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Research article

Quantification of steroid hormones in free-ranging Apennine wolf *Canis lupus italicus* hair samples collected post-mortem

Ilaria Troisio[1], Carmela Musto[1], Nicola Acquisti Casi[1], Nadia Govoni[1], Giuseppe Merialdi[2], Domenico Ventrella[1,3], Alberto Elmi[1,4], Mauro Delogu[1] and Maria Laura Bacci[1,3]

[1]Department of Veterinary Medical Sciences, Alma Mater Studiorum – University of Bologna, Ozzano dell'Emilia, Bologna, Italy
[2]Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Bologna, Italy
[3]Health Sciences and Technologies – Interdepartmental Center for Industrial Research (CIRI-SDV), Alma Mater Studiorum – University of Bologna, Bologna, Italy
[4]Department of Veterinary Medical Sciences, University of Pisa, Pisa, Italy

Correspondence: Alberto Elmi (alberto.elmi@unipi.it)

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After decades of dramatic reductions in their populations, Italian wolves have begun recolonizing parts of their historic range. This growth in populations can lead to potential conflicts with human activities, which remain the main cause of wolf mortality. With the aim of examining animal welfare and their health status, a hormonal dosage on hair was conducted on 20 wolf carcasses originating from the Tuscany and Emilia-Romagna regions. The concentrations of cortisol, testosterone, dehydroepiandrosterone (DHEA), progesterone, and oestradiol (E2) were measured using radioimmunoassay (RIA). Our results, listed as mean ± SD, were cortisol 1.81 ± 1.17, testosterone 2.74 ± 1.6, DHEA 62.92 ± 24.99, P4 53.22 ± 32.8 and E2 0.46 ± 0.23 pg/mg. The study employed a novel approach to analyse hormone levels in this species, but its opportunistic nature resulted in uneven data distribution across sex and age groups. Consequently, comparisons of hormone levels by these categories did not yield relevant differences. Further studies with larger sample sizes are necessary to analyse hormone trends throughout the year and explore differences based on factors such as sex, age, and causes of death.

Keywords: Apennine wolf, cortisol, dehydroepiandrosterone, hair, oestradiol, post-mortem analyses, progesterone, steroids, testosterone

Introduction

Grey wolf *Canis lupus* was once the most common large carnivore in the Northern Hemisphere (Szewczyk et al. 2019) as well as the most studied (Ripple et al. 2014), but several years of mistreatment have caused the decline of numerous wolf populations. During the 18th and 19th centuries, wolves were gradually exterminated from western Europe and the Alps (Breitenmoser 1998, Fabbri et al. 2007), leading to a

worldwide reduction in their historical territory by 68%, particularly in western and central Europe (Szewczyk et al. 2019). The southern Alps' Jura Mountains and Canton of Ticino were sites of the last surviving European populations (Breitenmoser 1998).

In fact, by the end of World War II, in Italy, wolves were almost extinct (Boitani 1984, 1992, Galaverni et al. 2016) but, in the last 40 years, Italian wolves have begun to recolonize parts of their historic range (Galaverni et al. 2016). As clarified by the 2021 survey conducted by the Italian Higher Institute for Environmental Protection and Research (ISPRA, Istituto Superiore per la Protezione e la Ricerca Ambientale), the total number of wolves in the Italian areal is estimated as between 2945 and 3608 divided as follows: 822–1099 wolves in the alpine area and 2020–2645 presenting the same genetic pool ('https://www.isprambiente.gov.it/it/attivita/biodiversita/monitoraggio-nazionale-del-lupo/link). Alongside the expansion of their range, despite wolves preferring areas distant from human settlements, they have been increasingly observed in urban areas (Dini et al. 2024), where the interaction with humans is more frequent and results in livestock depredations, killing of hunting dogs and dog hybridization (David Mech and Luigi 2006), leading to an increase in illegal killing and collision with vehicles (Musto et al. 2021), which mainly involves pups and juveniles (Galaverni et al. 2016). Given the extent of its habitat, the species exhibits significant variation in phenotypic traits. Such variety is also reflected in the taxonomy of the species, which is often updated and revised (Mech 1981, David Mech and Luigi 2006). In Italy, the presence of a local subspecies with unique physical and genetic features, known as the Apennine wolf *Canis lupus italicus*, has been verified (Nowak and Federoff 2002, Montana et al. 2017).

The hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-adrenal (HPA) axes are the key pathways involved in behavioural modulation (Elmi et al. 2020). The study of stressors and animal welfare provides for the standard approach of the evaluation of activity of the HPA axis. On the other hand, sexual steroids are useful to evaluate the health status of reproductive tissues, necessary for the growth and maintenance of secondary sexual characteristics allowing for successful reproduction. Measuring their concentrations would allow us to assess the activity of the hypothalamic-pituitary-gonadal (HPG) axis (Norman and Litwack 1987). The most prominent indicator of stress is cortisol (CORT) (Ralph and Tilbrook 2016), a stress-reactive hormone (Norman and Litwack 1987), also because it affects the cardiovascular system increasing vasoconstriction, cardiac output, thus mediating the fight or flight response under acute stress (Knezevic et al. 2023). Nonetheless, it is also secreted under favourable circumstances (Bacci et al. 2014). Dehydroepiandrosterone (DHEA), instead, is mainly known as an androgen precursor and for its role in reducing aggressive behaviour (Elmi et al. 2020). In addition, a very wide range of effects are reported: in humans, for example, the presence of specific DHEA receptors in different tissues suggests a potential neuroprotective, anxiolytic and antidepressant role (Nenezic et al. 2023).

Finally, there seems to be an association between lower DHEA levels in older specimens with reduced bone mineral density typical of osteopenia and osteoporosis (Zhou and Glowacki 2018). Another crucial hormone for skeleton and muscle growth is testosterone, also pivotal for the development and differentiation of reproductive tissues (Norman and Litwack 1987). Progesterone (P4) and oestradiol (E2), on the other hand, are mainly known as female steroids. Nevertheless, despite common belief, progesterone also serves important functions in male physiology (Oettel and Mukhopadhyay 2004). Progesterone is synthetized in ovaries by the corpus luteum and, in pregnancy, by the placenta too, although in less measure it is also produced by the adrenal cortex and Leydig cells, testes and in adipose tissue in male specimens (Kolatorova et al. 2022). Lastly, oestradiol is the most common circulating oestrogen, which are primarily produced by ovaries and then adrenal glands and adipose tissue (Vrtačnik et al. 2014, Fuentes and Silveyra 2019). Furthermore, it promotes epithelial proliferation in the endometrium and prepares the mammary gland for lactation during late-phase pregnancy. In males, lower levels of oestrogens are indicative of healthy sperm maturation and erectile function, specifically in humans (Fuentes and Silveyra 2019).

To detect the concentration of hormone accumulation, the use of alternative matrices is becoming increasingly common in a great number of species including wildlife, because these can give information about a longer timespan (Stalder and Kirschbaum 2012, Elmi et al. 2020, Olvera-Maneu et al. 2021). Hair presents many potential applications, mainly in monitoring chronic stress of wild animals (Ghassemi Nejad et al. 2022). The best-known hypothesis that tries to explain how incorporation of hormones in the hair occurs is based upon the same thesis that explains drug incorporation: via passive diffusion from blood at the site of the medulla (Boumba et al. 2006, Russell et al. 2012).

The aim of this work was to analyse the steroid profile of cortisol, testosterone, dehydroepiandrosterone, progesterone and oestradiol, using radioimmunology technique after methanol extraction, in 20 carcasses of Apennine wolf, to evaluate the activity of hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes.

Material and methods

Animals and sampling

Twenty (n = 20) Apennine wolves *Canis lupus italicus*, nine females and 11 males, originating from the Tuscany and Emilia-Romagna regions, were analysed after their demise at the Wildlife and Exotic Fauna Service of the University of Bologna and Experimental Zooprophylactic Institute of Lombardy and Emilia-Romagna of Bologna (IZSLER). The exact recovery location of each carcass is shown in Fig. 1.

Most of the carcasses (13/20, 65%) were retrieved during autumn; out of the remaining ones, six were retrieved in winter (30%) and only one in spring (5%). This pattern aligns closely with the dispersal period of young individuals. The condition of the carcasses was generally good, mainly due to

