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Assessment of cortisol concentrations from wolf hair samples in relation to age, sex, and health status of individuals

Diploma thesis

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Abstract

Studying cortisol concentrations is important for assessing stress levels in wild animals like wolves. While several studies used scats for hormone assessment, measuring cortisol levels from hair samples may be more suitable for analysing long-term physiological changes of an individual. In this thesis, the hair cortisol concentrations of free-ranging wolves above the age of one year were studied. The hair samples were collected from wolf carcasses recovered in Italy. The main aim was to determine whether sex, age, or health status (determined by body condition and signs of diseases) would have an effect on hair cortisol concentrations. Based on former studies, it was predicted that wolves with poor health status would have higher hair cortisol concentrations than healthy individuals. Concerning sex and age, previous studies did not reveal consistent effects on hair cortisol levels. Additionally, for this thesis, other factors were also considered, such as body mass, season of death, and the province where the individual had been found. Cortisol was extracted and assessed from each hair sample using enzyme immunoassays, and the results were statistically analysed with linear mixed models. Out of all considered factors, only health status was found to affect hair cortisol concentrations significantly - confirming that wolves with poor health status have elevated hair cortisol levels compared to healthier individuals. Neither sex, nor age had a significant effect on hair cortisol concentrations in this study population, and no correlation was discovered with any of the other considered factors.

Keywords: wolf, hair cortisol concentration, glucocorticoid, steroid hormone, chronic stress

Zusammenfassung

Die Messung von Kortisol-Konzentrationen ist ein wichtiger Bestandteil für die Beurteilung von Stress bei Wildtieren wie Wölfen. Einige Studien verwenden Kotproben zur Hormonbestimmung, aber die Messung von Kortisol aus Haarproben eignet sich möglicherweise besser um bereits länger bestehende Stresszustände zu bestimmen. Im Rahmen dieser Arbeit wurden die Kortisol-Konzentrationen im Fell freilebender Wölfe im Alter von über einem Jahr untersucht. Dafür wurden von Wolfskadavern, die in Italien gefunden wurden, Haarproben entnommen. Das Hauptziel bestand darin, festzustellen, ob Geschlecht, Alter, oder Gesundheitszustand (gemessen am Körperzustand und der An- oder Abwesenheit von Krankheitsanzeichen) einen Einfluss auf den Kortisolspiegel im Fell haben. Basierend auf früheren Studien wurde angenommen, dass Wölfe mit einem schlechten Gesundheitszustand höhere Haarkortisol-Konzentrationen aufweisen würden als gesunde Individuen. Was die möglichen Auswirkungen von Geschlecht und Alter betrifft, so zeigten frühere Studien keinen eindeutigen Einfluss auf den Kortisolspiegel im Fell an. Andere Aspekte, die in der vorliegenden Arbeit berücksichtigt wurden, waren zudem Körpergewicht, Todeszeitpunkt (Jahreszeit) und die Provinz, wo der Kadaver gefunden wurde. Kortisol wurde aus den Haarproben extrahiert und mittels Enzymimmunoassays quantifiziert. Die Ergebnisse wurden statistisch mithilfe von Linearen Gemischten Modellen (linear mixed models) analysiert. Es wurde festgestellt, dass von den genannten Faktoren nur der Gesundheitszustand die Kortisol-Konzentrationen im Fell signifikant beeinflusst. Wölfe mit schlechtem Gesundheitszustand wiesen, im Vergleich zu gesünderen Individuen, erhöhte Kortisolspiegel auf. Weder Geschlecht noch Alter hatten einen signifikanten Einfluss auf die Kortisol-Konzentrationen in dieser Studienpopulation, und es wurden keine Korrelationen mit den anderen berücksichtigten Faktoren festgestellt.

Abbreviations

AB: antibody

BC: body condition

CV: coefficient of variance

df: degrees of freedom

EIA: enzyme immunoassay

EL: enzyme label

Fig: figure

GC: glucocorticoid

HCC: hair cortisol concentration(s)

HPA: hypothalamic-pituitary-adrenal

LMM: linear mixed effects model

LRT: likelihood-ratio test

n: number of individuals

NSB: non-significant binding

p: probability

POD: peroxidase

S: standards

SD: standard deviation

TMB: Tetramethylbenzidine

vif: variance inflation factor

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1. Introduction

For the past few decades, the importance of wildlife conservation has been gaining more and more attention not only from scientific researchers, but also from the public. Although there have already been some significant developments in several different areas of wildlife research, there are still vast areas of uncharted territory that need further investigation as well as the urgent need to develop more accurate methods of monitoring and observation, with special regard to the lives of free-ranging wild animals and their frequent interactions with humans due to rapidly urbanising environments.

Wolves are widespread predators all over Europe. Due to their protected status, their numbers have been rapidly increasing over the past few years in most European countries (1). However, as they are moving much closer to human-dominated areas than before, the conservation of the species is becoming more and more debated (1, 2). Studying these carnivores in regard to their behaviour and physiology could help maintain their protected status and increasing numbers by mitigation of human-wildlife conflict.

The present thesis focuses on the endocrinology of free-ranging wolves in their natural habitats, with particular emphasis on cortisol, which is the most commonly assessed glucocorticoid (GC) hormone in mammals. Cortisol has long been studied as a stress hormone in many species, however, in the case of wild animals, it is usually assessed from faecal samples, which have a certain number of limitations regarding both storability and the accuracy of the reflected hormone contents. Lately, the use of hair samples for hormone assessments has become a popular alternative for wildlife testing, mostly because hairs - in contrast to other substrates - have the ability to reflect long-term hormonal changes of the body (3, 4), making them ideal for certain study questions, like investigating the correlation between cortisol levels and chronic diseases or long-term stress exposure. Furthermore, hair samples also have other advantages, for example, they can be collected non-invasively (by carcass-sampling or by using hair-traps), after which they are easily stored and transported without requiring special handling, like cooling or freezing (3, 4). Regarding wolves, cortisol has rarely been extracted from hair samples before, which makes the findings of this thesis essential for contributing to the validation of using hair samples to study the hormonal patterns of wild wolves and ultimately, to investigate the physiological consequences of habitat loss and urbanisation, which makes these predators cross paths with humans more and more frequently.

