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Research Article

Cortisol concentrations in hair are biomarkers of health status and seasonal change, but not urbanisation, in wolves

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Wolves have reclaimed much of their former range in Europe, including areas with a high human footprint, yet the physiological impacts of human-associated stressors remain poorly understood. We first examined methodological factors affecting hair cortisol concentrations (HCC) in wolves by using a 'shave/re-shave' design to assess hair growth rate and seasonal variation in four captive individuals. We also evaluated the effects of body region, collection method, and the relationship between faecal glucocorticoid metabolites (FGCMs) and HCC. Hair grew faster in summer, but HCC peaked in hair grown during winter, likely reflecting seasonal elevations in glucocorticoids (GCs) due to reproduction and varying metabolic demands. FGCMs showed comparable seasonal fluctuations and were significantly correlated with HCC. Additional samples collected by brushing the belly showed higher HCC than those from the back. To then assess the effects of urbanisation on HCC in free-ranging wolves, we analysed hair samples from 254 carcasses from Italy and quantified human influence using the human footprint index (HFI). Contrary to our prediction, the HFI did not explain variation in HCC. This result may reflect the absence of individuals exposed to extreme urbanisation, uncertainty regarding whether carcass locations represented true home ranges, and the possibility that broad landscape indices such as the HFI fail to capture more immediate anthropogenic stressors. In contrast, health status and season significantly predicted HCC: wolves with chronic injuries or infectious diseases exhibited elevated HCC, and individuals that died in winter had higher

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HCC than those that died in other seasons. We discuss these results in the context of the wolf breeding season and dispersal period, and highlight the importance of considering seasonal demands, health status, and methodological constraints when interpreting GC patterns in wolves.

Keywords : cortisol, hair growth cycle, health status, stress, urbanization, wolf

Introduction

Urbanisation increasingly transforms natural habitats into human-dominated landscapes, introducing novel challenges for wildlife, such as increased noise, light pollution, and habitat fragmentation (Berger-Tal and Saltz 2019). It is not just a change in landscape but a profound shift in the ecological dynamics that wildlife must navigate. The constant presence of humans, altered food sources, and new environmental stressors compel animals to adapt rapidly, which has widespread consequences from genes to behaviour and physiology (Atwell et al. 2012, Müller et al. 2013, Mascarenhas et al. 2023).

In canids, recent work on the effects of urbanisation has focused on red foxes *Vulpes vulpes* (DeCandia et al. 2019, Lazzaroni et al. 2024), grey foxes *Urocyon cinereoargenteus*, coyotes *Canis latrans* (LeFlore et al. 2019, Parsons et al. 2022, Robertson et al. 2023), and grey wolves (Lazzaroni et al. 2026). Living or moving closer to urban environments exposes canids to significant risks, such as lethal control measures and vehicle collisions (Stenglein et al. 2018, Margenau et al. 2023), but it may also offer opportunities. For instance, coyote survival rates were found to be higher in more densely populated areas in the Chicago metropolitan area (Zepeda et al. 2024). The authors link this unexpected finding to the reduction in hunting and trapping in urban compared to rural areas. Furthermore, anthropogenic structures may provide shelter from low temperatures during winter, an especially important factor given many coyotes in that study area suffered from sarcoptic mange, a condition which renders them unable to effectively thermoregulate (Zepeda et al. 2024). Finally, easily accessible anthropogenic food sources may represent crucial subsidies and facilitate urban colonization of canids such as foxes (Contesse et al. 2004) and coyotes (Petroelje et al. 2019).

City life thus appears to have complex effects on canid health and survival, with benefits that may offset some of the costs. The endocrine system provides a window to gauge the impact of stressors, such as those that come with living along the urbanisation gradient, on an individual's body. In particular, the hypothalamic-pituitary-adrenal (HPA) axis is a key regulatory system that governs how individuals respond to stressors. In the short term, this response is adaptive and beneficial by mobilising energy to respond to variation in metabolic demands, but when stress becomes constant, the system stays activated, and may suppress immune function, slow down healing, impair reproduction, and ultimately reduce survival (McEwen 2017). However, many wild-living species exhibit prolonged periods of naturally elevated glucocorticoids (GCs), for example during reproduction, migration, or winter acclimatisation without apparent fitness costs, wherein GCs are vital mediators of these life-history events (Crespi et al. 2013). Hence, GCs may be viewed primarily as metabolic regulators helping individuals to adapt to a wide range of different challenges and environments (Nelson et al. 2015, Romero and Beattie 2022), and elevated levels should be interpreted with this caveat in mind rather than assuming inherently detrimental effects.

Recent studies investigating the effects of urbanisation on mammal physiology have mostly measured GCs using faecal (Brunton et al. 2020, Carbillet et al. 2020, Ouellette et al. 2023) and hair samples (Potratz et al. 2019, Cordeschi et al. 2021), but only a handful of studies to date have attempted to link urban environments with physiological parameters in wild canids. Robertson et al. (2023) found that in coyotes, living in more urban environments was associated with higher stress levels as measured by cortisol extracted from hair samples (hair cortisol concentrations, HCC), despite considerable individual variation. Specifically, having poor body condition and a social status as a transient rather than resident alpha or subordinate individual aggravated this effect. In another study on endangered San Joaquin kit foxes *Vulpes macrotis*, faecal glucocorticoid metabolites (FGCMs) were found to be significantly higher among urban than rural foxes, a possible sign of chronic stress (Nelson et al. 2015). However, the authors note that the variation within urban individuals was relatively high, suggesting that other factors (such as health status or body condition) may also play a part. Actual survival and reproductive rates were higher among the urban than the rural foxes (Nelson et al. 2015), and recent evidence from meta-analyses shows that human disturbance per se does not cause a consistent increase in GC levels across wild vertebrates (Pérez-Ortega and Hendry 2023), promoting a more nuanced picture.

Following grey wolves' *Canis lupus* successful recovery (Chapron et al. 2014, Di Bernardi et al. 2025), their presence in more heavily urbanised regions of Europe has increased substantially in recent years (Zanni et al. 2023, Guimarães et al. 2025, Lazzaroni et al. 2026) with new potential challenges arising (Kuijper et al. 2024, Brogi et al. 2025a). Anthropogenic factors such as (illegal) killing and traffic accidents rank highest in the list of causes of wolf mortality in both North America (Treves et al. 2017, Morales-González et al. 2025) and Europe (Lovari et al. 2007, Musto et al. 2021, Morales-González et al. 2025). Furthermore, wolves living in more urbanised areas are more likely to test positive to one or more rodenticides (Musto et al. 2024). Although in some other canid species (e.g. red foxes, Stepkovitch et al. 2019)

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urbanisation is associated with larger body mass, this does not appear to be the case in wolves (Cerri et al. 2023). No studies, to our knowledge, have investigated the effect of urbanisation on stress hormones in wolves, although several studies have shown effects of other significant and potentially correlated stressors on wolves' physiological responses. For example, HCC were shown to be higher in a wolf population experiencing heavy rates of hunting than one not experiencing any hunting, likely reflecting increased stress levels due to social instability and pack disruptions (Bryan et al. 2015). Pereira and colleagues (2022) confirmed this effect for European wolf populations, finding that HCC were higher in wolves from regions implementing lethal management compared to wolves from protected regions. Further, they described an effect of the wolves' health: in particular, wolves affected by sarcoptic mange had the highest HCC. Seasonality has also been shown to affect HCC in wolves, with higher levels coinciding with wolves' winter breeding season (Eggermann et al. 2013) and late autumn dispersal time (Pereira et al. 2022); whereas multiple studies have shown no effect of sex and age (Sands and Creel 2004, Bryan et al. 2015, Molnar et al. 2015, Pereira et al. 2022, Roffler et al. 2022, Troisio et al. 2025).

Taken together, HCC appears to be a promising tool to investigate stress responses in free-ranging wolves, but some crucial methodological aspects remain unclear. One important consideration concerns the temporal resolution of hair samples. Hair is thought to integrate steroid hormones over longer time periods (weeks to months, depending on a species' hair growth cycle), and mainly, if not exclusively, during the anagen (active growth) phase of the hair (Greff et al. 2019). Thus, it has been argued that hair samples may provide a long-term, temporally stable signature of GC levels (Heimbürge et al. 2019, Koren et al. 2019, Gormally and Romero 2020). Hence, knowledge of a species' annual hair growth cycle, particularly when large seasonal differences in hair growth speed and moulting periods occur, is crucial for meaningful interpretation of findings. Contrastingly, there is recent experimental and meta-analytic evidence suggesting that GCs diffuse into, along, and out of hair shafts more flexibly, and in fact may be better markers for ongoing rather than past stress (Kalliokoski et al. 2019, Colding-Jørgensen et al. 2023). By accurately describing wolves' annual hair growth cycle, measuring the associated hormonal levels using a 'shave/re-shave' design, and linking them to simultaneously measured FGCM levels, we attempted to elucidate temporal interpretation of HCC in wolves.

