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Original

Availability:

This version is available <http://hdl.handle.net/11390/1169643> since 2019-11-11T15:53:32Z

Publisher:

Published

DOI:10.1007/s10344-019-1330-2

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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 ± 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**

42 We wish to thank both the staff working for the Province of Gorizia and the Natural History Museums of Pordenone,
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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1. Introduction

The ability of an organism to adapt to changes in environmental conditions has been receiving increased attention in recent years (Koolhaas *et al.* 1999; Janczak *et al.* 2003; Natoli *et al.* 2005; David *et al.* 2011; Ruiz-Gomez *et al.* 2011; Montiglio *et al.* 2012; Dingemanse and Reale 2018). Abiotic and biotic changes are common within the environment, and animals may respond through temporal variation in their vital rate and/or an alteration in their physiological response (Darlington *et al.* 1990). The activity of the hypothalamic-pituitary-adrenal (HPA) gland axis leads to the release of glucocorticoids (GCs), which are commonly used as indicators of physiological stress (Wingfield and Romero 2001; Wikelski and Cooke 2006). When animals are subjected to a stressor, the hypothalamus releases corticotropin-releasing hormones (CRHs) which signal to the adrenal cortex to release steroid hormones (such as GCs) to overcome stressful situations (Sapolsky *et al.* 2000) and restore homeostasis (Möstl and Palme 2002). This said, prolonged exposure to stressors may lead to chronic GC accumulation leading to chronic stress (Romero 2004) which is detrimental. Chronic stress negatively affects individuals' fitness in terms of the depression of immune responses, reduced reproductive success and growth suppression (Romero 2004). The quantification of glucocorticoid metabolites is a non-invasive tool that provides important information regarding endocrine status, and is a valuable method to study the potential stressors that may affect carnivores under natural conditions (Barja *et al.* 2007; Sheriff *et al.* 2011; Piñeiro *et al.* 2012; Schell *et al.* 2017). Cortisol is the main glucocorticoid hormone of the HPA gland axis and is considered the standard stress indicator for animal welfare (Mormède *et al.* 2007). Indeed, despite the various autonomic and endocrine responses that occur when an organism faces a stressful situation, cortisol has become commonly known as the stress hormone. As part of the stress response, cortisol acts on various metabolic pathways providing energy during a stressful situation. Cortisol's role in the endocrine system is metabolic however, and it is also released in response to arousal situations such as during sexual activity (Hamilton *et al.* 2008). Cortisol levels can be measured using both invasive (e.g. blood) and non-invasive methods in faecal samples (Dehnhard *et al.* 2001; Millspaugh *et al.* 2002; Huber *et al.* 2003; Ashley *et al.* 2011), urine (Rehbinder and Hau 2006), milk (Gygax *et al.* 2006) and saliva (Negrão *et al.* 2004). However, all these methods provide information in relation to short-term cortisol variations (within 12-24 h) (Sheriff *et al.* 2011; Russell *et al.* 2012). Hair and fur have been recognized as a relatively stable matrix that does not decompose as rapidly as other body fluids or tissues (Balíková 2005), and in which the incorporation of blood-borne hormones occurs through their passive diffusion from blood capillaries present on the basement membrane during its active growth phase (Pragst and Balíkova 2006). These hormones accumulate over a period of weeks or months (Davenport *et al.* 2006; Macbeth *et al.* 2010) and may remain detectable for long periods (Kintz *et al.* 2006; Webb *et al.* 2010) as the cortisol in hair is unaffected by variations in circadian hormone or by factors that induce short-term variations (Caslini *et al.* 2016). In this sense, measuring cortisol accumulating in hair or fur represents a valuable method to trace rates of long-term stress in both domestic (Comin *et al.* 2011, 2013, 2014; Peric *et al.* 2016, 2017, 2018; Stradaoli *et al.* 2017) and wildlife species (Bechshøft *et al.* 2011, 2012, 2015; Caslini *et al.* 2016; Weisser *et al.* 2016; Prandi *et al.* 2018). However, the technique's main limit is related to hair's slow growth rate which does not permit fine monitoring over short periods as it does not reflect daily or hourly fluctuations in circulating hormones (Koren *et al.* 2002). Although the dynamics of stress physiology have been studied in a range of taxa, to date, limited information is available concerning adrenal activity variations in response to environmental conditions, life-history stages or among individuals (Boonstra 2004; Romero 2004; Palme 2005; Wielebnowski and Watters 2007). GC concentration has been shown to change in relation to sex, age class, day time, season and reproductive status (Ziegler and Snowdon 1995; Gardiner and Hall 1997; 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 ± 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 ± 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 ± 4.48 pg cortisol/mg hair, while in feral ones we recorded an
244 average (\pm sd) level corresponding to 3.57 ± 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 ± 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