1.1. Stress and cortisol

To develop accurate long-term strategies of wildlife conservation, we must consider various aspects that may affect the lives and fitness of wild animals. One such aspect may be chronic stress. While acute stress response is mostly beneficial for the organism, chronically elevated stress levels can greatly affect the fitness and survival of an individual. Acute stress is defined as a physiological response to a short-term threat that requires increased energy mobilisation from an individual in order to ensure its survival in a particular situation (5). The physiological reaction to a stressor of any kind includes the activation of the so-called hypothalamic-pituitary-adrenal (HPA) axis, through which an elevated release of cortisol from the adrenal glands is initiated (6). Besides its basic energy regulatory function of releasing glucose (7), cortisol suppresses inflammation and promotes immune-cell proliferation on a short-term basis (8). These changes - evoked by acute stress response - are required for the organism to ensure its survival in threatening situations. However, long-term exposure to elevated cortisol levels is far less beneficial, with suspectedly increased vulnerability to certain infections (9) due to the stimulation of shifting from a cellular to a humoral type of immunity, which could even evoke autoimmune diseases and the growth of neoplasms or metastases (10, 11). Furthermore, nutritional and wound healing deficiencies have been observed following prolonged exposure to high levels of cortisol (5). There is also evidence that cortisol deteriorates the reproductive capacity of females by inhibiting oestradiol and luteinising hormone effects and thus reducing mating and ovulation (12). In addition, being under prolonged influence of high sympathetic activity may lead to certain cardiovascular deficits like hypertension or endothelial damage (13).

1.2. Cortisol and diseases

While stress-induced long-term rises in cortisol levels could be responsible for some of the above-mentioned health conditions, previous studies indicate that this directionality can also be reversed, meaning that battling certain illnesses brings about detectable changes in the body’s cortisol production (14). Investigating the correlation between health status and cortisol levels in wild wolves is a vital aim of this study. Painful conditions or unpleasant symptoms can be significant sources of stress, which can even be directly stimulated by the release of cytokines in the case of inflammatory diseases (15). While most studies on other species clearly indicate elevated cortisol levels when chronic diseases are present (16, 17),

one study on dogs revealed no such differences of hair cortisol concentrations (HCC) between healthy and chronically ill individuals (18). Regarding wild canids, a recent study found higher average HCC in coyotes with diseases than individuals that had died more suddenly - for example, from vehicle collision (19), and in wolves, viral, bacterial or parasitic infections as well as neoplasms have also been shown to extremely elevate HCC (14).

1.3. Role of biological factors

In addition, this thesis sets out to determine whether biological factors such as sex or age are linked to changes in HCC in wolves. Concerning the matter of sex in other species, previous studies on lynx (7, 20) and deer (21) found no significant differences of HCC between male and female individuals. A study on feral horses, however, revealed that males had significantly higher HCC than females (22), which is most likely related to the socio-ecology of the species, where stallions often need to fight off other rival individuals in order to defend the mares of their own harem group. Regarding canids, studies on dogs either found no differences of HCC between sexes (18), or showed higher HCC in females (23). In coyotes, however, either males were found to have higher HCC (24), or there were no differences between the two sexes (19). Considering possible differences in cortisol concentrations between male and female wolves, the results of previous studies are somewhat inconclusive, with either no significant differences between sexes (25–28), or male individuals having slightly higher cortisol levels (14). Based on these findings, it appears likely that the link between HCC and sex is not straightforward and may be influenced by other factors, like age, reproductive activity, or body condition for example.

Finally, age is an important biological factor that needs to be taken into consideration when examining cortisol levels, even though former research reveals some contradictory results here as well. Regarding other species, HCC did not seem to be affected by age in lynx (7), deer (21), dogs (18, 29) and coyotes (19). Considering wolves, one study revealed no effect of age on cortisol levels (27), whereas in several other cases, juveniles under two years of age had lower cortisol concentrations than adults (14, 25, 28). Interestingly, yet another study showed the exact opposite by measuring higher cortisol levels in subadult wolves leaving their homesites or natal pack (30). These findings indicate that increased cortisol levels in juvenile wolves may be the effect of dispersal from the pack. After being born, most wolves remain with their family packs only for about one or two years, after which time they

tend to leave them and wander off on their own, searching for new territories and potential mating partners. This is naturally a very stressful time for young wolves and is likely to be related to rising cortisol levels.

1.4. Substrates for the assessment of cortisol

Like most other steroid hormones, cortisol can be assessed from blood samples or any other bodily fluids (31, 32), but measurement following extraction from faeces (5, 27, 28, 33, 34), nail-clippings (8, 25) or hair samples (3, 4, 7, 14, 16, 18–26) can also provide valid results. Whilst blood drawing is rarely a viable option in the case of free-ranging animals, its most widely used alternative, faecal samples also have a number of limitations. For instance, short-term acute stressors or even changes in diet can cause variations in hormone contents, which reflect stimuli from approx. 24 hours before defecation, but this period can be longer or shorter, depending on the gut transition time of the species (34). In addition, collection and storage of faeces is somewhat complicated, as samples can easily degrade if not collected and stored appropriately within a few hours after excretion (33), not to mention that attributing samples to specific individuals is extremely difficult in free-ranging, elusive species, even if camera traps are installed (35).

1.5. Extraction of cortisol from hair

Extracting cortisol and potentially other steroid hormones from hair samples may be a useful alternative and more suitable for studying wild animals, considering that the results thus obtained are robust to acute, transient stressors (14) and are thought to reflect long-term stress exposure over several weeks or months (3, 4). This could be ideal for certain study questions, for instance, investigating longer and on-going disturbances or chronic health conditions. Furthermore, hair samples can be stored and transported at room temperature and hormone concentrations in hair remain stable over long periods of time (3, 4), thus making them ideal for field work where access to freezers can be difficult or unattainable.

There have been a few studies attempting to validate the assessment of GC hormones from hair samples in canids, with dogs being the overrepresented subject species (18, 23, 29), but coyotes have also been studied (19, 24). Regarding wolves, to this day, there have only been three studies assessing HCC (14, 25, 26), which certifies the need to promote this

method for future studies focusing both on captive and free-roaming populations. In addition to GC hormones, extracting reproductive (steroid) hormones from hair samples is a likewise validated approach to the investigation of hormonal patterns in canids (24–26), which, along with hair cortisol levels, could be a powerful tool for understanding more about wild wolves.

