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# Cortisol in hair: a comparison between wild and feral cats in the north-eastern Alps

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## Abstract

The quantification of glucocorticoid metabolites in hair is a non-invasive tool that provides important information regarding the endocrine status and represents a valuable method for studying potential stressors that may affect carnivores under both natural and non-natural conditions. Cortisol is the main glucocorticoid hormone of the hypothalamic-pituitary-adrenal gland axis and is considered a standard stress indicator for animal welfare. The current study aimed to compare cortisol levels extracted from hair of both dead, frozen European wildcats (*Felis silvestris silvestris*) and living feral individuals (*Felis silvestris catus*) living in different environmental conditions. The results obtained revealed that wild individuals exhibited a significantly ( $p < 0.001$ ) higher cortisol concentration ( $n = 15$ , mean  $\pm$  sd =  $8.91 \pm 4.48$  pg/mg) than feral ones ( $n = 10$ , mean  $\pm$  sd =  $3.57 \pm 1.25$  pg/mg), probably as a result of both the physiological and/or environmental factors to which each subspecies was subject. This is the first study in which cortisol concentrations have been compared within the *Felis silvestris* subspecies, thus enriching the scarce information available for the *Felidae*. Nevertheless, further research is needed to better understand the various physiological and ecological factors affecting the adrenocortical activity of species or populations living in different environmental contexts.

**Keywords:** wildcat, feral cat, *Felis silvestris*, hair cortisol, adrenocortical activity.

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43 Udine, and Trieste for the hair samples provided. Furthermore, the authors are indebted to all the wildlife technicians,  
44 interns, and volunteers involved in the collection of field data.

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## 81 **1. Introduction**

82 The ability of an organism to adapt to changes in environmental conditions has been receiving increased attention in  
83 recent years (Koolhaas *et al.* 1999; Janczak *et al.* 2003; Natoli *et al.* 2005; David *et al.* 2011; Ruiz-Gomez *et al.* 2011;  
84 Montiglio *et al.* 2012; Dingemanse and Reale 2018). Abiotic and biotic changes are common within the environment,  
85 and animals may respond through temporal variation in their vital rate and/or an alteration in their physiological  
86 response (Darlington *et al.* 1990). The activity of the hypothalamic-pituitary-adrenal (HPA) gland axis leads to the  
87 release of glucocorticoids (GCs), which are commonly used as indicators of physiological stress (Wingfield and  
88 Romero 2001; Wikelski and Cooke 2006). When animals are subjected to a stressor, the hypothalamus releases  
89 corticotropin-releasing hormones (CRHs) which signal to the adrenal cortex to release steroid hormones (such as GCs)  
90 to overcome stressful situations (Sapolsky *et al.* 2000) and restore homeostasis (Möstl and Palme 2002). This said,  
91 prolonged exposure to stressors may lead to chronic GC accumulation leading to chronic stress (Romero 2004) which is  
92 detrimental. Chronic stress negatively affects individuals' fitness in terms of the depression of immune responses,  
93 reduced reproductive success and growth suppression (Romero 2004). The quantification of glucocorticoid metabolites  
94 is a non-invasive tool that provides important information regarding endocrine status, and is a valuable method to study  
95 the potential stressors that may affect carnivores under natural conditions (Barja *et al.* 2007; Sheriff *et al.* 2011; Piñeiro  
96 *et al.* 2012; Schell *et al.* 2017). Cortisol is the main glucocorticoid hormone of the HPA gland axis and is considered the  
97 standard stress indicator for animal welfare (Mormède *et al.* 2007). Indeed, despite the various autonomic and endocrine  
98 responses that occur when an organism faces a stressful situation, cortisol has become commonly known as the stress  
99 hormone. As part of the stress response, cortisol acts on various metabolic pathways providing energy during a stressful  
100 situation. Cortisol's role in the endocrine system is metabolic however, and it is also released in response to arousal  
101 situations such as during sexual activity (Hamilton *et al.* 2008). Cortisol levels can be measured using both invasive  
102 (e.g. blood) and non-invasive methods in faecal samples (Dehnhard *et al.* 2001; Millspaugh *et al.* 2002; Huber *et al.*  
103 2003; Ashley *et al.* 2011), urine (Rehbinder and Hau 2006), milk (Gygax *et al.* 2006) and saliva (Negrão *et al.* 2004).  
104 However, all these methods provide information in relation to short-term cortisol variations (within 12-24 h) (Sheriff *et al.*  
105 *et al.* 2011; Russell *et al.* 2012). Hair and fur have been recognized as a relatively stable matrix that does not decompose  
106 as rapidly as other body fluids or tissues (Balíková 2005), and in which the incorporation of blood-borne hormones  
107 occurs through their passive diffusion from blood capillaries present on the basement membrane during its active  
108 growth phase (Pragst and Balikova 2006). These hormones accumulate over a period of weeks or months (Davenport *et al.*  
109 *et al.* 2006; Macbeth *et al.* 2010) and may remain detectable for long periods (Kintz *et al.* 2006; Webb *et al.* 2010) as the  
110 cortisol in hair is unaffected by variations in circadian hormone or by factors that induce short-term variations (Caslini  
111 *et al.* 2016). In this sense, measuring cortisol accumulating in hair or fur represents a valuable method to trace rates of  
112 long-term stress in both domestic (Comin *et al.* 2011, 2013, 2014; Peric *et al.* 2016, 2017, 2018; Stradaioli *et al.* 2017)  
113 and wildlife species (Bechshøft *et al.* 2011, 2012, 2015; Caslini *et al.* 2016; Weisser *et al.* 2016; Prandi *et al.* 2018).  
114 However, the technique's main limit is related to hair's slow growth rate which does not permit fine monitoring over  
115 short periods as it does not reflect daily or hourly fluctuations in circulating hormones (Koren *et al.* 2002). Although the  
116 dynamics of stress physiology have been studied in a range of taxa, to date, limited information is available concerning  
117 adrenal activity variations in response to environmental conditions, life-history stages or among individuals (Boonstra  
118 2004; Romero 2004; Palme 2005; Wielebnowski and Watters 2007). GC concentration has been shown to change in  
119 relation to sex, age class, day time, season and reproductive status (Ziegler and Snowdon 1995; Gardiner and Hall 1997;  
120 Cavigelli 1999; Romero 2002; Weingrill *et al.* 2004; Dantzer *et al.* 2010; Fanson *et al.* 2012). Furthermore, an existing

