From the Department of Interdisciplinary Life Sciences

At the University of Veterinary Medicine Vienna

Domestication Lab

Konrad Lorenz Institute of Ethology

Assessment of hair re-growth and the correlation between hair cortisol and faecal glucocorticoid metabolite concentrations in captive wolves

Diploma thesis

University of Veterinary Medicine Vienna

Submitted by

Yasmina Wukovich

Vienna, February 2025

Supervision: Priv.-Doz. Sarah Marshall-Pescini, PhD

Domestication Lab, Konrad Lorenz Institute of Ethology

Department of Interdisciplinary Life Sciences

University of Veterinary Medicine Vienna

Review: Ao. Univ.-Prof. Dr.med.vet. Rupert Palme

Unit of Experimental Endocrinology

Department of Biological Sciences and Pathobiology

University of Veterinary Medicine Vienna

Statutory Declaration

I hereby declare that I have composed this work by myself. No assistance or sources other than those permitted have been used for the creation of this thesis. Furthermore, all quotes and concepts from published sources have been identified as such and all participants assisting in the composition of this thesis have been given credit for their contribution to this work in the place indicated. This thesis has not been submitted or published elsewhere before, neither in current nor in any similar form.

14th February 2025, Vienna
Date and place

Signature

ABSTRACT

Given the growing interest in wild wolf populations, hormonal profiling is a valuable tool for conservation endocrinology. Hair samples appear as a reliable and practical substrate for hormonal analyses due to their non-invasiveness and storage advantages. As keratinous tissue incorporates hormones over a longer time (weeks to months), it seems suitable for long-term stress evaluation. The hair and faecal samples used for this thesis were collected from seven captive wolves. Their hair was collected using a shave/re-shave study design and additionally brushing. One of the study's aims was to conduct a comparison of concentrations of glucocorticoids, specifically cortisol, extracted from hair and faecal samples to investigate whether these represent an accurate reflection of systemic levels. In addition, possible confounding factors such as season, body region, or sampling method were analysed. Knowing the approximate time window during which hormonal incorporation into sampled hair occurs also improves their validity as a measure of potential stressors because elevations can be linked to a certain period or even specific stimuli. This requires knowledge of the hair growth pattern in wolves, which has not been investigated before and therefore was another goal of this study. The results demonstrate that hair growth in wolves is seasonally specific with a growth spurt in late summer and autumn. A significant, positive correlation between faecal and hair cortisol concentrations was established, demonstrating that hair cortisol does indeed reflect systemic levels. Body region and season were found to have a significant effect on hair cortisol and should thus be considered when analysing results. These findings add valuable information to the field of non-invasive endocrinology and contribute to future research investigating the systemic levels of cortisol in wild wolves in different contexts.

Keywords: wolves, glucocorticoids, hair cortisol concentration, faecal GC metabolites, stress

ZUSAMMENFASSUNG

Hinsichtlich des wachsenden Interesses an Wildtierpopulationen, insbesondere Wölfen, ist die Erstellung von Hormonprofilen ein wertvolles Mittel für Erhaltungs- und Artenschutzprojekte. Haarproben erweisen sich dafür als ein zuverlässiges und praktisches Substrat, da sie nicht-invasiv entnommen werden können und Vorteile bei der Lagerung bieten. Keratinhaltiges Gewebe lagert Hormone über einen längeren Zeitraum (Wochen bis Monate) ein, weshalb es besonders geeignet für eine Erhebung von chronischem Stress scheint. Die in dieser Studie verwendeten Haar- und Kotproben stammen von sieben Wölfen, die in Gehegen gehalten werden. Die Haare wurden mittels eines shave/re-shave Studiendesigns und Bürsten gewonnen und im Labor analysiert. Da bisher noch nicht ausreichend untersucht wurde, ob die extrahierten Glukokortikoide, vor allem Kortisol, aus den Wolfshaaren ein verlässliches Bild systemischer Konzentrationen widerspiegeln, war der Vergleich mit Glukokortikoid-Konzentrationen aus Kotproben ein Ziel der vorliegenden Studie. Zusätzlich wurden mögliche Einflussfaktoren wie Jahreszeit, Körperstelle und Entnahmemethode untersucht. Die Kenntnis über das Zeitfenster, in dem es zur Hormoneinlagerung in die Haare kommt, ist ein weiteres Kriterium zur Validierung von Haarproben und stellte eine weitere Fragestellung dieser Studie dar. Somit können die Ergebnisse in weiterer Folge mit einem Zeitraum, bestenfalls einem Stressor, in Verbindung gebracht werden. Dem vorausgesetzt ist Wissen über das Haarwachstumsmuster der Wölfe. Dies wurde bisher noch nicht untersucht. Die vorliegenden Ergebnisse zeigen, dass das Haarwachstum abhängig von den Jahreszeiten variiert, mit einem besonders hohen Wachstum im Spätsommer und Herbst. Zwischen den Kortisolkonzentrationen in Kot und Haar bestand weiters eine signifikant positive Korrelation. Die Körperstelle und Jahreszeit wurden ebenso als signifikante Einflussfaktoren auf Haarkortisolkonzentrationen identifiziert und müssen daher bei der Interpretation von Ergebnissen beachtet werden. Diese Resultate stellen wertvolle Informationen für die hormonelle Analyse von Haarproben dar und können zukünftige Forschungen unterstützen.

Abbreviations

AB: assay buffer

CRH: corticotropin-releasing hormone

df: degrees of freedom

FGCM: faecal glucocorticoid metabolites

GC: Glucocorticoid

GAM: generalized additive model

HCC: hair cortisol concentration

HPA: hypothalamic-pituitary-adrenocortical

LH: luteinizing hormone

LMM: Linear mixed model

POD: peroxidase

SD: standard deviation

TMB: tetramethylbenzidine

Table of contents

List of figures and tables

1. Introduction

With their recolonization, more precisely their growing population numbers in Austria and other European countries, wolves have long been a polarizing topic, leading to public debates calling coexistence into consideration, while still being subject to state protective measures in many European countries (1,2). Over the past few decades, the combination of the wolf's predatory nature and the circumstance of them settling closer to human-dominated landscapes caused increased research on large carnivores, in particular their behaviour and population dynamics (2,3), aiming to maintain a suitable habitat for wolves, as this is crucial for their health, genetic diversity and long-term viability (1).

Endocrinologists can contribute significantly to the understanding of the status of a population, especially regarding the growth of the population in relation to the health status of individuals, and to apprehend the mechanisms by which they cope with changes in the environment (4). Therefore, hormonal profiling is a valuable tool for wildlife conservation. To gain insight into that matter, steroid hormones linked to stress and reproduction are the most used measures. These measures can be obtained from samples of all body fluids – plasma, serum, saliva, urine – and even faeces and hair (5). However, there is an increasing preference for non-invasive sampling methods, particularly in studies concerning stress so that the sample taking itself does not become a stressor that could confound the results. Especially when studying wild species, non-invasive sample collecting methods (for example of scats and hair) are always preferred (5). However, it must also be considered which time interval hormonal levels (acute vs. chronic, basal vs. stimulated) of the samples reflect and how stable concentrations are if the samples must be stored. Hair samples, for example, provide a long-term record of endocrine activity over weeks or months (5) , whereas blood samples reflect a point in time and can be affected by acute stressors such as the capture and handling during sample collection (6). Thus, different types of samples may provide different information linked to the hormonal status of the animal.

In wild wolves, steroid hormones have been extracted and measured in hair (7) and faeces (8) before. Generally, both substrates are supposed to reflect a past state of the endocrine activity, while for hair it can be weeks to months, for faeces it is the state of hours to a maximum of one day prior (5). However, it has not yet been investigated whether these two give approximately the same information, more precisely, whether a link between the hormonal levels measured in those two substrates can be established. Due to potential variations in the seasonal hair

growth of wolves and the time at which hormones are incorporated into hair, this question needs further investigation.

The focus of this thesis is on the endocrine system of captive wolves. Those were housed at the Core Facility Wolf Science Center, an internationally recognized institution in which various research work has already been carried out on socialized wolves. The emphasis of the current thesis is on the steroid hormone cortisol – a “stress” hormone that has been studied widely before (9). Non-invasive methods are used for this purpose, measuring both faecal glucocorticoid metabolite (FGCM) and hair cortisol concentrations (HCC) to allow long-term monitoring of hormonal changes (10). However, considering both the fact that steroid hormones are only incorporated into growing hair and the fact that the hair growth rate within and among individuals might show differences, the interpretation of the hormonal levels might be more difficult (6,11). The annual hair cycle of wolves is another focus of this thesis, since knowing how rapidly wolf hair grows and in which specific seasons of the year the growth is at its maximum rate, a time window of hormonal incorporation can be estimated more precisely.

1.1. Glucocorticoids and their assessment

Glucocorticoids (GC) are steroid hormones produced in the adrenal cortex. Their release results from the activation of the hypothalamic-pituitary-adrenocortical (HPA) axis. Generally, the secretion of GC follows a circadian rhythm, but acute activation of the HPA axis in response to stressors also releases GC (12). Specifically, when the hypothalamus releases corticotropin-releasing hormone (CRH), this subsequently leads to the secretion of adrenocorticotropic hormone (ACTH) from the pituitary. ACTH then stimulates the adrenal cortex to produce cortisol and other GCs (cortisol being the predominant GC in wolves). GC levels are regulated via a negative feedback loop that acts on CRH and ACTH secretion and ultimately their production, resulting in the inhibition of the catabolic, lipogenic, anti-reproductive and immunosuppressive effects caused by high levels of GCs and return to homeostasis (13,14). Considering that cortisol is a stress hormone and short-term elevations of cortisol help individuals to cope with unexpected or even life-threatening situations, it is crucial to differentiate between the impacts of short-term in contrast to prolonged exposure to elevated GC. Chronically elevated GC concentrations, and therefore prolonged activation of the HPA axis, are less beneficial to the organism, as they exert a negative effect on the individuals’ health status (5,14). This is one of the reasons why GC concentrations are widely used in wildlife research as indices of animal welfare.

If environmental circumstances exceed the individual's capacity to adapt, stress occurs. From an evolutionary perspective, an appropriate stress response is of crucial importance (15). However, chronic stress can have detrimental effects, as chronically elevated cortisol concentrations are noxious to an individual's health.

Partially, the end products of the HPA axis repress cellular immunity and favour humoral immunity. However since the endogenous defence primarily functions through cellular immune responses, this affects the susceptibility to infections but also leads to restricted wound healing, an increased tendency for autoimmune diseases, and an improved vulnerability for tumour formation (16). Although there has been an association between clinically verified upper respiratory illnesses and elevated stress perception in humans (15), a previous study in wolves indicated that viral, bacterial, and ectoparasitic infections were linked to increased HCC (7). This study also revealed heightened HCC in wolves with neoplasia. Additionally, stress activates the sympathetic nervous system, which ultimately affects the circulatory system (e.g., increased heart rate, vasoconstriction, raised blood pressure) and therefore leads to an elevated risk of cardiovascular diseases such as hypertension, myocardial infarction, or atherosclerosis (17). This is also associated with increased HCC (18). Furthermore, chronic stress is shown to interfere with the positive feedback mechanism of oestradiol, which naturally generates elevation of the luteinizing hormone (LH), hence inducing ovulation. Therefore, chronically elevated cortisol concentrations can cause delayed LH surge and a diminished amplitude (19), possibly affecting reproduction.

Blood samples (20,21) and other body fluids such as saliva (20–22) are well-suited for the assessment of cortisol. However, other substrates, including faeces (23,24) , urine (21,25), claws (26), or hair (6,10,22,24,26–29) also contain cortisol or its metabolites (30), which can be quantified following extraction. Given this broad variety of potential sampling substrates, choosing the most suitable one should be done carefully according to different criteria. One such consideration concerns the timeframe that is reflected in the sample. While hormone concentrations in blood samples provide a point-in-time measurement of acute levels (minutes) (6), the cortisol concentrations in hair samples reflect long-term, integrated, and chronic stress levels of several weeks or months (10). In addition, considering how invasive collection techniques such as blood sampling might result in a stress reaction, it is of special importance, especially when sampling wildlife populations, to consider less invasive or non-invasive techniques, such as saliva, urine, faeces, or hair samples that can be collected almost without any manipulation of the animals (9).

1.2. Benefits of non-invasive sampling methods

In general, but especially in wild populations, non-invasive sampling such as hair collection offers a stress-free option for hormonal monitoring, providing significant insight into a population's status over an extended period and a retrospective view of hormonal changes during life history (10). Moreover, considering that collecting blood or saliva samples is not feasible in most field studies involving free-roaming animals, as the procedure is rather invasive and potentially influences the validity of the results, the use of non-invasively collected samples appears far more beneficial (23). Faecal and hair samples prove to be particularly popular. Both measurements of faecal GCMs as well as keratinous GC concentrations (hair, claws) provide an overview of prior HPA activity. Therefore, when assessing these values, it must be considered that they do not represent the specific time point of sampling.