1.6. Predictions and hypotheses

Based on previous studies outlined above, the most important prediction of this thesis is that wolves with chronic diseases or high parasitic loads will have significantly higher hair cortisol levels than healthy ones. This prediction corresponds to the results of similar studies on wolves (14) and coyotes (19), which investigated the effects of health status on HCC.

Considering the role of biological factors, sex is not expected to significantly influence the hair cortisol concentrations of wolves in this study. Regarding former studies on wild wolves, there were either no differences of HCC between the two sexes (25, 26), or males appeared to have slightly higher HCC (14).

Concerning age, juvenile wolves are predicted to have higher hair cortisol levels than adult individuals, because they tend to leave their natal pack around the age of one or two years, which is a very stressful event for them and could contribute to the elevation of their cortisol levels. This assumption is also supported by the findings of another study on wild wolves, in which cortisol was assessed from faecal samples (30). However, other studies on hair samples of free-ranging wolves obtained the opposite result, revealing higher HCC in adult wolves compared to juvenile individuals (14, 25).

1.7. Significance

By understanding how these basic factors (health status, sex, and age) relate to hair cortisol concentrations in wild wolves, the findings of this thesis will deliver crucial information on the validity and usefulness of hair samples to assess the hormonal status of wolves under certain conditions. In addition, limitations and confounds of the measurement of hormones from hair samples shall also be considered. Potential future applications may include the investigation of how anthropogenic factors and the urbanisation gradient affect the endocrinology of wolf populations in Europe.

2. Material and methods

2.1. Wolves and samples

For this thesis, hair samples of individual wolf carcasses - necropsied by the Veterinary Pathology Department at the University of Bologna - were collected. The carcasses included puppies, juveniles, and adult wolves of both sexes, all of which had been recovered from different regions of Italy (mostly Emilia-Romagna, Tuscany and Umbria). Initially, the hair samples of 166 wolves were used for cortisol extraction, however, puppies under one year and individuals with missing data were excluded from the subsequent statistical analysis, resulting in a final sample size of 114 wolves. A detailed list of all animals was created, containing important data such as the estimated age, sex, reproductive status and body mass of each animal. Other key parameters - like region and province where the carcass had been found, approximate time and cause of death, signs of diseases, body condition and stomach contents - were also noted.

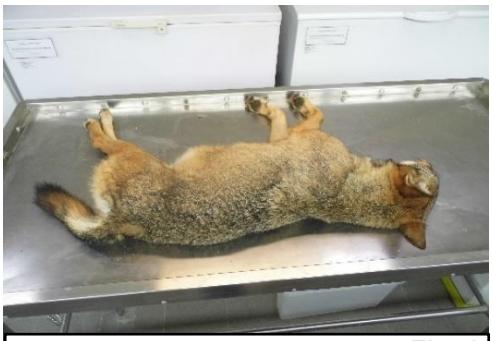

Fig. 1.
Photo of a wolf carcass at the Veterinary Pathology Department in Bologna.
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The hair samples were cut with scissors close to the skin from different body regions (shoulder, belly, upper and lower back) and then individually wrapped in aluminium foil and placed into paper envelopes labelled with the wolves' individual identification number and the body region from which the hair was collected. Each sample consisted of guard hairs as well as undercoat. For this study, mainly hairs of the shoulder region were used, or if not available, the upper back region, since these areas of the body are most likely to be used for hair collection in live animals.

2.2. Extraction protocol

The extraction from the hair samples was carried out by following the instructions of a previously validated extraction protocol (Appendix 7.1.) at the Experimental Endocrinology Unit of the Institute of Physiology, Pathophysiology and Biophysics at the University of Veterinary Medicine in Vienna between March and April 2023.

Briefly: On the first day, 200 mg of each hair sample were cut with scissors and weighed into glass vials, washed, and degreased by adding 7 ml n-hexane to each, then vortexed shortly and decanted. The hair samples were then left to dry overnight, and on the following day, about 100 mg from each sample were weighed into fresh glass tubes. Then, after adding 5 ml of 100 % methanol to each, the vials were plugged tightly and immersed in a water bath at 37 °C for approximately 24 hours. On the last day, the samples were centrifuged for 15 minutes at 2500 *g* at room temperature, and then 2.5 ml from each sample were transferred into clean glass tubes and dried under a nitrogen stream on a heating block at 60 °C. After adding 0.5 ml of assay-buffer (pH 7.5) to each sample and shaking them for 30 minutes, the extracts were transferred into small plastic tubes and stored in a freezer at -20 °C until analysis.

Fig. 2.
Photo of hair samples in glass vials.

2.3. Assay protocol

For the hormonal analysis of the extracted samples, a validated, in-house enzyme immunoassay (EIA) for cortisol was used. (Appendix 7.2.) Briefly: On the first day, the samples were thawed and pipetted into the appropriate wells of coated plates that had been washed before. The plates were then placed on a shaker in a cold room and incubated overnight. On the next day, the plates were washed, and a mixture of peroxidase and assay-buffer was added into the wells. The plates were incubated on a shaker in a cold room for 45 minutes, and then washed again. After that, a mixture of TMB (Tetramethylbenzidine), peroxidase and TMB-buffer was added into the wells, the plate was incubated again on a shaker in a cold room for 45 minutes. As a next step, 50 *µl* of sulfuric acid were pipetted into the wells, the plates were placed on a shaker at normal room temperature for 30 minutes, and then were read using a photometer at 450 *nm*. All samples were measured in duplicates and repeated if the coefficient of variance (%CV) of duplicates was above 10 %. Initially, 10 *µl* of sample were used on the assay, but due to low concentrations, samples were re-measured at 30 and 50 *µl*, respectively, and accepted only when falling within the linear range of the standard curve (20–80 % binding). Two samples had to be pre-diluted (1:10) before falling within the linear range. The standard curve ranged from 0.3–80 ng/well. Intra-assay variance was 5.7 % on average, inter-assay variance was 16.6 %. All values were converted into ng/g considering the sample volume used on the assay plate.