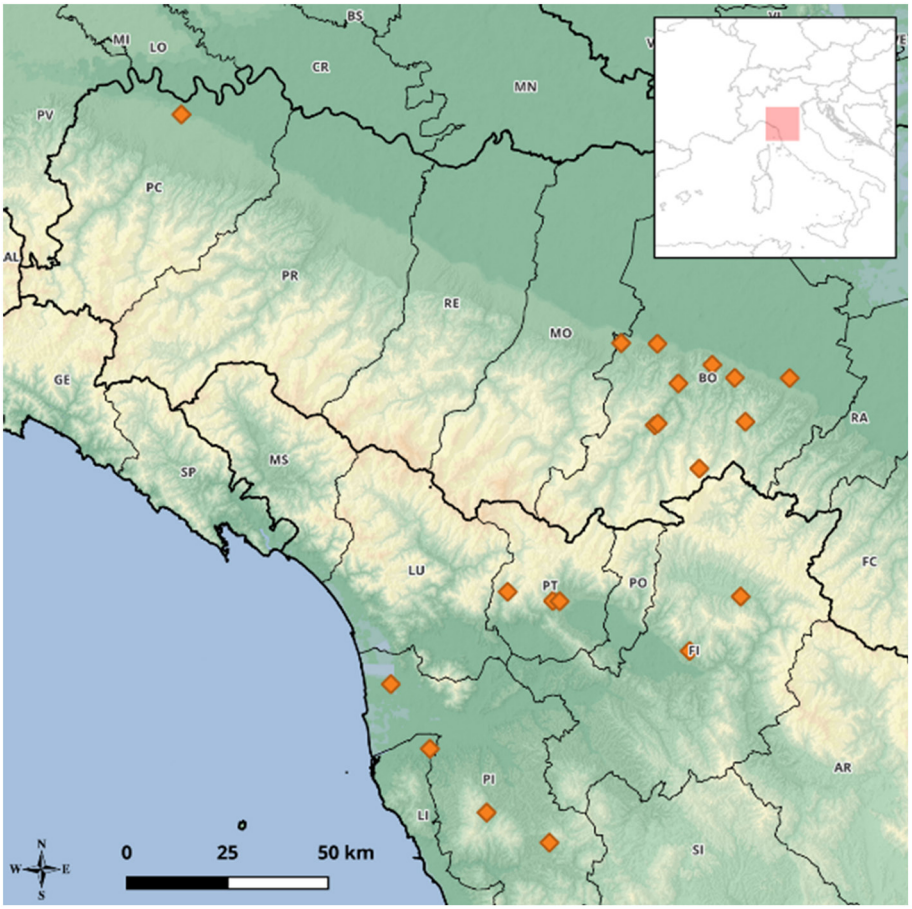

Figure 1. Localization of Apennine wolves' carcasses (map background was sourced from www.mapsforeurope.org).

the cold temperatures slowing down the decomposition processes. On average, carcasses were collected and delivered by the authorities within two to three days of being discovered.

Each wolf underwent a comprehensive necropsy examination for signalling, biometry, phenotypic markers, causes of death body size and weight. Age was estimated based on dental development (Brasington et al. 2023). Each individual was tested for the presence of toxic substances, including pesticides, rodenticides, insecticides, and other common poisons. However, individuals classified as 'poisoned' exhibited both pathological signs consistent with poisoning – acquired coagulopathies, pale mucous membranes, hemothorax, hemoperitoneum, and pulmonary edema – and laboratory confirmation of one or more toxic compounds. Examination of stomach contents was generally of limited diagnostic value, as poisoned individuals often had not eaten for several days prior to death.

Since hair from different body regions grows at varying rates and times, and hormone incorporation may differ across the body (Pereira et al. 2022), each wolf was shaved on a specific section of the rump area measuring 20 × 20 cm. Hair samples were then refrigerated at +4°C in the physiology laboratories (ANFI-ASA) of the Department of Veterinary Medical Sciences at the University of Bologna.

Hair washing and steroid extraction

Hair samples were analysed as previously described (Elmi et al. 2020). Briefly, they were washed with distilled water and isopropanol (Carlo Erba Reagents s.r.l.) to effectively remove organic residues; once dried, hair was pulverized with liquid nitrogen, and then an analytic scale was used to collect the necessary quantity for extraction. Samples were then added to methanol (Carlo Erba Reagents s.r.l.) and incubated overnight for steroids extraction. The ratio between the volume of methanol and the pulverized samples was adjusted on the bases of preliminary tests run in the lab: 1 ml/30 mg for cortisol, testosterone and DHEA, and 1 ml/50 mg for progesterone and oestradiol. Samples were then centrifuged for 30 minutes at 1500 g, and, under a chemical hood, methanol was collected and evaporated to dryness. The quantities of methanol recovered for each hormone analysed were as follows: cortisol: 3 ml; testosterone: 4.5 ml; dehydroepiandrosterone: 4.5 ml; progesterone: 7 ml; oestradiol: 7 ml.

Radioimmunoassay quantification of cortisol, testosterone, and DHEA

The dry extracts were stored at −20°C until suspension in 0.3 ml of RIA buffer (74.26 mmol/l Na_2HPO_4, 12.49 mmol/l EDTA Na, 7.69 mmol/l NaN_3) with 0.1% di BSA (pH 7.5);

