphere, two of the slides were used for measuring MR mRNA and two slides were used for measuring GR mRNA.

The radioactive slides were developed on a light sensitive film. After development of this film, the slides were scanned and quantified using imageJ software. When quantifying the CA1, CA3 and DG regions individually, the ROI manager function of the software was used. Using the marker values of each film and a polynomial regression function calculated by http://www.xuru.org/rt/pr.asp. The results given by image J software were transformed into Becquerel values. For the GR mRNA and the MR mRNA results, a value based on the greyscale of the film was also determined, as this will possibly exclude an error that goes into the calculation of the Becquerel value. For each sub region, the mean of the 4 measurements of one sub region that came from both hemispheres, and two slides per animal, was calculated. The quantification of the films was performed by two students, as a control and to compare the results.

2.3 Analysis cortisol from hair

The complete protocol for the analysis of cortisol from hair, which was done following a standard operating protocol, can be found in the appendix. The analysis of cortisol from hair was already performed before this specific study, the results were already available for statistical analysis.
After recovering the shaven hairs that were stored in tin foil at room temperature in the dark, they were put in a 50 mL tube, filling it for 50%. 20 mL PBS was added to the tube, and the tube was roughly shaken to remove most blood contamination. After the PBS wash, the hairs were washed twice in 20 mL isopropanol, using new isopropanol each time, 3 minutes per wash. The hairs were then dried in a stove at 37°C for 96 hours. Washing the hairs was necessary, as this step eliminates the cortisol that derives from sweat or saliva. Washing with isopropanol preserves more cortisol from the hairs than washing the hairs with methanol, as a study in polar bears and grizzly bears showed (Kroshko et al., 2017).
After washing, 60-70 mg of the hairs was placed in a 2 mL Eppendorf tube. The hairs were grinded using a Tissue Lyser II and three 5 mm steel beads, which were placed in the Eppendorf tube. After centrifugation, 35±2 mg of the pulverized hairs was weighed and put into a clean 2 mL Stardust cup with cap. 1,5 mL of methanol was added, and was incubated overnight on an end-over-end roller at room temperature to extract the steroids. Then after repeated centrifugation, the samples were dried in a stove. The amount of picogram (pg) cortisol was measured using a High Sensitivity Salivary Cortisol ELISA kit (Salimetrics). This was then used in further data analysis.

2.4 Data analysis

For all statistical analyses of the data of the UK deer, SPSS 24.0.0.2 for windows was used. A linear mixed model was used to determine if there was a relation between the outcome variables (HCC, GR mRNA, MR mRNA) and the park factors. MR mRNA was not included in the results, as no relations were found. Because the data was not normally distributed, the data of the variables was first log transformed to achieve a normal distribution (Boleij et al., 2014).
First, scatterplots were made for visual inspection of the data to determine which factors may contribute to the variation in HCC and GR mRNA. All the factors that seemed to show a relation with these parameters, were included in the linear mixed model. The covariance structure was explored and modeled appropriately. Then, all the factors that showed no significance were deleted from the model, following a backwards strategy (Boleij et al., 2014). Lastly, all the factors that remained in the model were tested for correlation with the variable using Spearman's rho correlation test.

For the secondary aim of this study to evaluate whether HCC is an accurate indicator for chronic stress, in comparison to GR mRNA and MR mRNA levels in the hippocampus, a correlation test using Spearman's rho was also conducted. We also took the MR mRNA levels into account, as a decrease in GR mRNA expression is often accompanied by a decrease in MR mRNA expression (Boleij et al., 2014).
The Spearman's rho was used, as not all data was normally distributed. Significance was accepted at $p<0,05$.

3. Results

3.1 AWD hippocampi

A total of 12 AWD hippocampi sets were sliced with the cryostat, of which 7 were male and 5 were female. All the sliced tissues containing female hippocampi, the left as well as the right half, are fit for future ISH. For one of the male hippocampi sets, only the left half was available for slicing. Two other sets of male hippocampi are unfit for ISH, due to formation of ice crystals in the brain tissue. Therefore, a total of 9 sets of hippocampi, plus one left hippocampus, are available for future ISH.
In figure 3, a few examples of the sliced hippocampi after Nissl coloring are visible.

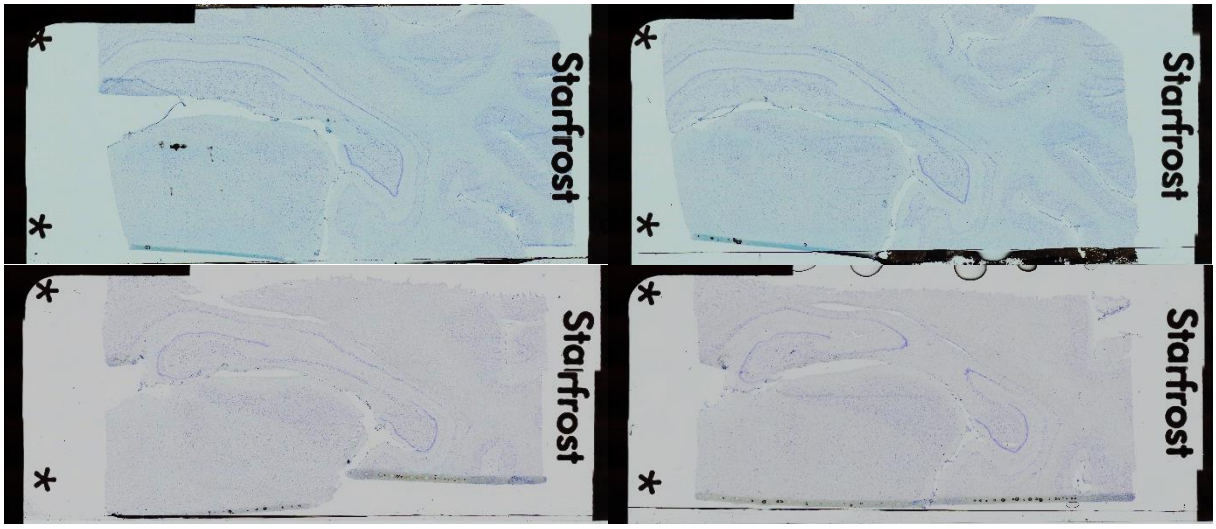


Figure 3 Examples of Nissl colored slices of two different male fallow deer (male 1 above, male 2 below). Left, the hippocampus near the A3/A2 transition, right the hippocampus near the A2/A1 transition.

