

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 ± 4.48 pg/mg) than feral ones ($n = 10$, mean \pm sd = 3.57 ± 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.

41 **Acknowledgments**

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43 Udine, and Trieste for the hair samples provided. Furthermore, the authors are indebted to all the wildlife technicians,
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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 20th 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 γ -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 μ 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 μ 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 ± 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 ± 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 ± 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 ± 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 ± 4.81 pg
347 cortisol/mg hair for males; mean \pm sd = 6.78 ± 2.87 pg cortisol/mg hair for females) than in feral ones (mean \pm sd =
348 3.76 ± 1.33 pg cortisol/mg hair for males; mean \pm sd = 2.81 ± 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.

391 **6. References**

392 Accorsi PA, Carloni E, Valsecchi P, Viaggaini R, Gamberoni M, Tamanini C, Seren E (2008) Cortisol determination in
393 hair and faeces from domestic cats and dogs. Gen Comp Endocrinol 155:398-402. doi:

394 10.1016/j.ygcen.2007.07.002

395 Angelini P, Augello R, Bagnaia R, Bianco P, Capogrossi R, Cardillo A, Ercole S, Francescato C, Giacanelli V, Laureti
396 L, Luger F, Luger N, Novellino E, Oriolo G, Papallo O, Serra B (2009) Il Progetto Carta della Natura. Linee
397 guida per la cartografia e la valutazione degli habitat alla scala 1:50.000
398 <http://www.isprambiente.gov.it/files/carta-della-natura/cdn-manuale.pdf>

399 Anile S, Bizzarri L, Lacrimini M, Sforzi A, Ragni B, Devillard S (2017) Home-range size of the European wildcat
400 (*Felis silvestris silvestris*): a report from two areas in Central Italy. *Mammalia* 82(1):1-11. doi:
401 10.1515/mammalia-2016-0045

402 Apostolico F, Vercillo F, La Porta G, Ragni B (2016) Long-term changes in diet and trophic niche of the European
403 wildcat (*Felis silvestris silvestris*) in Italy. *Mammal Res* 61:109-119. doi: 10.1007/s13364-015-0255-8

404 Ashley NT, Barboza PS, Macbeth BJ, Janz DM, Cattet MRL, Both RK, Wasser SK (2011) Glucocorticosteroid
405 concentrations in feces and hair of captive caribou and reindeer following adrenocorticotrophic hormone challenge.
406 *Gen Comp Endocrinol* 172:382-391. doi: 10.1016/j.ygcen.2011.03.029

407 Balíková M (2005) Hair analysis for drugs of abuse. Plausibility of interpretation. *Biomed Pap Med Fac Univ Palacky*
408 *Olomouc Czech Repub* 149:199-207. doi: 10.5507/bp.2005.026

409 Ballesteros-Duperón E, Virgós E, Moleón M, Barea-Azcón JM, Gil-Sánchez JM (2015) How accurate are coat traits for
410 discriminating wild and hybrid forms of *Felis silvestris*? *Mammalia* 79:101-110. doi: 10.1515/mammalia-2013-
411 0026

412 Barja I, Silván G, Rosellini S, Piñeiro A, González-Gil A, Camacho L, Illera JC (2007) Stress physiological responses
413 to tourist pressure in a wild population of European pine marten. *J Steroid Biochem Mol Biol* 104:136-142. doi:
414 10.1016/j.jsbmb.2007.03.008

415 Bayazit V (2009) Evaluation of Cortisol and Stress in Captive Animal. *Aust J Basic Appl Sci* 3:1022-1031.
416 <https://pdfs.semanticscholar.org/3f05/c6638addc7dcaa243cf74061969a9fca3854.pdf>

417 Bechshøft TØ, Derocher AE, Richardson E, Mislan P, Lunn NJ, Sonne C, Dietz R, Janz DM, St Louis VL (2015)
418 Mercury and cortisol in Western Hudson Bay polar bear hair. *Ecotoxicology* 24:1315-1321. doi: 10.1007/s10646-
419 015-1506-9

420 Bechshøft TØ, Rigét FF, Sonne C, Letcher RJ, Muir DCG, Novak MA, Henchey E, Meyer JS, Eulaers I, Jaspers VLB,
421 Eens M, Covaci A, Dietz R (2012) Measuring environmental stress in East Greenland polar bears, 1892–1927 and
422 1988–2009: What does hair cortisol tell us? *Environ Int* 45:15-21. doi: 10.1016/j.envint.2012.04.005

423 Bechshøft TØ, Sonne C, Dietz R, Born EW, Novak MA, Henchey E, Meyer JS (2011) Cortisol levels in hair of East
424 Greenland polar bears. *Sci Total Environ* 409:831-834. doi: 10.1016/j.scitotenv.2010.10.047

425 Berger EM, Leus K, Vercammen P, Schwarzenberger F (2006) Faecal steroid metabolites for non-invasive assessment
426 of reproduction in common warthogs (*Phacochoerus africanus*), red river hogs (*Potamochoerus porcus*) and
427 babirusa (*Babirusa babirusa*). *Anim Reprod Sci* 91:155-171. doi: 10.1016/j.anireprosci.2005.03.009

428 Boonstra R (2004) Coping with Changing Northern Environments: The Role of the Stress Axis in Birds and Mammals.
429 *Integr Comp Biol* 44:95-108. doi: 10.1093/icb/44.2.95

430 Burbonnais ML, Nelson TA, Cattet MR, Darimont CT, Stenhouse GB (2013) Spatial Analysis of Factors Influencing
431 Long-Term Stress in the Grizzly Bear (*Ursus arctos*) Population of Alberta, Canada. *PLoS ONE* 8(12):e83768.
432 doi: 10.1371/journal.pone.0083768

433 Brown JL, Wildt DE (1997) Assessing reproductive status in wild felids by non-invasive faecal steroid monitoring. *Int*
434 *Zoo Yh* 35:173-191 doi: 10.1111/j.1748-1090.1997.tb01208.x