100 µl were used cortisol and testosterone, while 10 µl for DHEA (30 mg hair equivalent for cortisol, 50 mg hair equivalent testosterone and 4.5 mg for DHEA). Tritiated antigens were added (30 pg/0.1ml; PerkinElmer Inc.) along with polyclonal anti-DHEA antibodies (0.1 ml, 1:10000), anti-cortisol antibodies (0.1ml, 1:20000), and anti-testosterone antibodies (0.1 ml, 1:50000), all produced in our laboratory in rabbits. After an overnight incubation at +4°C, a solution of charcoal-dextran (charcoal 0.25%, dextran 0.02% in phosphate buffer) was added to separate the steroids not bound to antibodies, followed by centrifugation (15 min, 3000 g). The supernatant was transferred into vials with scintillation fluid, and radioactivity was measured using a beta counter (Packard C1600). The sensitivity of the cortisol, DHEA, and testosterone assays was 6.02 pg/tube, 2.58 pg/tube, and 1.07 pg/tube, respectively, with intra-assay coefficients of variation of 4.9, 5.7, and 5.2%, respectively. Cross-reactions of various steroids with the rabbit anti-cortisol antibody, obtained using a well-established method (Abraham 1974), were cortisol (100%), cortisone (5.3%), 11α-deoxycortisol (5.0%), corticosterone (9.5%), 20α-dihydrocortisone (0.4%), prednisolone (4.60%), progesterone, and testosterone (< 0.001%). Cross-reactions of the anti-DHEA antibody were: dehydroepiandrosterone (100%), dehydroepiandrosterone sulfate (39%), androstenedione (10%), testosterone (0.25%), progesterone, and cortisol (< 0.001%). Cross-reactions for the anti-testosterone antibody were testosterone (100%), dihydrotestosterone (25.4%), androstenedione (0.43%), cortisol, and progesterone (< 0.001%).

To determinate the parallelism between the standard calibration curves and endogenous hormones in the hair, serial dilutions with RIA buffer as above-described (1:1–1:8) were made. Regression analysis was used to determinate the parallelism between the two hormone levels in the same assay. The results of the analyses were expressed in pg/mg of hair.

Radioimmunoassay quantification of oestradiol and progesterone

Samples for oestradiol and progesterone analyses were suspended in 0.4 ml of RIA buffer and subsequently loaded into RIA tubes, 100 µl for E2 (100 mg hair equivalent) and 10 µl for P4 (10 mg hair equivalent). The above-described analysis was then performed using specific polyclonal antibodies produced in-house in rabbits (1:10000 for P4; 1:20000 for E2). The sensitivity of the oestradiol and P4 assays was 1.63 pg/tube and 2.16 pg/tube, respectively. Cross-reactions of the anti-E2 antibody, assessed using the previously mentioned method, were: oestradiol 17b (100%), estrone-estriol (1.2%), oestradiol 17a (0.62%), testosterone, and progesterone (< 0.0001%). Cross-reactions of the anti-P4 antibody were observed: progesterone (100%), 11-a-OH-progesterone (9.7%), 20-a-OH-progesterone (0.3%), 17-a-OH-progesterone (1.5%), testosterone, and oestradiol (< 0.001%). The results of the analyses were expressed in pg/mg of hair.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism ver. 8 software (GraphPad Software Inc.). Descriptive analysis results were presented as mean and standard deviation for continuous variables and as a percentage of the total sampled subjects for qualitative variables. Data distribution was evaluated using the Shapiro–Wilk test. For inferential analysis of each hormone, subjects were categorized by sex and age classes. The Mann–Whitney non-parametric test was used for sex, while the Kruskal–Wallis non-parametric test was used for age, categorized as young, subadult, and adult. To evaluate the relationships between the different analysed hormones, a non-parametric Spearman rank correlation test was performed. The level of statistical significance for all analyses was set at $p < 0.05$.

Results

The data that support the findings of this study are openly available in AMSActa Institutional Research Repository at http://doi.org/10.6092/unibo/amsacta/7786.

Sampled population specifics

Table 1 reports the generic data of the animals received for necropsy investigation, including age category, gender, weight and cause of death.

The sampled population consisted of 11 male (55%) and nine female (45%) specimens. The age was categorized into three age groups: young (< 12 months, 35%), subadult (> 12 < 24 months, 35%) and adult (> 24 months, 30%).

As for the cause of death, 55% of the animals died due to vehicular trauma, 10% due to poisoning, 10% from bite wound trauma, 10% because of gunshot trauma, 10% passed away from combined poisoning and vehicle trauma and, finally, 5% died of cachexia.

Table 1. Specifics of the sampled population.

ID	Age	Gender	Weight (kg)	Cause of death
1	Young	Male	25.0	Poisoning
2	Subadult	Male	20.4	Cachexia
3	Young	Male	16.2	Bite wound trauma
4	Young	Female	16.5	Vehicular trauma
5	Subadult	Male	29.4	Vehicular trauma
6	Adult	Male	37.8	Bite wound trauma
7	Subadult	Female	27.2	Vehicular trauma
8	Adult	Male	34.5	Vehicular trauma
9	Adult	Male	34.7	Poisoning
10	Subadult	Male	30.5	Gunshot trauma
11	Adult	Female	26.4	Vehicular trauma
12	Young	Female	29.0	Vehicular trauma
13	Subadult	Female	29.2	Vehicular trauma
14	Subadult	Male	28.0	Vehicular trauma
15	Young	Female	21.0	Vehicular trauma
16	Young	Female	23.0	Vehicular trauma and poisoning
17	Adult	Male	37.0	Vehicular trauma and poisoning
18	Young	Female	21.0	Vehicular trauma
19	Subadult	Female	28.1	Gunshot trauma
20	Adult	Male	36.0	Vehicular trauma

Young 0–12 months; subadult 12–24 months; adult > 24 months. To every specimen has been associated an animal ID number.

