Does coat color affect cortisol levels in Border collie dogs?

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Title/Title: Does coat color affect cortisol levels in Border collie dogs?

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Abstract/Sammanfattning:

Cortisol is a stress hormone which is released from the adrenals in the Hypothalamic-Pituitary-Adrenal (HPA) axis and plays a major role in animal stress response. Cortisol is used as a stress marker and can be sampled using different methods. A good non-invasive method and a good measure of chronic stress is to measure cortisol through hair. Cortisol is stored in hair for months and therefore reflects chronic stress. The aim of this study was to investigate if cortisol concentration differs depending on coat color. Hair samples from 20 black and white Border collie dogs was analysed and used in this study. Cortisol was extracted with methanol and analysed with ELISA. The results showed no significant difference between black and white coat color within the population while there were individual differences. The results also showed that the sexes do not affect the cortisol concentration. In summary, coat color (black and white) has an effect on cortisol concentration which means that the factor color does need to be taken into account when measuring cortisol through hair.

Keywords/Nyckelord:
Hair cortisol; Dog; Coat color; Stress; Welfare; Canis
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1 Abstract

Cortisol is a stress hormone which is released from the adrenals in the Hypothalamic-Pituitary-Adrenal (HPA) axis and plays a major role in animal stress response. Cortisol is used as a stress marker and can be sampled using different methods. A good non-invasive method and a good measure of chronic stress is to measure cortisol through hair. Cortisol is stored in hair for months and therefore reflects chronic stress. The aim of this study was to investigate if cortisol concentration differs depending on coat color. Hair samples from 20 black and white Border collie dogs was analysed and used in this study. Cortisol was extracted with methanol and analysed with ELISA. The results showed no significant difference between black and white coat color within the population while there were individual differences. The results also showed that the sexes do not affect the cortisol concentration. In summary, coat color (black and white) has an effect on cortisol concentration which means that the factor color does need to be taken into account when measuring cortisol through hair.

2 Introduction

Cortisol is the main hormone released from the adrenal gland in the Hypothalamic-Pituitary-Adrenal (HPA) axis (figure 1) (Murri et al. 2016). This system is involved in the stress response in mammals and cortisol has regulatory effects on various body functions, i.e. blood pressure, sleep and immune system (Geber et al. 2012).

![Figure 1. Schematic figure of how cortisol is released by HPA axis. (Description by Malek et al. (2015))](image)

Stress can be due to different situations, both environmental and biological (Fourie et al. 2016). Increased cortisol levels in short term can be adaptive and help the animal in acute situations while long term increase of cortisol has negative effects on animal’s body and mental health (Bennet & Hayssen 2010). The negative effects that are caused from this long term stress, including decreased growth and weaker immune response which can lead to several autoimmune, physical and psychological disorders (Bennet & Hayssen 2010). Since cortisol plays a major role in the stress response, it is often used as a stress marker.

Cortisol concentration can be measured by different methods, using saliva, blood, urine or fecal samples (Bennet & Hayssen 2010). These sampling methods give a good measure of short term stress, because cortisol in these methods reflects stress from minutes to days (Fourie et al. 2016). Invasive sampling methods, for example, blood sample, can itself act like a stressor to the animal (Hennessy et al. 1998). Since the sampling methods blood and saliva correlate with each other, a noninvasive method like saliva is a better alternative to avoid stressing the animal (Kobelt et al. 2003). However, measure chronic stress by any of these methods needs multiple samplings and therefore is none of these methods suitable for that purpose (Fourie et al. 2016). Another alternative noninvasive sampling method to measure chronic stress is by extracting cortisol from hair (Bryan et al. 2013).
Cortisol in hair is stored for months, which is convenient for measurement of chronic stress since only one sample is needed. The hair is growing from the hair follicle and the intercalation of cortisol is chronological. It has also been shown that cortisol is incorporated differently in different hair types, that the guard hair containing more cortisol than wool hair (Macbeth et al. 2010). This sampling method is also noninvasive and if the animal gets stressed, the acute stress will not affect the results (Bryan et al. 2013). Hair can as well be saved and stored for a long period of time without breaking (Bryan et al. 2013).

One previous study (Bennet & Hayssen 2010) has shown that there is a difference in cortisol concentration due to the color of the coat in dogs (Canis lupus familiaris). The authors discovered that black hair and yellow hair on the same individual had different concentrations of cortisol; black hair contained lower concentrations of cortisol than yellow hair. Black hair contains more pigment than yellow hair, and that could be a reason to why black hair could not store as much cortisol as yellow hair.