Nevertheless, the concentration of GCMs in faecal samples is influenced by animal-related factors such as diet or the time of intestinal passage on the one hand and sample-related factors such as age and composition of the sample, bacterial activity, and errors in handling, storage, and processing of the sample on the other (23). The period reflected by the sample's values can be described relatively well by the time between two consecutive defecations. The steroid hormones are metabolized in the liver and released into the intestine via the bile and thus excreted in the faeces, which is why the time of intestinal passage serves as a good approximate measure of the delay (9). In most species, faecal GC measures therefore represent the systemic GC concentration from hours to one day prior to sampling (5). Comparatively soon after defecation, degradation due to bacterial activity begins, as the metabolites are metabolized by bacterial enzymes and can therefore no longer be measured accurately, resulting in distorted values (9). In addition, the effect of prolonged exposure to increased temperature and precipitation might also hinder accurate measurements (23). Ideally, the samples should therefore be collected immediately after defecation and stored correctly, i.e., dried or frozen until they are processed further (23).

As cortisol is incorporated into growing hair, the measured values reflect a longer period, lasting weeks to months, depending on the hair growth rate. Thus, hair might be a powerful substrate for long-term HPA activity monitoring and, therefore, a proxy for chronic stress. Repeated sampling allows to link elevated cortisol concentrations to seasonal variations and other events. Due to relatively slow hair growth rates, consequently, daily or hourly fluctuations are not taken into account when measuring GC from hair, which – depending on the study question – can be both a benefit or a limitation (24). A benefit of keratinous tissue is that GC

concentrations remain stable for long periods and do not require special handling, as they can be stored long-term at room temperature, ideally under dark and dry conditions (26). Thus, preference for hair samples is emerging due to these aspects. However, it is important to note that there is not only a systemic HPA axis, which response to stress and has systemic effects, but also local production of cortisol in the hair follicle itself. It responds to localized stressors such as UV exposure, extensive brushing, or dexamethasone exposure (24,31). Therefore, the interpretation of values obtained from hair samples, especially when those also contain follicles (e.g., brushed hair in contrast to clipped or cut hair), is not straightforward, as the contained levels may be a mix of systemic and local origin. This should lead to caution in the interpretation, and further investigations are indispensable (24,31,32).

Naturally, it seems plausible to assume that GC concentrations measured in the hair reflect a longer period, as they are absorbed into growing hair. However, recent studies have questioned this and tended to assess HCC as an acute stress indicator. A study showed that although HCC cannot be used as such an acute marker as blood and saliva (as they change within a short time), HCCs were altered just hours after induced stress. The values correlated best with values of faecal samples (33), which suggests that these may represent a similar time period. Another study on rats (34) showed that GC concentrations in the rats' hair increased hours after an injection with corticosterone, which contradicts with the assumption that HCC can be used as a marker for chronic stress and additionally suggest that GCs are absorbed into the hair shaft much faster than generally assumed. In addition, this study discussed the possibility of GCs diffusing through the hair shaft alongside GC incorporation. As this would explain the rapid changes in HCCs. This altogether, emphasises the necessity for further research to refine its application in stress assessment.

The collection of hair samples in the field is primarily conducted using specific hair-collecting devices, but species differences should be considered when deciding which method to use. Hair traps or snares are easy to install and depend on the fact that hair gets stuck to them or is pulled out when animals walk past, rub, or scratch against these objects. Hairs are collected on rub pads, brushes, or screws that are attached to objects in the animals' surroundings. However, cage traps can also be set up, which operate on the same principle; for example, adhesive pads, tapes, or brushes placed inside collect the hairs (35). Nevertheless, hair samples can be taken directly from the animals as well, by clipping, brushing, or plucking. Although this is usually performed on wildlife carcasses (7), another study on wolves used

leghold traps and attractants to capture the animals, and samples were taken by plucking and cutting the hair (26).

Past studies that have focused on evaluating the reliability of cortisol extracted from hair as a proxy for systemic HPA axis activity determined the correlation between faecal and hair cortisol concentrations in dogs and cats (24), mountain goats (36), as well as dairy cattle (37), and showed significant positive associations. This, however, has not yet been established for wolves.

1.3. Factors influencing cortisol levels

Subject-related factors such as sex and age have already been investigated in numerous studies in the past, although the results vary. Previous research on mountain goats (36), coyotes (38), and lynxes (39,40) showed no significant effect of sex. Therefore, whether the animal is male or female, did not significantly affect HCC. However, other studies focusing on lynxes (28) and dogs (41) revealed higher hair cortisol concentrations in females. In contrast, black bears (42) and feral horses (43) showed increased concentrations in males. In wolves, no significant link between sex and cortisol levels was found (7,26,30). These inconclusive results from previous studies suggest that not only sex itself, but rather sex in combination with other factors such as the species' social structure or reproductive system influences cortisol concentrations.

Furthermore, age should also be considered a potentially important factor, although many previous studies have not found an effect. For felids (39,40), canids (22,38), and horses (29), cortisol levels did not differ significantly between age groups. Conversely, studies in juvenile lynxes (28) and mountain goats (36) reported increased values compared to adult individuals. Regarding wolves, the results are inconsistent, with one study (30) finding no correlation, but others reporting (7,26) higher concentrations in adults than in juveniles and younger individuals. This contrasts with the results of studies on other species. A possible explanation could be that the elevated levels in adults may be due to a combination of several factors, including social status and seasonal ecological stress (38).

Considering social status, the studies for canids are in agreement, dominant individuals in both coyotes (38) and wolves (8,44) showed increased cortisol concentrations. It can be assumed that this is directly related to maintaining a high rank in the pack and that the individuals therefore experience a higher level of stress.

Attempting to improve the work with hair samples and enable better interpretation, the effects of various hair characteristics are also widely investigated. The analysis of hair type for mountain goats (36) showed that guard hair tends to contain more cortisol than underwool. However, this contrasts with other studies on wolves (26) and dogs (27), in which the different hair types contained similar levels, thus highlighting the need for more specific investigations.

The consideration of coat/hair colour does not reveal consistent results either. A study on dogs (22) found that black hair had lower cortisol concentrations, which may be explained by the fact that dark hair has less capacity as a storage site for cortisol than lighter hair, as the pigment already takes up that space. However, studies on horses (43) and cows (37) showed the opposite pattern (i.e., lighter hair having lower cortisol levels). The latter study, however, collected samples of black hair from different body regions, which could have influenced the results. Moreover, in several studies on canids (27,45) and felids (40), the body site from which the sample was taken was not considered, although studies on lynxes (39) and wolves (7) showed differences according to the sampled body areas of the animals. This may indicate an influence of natural sunlight (natural UV light) on GC concentrations (degradation) that is greater in certain body regions than others, thereby resulting in reduced GC concentrations primarily in hair from dorsal regions (32).

The effect of natural sunlight varies naturally depending on the season. In this regard, it is essential to consider that the season could potentially influence cortisol levels both systemically (by affecting, for example, food availability or reproduction) and externally, through fluctuating UV exposure. High exposure to UV radiation during summer would, therefore, decrease the values, while less UV exposure during winter may be associated with a higher amount of GC in the hair. These seasonal differences have been investigated in a multitude of studies on canids (7,27,30,46) and revealed a maximum GC concentration in winter, more precisely in January. However, in wolves, the mating season also coincides with the winter months, which could explain the increased cortisol levels as induced by mating and increased competition. In conclusion, these effects are interrelated and could potentially also cancel each other out. Collecting additional information about the population and individuals in question (e.g., availability of feeding resources, mating competition, presence of puppies, and so on) is essential in attempting to disentangle at least some of these effects. Testing specifically the effect of UV light on HCC is another step for future research towards validating hair samples for endocrine studies.

1.4. Hair growth in wolves

Hair growth in wolves generally follows the same pattern observed in many other mammals. After completing morphogenesis, the hair follicles show cyclical growth activity. Each cycle is classified into three distinct phases: anagen, catagen, and telogen. The anagen phase represents the period of active hair growth and is thus thought to be the time of hormonal incorporation (7,47). This is followed by the catagen phase, during which active hair growth ends and the follicle regresses. Quiescence dominates in the telogen phase, wherein the follicle shortens at some point, leading to the shedding of the old hair and the follicles enter a new cycle (47). Hair replacement thus follows a mosaic pattern in order to prevent local hair loss, consequently, neighbouring follicles undergo different phases (11). Hair grows to a predetermined length, varying based on the body region, and no further hair growth occurs after reaching the maximum and genetically defined length, resulting in species- and breed-specific variations regarding the phenotypic coat. However, hair growth may differ among individuals of the same breed and the different body regions. Predefined maximum hair length and hair growth at a body region are directly proportional to one other (11,48). Both endogenous and exogenous factors control the hair cycle, causing seasonal variations in growth rates. Endogenously, cytokines and growth regulators create a local impact, whereas hormones cause systemic effects influencing hair growth. Nonetheless, environmental aspects regarding the duration of photoperiod and temperature are increasingly considered to have a high impact on the seasonality of hair growth, hence those factors affect the secretion of both melatonin as well as prolactin, which control the hair follicles cyclical activity (47,48). Therefore, shedding follows an acceleration of the hair cycle, prompted by hormonal shifts stimulated by deviations in photoperiod (11) and temperature alterations (49). During moult, commonly in spring and autumn, the number of hairs that reach the telogen phase increases rapidly, causing them to shed for the change of coat in a higher quantity in comparison to the remaining year (11). The general idea is that the hair starts to grow again after moult. Therefore, since hormones are expected to be incorporated into growing hair via the surrounding blood vessels and glands, it can be assumed that the GC concentrations measured reflect the circulating values at that time period (7).

So far, previous studies on dogs have revealed rapid hair growth during the summer months compared to the winter season, as the low temperatures are believed to inhibit hair growth (11,50). In contrast, results from another study also conducted on dogs, show no significant

seasonal impact on hair growth rates (47). These issues have not yet been addressed explicitly for wolves, a gap that will be closed by this thesis.

1.5. Hypotheses and predictions

Building on the existing literature outlined above, the main hypothesis and prediction of this thesis concern the seasonality of the annual wolf hair growth cycle. Shedding is most likely to occur in spring and autumn. Thereafter, as soon as the anagen phase starts again, hormones should be incorporated into the growing hair. Thus, in summer, the hair should have a higher growth rate than during winter, as the cold temperatures have a negative influence, and therefore minimal growth can be assumed during the colder season (11,50).

As mentioned above, the link between systemic (faecal) and HCC in wolves has not yet been demonstrated. This is particularly relevant considering an additional possibility of hormone incorporation in the hair via the cutaneous HPA axis (31). If hair samples are to be considered meaningful proxies of systemic levels, a significant positive correlation between the two substrates needs to be established. Nevertheless, this correlation alone does not suffice, therefore demonstrating changes in hair sample levels (increases and decreases) during well-defined situations is additionally necessary to confirm their reliability.

Moreover, this thesis attempted to identify other confounding variables and thus evaluated also additional factors and more specifically: body region, season, and collection method (clipping vs. brushing). Even though coat colour appears to be an important factor and contradictory findings exist in the literature – for example, it is not clear whether black hair generally contains lower hormone concentrations than lighter hair (22) – this thesis could not test this due to the lack of more black-coated individuals (i.e., the available study population included only one black-coated female wolf). Similarly, the factor age could not be tested as the available study population comprised only adult individuals of roughly the same age. However, the factor body region could be tested and was predicted to have an impact on cortisol concentration, based on literature describing that sunlight/prolonged UV radiation can lead to a decrease in values. Thereby, certain body parts being exposed for a longer time period than others should exhibit decreased cortisol (more specifically, HCC in hair from the belly are expected to be higher than samples from the back) (32). Lastly, higher cortisol concentrations may be predicted in wolves during the winter compared to summer months, partially due to their seasonal mating period that stretches from January to March but also because of the possible influence of natural sunlight and UV radiation on hair (which is stronger during spring and summer months), which

could have a degrading effect (27,30). However, no formal test of the effect of controlled UV radiation on HCC was conducted for this study.

1.6. Statement of significance

Given that cortisol concentrations help to determine the health status of an individual or a population, identifying confounding factors affecting cortisol levels, as much as the evaluation and validation of HCC as proxies for systemic stress levels are essential. This knowledge can, therefore, be included in the subsequent data analysis. Additionally, concerning wolves, there are no studies on their hair growth, which makes the findings of this thesis valuable for validating hair samples from wolves. Furthermore, it provides information for future applications to better understand the welfare of wolf populations and to study the potential consequences of novel and anthropogenic stressors such as urbanization.

2. Material and methods

2.1. Study population and sampling process

All samples were obtained from a population of human-socialized and trained wolves, living in dyads and small packs at the Core Facility Wolf Science Center (CF-WSC) in Ernstbrunn, Austria. The controlled environment provides optimal conditions to conduct a shave/re-shave study design to assess hair re-growth in wolves. All samples were collected using non-invasive methods. Animals participated voluntarily during their regular grooming sessions carried out by the animal trainers.