121 difference in terms of circulating GC concentrations among species has been demonstrated (Roth *et al.* 2001). Cortisol  
122 level comparisons among species or subspecies have received little attention in recent years, especially as far as the  
123 *Felidae* (Fanson *et al.* 2012; Narayan *et al.* 2013; Naidenko *et al.* 2011, 2019) are concerned, and information relating  
124 to adrenocortical activity expression in the European wildcat (*Felis silvestris silvestris*) is still limited and mostly  
125 focused on faecal cortisol analyses (Piñeiro *et al.* 2012, 2015). Indeed, to the best of our knowledge, there are no  
126 previous studies aimed at assessing the potential differences in GC concentrations between wild and feral cats (*Felis*  
127 *silvestris catus*).

128 The European wildcat is a medium-sized carnivore widely but patchily distributed throughout Europe (Driscoll and  
129 Nowell 2010; Lozano and Malo 2012). It is a protected species listed within the Annex IV of the European Union  
130 Council Directive 92/43/EEC, which states that a strict protection regime must be applied across its entire natural range  
131 within the EU, both within and outside *Natura 2000* sites. The conservation of this carnivore requires considerable  
132 effort due to its elusive behaviour, low population density and sensitivity toward habitat loss and human persecution  
133 (Yamaguchi *et al.* 2015; Apostolico *et al.* 2016). Three fragmented populations are present along the Italian peninsula  
134 (thus, excluding those on Sardinia and Sicily): one in each of central and southern Italy, respectively, and another in the  
135 Eastern Alps, which is perhaps conjunctive with the Slovenian and Croatian populations (Mattucci *et al.* 2013). In Friuli  
136 Venezia Giulia the species is well distributed, particularly in the Julian Pre-Alps, but has also exhibited a positive  
137 dispersal trend even into lowland areas (Lapini 2006). The tendency to expand both north and southwards seems to have  
138 begun in the middle of 20<sup>th</sup> century, presumably favoured by two factors: (i) the recent spread of woodland in both the  
139 Alps and the Karst, and (ii) the legal protection of the species in 1977 (N. L. 968/1977), subsequently confirmed and  
140 enhanced in 1992 (N. L. 157/1992). To date, it is estimated that within the Region the species numbers about 220  
141 individuals (range: 148-296) (Lapini 2006).

142 Understanding the mechanisms of adrenal activity is essential to provide important insights into comparative  
143 physiology. Comparing HPA gland axis activity between species or subspecies living in different environmental  
144 settings may help to cast light on the complex normative patterns of GC expression in various situations, for instance,  
145 establishing basic information regarding adrenal activity in endangered species living in both wild and captive  
146 conditions, providing a foundation for future studies on stress physiology and may help to enhance *ex-situ* and *in-situ*  
147 management plans (Fanson *et al.* 2012). Moreover, the comparison of physiological activities between subspecies or  
148 populations living in different environmental contexts (i.e. wild and feral populations) may be useful in understanding  
149 the complexity of ecological factors (e.g. active movements, hunting, competition) involved in the higher metabolic  
150 levels recorded in wild animals (Naidenko *et al.* 2011).

151 The present pilot study sought to compare hair cortisol concentrations (HCCs) between wild and feral cats. Our  
152 hypothesis was that the two subspecies had different physiological responses and were subject to different  
153 environmental pressures. Thus, we predicted that the HCCs recorded in wild individuals would be different compared to  
154 those measured in feral ones.

## 155 **2. Materials and Methods**

### 156 **2.1 Study area**

157 Friuli Venezia Giulia is the north-easternmost Italian Region, which borders Austria to the north and Slovenia to the  
158 east. It is bordered to the south by the Adriatic Sea, while to the west it adjoins the Veneto Region (**Fig1**). Its climate is  
159 characterized by an average annual temperature of about 14.5°C with abundant precipitation (as much as 3,000

160 mm/year in the Pre-alps), especially during autumn (~ 1,200-1,400 mm/year). Habitat varies with location, with forests  
161 and open habitats common in Alpine and pre-Alpine territories, while agricultural lands are most abundant in lowland  
162 areas (the official website of Friuli Venezia Giulia region, n.d.).

## 163 **2.2 Data collection**

164 Hair samples from 15 road-kill wildcats (11 males and four females) and ten living feral individuals (eight males and  
165 two females) were included in the present study. The wildcat carcasses were opportunistically collected between 2006  
166 and 2014 and deep-frozen to prevent decay. Five individuals (four males and a female) were collected during the mating  
167 season (from January to March) while ten (seven males and three females) were found outside the mating season, of  
168 which three (a male and two females) were found during the period (from April to May) (Killshaw 2011) when the  
169 kittens are born. Hair samples belonging to the *Felis silvestris catus* subspecies were collected from individuals living  
170 in colonies near urban settlements (small villages), in which contacts with humans were solely related to feeding  
171 behaviour. Nevertheless, no information concerning either the season or year of data collection was available.  
172 Furthermore, no information regarding their health status or whether the animals were pregnant or not were obtained for  
173 either subspecies, though no evident signs of reproduction were noted. Hair samples were removed from the scruff of  
174 the neck of both wild and feral cats for two reasons: (i) it represents an area with less contamination by external agents  
175 such as saliva and/or soil and (ii) has a uniform hair growth rate. Finally, the samples collected were stored in paper  
176 envelopes.