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

5. Conclusions

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

6. References

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

10.1016/j.ygcen.2007.07.002

- Angelini P, Augello R, Bagnaia R, Bianco P, Capogrossi R, Cardillo A, Ercole S, Francescato C, Giacanelli V, Laureti L, Luger F, Luger N, Novellino E, Oriolo G, Papallo O, Serra B (2009) Il Progetto Carta della Natura. Linee guida per la cartografia e la valutazione degli habitat alla scala 1:50.000 <http://www.isprambiente.gov.it/files/carta-della-natura/cdn-manuale.pdf>
- Anile S, Bizzarri L, Lacrimini M, Sforzi A, Ragni B, Devillard S (2017) Home-range size of the European wildcat (*Felis silvestris silvestris*): a report from two areas in Central Italy. *Mammalia* 82(1):1-11. doi: 10.1515/mammalia-2016-0045
- Apostolico F, Vercillo F, La Porta G, Ragni B (2016) Long-term changes in diet and trophic niche of the European wildcat (*Felis silvestris silvestris*) in Italy. *Mammal Res* 61:109-119. doi: 10.1007/s13364-015-0255-8
- Ashley NT, Barboza PS, Macbeth BJ, Janz DM, Cattet MRL, Both RK, Wasser SK (2011) Glucocorticosteroid concentrations in feces and hair of captive caribou and reindeer following adrenocorticotrophic hormone challenge. *Gen Comp Endocrinol* 172:382-391. doi: 10.1016/j.ygcen.2011.03.029
- Balíková M (2005) Hair analysis for drugs of abuse. Plausibility of interpretation. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* 149:199-207. doi: 10.5507/bp.2005.026
- Ballesteros-Duperón E, Virgós E, Moleón M, Barea-Azcón JM, Gil-Sánchez JM (2015) How accurate are coat traits for discriminating wild and hybrid forms of *Felis silvestris*? *Mammalia* 79:101-110. doi: 10.1515/mammalia-2013-0026
- Barja I, Silván G, Rosellini S, Piñeiro A, González-Gil A, Camacho L, Illera JC (2007) Stress physiological responses to tourist pressure in a wild population of European pine marten. *J Steroid Biochem Mol Biol* 104:136-142. doi: 10.1016/j.jsbmb.2007.03.008
- Bayazit V (2009) Evaluation of Cortisol and Stress in Captive Animal. *Aust J Basic Appl Sci* 3:1022-1031. <https://pdfs.semanticscholar.org/3f05/c6638addc7dcaa243cf74061969a9fca3854.pdf>
- Bechshøft TØ, Derocher AE, Richardson E, Mislan P, Lunn NJ, Sonne C, Dietz R, Janz DM, St Louis VL (2015) Mercury and cortisol in Western Hudson Bay polar bear hair. *Ecotoxicology* 24:1315-1321. doi: 10.1007/s10646-015-1506-9
- Bechshøft TØ, Rigét FF, Sonne C, Letcher RJ, Muir DCG, Novak MA, Henchey E, Meyer JS, Eulaers I, Jaspers VLB, Eens M, Covaci A, Dietz R (2012) Measuring environmental stress in East Greenland polar bears, 1892–1927 and 1988–2009: What does hair cortisol tell us? *Environ Int* 45:15-21. doi: 10.1016/j.envint.2012.04.005
- Bechshøft TØ, Sonne C, Dietz R, Born EW, Novak MA, Henchey E, Meyer JS (2011) Cortisol levels in hair of East Greenland polar bears. *Sci Total Environ* 409:831-834. doi: 10.1016/j.scitotenv.2010.10.047
- Berger EM, Leus K, Vercammen P, Schwarzenberger F (2006) Faecal steroid metabolites for non-invasive assessment of reproduction in common warthogs (*Phacochoerus africanus*), red river hogs (*Potamochoerus porcus*) and babirusa (*Babirusa babirusa*). *Anim Reprod Sci* 91:155-171. doi: 10.1016/j.anireprosci.2005.03.009
- Boonstra R (2004) Coping with Changing Northern Environments: The Role of the Stress Axis in Birds and Mammals. *Integr Comp Biol* 44:95-108. doi: 10.1093/icb/44.2.95
- Burbonnais ML, Nelson TA, Cattet MR, Darimont CT, Stenhouse GB (2013) Spatial Analysis of Factors Influencing Long-Term Stress in the Grizzly Bear (*Ursus arctos*) Population of Alberta, Canada. *PLoS ONE* 8(12):e83768. doi: 10.1371/journal.pone.0083768
- Brown JL, Wildt DE (1997) Assessing reproductive status in wild felids by non-invasive faecal steroid monitoring. *Int 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 Dingemanse 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, *Crocota 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 Progesterones in Feces and Serum. Zoo Biol 20:227-236. doi: 10.1002/zoo.1022