Samples were initially collected from seven wolves (four adult males, and three adult females), but only six wolves continued with the study since one female was no longer willing to participate after the first sample collection. Unfortunately, another female died shortly after providing the first hair sample, and one male died following the second hair collection session, resulting in only four individuals (three males, one female) completing the full study year.

Hair was clipped using electric clippers designed for animals on the left or right shoulder (depending on which side the animal preferred during habituation training to the clipping machine) on two predefined locations marked by the bone structure (scapula spine) of the shoulder blade (cranial and caudal). The second spot served as a substitute in case of little or no hair growth in the first spot in the following sampling session. Additionally, hair was brushed from several body regions: left/right shoulder (next to the clipped spots), belly, upper back, and lower back (upper back being between the shoulder blades and lower back being at the base of the tail/on top of the hip bones). To assess hair re-growth, repeated sampling of wolf hair from the same spots was conducted for a year, from October 2021 to October 2022 (see *Fig. 1.* for a visual timeline). Collectively, there were five sampling sessions for each wolf, with a three-month interval between them. Therefore, each clipped hair sample reflected the previous, three-month period since the last clipping.

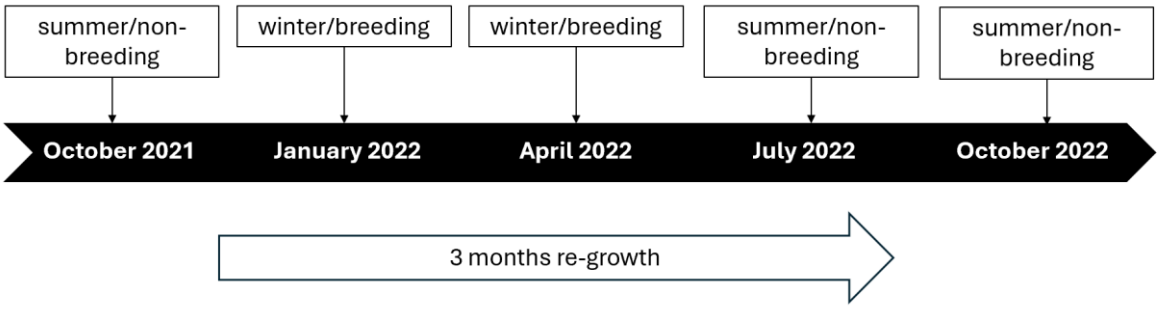

Figure 1. A visual timeline of the sampling regime and represented season.

Considering that, regarding two female wolves (Yukon and Tala), only the first sample of the shave/re-shave study was collected, and therefore no relevant data for evaluation was provided, they were not included in the assessment of hair re-growth or the correlation between hair and faecal cortisol concentrations. This reduced the number of individuals (n) for formal statistical analysis to five.

Hair re-growth was recorded visually with photographs and measurements of individual hair lengths. All gathered samples were individually stored in paper bags, labelled with the running sample number, subject ID, collection date, collection method (clipped/brushed), sample weight, and body region from which the hair was collected. Samples were stored at room temperature in a dry and dark room until further processing. In total, 152 hair samples were obtained, of which three could not be used further since they did not meet the necessary weight requirement (no less than 10 mg) for the cortisol extraction.

Ultimately, 149 hair samples had a sufficient weight for further processing, 41 of them were ‘clipped’ and 108 were ‘brushed’. First, the hair was evaluated macroscopically by measuring the average hair length and categorizing the samples based on their composition. The average hair length was determined by calculating the mean value of three hair strands. Due to the sampling technique, it was not possible to separate guard hair and undercoat.

Simultaneously, during the entire duration of the hair sampling, faecal samples were collected weekly from each wolf during leashed walks. The trainers collected the samples fresh immediately after defecation. The samples were placed into plastic tubes, again labelled with a running sample number, subject ID, collection date, and sample weight, and stored at -20 °C until steroid measurements. In total 227 faecal samples were collected.

2.2. Extraction protocol

The Experimental Endocrinology Unit of the Department of Biological Sciences and Pathobiology at the University of Veterinary Medicine provided a previously validated extraction protocol for steroid hormones (*Appendix 7.1.*). Following the macroscopic evaluation, approximately 150-200 mg of each hair sample were cut with scissors into approximately 3 mm-long pieces and weighed into a glass vial. To degrease the hair, 7 ml of n-hexane was added and decanted after shaking shortly on a hand vortex. Further processing required the samples to be dry, so they were left under the hood to evaporate the remaining moisture overnight. On the second day of extraction, 100 mg of each sample was weighed into a clean glass vial and mixed with 5 ml of methanol (100 %). In case of a differing sample quantity, the added methanol quantity was adapted accordingly (e.g., 50 mg of sample plus 2.5 ml of methanol). After plugging, the vials were placed into a heated water bath with a constant temperature of 37 °C for 24 hours. Then, the samples were centrifuged for 15 minutes at 2500 g, and 2.5 ml (or 1.25 ml in case of lower methanol volume) of each sample were transferred into a fresh glass vial, dried under a nitrogen air stream at 50-60 °C. To redissolve, 0.5 ml of buffer was added to the samples and shaken for 30 minutes. The extracts were transferred into plastic tubes and stored at -20 °C until analysis by enzyme immunoassay (EIA).

The results from the faecal samples had already been obtained before, therefore, the extraction process is outlined here only briefly for completeness. The frozen faecal samples were thawed, and then 0.5 g of each sample was transferred into a clean glass tube. Then, 5 ml of methanol (80 %) was added to each sample and placed onto the shaker for 30 minutes. This was followed by 10 minutes in the centrifuge at 2500 g. Afterward, 0.7 ml of the supernatant was transferred into plastic tubes, diluted at 1:10, and stored at -20 °C until analysis.

2.3. Enzyme immunoassay protocol

Regarding the hormonal analysis, a previously validated in-house EIA for cortisol (for details see Palme and Möstl (51)) was performed (*Appendix 7.2.*). Day 1 started by thawing the extracted hair samples and washing precoated plates, in which, according to a customized template, the samples were then pipetted in duplicates into the appropriate wells. The amount of sample used on the assay plate was adjusted according to the quantity weighed in on the second day of extraction. Specifically, for samples extracted with 100 mg, 10 µl of sample were pipetted onto the plate, however, if the extracted sample quantity was lower than 100 mg, the

amount was adjusted to 30 µl (for 50 mg) or 50 µl (for 25 mg) to ensure that measured concentrations would fall within the linear range of the assay. After adding the assay substrates, the plates were incubated for 24 hours at 4 °C while being placed on a shaker. On day 2, after washing the plates, a mix of peroxidase (POD) and assay buffer (AB) was added to each well. After a 45-minute-long incubation, the plates were washed once more, followed by adding a mix of tetramethylbenzidine (TMB) and buffer before incubating again on a shaker for 45 minutes at 4 °C. To stop the reaction and read the plate, 50 µl of sulfuric acid was pipetted into the wells and shaken for 15 minutes at room temperature.

A photometer was used to read the plates at 450 nm. In cases where the optical densities (OD) of the duplicates deviated by more than 10% coefficient of variation (% CV), the measurements were repeated (five samples). If samples fell above or below the linear range of the standard curve (binding below 20 % or above 80 %), they were re-measured with adjusted quantities (two samples). All samples from one individual were measured on the same assay plate. Intra-assay CVs were below 5 % (average % CV of the duplicates' OD), and inter-assay CVs were below 15 % and 20 %, respectively for hair and faeces (n = 5 plates of hair samples, n = 6 plates of faecal samples).

2.4. Statistical analysis

For statistical analysis, five faecal samples had to be excluded due to diarrhoea or altered faecal consistency which could bias hormonal concentrations. One faecal sample from a male wolf was excluded as it revealed extremely high cortisol concentrations and was collected shortly before his cancer-related death. Other than that, no samples were excluded. As mentioned above, two wolves had no matching hair samples for the assessment of the correlation between FCGM and HCC (one died and one dropped out of the study early after not cooperating anymore during shaving and brushing), which reduced the total sample size for statistical analyses to 193 faecal and 149 hair samples of five wolves.

All statistical analyses were conducted in R Statistical Software (Version 4.4.1; R Core Team 2024), fitting linear mixed effect models (LMM) with a Gaussian error distribution and implementing full-null model comparisons to avoid inflated type I error rates. Subject ID was added to all models as a random intercept effect to avoid pseudo-replication, since multiple samples per wolf were collected. Furthermore, to fulfil model assumptions of normally distributed residuals, hormones concentrations were log-transformed prior to model fitting. Since the wolves in this study were all adults, approximately in the same age range, the factor

age was not included in this evaluation. Beyond that, coat colour and hair type only appear descriptively, given the circumstance that just one wolf featured dark hair and most of the hair samples were a mixture of guard hair and underwool.

First, to assess hair re-growth in the clipped samples from the shoulder, the average hair length was set as the response variable, with the season reflected by the regrown hair (i.e., the period since the last sampling and thus the period of re-growth) as the fixed effect test predictor.

The data subset comprising the average FGCM and matching HCC (from clipped hair) was used to test for a correlation between faecal and hair concentrations, using a repeated measures correlation test (package “rmcorr”) (52).

Next, to test the effect of body region on HCC, the dataset comprising of the brushed hair samples was used with HCC as the response and body region as the test predictor.

To then investigate the effect of season on the HCC in the clipped samples from the shoulder, another model was fitted with HCC (log-transformed) as the response, and season reflected by the regrown hair (i.e., the period since the last sampling and thus the period of re-growth) as the fixed-effect test predictor.

To compare the effect of sampling method (brushing vs. clipping), the subject of samples from the shoulder was used, with HCC as the response and sampling method as the test predictor. Only the first samples were considered, as subsequent clipping/brushing of the same spot could have affected the next samples differently and to account for differences in hair length between subsequent brushed and clipped samples.

Lastly, to evaluate potential confounding factors on faecal cortisol levels, another model was fitted using log-transformed FGCM as the response and season in interaction with sex (due to possibly sex-mediated effects of the reproductive season) as the test predictor.

3. Results

3.1. Assessment of hair re-growth

The average length and standard deviation (SD, in brackets) of the clipped hair samples from the shoulder in autumn 2021 (first collection), was 62 (15.98) mm (n = 5, range = 33–77 mm). These initial values were not included in the following hair growth rate assessment, as they do not reflect re-growth but rather maximum length when left alone. However, they are mentioned to facilitate comparison with the subsequent samples. The data collected for hair re-growth over the period of one year are listed in *Tab. 1.* and shown in *Fig. 2.*

Table 1. Hair re-growth (mm) by season, method (clipped) and body region (shoulder).

	autumn	**winter**	**spring**	**summer**	**autumn**
Tekoa	75	26	15	60	44
Taima	33	25	16	23	44
Gero	68		27	76	68
Chitto	57	39			
Amarok	76	36	79	69	63
mean length (mm)	62	31	34	57	55
SD	15.98	6.02	26.38	20.48	10.86
re-growth/day		0.35	0.37	0.63	0.60

The rate of hair growth differed between individual wolves. Moreover, there was a significant difference between the means of summer and winter growth rates. The re-growth rate for the sample dates four (summer) and five (autumn) was significantly higher than the measurements of the other dates. For winter, the mean length (SD) of 31 (6.02) mm (range = 25–31 mm),

resulted in a daily growth rate of 0.35 mm. On the contrary, during summer, growth was significantly higher with a mean length (SD) of 57 (20.48) mm (range = 23–76 mm). Except for one wolf (Amarok), the third sample revealed the smallest re-growth for all individuals. This period, late winter and spring (February, March, April) had a mean length (SD) of 34 (26.38) mm and a growth rate of 0.37 mm per day.