Table 2. Descriptive statistics of the quantified hormones.

ID	Cortisol (pg/mg)	Testosterone (pg/mg)	DHEA (pg/mg)	P4 (pg/mg)	E2 (pg/mg)
1	0.61	4.68	110.51	94.72	0.89
2	2.50	0.95	33.42	21.72	0.19
3	4.90	1.84	57.34	40.35	0.25
4	2.04	2.27	60.54	30.35	0.36
5	0.95	4.59	70.20	64.76	0.35
6	0.98	2.80	75.95	29.73	0.83
7	0.77	1.96	33.13	54.23	0.78
8	1.46	1.21	45.21	26.59	0.34
9	1.20	3.21	72.75	42.69	0.37
10	2.24	3.15	34.55	76.90	0.67
11	1.62	1.52	43.68	42.71	0.51
12	1.40	1.25	45.71	/	/
13	3.08	1.67	49.42	35.65	0.42
14	1.45	4.00	98.43	95.67	0.54
15	1.35	1.74	57.85	37.33	0.21
16	1.86	1.15	33.77	20.25	0.16
17	1.25	2.76	58.85	22.12	0.27
18	1.77	6.23	93.82	126.68	0.84
19	0.35	6.06	111.22	113.58	0.51
20	4.38	1.80	71.96	35.23	0.53

DHEA: dehydroepiandrosterone; P4: progesterone: E2: oestradiol.

Female specimens' weight ranged from 16.50 to 29.20 kg, with a mean of 24.60 ± 4.43 kg; while males ranged between 16.20 and 37.80 kg, with mean of 29.95 ± 7.07 kg.

Hormone concentrations

Table 2 reports the concentrations, expressed in pg/mg of hair, of the five hormones of this study.

Mean concentrations ± SD of hormones were: 1.81 ± 1.17 pg/mg for cortisol, 2.74 ± 1.6 pg/mg for testosterone, 62.92 ± 24.99 pg/mg for DHEA, 53.22 ± 32.8 pg/mg for P4 and, finally, 0.46 ± 0.23 pg/mg for E2.

Hormone concentration categorized by sex

The Mann–Whitney non-parametric test highlighted no significative differences when comparing male (n = 11) versus female (n = 9) specimens for all hormones, as graphically shown in Fig. 2.

Hormone concentration categorized by age

The Kruskal–Wallis non-parametric test was used to evaluate potential differences between age groups; no differences were recorded, as shown in Fig. 3.

Spearman's rank correlation analysis

The correlation coefficients (ρ) between hormones are represented in Fig. 4.

Out of the analysed hormones, cortisol was the only one not showing correlations to other analytes. On the other hand, TEST was positively correlated to DHEA ($\rho = 0.78$, $p < 0.0001$), P4 ($\rho = 0.78$, $p < 0.0001$) and E2 ($\rho = 0.63$, $p = 0.004$). DHEA was also positively correlated to P4 and E2, yet less strongly (respectively, $\rho = 0.51$, $p = 0.027$ and $\rho = 0.46$, $p = 0.046$). Finally, P4 and E2 were also positively correlated ($\rho = 0.66$, $p = 0.002$).

Discussion

The present work was aimed at quantifying cortisol, testosterone, dehydroepiandrosterone, progesterone and oestradiol, by means of RIA methods, in hair samples collected on Apennine wolf carcasses, to evaluate the activity of hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes.

The first article of the Declaration of Principle of Wolf Conservation adopted by the Wolf Specialist Group of the Species Survival Commission of International Union for Conservation of Nature (IUCN) in 1973 states that 'Wolves have a right to exist in a wild state in viable populations'. This fundamental right does not reside in the value to mankind, but rather on the right to co-exist as a part of the natural ecosystem, as any other animal species (Boitani 2000).

To date, the wolf has regained its lost areal and such recolonization has seen the involvement of peri-urban territories, thus increasing interactions with human activities. In fact, in 2016 70% of packs included at least one urban settlement within the expected home range in the European region (Zanni et al. 2023). The increasing interactions with man have led to an increment in anthropogenic origin mortality (David Mech and Luigi 2006, Musto et al. 2021). This is reflected in the collection of carcasses for this study, which are often difficult to detect because specimens have been victims of illegal killing (Musto et al. 2021). In fact, out of the total of twenty specimens, only 15% died of natural causes (intraspecific aggression, cachexia) not imputable to humans.