3.2 Hair cortisol

For the UK dataset, the first step we took in data analysis was to visually inspect the data and determine if there were potential relations between specific park factors and HCC. For this purpose, scatterplots were made. First, scatterplots including all the available data were made. These showed a visual relation between HCC and the average temperature in May-June as well as with pasture density. Then, a new set of scatterplots were created after splitting the data by gender. These scatterplots showed, on top of the park factors named before, a relation between HCC and the factors fallow density and altitude. The scatterplots that seemed to show a visual relation, and were ultimately used in the linear mixed model, are presented in figures 4 and 5.

Based on this first inspection of the data, the mixed linear model was made, with the factors fallow density, pasture density, average temperature in May-June and altitude as covariates and park number as the random factor. Because on the fact that the scatterplots revealed a visual difference between the sexes, a linear mixed model was also made based on interactions between gender and the covariates.

First, we looked at the linear mixed model that encompassed all available data and analyzed the main effects between the park factors. No significant relation of the park factors and HCC was found in this model ($p>0,05$).

When the model was altered to take the interaction of park factors with gender into account, this revealed gender dependent effects of pasture density ($F_{(2,72)}=_{(sex\_num\ x\ pasture\_dens)}$ 6,1; p=0,004), fallow density ($F_{(2,72)}=_{(sex\_num\ x\ fallow\_dens)}$ 7,4; p=0,001), average temperature in May-June ($F_{(2,72)}=_{(sex\_num\ x\ AVGtempmay\_june)}$ 16,8; p=0,000) and altitude ($F_{(2,72)}=_{(sex\_num\ x\ altitude)}$ 16,8; p=0,000),

The next step was to determine the effects of these factors, e.g. pasture density, fallow density, average temperature in May-June, for males and females in separate analyses. This revealed that pasture density ($F_{(1,47)}=_{(pasture\_dens)}$ 9,8; p=0,003), fallow density ($F_{(1,47)}=_{(fallow\_dens)}$ 6,5; p=0,014) and the average temperature in May-June ($F_{(1,47)}=_{(AVGtempmay\_june)}$ 20,6; p=0,000) are significantly related to the HCC in this model for the female population.
For the male population, we found that the park factors fallow density ($F_{(1,25)}=_{(fallow\_dens)}$ 7,1; p=0,013), average temperature in May-June ($F_{(1,25)}=_{(AVGtempmay\_june)}$ 5,3; p=0,030) and altitude ($F_{(1,25)}=_{(altitude)}$ 10,9; p=0,003) are significantly related to HCC in this model.

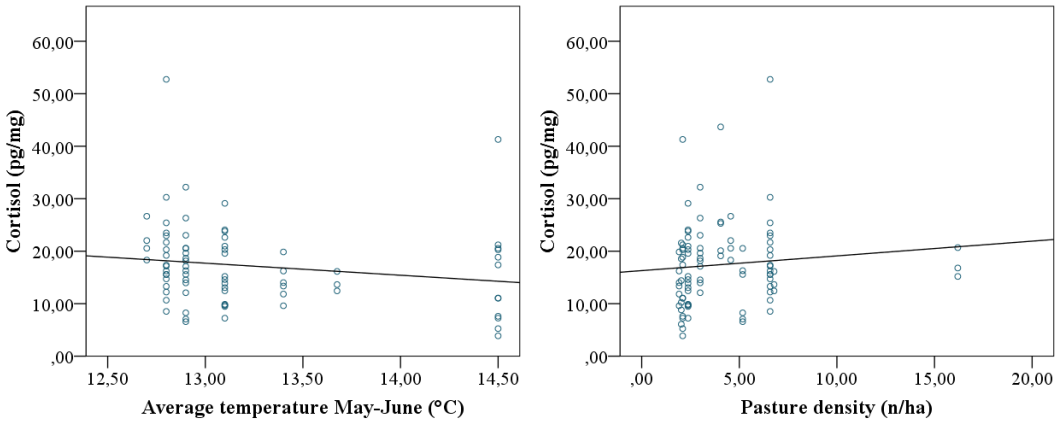


Figure 4 Scatterplots that encompasses all the HCC data, without sorting by gender. Left the relation between HCC (pg/mg) and the average temperature in May-June (°C). Right the relation between HCC (pg/mg) and pasture density (n/ha)