## 177 **2.3 Data analysis**

178 The attribution to the subspecies *silvestris* was carried out using the system proposed by Ragni and Possenti (1996),  
179 modified by Ballesteros-Duperón *et al.* (2015). Potential hybrids and feral cats were considered as a single group  
180 because hair samples were collected from individuals living within the same colonies. Using QGIS Software (version  
181 2.18) we applied a buffer with a radius of 1,820 m for each point representing the recovery coordinates of each road-  
182 killed wildcat. The choice to apply such a buffer was made to reproduce the average home-range size of a wildcat  
183 according to Anile *et al.* (2017). Within the buffer area, we calculated a habitat suitability (HS) value, ranging from 0  
184 (unsuitable habitat) to 100 (optimal habitat), by multiplying the percentage of landscape (PLAND) calculated for each  
185 habitat with a habitat suitability index (HSI) varying from 0 to 1 (0 = unsuitable habitat; 0.33 = little suitable habitat;  
186 0.66 = suitable habitat; 1 = optimal habitat), randomly defined. Based on the habitat legend obtained from the attribute  
187 table of the shapefile named the “*Carta della Natura del Friuli Venezia Giulia (2007)*” and freely downloadable from  
188 the Regional IRDAT website, we determined the suitability or unsuitability of each habitat following Lozano *et al.*  
189 (2003), Lozano (2010), Sarmiento *et al.* (2006), and Klar *et al.* (2008). Finally, we extracted an index of anthropic  
190 pressure (IAP) within each buffer, obtained from the attribute table of the “*Carta Natura*” shapefile and calculated it  
191 following the method proposed by Angelini *et al.* (2009).

### 192 **2.3.1 Cortisol radioimmunoassay**

193 Cortisol extraction was carried out using a radioimmunoassay protocol. Strands of hair were washed in 3 mL  
194 isopropanol and dried. Unminced hair (5 mg) was extracted in a glass vial using 3 mL of methanol. The vials were then  
195 incubated at 37°C for 18 h. After this the methanol was decanted into a separate vial from which it was evaporated to  
196 dryness. Next, the liquid in the vial was evaporated to dryness at 37°C under an airstream suction hood. The remaining  
197 residue was dissolved in 0.35 mL of phosphate-buffered saline (PBS), 0.05 M, pH 7.5 (RIA buffer). The cortisol in the

198 hair was measured using a solid-phase microtitre RIA procedure. In brief, a 96-well microtitre plate (OptiPlate, Perkin-  
199 Elmer Life Science, Boston, MA, USA) was coated with goat anti-rabbit  $\gamma$ -globulin serum, diluted 1:1000 in 0.15 mM  
200 sodium acetate buffer, pH 9, and incubated overnight at 4°C. The plate was washed twice with RIA buffer, pH 7.4, and  
201 incubated overnight at 4°C with 200  $\mu$ L of the anti-cortisol serum diluted 1:20000. The rabbit anti-cortisol antibody  
202 used was obtained from Biogenesis (Poole, UK) as described by Leboulenger *et al.* (1982). The cross-reactivities of this  
203 antibody with other steroids are as follows: cortisol 100%, corticosterone 1.8% and aldosterone < 0.02%. After washing  
204 the plate with RIA buffer, standards (5 - 200 pg/well), a quality control extract, the test extracts and tracer  
205 (Hydrocortisone (Cortisol, [1,2,6,7-3H (N)]-), Perkin-Elmer Life Sciences, Boston, MA, USA) were added, and the  
206 plate was incubated overnight at 4°C. The bound hormone was separated from the free hormone by decanting the  
207 extract and washing the wells in RIA buffer. After the addition of 200  $\mu$ L scintillation cocktail, the plate was counted on  
208 a beta-counter (Top-Count, Perkin-Elmer Life Sciences, Boston, MA, USA). The intra-assay and inter-assay  
209 coefficients of variation were 3.6% and 9.8%, respectively. The assay sensitivity (defined as the hormone concentration  
210 resulting in a displacement of the labeled hormone at least 2 standard deviations from maximal binding) was 1.23  
211 pg/well. To determine the comparability between cortisol standards and endogenous cortisol in cats, hair samples  
212 containing high concentrations of endogenous cortisol were serially diluted in 0.05 M PBS, pH 7.5. The relationship  
213 between hair cortisol concentrations and the standard cortisol curve, determined through linear regression, was linear:  
214 the correlation coefficient (r) was 0.99 and the model was provided by the equation  $y = 0.9796x + 1.68$ .

### 215 2.3.2 Statistical analysis

216 Statistical analysis was performed through R Software (version 3.5) and the alpha value was set at 0.05. Data were  
217 analyzed in terms of HCC in which subspecies and sex were considered as independent variables and cortisol  
218 concentration as a dependent variable.

219 To determine the effect of subspecies and sex on HCC, Generalized Linear Models (GLMs) following Zuur *et al.*  
220 (2009) were used in which the family distribution of the dependent variable was assessed using the R package  
221 “fitdistrplus”. Models ranking was done based on the Akaike’s Information Criterion corrected (AICc) to best fit with  
222 reduced sample sizes.

## 223 3. Results

224 The average PLAND value calculated within each buffer area (radius = 1,820 m) revealed that habitat was mainly  
225 composed of forests and semi-natural areas (47.03 %) followed by agricultural areas (34.95 %), artificial surfaces  
226 (16.67 %), water bodies (0.76 %) and wetlands (0.59 %). The average calculated HS index was equal to 51.53 revealing  
227 a habitat less suitable for the wild subspecies, while the IAP was equal to 0.66 indicating a notable anthropic pressure in  
228 each area.