2.4. Health score and age estimation

To define health status, each wolf was given a score on a scale of *one* to *four*: a health score of *four* meant best possible health status, while score *one* indicated the worst possible health status. Initially, all individuals had a score of *four*, from which - if necessary - points were subtracted, based on two different aspects. Firstly, the presence of any signs of disease at the time of necropsy was considered: if an animal had none, its score remained *four*; however, in case it *did show* signs of disease, one point was subtracted, resulting in a score of *three*. The next aspect that was considered for scoring, was body condition (BC), which was determined by weight, age class and nutritional status of the animal, ranging from good to cachectic: as for wolves with good BC, no points were subtracted; for medium BC, one point was subtracted; and in case of poor BC or cachexy, two points were subtracted. Based on this scoring system, for instance, a wolf *with* disease *and* cachexy was given a health score of *one*, while another individual *with* disease *and* medium BC received a score of *two,* thus matching the score of another wolf that *did not have* obvious signs of disease *but* still had poor BC. A score of *three* was given to wolves that *did have* a disease *and* had good BC, and also to the individuals that *did not have* a disease *but* had medium BC. Healthy-appearing animals *with* good BC were given a health score of *four*.

The age of the wolves was estimated by assessing weight, muscular development and dentition, respectively (2). Based on these aspects, three age groups were created: puppies (class 1; under one year old), subadults or juveniles (class 2; between one and two years old), and adults (class 3; above two years old). Puppies were easily recognised due to their small body size and low weight, and their thin, sharp teeth. *(Fig. 3* and *4.)* Juvenile wolves were identified by slender body and underdeveloped skeletal muscles, but also by their lily-shaped incisors. *(Fig. 3* and *4.)* Adult individuals were distinguished upon higher weight, more massive body dimensions and fully developed muscle mass. *(Fig. 3.)* Furthermore, they had a wide forehead, and regarding dentition, the distance between their canines was more than 4 cm, and the lily flower shapes on the incisors were no longer recognisable. As age progresses, dental inspections usually reveal worn teeth, with older individuals having shorter incisors, or even completely worn incisors and broken canines in the case of very old subjects. *(Fig. 4.)*

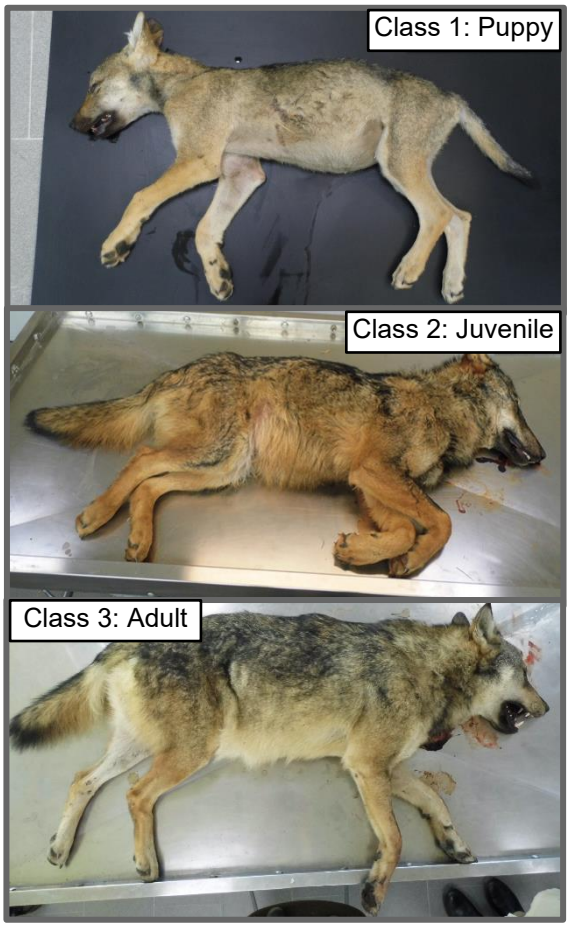

Fig. 3.
Photos of wolf carcasses of different age groups.

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Fig. 4.
Photos of the dentition of wolves in different ages.
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2.5. Statistical analysis

For the statistical analysis, juvenile (class 2, n = 49) and adult (class 3, n = 71) wolves of both sexes were considered, excluding puppies (class 1, n = 46). This was primarily done to simplify the analysis, as the age class 1 (puppies) in this population represented a very heterogenous subgroup, given their ages could range between a few days or weeks and one year. Some other individuals also had to be excluded because of missing information about the carcasses (n = 6). This resulted in a final sample size of 114 wolves used to fit the statistical model, out of which 77 were male (27 juveniles and 50 adults), and 37 were female (19 juveniles and 18 adults).

To investigate the effects of age, sex, and health status (health score) on wolves' HCC, a linear mixed effects model (LMM) with Gaussian error structure was fitted (R statistical software, version 4.2.1; R Core Team, 2022) using the function *lmer* of the R package *lme4* (version 1.1-31) with the optimizer '*bobyqa*'. The response variable (HCC) was log-transformed to comply with model assumptions of homogenous and normally distributed residuals. Age class, sex, and health score were included as fixed effects (test predictors). Weight and season of death were included as control predictors to account for the potential effects of body mass and season (summer vs. winter coat) on HCC. Furthermore, in the full model, two-way interactions of age class and sex, and age class and season of death were included. Plate id (assay plate) and province (in which the carcass had been found, n = 25) were included as random factors. The co-variate (weight in kg) was z-transformed to ease model convergence and interpretation of results. Reference levels of the factors are listed in Appendix 7.3. *(Table 7.3.2.)* To keep type I error rate at the nominal level of 5 %, all theoretically identifiable random slopes components were included and manually dummy coded. As an overall test of the effect of the three test predictors (sex, age class, health score) and to avoid problems arising from multiple testing, a full-null model comparison was performed. Specifically, the full model was compared to a null model lacking the test predictors but comprising the same control predictors and random effect structure as the full model (36) using a likelihood ratio test (LRT); (37). In case the interaction terms did not reveal significance, a reduced model was fitted to determine the significance of the main effects. To rule out collinearity, the variance inflation factor (vif) was determined which revealed no higher values than 1.58 (weight), indicating that collinearity was not an issue (38). Model stability was assessed by comparing the estimates obtained from the model based on all data with those obtained from models with levels of the random effects excluded one at a time. This revealed overall good model stability. (Appendix 7.4., *Table 7.4.2.*) Confidence intervals were derived using the function *bootMer* of the package *lme4*, using 1000 parametric bootstraps. Tests of the individual fixed effects were derived using likelihood ratio tests (39); (R function *drop1* with argument 'test' set to '*Chisq*').