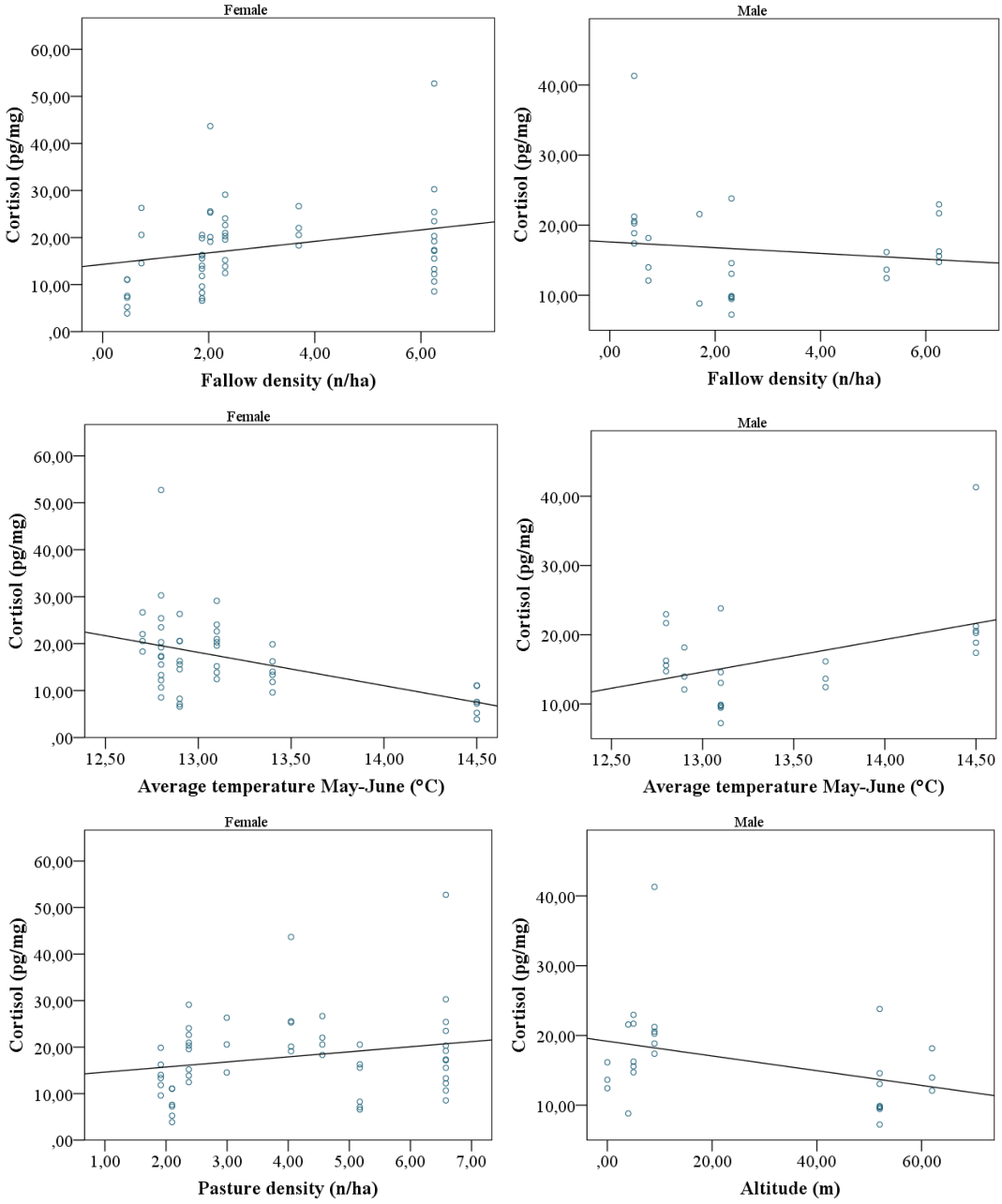


Figure 5 Scatterplots that show the relation of HCC (pg/mg) with the park factors fallow density (n/ha), average temperature in May-June (°C), pasture density (n/ha) and altitude (m), sorted by gender.

Finally, we performed correlation analyses between the HCC and the different park factors that were revealed to be associated with HCC levels based on the linear mixed model analyses. For the correlation analysis where no distinction in gender was made, the HCC data that had no assigned gender was included. No correlation was found for pasture density and average temperature in May-June ($p>0,05$). When looking at the data after it was sorted by gender, the correlation analysis confirmed a correlation in the female data for fallow density (Spearman = 0,404 $p=0,003$) and average temperature in May-June (Spearman = -0,454 $p=0,001$) with HCC levels. However, no correlation was found for the park factor altitude with HCC using Spearman's rho.

3.3 GR mRNA

For the analysis of GR mRNA, the data of a total of 33 UK samples were available, of which 5 were male and 28 were female. For this analysis, because of the limited amount of data, the linear mixed model was not based on scatterplots, but the best fit linear mixed model, used for the HCC analysis, was used. The linear mixed model was applied to all the different hippocampal regions, the CA1, CA3 and the DG.
First, we looked at the linear mixed model in which the dependent variable was the log greyscale data of the CA1 region and the covariates that were the factors fallow density, pasture density, average temperature in May-June and altitude. Park number was entered as the random factor. First, we looked at the main effects between the covariates. No significant relations of the park factors with the amount of GR mRNA in the CA1 region was found ($p>0,05$). Subsequently, we did the same analysis for the CA3 and the DG regions in the hippocampus. No significant relations were found between the park factors and the CA3 or DG regions ($p>0,05$).

Then, we altered the model to take the interaction between gender and the park factors into account. We found no significant gender dependent relations between the park factors and the amount of GR mRNA in the CA1, CA3 and DG regions ($p>0,05$).

3.4 Hair cortisol and GR mRNA

For the secondary aim of this study to evaluate whether HCC is an accurate indicator for chronic stress, in comparison to GR mRNA levels in the hippocampus, correlation analyses between HCC and the data from the greyscale values of GR_CA1, GR_CA3, GR_DG MR_CA1, MR_CA3 and MR_DG were executed. No significant correlations were found between HCC and the different hippocampal regions. The correlation of HCC with GR_DG, however, came close to significance with $p=0,056$.

4. Discussion

The aim of this study was to determine which factors in wildlife park management influence chronic stress levels of fallow deer. For this project, (1) brain slices were cut from AWD samples for future analysis and (2) statistical analysis of the cumulative data for HCC and GR mRNA levels for UK samples were performed. The latter results, based on the results of the HCC data, provide evidence for the impact of pasture density, fallow density and the temperature in May-June on markers for chronic stress in fallow deer.

4.1 UK deer data

When we look at the whole dataset, it shows that fallow deer have a higher HCC when the pasture density is higher. It also shows that the fallow deer have a lower HCC when the average temperature in May-June is higher. The influence of these factors prove to be more pronounced in the female population than the male population.

Female fallow deer show a lower HCC when the average temperature in May-June is higher. The opposite effect can be seen in male fallow deer. Warmer temperatures in this period, may have an influence on the abundance and quality of their food. These gender differences may be related to the grouping system of fallow deer. The grouping system of fallow deer depends on the season, but mostly on the environment they live in (Putman 1986). Usually, when the habitat

consists of many large and open areas, groups tend to be larger, and fallow deer of both genders may live together throughout the year. In woodland areas, the sexes mostly live separate (Putman 1986). In a study performed by Thirgood (1995), it was found that female fallow deer groups in the New Forest tend to live in woodlands in the autumn and in winter, while in the spring and summer time they preferred to live on open grasslands. Not enough data was collected to determine the seasonal differences by individuals in male and mixed groups. In another study of Putman et al. (1993) performed in fallow deer in parks in the United Kingdom, it was found that, based on fecal samples, males had a diet of better quality in the winter, while females had higher quality diets in the spring time, possibly because of the forming of female-young feeding aggregations during spring season. A study of Main (2008) reviewing sexual segregation in ungulates, showed that females typically alter their feeding behavior based on increasing the likelihood of survival of their offspring. They therefore sometimes choose the habitat of poorer quality, if that means they can flee easily when needed. If there was a lower risk of predation, females choose the higher quality food. Males then, avoided these areas preferred by females. The results of these studies, combined with the notion that there is more growth of vegetation in higher temperatures in springtime, could explain why the HCC in females are lower for those deer that live in parks where the average temperature in May-June is higher compared to those who live in parks with lower temperatures in late spring/early summer. That the HCC in males increases when average temperature is higher in this period, could possibly be because males typically do not live near the females during this period, and may have to resort to less vegetated areas.