229 From the model ranking using the AICc value as reference parameter, we discerned that the best model was the one in  
230 which only the effect of the “subspecies” variable was considered (**Tab1**). Results obtained showed a significant  
231 difference ( $p < 0.001$ ) between subspecies. Cortisol concentrations measured in the hair of frozen wildcats ( $n = 15$ )  
232 ranged from 3.90 to 19.30 pg cortisol/mg hair (mean  $\pm$  sd =  $8.91 \pm 4.48$  pg/mg), while those in live feral individuals ( $n$   
233 = 10) varied from 2.20 to 6.50 pg cortisol/mg hair (mean  $\pm$  sd =  $3.57 \pm 1.25$  pg/mg) (**Tab2; Fig2**). Cortisol levels  
234 encountered in wild males ( $n = 11$ ) ranged from 4.30 to 19.30 pg cortisol/mg hair (mean  $\pm$  sd =  $9.69 \pm 4.81$  pg/mg)  
235 while those in wild females ( $n = 4$ ) varied from 3.90 to 10.50 pg cortisol/mg hair (mean  $\pm$  sd =  $6.78 \pm 2.87$  pg/mg)

236 (Tab3; Fig3). Cortisol concentrations measured in feral males (n = 8) ranged from 2.20 to 6.50 pg cortisol/mg hair  
237 (mean  $\pm$  sd = 3.76  $\pm$  1.33 pg/mg) while those in feral females (n = 2) varied from 2.50 to 3.20 pg cortisol/mg hair (mean  
238  $\pm$  sd = 2.81  $\pm$  0.50 pg/mg) (Tab3; Fig3). Nevertheless, no significant difference was found between sexes in either wild  
239 or feral individuals.

## 240 4. Discussion

### 241 4.1 Differences in cortisol levels between wild and feral cats

242 Using a radioimmunoassay protocol, we were able to detect good levels of hair cortisol in both subspecies. In wild  
243 individuals, we obtained an average ( $\pm$  sd) value of 8.91  $\pm$  4.48 pg cortisol/mg hair, while in feral ones we recorded an  
244 average ( $\pm$  sd) level corresponding to 3.57  $\pm$  1.25 pg cortisol/mg hair. It is important to specify that road-kill events do  
245 not affect hair cortisol accumulation for two reasons: (i) cortisol enters the hair shaft through passive diffusion from  
246 blood vessels and thus requires a certain amount of time, (ii) the freezing process blocks cortisol accumulation in hair  
247 when the blood freezes. Therefore, the potential negative effect of the freezing process can be excluded as the  
248 cholesterol substrate inside the hair prevents cortisol degradation (Prandi *unpub. data*). Average HCC measured in feral  
249 cats was similar to that one obtained by Accorsi *et al.* (2008) in domestic cats (3.32  $\pm$  0.27 pg cortisol/mg HCC). This  
250 said, the levels measured when compared with those recorded in wild individuals was remarkable, a significant  
251 difference being found between HCCs in wild and those in feral cats. Because of the existence of species-specific  
252 differences in the secretion of metabolic hormones, the radio-immunological protocols used during the extraction of  
253 certain groups of steroid hormones should be performed separately, even in the case of phylogenetically-related species  
254 (Palme *et al.* 1996; Schwarzenberger *et al.* 1996; Schwarzenberger and Palme 1997; Graham *et al.* 2001; Möstl and  
255 Palme 2002; Young *et al.* 2004; Palme 2005; Berger *et al.* 2006; Heistermann *et al.* 2006). An example of how different  
256 reproductive and endocrine physiology may be, even among phylogenetically close species, was demonstrated by  
257 endocrine studies performed on four rhino species: the white rhino (*Ceratotherium simum*), black rhino (*Diceros*  
258 *bicornis*), Indian or greater one-horned rhino (*Rhinoceros unicornis*) and the Sumatran rhino (*Dicerorhinus*  
259 *sumatrensis*). Throughout the use of faecal, urine and saliva steroid analysis, authors showed that not one of the four  
260 species exhibited reproductive cycles of similar length. What was more, faecal steroid metabolites excreted varied  
261 considerably underling the necessity to carry out species-specific endocrine tests (Roth *et al.* 2001; Schwarzenberger  
262 2007).

263 Hair cortisol levels comparison between subspecies has received little attention in recent years, especially as far as the  
264 *Felidae* are concerned (Narayan *et al.* 2013, Naidenko *et al.* 2019). For instance, Narayan *et al.* (2013) compared Faecal  
265 Cortisol Metabolites (FCMs) in captive Bengal (*Panthera tigris tigris*) and Sumatran (*Panthera tigris sumatrae*) but no  
266 significant difference in terms of cortisol levels between subspecies was found. However, the main differences with our  
267 study were firstly related to the matrix used for cortisol extraction and, secondly, to the captive condition of the two  
268 tiger populations considered which may have affected faecal cortisol concentrations differently. Despite faecal-based  
269 immunoassay being considered one of the most valuable methods for mammals stress assessment, especially for  
270 endangered and managed populations, due to its non-invasiveness (Schatz and Palme 2001; Young *et al.* 2004; Palme  
271 2005; Keay *et al.* 2006; Bayazit 2009) it presents some limitations given that FCMs analysis provides a pooled value of  
272 GC activity over the short term while HCCs provide a measure of past long-term HPA gland axis activity which cannot  
273 be obtained using other analyses (Lafferty *et al.* 2015). The effect of captivity on GC concentration comparing wild and  
274 captive animals has been examined but the results obtained are controversial. For instance, Naidenko *et al.* (2011)



275 obtained a significant difference in terms of HPA gland axis activity between wild and captive Amur tigers (*Panthera*  
276 *tigris altaica*) where wild specimens, probably due to unfavourable environmental conditions in which they lived,  
277 showed significantly higher cortisol levels compared to captive ones. Contrariwise, Fanson *et al.* (2012) showed that  
278 captive Canada lynx (*Lynx canadensis*) had higher FGM concentrations than wild lynx. In our case, we could not  
279 perform a comparison with such studies as a feral existence is not the same as captivity. Nevertheless, differences  
280 recorded in terms of HCCs between wild and feral cats may be related to various factors. The first explanation could be  
281 related to underlying differences in metabolism, diet, and/or energy regulation which may have affected steroid  
282 production. The impact of these factors on steroid production and excretion has also been recorded in other species (von  
283 der Ohe and Servheen 2002; Hajamor *et al.* 2003; Goymann 2005). Moreover, GCs are linked to energy regulation  
284 (Romero *et al.* 2009), so that the major energetic differences between wild and feral individuals could explain the  
285 difference recorded in HCCs. Essentially, there is a complex network of interactions between GCs, steroids, and  
286 metabolic hormones which means that disturbances in this web may produce a cascade effect on other physiological  
287 systems (Fanson *et al.* 2012).