Furthermore, HCC differed by body region in wolves (Pereira et al. 2022), with similar findings also described in other species (chimps *Pan troglodytes*, Carlitz et al. 2015 and grizzly bears *Ursus arctos horribilis*, Macbeth et al. 2010), but not in coyote pups aged five weeks (Schell et al. 2017). Bryan et al. (2013) also found no significant variation in cortisol concentrations between hair collected from the left and right shoulder in pet dogs. While the reported body region effects could be related to differences in patterns and timing of hair growth across the body (Macbeth et al. 2010), or variation in skin blood flow (Carlitz et al. 2015), they may also be linked to differences in exposure to environmental factors such as UV light, which can degrade steroid hormones (Wester et al. 2016). Last, the presence or absence of follicles (Sergiel et al. 2020) and mechanical irritation of the skin can affect cortisol concentrations (Salaberger et al. 2016) which has implications for the way hair samples are collected (i.e. cutting with scissors, clipping with an electric clipping machine, plucking, or brushing). We therefore tested for an effect of body region as well as collection method in the current study.

Thus, in the current study we had two main aims: first, to facilitate interpretation of HCC in wolf studies, we investigated the link between HCC and the annual hair growth pattern in captive wolves. To do so, we used a 'shave/re-shave' design to repeatedly sample the same wolves housed at the Core Facility Wolf Science Center Austria (CF-WSC, University of Veterinary Medicine Vienna, Austria, www.wolfscience.at/en), over the course of one year. In addition, we tested for effects of season, body region, and collection method (brushed versus clipped), and the correlation between HCC and FGCMs (Study 1).

The second aim was to assess the potential effect of urban environments, while considering also season and health status, on wolves' stress physiology. Here, we focused on the Italian wolf subpopulation *Canis lupus italicus*, which has expanded into areas with a high degree of human development, including cities (Zanni et al. 2023, Lazzaroni et al. 2026) (Study 2). We analysed hair samples collected from wolf carcasses found in Italy and delivered to the University of Bologna, the Experimental Zooprophylactic Institutes of Lombardy and Emilia-Romagna, Umbria and Marche, and the Mezzogiorno Institute for post-mortem examinations. We hypothesized that, if human-modified environments are indeed more challenging for wolves, we would find a positive association between the urbanisation gradient (as measured by the human footprint index, HFI; Mu et al. 2022), and wolf HCC. Wolves are a species in which typically young adults disperse for considerable distances, introducing uncertainty in relation to the extent that carcass location can reflect the living environment of the individual. In an effort to account for this, we included age category (class 1: < 12 months; class 2: 1–2 years; class 3: > 2 years) in interaction with the urbanisation gradient, with the assumption that younger and older individuals may be more likely to have been 'residents', and hence potential effects of the HFI on GC levels may be more evident in these categories. Further, we predicted higher HCC in individuals with poor health and chronic conditions such as sarcoptic mange (Pereira et al. 2022), and an effect of season with higher HCC in winter, coinciding with the wolves' breeding season, than in summer (Eggermann et al. 2013, Pereira et al. 2022).

Material and methods – Study 1 (captive wolves)

Study population

Seven grey wolves *Canis lupus* (four males, three females, all adults aged 5–12 years at the beginning of the study) initially

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participated in Study 1. The males were vasectomized and unable to sire offspring but hormonally intact, thus showing all physiological and behavioural changes related to the reproductive season in wolves. All females were intact. Unfortunately, one female wolf refused to participate after the first sampling session, and a second female died shortly after providing the first hair sample due to unrelated health problems. One male died following the second hair sampling session, resulting in only four individuals (three males, one female) completing the full collection year. All animals came from zoo populations (i.e. born in captivity) and were hand-raised by professional animal trainers from the age of 2 weeks. All animals were housed outside in large enclosures in conspecific dyads and small packs at the CF-WSC. Their housing conditions and pack compositions did not change during the study period, except for the two packs which each lost one member. Their life experiences, which include regular, positive contact with humans, made it possible to habituate them to the hair sampling procedure. The animals subsequently participated voluntarily during their regular grooming sessions carried out by the animal trainers.

Sample collection

Hair collection

Depending on which side the animal preferably offered during habituation to the clipping machine, hair was always collected from the same side (left or right) from a predefined location marked by the bone structure of the shoulder blade (one finger's width cranial of the scapula spine) (video in the Supporting information). Hair samples were collected from each animal five times throughout the study year (October 2021–October 2022), at 3-month intervals: samples were therefore collected at the end of October, the end of January, the end of April, the end of July, and finally the end of October. Each clipped hair sample (except the first one) thus reflected the regrown hair of the previous 3-month period since the last collection. Simultaneously, hair was collected by brushing from four different body areas (the shoulder area next to the clipped spot, the upper back between the shoulder blades, the lower back at the base of the tail/above the hip bones, and the belly). Immediately following collection, all samples were stored in paper bags at room temperature in a dry and dark place until further processing. Ten hair samples could not be processed further because they did not reach the minimum quantity for extraction (10 mg), resulting in 121 hair samples for analysis.

Faeces collection

Faecal samples were collected once per week from the same animals throughout the whole study period (resulting in > 40 samples per animal). Animals were leashed and taken on walks through the park either in the morning or afternoon, and faecal samples were collected immediately upon defecation and frozen at −20°C until extraction and analysis. In total, 230 faecal samples were collected. Three samples were excluded because of altered faecal consistency, which could affect hormonal content, and one sample from a male wolf was excluded because it was collected shortly before his death. This resulted in 226 faecal samples for analysis.

Laboratory work

Extraction protocol (hair samples)

Before proceeding with the extraction process, to determine hair regrowth, the clipped hair samples were evaluated macroscopically. Three individual guard hairs per sample were measured and their lengths averaged. Then, 150–200 mg of each sample were cut with scissors into approx. 3 mm pieces and transferred into a glass vial (volume 10 ml). To wash and degrease (Salaberger et al. 2016), 7 ml of n-hexane (Merck/Supelco: 1.04367.2500) was added and decanted after vortexing for 1 min. Samples were dried under the hood overnight. On the second day, 100 mg of each sample were weighed into a new, clean glass vial (volume 10 ml) and mixed with 5 ml of methanol (100%; Merck/Supelco: 1.6009.2500). In case of insufficient sample quantity (i.e. lower than 100 mg but at least 10 mg), the added methanol quantity was adapted accordingly (e.g. 50 mg of sample plus 2.5 ml of methanol, 25 mg of sample plus 1.25 ml of methanol). The closed vials were placed into a heated water bath at a constant temperature of 37°C for 24 h. Then, the samples were centrifuged for 15 min at 2500 g, and 2.5 ml (1.25 ml and 0.625 ml in case of lower methanol volume) of each sample were transferred into a new vial, and dried under a nitrogen air stream at 50–60°C. To redissolve, 0.5 ml of assay buffer (pH = 7.5) was added to the samples, and they were placed on a shaker for 30 min. The extracts were transferred into plastic tubes and stored at −20°C until analysis by a cortisol enzyme immunoassay (EIA).

Extraction protocol (faecal samples)

The frozen faecal samples were thawed at room temperature, and 0.5 g of each sample was transferred into a clean glass tube (volume 10 ml). Then, 5 ml of methanol (80%; Merck/Supelco: 1.6009.2500, diluted with aqua bidest.) was added to each tube, and they were placed onto the shaker for 30 min. Next, samples were placed in the centrifuge at 2500 g for 10 min (Palme et al. 2013). Last, 0.7 ml of the supernatant was transferred into plastic tubes, diluted at 1 + 9 with assay buffer (pH = 7.5), and stored at −20°C until analysis by EIA.