The relation between pasture or fallow density and HCC is in this study also gender dependent in that female fallow deer that live in a situation where the fallow density as well as pasture density is higher, show a higher HCC. Male fallow deer however, show a decrease in HCC when the fallow density is higher. No significant effect was seen for pasture density in the male fallow deer. The increase of HCC when population density is high, is in line with previous studies (Caslini et al., 2016; Ashley et al., 2011). These studies did however show no significant difference between females and males. Ashley et al. did however state that differences based on gender could not be ruled out based on their study. Early results of this overarching study performed in the AWD also show that cortisol levels of females decline simultaneously with the declining of the population density, even though this effect isn't seen in males (Lesscher et al. unpublished findings). Sexual dimorphism in the responsiveness of the HPA-axis has been well recorded in rodents, in which males show lower adrenocortical responses than females (Ferin, 2006). In our study, as the number of male subjects was relatively low, a definite conclusion regarding the fallow density based on gender cannot be made, though it does suggest an effect. This effect could as well be explained by the gender dependent grouping system, and therefore the different availability of food, as explained before. When population density is higher, the fallow deer also may have to compete with other animal species for the preferred dietary items, which creates a stressful situation (Main, 2008). Males may be less affected by this, as they show less site fidelity, and because they are not restricted by young. Females with young tend to be concentrated over localized areas, as they form larger groups and show more site fidelity. Sexual segregation in this prevents competition between the male and female populations (Main, 2008).

Results also show that, in the model that has been used in this study, when male fallow deer live in an area on a higher altitude, they possess a higher HCC. However, no correlation was found using Spearman's rho. It has been recorded that animals that live on higher altitudes usually have to deal with more difficult climatic conditions, because of e.g. less vegetation (Caslini et al., 2016). However, the altitudes of the parks participating in this study, while giving

a suggestion to this effect, seem to be not sufficiently high enough to significantly influence the HCC. A more extensive dataset may be required to determine whether altitude indeed contributes to chronic stress among fallow deer. Perhaps the degree of variation in altitude was also not sufficiently high for the selected parks to draw conclusions regarding the potential impact of altitude on HCC levels.

No significant relations correlations have been found between the covariates and the GR mRNA data. Previous studies do however show that a decline in GR mRNA is an adequate indicator for chronic stress in different animal species (Boleij et al., 2014; Dickens et al., 2009; Gomez et al., 1996; Patel et al., 2008; Sterleman et al. 2008). Early results from this ongoing study do also indicate that GR levels are lower in fallow deer in a bad body condition than deer in an adequate body condition (Lesscher, unpublished findings). The fact that this effect does not show in this study, can be explained by the shortage of data, as some hippocampi still have to be processed, with a total of 38 samples of which 28 are female, 5 male and 5 of an unknown gender.

For all the male fallow deer results, it must be said that these are based on little data, as for the HCC data 27 males were available and for the GR mRNA data 5 males were available. Especially when comparing them to the female fallow deer data, of which 52 were available for the HCC data, and 28 for the GR mRNA data. The most plausible reason for this difference is that when culling females, the possibility for an offspring is also reduced. Therefore, culling females is a more effective way to manage the population than culling males. For a study with more accuracy, it is necessary to increase the amount of subjects in the study. Moreover, the results will be more reliable when there is an even distribution of data between the genders.
There are differences in quantity in the data from the different parks, as well as uneven contributions per year. In the year 2014, a total of 60 fallow deer have been delivered for this study. From 2015, this amount was 21, and from 2016, this amount was 10. Moreover, as each park did not contribute the same amount of deer, there could be an uneven distribution of the park factors that are being examined. This together with the fact that the overall numbers for deer from the different parks are generally still rather low (N = 3 - 17), this study would benefit from more samples for the parks involved, and a better gender balance.
Because of the fact that this is a project that has been ongoing for several years, many different students as well as other staff members have been working on the processing of the tissues and analyses. This could lead to small inconsistencies in the results.

For the continuation of this study, and to improve other similar studies, a few suggestions can be made. Foremost, there has to be an expansion of the data, and the data should be evenly distributed between the genders as much as possible to get a clear view of potential differences between the sexes. It is possible that some results, both in the male and female dataset, are carried by a few outliers. However, this is more relevant in the male dataset, as there is less data. For this purpose, it is necessary to have knowledge of the gender of all culled deer, and the aim must be an equal distribution between the sexes. When more data from the GR mRNA is available, it will also be interesting to see if any conclusions regarding the relative value of HCC and GR mRNA as markers for chronic stress and welfare. Furthermore, when more data is available, it is possible that an alteration to the statistical model has to be made, as it has been based on the factors that seemed to be related to the increase of HCC in this study.