One adult male wolf (sample number 68) had extremely high HCC (approx. 45 times higher than the average of all other individuals), therefore, two identical models were fitted, one including this outlier (first model) and one excluding it (second model). Model output tables for both models can be found in Appendix 7.3. and 7.4.

3. Results

Overall, the entire study population (n = 114; first statistical model) had a mean (SD) HCC of 2.79 (8.79) ng/g (range = 0.03–91.40 ng/g), and after excluding the outlier sample, the remaining individuals (n = 113; second model) had a mean (SD) HCC of 2.01 (2.70) ng/g (range = 0.03–20.18 ng/g). Although both models had similar statistical outcomes, only the results of the second one (without the outlier) will be considered hereinafter. Details of the first model can be found in Appendix 7.4., and the curious case of the outlier sample will be discussed separately later in this thesis.

3.1. Effects of health status

Of the test predictors included in the model, only health score was found to have an effect on the hair cortisol concentrations of wolves. *(Tables 7.3.1* and *7.4.1* in the appendix.*)* The higher the health score, the healthier the animal was considered. The results obtained from the second model lacking the outlier indicated that wolves with higher health scores tended to have somewhat lower HCC (LRT = 3.576, df = 1, p = 0.059) than individuals with poorer health scores *(Fig. 5.)*, in line with the predictions of this thesis.

Individuals with a health score of *three* or *four* (n = 93) had a mean (SD) HCC of 1.87 (2.71) ng/g (range = 0.03–20.18 ng/g), while animals with a score of *one* or *two* (n = 20) had a mean (SD) HCC of 2.66 (2.65) ng/g (range = 0.16–9.25 ng/g) *(Table 1.)*.

Table 1. HCC of wolves (n = 113) in different health score classes.
(Mean and standard deviation.)

HCC (ng/g)	*poorest* Health score *healthiest*			
	1 *(n = 17)*	**2** *(n = 3)*	**3** *(n = 21)*	**4** *(n = 72)*
Mean	**2,48**	**3,69**	**2,02**	**1,82**
SD	*2,28*	*4,81*	*3,48*	*2,47*

When only considering the presence of diseases, healthy appearing wolves (n = 88) had a mean (SD) HCC of 1.69 (2.28) ng/g, in contrast to individuals with apparent signs of diseases (n = 25), which had a mean (SD) HCC of 3.12 (3.70) ng/g. *(Table 2.)*

Wolves with diseases either had sarcoptic mange (n = 8) - and a mean (SD) HCC of 2.67 (2.66) ng/g, or other disease (n = 17) - resulting in a mean (SD) HCC of 3.33 (4.15) ng/g. *(Table 2.)*

Table 2. HCC of wolves (n = 113) based on 'signs of diseases'.
(Mean and standard deviation.)

HCC (ng/g)	(n = 88)	(n = 25)		
	No disease	Disease	(n = 8) Sarcoptes	(n = 17) Other disease
Mean	**1,69**	**3,12**	**2,67**	**3,33**
SD	*2,28*	*3,70*	*2,66*	*4,15*

Regarding body condition (BC), wolves with good or medium BC (n = 95) had a mean (SD) HCC of 1.94 (2.79) ng/g, while poor or cachectic BC (n = 18) resulted in a mean (SD) HCC of 2.39 (2.25) ng/g.

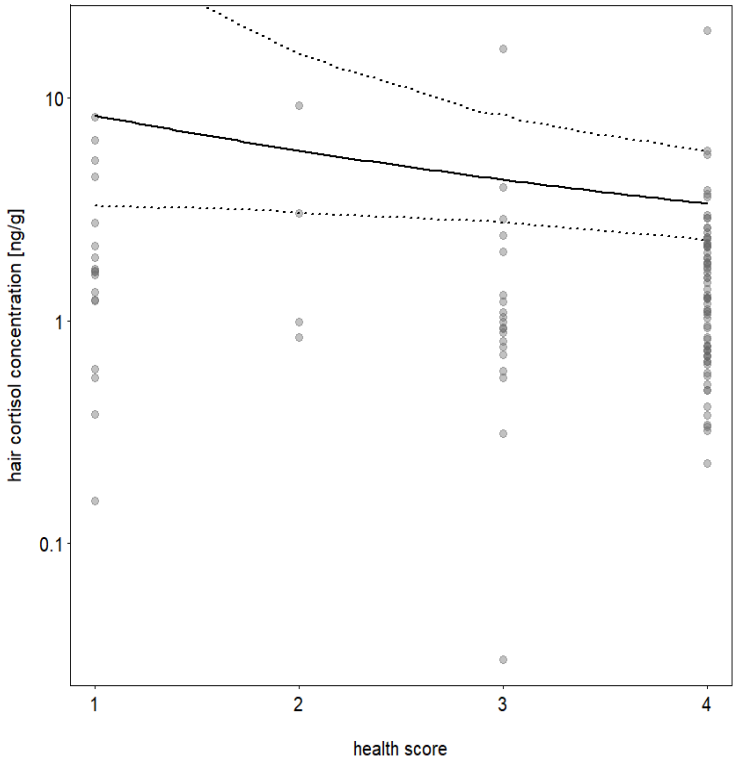

Fig. 5. Hair cortisol concentrations of wolves (n = 113) in relation to their health score.
The figure shows the fitted model (solid line), its upper and lower 95% confidence intervals (dotted lines), and the data (grey dots).

3.2. Effects of sex and age

Sex did not significantly affect HCC (LRT = 0.254, df = 1, p = 0.614).

Male wolves had a mean (SD) HCC of 1.88 (2.27) ng/g (n = 76, range = 0.23–16.67 ng/g), and females had a mean (SD) HCC of 2.28 (3.45) ng/g (n = 37, range = 0.03–20.18 ng/g). *(Table 3.)*

Age also did not have a significant effect on HCC (LRT = 0.662, df = 1, p = 0.416) in this study population, which consisted only of juvenile wolves (i.e., with estimated age between one and two years) and adult (above two years old) individuals.

Juveniles had a mean (SD) HCC of 2.02 (3.05) ng/g (n = 46, range = 0.34–20.18 ng/g), and adults had a mean (SD) HCC of 2.00 (2.47) ng/g (n = 67, range = 0.03–16.67 ng/g). *(Table 3.)*

Table 3. HCC of wolves (n = 113) based on sex and age group.
(Mean and standard deviation.)