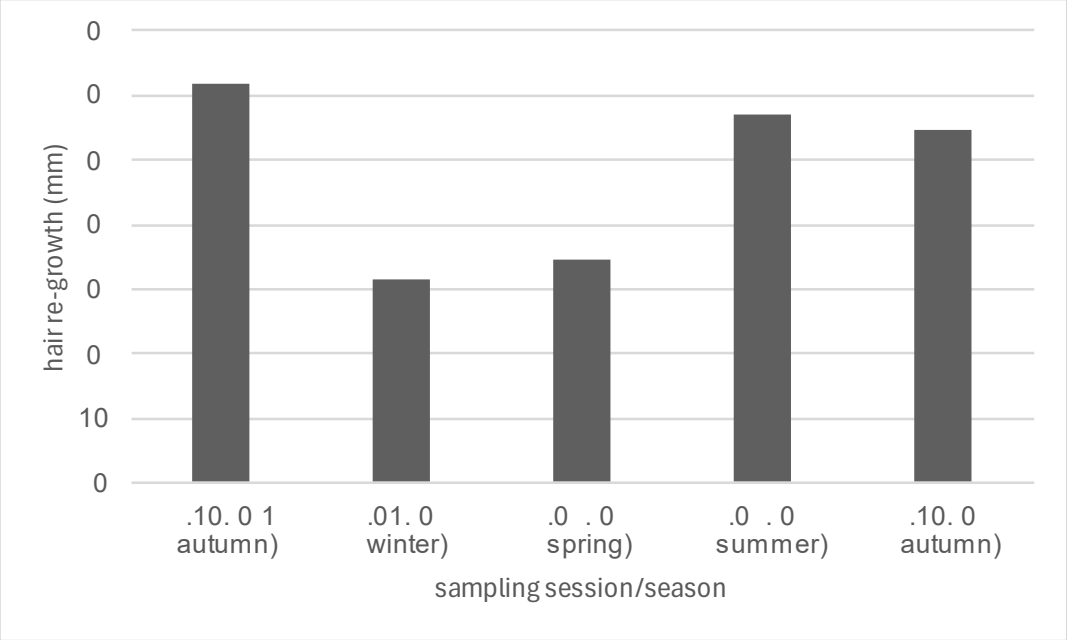

Figure 2. Average hair re-growth (mm) by sampling date and season for n = 5 wolves (samples clipped from shoulder). First sample (27.10.2021) represents the initial hair length without prior manipulation.

A peak between the third (spring) and fourth sample (summer) was observed, i.e., in spring and early summer (May, June, July), re-grown hair had almost reached the length observed at the beginning of the study. In summary, season had a significant effect on hair re-growth (LMM, ChiSq = 9.24, df = 3, p = 0.03). The different growth rates were most pronounced between the fourth and second sample (post-hoc testing, Tukey adjusted p-value = 0.04), the fourth and third sample (post-hoc testing, Tukey adjusted p-value = 0.02), as well as the fifth and third sample (post-hoc testing, Tukey adjusted p-value = 0.04, see *Appendix Fig. 7.3.1.*).

3.2. Correlation between hair cortisol and faecal GCM concentrations

Only clipped samples were used for this analysis, which resulted in some missing HCC data due to a lack of hair re-growth and therefore in-sufficient sample amounts for cortisol extraction *(Tab. 2.)*. This is particularly evident for the period January–April, where no HCC could be measured for any of the wolves. Concordantly, this period revealed the lowest rate of hair re-growth for the majority of the wolves.

Table 2. Corresponding average FGCM levels and HCC (clipped, shoulder) by subject.

		mean FGCM (**ng/g**)	**HCC** (**ng/g**)
Tekoa	October – January	35.09	13.47
	January – April	31.06	
	April – July	8.31	2.73
	July - October	27.49	4.92
Taima	October – January	106.30	9.13
	January – April	99.61	
	April – July	28.25	3.52
	July - October	53.16	12.04
Gero	October – January	16.35	
	January – April	6.10	
	April – July	6.33	1.75
	July - October	5.57	3.78
Amarok	October – January	11.45	4.72
	January – April	15.87	
	April – July	8.96	2.89
	July - October	7.68	4.11
Chitto	October – January	17.2	3.33
	January – April	100.09	

There was a significant positive correlation between FGCM levels and HCC (repeated measures correlation, r = 0.72, df = 6, p = 0.04, 95 % CI [-0.134, 0.979], *Fig. 3.*).

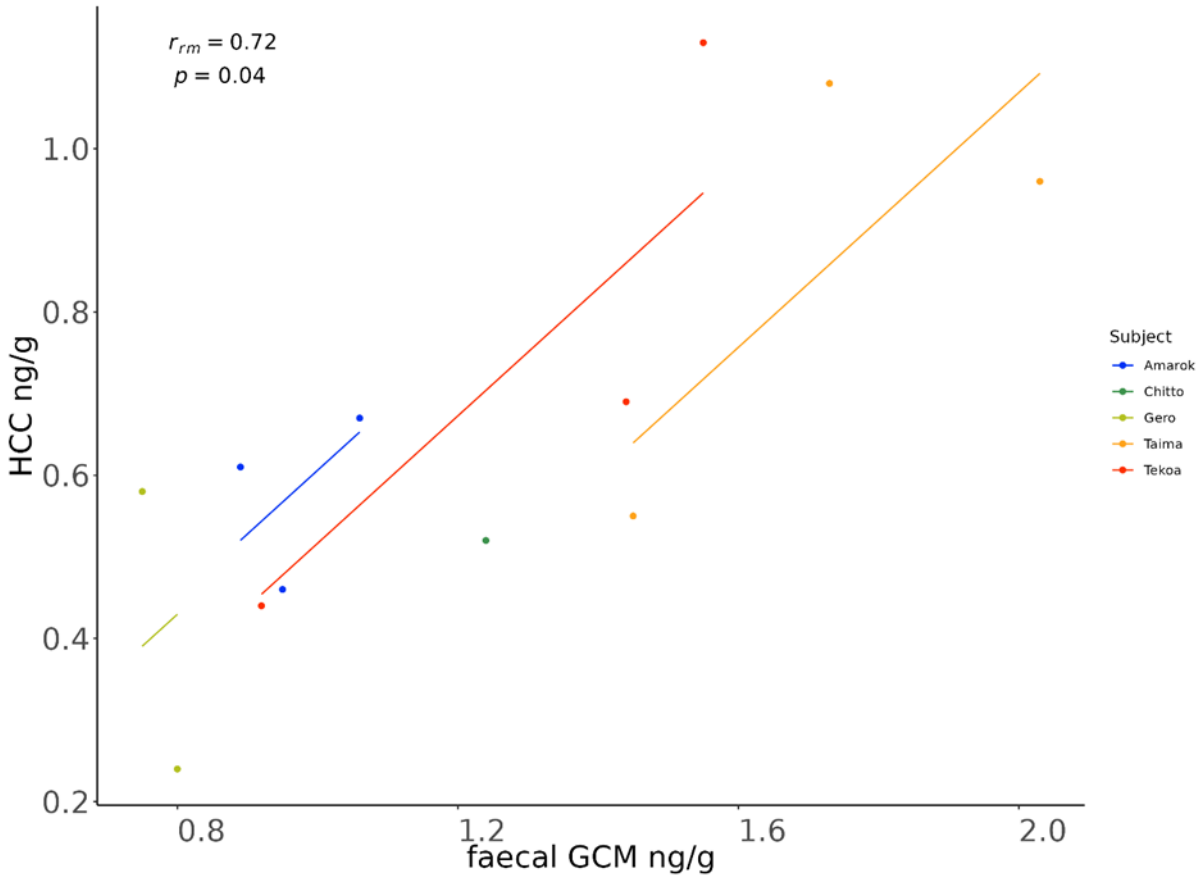

Figure 3. Repeated measures correlation plot of hair (HCC) and FGCM concentrations (log-transformed) of n = 5 wolves sampled repeatedly and represented by different colours (one wolf, Chitto, green dot, only provided one sample pair and thus did not contribute to the correlation analysis).

The mean FGCM (SD) for the period October–January was 38.44 (66.90) ng/g (n = 47, range = 0.77–374.99 ng/g). The corresponding mean (SD) HCC was 7.66 (3.98) ng/g (n = 4, range = 3.33–13.47 ng/g). Skipping the second sample (January–April) (n = 51) since no HCC could be assessed, the mean (SD) FGCM for the third sample (April–July) was 12.60 (14.29) ng/g (n = 47, range = 0.84–60.61 ng/g) and related mean (SD) HCC was 2.72 (0.63) ng/g (n = 4, range = 1.75–3.52 ng/g). For the last sample (July–October) the mean (SD) FGCM revealed 24.92 (43.23) ng/g (n = 48, range = 0.66–237.71 ng/g), and the mean (SD) HCC was 6.21 (3.39) ng/g (n = 4, range = 3.78–12.04 ng/g) (see *Appendix Tab. 7.3.1.* for visualisation).

3.3. Assessment of confounding factors in hair and faeces

3.3.1. Effect of body region on brushed hair samples

The test predictor body region had a significant effect on the cortisol concentration extracted from hair ((LMM, ChiSq = 21.84, df = 3, p = 0.00), see *Fig. 4*. for visualisation). Samples taken from the shoulder (n = 22) revealed a mean (SD) HCC of 5.16 (3.66) ng/g (range = 1.55–15.35 ng/g). Belly samples (n = 25) were almost as high, with a mean (SD) HCC of 4.66 (2.77) ng/g (range = 1.69–11.12 ng/g), but samples from the upper (n = 27) and lower (n = 27) back exhibited lower HCC. The mean upper back (SD) HCC was at 3.43 (2.38) ng/g (range = 0.50–12.41 ng/g) and the mean lower back (SD) HCC was at 3.37 (1.93) ng/g (range = 0.72–7.87 ng/g) (visualized in *Appendix Tab. 7.3.2.)*.

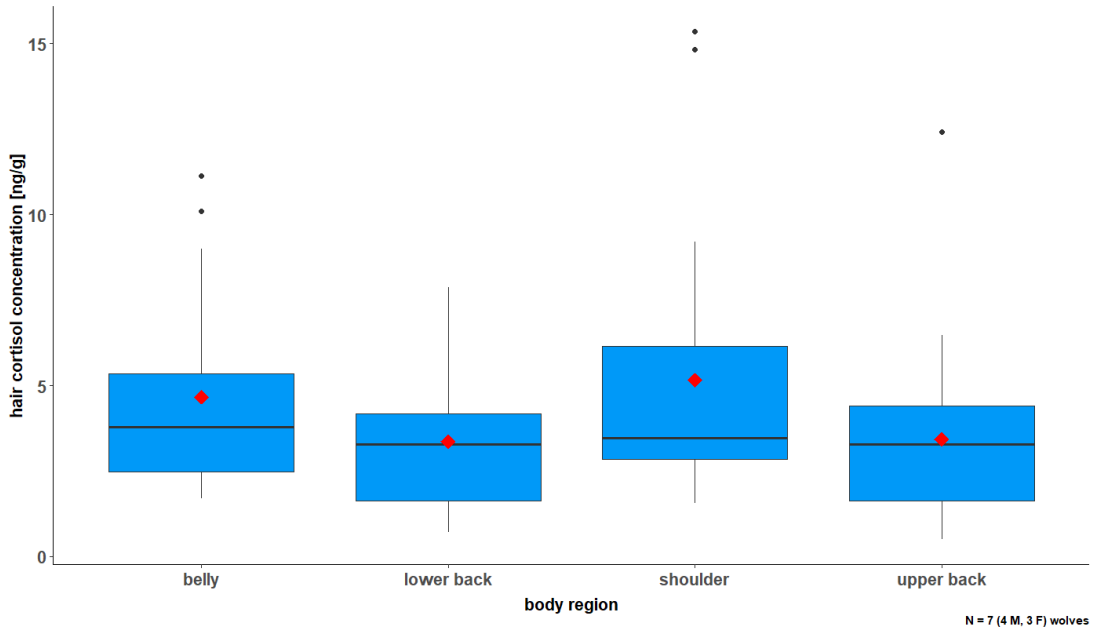

Figure 4. HCC in wolves (brushed samples) by body region. Blue boxes represent the interquartile range with black lines as the median and red dots as the mean values.

Post-hoc pairwise comparisons of the model output revealed significant differences between the lower back and belly (adj. p = 0.01) and the upper back and belly (adj. p = 0.01), as well as between the lower back and shoulder (adj. p < 0.01) and the upper back and shoulder (adj. p < 0.001). However, no significant difference was established between the shoulder and belly. The measured amount of cortisol, therefore, differs significantly depending on the body region, from which the sample was taken.

3.3.2. Effect of season on HCC and FGCM

Considering that cortisol levels differ depending on the body region, only hair collected from the shoulder region was used to test for an association between brushed HCC and season. There was no effect of season on HCC (LMM, ChiSq = 6.4, df = 8, p = 0.60).

The highest mean (SD) HCC for brushed samples was at 5.39 (3.40) ng/g (n = 16, range = 1.40–15.35 ng/g) for the fifth time point/autumn, which contains hormones from August to October. In comparison, the lowest values of mean HCC were recorded at sample time point four (summer, representing May, June, July): 2.78 (0.87) ng/g (n = 12, range = 1.50–4.46 ng/g) (values per season in *Appendix Tab. 7.3.3.).*

Season did, however, have a significant effect on HCC when only clipped hair was used (LMM, ChiSq = 11.42, df = 3, p = 0.01). Here, the highest mean (SD) HCC appeared in the second sample, which represents winter: 7.66 (3.98) ng/g (n = 4, range = 3.33–13.47 ng/g), reflecting hormone incorporation in November, December and January. Furthermore, there was a significant difference between the third and second sample (adj. p = 0.05) as well as the fourth and second sample (adj. p < 0.001). Thus, significantly less cortisol was detected in the samples representing spring and summer than in winter.