Hormonal quantification

First, the extracted samples were thawed and pipetted in duplicates onto the assay plates (in-house cortisol EIA with an antibody raised against cortisol-3-CMO:BSA and a biotinylated cortisol-3-CMO-DADOO-biotin label; Palme and Möstl 1997). For the hair samples, the amount was adjusted according to the quantity of original sample used during extraction. Specifically, for samples extracted with 100 mg, 10 µl of extract were pipetted onto the plate, and for 50 and 25 mg of extracted sample, the quantity was adjusted to 30 and 50 µl, respectively, to ensure that measured concentrations

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would fall within the linear range of the assay. For the faecal samples, 50 µl of the pre-diluted extract were pipetted onto the plate. The EIA has been previously validated for measuring FGCMs in wolves (Molnar et al. 2015), including a biological validation involving a standardized stressor (capture and veterinary examination) in three captive wolves (2 females, 1 male) which resulted in a marked increase in FGCM concentrations within 24–48 h. The analytical validation included demonstration of accuracy (recovery > 90%), precision and repeatability (intra- and inter-assay coefficients of variance (CV) < 10% and < 15%, respectively), and parallelism between faecal extract dilutions and the standard curve. For the present analysis, all faecal extracts were analysed at a single dilution to avoid analytical bias. For the hair, we performed additional analytical validation steps specific to this matrix. We tested for parallelism with serial dilutions of a dog and a wolf hair pool (1:1, 1:2, 1:4, 1:8, 1:16, 1:32; the latter two fell outside the linear range of the assay and were excluded from assessment of parallelism). Both hair extract pools (dog and wolf) formed curves parallel to the standard curve as confirmed by plotting optical density (OD) as a function of the $\log_{10}$ dilution factor (Supporting information). The slope (on log scale) and R^2 of the standard were 0.091 and 0.99; for the dog pool 0.086 and 0.98; and for the wolf pool 0.061 and 0.97, respectively. Precision and repeatability were confirmed by analysing all samples in duplicates and including two pooled quality control samples on all plates. The intra-assay CV was below 5% (average % CV of the sample duplicates' OD), and inter-assay CVs (derived from values of the same pools across all assay plates) were below 15 and 20%, respectively, for hair and faeces (total n = 5 plates of hair samples, n = 6 plates of faecal samples). Assay sensitivity was 0.7 ng g^{-1} faeces and 0.06 ng g^{-1} hair. Results are expressed in ng g^{-1} wet faeces or ng g^{-1} hair. When the difference between ODs of the duplicates exceeded 10%, the samples were re-analysed. This concerned 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.

Statistical analyses

All statistical analyses were conducted in R statistical software (ver. 4.2.1; www.r-project.org), fitting linear mixed effect models (LMM) with a Gaussian error distribution and implementing full-null model comparisons to avoid inflated type I error rates (Forstmeier and Schielzeth 2011). In brief, a full-null model comparison as performed here refers to the direct comparison by means of a likelihood-ratio test, of a full model including the test predictor and its interactions (if applicable), all control predictors, and the random effect structure, to a null model lacking only the test predictor (and its interactions), but retaining all control predictors and random effects. This tests whether the additional parameter(s) associated with the test predictor lead to a statistically reliable improvement in model fit.

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, hormone concentrations were log-transformed prior to model fitting when used as response variable. Post hoc pairwise comparisons of estimated marginal means were conducted using the *glht* function from the 'multcomp' package (Hothorn et al. 2008), with Tukey's method to adjust for multiple testing. All model outputs with significant results are reported in the Supporting information.

Since all wolves were adults and previous studies did not report age-related effects on HCC (Roffler et al. 2022), we did not include age as a variable. Coat colour and hair type were not considered statistically either, given the circumstance that just one wolf had a darker coat, and the hair samples comprised both guard hair and underwool. Previous work by Roffler and colleagues (2022) had shown no significant differences in wolf HCC between guard hair and underwool.

Annual hair growth rate

First, to assess hair re-growth rate of the clipped samples from the shoulder, the average hair length per sample was used as the response variable, with 'chronology' (i.e. the number of the sample representing the period since the last sampling and thus the period of hair re-growth) as the test predictor.

Correlation between faecal glucocorticoid metabolite levels and hair cortisol concentrations

Two wolves had no matching pair of hair and faecal samples for the assessment of the correlation between FGCM and HCC because they did not continue their participation in the study following the initial sample collection. The subset of individuals with matching faecal and hair samples was used to test for a correlation between FGCMs (i.e. average FGCMs of samples collected since the last clipping) and HCC (from clipped shoulder hair) using a repeated measures correlation test (package 'rmcorr', ver. 0.7.0, Bakdash and Marusich 2017), specifically designed for longitudinal data with multiple observations per individual accounting for their non-independence.

Seasonal effects on hair cortisol concentrations

To investigate annual differences in HCC in clipped samples, another model was fitted with HCC (log-transformed) as the response, and 'chronology' as the fixed-effect test predictor and excluding the very first sample (not re-grown hair). The same model was fitted but using HCC concentrations (log-transformed) from brushed hair samples of the shoulder area, collected concurrently with the clipped hair, as the response.

Effect of body region and collection method

To test the effect of body region on HCC, the dataset comprising the brushed hair samples was used with HCC as the response and body region as the test predictor. To compare hair samples collected by brushing versus clipping, only samples from the shoulder were used because clipped hair was

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only collected from the shoulder. HCC was the response and collection method the test predictor. Only the first samples were considered here to account for differences in hair length between subsequent brushed and clipped samples.

Effect of Julian date on faecal glucocorticoid metabolites

Last, to test for possible non-linear seasonal variations in FGCM concentrations, a generalized additive mixed model (GAMM) was fitted using log-transformed FGCM concentrations as the response and the Julian date (days 1–365) modelled with a cubic cyclic spline as the test predictor. Separate smooths by sex were included to account for possible sex-specific seasonal effects, possibly due to reproductive dynamics. Subject ID was included as a random intercept. Seasonal effects were assessed by comparing the full model to a null model lacking Julian date but otherwise identical.

Results – Study 1 (captive wolves)

Annual hair growth rate

The average length and SD of the clipped hair samples from the first collection was 66 mm (SD 11.8, range = 33–79 mm; Table 1). These initial values were not included in the following hair growth rate assessment, as they do not reflect re-growth, but represent the initial hair length before the study started. They are reported to facilitate comparison with the subsequent samples. There was a significant difference in growth rate across the samples and thus across the year ($\chi 2 = 9.24$, df = 3, p = 0.03; Supporting information). During winter, the mean hair length was lowest (mean 31, SD 12.3, range = 25–39 mm) and translated into a daily growth rate of 0.37 mm. Except for one male wolf (Amarok), the sample collected during spring revealed similarly low re-growth as during winter with a mean length (SD) of 34 (23.9) mm and a growth rate of 0.41 mm per day. The sample collected from Amarok represents a biologically valid outlier, not measurement error, and was therefore retained in the analysis. Without his sample, the mean hair length in spring would be 19 mm, the lowest overall. In summer, mean hair length was significantly higher, 57 mm (SD 11.9, range = 23–76 mm), than during winter and spring, resulting in a daily growth rate of 0.68 mm, and re-grown hair had almost reached the length observed at the beginning of the study.

Table 1. Mean and standard deviation (SD) of the hair length (mm) and mean hair re-growth rate (mm day^{-1}) by season and subject. NA = not available.

Subject	Autumn	Winter	Spring	Summer	Autumn
Tekoa	75	26	15	60	44
Taima	33	25	16	23	44
Gero	68	NA	27	76	68
Chitto	57	39	NA	NA	NA
Amarok	76	36	79	69	63
Tala	79	NA	NA	NA	NA
Yukon	77	NA	NA	NA	NA
Mean length	66	31	34	57	54
SD	11.8	12.3	23.9	11.9	9.0
Mean growth day^{-1}	NA	0.37	0.41	0.68	0.64

Correlation between faecal glucocorticoid metabolite levels and hair cortisol concentrations

There was a significant positive correlation between FGCM levels and HCC (R = 0.72, df = 6, p = 0.04, 95 % CI [−0.13, 0.98]; Fig. 1).