HCC (ng/g)	**Male** *(n = 76)*	**Female** *(n = 37)*	**Juvenile** *(n = 46)*	**Adult** *(n = 67)*
Mean	**1,88**	**2,28**	**2,02**	**2,00**
SD	*2,27*	*3,45*	*3,05*	*2,47*

Juvenile male wolves (n = 27) presented a mean (SD) HCC of 1.55 (1.07) ng/g, while juvenile females (n = 19) had a mean (SD) HCC of 2.70 (4.55) ng/g. *(Table 4.)*
Adult male individuals (n = 49) had a mean (SD) HCC of 2.06 (2.71) ng/g, while adult females (n = 18) had a mean (SD) HCC of 1.83 (1.67) ng/g. *(Table 4.)*

Table 4. HCC of wolves (n = 113) of different sexes in the two age groups.
(Mean and standard deviation.)

HCC (ng/g)	Juvenile		Adult	
	Male *(n = 27)*	**Female** *(n = 19)*	**Male** *(n = 49)*	**Female** *(n = 18)*
Mean	**1,55**	**2,70**	**2,06**	**1,83**
SD	*1,07*	*4,55*	*2,71*	*1,67*

4. Discussion

The aim of this study was to determine whether sex, age, or health status would have an effect on hair cortisol concentrations in wolves. Regarding health, the obtained results indicated that wolves with poor health status had higher HCC than healthy individuals, which corresponds to the predictions of this thesis and the results of previous studies as well. As predicted, sex had no significant effect on the HCC of wolves. Considering the two different age groups that were studied, no apparent influences of age on HCC were revealed. This is in contrast with the prediction deriving from the results of former studies indicating that juvenile wolves would have higher HCC than adult individuals, possibly due to dispersal. Overall, the present findings indicate that hair samples can be a useful substrate to investigate the endocrinology of wild wolves, and add to the current knowledge of the factors that may affect cortisol levels in wild wolf populations.

4.1. Health influences

Health status in the present study was determined by giving each individual a score on a scale of *one* to *four*. The highest score (*four*) indicated that the individual had good body condition and no signs of diseases, while the lowest score (*one*) stood for worst possible body condition with diseases. Looking closely at the results, health score (therefore, health status and body condition) was found to have an effect on hair cortisol levels. *(Fig. 5.)* To be exact, the higher the health score (i.e., the healthier the individual), the lower the HCC, meaning that wolves with painful conditions or health deficits had higher HCC than healthy individuals. The same connection between health status and HCC has been revealed before by studies on wolves (14), coyotes (19) and other species (16) as well.

In this study, health status (i.e., health score) was defined by the presence of diseases and body condition. Regarding diseases, sarcoptic mange is a common parasitic skin infection of wild wolf populations that can last up to several months or years (40). Although all diseases could be significant causes of stress for an animal, a previous study on wild wolves indicated that the highest HCC in samples were associated with sarcoptic mange (14). However, that was not confirmed by the results of the current thesis, which found similarly elevated mean hair cortisol levels in wolves suffering from sarcoptic mange as in the case of individuals with any other diseases.

Besides diseases, body condition is also a significant factor to be taken into account when assessing hair cortisol levels. Animals, which are generally healthy, are more likely to maintain their bodyweight and a physiological hormonal balance, while sick individuals tend to be subjected to weight loss, either because of hunting difficulties or as a direct result of the disease itself. In accordance with this, studies on coyotes (19) and other species (16, 21, 22) have revealed elevated HCC in animals with poor body condition compared to individuals with better BC. Despite that, the current thesis only discovered small differences in that department. Overall, there were 18 wolves with body condition deficits among the samples, and they appeared to have slightly higher mean hair cortisol levels than well-nourished individuals. However, this was not tested in further statistical analyses, meaning that any possible effect, which BC alone might have on the HCC of wolves, remains unclear.

4.2. Sex and age influences

The results of this current thesis do not indicate significant HCC differences between male and female wolves. Previous relevant studies - in which hair samples (14, 25, 26) or faeces (27, 28) of wild wolves were used for cortisol extraction - also had inconclusive results concerning the link between sex and cortisol concentrations. These studies either showed no obvious effect of sex on cortisol levels (25–28), or revealed male wolves to have slightly higher cortisol levels (14). Based on these findings, it is likely that the link between HCC and sex is not straightforward and could be influenced by several other factors, for example, body condition, reproductive activity or seasonal effects.

Regarding age, no link was found between hair cortisol levels and certain age classes of wolves. However, the study population only included individuals above the age of one year, meaning that younger animals (puppies) were not included in this thesis. Considering previous research which used hair samples (14, 25) or faeces (27, 28, 30) for cortisol assessment in wild wolves, some studies found that juvenile individuals had lower cortisol concentrations than adults (14, 25, 28), but in other cases, either the opposite results were found (30), or cortisol levels did not seem to be affected by age (27). These contradictory findings either indicate that detecting subtle differences of cortisol levels in hair samples is somewhat limited, or they point out the fact that HCC are not directly correlated with age and could be subjected to several other factors, like season, prey availability, time of dispersal from the pack, or hunting pressure in the area.

4.3. The outlier individual

Interestingly, one adult individual (included in the first statistical model; sample nr. 68) had a remarkably high HCC (91.40 ng/g), which was about 45 times higher than the average HCC of all other samples (2.01 ng/g). This wolf was a very old male (he had completely worn incisors and broken canines) and was discovered alive in Florence, Toscana in January, but died in a wildlife recovery centre a few days later, most likely as a result of starvation. He received a health score of *one* due to his poor body condition and apparent signs of diseases, such as sarcoptic mange. In addition, there was a piece of bone stuck on his hard palate *(Fig. 6.)*, which caused him prolonged pain and a serious infection, and - probably - difficulties in eating as well.

Fig. 6. Photo of sample nr. 68. *© Dr. Carmela Musto*

Although the extremely elevated HCC of sample nr. 68 might seem unusual, it is not surprising considering the cicumstances. Starvation is not only an incredibly stressful process for animals and a direct cause for cortisol level elevations (21), but on a long-term basis, circulating high cortisol concentrations can also be responsible for maintaining a poor body weight by using up all energy storages (21), and for increasing vulnerability to certain infections (9) which can lead to the development of other diseases, thus creating an unbreakable vicious cycle for the weakened animal.