Hair was the chosen matrix as it presents multiple advantages because its sampling may be considered one of the more appropriate solutions for post-mortem hormonal dosages. Although this is a preliminary and opportunistic study that has been conducted on dead specimens, the possible future

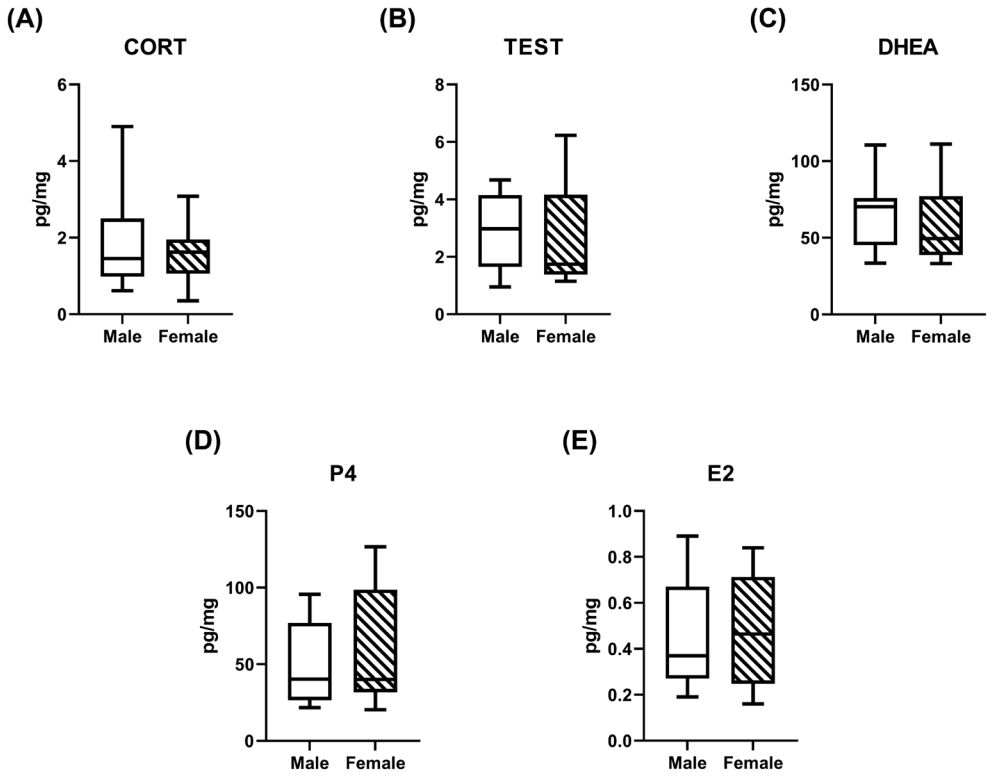

Figure 2. Concentration expressed in pg/mg of each hormone object of the study comparing by sex, using the Mann–Whitney non-parametric test: (A) cortisol - CORT, (B) testosterone - TEST, (C) DHEA, (D) progesterone - P4, (E) oestradiol - E2.