Seasonal effect on hair cortisol concentrations

HCC in the re-grown hair varied significantly across the annual sampling period ($\chi^2 = 11.42$, df = 3, p = 0.01; Supporting information). The highest HCC (13.47 ng g^{-1}) appeared in the second sample ('1st re-growth'), which represented re-growth during winter (Nov, Dec, Jan). There was a marginally significant difference (estimate = −0.88, SE = 0.35, p = 0.05; Supporting information) between the second ('1st re-growth') and third ('2nd re-growth') sample representing spring months (Feb, Mar, Apr), although it should be noted that this period is represented by only one sample which provided sufficient hair for hormone quantification. The second ('1st re-growth') and fourth ('3rd re-growth') samples representing summer months (May, June, July) differed significantly: specifically, significantly lower HCC were measured in the samples representing summer in comparison to winter samples (estimate = −0.96, SE = 0.22, p < 0.001; Supporting information). HCC in the fifth sample ('4th re-growth'), representing autumn (Aug, Sept, Oct), was

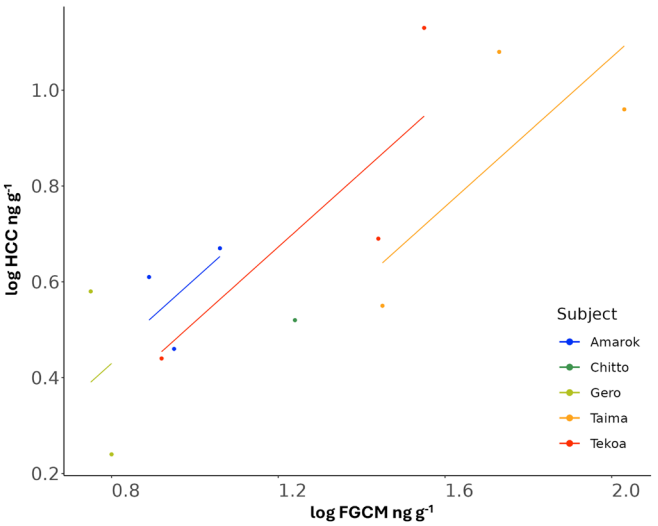


Figure 1. Correlation between hair cortisol concentrations (HCC) and average faecal glucocorticoid metabolite (FGCM) concentrations (log-transformed, in ng g^{-1}) of n = 5 captive wolves, represented by different colours. Each data point represents a paired measurement of HCC and FGCM concentrations, with individuals contributing 1–3 paired observations each. The number of faecal samples resulting in the average FGCM concentration ranged between 10 and 13 samples per individual, collected between clippings to match the hair re-growth period. The lines represent the individual-specific regression lines, illustrating how each wolf's HCC and FGCM concentrations co-vary over time.

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significantly higher compared to the sample re-grown during summer ('3rd re-growth') (estimate = 0.74, SE = 0.20, $p < 0.01$; Supporting information). The lowest HCC (1.75 ng g^{-1}) was found in summer samples ('3rd re-growth') (Fig. 2). For HCC measured in brushed hair no seasonal pattern was found ($\chi 2 = 6.4$, df = 3, $p = 0.6$).

Effect of body region and collection method

Body region had a significant effect on HCC ($\chi 2 = 21.84$, df = 3, $p < 0.001$). Specifically, samples from the shoulder had significantly higher HCC than samples from the lower and upper back (shoulder–lower back: estimate = 0.48, SE = 0.13, $p < 0.01$; shoulder–upper back: estimate = 0.51, SE = 0.13, $p < 0.001$; Supporting information). Samples from the upper and lower back had significantly lower HCC than belly samples (upper back–belly: estimate = −0.40, SE = 0.12, p = 0.01, lower back–belly: estimate = −0.37, SE = 0.12, p = 0.01; Supporting information, Fig. 3). No significant difference was found between shoulder and belly samples (estimate = 0.11, SE = 0.13, p = 0.85; Supporting information). Last, there was no effect of the collection method (clipping versus brushing) on HCC as measured in the samples collected at the start of the sampling period ($\chi 2 = 0.28$, df = 1, p = 0.59).

Effect of Julian date on faecal glucocorticoid metabolite levels

FGCM levels significantly varied along the year (Δdeviance = 33.55, df = 8.14, $p < 0.01$; Fig. 4, Supporting information). Samples collected in February–April showed higher FGCM concentrations than those collected in July–August, with maximum (16.12 ng g^{-1}) and minimum (5.63 ng g^{-1}) FGCM being predicted by the model for 7 March and 22 July, respectively.

Discussion – Study 1 (captive wolves)

By studying captive, human-socialized wolves in a 'shave–re-shave' design, we investigated the wolf hair growth cycle throughout one year to better understand the temporal structure of steroid hormone incorporation into wolf hair and improve our understanding of the link between HCC and wolf sociobiology. We found that wolf hair grows fastest during summer and autumn but slows down significantly during winter. This aligns closely with a study by McLaren and colleagues (2015), who report guard hair growth rates of a shaved patch located between the shoulder blades of three captive wolves of 1.0 ± 0.07 and 0.63 ± 0.23 mm/day for July–September and September–November, respectively. These results are similar to our findings of growth rates between 0.68 mm day^{-1} for summer months (May–July) and 0.64 mm day^{-1} for late summer/autumn (August–October); however, differences may be attributed to the variation in sampling windows (months, length) and climatic differences between study sites. Furthermore, published data on a closely related canid species, the domestic dog *Canis familiaris* (Butler and Wright 1981, Gunaratnam and Wilkinson 1983) also indicate the same seasonal patterns, but the seasonal effect was not evident in dogs housed indoors for 90% of their time (Diaz et al. 2004). In our sample, the wolves were housed near Vienna, Austria, and stayed outdoors the whole year around. Cold weather with low temperatures and considerably reduced hours of daylight set in around the end of October and last until the beginning of April in this area.

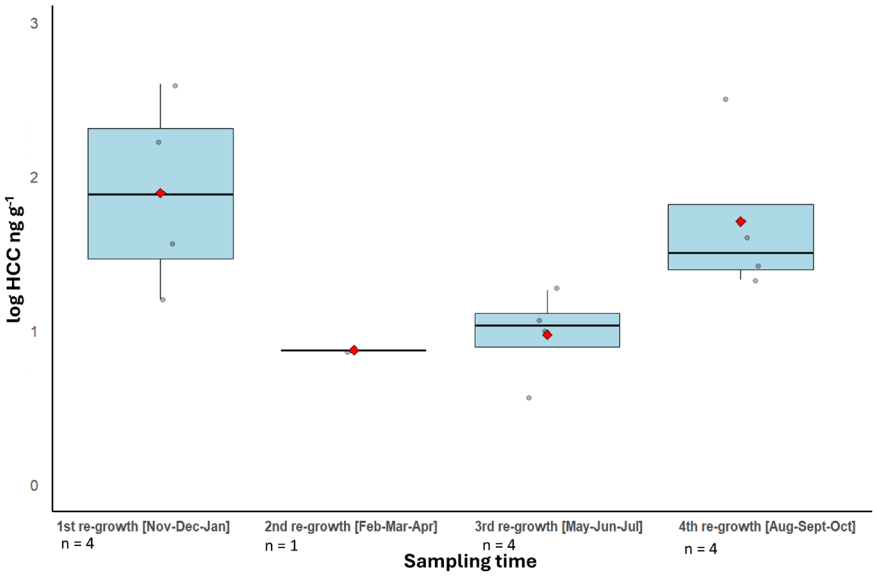
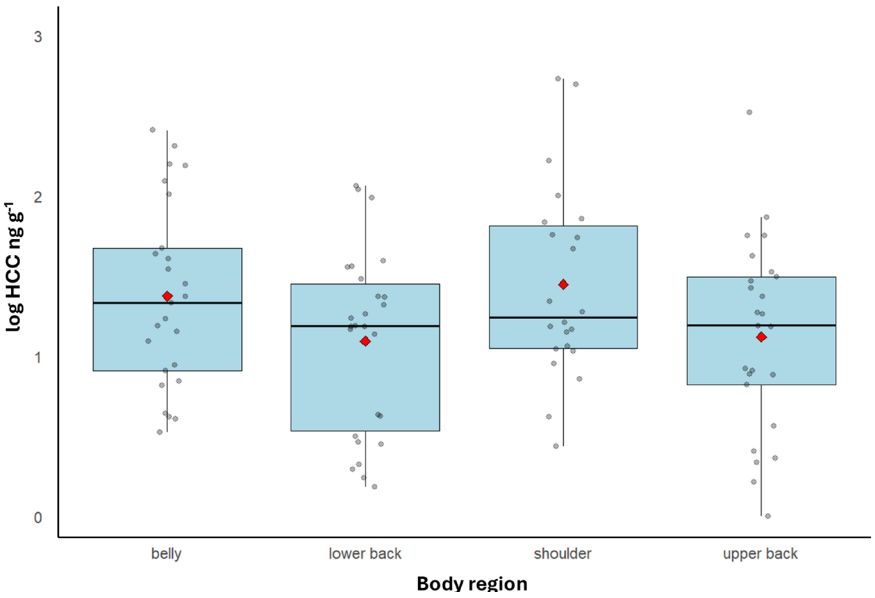


Figure 2. Hair cortisol concentrations (HCC) (log-transformed, in ng g^{-1}) by sampling time (season; samples obtained by clipping hair from shoulder of captive wolves; the number of samples is indicated below). Light blue boxes represent the interquartile range (IQR; 25–75th percentile) with the horizontal line indicating the median. Red diamonds indicate the mean. Raw data points are overlaid as individual jittered dots.