The elevated HCC in the fifth sample (autumn) compared to the sample collected in summer (adj. p = 0.00) indicates increased cortisol incorporation into hair during the months of August, September, and October. The lowest mean (SD) HCC for clipped hair samples was found in summer samples (fourth sample) with 2.72 (0.63) ng/g (n = 4, range = 1.75–3.52 ng/g) (values per season in *Appendix Tab. 7.3.4.*).

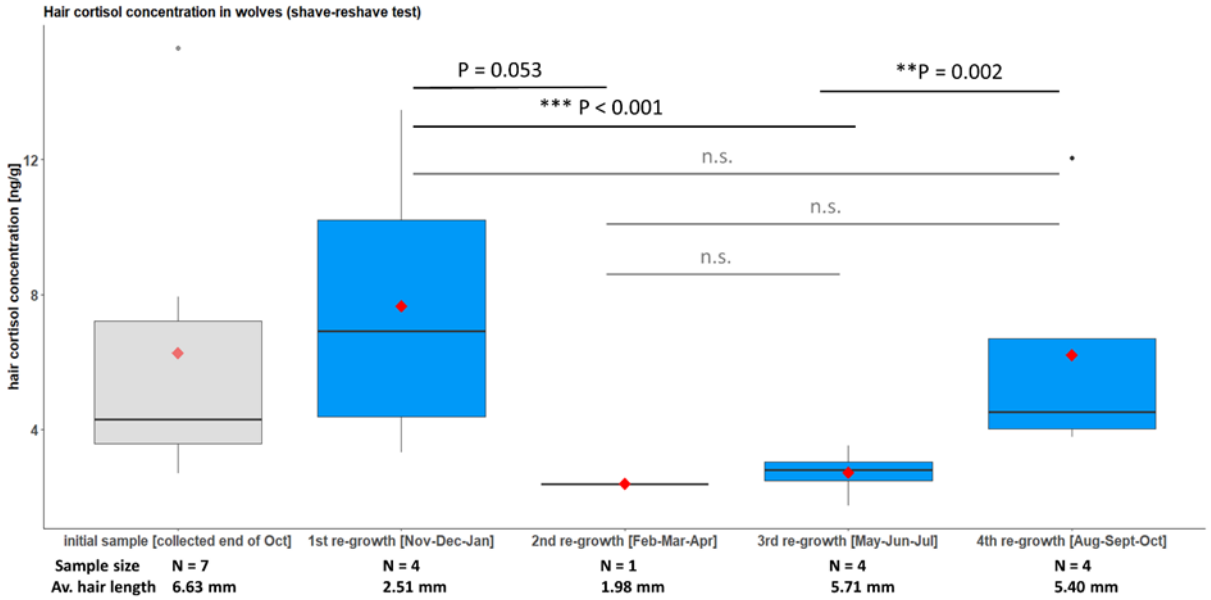

Figure 5. HCC in wolves (shave/re-shave test) by season. Blue boxes represent the interquartile range with black lines as the median and red dots as the mean values.

Additionally, season also had a significant main effect on FGCM (ChiSq = 20.96, df = 7, p = 0.00).

Samples collected during winter and spring exhibited significantly higher FGCM concentrations than summer samples. A significant difference was determined between summer and winter (adj. p = 0.00), summer and spring (adj. p < 0.001), and summer and autumn (adj. p = 0.05). The latter reflects FGCM concentrations in the months of August, September, and October.

The lowest mean (SD) FGCM concentration was found during summer at 15.04 (20.19) ng/g (n = 51, range = 0.84–110.99 ng/g), while the highest mean (SD) FGCM was in samples reflecting spring at 45.30 (58.58) ng/g (n = 59, range = 0.66–237.71 ng/g) (see faecal samples per season in *Appendix Tab. 7.3.5.*).

As visualized in *Fig. 6.*, a generalized additive model (GAM) was fitted to analyse data regarding seasonal differences, creating a nonlinear pattern.

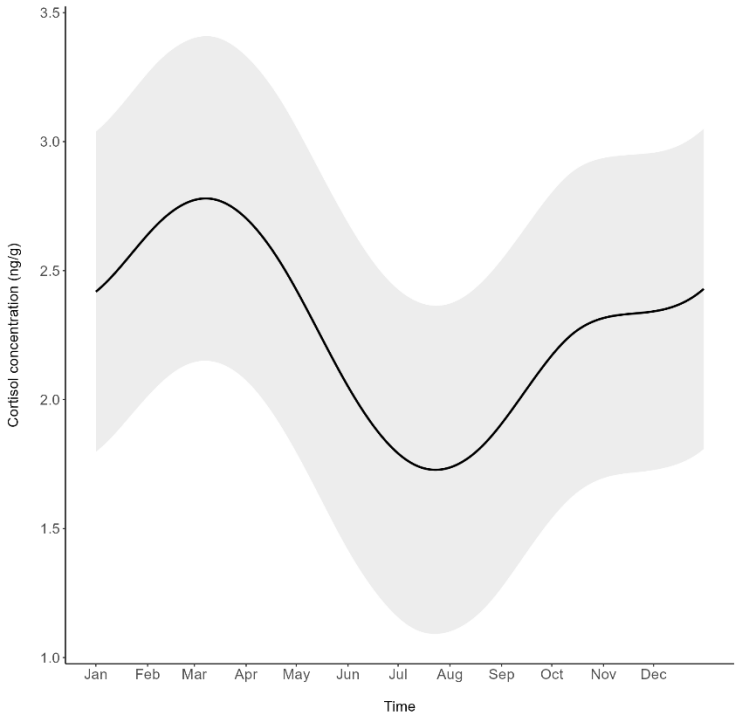

Figure 6. Seasonality of cortisol concentrations in WSC scats, predicted by a GAM model.

3.3.3. Hair collection method comparison

To test the possible influence of the collection method (brushed vs. clipped) on HCC, only samples from the shoulder and the first collection time were used to avoid other confounding factors (body region, season, hair length). There was no significant difference between HCC in brushed vs. clipped samples (LMM, ChiSq = 0.283, df = 1, p = 0.59) (*Appendix Fig. 7.3.2.*).

4. Discussion

The primary aims of this thesis were to assess the annual hair growth of wolves as well as to evaluate whether FGCM levels are reflected in the HCC found in wolf hair samples. Additionally, factors possibly influencing these HCCs were investigated. Together, the results of this study, conducted in captive wolves, aimed to provide information potentially useful for studies on cortisol concentration in wild wolf populations.

Considering the annual hair growth, the results show that season had a significant effect on hair re-growth, as there is a strong difference between summer (third and fourth re-growth in summer and autumn) and winter (first and second re-growth in winter and spring) growth rates. This aligns with the predictions of this thesis. For the correlation between hair and faecal GCM concentrations, the predicted outcome was also met, since FGCM and HCC were significantly positively linked. Regarding the confounding factors on HCC, the results show that the body region from which the hair sample was taken, had a significant effect on the HCC. Brushed samples from the shoulder and belly regions had higher cortisol concentrations than the samples from the upper and lower back. The seasonal effect on HCC of brushed samples was not significant. This contrasts with the prediction of this thesis. However, season had a significant effect when considering clipped hair and faecal samples. In both cases, and in line with the predictions, the highest concentrations were measured in the winter months. Lastly, the results for the hair collection method comparison showed no significant difference in HCC between brushed and clipped hair samples from the shoulder.

4.1. Hair re-growth

Hair growth was determined using clipped hair samples from the shoulder, which were repeatedly collected after a three-month period. As mentioned before, the first sample taken in October 2021 was exclusively used as a reference for assessing hair (re-)growth rates. The fourth sample, reflecting spring and early summer, exhibited the highest mean growth rate and was close to reaching the initial length (relative to sample one). Since the fifth sample clipped in autumn showed a similar (slightly lower) mean length and daily growth rate, these two time periods are in strong contrast to the other ones. These high growth rates may be due to the wolves growing their winter coat earlier than expected. Generally, the second sample (reflecting November, December, January) had the lowest mean growth rate, however, for all wolves but one (Amarok), the third sample (reflecting February, March, April) truly showed the lowest growth for the individuals (n = 3, 0.21 mm/day with a mean length (SD) of 19 (5.58) mm).

Considering this period was the fastest growth for the wolf missing from those numbers (Amarok), it explains the high standard deviation to the mean growth rate when including him in the results (*Tab. 1.*). This, however, highlights how individually different hair growth can be even within the same species (11).

The results show a seasonal variation in growth rates and therefore not only align with the predictions of this thesis but also with previous studies in dogs (11,50) and horses (29). The canid studies even revealed the same outcome, with higher growth rates in summer months, underlining the positive influence of natural sunlight and temperature on hair growth, which has been described in other studies as well (11,49,50). Considering the past meteorological data for Vienna (autumn 2021 and autumn 2022), obtained from © WeatherSpark (see *Appendix Fig. 7.3.3.–7.3.6.*), it may be assumed that the cold temperatures that set in around the end of October and lasted until the beginning of April had the described negative effect on hair growth. The same can be observed in the numbers for the hours of daylight, as the photoperiod almost halves at the end of November (compared to summer months) and only starts to increase again in March.

Overall, the results confirm that wolves at the WSC begin to develop a longer and denser winter coat in autumn, to protect themselves from the cold conditions, and their coats thin out during spring and summer to prevent the body from overheating. However, additional factors influencing hair growth, such as diet and nutritional and health status, must also be taken into consideration, especially when transferring these results from captive wolves to free-ranging populations.

4.2. Correlation between hair and faecal GCM concentrations

As predicted, the results of this thesis show a significant positive correlation (*Fig. 3.*) between the measures of FGCM and HCC in captive wolves, albeit with considerable individual variation resulting in rather large confidence intervals. Overall, in cases of high FGCM, elevated cortisol concentrations in hair in the corresponding samples were also detected. This supports the hypothesis that hair samples can be used as proxies for systemic stress levels. Similar results were established for dogs, cats (24), and cattle (37), emphasizing that hair, as a substrate, is suitable as a biomarker of chronic stress and thus animal welfare. Nonetheless, there is a cutaneous HPA axis that affects and influences HCC, which was not assessed in this study but needs to be considered, indicating that merely proving a positive correlation is not sufficient to prove the suitability of the method. To validate these approaches further, experimental

studies manipulating peripheral cortisol levels (either increasing or decreasing them) are inevitable. This is of special importance considering studies highlighting the acute increases in HCC (33,34), suggesting that further validation is required to confirm the method's effectiveness in different scenarios.

4.3. Confounding factors in hair and faeces

Four different body regions (shoulder, belly, upper back, and lower back) were sampled through brushing and compared in relation to the measured HCC to investigate whether body region serves as a confounding factor on the HCC.

Studies in wolves (7) and lynx (39) revealed differences in HCC between body regions. Considering the influence of UV radiation on steroid hormones (32), it is likely to have a greater impact on certain body regions that are more exposed to sunlight than others. The degrading effect of sunlight is supported by the results of this thesis (*Fig. 4*.), as the HCC values between the lower back and belly ($p = 0.01$), as well as those between the upper back and belly ($p = 0.01$), showed significant differences. The concentrations extracted from shoulder samples had the highest mean values (5.16 ng/g). These results are inconsistent with existing research. No effect of body region was observed in bobcats (40) and dogs (27,45). However, one study concerning dogs only compared the left and right shoulder region and since these areas generally receive the same amount of sunlight, likely, the concentrations do not differ greatly. Certainly, these results should not be interpreted solely as an effect of UV radiation. It must also be considered that the distinct body regions likely experience different types of manipulation (e.g., scratching or grooming), with one region being subjected to more than another, which possibly influences HCC by stimulating the cutaneous cortisol production or mild inflammatory processes (31). Additionally, this thesis did not determine if hair colour varied among the body regions of an individual, which could have impacted HCC as well, given that, for example, belly hair tends to differ in colour and composition form the hair on the back. Therefore, it is important to emphasize that further research is required to understand why these differences in HCC occur when comparing body regions.

Furthermore, the density, length, and composition of the hair sample influence HCC. Nevertheless, although the present thesis could not provide any conclusive information about the hair composition, differences in the contained HCC in the two hair structures were found in mountain goats (36). Guard hair generally revealed almost twice as much HCC than underwool. Additionally, the ratio of guard hair to undercoat differs depending on the body

region (e.g., the amount of guard hair in samples from the belly is lower than the amount in back samples), highlighting the necessity for standardized sample taking, regarding body region differences. However, studies in wolves (26) and dogs (27) could not report differences between the hair structures, which is precisely why more clarification regarding hair composition is required for future research and why, for this thesis, guard hair and underwool in the samples were not separated. Generally, the results of this thesis emphasize the importance of standardized sampling procedures, thereby utilizing the same body region for sample collection to reduce the possibility of variability.