4.2 Hair cortisol and GR mRNA

In this study, we looked if a correlation could be found between the data of HCC and the data of the level of GR and MR mRNA from different hippocampal regions, namely CA1, CA3 and the DG. We looked at potential correlations between HCC and GR mRNA, and between HCC and MR mRNA, as a decrease in GR mRNA expression is often accompanied by a decrease in MR mRNA expression (Boleij et al., 2014). No significant correlations were found after testing with Spearman's rho ($p>0,05$), the correlation of HCC with GR_DG, however, came close to significance with $p=0,056$. This is in line with a previous study, that show that the DG is the most sensitive area to stress (Sarabdjitsingh et al., 2008) The results of this study regarding population density align with previous ones (Caslini et al., 2016; Ashley et al., 2011). The question if HCC and GR mRNA expression reflect the same effects of chronic stress, does remain. It does, however, seem plausible for the GR mRNA expression to decline in the case of chronic stress, as a prolonged elevated levels of glucocorticoids, thus a high level of HCC, will downregulate the mRNA of the GRs and MRs, as GRs and eventually also MRs are sensitive to elevated glucocorticoid levels (Mizoguchi et al., 2001; Sapolsky et al., 1990). A reason why they do not reflect the same effect, could be because the GR receptors are less sensitive to elevated levels of glucocorticoids due to chronic stress, compared to storage of cortisol in hair. It could be that the amount of stress the fallow deer experience, is enough for cortisol to store in the hair, but not sufficient enough for glucocorticoids to downregulate the GR mRNA or MR mRNA. This is in line with a previous study in rats, which studied the effect of mild chronic stress on neurobiological parameters (Chen et al. 2016)

This study mainly gives information about the relation between HCC and the park factors that could influence the welfare of fallow deer. HCC shows to be an acceptable index on determining long-term HPA-axis activity. This is in line with findings from previous stress related studies (Caslini et al., 2016; Montillo et al., 2014). In this study hairs were collected post mortem, but hair samples can easily be collected in a non-invasive manner, for example, using barbwire. With that approach, the animals will not experience stress due to capturing and handling, although this should not affect HCC anyhow, as the cortisol in the hair accumulates over a longer period of time. A disadvantage of this method, is that the hairs are not sure to be from the same anatomical location. More studies have to be performed to determine if there are any irregularities in deer HCC when looking at e.g. hair color, location on the body, health status of the animal and other external factors, as a study performed in dogs showed significant differences regarding these factors (Mesarcova et al., 2017). This could be necessary knowledge, if a similar study as this one is to be performed on groups of fallow deer that are not being culled.

However, lastly, it is important to question and determine how chronic stress then translates to animal welfare. What should we measure in wildlife animals to be able to say that their welfare has been compromised? Accumulation of cortisol in hair does not necessarily mean that the animal's ability to adapt has worsened. A certain level of stress hormones can be seen as normal as stress is necessary for an adequate response to adversities. Chronic accumulation of cortisol, however, is not normal. An abnormal buildup of cortisol in hair could result from for example downregulation of GR mRNA in the hippocampus. Downregulation of GR mRNA translates to a malfunctioning HPA-axis, as the negative feedback loop is compromised. In that case, it can be concluded that the animal's welfare is affected, as their adaptability is compromised. Thus to adequately determine the animal's welfare, ideally, conclusions have to be drawn from data on the status of GR mRNA. For the continuation of this study, it is thus recommended that correlation between HCC and GR mRNA is continuously studied when more data is available.

5. Conclusion

As the interaction between humans and wildlife intensifies, there is an increasing interest in animal welfare. This study set out to determine which factors in wildlife park management influence chronic stress levels of fallow deer. This was achieved by analyzing data on the amount of expression of GR mRNA and HCC levels in fallow deer living in parks in the UK, which are indicative of chronic stress. Next to this, the AWD data was extended.
The main finding of this study is that pasture density, fallow density and the average temperature in May-June are important factors that predict if fallow deer experience chronic stress in their habitat. We found that these effects are gender dependent.
We evaluated whether HCC is an accurate indicator for chronic stress, in comparison to GR mRNA levels in the hippocampus. This was accomplished by looking for correlations between the data of HCC and the data of the level of GR and MR mRNA from different hippocampal regions, namely CA1, CA3 and the DG. No significant correlations were found ($p>0,05$).
Future studies are required to extend the dataset and include more GR mRNA measurements to confirm these initial results. Nevertheless, based on these initial findings we suggest that for the sake of animal welfare, population density should be considered in management strategies of wildlife parks, especially when it concerns female deer. This could possibly prevent suffering and improve the welfare of the fallow deer.

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Appendix

Nissl coulouring

Most recent adjustment made by: **Date:**

1. Introduction

2. Chemicals

Chemicals	Supplier	Product Nr.	Location
Cresyl Violet 0,14% aqueous	Klinipath	641571	066
Glacial acid			063, BVK
96% ethanol (technical)			
100% ethanol (technical)			
Xylene			
Entellan			

2. Solutions
When using the cresyl violet for the first time, add 1,77 ml glacial acid.
When the cresyl violet is not working well anymore add 1,77 ml glacial acid.
Indicate this on the bottle.

5. Procedures (perform this procedure in the fume hood)
For 20µM tissue sections (frozen or non-frozen but directly after sectioning).

- Allow tissue to dry.
- When dry, put the slides in the nissl holder in cresyl-violet for 45'- 4hr. (depending of cresyl-violet and tissue).
- Dip 2x 10 times in 96% Ethanol
- Dip 2x 10 times in 100% Ethanol
- Leave the slides 2*3 minutes in the xylene
- Add 3 drops of entellan on the tissue.
  Entellan is a synthetic resin soluble in xylene. Excess mounting medium diminishes microscopic clarity.
- Cover the slide with a coverslip.
  However, better quality pictures are obtained after the mounting medium has fully hardened, i.e. after more than 12 h.
- Keep the slides in the fumehood to dry for at least 12 hr.