- 435 Bryan HM, Darimont CT, Paquet PC, Wynne-Edwards KE, Smits JEG (2014) Stress and reproductive hormones reflect
436 inter-specific social and nutritional conditions mediated by resource availability in a bear-salmon system. *Conserv*
437 *Physiol* 2:1-18. doi: 10.1093/conphys/cou010
- 438 Carta della Natura del Friuli Venezia Giulia (2007). Retrieved 27 February 2019 from
439 <http://irdat.regione.fvg.it/WebGIS/>.
- 440 Caslini C, Comin A, Peric T, Prandi A, Pedrotti L, Mattiello S (2016) Use of hair cortisol analysis for comparing
441 population status in wild red deer (*Cervus elaphus*) living in areas with different characteristics. *Eur J Wildl Res*
442 62:713-723. doi: 10.1007/s10344-016-1049-2
- 443 Cattet M, Macbeth BJ, Janz DM, Zedrosser A, Swenson JE, Dumond M, Stenhouse GB (2014) Quantifying long-term
444 stress in brown bears with the hair cortisol concentration: a biomarker that may be confounded by rapid changes
445 in response to capture and handling. *Conserv Physiol* 2:1-15. doi: 10.1093/conphys/cou026
- 446 Cavigelli SA (1999) Behavioural patterns associated with faecal cortisol levels in free-ranging female ring-tailed
447 lemurs, *Lemur catta*. *Anim Behav* 57:935-944. doi: 10.1006/anbe.1998.1054
- 448 Comin A, Peric T, Magrin L, Corazzin M, Cornacchia G, Prandi A (2014) Study of progesterone and cortisol
449 concentrations in the Italian Friesian claw. *J Dairy Sci* 97:5491-5496. doi: 10.3168/jds.2014-7943
- 450 Comin A, Peric T, Corazzin M, Veronesi MC, Meloni T, Zufferli V, Cornacchia G, Prandi A (2013) Hair cortisol as a
451 marker of hypothalamic-pituitary-adrenal axis activation in Friesian dairy cows clinically or physiologically
452 compromised. *Livest Sci* 152:36-41. doi: 10.1016/j.livsci.2012.11.021
- 453 Comin A, Prandi A, Peric T, Corazzin M, Dovier S, Bovolenta S (2011) Hair cortisol levels in dairy cows from winter
454 housing to summer highland grazing. *Livest Sci* 138:69-73. doi: 10.1016/j.livsci.2010.12.009
- 455 Creel S, Christianson D, Schuette P (2013) Glucocorticoid stress responses of lions in relationship to group
456 composition, human land use, and proximity to people. *Conserv Physiol* 1:1-9. doi: 10.1093/conphys/cot021
- 457 Dantzer B, McAdam AG, Palme R, Fletcher QE, Boutin S, Humphries MM, Boonstra R (2010) Fecal cortisol
458 metabolite levels in free-ranging North American red squirrels: Assay validation and the effects of reproductive
459 condition. *Gen Comp Endocrinol* 167:279-286. doi: 10.1016/j.ygcen.2010.03.024
- 460 Darlington DN, Chew G, Ha T, Keil LC, Dallman MF (1990) Corticosterone, but not Glucose, Treatment Enables
461 Fasted Adrenalectomized Rats to Survive Moderate Hemorrhage. *Endocrinology* 127(2):766-772. doi:
462 10.1210/endo-127-2-766
- 463 Davenport MD, Tiefenbacher S, Lutz CK, Novak MA, Meyer JS (2006) Analysis of endogenous cortisol concentrations
464 in the hair of rhesus macaques. *Gen Comp Endocrinol* 147:255-261. doi: 10.1016/j.ygcen.2006.01.005
- 465 David M, Auclair Y, Cézilly F (2011) Personality predicts social dominance in female zebra finches, *Taeniopygia*
466 *guttata*, in a feeding context. *Anim Behav* 81:219-224. doi: 10.1016/j.anbehav.2010.10.008
- 467 Dehnhard M, Clauss M, Lechner-Doll M, Meyer HHD, Palme R (2001) Noninvasive monitoring of adrenocortical
468 activity in roe deer (*Capreolus capreolus*) by measurement of fecal cortisol metabolites. *Gen Comp Endocrinol*
469 123:111-120. doi: 10.1006/gcen.2001.7656
- 470 Deng H, Jin X, Hn D (2014) Fecal cortisol content of wild giant pandas (*Ailuropoda melanoleuca*) to monitor human
471 disturbance level in natural habitats. 64:75-86. doi: 10.1163/15707563-00002432
- 472 Dingemans NJ, Réale D (2005) Natural selection and animal personality. *Behaviour* 142:1159-1184. doi:
473 10.1163/156853905774539445
- 474 Driscoll C, Nowell K (2010) *Felis silvestris*. The IUCN Red List of Threatened Species. Version 2014.2.
475 <http://www.iucnredlist.org/details/8543/0>. Accessed on 04 November 2019