493 Gygax 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

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

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

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

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

Lapini L (2006) 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. 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

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

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

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

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

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

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

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

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

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

10.1016/j.anbehav.2012.08.010

- Mormède P, Andanson S, Aupérin B, Beerda B, Guémené D, Malmkvist J, Manteca X, Manteuffel G, Prunet P, van Reenen CG, Richard S, Vaissier I (2007) Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. *Physiol Behav* 92:317-339. doi: 10.1016/j.physbeh.2006.12.003
- Möstl E, Palme R (2002) Hormones as indicators of stress. *Domest Anim Endocrinol* 23:67-74. doi: 10.1016/S0739-7240(02)00146-7
- Naidenko SV, Berezhnoi MA, Kumar V, Umapathy G (2019) Comparison of tigers' fecal glucocorticoids level in two extreme habitats. *PLoS ONE* 14(4): e0214447. doi: 10.1371/journal.pone.0214447
- Naidenko SV, Ivanov EA, Lukarevskii VS, Hernandez-Balanco JA, Sorokin PA, Litvinov MN, Kotlyar AK, Rozhnov VV (2011) Activity of the Hypothalamic-Pituitary-Adrenal Axis in the Siberian Tiger (*Panthera tigris altaica*) in Captivity and in The Wild, and Its Dynamics throughout the Year. *Biol Bull Russ Acad Sci* 38(3):301-305. doi: 10.1134/S1062359011030095
- Narayan EJ, Parnell T, Clark G, Martin-Vegue P, Mucci A, Hero JM (2013) Faecal cortisol metabolites in Bengal (*Panthera tigris tigris*) and Sumatran tigers (*Panthera tigris sumatrae*). *Gen Comp Endocr* 194:318-325. doi: 10.1016/j.ygcen.2013.10.002
- Natoli E, Say L, Cafazzo S, Bonanni R, Schmidt M, Pontier D (2005) Bold attitude makes male urban feral domestic cats more vulnerable to Feline Immunodeficiency Virus. *Neurosci Biobehav Rev* 29:151-157. doi: 10.1016/j.neubiorev.2004.06.011
- Natoli E (1994) Urban feral cats (*Felis catus* L.): perspectives for a demographic control respecting the psychological welfare of the species. *Ann Ist Super Sanità* 30(2):223-227. https://www.researchgate.net/profile/Eugenia_Natoli/publication/15367635_Urban_feral_cats_Felis_catus_L_per_spectives_for_a_demographic_control_respecting_the_psychological_welfare_of_the_species/links/55117cb20cf21209d528a8ae.pdf
- Negrão JA, Porcionato MA, de Passillé AM, Rushen J (2004) Cortisol in Saliva and Plasma of Cattle After ACTH Administration and Milking. *J Dairy Sci* 87:1713-1718. doi: 10.3168/jds.S0022-0302(04)73324-X
- Official site of Friuli Venezia Giulia region - English Version (n.d.). Retrieved 28 February 2019 from http://www.regione.fvg.it/inglese/pagine_interne/welcome_history.asp.
- Palme R, Rettenbacher S, Touma C, El-Bahr SM, Möstl E (2005) Comparative Aspects Regarding Metabolism, Excretion, and Noninvasive Measurement in Faecal Samples. *Ann NY Acad Sci* 1040:162-171. doi: 10.1196/annals.1327.021
- Palme R (2005) Measuring fecal steroids: Guidelines for practical application. *Ann N Y Acad Sci* 1040:75-80. doi: 10.1196/annals.1343.007
- Palme R, Touma C, Sachser N, Erich M (2003) Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. *130:267-278*. doi: 10.1016/S0016-6480(02)00620-2
- Palme R, Fisher P, Schildorfer H, Ismail MN (1996) Excretion of infused ¹⁴C-steroid hormones via faeces and urine in domestic livestock. *Anim Reprod Sci* 43:43-46. doi: 10.1016/0378-4320(95)01458-6
- Peric T, Comin A, Corazzin M, Montillo M, Canavese F, Stebel M, Prandi A (2018) Hair cortisol concentrations in New Zealand white rabbits subjected to surgery. *Anim Welfare* 27:13-20. doi: 10.7120/09627286.27.1.013
- Peric T, Corazzin M, Romanzin A, Bovolenta S, Prandi A, Montillo M, Comin A (2017) Cortisol and DHEA concentrations in the hair of dairy cows managed indoor or on pasture. *Livest Sci* 202:39-43. doi: 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, Barja 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 Rehbindler 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:

10.1016/j.psyneuen.2011.09.009

- Sapolsky RM, Romero LM, Munck AU (2000) How Do Glucocorticoids Influence Stress Responses? Preparative Actions. *Endocr Rev* 21:55-89. doi: 10.1210/er.21.1.55
- Sarmiento P, Cruz J, Tarroso P, Fonseca C (2006) Space and Habitat Selection by Female European Wild Cats (*Felis silvestris silvestris*). *Wildl Biol Pract* 2(2):79-89. doi:10.2461/wbp.2006.2.10
- Schatz S, Palme R (2001) Measurement of Faecal Cortisol Metabolites in Cats and Dogs: A Non-invasive Method for Evaluating Adrenocortical Function. *Vet Res Commun* 25:271-287. doi: 10.1023/A:1010626608498
- Schell CJ, Young JK, Lonsdorf EV, Mateo JM, Santymire RM (2017) Investigation of techniques to measure cortisol and testosterone concentrations in coyote hair. *Zoo Biol* 36:220-225. doi: 10.1002/zoo.21359
- Schwarzenberger F (2007) The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species. *Int Zoo Yb* 41: 52-74. doi: 10.1111/j.1748-1090.2007.00017.x
- Schwarzenberger F, Palme R, Bamberg E, Möstl E (1997) A review of faecal progesterone metabolite analysis for non-invasive monitoring of reproductive function in mammals. *Int J Mamm Biol* 62:214-221. https://www.researchgate.net/publication/266084067_A_review_of_faecal_progesterone_metabolite_analysis_for_non-invasive_monitoring_of_reproductive_function_in_mammals
- Schwarzenberger F, Möstl E, Palme R (1996) Faecal steroid analysis for non-invasive monitor of reproductive status in farm, wild and zoo animals. *Anim Reprod Sci* 42:515-526. doi: 10.1016/0378-4320(96)01561-8
- Sheriff MJ, Dantzer B, Delehanty B, Palme R, Boonstra R (2011) Measuring stress in wildlife: Techniques for quantifying glucocorticoids. *Oecologia* 166:869-887. doi: 10.1007/s00442-011-1943-y
- Stradaoli G, Peric T, Montillo M, Comin A, Corazzin M, Veronesi MC, Prandi A (2017) Hair cortisol and testosterone concentrations and semen production of *Bos taurus* bulls. *Ital J Anim Sci* 16(4):631-639. doi: 10.1080/1828051X.2017.1303339
- von der Ohe CG, Servheen C (2002) Measuring stress in mammals using fecal glucocorticoids: opportunities and challenges. *Wildlife Soc B* 30(4):1215-1225. <https://www.jstor.org/stable/pdf/3784291.pdf>
- Webb E, Thomson S, Nelson A, White C, Koren G, Rieder M, Van Uum S (2010) Assessing individual systemic stress through cortisol analysis of archaeological hair. *J Archaeol Sci* 37:807-812. doi: 10.1016/j.jas.2009.11.010
- Weingrill T, Gray DA, Barrett L, Henzi SP (2004) Fecal cortisol levels in free-ranging female chacma baboons: relationship to dominance, reproductive state and environmental factors. *Horm Behav* 45:259-269. doi: 10.1016/j.yhbeh.2003.12.004
- Weisser JJ, Hansen M, Björklund E, Sonne C, Dietz R, Styrishave B (2016) A novel method for analysing key corticosteroids in polar bear (*Ursus maritimus*) hair using liquid chromatography tandem massspectrometry. *J Chromatogr B* 1017-1018:45-51. doi: 10.1016/j.jchromb.2016.02.029
- Wielebnowski and Watters (2007) Applying Faecal Endocrine Monitoring to Conservation and Behavior Studies of Wild Mammals: Important Considerations and Preliminary Tests. *Isr J Ecol Evol* 53:439-460. doi: 10.1560/IJEE.53.3.439
- Wikelski M, Cooke SJ (2006) Conservation physiology. *Trends Ecol Evol* 21:38-46. doi: 10.1016/j.tree.2005.10.018
- Yamaguchi N, Kitchener A, Driscoll C, Nussberger B (2015) *Felis silvestris*. IUCN Red List Threat Species 2015 8235:e.T60354712A50652361. doi: 10.2305/IUCN.UK.2015-2.RLTS.T60354712A50652361.en
- Young KM, Walker SL, Lanthier C, Waddell WT, Monfort SL, Brown JL (2004) Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analysis. *Gen Comp Endocrinol* 137:148-165. doi: 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.