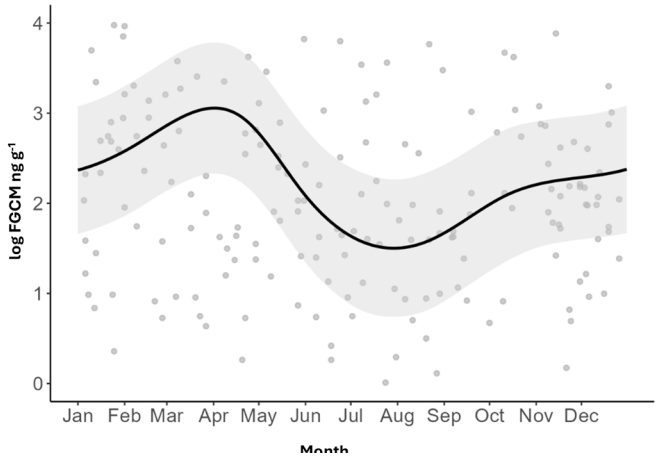
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Q2 Figure 3. Hair cortisol concentrations (HCC) (log-transformed, in ng g^{-1}) by body region (samples obtained by brushing from captive wolves over the whole sampling period). Light blue boxes represent the interquartile range (IQR; 25–75th percentile) with the horizontal line indicating the median. Red diamonds indicate the mean. Raw data points are overlaid as individual jittered dots.

Specifically, hours of daylight are almost halved by the end of November compared to summer months and start to increase only in March. Thus, our data confirm that photoperiod and ambient temperature affect hair growth rate in wolves, as in domestic dogs (Butler and Wright 1981, Gunaratnam and Wilkinson 1983) and other species (e.g. bears, Stern et al. 2025).

HCC and FGCM concentrations were positively correlated within subjects, demonstrating the validity of HCC as a proxy of systemic GC levels. There is local production of GCs in the skin, specifically the hair follicles (Keckeis et al. 2012, Salaberger et al. 2016, Sergiel et al. 2020), but the present data support the interpretation that HCC in wolves primarily incorporate circulating cortisol rather than being dominated by local follicular production. While previous papers provide partial validation data for dogs (hair and faeces, Accorsi et al. 2008; hair and urine, van der Laan et al. 2022; hair and saliva, Bennett and Hayssen 2010; hair and plasma, Ouschan et al. 2013), this is the first study to show a correlation between HCC and FGCM in wolves, thereby contributing to the validation of hair as a matrix for assessing HPA axis activity in wild canids. Further, linking HCC to FGCM patterns allows us to better understand the temporal resolution of hair samples as a means of measuring steroid hormone levels, which we discuss further below.



Q3 Figure 4. Faecal glucocorticoid metabolite (FGCM) concentrations (log-transformed, in ng g^{-1}) of captive wolves by month. Faecal samples were collected on a weekly basis. Solid black line indicates the model, with 95% confidence interval (shaded light grey area), and raw data points.

Next, we tested for an effect of body region on HCC. We found that samples from the dorsal upper and lower back regions had significantly lower HCC than samples from the shoulder and belly. This contrasts with Pereira et al. (2022), who found highest levels in the lumbar region and lowest in the ventral thorax. However, in their study, they sampled wolves from different subpopulations (Scandinavian, Iberian, Alpine, and Dinaric-Balkan) and analysed the effect of body region on HCC collectively. This could have masked an effect of UV exposure as it has been hypothesized that UV light degrades cortisol in hair (Grass et al. 2016, Wester et al. 2016), if hours of sunlight differ significantly between these regions. As we found higher HCC in winter than summer, a contributing effect of UV light may be conceivable, but given we found similar seasonal patterns also in FGCM, it cannot be the sole explanation. In addition to UV light, other possible explanations could account for HCC variation across body sites: for example, hair from the belly may be more likely to be contaminated with urine or saliva and thus could present erroneously increased cortisol concentrations. In our

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sample, we do not think this to be the case, given we carefully inspected and washed the samples prior to extraction. Second, different body regions may vary in hair pigmentation and melanin content, which may affect cortisol binding. This effect has previously been described in dogs (Bennett and Hayssen 2010). Third, body regions may also vary regarding the ratio of guard hairs to undercoat, which again may translate to differences in hormone content given their distinct growth dynamics. While the present data do not allow us to distinguish between those mechanisms, future studies should aim to isolate their relative contributions to the body region effect of HCC.

We did not find an effect of collection method (brushing versus clipping) on HCC in samples collected from the shoulder (only the first samples were used). However, given that the presence of hair follicles can affect hormone content in a hair sample (Sergiel et al. 2020), we strongly recommend keeping both collection method and body region consistent in studies using HCC. In addition, HCC measured in hair collected by brushing did not reflect seasonal patterns as found in clipped hair and faeces (discussed below). Brushed hair is more likely to have been in the non-growing phase at the time of collection, and thus represents a different period of endocrine activity, whereas re-grown hair reflects hormone incorporation during growth.

Finally, we tested whether HCC from clipped hair and FGCMs varied across the year and found peak values during winter and spring in both substrates, while summer yielded the lowest values. Further, hair samples regrown during winter displayed higher variability than samples from spring and summer (Fig. 2), which may suggest that winter imposes more variable metabolic challenges (e.g. thermoregulation, breeding season) which could amplify individual differences in HPA axis activity. Our results are consistent with an effect of the wolf breeding season (which starts in winter) on metabolic stress levels, and align with previously published findings (Eggermann et al. 2013, Pereira et al. 2022). Importantly, as our study population was kept in captivity and fed in the same way for the whole study year, higher HCC and FGCM levels during winter can be attributed to the wolf breeding season rather than changes in food availability and body condition. The reproductive period can be considered particularly demanding for the wolves in our study, as they see, smell, and hear all other animals from their neighbouring packs. In addition, they are not neutered and exhibit all physiological and behavioural changes related to the reproductive period, such as mating, denning, and mate guarding/mate provisioning (Dale et al. 2017) as soon as females become pseudo-pregnant. However, a similar seasonal effect on cortisol levels was also found in other species without a clear-cut annual breeding season, including domestic dogs and humans (Persson et al. 2008, Roth et al. 2016), with consistently higher cortisol concentrations during winter and spring than summer, which suggests that other factors such as daylight, activity rates, the need for thermoregulation, and other biological processes, also play a role.

In conclusion, results from Study 1 show that while hair growth rate is lowest in winter, HCC and FGCM peak during the winter months in wolves.

Material and methods – Study 2 (free-ranging wolf carcasses)

Study population

We obtained hair samples from wolf carcasses examined between 2021 and 2024 at the University of Bologna, the Experimental Zooprophylactic Institutes of Lombardy and Emilia-Romagna, Umbria and Marche, and the Mezzogiorno Institute. The sampled carcasses were retrieved from diverse regions across Italy, including Emilia-Romagna, Tuscany, Marche, Calabria, Lombardy, and Umbria, and encompassed different age groups, ranging from pups to older individuals. Upon arrival of each carcass to the institute, a form containing the following information was filled: subject's identification data with the attribution of a unique ID code, the discovery location (reported as GPS coordinates), circumstances of recovery, the sex, the weight (in kg), body condition, biometrics, signs of illness, and phenotypic pattern. The age of the animal was estimated based on dental development, body size, and weight (Brasington et al. 2023). Here, all individuals were aged using three categories: class 1: < 12 months; class 2: 1–2 years; class 3: > 2 years.