- 476 Fanson KW, Wielebnowski NC, Shenk TM, Lucas JR (2012) Comparative patterns of adrenal activity in captive and
477 wild Canada lynx (*Lynx canadensis*). *J Comp Physiol B* 182:157-165. doi: 10.1007/s00360-011-0597-8
- 478 Fourie NH, Turner TR, Brown JL, Pampush JD, Lorenz JG, Bernstein RM (2015) Variation in vervet (*Chlorocebus*
479 *aethiops*) hair cortisol concentrations reflects ecological disturbance by humans. *Primates* 56:365-373. doi:
480 10.1007/s10329-015-0486-y
- 481 Gardiner KJ, Hall AJ (1997) Diel and annual variation in plasma cortisol concentrations among wild and captive harbor
482 seals (*Phoca vitulina*). *Can J Zool* 75:1773-1780. doi: 10.1139/z97-806
- 483 Goymann W (2012) On the use of non-invasive hormone research in uncontrolled, natural environments: the problem
484 with sex, diet, metabolic rate and the individual. *Methods Ecol Evol* 3(4):757-765. doi: 10.1111/j.2041-
485 210X.2012.00203.x
- 486 Goymann W (2005) Non-invasive Monitoring of Hormones in Bird Droppings Physiological Validation, Sampling,
487 Extraction, Sex Differences, and the Influence of Diet on Hormone Metabolite Levels. *Ann NY Acad Sci* 1046:
488 35-53. doi: 10.1196/annals.1343.005
- 489 Goymann W, Möstl E, Van't Hof T, East ML, Hofer H (1999) Non-invasive Fecal Monitoring of Glucocorticoids in
490 Spotted Hyenas, *Crocuta crocuta*. *Gen Comp Endocr* 114:340-348. doi: 10.1006/gcen.1999.7268
- 491 Graham L, Schwarzenberger F, Möstl E, Galama W (2001) A Versatile Enzyme Immunoassay for the Determination of
492 Progesterogens in Feces and Serum. *Zoo Biol* 20:227-236. doi: 10.1002/zoo.1022
- 493 Gygas L, Neuffer I, Kaufmann C, Hauser R, Wechsler B (2006) Milk Cortisol Concentration in Automatic Milking
494 Systems Compared with Auto-Tandem Milking Parlors. *J Dairy Sci* 89:3447-3454. doi: 10.3168/jds.S0022-
495 0302(06)72382-7
- 496 Hajamor S, Despre J, Couillard C, Lemieux S, Tremblay A, Prud'homme D, Tchernof A (2003) Relationship Between
497 Sex Hormone-Binding Globulin Levels and Features of the Metabolic Syndrome. *Metabolism* 52(6):724-730.
498 doi: 10.1016/S0026-0495(03)00066-0
- 499 Hamilton LD, Rellini AH, Meston CM (2008) Cortisol, Sexual Arousal, and Affect in Response to Sexual Stimuli. *J*
500 *Sex Med* 5:2111-2118. doi: 10.1111/j.1743-6109.2008.00922.x
- 501 Heistermann M, Palme R, Ganswindt A (2006) Comparison of Different Enzymeimmunoassays for Assessment of
502 Adrenocortical Activity in Primates Based on Fecal Analysis. *Am J Primatol* 68:257-273. doi: 10.1002/ajp
- 503 Huber S, Palme R, Arnold W (2003) Effects of season, sex, and sample collection on concentrations of fecal cortisol
504 metabolites in red deer (*Cervus elaphus*). *Gen Comp Endocrinol* 130:48-54. doi: 10.1016/S0016-6480(02)00535-
505 X
- 506 Janczak AM, Pedersen LJ, Bakken M (2003) Aggression, fearfulness and coping styles in female pigs. *Appl Anim*
507 *Behav Sci* 81:13-28. doi: 10.1016/S0168-1591(02)00252-6
- 508 Jędrzejewski W, Jędrzejewska B (1992) Foraging and Diet of the Red Fox *Vulpes vulpes* in Relation to Variable Food
509 Resources in Białowieża National Park, Poland. *Ecography* 15(2):212-220. doi: 10.1111/j.1600-
510 0587.1992.tb00027.x
- 511 Keay JM, Singh J, Ph D, Gaunt MC (2006) Fecal glucocorticoids and their metabolites as indicators of stress in various
512 mammalian species : A literature review. *J Zoo Wildlife Med* 37(3): 234-244. doi: 10.1638/05-050.1
- 513 Killshaw K (2011) Scottish wildcats. Scottish Natural Heritage Publishing, Battleby.
- 514 Kintz P, Villain M, Cirimele V (2006) Hair analysis for drug detection. *Ther Drug Monit* 28:442-446. doi:
515 10.1097/01.ftd.0000211811.27558.b5
- 516 Klar N, Fernández N, Kramer-Shadt S, Herrmann M, Trinzen M, Büttner I, Niemitz C (2008) Habitat selection models

517 for European wildcat conservation. *Biol Conserv* 141:308-319. doi:10.1016/j.biocon.2007.10.004

518 Koolhaas JM, Korte SM, De Boer SF, Van Der Vegt BJ, Van Reenen CG, Hopster H, De Jong IC, Ruis MAW,
519 Blokhuis HJ (1999) Coping styles in animal: Current status in behavior and stress-physiology. *Neurosci Biobehav*
520 *Rev* 23:925-935. doi: 10.1016/S0149-7634(99)00026-3

521 Koren L, Mokady O, Karaskov T, Klein J, Koren G, Geffen E (2002) A novel method using hair for determining
522 hormonal levels in wildlife. *Anim Behav* 63:403-406. doi: 10.1006/anbe.2001.1907

523 Lafferty DJR, Laudenslager ML, Mowat G, Heard D, Belant JL (2015) Sex, diet, and the social environment: Factors
524 influencing hair cortisol concentration in free-ranging black bears (*Ursus americanus*). *PLoS One* 10(11):
525 e0141489. doi: 10.1371/journal.pone.0141489

526 Lapini L (2006) Attuale Distribuzione del Gatto Selvatico *Felis silvestris silvestris* Schreber, 1775 nell'Italia Nord-
527 Orientale (Mammalia: *Felidae*). *Boll Mus civ St nat Venezia* 57:221-234.
528 [https://www.researchgate.net/profile/Luca_Lapini2/publication/272096191_LAPINI_L_2006_A_Attuale_distribuzione_del_gatto_selvatico_Felis_silvestris_silvestris_SCHREBER_1775_nell'Italia_nord-](https://www.researchgate.net/profile/Luca_Lapini2/publication/272096191_LAPINI_L_2006_A_Attuale_distribuzione_del_gatto_selvatico_Felis_silvestris_silvestris_SCHREBER_1775_nell'Italia_nord-orientale_Mammalia_Felidae_Boll_Mus_civ_St_nat_Venezia_57_221-234/links/54db2b640cf2ba88a68f4f04.pdf)
529 [orientale_Mammalia_Felidae_Boll_Mus_civ_St_nat_Venezia_57_221-234/links/54db2b640cf2ba88a68f4f04.pdf](https://www.researchgate.net/profile/Luca_Lapini2/publication/272096191_LAPINI_L_2006_A_Attuale_distribuzione_del_gatto_selvatico_Felis_silvestris_silvestris_SCHREBER_1775_nell'Italia_nord-orientale_Mammalia_Felidae_Boll_Mus_civ_St_nat_Venezia_57_221-234/links/54db2b640cf2ba88a68f4f04.pdf)

530 Leboulenger F, Delarue C, Belanger A, Perroteau I, Netchitailo P, Leroux P, Jegou S, Tonon MC, Vaudry H (1982)
531 Direct Radioimmunoassays for Plasma Corticosterone and Aldosterone in Frog. I. Validation of the Methods and
532 Evidence for Daily Rhythms in a Natural Environment. *Gen Comp Endocr* 46:521-532. doi: 10.1016/0016-
533 6480(82)90108-3

534

535 Lozano J, Malo AF (2012) Conservation of European wildcat (*Felis silvestris*) in Mediterranean environments: a
536 reassessment of current threats. In: Williams GS (ed) *Mediterranean ecosystems: dynamics, management and*
537 *conservation*. Nova Science Publishers, Hauppauge, NY, pp 1-31.