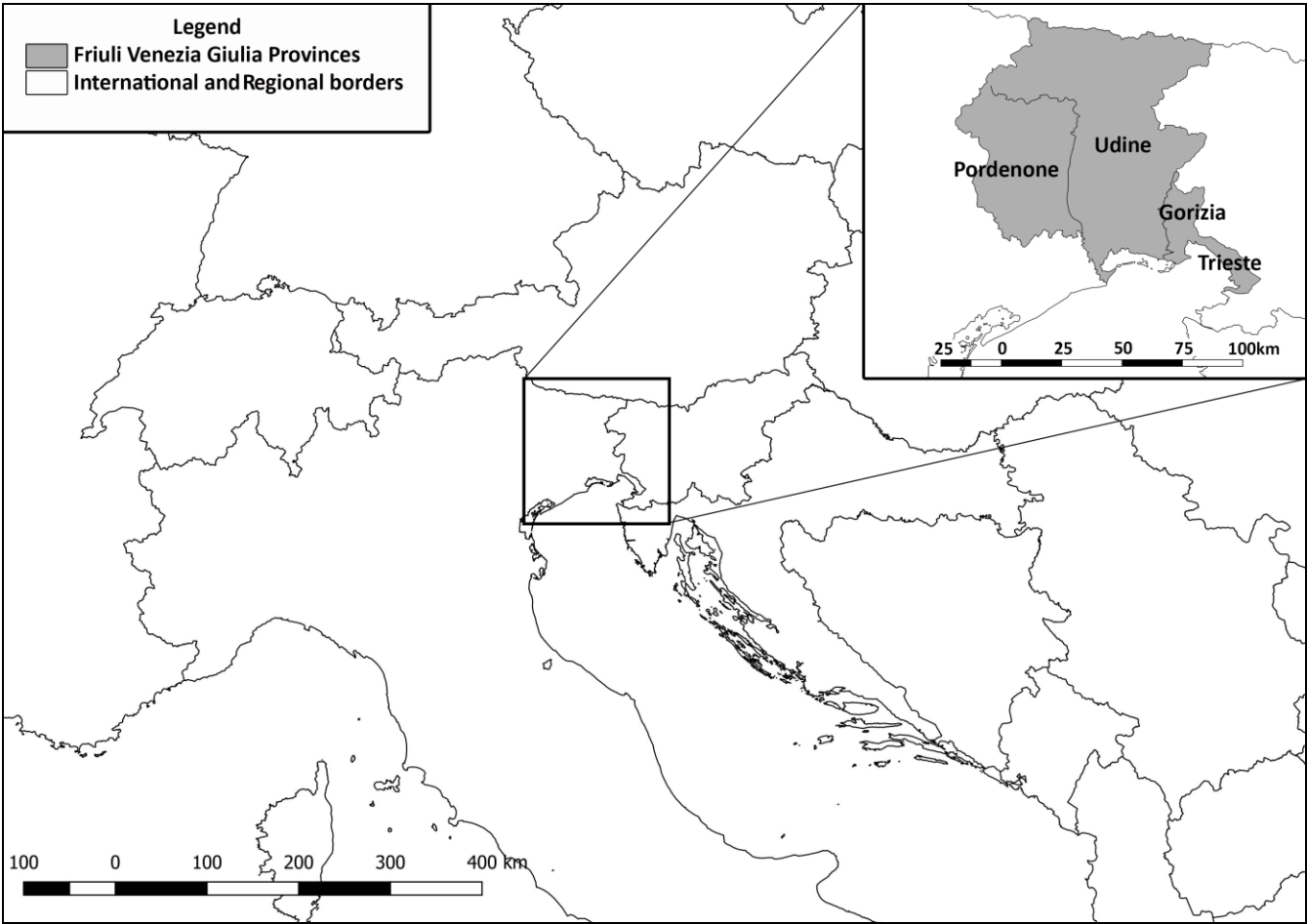


Fig2. Box-plot showing the difference in terms of hair cortisol concentration (pg cortisol/mg hair) between subspecies.