Moreover, the findings of the present study demonstrate seasonal variations in HCC of clipped hair samples (*Fig. 5.*). This is in agreement with the predictions of this thesis, as well as with previous studies on bobcats (40), dogs (27), and wolves (46). The second sample had the highest mean HCC (7.66 ng/g), representing winter, while the fourth sample (summer) had the lowest mean concentration. The studies on dogs and wolves described the same outcome. The mating period may be the most influential factor, resulting in higher concentrations in the winter months. This period is considered particularly stressful for the wolves, as despite the small packs at the WSC, they see, smell, and hear the other wolves from the neighbouring packs and may show higher levels of competitive behaviours such as marking, patrolling, and howling. In addition, as animals are not neutered but intact (females) or vasectomized (males), they also exhibit all physiological and behavioural changes related to the reproductive period, such as mating, denning, and mate guarding/mate provisioning as soon as females become pseudo-pregnant. However, it is worth mentioning that the study on bobcats (40) only found seasonal changes in HCC in wild but not in captive bobcats. One could thus assume that additional factors influenced GC concentrations, such as diet and food supply, respectively, which were kept constant in captivity. Similarly, in the present sample, food supply at the WSC is constant throughout winter, and a seasonal effect of nutritional stress on HCC is therefore unlikely. However, this may be different for free-ranging wolves in future studies.

The negative effect of natural sunlight on the GC concentration in hair may also be considered here since UV radiation causes the glucocorticoids to gradually disappear from the hair shaft, as has been revealed for humans and sheep (31,32). Considering the data on hours of daylight obtained from © WeatherSpark (see *Appendix Fig. 7.3.3*–*7.3.6.*), as beforementioned, the low values of the clipped hair samples in spring and summer (summer being the lowest at 2.72 ng/g) (February to July) overlap with the months that have the most daylight and

consequently the highest UV radiation. HCC was the highest in winter when the hours of daylight were much shorter.

Another study on wolves (8) found no seasonal pattern in faecal GCM concentrations, which concords with the findings of the brushed samples in this study. A possible reason could be insufficient sample quality and number. However, it is most probable that the brushed samples contained hairs from different growth phases and were analysed as a mix, leading to a distortion of the concentration values.

Seasonality was also evident in FGCM, with the lowest mean FGCM measured in summer (15.04 ng/g) and the highest mean FGCM in spring (45.30 ng/g) (*Fig. 6.*). A study on mountain goats (36) also revealed a seasonal pattern in FGCM (although different with the highest FGCM levels in summer and the lowest in autumn). The influence of UV radiation on this matrix can be ruled out, especially considering the applied collection method used in the present study. However, the mating period, as well as heavy weather changes (e.g., cold temperatures) are possible impacting factors. At this point though, it is necessary to highlight that the HCC and the FGCM peaks did not fully coincide, as the FGCM peak appeared later than the one in hair. Although the opposite might have been expected, considering the duration of hormonal incorporation into hair and the faster excretion via faeces. This, however, might also be a cautionary sign for HCC as an indicator for chronic stress and it is important to consider that it may also reflect more acute stress responses, requiring further investigation into its role in different stress contexts.

Lastly, to determine if the sample collection method had a significant effect on HCC, brushed shoulder and clipped shoulder samples were compared. However, since, as described above, not only the body region but also the season impacted HCC, only the first and fifth sample were suitable for comparison, as they were taken at the same time (autumn). However, to avoid the possible influence of manipulation (i.e., repeated clipping and brushing), which may have led to local cortisol production, only the first sample was considered valuable. No significant difference between the methods was found. Despite these results, the question of whether the presence of hair follicles influences the HCC remains interesting for future studies. While clipped samples do not contain follicles, the number of follicles is possibly greater in brushed samples (since they could be pulled out by the root). In a study in brown bears (53), a significant difference was found. Therefore, HCC was higher in samples that included the follicle. As this contrasts with the results of this thesis, it may be beneficial for the future efficiency of hair sampling to determine whether the presence of follicles significantly

influences HCC in wolves. After all, if the presence of follicles has no impact on HCC, all sampling methods (e.g., clipping, plucking, snagging) would appear comparable and interchangeable for long-term stress evaluation. This is of special importance when studying wildlife. However, with the present results, no further conclusion can be made on this topic, as it is not clear how many hairs from the brushed samples were collected with the follicle.

4.4. Limitations of this thesis

Although cortisol measurement from hair samples proved to be a useful method for steroid hormone assessment in wolves, the present study has certain limitations. The relatively limited sample size, due to the small available wolf population, could have affected generalisability and reduced the statistical power of this thesis' results. Additionally, the influence of the coat colour could not be tested in more detail, as the presence of only one black wolf prevented any conclusions. Previous studies produced inconsistent data. In dogs (22) black hair was mentioned in combination with lower HCC, while the opposite was found in horses (43) and cattle (37).

Furthermore, considering most of the used samples contained both guard hair and underwool and were not separated for extraction, this could have led to some inaccuracies of the concentration values. This is especially relevant given that the literature describes a variation in the growth of these hair types in wolves (26) and a difference in the contained HCC (36), while another study in wolves did not report differences (26).

Individual variations between the wolves partly resulted in large differences regarding the absolute cortisol concentrations in hair and faeces. One wolf (Taima) consistently exhibited higher cortisol levels than the other individuals and was in the upper range for every sample time point. Although she was the only black wolf participating in this study, she showed very high levels of both HCC and FGCM. This highlights the importance of other impacting factors (e.g., health status, metabolic reasons). Additionally, the setting of this thesis allowed a good insight into the characteristics of the wolves, as the number of individuals was manageable, and the wolves were under constant observation. Therefore, it can be assumed that personality and behaviour could also influence cortisol concentrations, particularly in the case of Taima, who is relatively nervous and easily stressed, leading to higher levels. Moreover, it should also be mentioned that she and Takoa, who also exhibited fairly high cortisol concentrations, are the only European wolf mixes included in the study population. Both display rather shy and nervous behaviour compared to the other wolves, which are pure North American wolves.

Those tend to be less shy and timid (54). Unfortunately, a more detailed investigation into this was not possible due to the small number of individuals, however, this could be interesting for future studies with larger populations including wild wolves from Europe and US/Canada.

Furthermore, another wolf (Yukon) received prednisolone treatment during the study period, which likely affected both hair and faeces measurements. Her samples were only included in the assessment of confounding factors (body region, method), as she died shortly after the second hair sampling session. As a synthetic GC, prednisolone has a similar effect to the body's natural cortisol and acts as an anti-inflammatory and immunosuppressive drug. It may lead to increased levels when administered initially, but a long-term use is described to suppress the negative feedback loop of the HPA axis (55). However, these decreased values would have been recorded by the used cortisol EIA. Overall, her sample values did not show lower concentrations compared to the other wolves. Certainly, there are no results that show what her values were before the medication was administered, therefore it could well be that she also exhibited higher levels in general and that the values measured were reduced to a lower level. However, there could also be other explanations for this. First, she could have reacted to other stressors – which then again might possibly suggest that HCC would be appropriate as an acute stress measurement. Additionally, considering that she could have developed a tolerance to the medication, therefore limiting the effectiveness, might have also caused the measured concentrations in this thesis. Lastly, the possibility of possible errors during the measurement cannot be ruled out either. Despite the many possibilities, it is ultimately important to mention that no exact reason could be established. The other wolves were treated with anti-parasitic drugs and spot-on treatments throughout the study period, and no effect on the cortisol concentration was expected, but it cannot be excluded completely.

Moreover, one cannot neglect the uncertainty regarding how much the cutaneous "HPA axis homolog" (56) influences the incorporation of cortisol into the hair. Although the present results show a significant correlation between HCC and systemic levels, one cannot infer about the influence of local cortisol production and mediation. Therefore, this question requires further clarification. Additionally, the extraction process may have introduced variance into the present results, considering that the hair and faecal samples were analysed by different people.

Finally, a substantial methodological issue in the interpretation of HCC as a biomarker for chronic stress lies in the character of correlative studies. Correlation does not imply causation, therefore, even though a link between GC concentrations in hair and faecal samples and season and body region was observed, it does not allow a direct conclusion drawn about the

causal relationship. Therefore, it is possible that other unconsidered factors have influenced the HCC values. Recalling the studies that suggested that HCCs do not exclusively reflect long-term HPA axis activity but rather short-term fluctuations (33,34), these results indicate that HCCs should be used cautiously as a proxy for chronic stress. To validate HCC as a reliable marker of chronic stress, targeted experimental studies are required in which HPA axis activity is systematically manipulated (increased/decreased) and the effects on HCC can be measured. Such experimental designs may provide more precise insights into the mechanisms of cortisol incorporation into hair.

4.5. Conclusion of the study

In conclusion, the findings of this thesis reveal that the hair growth of captive wolves is seasonally specific, with a peak during late summer and autumn, and suggest that clipped hair samples serve as a useful biomarker for assessing long-term stress levels in wolves. Additionally, the results show that influencing factors such as body region must be considered during the interpretation of the HCC. Overall, the results emphasize the need for a standardized sampling technique (e.g., body region, storage) to improve the significance and inference of the HCC data. Altogether, due to the relatively low invasiveness, and stability of hair samples, this method appears valuable for future research questions aimed at studying wildlife.

5. Acknowledgments

Firstly, I would like to thank to the trainers operating at the Wolf Science Center, who have made a significant contribution to this study through their efforts not only in sample collection but also in the preparation stages of this research, considering they had to practice with the wolves in advance. Without their participation, the study would not have been feasible.

Additionally, I would like to extend my appreciation and gratitude to my supervisor, Priv.-Doc. Sarah Marshall-Pescini, PhD. Her valuable thoughts and immeasurable knowledge in this research field, have greatly contributed to several thought-provoking moments on my part and, thus, significantly inspired the content of this thesis.

Ultimately, I am immensely grateful for the support of Mag.med.vet. Gwendolyn Wirobski, PhD, who took on the role of the Vienna-based supervisor and was always available for questions, problems, or feedback. The quick responses and attentiveness contributed to many aspects of this work. Her instructions in the laboratory processes and ongoing support in the statistical analysis, which were undoubtedly the most important aspects in the realisation of this study, would not have been possible without her help. With her broad knowledge and attention to detail, she also provided numerous of great ideas for the research, content, and structure of this thesis. I would also like to acknowledge her insightful feedback and constructive criticism, which greatly improved my work. Truly, she contributed to every step of this thesis, and I owe her special thanks for her continuous guidance and support.

6. References

1. Kuijper DPJ, Churski M, Trouwborst A, Heurich M, Smit C, Kerley GIH, et al. Keep the wolf from the door: How to conserve wolves in Europe's human-dominated landscapes? Biological Conservation. 2019;235:102–11.

2. Boitani L. Action Plan for the conservation of the wolves (*Canis lupus*) in Europe. Nature and environment, No. 113. Council of Europe; 2000.

3. Enserink M, Vogel G. The carnivore comeback. Science. 2006;(314):746–9.

4. McCormick SD, Romero LM. Conservation Endocrinology. BioScience. 2017;67(5):429–42.

5. Sheriff MJ, Dantzer B, Delehanty B, Palme R, Boonstra R. Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia. 2011;166(4):869–87.

6. Koren L, Bryan H, Matas D, Tinman S, Fahlman Å, Whiteside D, et al. Towards the validation of endogenous steroid testing in wildlife hair. Journal of Applied Ecology. 2019;56(3):547–61.

7. Pereira P, Fandos Esteruelas N, Nakamura M, Rio-Maior H, Krofel M, Di Blasio A, et al. Hair cortisol concentration reflects the life cycle and management of grey wolves across four European populations. Scientific Reports. 2022;12(1):5697.

8. Van Kesteren F, Sillero-Zubiri C, Millar R, Argaw K, Macdonald DW, Paris M. Sex, stress and social status: Patterns in fecal testosterone and glucocorticoid metabolites in male Ethiopian wolves. General and Comparative Endocrinology. 2012;179(1):30–7.

9. Palme R. Non-invasive measurement of glucocorticoids: Advances and problems. Physiology & Behavior. 2019;199:229–43.

10. Koren L, Mokady O, Karaskov T, Klein J, Koren G, Geffen E. A novel method using hair for determining hormonal levels in wildlife. Animal Behaviour. 2002;63(2):403–6.

11. Gunaratnam P, Wilkinson GT. A study of normal hair growth in the dog. Journal of Small Animal Practice. 1983;24(7):445–53.

12. Herman JP, Figueiredo H, Mueller NK, Ulrich-Lai Y, Ostrander MM, Choi DC, et al. Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo–pituitary–adrenocortical responsiveness. Frontiers in Neuroendocrinology. 2003;24(3):151–80.

13. Charmandari E, Kino T, Chrousos GP. Glucocorticoids and Their Actions: An Introduction. Annals of the New York Academy of Sciences. 2004;1024(1):1–8.

14. Sapolsky RM, Romero LM, Munck AU. How Do Glucocorticoids Influence Stress Responses? Integrating Permissive, Suppressive, Stimulatory, and Preparative Actions. Endocrine Reviews. 2000;21(1):55–89.

15. Gouin JP. Chronic Stress, Immune Dysregulation, and Health. American Journal of Lifestyle Medicine. 2011;5(6):476–85.