Hair sample collection

Samples were cut from the shoulder area of the carcass using scissors cutting as close as possible to the skin. When visible contamination was present, samples were gently rinsed with distilled water, then dried on absorbent paper and air-dried completely at room temperature before storage. Samples with extensive contamination that could not be removed by rinsing (e.g. heavily soiled or decomposed hair) were excluded from analysis. Once dry, samples were wrapped in aluminium foil and paper bags until transfer to the University of Veterinary Medicine Vienna, Laboratory for Experimental Endocrinology, for extraction and hormonal analysis.

Assessment of health status

To operationalize health status for statistical analyses, the veterinarians who performed the post-mortem examinations categorized each carcass according to the presence or absence of injuries and acute or chronic pathologies. The body condition of each individual was classified into three categories: 'good', 'intermediate', and 'poor'. Individuals assigned to the 'good' body condition category exhibited an adequate or optimal physical state relative to their age and the season of death, with no evidence of weight loss or significant reduction in muscle mass. The 'intermediate' body condition category included animals that, while not displaying severe physical impairment, fell below the expected body condition range for their age and the time of year. Finally, individuals classified as having a 'poor' body condition showed marked weight loss, marked muscular and/or visceral wasting, and an overall

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physical deterioration consistent with a prolonged period of discomfort prior to death. The health status of each individual was classified as either 'good' or 'poor' based on the presence of current or past pathological conditions. Individuals assigned to the 'poor' health status category exhibited chronic, debilitating, or disabling diseases, often associated with marked organic wasting and a prolonged period of stress or discomfort before death. A relevant example is represented by wolves affected by sarcoptic mange, which frequently displayed severe physical impairment also attributable to starvation. In the examined sample, individuals affected by the following conditions were also assigned in the 'poor' health category: 1) chronic or debilitating viral or parasitic infections, 2) previous bone fractures, 3) inguinal hernia, 4) megacolon, and 5) peritonitis. Animals that showed no chronic, debilitating, or ongoing pathological conditions were instead assigned to the 'good' health status category.

Human footprint index as a proxy for urbanisation

To quantify urbanisation, each sampling location (i.e. carcass site) was assigned an urbanisation score based on the human footprint index (HFI). We used the 2020 global HFI raster (1-km resolution) from Mu et al. (2022), which integrates multiple indicators of human presence (population density, built and cultivated land, transportation networks, and nighttime lights) into a continuous score from 0 (lowest) to 50 (highest). Urbanisation was characterized at a spatial scale relevant to wolves by calculating a distance-weighted mean HFI within a circular buffer (radius = 5.129 km; area = 82.65 km^2) centred on each sampling site, corresponding to the average home-range size reported for wolves in central Italy (Mattioli et al. 2018). We applied a single biologically grounded buffer size to all individuals to ensure a consistent characterization of the environmental context, as currently available data do not support robust sex-, age-, or season-specific home-range estimates for Italian wolves at the scale of our dataset. To avoid over-representation of peripheral zones, each pixel within the buffer was weighted linearly according to its distance from the site. The resulting HFI values assigned to each carcass ranged from 11 to 41 (see Fig. 5 for the distribution of carcasses along the HFI gradient).

Laboratory work

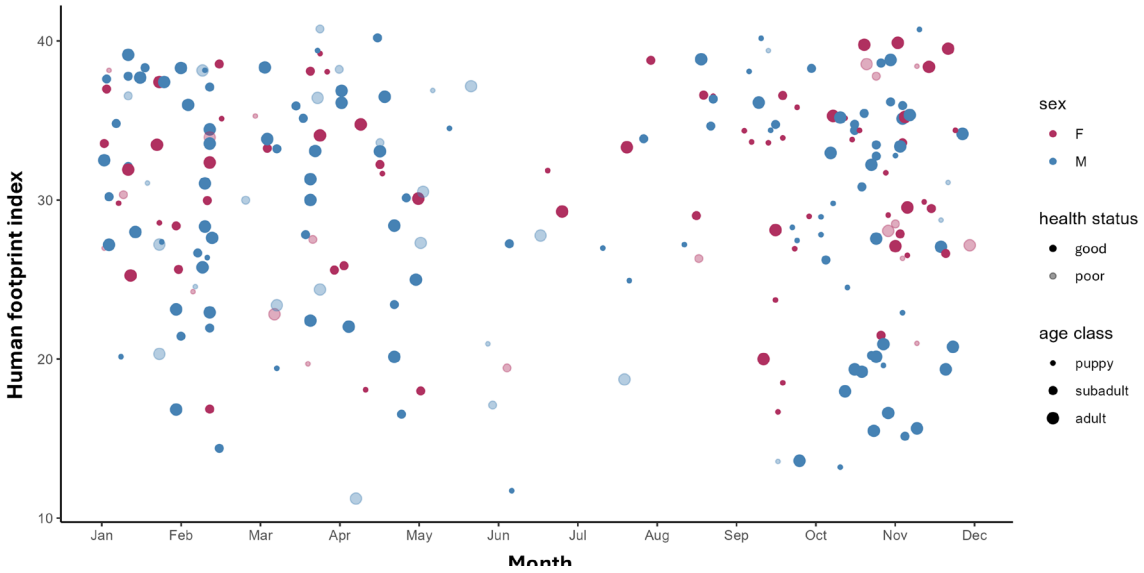
The hair extraction and assay protocol was identical to Study 1. Intra-assay CVs were below 5% and inter-assay CVs were below 10% (total n = 11 plates).

Statistical analysis

Of the original 254 wolves, three samples had to be excluded due to missing data. In addition, all individuals with atypical colouration or coat patterns were excluded. Atypical coat patterns represent one potential sign of dog–wolf hybridization which, considering differences in unstimulated urinary GC metabolite levels between wolves and dogs (Wirobski et al. 2021), may affect results. Although coat colouration may not be used to unequivocally determine hybridization, we chose to err on the side of caution and exclude these samples from further analyses. This resulted in a total of 232 wolves (143 males and 89 females) used for statistics.

Effect of urbanisation, health, and season (Julian date) on hair cortisol concentrations

To investigate the effects of urbanisation (as captured by the HFI), health status and seasonality (annual variation captured by the Julian date) on wolves' HCC, a linear mixed effects model (LMM) with Gaussian error structure was fitted (R statistical software) using the function lmer of the R package 'lme4' (ver. 2.0-1, Bates et al. 2015). The response variable



Q4 Figure 5. Distribution of free-ranging wolf carcasses along the human footprint index of their recovery location and estimated death date (month). Symbol colours, shape, and size indicate sex (F = female, M = male), health status ('good', 'poor'), and age class (puppy, subadult, adult), respectively.

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(HCC) was log transformed to comply with model assumptions of homogenous and normally distributed residuals.

We included the HFI, the age class, and their interaction to account for possible age-dependent differences, whereby we expected that pups and adults (age classes 1 and 3) would have been more likely residents, as opposed to dispersers and transient individuals, in the area their carcass was found in than subadults (age class 2). Health status was included as a factor with two levels ('good' versus 'poor'). To account for seasonality and the transition between December and January, the protocol by Stolwijk et al. (1999) was followed: the Julian date of the estimated death (ranging from 1 to 365) was first transformed into radians. Then, the sine and cosine of this resulting variable were included in the model as additive terms. We also included the standardized body mass, sex, and the latitude as control predictors. Standardized body mass was calculated as body mass divided by total body length (measured from the nose to the base of the tail), to account for differences in skeletal size among individuals, while latitude allowed us to account for possible regional effects on the average population stress level, leveraging the north–south extension of Italy. Assay plate ID was included as random factor.

To keep type I error rate at the nominal level of 0.05, we included random slopes (Schielzeth and Forstmeier 2009, Barr et al. 2013) of the average HFI, of the age class, of their interaction, of the health status, of both sine and cosine of the Julian date, of the standardized body mass, of the sex, and of the latitude within assay plate ID into the model. Prior to fitting the model, we assessed collinearity among predictors using pairwise correlations and variance inflation factors (VIFs), but no concerning levels of collinearity or multicollinearity were detected (all pairwise $|r| < 0.6$; all VIFs < 3). We z-transformed the numerical predictors (HFI, body mass, and latitude) to a mean of zero and a standard deviation of one to get comparable estimates and easier interpretable model results (Schielzeth 2010). We visually inspected model residual diagnostics, which were overall satisfactory, showing no marked heteroscedasticity, only moderate deviations from normality, and no clear evidence of strongly influential observations or pronounced outliers. As an overall test of the effect of the three test predictors (HFI, alone or in interaction with age class, health status, and seasonality) and to avoid 'cryptic multiple testing' we compared the fit of the full model as described above with that of a null model lacking the HFI, health status, and seasonality but being otherwise identical, by means of a likelihood ratio test (*anova()* R function) (Forstmeier and Schielzeth 2011). When this overall comparison was significant, we additionally tested the overall HFI effect (alone and in interaction with the age class) by comparing the full model with a reduced model lacking both terms, while keeping all other terms unchanged. We applied the same approach to test the overall effect of seasonality, by comparing the full model with a reduced model lacking both sine and cosine of the Julian date. All model outputs with significant results are reported in the Supporting information.