288 A second explanation might be related to differing degrees of tolerance of each subspecies toward anthropic disturbance  
289 or towards other con(sub)specifics. Feral cats are known to live in close contact with humans (and each other), although  
290 only in relation to foraging behaviour (Natoli 1994). In this sense, they may be more tolerant toward humans and  
291 intraspecific presence than wild individuals. Anecdotal evidence suggests some “feral” cats behave as wildcats. An  
292 animal closely resembling a wildcat or a hybrid currently (August 2019, and since at least 2011) lives in a “wild”  
293 setting, amongst rocks in woodland beneath a road close to a village in the Italian Karst (in an area where true wildcats  
294 are present). Only 50 metres from the nearest houses, it does not frequent the feral cat colony less than 75 metres from  
295 its earth and has never been observed feeding on food put out for these animals, even in cold weather (Paul Tout, *pers.*  
296 *comm.*). The Friuli Venezia Giulia Region is characterized by a marked anthropic influence due to the presence of  
297 infrastructure across much of its surface area, leading to a reduction in the ecological value of each area affected  
298 (Angelini *et al.* 2009). Consequently, wildcats are forced to live in areas with less suitable or unsuitable habitats. Our  
299 results showed that, based on the average PLAND value calculated within each buffer, the habitat was composed of a  
300 notable percentage of both agricultural (34.95 %) and built-up areas (16.67 %) which, as reported by Lozano *et al.*  
301 (2003, 2010), Sarmiento *et al.* (2006), and Klar *et al.* (2008) are considered as less suitable or unsuitable habitat for the  
302 wildcat. What is more, the average IAP calculated within each buffer showed that the area was influenced by a marked  
303 anthropic pressure, which may have affected individual wildcat welfare. The effect of anthropic disturbance on cortisol  
304 accumulation has received considerable attention in recent years and has been studied in various species (Rangel-Negrín  
305 *et al.* 2009; Zwijacz-Kozica *et al.* 2012; Burbonnais *et al.* 2013; Creel *et al.* 2013; Deng *et al.* 2014; Fourie *et al.* 2015)  
306 including tigers (Naidenko *et al.* 2019) and wildcats (Piñeiro *et al.* 2012). For instance, Naidenko *et al.* (2019)  
307 compared faecal glucocorticoid levels between two tiger subspecies, the Amur tiger and Bengal tiger living in two  
308 extreme habitats. From the analysis, they recorded that FCMs were significantly higher in Bengal tigers living in India  
309 than in Amur tigers living in the Russian Far East and, as explained by the authors, these reasons might be related to  
310 tiger density or anthropogenic disturbance. A further study performed by Rangel-Negrín *et al.* (2009) showed that forest  
311 fragmentation may create long-term stressors for spider monkeys (*Ateles geoffroyi yucatanensis*) affecting population  
312 viabilities. Deng *et al.* (2014) revealed a significant positive correlation between FCM levels recorded in giant pandas  
313 (*Ailuropoda melanoleuca*) and the degree of human disturbance within their habitat. Fourie *et al.* (2015) showed that  
314 human impacts on vervet monkeys’ (*Chlorocebus aethiops*) behavioural ecology appeared to be a significant source of  
315 stress, especially for males. Furthermore, it was demonstrated that even tourism may exert a negative effect on animal

316 welfare. Zwijacz-Kozica *et al.* (2012), studying the concentration of FCMs in chamois (*Rupicapra rupicapra*) in  
317 relation to tourist pressure in Tatra National Park (South Poland), showed that stress levels increased in relation to  
318 increasing numbers of visitors, exhibiting a peak during the summer months in areas where tourists were common. The  
319 same result was obtained by Piñeiro *et al.* (2012) measuring cortisol metabolites in fresh wildcat faecal samples within  
320 the Natural Park Montes do Invernadeiro (north-western Spain). From their study, they showed that cortisol metabolite  
321 concentrations were higher in certain park areas where tourism intensity was higher.

322 A third explanation may be related to differences in terms of environmental pressures to which each subspecies was  
323 subjected. For instance, Naidenko *et al.* (2011) compared cortisol levels between wild and captive Amur tigers, showing  
324 that wild tigers had significantly higher cortisol concentrations compared to captive ones and that the reason might be  
325 related to the unfavourable influences of low temperatures and deep snow cover. As the feral cats lived in colonies in  
326 close contact with human beings who regularly supplied them with food, they were not subjected to stressors (i.e.  
327 hunting for food) which might have led to increased HPA gland axis activity. Moreover, factors such as interspecific  
328 and intraspecific competition might have affected individual wildcats' welfare resulting in higher hair cortisol  
329 accumulations. In Friuli Venezia Giulia, the main medium-sized carnivores which may compete with the wildcat for  
330 territory and/or food resources are the beech marten (*Martes foina*), the pine marten (*Martes martes*), the red fox  
331 (*Vulpes vulpes*), and the golden jackal (*Canis aureus*). However, research focused on assessing the impact of  
332 interspecific competition on wildcat welfare is still rather sparse. To the best of our knowledge, the only study carried  
333 was that by Piñeiro *et al.* (2015) in which FCM levels in a free-living population of wildcats in northwest Spain were  
334 analyzed and showed that the presence of competitors such as pine martens and red foxes did not significantly affect  
335 cortisol concentration. As reported by the authors, the absence of a significant effect in relation to red fox presence may  
336 be attributable to the generalist behaviour of that species, both in terms of diet (Jędrzejewski and Jędrzejewska 1992)  
337 and habitat selection (Lucherini *et al.* 1995), with subsequently reduced competition. As far as the presence of pine  
338 martens is concerned, despite no significant effects being detected, the authors showed that there was a trend for raised  
339 cortisol levels measured in wildcats living in habitat selected by the pine marten (i.e. pine forests and shrublands).