4.4. Limitations of the study

Although extracting glucocorticoids from hair samples is becoming increasingly popular for hormone assessment in free-roaming animals, there may still be some limitations regarding the accuracy of the results obtained through this method. In order to correctly interpret the findings of this thesis, we must consider several different factors that could influence the variations of HCC among certain individuals.

While hair samples can be suitable for detecting significantly elevated cortisol levels in stressed or sick animals, they may not be able to reflect the small differences of cortisol levels possibly influenced by sex or age. In contrast to other substrates commonly used for cortisol assessment (like blood or saliva), hairs may not be sensitive enough to reflect subtle

changes in cortisol levels, mostly because of their low temporal resolution or the difficulties of the analytical method (including the extraction process) which could lead to the loss of some metabolites. Based on these considerations, the use of hair samples for cortisol assessment is probably unable to the reflect the subtle effects of some of the considered factors (like sex and age), and is only suitable for detecting major hormonal changes, like the effects of diseases or chronic stress, which can hugely elevate systemic cortisol levels.

Although this thesis did not investigate whether (or if yes, how) the differences of hair samples could affect HCC assessments, it is possible that various hair attributes could also influence cortisol level measurements. For instance, the inhomogeneous composition of hair samples used for the extraction could be an influencing factor. Even though guard hairs and the underlying wool hairs from each wolf were both used for extraction, these two different hair types were not equally distributed amongst the samples, meaning that in case of some individuals, for example, there may have been either more guard hairs or more wool used for the analysis than in case of other animals. However, a recent study on wolves found no differences between the cortisol levels of guard hair and underwool (25), and in another study on dogs, the HCC of various hair types - collected from the same body region - were also highly correlated (29). Considering possible influences of hair colour, a study on German Shepherd dogs found black hairs to have lower cortisol levels than lighter coloured hairs of the same body region (29), however, that was not consistent with the findings of a study on wolves, which found no such correlation and no effect of hair colour on HCC (26).

Regarding possible differences between the hormone contents of winter and summer coat, ‘season of death’ was an important factor which was included in the statistical model as well, but appeared not to have affected the HCC of the samples. Another potential outcome in that matter could have been either a lower HCC in case of the carcasses found during the summer season - as prolonged exposure to sunlight has been found to reduce hair cortisol levels in other species (22, 41), or an elevated HCC in case of wolves found in winter - possibly invoked by the start of the mating season (42). As temperatures begin to drop in autumn and winter, reduced prey availability could also cause stress for predators and lead to the elevation of their cortisol levels. In accordance with this assumption, a study with bobcats has shown that in autumn, HCC were greater in wild bobcats than in captive ones (7), which was attributed to the fact that captive bobcats were fed consistently and thus did not have to face nutrition scarcity like the free-roaming individuals did (7). However,

the current thesis found no such correlations regarding the HCC of wolves in this study population, which could be explained by the fairly stable prey density of the mild winter seasons of Italy. Despite that, there had been remarkably more carcasses discovered between December and March than in other seasons. *(Table 5.)* The most likely reason for this could again be the dispersion of young adult wolves from their natal pack during this season, which often leads to their death, most commonly caused by anthropogenic reasons, such as vehicle collision (n = 77), poisoning (n = 15), or gunshot (n = 10).

Table 5. HCC of wolves (n = 113) based on season of death.
(Mean and standard deviation.)

HCC (ng/g)	Season of death		
	Dec-March *(n = 61)*	April-July *(n = 22)*	Aug-Nov *(n = 30)*
Mean	**1,93**	**2,01**	**2,16**
SD	*2,84*	*2,14*	*2,86*

Apart from the possible effects of seasons, wolves in certain regions might be subjected to different amounts and sorts of stimuli than others. For instance, an area with high pack density is clearly a great source of stress for an individual, but hunting pressure or human activities in rapidly urbanising environments can also disturb the free-roaming predators nearby (26). This could not have been explored as part of the current thesis, as it was not possible to determine if wolves were actual residents of an area or whether they were just moving through. However, future research may take the opportunity to also investigate the effects of urbanised environments on wild wolves, by monitoring their movements along different regions of Europe, for example by using stable isotope analyses. This would enable a more accurate interpretation of hormone levels assessed from hair samples.

4.5. Conclusions of the study

The findings of this thesis confirm that the use of hair samples can be a powerful tool for studying the correlation between chronic diseases and the hormonal status of wild wolves. Although there are still some limitations to this method, it seems applicable for future studies which aim to investigate the potential effects of urbanisation or other anthropogenic factors on the endocrinology of wild wolves by assessing various hormones from their hair samples.

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7. Appendix

7.1. Extraction protocol of cortisol

Day 1:

Sorting samples in batches of n = 36

- Cutting hair samples (~2–3 mm short).
- Weighing ~0.2 g hair into each glass vial.

Washing (degreasing)

- Adding 7 ml n-hexane to each vial.
- Shaking vials on a hand vortex (~1 min).
- Decanting n-hexane.
- Leaving hair samples to dry overnight.

Day 2:

Extraction (part 1)

- Weighing 0.1 g hair (0.100 ± 0.0005 g) from each vial into an eprouvette.
- Adding 5 ml methanol (100 %) to each vial.
- Plugging vials tightly.
- Incubating in thermomixer at 37 °C (~24 hours).

Day 3:

Extraction (part 2)

- Centrifuging vials (15 min at 2500 *g*, at room temperature).
- Transferring 2.5 ml of each extract into a new eprouvette.
- Drying under a stream of nitrogen at 50°–60° C (~45 min).
- Re-dissolving in EIA buffer (0.5 ml each).
- Shaking for 30 min.
- Transferring extracts into *'Biorad'* tubes (diluting 1:10 if necessary).
- Storing in freezer at -20° C until analysis.

7.2. Enzyme immunoassay (EIA) protocol for cortisol

Day 1:

- Thawing extracts at room temperature.
- Washing coated plates with cold wash-solution.
- Thawing *Enzyme label* (EL), *Antibody* (AB) and *Standards* (S).
- *Pipetting plates:*
  - S1–S7: 10 *µl Standard* + 40 *µl* assay-buffer
    - 100 *µl* EL
    - 100 *µl* AB
  - P1–P*x*: 50 *µl* sample (or 30 *µl sample* + 20 *µl* assay-buffer)
    - 100 *µl* EL
    - 100 *µl* AB
  - NSB: 150 *µl* assay-buffer
    - 100 *µl* EL
  - B0: 50 *µl* assay-buffer
    - 100 *µl* EL
    - 100 *µl* AB
  - Incubating plates on shaker in a cold room overnight.