Another previous study (Yamanashi et al. 2013) found a difference in cortisol levels between black and white hair on captive chimpanzees (Pan troglodytes). They observed that cortisol concentrations were lower in black hair compared to white, even when the hair was collected from the same part of the body. In the article, the authors concluded that white hair should be avoided when measure cortisol in chimpanzees.

Studies have also found that coat color does not affect cortisol levels. In one study in which dogs were used (Nicholson & Meredith 2015), the authors concluded that there was no difference in cortisol concentration between black hair and non-black hair in a population. Two studies (Sauvé et al. 2007, Manenschijn et al. 2011) done on human also showed that natural hair color does not affect how cortisol is stored in the hair.

The aim of the present study was to investigate if there is a difference in cortisol concentration between black and white dog hair. Based on the findings from the previous studies (Bennet & Hayssen 2010), the hypothesis is that black hair contains lower cortisol concentration than white hair.

3 Material & methods

3.1 Animals
20 border collie dogs in the ages from 17 months to 12 years old were used in this study (9 females and 11 males). Border collie dogs were used as study animals due to its coat, they are colored black and white and has long guard hair. The dogs came from owners in Östergötland (N=19) and in Västmanland (N=1) that voluntary set up for this study and were found using social media. All of the dogs had black and white coat color and some of the dogs (N=6) had three colors, black, white and brown. Hair was only collected from places where both black and white hair was present. Two of the dogs were a crossing between a border collie and a flatcoated retriever.

3.2 Hair sampling
Hair samples were taken in the shift between black and white coat colors, around the neck. The hair was cut as close to the skin as possible with a scissor. Approximate 1 gram of each color was cut and stored in small plastic bags. Further, date, sex and age were noted on the plastic bag.
3.3 Hair preparation

Hair samples were stored in room temperature until preparing. Hair from 20 dogs was divided into black- and white colored hair. The hair was prepared using a milligram scale, sample tubes, scissor, tweezers and baking tins. The wool hair was removed from the guard hair with a tweezer. 2 cm from the bottom of the guard hair (the hair that is closest to the skin) was cut into small pieces (<3-4mm) in the baking tins. The hair pieces were then placed in a sampling tube containing small stainless steel beads and weighted on a scale. Approximate 7-8 mg of guard hair was used in all samples, but some of the samples only contained 3-6 mg of hair due to lack of guard hair.

3.4 Freezing and pulverization of the hair

Tubes containing hair and stainless steel beads were put into metal containers and the containers with tubes were thereafter put into liquid nitrogen for 2 minutes. A Tissuelyzer II (Qiagen, Hilden, Germany) was used to powder the hair after it has been frozen (23 Hz for 2 minutes).

3.5 Cortisol extraction

The cortisol was extracted using 100 % methanol (Fisher Scientific, Loughborough, UK). 1 ml 100 % methanol was added to each sampling tube. Para film were then put over the tube caps to prevent any leakage. The tubes were put to shake overnight in a Grant-bio PTR-60 (Grant-bio, Cambridgeshire, England).

The tubes were thereafter centrifuged in a Micro 22OR (Hettich Zentrifugen, Tuttlingen, Germany) at 23 x g for 1 minutes in 4˚C. 800 µl of the supernatant was transferred to new tubes and put in a speed-vac (Genevac, NY, USA) for 1.5 hours to evaporate the methanol. The tubes were then closed and stored in the fridge until coming ELISA analysis.

3.6 ELISA

The pellet obtained after the speed-vac was solved by adding 30 µl of 99.5 % ethanol (Solveco, Rosenberg, Sweden) followed by adding 220 µl of assay buffer. Cortisol levels were thereafter detected using a commercial cortisol enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, NY, USA). All samples were tested in duplicate following a standard protocol (using an assay buffer containing 12% ethanol).

In short, samples were added to antibody-coated wells in a 96-well plate and incubated for 2 hours in a shaker equipment. The plate was washed and substrates added before incubated for 1 hour in room temperature. Stop solution was added to all wells and the plate was then read in the microplate reader, ASYS uvm-340 (Biochrom, Cambridge, England) at 405 nm. The results were saved for data analysis.