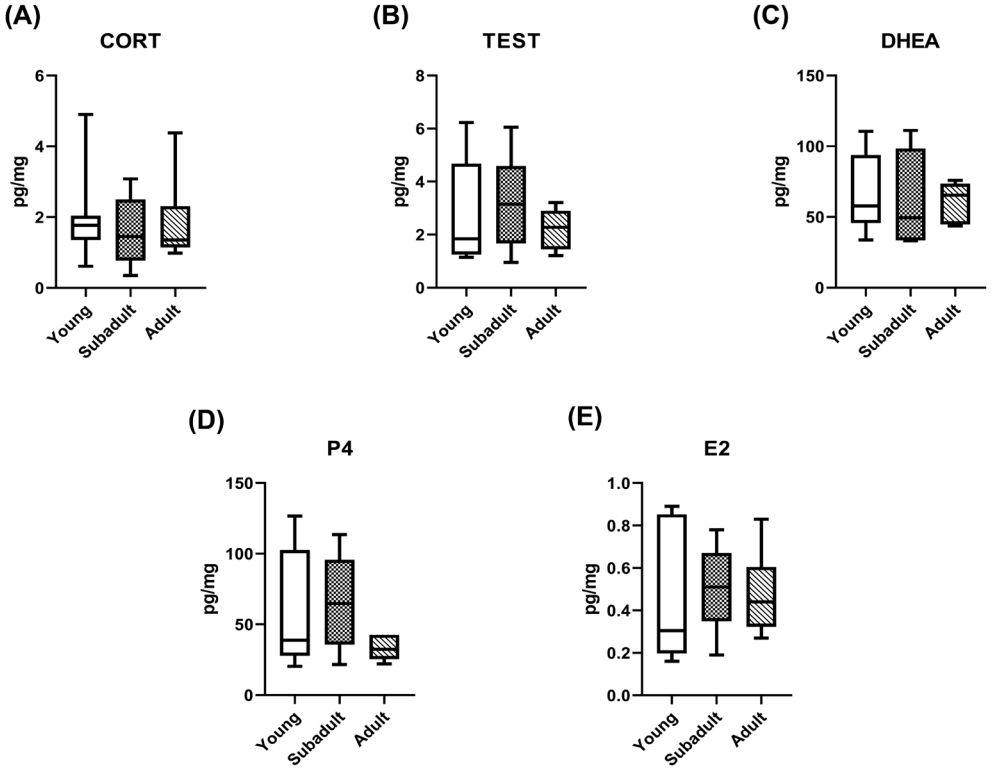

Figure 3. Concentration expressed in pg/mg of each hormone object of the study comparing by age, using the Kruskal–Wallis non-parametric test: (A) cortisol - CORT, (B) testosterone - TEST, (C) DHEA, (D) progesterone - P4, (E) oestradiol - E2.

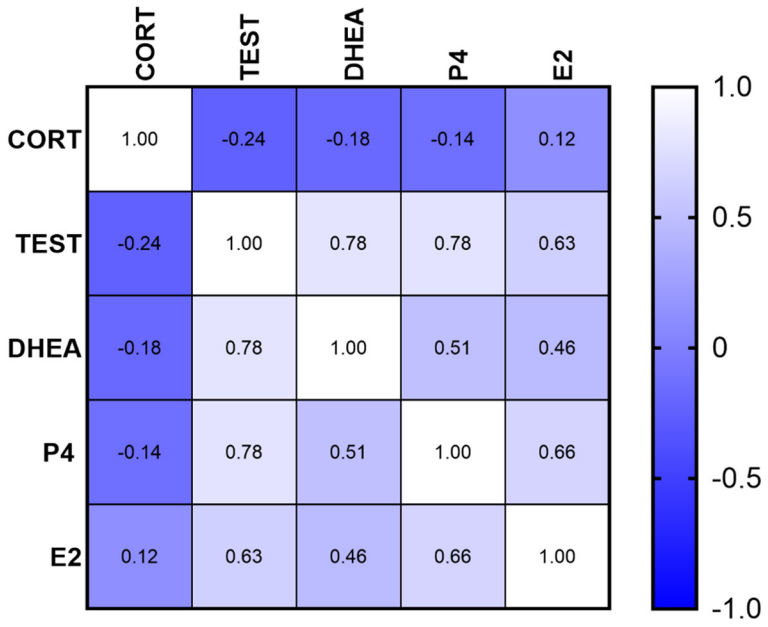

Figure 4. Spearman rank correlation coefficients (ρ) table for all the analysed parameters.

application with a living collection requires a methodology validation. The most common matrices in hormonal dosages are blood and saliva collection, which require containment actions during living sample collection. The other non-invasive matrices are faeces and urine, but faecal samples are rich in many metabolites, while urine provides a measure of secretion of hormones over a range of 12–24 hours (Ataallahi et al. 2022). Moreover, both have shorter storage requirement times, whereas hair can be stored at room temperature. Each matrix may serve its own purpose, and hair can be one of the most stable and reliable when the goal is to assess the influence of the environment on wildlife biology over a longer time span. Indeed, in the last two decades this matrix has become extremely popular in wild species (Caslini et al. 2016, Ventrella et al. 2020), and in wolf (Bryan et al. 2015, Pereira et al. 2022, Roffler et al. 2022), due to its utility in long-term evaluation of HPG axis activity. Furthermore, the use of hair could promote studies on demography (population size, reproduction, mortality), ecology (distribution, habitat use), health status of the population itself and genetic determination of species.

This study aimed at demonstrating the feasibility of hair sampling and hormone quantification methods in the Apennine wolf *Canis lupus italicus* and, at a preliminary stage, to understand if there were differences among age and sex classes. As for the first objective, the results confirm the efficacy of hair as a non-invasive matrix for hormonal dosage in wolf using radioimmunoassay. The in-house antibodies showed high performance except for the progesterone one, which showed significant cross-reactions with other progestans. No statistically significant differences were observed between individuals, either categorized by sex or age, probably because of the small sample size, resulting in a low frequency of adult individuals (35%) and a high frequency of sexually immature individuals (65%). Nonetheless, the same lack of statistical differences for cortisol between wolves divided per sex are reported in other studies (Pereira et al. 2022, Roffler et al. 2022).