ISH with radioactive probes

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

1. Introduction

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

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

2. Chemicals

Chemicals	Supplier	Product Nr.	Location
10x transcription buffer	Roche	11130900	Lab 383, Fridge #7
ATP (100 mM, 25 µmol)	Fermentas	#R0441	Lab 383, Fridge #7
CTP (100 mM, 25 µmol)	Fermentas	#R0451	Lab 383, Fridge #7
GTP (100 mM, 25 µmol)	Fermentas	#R0461	Lab 383, Fridge #7
UTP (100 mM, 25 µmol)	Fermentas	#R0471	Lab 383, Fridge #7

DTT	Sigma	D0632	Lab 383, Fridge #8
RNAse inhibitor (20 U/µl)	Roche	M14764	Lab 383, Fridge #7
DEPC	Calbiochem la Jolla, CA	298711	Lab 385, Fridge #4
$33_{P\ UTP\ (100\ \mu l)\ enough\ for}$ 20 labeling reactions	Perkin Elmer	NEG607H001MC	RA Lab
$33_{P\ UTP\ (25\ \mu l)\ enough\ for\ 5}$ labeling reactions	Perkin Emer	NEG607H250UC	RA Lab
T7 RNA polymerase	Fermentas	#EP0111	Lab 383, Fridge #7
T3 RNA polymerase	Fermentas	#EP0101	Lab 383, Fridge #7
DNAse (RNAse free 40 U/µl)	Fermentas	#EN0521	Lab 383, Fridge #7
tRNA	Roche	10109525001	Lab 383, Fridge #7
Sodium acetate	Sigma	11OH-072015	Lab 383, Chemical cabinet #1
Ethanol 100%	Merck	1.00983.2500	Lab 383, fume cupboard
Paraformaldehyde (PFA)	Merck	PROL28793.292	Lab 385, Fridge #4
Acetate anhydride (pure)	Merck	1.00042.1000 1.00041.1000	Lab 383, fume cupboard #4
HCl (37.5%)	Merck	1.00317.1000	Lab 383, fume cupboard #6
Triethanolamine (TEA)	Merck	1.08379.0250	Lab 383, fume cupboard #4
Formamide	Roche	11814320001	Lab 383, Fridge #6

Dextran sulfate	Sigma	D-8906	Lab 383, Chemical cabinet #1
tRNA Bakers yeast	Roche	10954821	Lab 383, Fridge #7
Sonificated Salmon Sperm DNA	Stratagene	201190	Lab 383, Fridge #8
Ultima gold scintillation Cocktail	Perkin Elmer Precisely	6013119	RA Lab
Ficoll PM 400	GE Healthecare Diegem	17030050	Lab 383, Chemical cabinet #1
Polyvinylpyrrolidone	Sigma	P5288	Lab 383, Chemical cabinet #2
BSA-fraction V	Merck	126609-5GM	Lab 056, cold room
Acetic acid	Merck	1.0063.1000	Lab 63, fume cupboard
NaCl	Merck	1.04873.1000	Lab 63, chemical cabinet
KCl	Sigma	P9541	Lab 63, chemical cabinet #2
di-sodium hydrogen phosphate dihydrate	Merck	1.06580.1000	Lab 63, chemical cabinet
$Na_{2HPO4.2H2O}$ Sodium citrate ($C_{6H5Na3O7.2H2O)}$	Backer J.T. Deventer	0280	Lab 63, chemical cabinet
RNAse A	Roche	10109142001	Lab 383, fridge #8
Acetic anhydride (pure)	Merck	1.00042.1000 1.00041.1000	Lab 383, fume cupboard #4
NaOH	Merck	6498.1000	Lab 63, Chemical cabinet #1

pH calibration buffers pH 7	Radiometer Copenhagen	943-112	Lab 385, cabinet above pH-measurement system
pH calibration buffer pH 4.1	Radiometer Copenhagen	943-111	Lab 385, cabinet above pH measurement system
pH calibration buffer pH 10.0	Radiometer Copenhagen	S 1336	Lab 385, cabinet above pH measurement system
Potassium dihydrogen Phosphate ($KH_{2PO4)}$	Merck	1.04873.1000	Lab 63, Chemical cabinet #1

3. Materials and equipment

3.1 Materials

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

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

3.2 Equipment

Material	**Supplier**	**Article number**
Water bath or stove (37 °C)	Lab companion	BW-05G
Water bath (65 °C)	Lab companion	BS-11
Hybridization stove (55 °C) Set point 56°C	Appligene	Notatia system detached
Stove (>200 °C)	Dépex b.v. De Bilt	Memmert ULE 500 Heating
block (>90 °C)	Beun DeRonde	Lab line multi-blok heater 2000-ICE
Vortex	Scientific Industries	Vortex genie 2

Magnetic stirring device incl. heater (3x)	Tamson	IKA MAG RCT
pH-measurement system	Beun DeRonde	Consort C231
β- counter	Packard Tricarb	1900CA

4. Solutions

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

DEPC water

1 ml DEPC/litre
Keep stirring for 2 h or O/N
Autoclave
(For 20 slides ± 3 liters needed)

10x PBS (1000 ml)
In DEPC

80 gr. NaCl, 2.44 gr. KH_2PO_4,
17.8 gr.$Na_2HPO_4.2H_2O$, 2.0 gr. KCl
Fill up till 900 ml with Milli Q and set pH: 7.5 (met NaOH - juli 2014)
End volume of 1000 ml
Add 1 ml of DEPC
Keep stirring for 2h or O/N
Autoclave

20x SSC (1000 ml)
(3M NaCl/300mM sodiumcitraat)
In DEPC

NaCl 175.3 gr.
Sodium citraat dihydraat 88.2 gr.
Fill up to 900 ml with Milli Q and set pH:7.0
End volume of 1000 ml
Add 1 ml of DEPC
Keep stirring for 2h or O/N
Autoclave

50x Denhardt's solution (50ml)

0.5 gr. Ficoll-400;
0.5 gr.Polyvinylpyrrolidone;
0.5 gr.BSA-fraction V
Dissolve in 50 ml DEPC (in 50 ml tube), by shaking for 10 minutes at 37 °C.
Filtrate over 0.45 µm filter and fill out in eppendorf tubes (50x 1ml)
Store at- 20 °C

Hybridization buffer (500 ml)

100% formamide	250 ml
20x SSC	125 ml
50x Denhardts	50 ml
12.5 mg/ml tRNA bakers yeast	10 ml
10 mg/mlSonificated Salmon Sperm DNA (ssDNA)	25 ml

RNAse A Stock

Stock solution 500 units/ml (100x)
(50-60U/mg)
Dissolve RNAse A powder in Tris buffer:
For 50 ml Tris Buffer:
60.6 mg Tris,
43.8 mg NaCl in MilliQ
pH 7.5

	Boil for 15 minutes at 100 °C Cool down slowly Store at -20 °C
1M Tris pH 8	121,14 g / liter MilliQ Set to pH 8,0
5 M NaCl	292,2 g/liter MilliQ
1M DTT	Dissolve 154.25 mg in 1 ml Milli Q Store at -20°C
tRNA 10 mg/ml	Dissolve 10 mg in 1 ml DEPC-H2O Store at-20°C
3M SodiumAcetate	Dissolve 24.6 gr in 70 ml Milli Q Set pH to 5.2 with Acetic Acid Fill up till 100 ml with Milli Q Autoclave
50% ethanol	Dilute 125 ml 100% ethanol using Milli Q (RNAse free) End volume of 250 ml
70% ethanol	Dilute 175 ml 100% ethanol using Milli Q (RNAse free) End volume of 250 ml
80% ethanol	Dilute 200 ml 100% using Milli Q (RNAse free) End volume of 250 ml
10M NaOH (100 ml)	Mw: 40.00 g/mol Dissolve 40 gr. of NaOH in 100 ml MilliQ

5. Procedures

Probe synthesis

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

RNAse free! Use DEPC-H2O and baked end/or autoclaved glasswork!