Results – Study 2 (free-ranging wolf carcasses)

Urbanisation effect

The full-null model comparison was significant (full-null likelihood ratio test: $\chi2 = 15.61$, df = 6, $p = 0.02$), revealing that the set of test predictors explained a non-negligible portion of wolves' HCC variation. However, neither HFI alone nor its interaction with age class had a significant effect on wolves' HCC (full-reduced likelihood ratio test: $\chi2 = 1.94$, df = 3, $p = 0.56$; Supporting information).

Effect of health status

Wolves in the 'poor' health category had significantly higher HCC than wolves in the 'good' health category (beta coefficient = −0.61, df = 8.73, $p = 0.04$; Fig. 6, Supporting information).

Seasonality (Julian date)

Wolf carcasses retrieved in autumn and winter had significantly higher HCC than those retrieved in spring and summer (full-reduced likelihood ratio test: $\chi2 = 8.88$, df = 2, $p = 0.01$; Fig. 7, Supporting information).

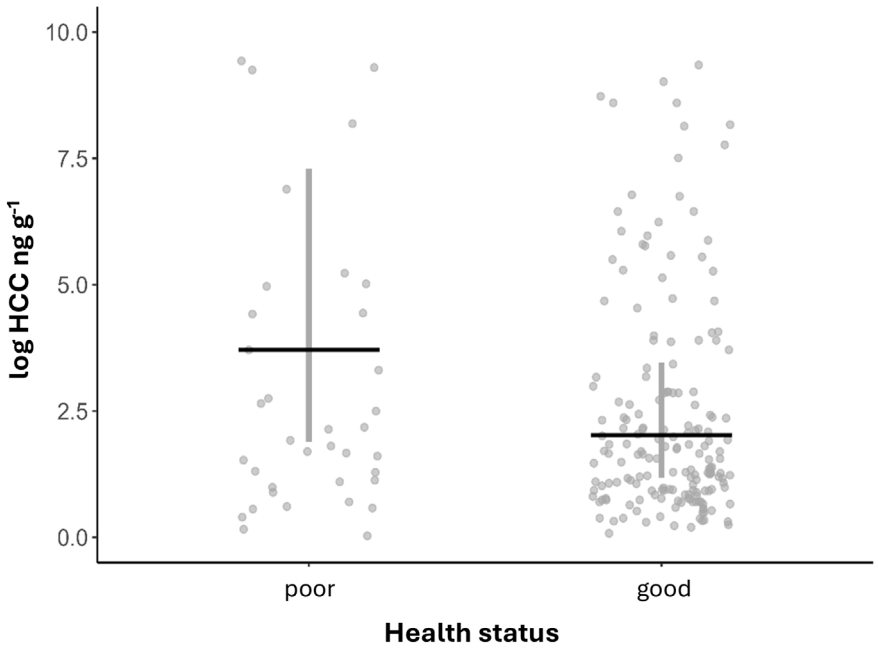
Discussion – Study 2 (free-ranging wolf carcasses)

In our second study, we built on the expertise gained from sampling the captive wolves in Study 1 and took advantage of the same analytical pipeline. Hence, we measured HCC in samples collected from free-ranging wolf carcasses (all cut with scissors from the same body region) and tested for the effects of urbanisation (as measured by the HFI), health status, and day of the year. Both health and day of the year (i.e. seasonality) significantly affected wolves' HCC, although urbanisation did not.

In line with our predictions, we found that wolves with poor health (chronic injuries or mange) had significantly higher HCC than wolves with good health that died from acute reasons such as vehicle collision or gunshot trauma. Mange infection was also one of the determinants of the highest HCC reported in a previous wolf (Pereira et al. 2022) and coyote study (Robertson et al. 2023). Overall, HCC can therefore be informative of the health status of a population and may be useful when other forms of sampling are not feasible. Yet, it must be noted that a number of other intrinsic and extrinsic factors influence HCC, and interpretation must be done extremely carefully. Further, mange appears to have a strong effect on HCC, which may be due to the chronic nature of the infection and its consequences on the animals' energy metabolism, and/or by damaging skin and follicles directly, it might cause alterations in the way cortisol is incorporated into hair.

To collect hair samples from free-ranging wolves non-invasively and without the need to trap or capture the animals, hair traps have been successfully used (Ausband et al.

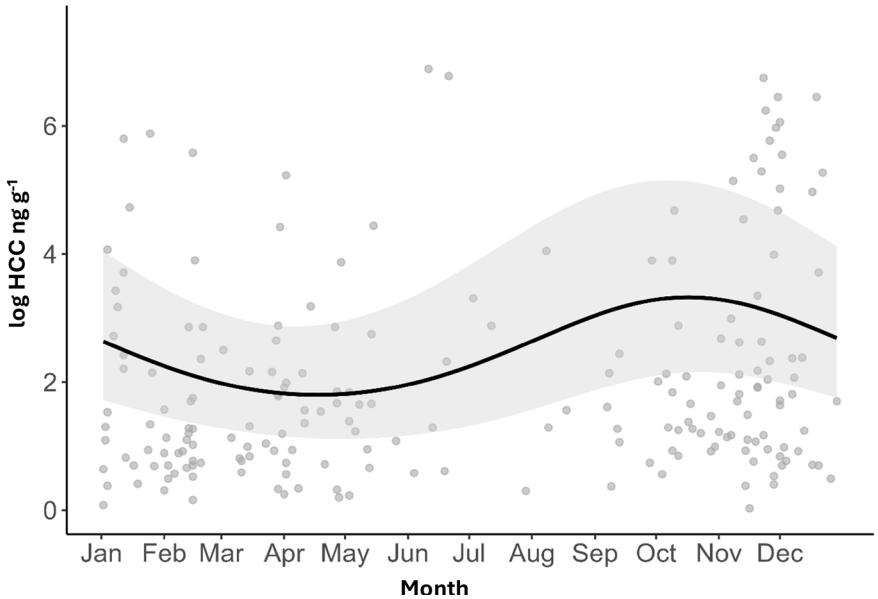
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Q5 Figure 6. HCC (log-transformed, in ng g^{-1}) of free-ranging wolf carcasses by health status ('good', n = 186; 'poor', n = 46). Indicated are the model (solid black line) and 95% confidence interval with overlaid jittered raw data points.

2011). However, in light of the minimum amount of hair needed for hormonal assays (in the present study: 10 mg), hair traps should be thoroughly tested for suitability and may need improvements, if collected amounts turn out to be insufficient.

As regards the annual variation in HCC (i.e. indicated by the Julian date of estimated death), we report that wolves dying in autumn and winter had higher HCC than those dying in spring. The highest HCC in our sample were evident in carcasses retrieved in December (Fig. 7), as in Pereira et al. (2022), and thus coinciding with the early phase of the wolf breeding period, a time of potentially higher social instability and competition. Although mating generally occurs only in January to March in European wolves (Schmidt et al. 2008), data collected at the CF-WSC show a sharp increase in male wolves' urinary testosterone levels as soon as



Q6 Figure 7. Hair cortisol concentrations (HCC) (log-transformed, in ng g^{-1}) of free-ranging wolf carcasses by month (calendar day indicating the estimated time of death). Solid black line indicates the model with the 95% confidence interval as shaded light grey area and raw data points.

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November, accompanied by the start of behavioural changes related to the breeding period (unpubl.). Furthermore, late winter is also the period of increased dispersal in wolves (Morales-González et al. 2022) and increased human-reported wolf sightings (Brogi et al. 2025b), and has been linked to increased human-induced mortality (Musto et al. 2021). In addition, seasonal metabolic adjustments driven by photoperiod, thermoregulatory demands, and food availability likely contribute to the observed patterns. Last, the results align with Study 1 (wolves housed at the CF-WSC), wherein hair samples re-grown during winter had significantly higher HCC than samples re-grown during summer.