538 Lozano J (2010) Habitat use by European wildcats (*Felis silvestris*) in central Spain: what is the relative importance of
539 forest variables? *Anim Biodiv Conserv* 33(2):143-150.
540 https://www.researchgate.net/publication/49592464_Habitat_use_by_European_wildcats_Felis_silvestris_in_central_Spain_what_is_the_relative_importance_of_forest_variables

541

542 Lozano J, Virgós E, Malo AF, Huertas DL, Casanovas JG (2003) Importance of scrub-pastureland mosaics for
543 wildliving cats occurrence in a Mediterranean area: implications for the conservation of the wildcat (*Felis*
544 *silvestris*). *Biodiv Conserv* 12: 921-935. doi: 10.1023/A:1022821708594

545 Lucherini M, Lovari S, Crema G (1995) Habitat use and ranging behaviour of the red fox *Vulpes vulpes* in a
546 Mediterranean rural area: is shelter availability a key factor? *J Zool* 237:577-591. doi: 10.1111/j.1469-
547 7998.1995.tb05016.x

548 Macbeth BJ, Cattet MRL, Stenhouse GB, Gibeau ML, Janz DM (2010) Hair cortisol concentration as a noninvasive
549 measure of long-term stress in free-ranging grizzly bears (*Ursus arctos*): considerations with implications for
550 other wildlife. *Can J Zool* 88:935-949. doi: 10.1139/Z10-057

551 Mattucci F, Oliveira R, Bizzarri L, Vercillo F, Anile S, Ragni B, Lapini L, Sforzi A, Alves PC, Lyons LA, Randi E
552 (2013) Genetic structure of wildcat (*Felis silvestris*) populations in Italy. *Ecol Evol* 3:2443-2458. doi:
553 10.1002/ece3.569

554 Millspaugh JJ, Washburn BE, Milanick MA, Beringer J, Hansen LP, Meyer TM (2002) Non-Invasive Techniques for
555 Stress Assessment in White-Tailed Deer. *Source Wildl Soc Bull* 30(3):899-907. doi: 10.2307/3784245

556 Montiglio PO, Garant D, Pelletier F, Réale D (2012) Personality differences are related to long-term stress reactivity in
557 a population of wild eastern chipmunks, *Tamias striatus*. *Anim Behav* 84:1071-1079. doi:

558 10.1016/j.anbehav.2012.08.010

559 Mormède P, Andanson S, Aupérin B, Beerda B, Guémené D, Malmkvist J, Manteca X, Manteuffel G, Prunet P, van
560 Reenen CG, Richard S, Vaissier I (2007) Exploration of the hypothalamic-pituitary-adrenal function as a tool to
561 evaluate animal welfare. *Physiol Behav* 92:317-339. doi: 10.1016/j.physbeh.2006.12.003

562 Möstl E, Palme R (2002) Hormones as indicators of stress. *Domest Anim Endocrinol* 23:67-74. doi: 10.1016/S0739-
563 7240(02)00146-7

564 Naidenko SV, Berezhnoi MA, Kumar V, Umapathy G (2019) Comparison of tigers' fecal glucocorticoids level in two
565 extreme habitats. *PLoS ONE* 14(4): e0214447. doi: 10.1371/journal.pone.0214447

566 Naidenko SV, Ivanov EA, Lukarevskii VS, Hernandez-Balanco JA, Sorokin PA, Litvinov MN, Kotlyar AK, Rozhnov
567 VV (2011) Activity of the Hypothalamic-Pituitary-Adrenal Axis in the Siberian Tiger (*Panthera tigris altaica*) in
568 Captivity and in The Wild, and Its Dynamics throughout the Year. *Biol Bull Russ Acad Sci* 38(3):301-305. doi:
569 10.1134/S1062359011030095

570 Narayan EJ, Parnell T, Clark G, Martin-Vegue P, Mucci A, Hero JM (2013) Faecal cortisol metabolites in Bengal
571 (*Panthera tigris tigris*) and Sumatran tigers (*Panthera tigris sumatrae*). *Gen Comp Endocr* 194:318-325. doi:
572 10.1016/j.ygcen.2013.10.002

573 Natoli E, Say L, Cafazzo S, Bonanni R, Schmidt M, Pontier D (2005) Bold attitude makes male urban feral domestic
574 cats more vulnerable to Feline Immunodeficiency Virus. *Neurosci Biobehav Rev* 29:151-157. doi:
575 10.1016/j.neubiorev.2004.06.011

576 Natoli E (1994) Urban feral cats (*Felis catus* L.): perspectives for a demographic control respecting the psycho-
577 biological welfare of the species. *Ann Ist Super Sanità* 30(2):223-227.
578 [https://www.researchgate.net/profile/Eugenia_Natoli/publication/15367635_Urban_feral_cats_Felis_catus_L_per](https://www.researchgate.net/profile/Eugenia_Natoli/publication/15367635_Urban_feral_cats_Felis_catus_L_per_spectives_for_a_demographic_control_respecting_the_psycho-biological_welfare_of_the_species/links/55117cb20cf21209d528a8ae.pdf)
579 [spectives_for_a_demographic_control_respecting_the_psycho-](https://www.researchgate.net/profile/Eugenia_Natoli/publication/15367635_Urban_feral_cats_Felis_catus_L_per_spectives_for_a_demographic_control_respecting_the_psycho-biological_welfare_of_the_species/links/55117cb20cf21209d528a8ae.pdf)
580 [biological_welfare_of_the_species/links/55117cb20cf21209d528a8ae.pdf](https://www.researchgate.net/profile/Eugenia_Natoli/publication/15367635_Urban_feral_cats_Felis_catus_L_per_spectives_for_a_demographic_control_respecting_the_psycho-biological_welfare_of_the_species/links/55117cb20cf21209d528a8ae.pdf)

581 Negrão JA, Porcionato MA, de Passillé AM, Rushen J (2004) Cortisol in Saliva and Plasma of Cattle After ACTH
582 Administration and Milking. *J Dairy Sci* 87:1713-1718. doi: 10.3168/jds.S0022-0302(04)73324-X

583 Official site of Friuli Venezia Giulia region - English Version (n.d.). Retrived 28 February 2019 from
584 http://www.regione.fvg.it/inglese/pagine_interne/welcome_history.asp.