Prepare fresh:

- 100 mM DTT
    - Dilute from stock solution 1M DTT with autoclaved Milli Q water
- 10 mM NTP's (ATP, CTP, GTP, UTP)
    - Dilute from stock solution 100 mM with autoclaved Milli Q
- 10 mg/ml tRNA
    - Dissolve 10 mg in 1 ml DEPC-H2O

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

- Labeling reaction pipetting scheme

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

- Incubate at 37 °C for 60' – 90'
- Add 0.75 µl DNAse (RNAse free, 40 U/µl)
- Incubate at 37 °C for 30'
- Add the following and mix by vortexing
  - 80.0 µl ddH_{2O}
  - 1.0 µl tRNA (10 mg/ml)
  - 10.0 µl 3M NaAc (pH=5.2)
  - 270 µl Absolute ethanol
- Freeze at -80 °C for 15'- 30' or O/N at -20°C
- Centrifuge at maximum speed (130000 rpm) for 20' at 4°C
- Collect supernatant in tubes (SN1)
- Add 500 µl 70-80% ethanol and centrifuge for 5-10' at maximal speed at 4°C
- Collect the supernatant in tubes (SN2)
- Air-dry the pellet
- Dissolve pellet at 49 µl DEPC and 1 µl 1M DTT. Store at -20 °C until use
- Measure the efficiency in vitro transcription:
  - Pipette 1 µl of labelled riboprobe in scintillation tube
  - Pipette 7.6 µl of SN1 in scintillation tube (in general 1/50 of total volume)
  - Add 3 ml Ultima gold scintillation fluid. Shake solution thoroughly
  - Measure in β-counter

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

Day 1: Prehybridization and hybridization
Work RNAse free! Use DEPC-H_2O and baked end/or autoclaved glasswork!

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

- 4% Paraformaldehyde (PFA):
  - Dissolve 10 gram of PFA in 225 ml DEPC and 25 ml 10x PBS at 60-65 °C using a heating plate and magnetic stirrer (do not heat excessively as PFA breaks down!).
  - Weigh the PFA under a fume hood!!
  - If the PFA doesn't dissolve at 60-65°C, add a few drops of NaOH
  - Set the pH to 7.4
- 1x PBS:
  - Dilute from 10x PBS using DEPC. Make 500 ml.
- Triethanolamine (TEA):
  - 3.3 ml triethanolamine
  - 0,438 ml HCl (36%, fuming)
  - 250 ml MQ.
  - 0.625 ml Acetic anhydride (drop wise adding)
- 2x SSC
  - Dilute from 20x SSC using DEPC
- Alcohol series:
  - 50%, 80%, 100%.
  - Dilute 100% ethanol with DEPC water, to 50% and 80%

Preparations

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

Protocol

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

Dehydrate:

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

Hybridization

Table 1 Hybridization mix

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

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

Preparations for the next day:

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

Day 2: Posthybridization

The use of DEPC is not necessary.

Preparations:

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

- 0.2x SSC
    - Dilute from 20x SSC, make at least 300 ml
    - Preheat to 65 °C
- 0.1x SSC
    - Dilute from 20x SSC, make at least 600 ml
    - Preheat to 65 °C
- 0.5x SSC
    - Dilute from 20x SSC, make at least 300 ml
    - Preheat 65 °C
- Alcohol series
    - Dilute 100% ethanol (technical) with Milli Q water, to 80% and 50%

Protocol:

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

Wash steps:

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

Dehydrate:

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

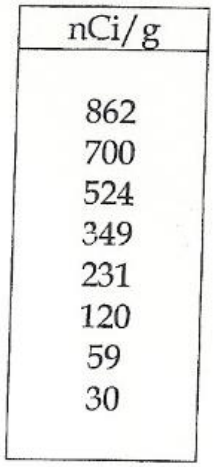
- Air dry the slides
- Clean the autoradiography cassettes with ethanol and put filterpaper in the cassette
- Spray the filter paper with glue and paste the slides on the filterpaper and put it in the cassette
- In a dark room put film on top of the slides and expose the slides for at least 2 weeks

Important:

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

Attachments

Attachment 1:
Figure of the Carbon - 14 marker



Cortisol/corticosterone analysis from hair

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

1. Introduction

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

Protocol adapted from a protocol from the BPRC adapted from a protocol by Davenport, 2006).

2. Chemicals

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

3. Materials

Accurate scale for minimum amounts (mg)
Aluminium foil + tape (for the mark)
Beads (Lab Services BV Biospec Products, 3.2 mm no 11079132)
Centrifuge
Combi-tip 25 mL Eppendorf
End-over-end roller
Eppendorf centrifuge tubes 2 mL(!!!), sure cap
Gloves
Instruments: Forceps, scissor and a scoop

Petri dishes
Pipet 1mL + tips and 200µL tips
Reaction vials 1,5mL, sure cap
Repeater-pipet
Sartstedt Micro tube with cap 2ml (ref 72.694)
Speed Vac Concentrator, Labconco
Stove at 37 oC
Thermomixer, Eppendorf
Tissue Lyser II (Cat. No. 85300, Quiagen)
Tubes (50 mL)