Day 2:

- Washing plates.
- Mixing POD (peroxidase) and assay-buffer, then adding 100 *µl* into each well.
- Incubating plates on shaker in a cold room for 45 minutes.
- Washing plates.
- Mixing TMB (Tetramethylbenzidine), H_2O_2 and TMB-buffer, then adding 100 *µl* into each well.
- Incubating plates on shaker in a cold room for 45 minutes.
- Adding 50 *µl* of H_2SO_4 (Sulfuric acid) into each well.
- Shaking plates at room temperature for 30 minutes.
- Reading the plates with a photometer at 450 *nm.*

7.3. Statistical model lacking the outlier (second model, 113 samples)

Table 7.3.1. Reduced (non-significant interactions dropped) model output table of the second model (lacking the outlier).
(Legend: npar = df (degrees of freedom), AIC = Akaike information criterion, LRT = likelihood ratio test, Pr(Chi) = p-value, X2.5 = lower confidence limit, X97.5 = upper confidence limit.)

	Estimate	Std. Error	t value	npar	AIC	LRT	Pr(Chi)	X2.5.	X97.5.
(Intercept)	0,805	0,460	1,748	NA	330,961	NA	NA	-0,138	1,777
sexM	0,097	0,184	0,529	1	329,216	0,254	0,614	-0,294	0,479
age_classclass_3	-0,171	0,205	-0,836	1	329,623	0,662	0,416	-0,600	0,256
health_score	-0,187	0,095	-1,963	1	332,537	3,576	*0,059*	-0,369	0,000
z.weight_kg	0,111	0,129	0,860	1	329,647	0,686	0,408	-0,144	0,379
season_of_deathaug_nov	0,340	0,298	1,141	2	328,166	1,204	0,548	-0,295	0,979
season_of_deathdec_mar	0,151	0,318	0,476					-0,515	0,824

Table 7.3.2. Reference levels of the test predictors.
(Legend: F = female; class 2 = juveniles; apr_jul = April, May, June, July.)

Reference levels:	
sex	F
age_class	class_2
season_of_death	apr_jul

Table 7.3.3. Model stability parameters of the second model.
(Legend: Orig = original estimate, min = minimum, max = maximum.)

	orig	min	max
(Intercept)	0,805	0,471	1,195
sexM	0,097	-0,097	0,240
age_classclass_3	-0,171	-0,342	0,057
health_score	-0,187	-0,290	-0,111
z.weight_kg	0,111	0,009	0,249
season_of_deathaug_nov	0,340	0,084	0,591
season_of_deathdec_mar	0,151	-0,163	0,330
province@z.weight_kg@NA	0,000	0,000	0,000
province@season_of_death.dec_mar@NA	0,542	0,000	0,855
province@season_of_death.aug_nov@NA	0,000	0,000	0,918
province@age_class.class_3@NA	0,000	0,000	0,000
province@sex.M@NA	0,000	0,000	0,184
province@(Intercept)@NA	0,102	0,000	0,271
plate_id@z.weight_kg@NA	0,000	0,000	0,000
plate_id@season_of_death.dec_mar@NA	0,368	0,000	0,508
plate_id@season_of_death.aug_nov@NA	0,000	0,000	0,352
plate_id@age_class.class_3@NA	0,000	0,000	0,000
plate_id@sex.M@NA	0,000	0,000	0,220
plate_id@(Intercept)@NA	0,372	0,196	0,499
Residual	0,791	0,633	0,831

7.4. Statistical model with the outlier (first model, 114 samples)

Table 7.4.1. Reduced model output table of the first model (including the outlier).
(Legend: npar = df (degrees of freedom), AIC = Akaike information criterion, LRT = likelihood ratio test, Pr(Chi) = p-value, X2.5 = lower confidence limit, X97.5 = upper confidence limit.)

	Estimate	Std. Error	t value	npar	AIC	LRT	Pr(Chi)	X2.5.	X97.5.
(Intercept)	1,017	0,491	2,072	NA	345,805	NA	NA	0,118	1,983
sexM	0,136	0,193	0,708	1	344,262	0,457	0,499	-0,248	0,508
age_classclass_3	-0,126	0,214	-0,590	1	344,137	0,332	0,565	-0,564	0,303
health_score	-0,268	0,098	-2,729	1	350,794	6,989	0,008	-0,462	-0,056
z.weight_kg	0,110	0,135	0,818	1	344,435	0,630	0,427	-0,171	0,378
season_of_deathaug_nov	0,384	0,308	1,245	2	343,054	1,249	0,536	-0,241	1,006
season_of_deathdec_mar	0,237	0,307	0,773					-0,355	0,875

Table 7.4.2. Model stability parameters of the first model.
(Legend: Orig = original estimate, min = minimum, max = maximum.)

	orig	min	max
(Intercept)	1,017	0,472	1,533
sexM	0,136	-0,094	0,350
age_classclass_3	-0,126	-0,274	0,111
health_score	-0,268	-0,378	-0,161
z.weight_kg	0,110	-0,019	0,256
season_of_deathaug_nov	0,384	0,220	0,595
season_of_deathdec_mar	0,237	-0,013	0,447
province@z.weight_kg@NA	0	0	0,000
province@season_of_death.dec_mar@NA	0,503	0	0,733
province@season_of_death.aug_nov@NA	0	0	0,942
province@age_class.class_3@NA	0	0	0,146
province@sex.M@NA	0,000	0	0,000
province@(Intercept)@NA	0,159	0	0,276
plate_id@z.weight_kg@NA	0	0	1,84E-08
plate_id@season_of_death.dec_mar@NA	0,297	0	0,511
plate_id@season_of_death.aug_nov@NA	0	0	0,346
plate_id@age_class.class_3@NA	0	0	0
plate_id@sex.M@NA	0	0	3E-01
plate_id@(Intercept)@NA	0,488	0,194388	6E-01
Residual	0,831	0,705	9E-01