16. Elenkov IJ, Chrousos GP. Stress, cytokine patterns and susceptibility to disease. Baillière's Clinical Endocrinology & Metabolism. 1999;13(4):583–95.

17. Esch T, Stefano GB, Fricchione GL, Benson H. Stress in cardiovascular diseases. Medical Science Monitor. 2002;8(5):RA93–101.

18. Manenschijn L, Schaap L, Van Schoor NM, Van Der Pas S, Peeters GMEE, Lips P, et al. High Long-Term Cortisol Levels, Measured in Scalp Hair, Are Associated With a History of Cardiovascular Disease. The Journal of Clinical Endocrinology & Metabolism. 2013;98(5):2078–83.

19. Ralph CR, Lehman MN, Goodman RL, Tilbrook AJ. Impact of psychosocial stress on gonadotrophins and sexual behaviour in females: role for cortisol? Reproduction. 2016;152(1):R1–14.

20. Vincent IC, Michell AR. Comparison of cortisol concentrations in saliva and plasma of dogs. Research in Veterinary Science. 1992;53(3):342–5.

21. Neary JP, Malbon L, McKenzie DC. Relationship between serum, saliva and urinary cortisol and its implication during recovery from training. Journal of Science and Medicine in Sport. 2002;5(2):108–14.

22. Bennett A, Hayssen V. Measuring cortisol in hair and saliva from dogs: coat color and pigment differences. Domestic Animal Endocrinology. 2010;39(3):171–80.

23. Millspaugh JJ, Washburn BE. Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. General and Comparative Endocrinology. 2004;138(3):189–99.

24. Accorsi PA, Carloni E, Valsecchi P, Viggiani R, Gamberoni M, Tamanini C, et al. Cortisol determination in hair and faeces from domestic cats and dogs. General and Comparative Endocrinology. 2008;155(2):398–402.

25. McLeod PJ, Moger WH, Ryon J, Gadbois S, Fentress JC. The relation between urinary cortisol levels and social behaviour in captive timber wolves. Canadian Journal of Zoology. 1996;74(2):209–16.

26. Roffler GH, Karpovich S, Charapata P, Keogh MJ. Validation and measurement of physiological stress and reproductive hormones in wolf hair and claws. Wildlife Society Bulletin. 2022;46(4):e1330.

27. Roth LSV, Faresjö Å, Theodorsson E, Jensen P. Hair cortisol varies with season and lifestyle and relates to human interactions in German shepherd dogs. Scientific Reports. 2016;6(1):19631.

28. Azevedo A, Wauters J, Kirschbaum C, Serra R, Rivas A, Jewgenow K. Sex steroids and glucocorticoid ratios in Iberian lynx hair. Cooke S, editor. Conservation Physiology. 2020;8(1):1–14.

29. Gardela J, Carbajal A, Tallo-Parra O, Olvera-Maneu S, Álvarez-Rodríguez M, Jose-Cunilleras E, et al. Temporary Relocation during Rest Periods: Relocation Stress and Other Factors Influence Hair Cortisol Concentrations in Horses. Animals. 2020;10(4):642.

30. Molnar B, Fattebert J, Palme R, Ciucci P, Betschart B, Smith DW, et al. Environmental and Intrinsic Correlates of Stress in Free-Ranging Wolves. PLOS ONE. 2015;10(9):e0137378.

31. Salaberger T, Millard M, Makarem SE, Möstl E, Grünberger V, Krametter-Frötscher R, et al. Influence of external factors on hair cortisol concentrations. General and Comparative Endocrinology. 2016;233:73–8.

32. Wester VL, Van Der Wulp NRP, Koper JW, De Rijke YB, Van Rossum EFC. Hair cortisol and cortisone are decreased by natural sunlight. Psychoneuroendocrinology. 2016;72:94–6.

33. Kalliokoski O, Jellestad FK, Murison R. A systematic review of studies utilizing hair glucocorticoids as a measure of stress suggests the marker is more appropriate for quantifying short-term stressors. Sci Rep. 2019 Aug 19;9(1):11997.

34. Colding-Jørgensen P, Hestehave S, Abelson KSP, Kalliokoski O. Hair glucocorticoids are not a historical marker of stress – Exploring the time-scale of corticosterone incorporation into hairs in a rat model. General and Comparative Endocrinology. 2023;341:114335.

35. Patkó L, Ujhegyi N, Szabó L, Péter F, Schally G, Tóth M, et al. Even a hair casts its shadow: review and testing of noninvasive hair collecting methods ofcarnivore species. North-Western Journal of Zoology. 2016;12(1):130–40.

36. Dulude-de Broin F, Côté SD, Whiteside DP, Mastromonaco GF. Faecal metabolites and hair cortisol as biological markers of HPA-axis activity in the Rocky mountain goat. General and Comparative Endocrinology. 2019;280:147–57.

37. Tallo-Parra O, Manteca X, Sabes-Alsina M, Carbajal A, Lopez-Bejar M. Hair cortisol detection in dairy cattle by using EIA: protocol validation and correlation with faecal cortisol metabolites. Animal. 2015;9(6):1059–64.

38. Robertson KE, Ellington EH, Tonra CM, Gehrt SD. Stress in the city? Coyote hair cortisol varies with intrinsic and extrinsic factors within a heavily urbanized landscape. Science of The Total Environment. 2023;901:165965.

39. Terwissen CV, Mastromonaco GF, Murray DL. Influence of adrenocorticotrophin hormone challenge and external factors (age, sex, and body region) on hair cortisol concentration in Canada lynx (Lynx canadensis). General and Comparative Endocrinology. 2013;194:162–7.

40. Carroll RP, Litvaitis MK, Foxall T. Bobcat Hair Cortisol Correlates with Land Use and Climate. The Journal of Wildlife Management. 2021;85(4):772–81.

41. Van Der Laan JE, Vinke CM, Arndt SS. Evaluation of hair cortisol as an indicator of long-term stress responses in dogs in an animal shelter and after subsequent adoption. Scientific Reports. 2022;12(1):5117.

42. Lafferty DJR, Laudenslager ML, Mowat G, Heard D, Belant JL. Sex, Diet, and the Social Environment: Factors Influencing Hair Cortisol Concentration in Free-Ranging Black Bears (Ursus americanus). PLOS ONE. 2015;10(11):e0141489.

43. Medill SA, Janz DM, McLoughlin PD. Hair Cortisol Concentrations in Feral Horses and the Influence of Physiological and Social Factors. Animals. 2023;13(13):2133.

44. Creel S. Dominance, aggression, and glucocorticoid levels in social carnivores. Journal of Mammalogy. 2005;86(2):255–64.

45. Bryan HM, Adams AG, Invik RM, Wynne-Edwards KE, Smits JE. Hair as a Meaningful Measure of Baseline Cortisol Levels over Time in Dogs. Journal of the American Association for Laboratory Animal Science. 2013;52(2).

46. Eggermann J, Theuerkauf J, Pirga B, Milanowski A, Gula R. Stress-Hormone Levels of Wolves in Relation to Breeding Season, Pack Size, Human Activity, and Prey Density. Annales Zoologici Fennici. 2013;50(3):170–5.

47. Diaz SF, Torres SMF, Dunstan RW, Lekcharoensuk C. An analysis of canine hair re-growth after clipping for a surgical procedure. Veterinary Dermatology. 2004;(15):25–30.

48. Diaz SF, Torres SMF, Nogueira SAF, Gilbert S, Jessen CR. The impact of body site, topical melatonin and brushing on hair regrowth after clipping normal Siberian Husky dogs. Veterinary Dermatology. 2006 Feb;17(1):45–50.

49. Schmidt K, Deichsel K, De Oliveira RA, Aurich J, Ille N, Aurich C. Effects of environmental temperature and season on hair coat characteristics, physiologic and reproductive parameters in Shetland pony stallions. Theriogenology. 2017;97:170–8.

50. Butler WF, Wright AI. Hair growth in the greyhound. Journal of Small Animal Practice. 1981;22(10):655–61.

51. Palme R, Möstl E. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. International Journal of Mammalian Biology. 1997;62, Suppl. 2:192–7.

52. Bakdash JZ, Marusich LR. Repeated Measures Correlation. Frontiers in Psychology. 2017;8:456.

53. Sergiel A, Cattet M, Kapronczai L, Janz DM, Selva N, Bartoń KA, et al. Do follicles matter? Testing the effect of follicles on hair cortisol levels. Conservation Physiology. 2020;8(1):coaa003.

54. Tebelmann H, Ganslosser U. Differences in boldness between Eurasian and American wolves (*Canis lupus*) might be based on adaptive mechanisms. Ecology and Evolution. 2024;14(8):e70178.

55. Munck A, Guyre PM. Glucocorticoid Physiology, Pharmacology and Stress. In: Chrousos GP, Loriaux DL, Lipsett MB, editors. Steroid Hormone Resistance. Boston, MA: Springer US; 1986. p. 81–96.

56. Skobowiat C, Dowdy JC, Sayre RM, Tuckey RC, Slominski A. Cutaneous hypothalamic-pituitary-adrenal axis homolog: regulation by ultraviolet radiation. American Journal of Physiology-Endocrinology and Metabolism. 2011;301(3):E484–93.

Appendix

7. Appendix

7.1. Extraction protocol for cortisol

Day 1:

- Sort samples in batches of n = 36 (= 1 plate)
- Cut hair samples (~2–3 mm)
- Weighing ~0.2 g hair into a glass vial

Washing step (degreasing)

- Add 7 ml n-hexane
- Shake vials on hand vortex (~1 min)
- Decant n-hexane
- dry hair samples overnight

Day 2:

Extraction (1st part)

- Weigh 100 mg hair (0.100 ± 0.0005 g) from each vial into an eprouvette
- 50 mg – 25 mg – 10 mg
- Add 5 ml methanol (100 %)
- 2.5 ml – 1.25 ml – 0.5 ml
- Plug vials tightly
- 24h incubation in thermomixer at 37 °C

Day 3:

Extraction (2nd part)

- Centrifuge (15 min at 2500 *g*)
- Transfer 2.5 ml into a new eprouvette
- 1.25 ml – 0.625 ml – 0.25 ml
- Dry down under a stream of nitrogen at 50°–60° C
- Re-dissolve in EIA buffer (0.5 ml; shaker for 30min)
- Always add 0.5 ml of EIA buffer regardless of sample amount
- Transfer extracts into *'Biorad'* tubes
- Store in freezer at -20° C until analysis

7.2. Enzyme immunoassay (EIA) protocol for cortisol

Day 1:

- Thaw extracts
- Wash coated plates
- Thaw *Enzyme label* (EL), *Antibody* (AB) and *Standards* (S).
- Pipet plates:
    - S1–S7: 10 *µl* S*tandard* + 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
    - Incubate plates on a shaker in a cold room overnight

Day 2:

- Wash plates
- Mix POD (peroxidase) and assay-buffer, then add 100 *µl* into each well
- Incubate plates on a shaker in a cold room for 45 minutes
- Wash plates
- Mix TMB (Tetramethylbenzidine), H2O2 and TMB-buffer, then add 100 *µl* into each well
- Incubate plates on a shaker in a cold room for 45 minutes
- Add 0 µl of H S O Sulfuric acid) into each well
- Shake plates at room temperature for 30 minutes
- Read plates with a photometer at 450 nm

7.3. Additional figures and tables

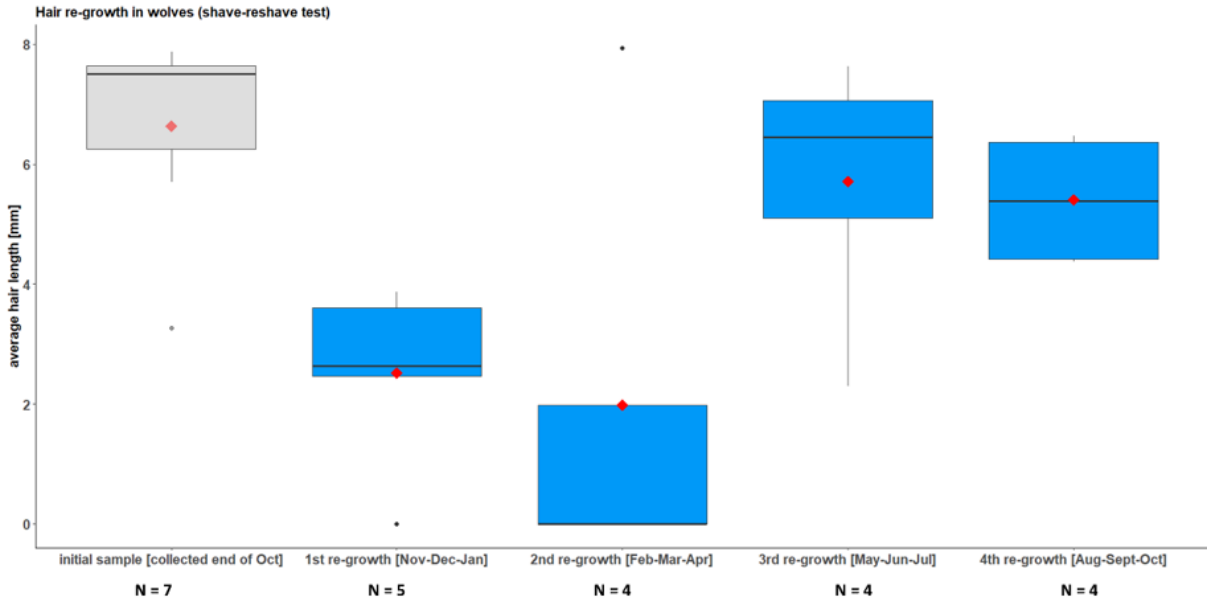

Figure 7.3.1. Hair re-growth cycle in wolves (shave/re-shave test). Blue boxes represent the interquartile range with black lines as the median and red dots as the mean values.