4. Solutions
Dilution solutions provided with the respective kits

5. Procedures
During whole protocol: keep samples in the dark! & wear gloves

1. Shave at least 500 mg hair from the back of the neck
2. Store in aluminium foil, in the dark, at room temperature
3. Put some hair sample (approx. 50% of the tube) in a 50mL tube
   Mark the tube, twice: one on top and one on the side (because work with –ol's), repeat this marking for all the tubes or reaction fails, during this protocol.
1. Wash the hair once in 20mL 1x PBS by roughly shaking (by hand) at room temperature for 2 minutes and decant the PBS, at this step; most blood will be removed from the hair.
   After the PBS wash, wash the hair twice with 20 mL isopropanol by gently mixing for 3 minutes per wash, decant the isopropanol and add new isopropanol for the second wash
1. After the wash, cover a petri dish with aluminium foil (with a mark!). Empty the tube above the dish.
2. Dry the hairs for 96h in a stove at 37°C (has to be thoroughly dry)
3. Remove the hair from the stove. If continue: Step 8.
   For storage: Fold the aluminium foil and make sure the mark (the deer number) is visible. Store in the dark at room temperature
1. Cut the hair with a scissor into pieces (as small as possible) on aluminium foil (approx. 80-90mg). Use a forceps to hold the hairs to minimize hair loss.
2. Put 3 beads(Lab services BV Biospec products, 3.2 mm no 11079132) into an marked Eppendorf tube of 2 mL(2 mL!!!- in 1,5 ml the balls cannot grind at the bottom of the tube). Weigh this on an accurate scale. Grab the hair with the forceps and put the hair in the Eppendorf tube. Weigh again and calculate hair weight. Place 60-70mg washed hair in sure cap Eppendorf centrifuge tubes.
   Clean the scissor and forceps with 70% ethanol, let it dry and collect the next sample.
1. Grind the hair samples with a Tissue Lyser II (Cat. No. 85300, 100–120/220–240 V, 50/60Hz, Quiagen) at 30 Hz during 15 minutes. Centrifuge (3 min, room temperature, 14000 rpm) and repeat for another 15 minutes. Centrifuge (Short spin). Ensure afterwards no clots of hairs are still present in the tubes. If necessary repeat until ground to powder.
2. Weigh in 35±2 mg hair (mink, deer) powder into a clean 2 mL Sarstedt tube with cap. (for rat and monkey (Rhesus and Java) use 50 mg hair). Note the exact weights.
   Clean the spatula with ethanol 70%, let it dry and weigh the next sample.
1. Add 1.5 ml methanol (using a combitip 25mL Eppendorf, on a repeater-pipet with a 200µl pipet-tip attached) and incubate the tubes at room temperature overnight on an end-over-end roller to extract the steroids.
   Make sure that the Sartstedt tubes are completely closed! Put 3 or 4 Sartstedt tubes in one 50mL tube.
   End-over-end roller present at third floor at room 384 in Nieuw Gildestein building Utrecht

2. Centrifuge (5 min, room temperature, 14000rpm) and pipet twice 650µL of the extract (1 mL pipet) into a clean reaction vial (1.5 mL). Centrifuge again (5 min, room temperature, 14000rpm) and use reverse pipetting to put exact 1 mL of the extract in a new reaction vial (1.5 mL).
3. Dry the methanol from the tubes in a Speed Vac Concentrator (CentriVap Concentrator Labconco) at 42oC for 2.5 hours. The lids of the tubes have to be open.
   Speed Vac Concentrator present at second floor at room 272 in radioactive laboratory in Nieuw Gildestein building Utrecht
1. Dissolve the dried extracts of ferret and deer in 60µl phosphate buffer(assay buffer) that is provided in the essay kit (High Sensitivity Salivary Cortisol ELISA kit (Salimetrics) using the thermomixer at 50 oC at 1400 rpm for 30 minutes with the lids of the tubes closed.
   (The rat extract were dissolved in 1 mL Diluent included in the corticosterone RIA kit MP Biomedicals, Germany).
1. Centrifuge the samples at room temperature for a short spin till 14000 rpm. Measure the cortisol with using the previous described ELISA kit by following the accessory protocol. Analysis are in duplicate.
   Plate mixer (DPC Micromix 5) present at laboratory of the Department of Animal in Science and Society, ground floor in Nieuw Gildestein building Utrecht; ELISA reader (Softmax Pro 5, Versa max microplate reader) present at second floor at room 236 in Nieuw Gildestein building Utrecht
   (Rat extracts were diluted 1:4 in diluent included in the RIA kit.)
1. Calculate the pg cortisol/ mg hair used (ng corticosterone/mg hair). (As the RIA from MP Biomedical is for plasma and all plasma samples are diluted 1:200 and the kit corrects for that, the results of the RIA analysis have to be DIVIDED by 200 to get the absolute pg cort/ hair).
   In EXCEL calculate OD 450nm (optical density) - OD 490nm and organize the data so the sample ID corresponds to the OD.
   Subtract the average OD NSBW (non-specific-binding-wells) from all the OD-values.
   Calculate log-values of the cortisol-standards' concentrations
   Open the program 'graph pad' -> use tutorial ELISA or RIA, follow this tutorial and, and put those log-values as known x-values in the program.
   The y-values are the OD's. Let the program calculate the X-values, this will be the log of the µg/dL, copy those to your EXCEL file
   Calculate the inverse log so the unknowns samples are now in µg/dL.
   <u>Compare the kit controls</u>, if it's in the range, continue, if not: re-do the whole protocol.
   The result (µg/dL) are multiplied by the amount of phosphate buffer (step 15) divided by 100 to convert units to [ng/µL]
   Multiply this by 1.5 (to control for loss; step12: 1.5mL -> 1mL (step 13)). So the outcome will be the total cort (ng) extracted
   Divide total cort [ng] by hair weight [mg] (step 11)
   Multiply this by 1000 to convert units to pg cortisol/ mg hair.
1. Clean the beads for re-use. After removing the beads from the tubes wash them by the following steps:
   1)Wash the dirty beads in a petri dish with tap water until they are visibly clean
   2)Put the beads in a 50 mL tube and add methanol
   3)Incubate this tube on a gentle mixer overnight
   4)Decant methanol and add pure ethanol
   5)Dry the beads and put them again in a clean petri dish

6. Reference

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