340 Here we encounter a potential explanation for the difference in terms of cortisol levels recorded between wild and feral  
341 cats, thus corroborating our hypothesis. Nevertheless, there might be further ecological and physiological factors (e.g.  
342 individuals' health status or pregnancy condition) not considered in the current study which could affect HPA gland  
343 axis activity in both subspecies.

#### 344 **4.2 Hair cortisol levels comparison between sexes in wild and feral individuals**

345 No significant difference between sexes was found in either wild or feral individuals. In general, males showed higher  
346 cortisol levels than females and this difference was more evident in wild individuals (mean  $\pm$  sd =  $9.69 \pm 4.81$  pg  
347 cortisol/mg hair for males; mean  $\pm$  sd =  $6.78 \pm 2.87$  pg cortisol/mg hair for females) than in feral ones (mean  $\pm$  sd =  
348  $3.76 \pm 1.33$  pg cortisol/mg hair for males; mean  $\pm$  sd =  $2.81 \pm 0.50$  pg cortisol/mg hair for females). Sex differences in  
349 cortisol accumulation have been documented in felids (Brown and Wildt 1997; Narayan *et al.* 2013; Cattet *et al.* 2014)  
350 highlighting the underlying differences in steroid metabolism, excretion routes, and HPA gland axis responses  
351 (Goymann 2012). For instance, Narayan *et al.* (2013) observed a significant difference in terms of FCMs in two captive  
352 populations of Bengal and Sumatran tigers. Following the authors, this difference might be due to the females'  
353 reproductive hormone cycle (i.e. oestrogen and progesterone) which could have affected the expression of FCMs  
354 (Palme *et al.* 2005). Hair cortisol comparison between sexes has also been identified in other mammal taxa (Dantzer *et*  
355 *al.* 2010; Creel *et al.* 2013; Bryan *et al.* 2014; Lafferty *et al.* 2015) but the results obtained were sometimes

356 controversial. For example, Bryan *et al.* (2014) found no significant differences in HCCs between sexes in American  
357 black bears (*Ursus americanus*) and/or grizzly bears (*Ursus arctos horribilis*), Creel *et al.* (2013) found that male lions  
358 had higher faecal cortisol metabolite levels than females. On the contrary, Dantzer *et al.* (2010), comparing faecal  
359 glucocorticoids levels between sexes in North American red squirrels (*Tamiasciurus hudsonicus*), showed that females  
360 exhibited higher cortisol levels than males. Conflicting results among studies suggest that multiple factors may thus  
361 contribute to the observed sex-based difference in stress hormone levels. For example, intraspecific dominance and  
362 competition among males may induce a stress situation, but reproduction also seems to play a key role. Adrenal activity  
363 has been shown to vary with female reproductive status in different species. In fact, pregnant females have significantly  
364 higher levels of GCs than non-pregnant ones (Ziegler and Snowdon 1995; Gardiner and Hall 1997; Cavigelli 1999;  
365 Weingrill *et al.* 2004; Dantzer *et al.* 2010; Fanson *et al.* 2012). Gardiner and Hall (1997) obtained a significant  
366 difference in plasma cortisol concentrations between males and females in harbour seal (*Phoca vitulina*) within the  
367 reproductive period. Weingrill *et al.* (2004) observed that faecal cortisol levels in free-ranging female chacma baboons  
368 (*Papio hamadryas ursinus*) were significantly higher in females than in males. Dantzer *et al.* (2010) showed that a  
369 reproduction-related condition (i.e. pregnancy, lactation, post-lactation) significantly affected FCM levels in free-  
370 ranging female North American red squirrels. Fanson *et al.* (2012) observed that males and females in Canada lynx  
371 showed differing seasonal pattern in FGM concentrations. In males, FCMs peaked during the breeding season and then  
372 decreased during summer. Conversely, FCMs in females were lower in winter/early spring and increased toward the  
373 end of the breeding season. Thus, in general, the increased metabolic demands associated with reproduction could be  
374 driving GC concentrations during reproductive phases (Cavigelli 1999; Goymann *et al.* 1999; Palme *et al.* 2003)  
375 leading to higher cortisol concentrations in females. In our case, we did not know the reproductive status of each female  
376 monitored as no hormonal analysis was performed. However, no evident signs of reproduction were detected. To sum  
377 up, therefore, we could say that the difference in cortisol secretion between males and females might be related to both  
378 physiological and individual behavioural characteristics.

## 379 **5. Conclusions**

380 The results presented in this study revealed that wildcats showed significantly higher HCCs than feral individuals, and  
381 no significant difference in terms of cortisol levels were obtained between sexes in either population. We are aware that  
382 this research presents some limitations in terms of reduced sample size, lack of information regarding the individuals'  
383 health and reproductive status and the higher number of males sampled compared to females in both groups, which may  
384 have biased the lack of significance of some results achieved. Nevertheless, despite such limitations, this is the first time  
385 in which hair cortisol levels have been compared between wild and feral cats, thus increasing the limited information  
386 available regarding the physiological response of felids exposed to different environmental pressures. The findings  
387 presented may thus contribute to laying the foundation for future works focused in assessing the various physiological  
388 and ecological factors affecting the HPA gland axis activity of those populations living under a range of environmental  
389 conditions, thus leading to the establishment of adequate conservation plans toward those species (or subspecies) which  
390 are classified as endangered or critically endangered.