585 Palme R, Rettenbacher S, Touma C, El-Bahr SM, Möstl E (2005) Comparative Aspects Regarding Metabolism,
586 Excretion, and Noninvasive Measurement in Faecal Samples. *Ann NY Acad Sci* 1040:162-171. doi:
587 10.1196/annals.1327.021

588 Palme R (2005) Measuring fecal steroids: Guidelines for practical application. *Ann N Y Acad Sci* 1040:75-80. doi:
589 10.1196/annals.1343.007

590 Palme R, Touma C, Sachser N, Erich M (2003) Effects of sex and time of day on metabolism and excretion of
591 corticosterone in urine and feces of mice. *130:267-278*. doi: 10.1016/S0016-6480(02)00620-2

592 Palme R, Fisher P, Schildorfer H, Ismail MN (1996) Excretion of infused ¹⁴C-steroid hormones via faeces and urine in
593 domestic livestock. *Anim Reprod Sci* 43:43-46. doi: 10.1016/0378-4320(95)01458-6

594 Peric T, Comin A, Corazzin M, Montillo M, Canavese F, Stebel M, Prandi A (2018) Hair cortisol concentrations in
595 New Zealand white rabbits subjected to surgery. *Anim Welfare* 27:13-20. doi: 10.7120/09627286.27.1.013

596 Peric T, Corazzin M, Romanzin A, Bovolenta S, Prandi A, Montillo M, Comin A (2017) Cortisol and DHEA
597 concentrations in the hair of dairy cows managed indoor or on pasture. *Livest Sci* 202:39-43. doi:
598 10.1016/j.livsci.2017.05.020

- 599 Peric T, Comin A, Corazzin M, Montillo M, Canavese F, Stebel M, Prandi A (2016) Relocation and Hair Cortisol
600 Concentrations in New Zealand White Rabbits. *J Appl Anim Welf Sci* 20(1):1-8. doi:
601 10.1080/10888705.2016.1183489
- 602 Piñeiro A, Barja I, Otero GP, Silván G, Illera JC (2015). No effects of habitat, prey abundance and competitor carnivore
603 abundance on faecal cortisol metabolite levels in wildcats (*Felis silvestris*). *Ann Zool Fennici* 52:90-102. doi:
604 10.5735/086.052.0208
- 605 Piñeiro A, Bárja I, Silván G, Illera JC (2012) Effects of tourist pressure and reproduction on physiological stress
606 response in wildcats: Management implications for species conservation. *Wildl Res* 39:532-539. doi:
607 10.1071/WR10218
- 608 Pragst F, Balikova MA (2006) State of the art in hair analysis for detection of drug and alcohol abuse. *Clin Chim Acta*
609 370:17-49. doi: 10.1016/j.cca.2006.02.019
- 610 Prandi A, Peric T, Corazzin M, Comin A, Colitti M (2018) A first survey on hair cortisol of an Alpine ibex (*Capra ibex*
611 *ibex*) population. *Anim Sci Pap Rep* 36(1):57-74.
612 [https://www.researchgate.net/publication/323705058_A_first_survey_on_hair_cortisol_of_an_alpine_ibex_Capra](https://www.researchgate.net/publication/323705058_A_first_survey_on_hair_cortisol_of_an_alpine_ibex_Capra_ibex_ibex_population)
613 [_ibex_ibex_population](https://www.researchgate.net/publication/323705058_A_first_survey_on_hair_cortisol_of_an_alpine_ibex_Capra_ibex_ibex_population)
- 614 Ragni B, Possenti M (1996) Variability of coat-colour and markings system in *Felis silvestris*. *Ital J Zool* 63:285-292.
615 doi: 10.1080/11250009609356146
- 616 Rangel-Negrín A, Alfaro JL, Valdez RA, Romano MC, Serio-Silva JC (2009) Stress in Yucatan spider monkeys:
617 Effects of environmental conditions on fecal cortisol levels in wild and captive populations. *Anim Conserv*
618 12:496-502. doi: 10.1111/j.1469-1795.2009.00280.x
- 619 Rehbinder C, Hau J (2006) Quantification of cortisol, cortisol immunoreactive metabolites, and immunoglobulin A in
620 serum, saliva, urine, and feces for non-invasive assessment of stress in reindeer. *Can J Vet Res* 70:151-154.
621 [https://www.researchgate.net/publication/7141019_Quantification_of_cortisol_cortisol_immunoreactive_metabol](https://www.researchgate.net/publication/7141019_Quantification_of_cortisol_cortisol_immunoreactive_metabolites_and_immunoglobulin_A_in_serum_saliva_urine_and_feces_for_noninvasive_assessment_of_stress_in_reindeer)
622 [ites_and_immunoglobulin_A_in_serum_saliva_urine_and_feces_for_noninvasive_assessment_of_stress_in_reind](https://www.researchgate.net/publication/7141019_Quantification_of_cortisol_cortisol_immunoreactive_metabolites_and_immunoglobulin_A_in_serum_saliva_urine_and_feces_for_noninvasive_assessment_of_stress_in_reindeer)
623 [eer](https://www.researchgate.net/publication/7141019_Quantification_of_cortisol_cortisol_immunoreactive_metabolites_and_immunoglobulin_A_in_serum_saliva_urine_and_feces_for_noninvasive_assessment_of_stress_in_reindeer)
- 624 Romero LM, Dickens MJ, Cyr NE (2009) Hormones and Behavior The reactive scope model - A new model integrating
625 homeostasis, allostasis, and stress. *Horm Behav* 55:375-389. doi: 10.1016/j.yhbeh.2008.12.009
- 626 Romero LM (2004) Physiological stress in ecology: Lessons from biomedical research. *Trends Ecol Evol* 19:249-255.
627 doi: 10.1016/j.tree.2004.03.008
- 628 Romero LM (2002) Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. *Gen Comp*
629 *Endocr* 128:1-24. doi: 10.1016/S0016-6480(02)00064-3
- 630 Romero LM, Wingfield JC (2001) Regulation of the hypothalamic-pituitary-adrenal axis in free-living pigeons. *J Comp*
631 *Physiol B* 171:231-235. doi: 10.1007/s003600000167
- 632 Roth TL, Brien JKO, Mcrae MA, Bellem AC, Romo SJ, Kroll JL, Brown JL (2001) Ultrasound and endocrine
633 evaluation of the ovarian cycle and early pregnancy in the Sumatran rhinoceros, *Dicerorhinus sumatrensis*.
634 *Reproduction* 121:139-149. doi: 10.1530/reprod/121.1.139
- 635 Ruiz-Gomez MDL, Huntingford FA, Øverli Ø, Thörnqvist PO, Höglund E (2011) Response to environmental change in
636 rainbow trout selected for divergent stress coping styles. *Physiol Behav* 102:317-322. doi:
637 10.1016/j.physbeh.2010.11.023
- 638 Russell E, Koren G, Rieder M, Van Uum S (2012) Hair cortisol as a biological marker of chronic stress: Current status,
639 future directions and unanswered questions. *Psychoneuroendocrinology* 37:589-601. doi:

640 10.1016/j.psyneuen.2011.09.009

641 Sapolsky RM, Romero LM, Munck AU (2000) How Do Glucocorticoids Influence Stress Responses? Preparative
642 Actions. *Endocr Rev* 21:55-89. doi: 10.1210/er.21.1.55

643 Sarmiento P, Cruz J, Tarroso P, Fonseca C (2006) Space and Habitat Selection by Female European Wild Cats (*Felis*
644 *silvestris silvestris*). *Wildl Biol Pract* 2(2):79-89. doi:10.2461/wbp.2006.2.10

645 Schatz S, Palme R (2001) Measurement of Faecal Cortisol Metabolites in Cats and Dogs: A Non-invasive Method for
646 Evaluating Adrenocortical Function. *Vet Res Commun* 25:271-287. doi: 10.1023/A:1010626608498

647 Schell CJ, Young JK, Lonsdorf EV, Mateo JM, Santymire RM (2017) Investigation of techniques to measure cortisol
648 and testosterone concentrations in coyote hair. *Zoo Biol* 36:220-225. doi: 10.1002/zoo.21359

649 Schwarzenberger F (2007) The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species. *Int Zoo*
650 *Yb* 41: 52-74. doi: 10.1111/j.1748-1090.2007.00017.x

651 Schwarzenberger F, Palme R, Bamberg E, Möstl E (1997) A review of faecal progesterone metabolite analysis for non-
652 invasive monitoring of reproductive function in mammals. *Int J Mamm Biol* 62:214-221.
653 [https://www.researchgate.net/publication/266084067_A_review_of_faecal_progesterone_metabolite_analysis_for](https://www.researchgate.net/publication/266084067_A_review_of_faecal_progesterone_metabolite_analysis_for_non-invasive_monitoring_of_reproductive_function_in_mammals)
654 [_non-invasive_monitoring_of_reproductive_function_in_mammals](https://www.researchgate.net/publication/266084067_A_review_of_faecal_progesterone_metabolite_analysis_for_non-invasive_monitoring_of_reproductive_function_in_mammals)

655 Schwarzenberger F, Möstl E, Palme R (1996) Faecal steroid analysis for non-invasive monitor of reproductive status in
656 farm, wild and zoo animals. *Anim Reprod Sci* 42:515-526. doi: 10.1016/0378-4320(96)01561-8

657 Sheriff MJ, Dantzer B, Delehanty B, Palme R, Boonstra R (2011) Measuring stress in wildlife: Techniques for
658 quantifying glucocorticoids. *Oecologia* 166:869-887. doi: 10.1007/s00442-011-1943-y

659 Stradaoli G, Peric T, Montillo M, Comin A, Corazzin M, Veronesi MC, Prandi A (2017) Hair cortisol and testosterone
660 concentrations and semen production of *Bos taurus* bulls. *Ital J Anim Sci* 16(4):631-639. doi:
661 10.1080/1828051X.2017.1303339

662 von der Ohe CG, Servheen C (2002) Measuring stress in mammals using fecal glucocorticoids: opportunities and
663 challenges. *Wildlife Soc B* 30(4):1215-1225. <https://www.jstor.org/stable/pdf/3784291.pdf>

664 Webb E, Thomson S, Nelson A, White C, Koren G, Rieder M, Van Uum S (2010) Assessing individual systemic stress
665 through cortisol analysis of archaeological hair. *J Archaeol Sci* 37:807-812. doi: 10.1016/j.jas.2009.11.010

666 Weingrill T, Gray DA, Barrett L, Henzi SP (2004) Fecal cortisol levels in free-ranging female chacma baboons:
667 relationship to dominance, reproductive state and environmental factors. *Horm Behav* 45:259-269. doi:
668 10.1016/j.yhbeh.2003.12.004

669 Weisser JJ, Hansen M, Björklund E, Sonne C, Dietz R, Styrihave B (2016) A novel method for analysing key
670 corticosteroids in polar bear (*Ursus maritimus*) hair using liquid chromatography tandem massspectrometry. *J*
671 *Chromatogr B* 1017-1018:45-51. doi: 10.1016/j.jchromb.2016.02.029

672 Wielebnowski and Watters (2007) Applying Faecal Endocrine Monitoring to Conservation and Behavior Studies of
673 Wild Mammals: Important Considerations and Preliminary Tests. *Isr J Ecol Evol* 53:439-460. doi:
674 10.1560/IJEE.53.3.439