Cortisol levels in animals are influenced by temperature and physiological states. In red deer, lower cortisol values are observed during warmer months, with an increase in late pregnancy (Ventrella et al. 2020). Similarly, roe deer males experience elevated cortisol due to stress from territorial fights in spring (Ventrella et al. 2018), as do wolves in physical conflict during the mating season (Eggermann et al. 2013). Hair cortisol concentration in this species, in fact, peaks in winter due to increased social instability during the mating season, while the lowest levels occur in May when competition and instability decrease (Pereira et al. 2022). Moreover, another study of 2015 stated that higher levels of stress and reproductive hormones likely reflect different environmental conditions, including human-caused mortality in wolf (Bryan et al. 2015). Our results are similar to the ones detected in previous studies conducted on wolves using the same matrix (Roffler et al. 2022), somewhat proving the consistency of the method. The Spearman's rank correlation analysis showed that cortisol was the only hormone, out of the analysed ones, not correlating to others. This finding may be imputable to the fact that, within the steroidogenic pathway, it is the only glucocorticoid, while the other chosen analytes are either androgens or estrogens, more deeply connected to the reproductive physiology.

The second hormone was testosterone, which has a seasonal trend in seasonal breeder animals with the highest peaks occurring in the blood during reproductive activity (Kozioł and Koziorowski 2013). Another decisive impact on testosterone levels is played by age of individuals, where increasing concentrations are expected when sampling occurs in animals

of reproductive age and even more in the period leading up to mating. These age- and season-related fluctuations are also clearly shown in studies involving boars with radioimmunology quantification (Macchi et al. 2010, Aniballi et al. 2023). Our results, instead, do not show higher testosterone levels in adults. It is nonetheless important to highlight that the age categorization performed in this study led to very small groups, with the added confounding effect represented by the inclusion of both sexes. Indeed, consistent with what seen in boars and roe deer, differences in testosterone levels between males and females have also been highlighted through hair matrix in domestic dogs, where testosterone was higher in uncastrated males compared to females and neutered males (Calamari et al. 2020). Despite the lack of statistical significance, once again probably imputable to the relatively low sample size, the female animals analysed in this study also showed lower mean values than males.

Our progesterone and oestrogen concentrations are somewhat hard to analyse too, due to the lack of background data and physiological in-depth investigations. Our P4 data fall within the range concentration already proposed by others (Roffler et al. 2022), strengthening the validity of the obtained values. In a previous study conducted on wolf, progesterone and testosterone were positively correlated, so that the authors hypothesized a correlation with reproductive activity too, probably due to social disturbances for anthropogenic reasons (Roffler et al. 2022). These data are also supported by another study in which authors found higher progesterone levels in tundra–taiga wolves, especially females. The authors correlated a higher human presence in the environment with an increased concentration of progesterone in female specimens (Bryan et al. 2015). Additionally, other studies have highlighted differences in progesterone levels linked to different stages of pregnancy using the same matrix and quantification technique, but in different wild species (Ventrella et al. 2020). Our results show a strong positive correlation of both P4 and E2 with testosterone, in complete alignment with the above-mentioned existing literature. A milder positive correlation was also noted with DHEA and between P4 and E2 themselves. Being DHEA a testosterone precursor, such correlation may be reflecting the one to testosterone, stronger yet positive.

No other considerations can be made for DHEA, given that we do not have consistent data to compare with in literature. However, our results did not show any significant variations among sex and ages. Since the literature highlighted a negative correlation between DHEA and aging (Zhou and Glowacki 2018), further investigation is needed to deepen our knowledge. As expected, in the present study it was strongly positively correlated to testosterone, being its precursor.

To conclude, this preliminary and opportunistic study conducted on the Apennine wolf allowed us to understand the feasibility of the steroid quantification technique through radioimmunoassay in hair samples collected post-mortem. The results hereby shown are consistent and could be used to propose further studies, with a larger and better distributed sample population in terms of sex and age, to better understand the reproductive cycle and physiological status of Apennine wolves, as well as to evaluate the relationship with the surrounding environment, providing new information on the physiological response to chronic stressors such as increased anthropogenic pressure.

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Author contributions

Ilaria Troisio: Formal analysis (equal); Writing – original draft (equal). **Carmela Musto**: Conceptualization (equal); Writing – review and editing (equal). **Nicola Acquisti Casi**: Methodology (equal); Writing – original draft (equal). **Nadia Govoni**: Methodology (equal); Writing – review and editing (equal). **Giuseppe Merialdi**: Methodology (equal); Resources (equal). **Domenico Ventrella**: Formal analysis (equal); Writing – review and editing (equal). **Alberto Elmi**: Conceptualization (equal); Formal analysis (equal); Project administration (equal). **Mauro Delogu**: Resources (equal); Supervision (equal). **Maria Laura Bacci**: Resources (equal); Supervision (equal).

Data availability statement

Data are available from the AMSActa Institutional Research Repository of the University of Bologna: https://amsacta.unibo.it/id/eprint/7786 (Troisio et al. 2025).

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