3.7 Data analysis

Microsoft Excel (2016) was used to structuring the raw data from the ELISA analysis. The blank sample was removed from both duplicate standard 1-7 samples and hair samples to remove all disturbances. This data was then inserted in Graph pad prism 7 (Graph Pad software, San Diego, CA, USA) to calculate the cortisol concentration in pg/ml.
The data from graph pad were insert once again in Microsoft excel. By an equation with factors: amount of resuspended pellet (250 µl), milligram of hair in the sample and the normalized ratio (0.8), the cortisol concentration in pg/mg dog hair was calculated for each sample. The equation for cortisol in a sample is followed:

\[
\frac{\text{Cortisol concentration for each sample in } \text{pg/mg} \times \text{amount of resuspend pellet in } \text{ml, 0.25 ml}}{(\text{dog hair in } \text{mg} \times \text{normalized ratio, 0.8})}
\]

A Wilcoxon matched-pairs signed rank test in graph pad was performed to calculate if there was a difference in cortisol concentration in this population between black and white coat colors. A Mann-Whitney U test was performed to investigate if there was a difference in cortisol between females and males. A correlation graph was made in excel to get an R² value on the correlation between black and white coat color and a Spearman rank correlation test was performed in graph pad to investigate if there was a significant correlation. A p-value < 0.05 was considered to be significant.

4 Results

Cortisol was extracted from dog hair from 20 border collies (11 males and 9 females). The extracting method which was used is present in Roth et al. (2015). Cortisol levels were thereafter measured using an ELISA and the results is present in figure 2. The results from statistical analyses showed no significant difference (p= 0.52, W= -29.00) in cortisol concentration in this population between black and white coat color (N=17) (figure 2). A correlation graph (figure 3) with an R² value of 0.10 indicate that there is no strong correlation between black and white coat color. A Spearman rank correlation test (p= 0.21, r= 0.32) also showed that there was no significant correlation between the two colors.

3 of 20 dogs were removed from the study (number 1, 9 and 16). The results were not trustworthy due to lack of guard hair in the samples which affected the results from ELISA (table 1).

![Figure 2. Hair cortisol concentration (pg/mg) with SEM in black and white coat color from 17 border collie dogs. The cortisol is extracted by methods from Roth et al. (2015) and analyzed with ELISA. A Wilcoxon test showed no significant difference (p= 0.52) in cortisol concentration between black and white coat color.](image-url)
Table 1 shows the levels of cortisol in the dogs in black and white coat color. This study found that the cortisol levels varied between the dogs, some dogs had higher cortisol levels in their white hair compared to the levels in the black hair (3, 4, 6, 8 and 10). Some dogs show the opposite tendency, i.e. lower levels of cortisol in their white hair compared to the levels of cortisol in their black hair (5, 14, 19 and 20). Some of the dogs had equal amount of cortisol in their white and black hair (2, 7, 11, 12, 13, 15, 17 and 18).
Table 1. Border collie dogs used in the study when investigate if black hair and white hair contain different amount of cortisol. The red marked dogs were removed from the study due to lack of guard hair in the samples which affected the results.

<table>
<thead>
<tr>
<th>Dog No</th>
<th>Sex</th>
<th>Age (year)</th>
<th>Coat color</th>
<th>Hair (mg)</th>
<th>Cortisol (pg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>8</td>
<td>Black</td>
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<td>11.97</td>
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<td>8.23</td>
</tr>
<tr>
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<td></td>
<td></td>
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<td>6.7</td>
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</tr>
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<td>8.1</td>
<td>7.91</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
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<td>Black</td>
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</tr>
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<td></td>
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</tr>
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<td>7</td>
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<td>9.98</td>
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<td></td>
<td></td>
<td>White</td>
<td>7.9</td>
<td>20.96</td>
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</tr>
<tr>
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<td></td>
<td></td>
<td>White</td>
<td>7.9</td>
<td>6.97</td>
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<tr>
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<td>10</td>
<td>Black</td>
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<td>8.84</td>
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</tr>
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<td>8.1</td>
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<tr>
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<td></td>
<td></td>
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<td>7.28</td>
</tr>
<tr>
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<td>Black</td>
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</tr>
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<td>10.11</td>
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<tr>
<td>16</td>
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<tr>
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<td></td>
<td></td>
<td>White</td>
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<td>3.77</td>
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<tr>
<td>17</td>
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<td>3</td>
<td>Black</td>
<td>8.1</td>
<td>4.23</td>
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<tr>
<td></td>
<td></td>
<td></td>
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<tr>
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<td></td>
<td>White</td>
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<td>7.69</td>
</tr>
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<td>Black</td>
<td>7.0</td>
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<td>Black</td>
<td>7.9</td>
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<td></td>
<td></td>
<td></td>
<td>White</td>
<td>8.1</td>
<td>5.70</td>
</tr>
</tbody>
</table>

This study was also interested to see whether or not sex affected cortisol levels (figure 4). The study found no differences in cortisol levels between males and females (p=0.60 U=29.00).
Figure 4. Cortisol concentration (pg/mg) with SD measured using ELISA in both coat color between males (N=10) and females (N=7). A Mann-Whitney U test showed no significant difference (p=0.60) in cortisol between the sexes.