The main aim of this investigation, however, was to uncover a hypothesized association between GCs and living in more urbanised settings in free-ranging wolves. For this reason we predicted higher HCC in wolf carcasses from areas with a higher HFI, particularly in younger (< 1 year) and older (> 2 years) individuals more likely to be resident in the area surrounding where the carcass was found. However, in contrast to findings of urban coyotes (Robertson et al. 2023), we did not find an effect of HFI on HCC in our study sample. This aligns with meta-analytic findings from other species that showed no differences in GC levels between urban and rural populations of vertebrates (Iglesias-Carrasco et al. 2020) and no consistent effect of human disturbance on GC hormones (Pérez-Ortega and Hendry 2023). There may be several potential explanations for the lack of an effect in the present study. First, it is undeniable that, in comparison to the coyotes tested in the study by Robertson et al. (2023), which were sampled in the Chicago Metropolitan Area (a heavily developed landscape with more than 5 million residents in just one of the counties included in this area), the wolves sampled in our study would not have experienced such extremely high levels of urbanisation. While the range of HFI in our study still represents a variety of habitats with differing human influence, we did not have carcasses assigned to the extreme ends of the spectrum (HFI < 11 or > 41). Second, there is an important methodological limitation to the collection of samples from carcasses. Namely, we could not determine with certainty whether the location of the carcass (which was used to calculate the HFI) really corresponded to the home territory of the animal when it was still alive. Although pups and adult females that recently gave birth (i.e. resident females) would have most likely also lived near the location in which they died, juveniles/subadults and adult, non-reproductive females and males may have been in dispersal at the time of their death. Yet, we did not find an effect of the HFI on HCC in any of the age classes. Third, we should consider that the HFI might not have adequately captured the factors that really impact wolves' stress physiology. For example, snowmobile activity (Creel et al. 2002) and being hunted were shown to significantly impact wolves' GC response (Bryan et al. 2015), presumably because these specific stressors target wolves' livelihood much more directly and intensely. Importantly, however, two recent studies experimentally confirmed that wolves show strong fear and avoidance responses to merely hearing human voices on playbacks (Kasper et al. 2025, Lazzaroni et al. 2026), which demonstrates that they do remain highly reactive to mere human presence, even in protected areas where they are not hunted. Still, wolves may cope better than we think with living near humans, and they appear to be more resilient than previously assumed. Thus, we may be overestimating the negative impact of urbanisation on certain species that have already shown that they are able to survive and even reproduce in urbanised areas ('urban visitors' and 'urban dwellers'), including several canid species such as red foxes, coyotes, golden jackals *Canis aureus*, and wolves (Santini et al. 2019, Lazzaroni et al. 2026). Indeed, human activity and urban development per se may not have a large impact on animals' lives if they are able to adapt behaviourally and exploit the newly created niches (Zenth et al. 2025). Additionally, wolves living in areas with a high HFI may select habitats in ways that still buffer them from intense exposure to anthropogenic stressors at a finer spatial scale (spatial avoidance) or allocate activity to the time of day when fewer or no humans are around (temporal avoidance) (Lesmerises et al. 2012, Gaynor et al. 2018, Vorel et al. 2024), as has also been shown in other large carnivores (lions: Mills et al. 2023, and pumas: Barceló et al. 2025).

General discussion

The present study sought to deepen our understanding of the significance of physiological measurements by focusing on HCC in wolves as a key parameter. For physiological metrics to contribute meaningfully to ecological, behavioural, and cognitive research, they must serve as reliable indicators of systemic and metabolic processes, with their temporal dynamics thoroughly characterized. Such investigations often necessitate repeated sampling of the same individuals under controlled conditions, highlighting the critical need to test and validate methodologies in captive settings before applying them to free-ranging populations. Importantly, the integration of findings from captive and wild environments hinges on the consistency of the methods employed. This study was designed to address some of these challenges, bridging the gap between controlled experimentation and field application.

From our captive population (Study 1), we found that wolf hair growth is fastest in summer and autumn (May–October) and slows down during winter, which is supported by findings from the closely related domestic dog (Butler and Wright 1981, Gunaratnam and Wilkinson 1983), and that HCC were significantly higher in hair grown during winter than summer. What does that imply in relation to the interpretation of findings from free-ranging wolves (Study 2)? Wolves that died and whose carcasses were recovered and sampled during winter had the highest HCC. Those individuals had grown their coat over the previous summer and autumn months. Hence, if only growing hair incorporates cortisol (Meyer and Novak 2012, Heimbürge et al. 2019), we would assume that HCC accumulated and dated back to events during the summer and autumn months. However,

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if cortisol, in fact, diffuses in and out of the hair shaft in a more flexible manner in response to acute and on-going stress (Kalliokoski et al. 2019, Colding-Jørgensen et al. 2023), we may reach a different conclusion, and we could relate those higher HCC to increased metabolic demands during the winter months. This outcome is consistent with our findings from the captive animals whose re-grown hair during winter showed the highest HCC, which – in combination with the data from the faeces – strongly suggests a link with the start of the winter and reproductive season, as well as dispersal period in wolves. In the free-ranging population, we found higher HCC in animals whose health was chronically impacted by injuries and infectious diseases at the time of their deaths. Taken together, our results highlight how combined findings from captive and wild settings provide complementary perspectives and strengthen inferences about natural behaviour and physiology.

For the future, to advance our understanding of how urbanisation shapes a species' behaviour, physiology and, ultimately, fitness, we will need to integrate hormonal data with broader measures of biological condition, from dietary breadth and microbiome dynamics (Sugden et al. 2020) to parasite load (Dini et al. 2025). Additionally, it would be most informative to employ a longitudinal approach whereby the same individuals are sampled multiple times throughout their lives as they move along the urbanisation gradient. This may be achieved by tracking the same individuals over time using GPS collars and could help to disentangle whether (and which type of) human activity affects wolf stress physiology at a certain stage of life. Furthermore, if it does, the directionality of the effect could be measured: do individuals in worse condition (chronically infected, ill, or injured, with higher levels of metabolic stress) approach more urbanised areas because they are unable to obtain a more preferred, rural territory (Zanni et al. 2023), or do they arrive in the urbanised territory healthy and with low metabolic stress levels, which subsequently decline due to the novel conditions and stressors they have to face?

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Permits – Study 1 received ethical approval from the 'Ethik und Tierschutzkommission' of the University of Veterinary Medicine Vienna (reference no. ETK-024/02/2021) and was conducted in accordance with Good Scientific Practice and ARRIVE guidelines and national as well as EU legislation. All necropsies to obtain samples for Study 2 were conducted at specialized centres dedicated to wildlife disease control. No specific permission was required to perform the sampling, in accordance with the Italian national Legislative Decree 26/2014.

Author contributions

Gwendolyn Wirobski: Conceptualization; Methodology; Validation; Formal analysis; Investigation; Writing – original draft; Writing – review and editing; Visualization; Project administration. **Carmela Musto**: Methodology; Validation; Investigation; Resources; Writing – review and editing. **Rudy Brogi**: Formal analysis; Writing – review and editing; Visualization. **Martina Lazzaroni**: Project administration; Writing – review and editing. **Rupert Palme**: Methodology; Resources; Writing – review and editing; Supervision. **Yasmina Wukowich**: Investigation; Writing – review and editing. **Fanni Kertesz**: Investigation; Writing – review and editing. **Agnes Szabo**: Investigation; Writing – review and editing. **Chiara Garbarino**: Investigation; Resources. **Marco Gobbi**: Investigation; Resources. **Lorella Barca**: Investigation; Resources. **Mauro Delogu**: Resources; Funding acquisition. **The Wolf Network**: Investigation; Resources. **Friederike Range**: Resources; Writing – review and editing; Supervision; Funding acquisition. **Marco Apollonio**: Resources; Writing – review and editing; Supervision; Funding acquisition. **Sarah Marshall-Pescini**: Conceptualization; Methodology; Writing – review and editing; Project administration; Resources; Funding acquisition.

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Data availability statement

Data are available from Zenodo: https://doi.org/10.5281/zenodo.17976692 (Wirobski et al. 2026).

Supporting information

The Supporting information associated with this article is available with the online version.

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