675 Wikelski M, Cooke SJ (2006) Conservation physiology. *Trends Ecol Evol* 21:38-46. doi: 10.1016/j.tree.2005.10.018

676 Yamaguchi N, Kitchener A, Driscoll C, Nussberger B (2015) *Felis silvestris*. IUCN Red List Threat Species 2015
677 8235:e.T60354712A50652361. doi: 10.2305/IUCN.UK.2015-2.RLTS.T60354712A50652361.en

678 Young KM, Walker SL, Lanthier C, Waddell WT, Monfort SL, Brown JL (2004) Noninvasive monitoring of
679 adrenocortical activity in carnivores by fecal glucocorticoid analysis. *Gen Comp Endocrinol* 137:148-165. doi:
680 10.1016/j.ygcen.2004.02.016

- 681 Ziegler TE, Snowdon CT (1995) The Relationship of Cortisol Levels to Social Environment and Reproductive
682 Functioning in Female Cotton-Top Tamarins, *Saguinus oedipus*. *Horm Behav* 29:407-424. doi:
683 10.1006/hbeh.1995.1028
- 684 Zuur AF, Ieno EN, Walker NJ, Saveliev AA, Smith GM (2009) Mixed effects models and extensions in ecology with R.
685 New York, NY: Springer.
- 686 Zwijacz-Kozica T, Selva N, Barja I, Silván G, Martínez-Fernández L, Illera JC, Jodlowski M (2012) Concentration of
687 faecal cortisol metabolites in chamois in relation to tourist pressure in Tatra National Park (South Poland). *Acta*
688 *Theriol* 58(2):215-222. doi: 10.1007/s13364-012-0108-7

Fig1. Location of the study area.

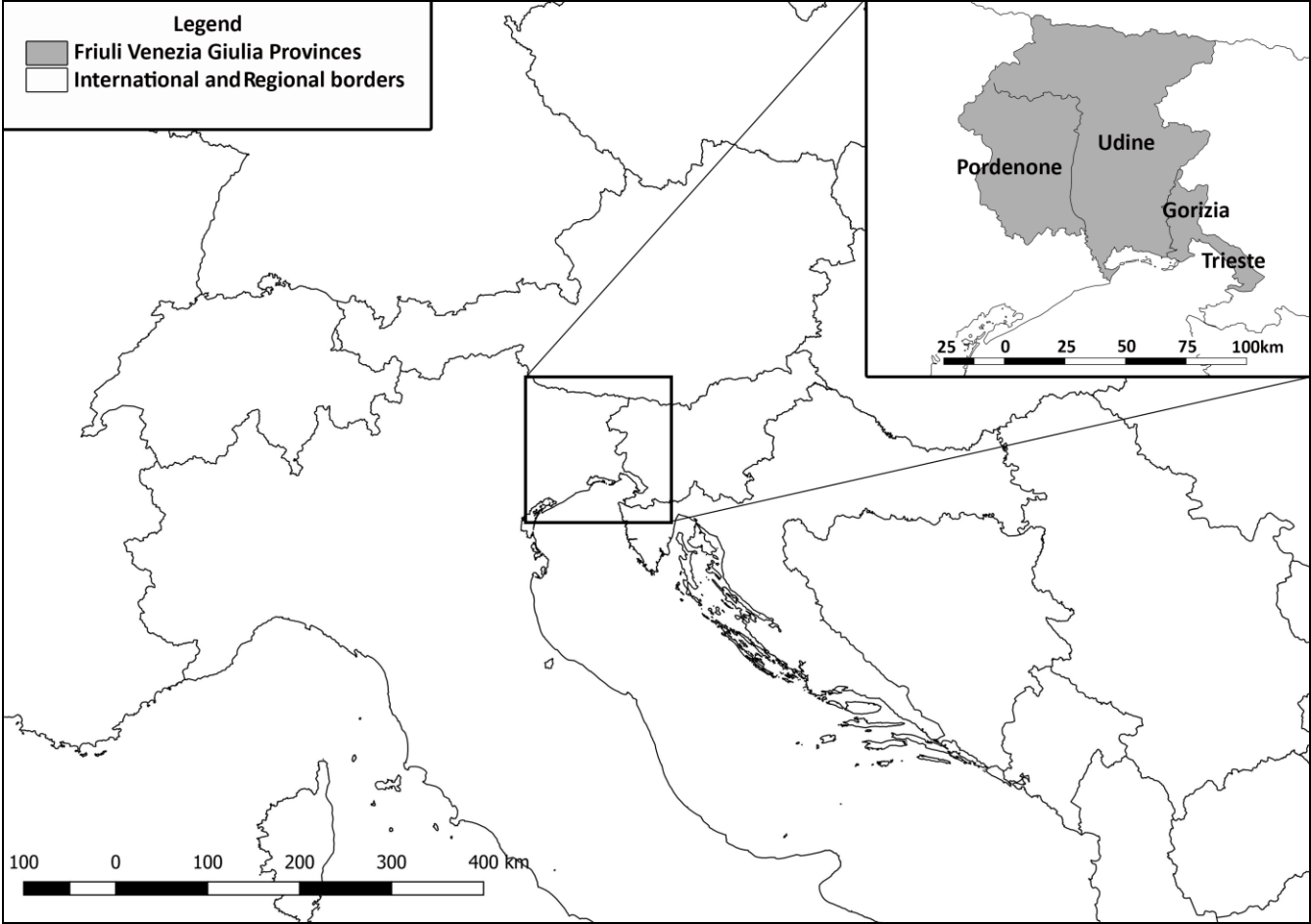


Fig2. Box-plot showing the difference in terms of hair cortisol concentration (pg cortisol/mg hair) between subspecies.