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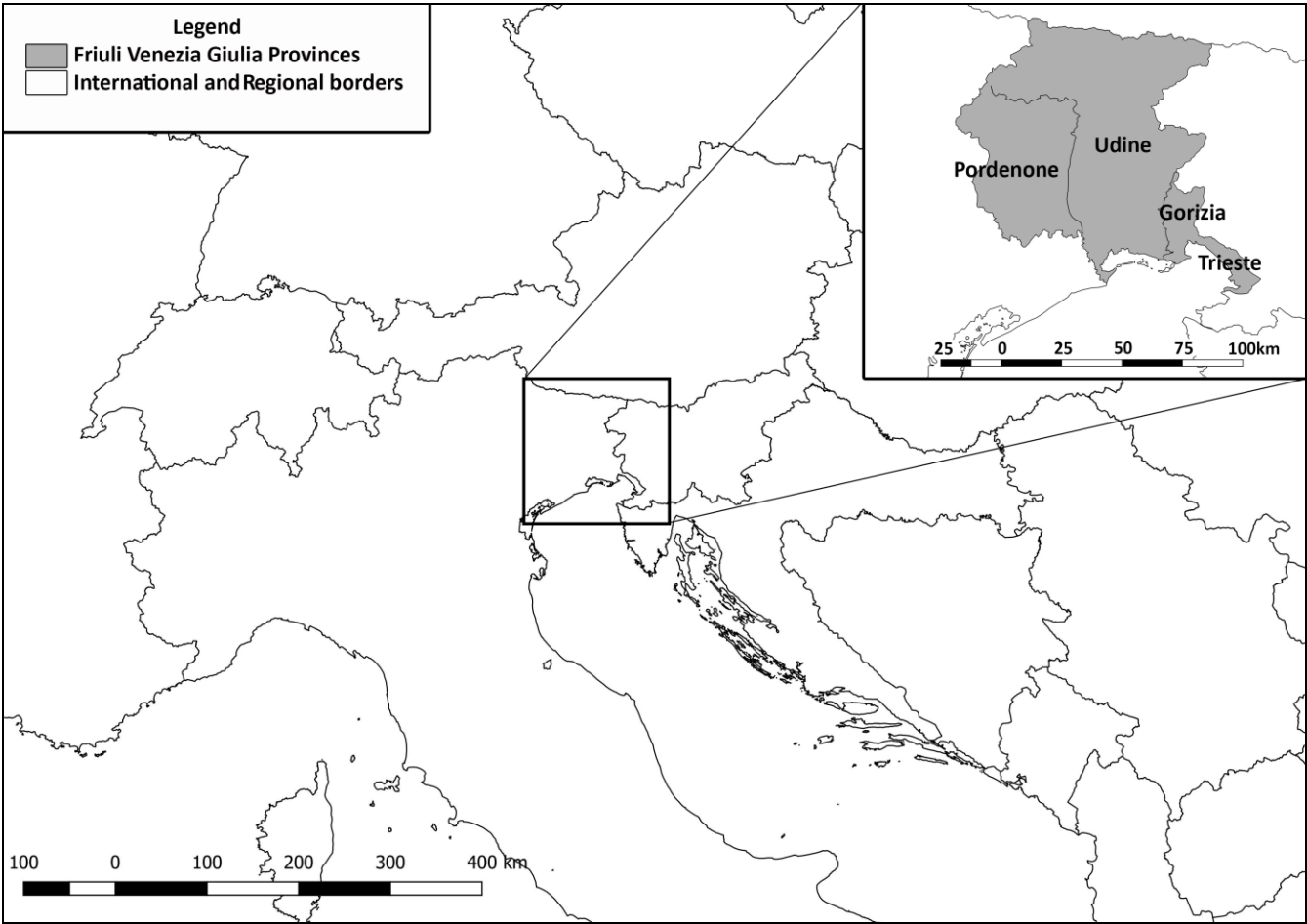
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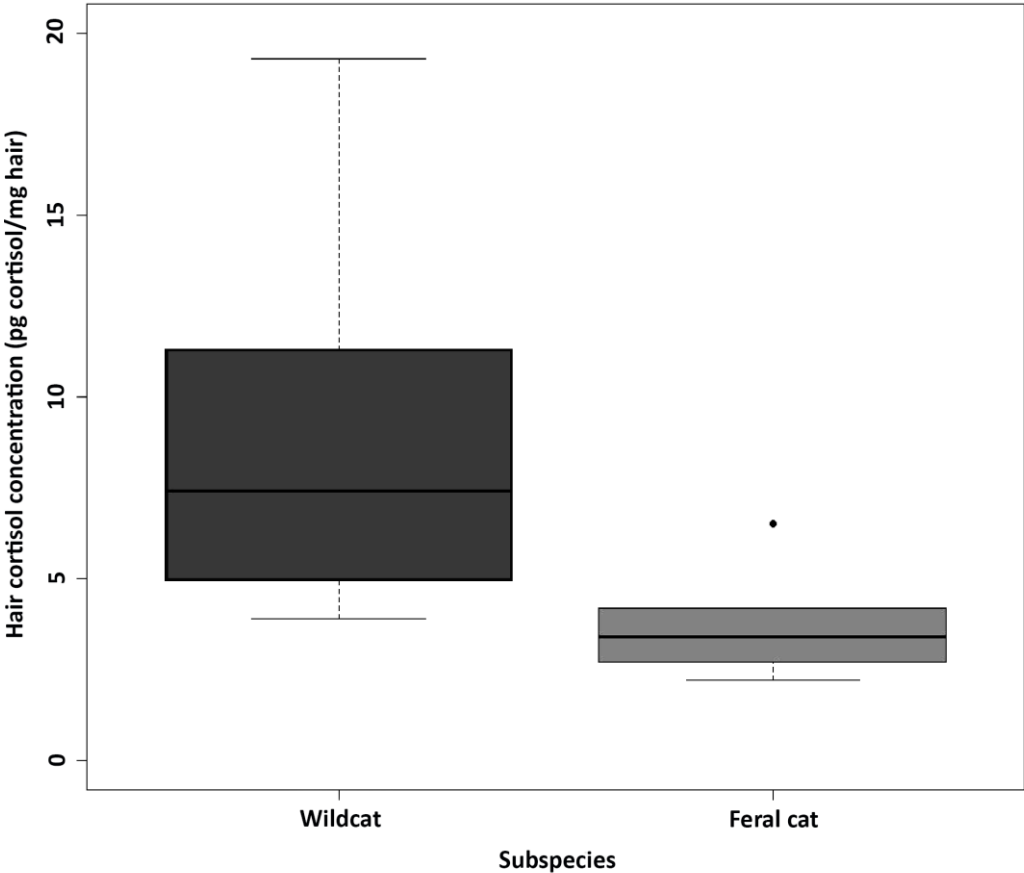
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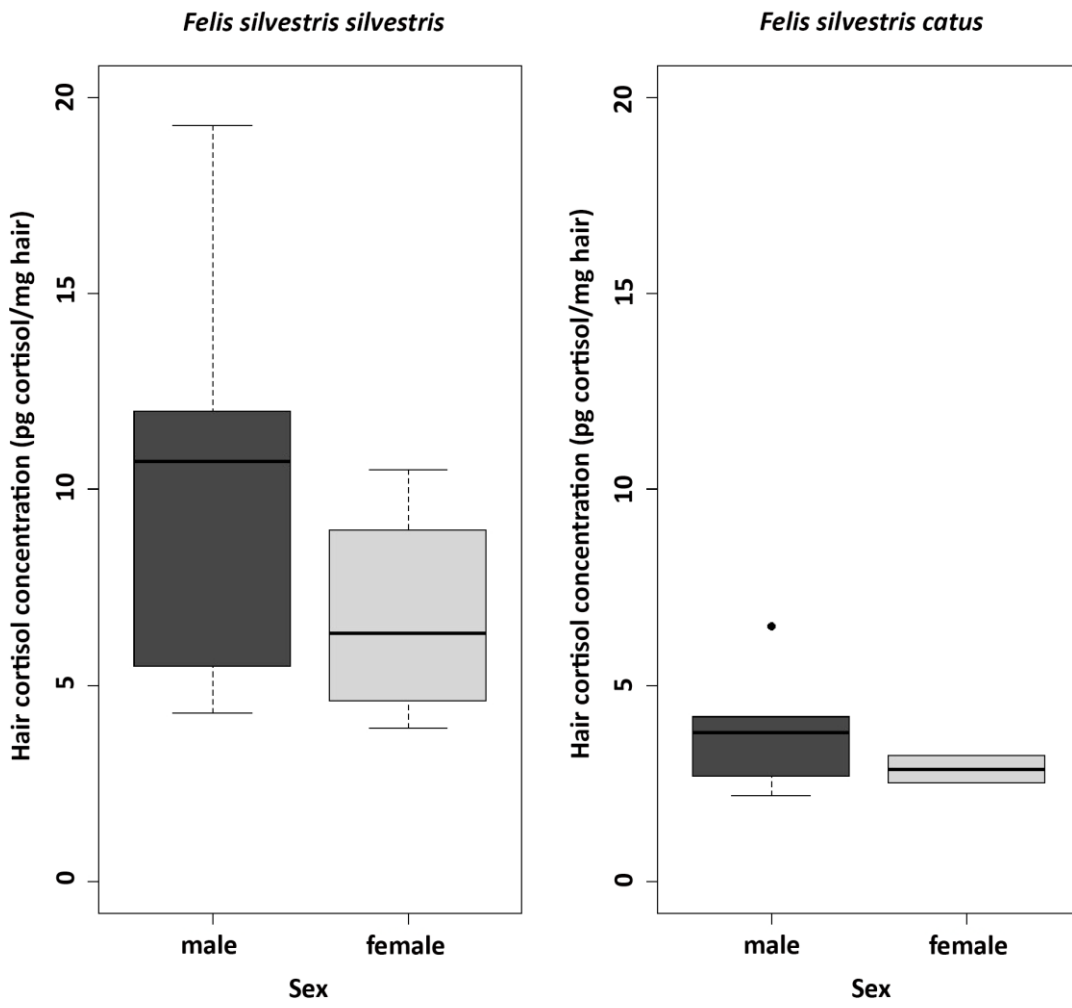
**Fig1.** Location of the study area.