5 Discussion

The results showed no difference in cortisol concentration in black and white hair within this population or between females and males. The results also showed no correlation between the two colors within the population and differences in cortisol levels between individuals. This indicates that storing of cortisol in hair is affected by black and white coat color.

The results from the present study is not fully consistent with previous studies where it has been shown that black hair in German shepherd dogs contained lower amount of cortisol than non-black hair, in this case yellow (Bennet & Hayssen 2010). However, one factor that must be considered is that their study was done on German shepherd dogs which is not the same breed used in this study. The difference in dog breeds can have an effect on the results since the finding from an earlier study done by Nicholson and Meredith (2015) is consistent with the finding in the present study. Nicholson and Meredith (2015) analyzed cortisol levels in dogs with black hair and dogs with another color of hair in different breeds (Labrador retriever, springer spaniel, shih Tzu, king Charles spaniel and jack russell terrier) and crossbreeds. They did not observe any difference in cortisol concentration in the population between black and non-black hair, when compare dogs with each other. This reflects the present study were no difference could be found on a population level. Cortisol levels in breeds with other coat color, e.g. grey or brown remains to be determined. Some breeds, e.g. German Shepheard dog may have more cortisol in their non-black hair compare to their black hair (Bennet & Hayssen 2010).

We did not observe any statistical difference between black and white coat color within the population, but there was still some variation between the dogs; some dogs had higher cortisol concentration in black hair compare to others that had the opposite, more cortisol in white hair. In the correlation graph, we cannot see any correlation between black and white coat color which indicate that there is a variation in the two colors between individuals. We cannot either detect if black or white hair containing more cortisol than the other due to this non correlation. This variation between the dogs is not observed in Bennet
and Hayssen (2010) there all of their dogs had lower cortisol concentration in their black hair. The effect of other coat color (than black and white) and different breeds in cortisol concentrations could also be of interest for future studies.

Since there was no difference in cortisol concentration in coat color within the population, we were interested to investigate if cortisol levels are affected by sex. The result showed that none of the sexes had higher or lower cortisol than the other. This finding indicates that the sexes does not need to be taken into account when extracting cortisol through hair. This is consistent with the study done on humans by Manenschijn et al. (2011) where it was no differences in cortisol concentration depending on the gender. This was also confirmed by Nicholson and Meredith (2015) that used dogs in their study. One other study done on humans by Dettenborn et al, (2012) showed that males in ages 1-9 and 18-49 had higher hair cortisol concentration than females in the same ages.

If using hair as a method to measure cortisol from a single individual, several factors are needed or not needed to be taken into account. This study, together with others (Manenschijn et al. 2011, Nicholson & Meredith 2015) has shown that if measure cortisol from a single individual, both females and males can be treated equally. Coat color, black and white, has shown no correlation with each other and therefore, there is a variation in these two colors within and between individuals. This means that black and white coat color cannot be treated equally when measure cortisol through hair from a single individual.

### 5.1 Societal & ethical considerations

Stress is used as a marker to investigate if an animal is not feeling well and is important in an animal welfare perspective. Stress is often used in studies when investigate if different factors, i.e. situations, smells or sounds, affecting an animal. Therefore, it is important to have good and suitable cortisol sampling methods. Sampling methods using material such as saliva, blood, urine or fecal samples give a good measure of cortisol on short term stress. However, since these sampling methods are invasive they might act like a stressor to the animal. Also, measuring chronic stress by any of these methods needs multiple sampling and therefore are none of these methods suitable for that purpose. Researchers have therefore started extracting cortisol from hair since this gives a measurement of chronic stress while being a less invasive sampling method at the same time. Measure cortisol in hair has several advantages, the hair will grow back, it can be repeated, hair is easy to store, it is possible to measure chronic stress in one sample and this contributes to a better welfare for the animals.

### 5.2 Conclusion

We could detect and measure cortisol in hair from Border collies using a non-invasive sampling method followed by ELISA analysis. We found that cortisol concentration in hair from border collies is not affected by sexes or coat color on a population level. However, we also observed no correlation between the two colors and individual differences in cortisol concentration in black and white hair. This finding indicates that black and white coat color in dogs cannot be treated equally.
6 Acknowledgement

I would like to thank my supervisors, Per Jensen and Ann-Charlotte Svensson Holm, for their support during this study. I am very grateful to Ann-Charlotte Svensson Holm for all support when written this report and for the help with the lab work. I would also thank the laboratory staff for other help in the lab and my examiner Hanne Lövlie for feedback and approval of the report. I would like to send my warmest thanks to all dog owners that volunteers with their dog or dogs to this study. At last, I will give a great thanks to all other, that in one way or another participated in this study.

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