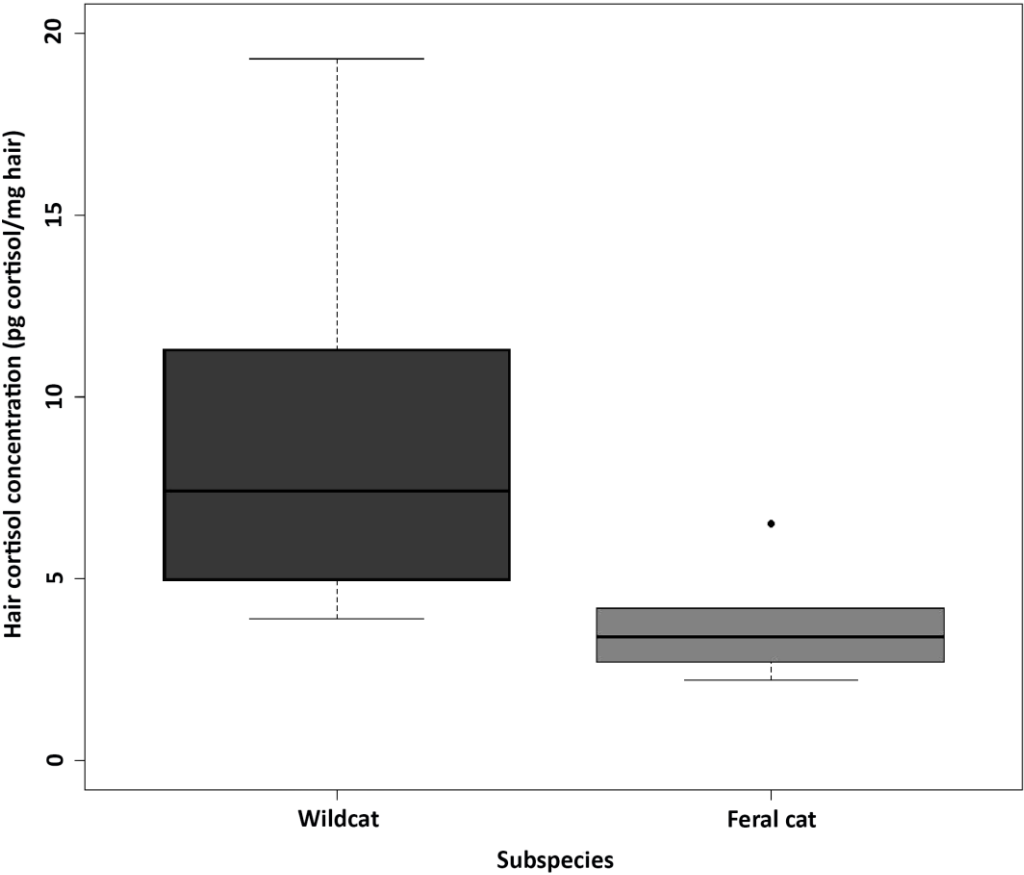
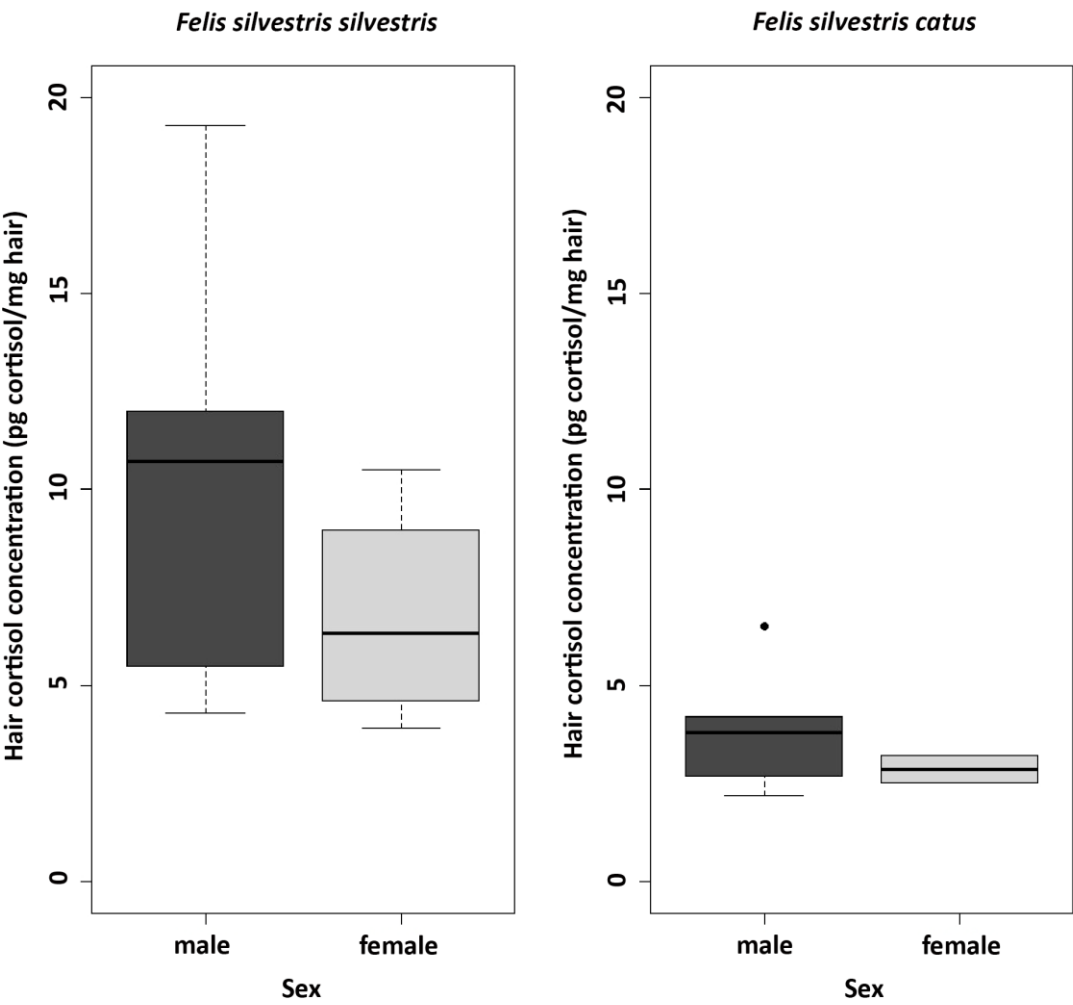


Fig3. Box-plot showing the difference in terms of hair cortisol levels (pg cortisol/mg hair) between sexes in wild (*Felis silvestris silvestris*) and feral (*Felis silvestris catus*) cats.



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

ID model	Independent variables	K	-2 logLik	AICc	Δ AICc	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