**Fig2.** Box-plot showing the difference in terms of hair cortisol concentration (pg cortisol/mg hair) between subspecies.



**Fig3.** Box-plot showing the difference in terms of hair cortisol levels (pg cortisol/mg hair) between sexes in wild (*Felis silvestris silvestris*) and feral (*Felis silvestris catus*) cats.



**Tab1.** Generalized Linear Models (GLMs) ranking with the best model marked in bold. Abbreviations:  $K$  = number of parameters;  $\log\text{Lik}$  =log-likelihood;  $\text{AICc}$  = Akaike’s Information Criterion corrected;  $\omega_i$  = Akaike’s weight.

ID model	Independent variables	$K$	-2 $\log\text{Lik}$	$\text{AICc}$	$\Delta\text{AICc}$	$\omega_i$
<b>1</b>	<b>subspecies</b>	<b>3</b>	<b>119.02</b>	<b>126.16</b>	<b>0</b>	<b>0.70</b>
2	subspecies, sex	5	114.67	127.83	1.67	0.30
3	subspecies, sex, subspecies:sex	9	108.65	138.65	12.49	0.00
4	sex	3	138.04	145.18	19.02	0.00

**Tab2.** Hair cortisol concentration (pg cortisol/mg hair) comparison between subspecies.  $n$  = number of individuals.

Subspecies	n	n (%)	Mean	SD	Median
Wildcat	15	60	8.91	4.48	7.40
Feral cat	10	40	3.57	1.25	3.39

**Tab3.** Hair cortisol levels (pg cortisol/mg hair) comparison between sexes in wild (*Felis silvestris silvestris*) and feral (*Felis silvestris catus*) cats.  $n$  = number of individuals.

Subspecies	Sex	n	n (%)	Mean	SD	Median
<i>Felis silvestris silvestris</i>	Male	11	73.33	9.69	4.81	10.70
	Female	4	26.67	6.78	2.87	6.35
<i>Felis silvestris catus</i>	Male	8	80	3.76	1.33	3.82
	Female	2	20	2.81	0.50	2.81