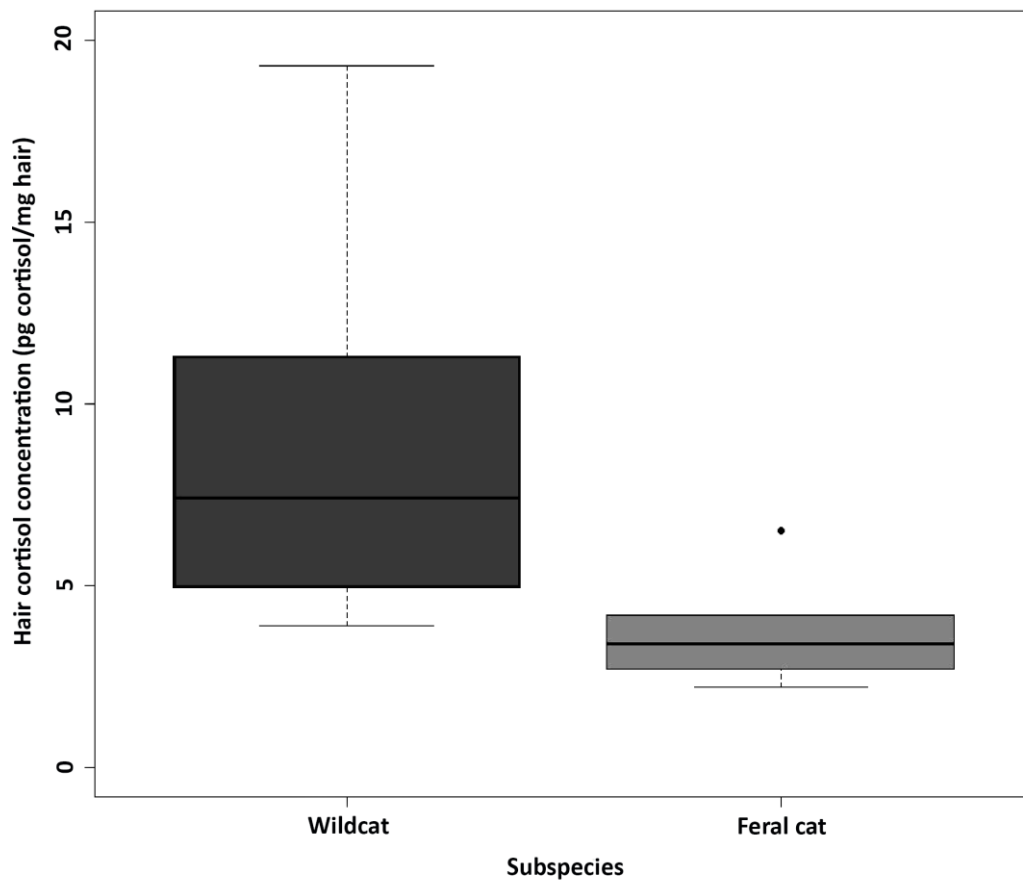
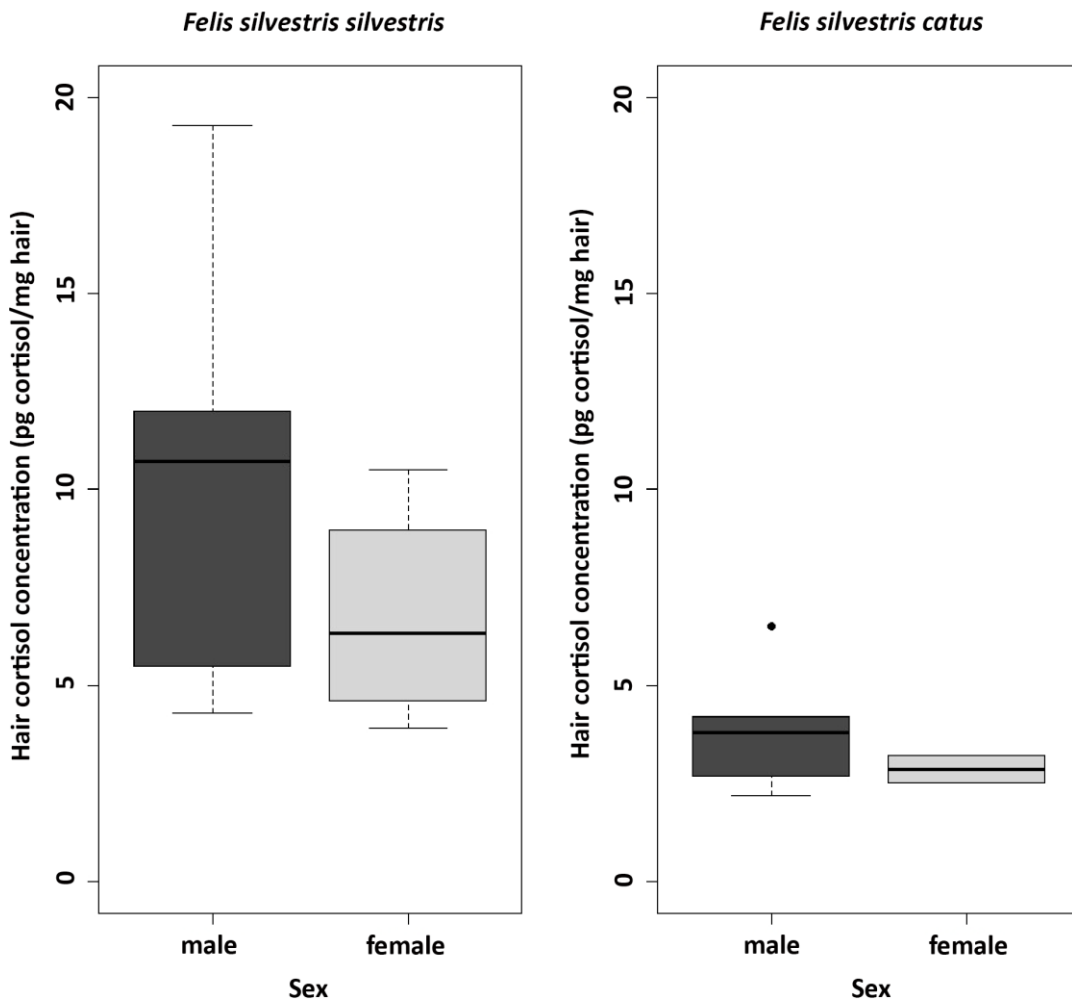


Fig3. Box-plot showing the difference in terms of hair cortisol levels (pg cortisol/mg hair) between sexes in wild (*Felis silvestris silvestris*) and feral (*Felis silvestris catus*) cats.



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

ID model	Independent variables	K	-2 $\log\text{Lik}$	AICc	ΔAICc	ω_i
1	subspecies	3	119.02	126.16	0	0.70
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