Table 7.3.1. Comparison of mean FGCM and HCC per sampling time.

	Oct.–Jan.	Jan.–April	April–July	July–Oct.
n	47	51	47	48
mean FGCM (ng/g)	38.44	47.44	12.60	24.92
SD	66.90	59.25	14.29	43.23
minimum	0.77	0.97	0.84	0.66
maximum	374.99	223.74	60.61	237.71
n	4		4	4
mean HCC (ng/g)	7.66		2.72	6.21
SD	3.98		0.63	3.39
minimum	3.33		1.75	3.78
maximum	13.47		3.52	12.04

Table 7.3.2. Comparison of body positions based on HCC.

	shoulder	belly	upper back	lower back
n	22	25	27	27
mean HCC (ng/g)	5.16	4.66	3.43	3.37
SD	3.66	2.77	2.38	1.93
minimum	1.55	1.69	0.50	0.72
maximum	15.35	11.12	12.41	7.87

Table 7.3.3. Brushed hair samples per season.

	Autumn	Winter	Spring	Summer	Autumn
n	28	22	23	12	16
mean HCC (ng/g)	4.47	3.61	3.89	2.78	5.39
SD	3.46	2.33	2.00	0.87	3.40
minimum	0.98	0.50	1.38	1.50	1.40
maximum	14.83	8.93	9.20	4.46	15.35

Table 7.3.4. Clipped hair samples per season.

	Autumn	Winter	Spring	Summer	Autumn
n	13	4	4	4	12
mean HCC (ng/g)	6.47	7.66	4.45	2.72	5.52
SD	4.03	3.98	1.99	0.63	2.93
minimum	2.70	3.33	2.39	1.75	2.00
maximum	15.31	13.47	7.40	3.52	12.04

Table 7.3.5. Faecal samples per season.

	Winter	Spring	Summer	Autumn
n	60	59	51	48
mean FGCM (ng/g)	26.70	45.30	15.04	24.92
SD	55.35	58.58	20.19	43.23
minimum	0.77	0.97	0.84	0.66
maximum	374.99	223.74	110.99	237.71

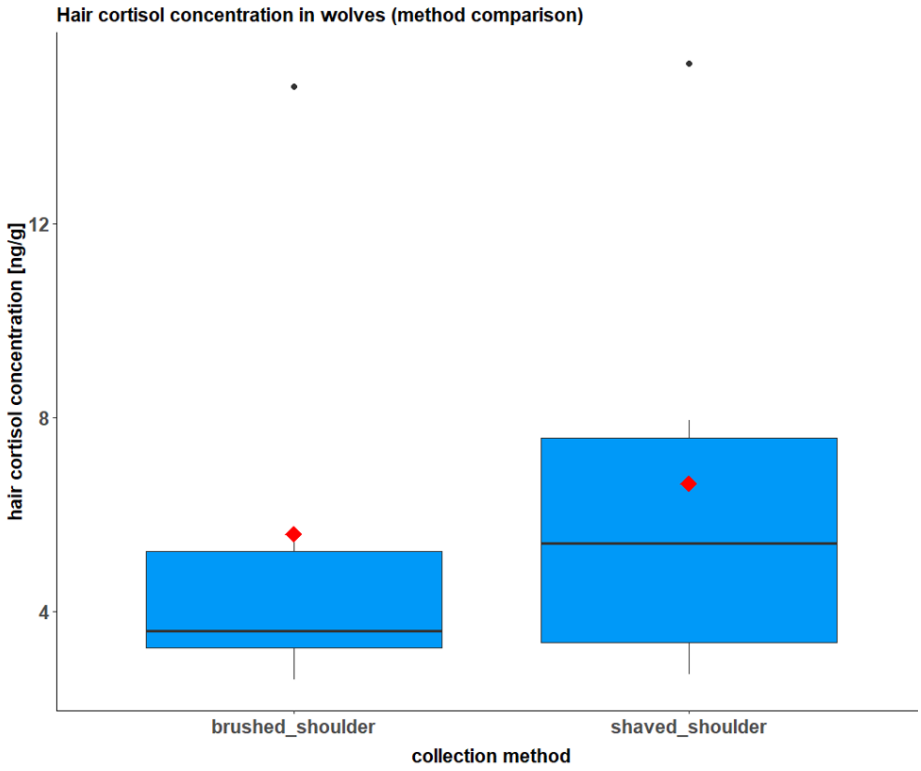

Figure 7.3.2. HCC in wolves (brushed vs clipped samples). Blue boxes represent the interquartile range with black lines as the median and red dots as the mean values.

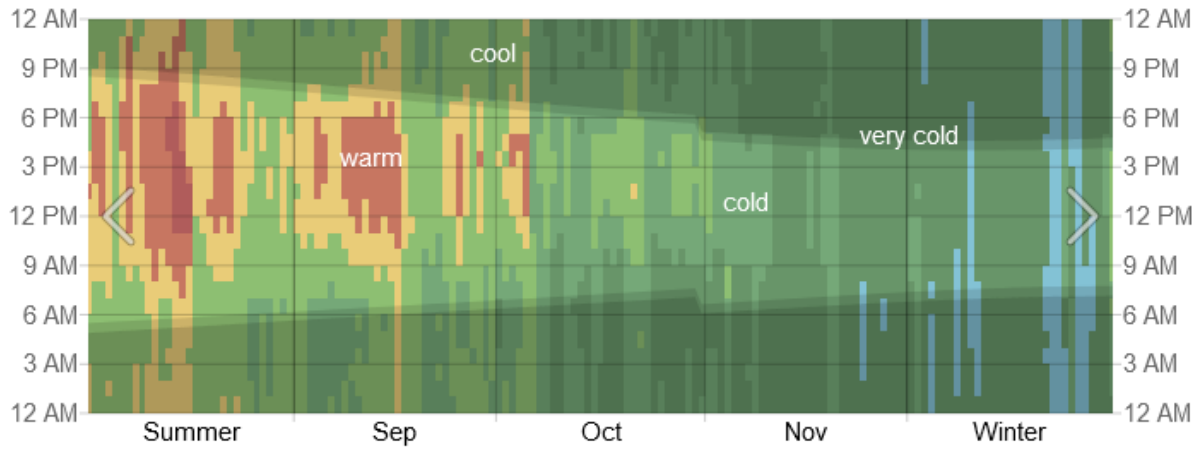

Figure 7.3.3. Hourly Temperature in Fall 2021 in Vienna (© WeatherSpark.com)
The hourly reported temperature, colour coded into bands. The shaded overlays indicate night and civil twilight.

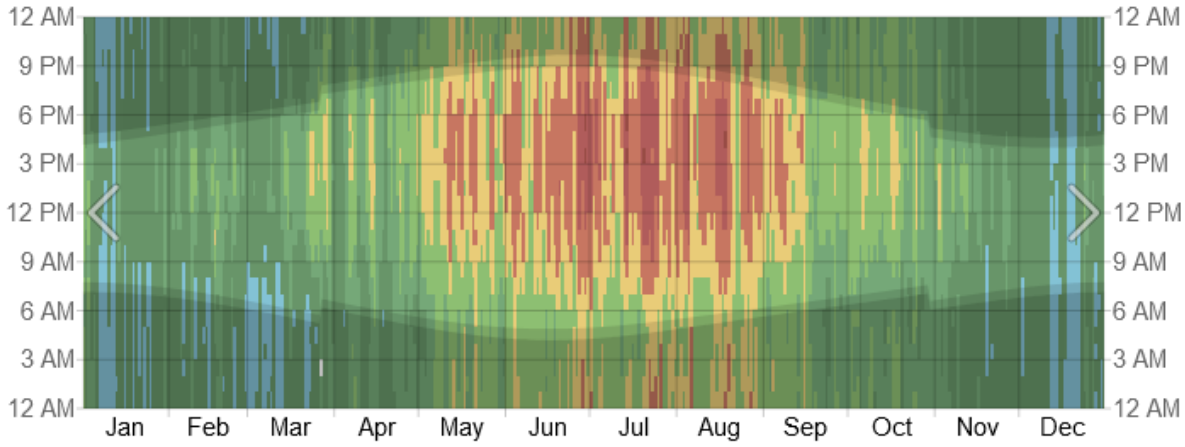

Figure 7.3.4. Hourly temperature in 2022 in Vienna (© WeatherSpark.com)
The hourly reported temperature, colour coded into bands. The shaded overlays indicate night and civil twilight.

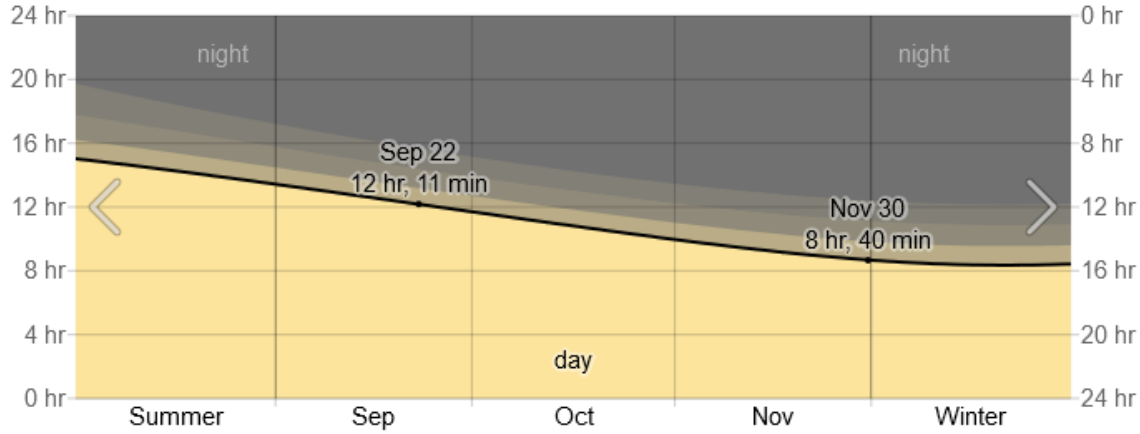

Figure 7.3.5. Hours of daylight and twilight in Fall 2021 in Vienna (© WeatherSpark.com)
The number of hours during which the sun is visible (black line). From bottom (most yellow) to top (most grey), the colour bands indicate: full daylight, twilight, and full night.

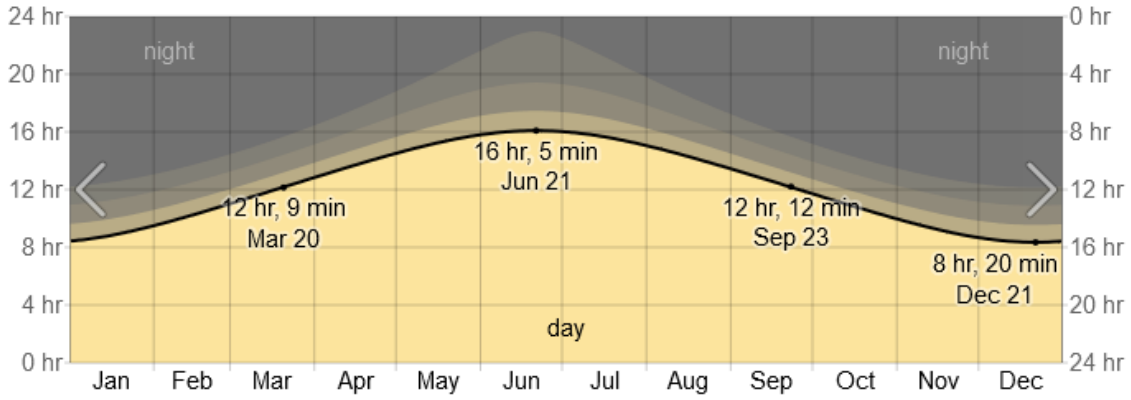

Figure 7.3.6. Hours of daylight and twilight in 2022 in Vienna (© WeatherSpark.com)
The number of hours during which the sun is visible (black line). From bottom (most yellow) to top (most grey), the colour bands indicate: full daylight, twilight, and full night.