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1 **Corticosterone mediates telomere length in raptor chicks exposed**
2 **to chemical mixture**

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17
18 *Keywords: pollutant ; telomere ; corticosterone ; Red kite ; stress ; cocktail effect*

19 Running head : Raptor chicks exposition to chemical mixture

20
21 **Authors' Contributions Statement**

22 M.C. designed the study. C.M. and M.C. managed and carried out fieldwork and sampling. N.C.
23 conducted the metal analysis. S.Z., S.M. and N.B. supervised and conducted the TL measurements.
24 I.F. conducted the rodenticide analysis and T.G.P. conducted the CORT analysis. M.C., T.P. and N.B.
25 analyzed and interpreted the data and wrote the manuscript. All co-authors commented on the
26 manuscript and accepted the final version.
27

28 **Abstract**

29 Stressors experience early in life by animals may have carry over impacts on life-traits over the life
30 cycle. Accelerated telomere attrition induced by stress during development and growth could play a
31 role in such delayed effects. Among stressors, exposure to chemicals may modify telomere dynamic
32 but, to date, the trends evidenced between exposure and telomere shortening remains inconsistent.
33 Moreover, the role of corticosterone as a possible mediator of chemical impact on telomere is not
34 yet clearly established. Here, we investigated in wild populations of Red kite whether nestling
35 exposure to metals and pesticides was related to corticosterone concentrations in feathers and
36 telomere length measured in 47 individuals. Lead and mercury concentrations in blood ranged from
37 2.3 to 59.0 $\mu\text{g L}^{-1}$ and to 1.4 to 115.7 $\mu\text{g L}^{-1}$, respectively, and were below the toxicity thresholds
38 proposed for wildlife. Rodenticides were detected in 30% of the chicks. Corticosterone increased
39 with mercury and lead in interaction, showing a synergistic effect of these 2 non-essential metals on
40 this stress hormone. Telomere length was not linked to metals and/or rodenticide exposure while it
41 was related negatively to corticosterone. The relationship between telomere and corticosterone was
42 modulated by nestling's age, which suggests that the rate of telomere shortening is higher when
43 corticosterone increases. Our findings propose an effect of low exposure of Red Kite nestlings to
44 mercury and lead mixture to raise baseline corticosterone in feathers. The relationships established
45 suggest the hypothesis that telomere attrition could be an indirect consequence of metal exposure
46 mediated by corticosterone.

47

48 1. INTRODUCTION

49 Stressors experienced during the early stages of an animal's life may impact upon its development
50 and growth but can also have carry over consequences on other life-history traits, such as
51 reproduction and/or longevity, and thus, fitness (Monaghan, 2008). Recently, interest has been
52 growing as to whether environmental stressors, including exposure to pollutants, accelerate
53 telomere attrition (Angelier et al., 2018; Blévin et al., 2016). Telomeres are sequences of non-coding
54 DNA located at the end of chromosomes that shorten naturally at each cell division (Monaghan and
55 Haussmann, 2006). When telomeres are shortened, coding DNA may be damage during replication
56 which leads to cell senescence and telomere shortening has been related to health status, individual
57 quality and lifespan in mammals and birds (Bize et al., 2009; Boonekamp et al., 2014; Angelier, et al.,
58 2019). Telomere attrition may be accelerated when individuals are exposed to pollutants as
59 demonstrated for humans (Hoxha et al., 2009). The few studies dealing with telomere shortening
60 related to pollutant exposure in birds did not reveal any consistent trend (Blévin et al., 2016; Sletten
61 et al., 2016; Stauffer et al., 2017). For instance, in female kittiwakes, telomere length is shorter with
62 oxychlorane exposure (Blévin et al., 2016) while it is longer in birds exposed to perfluoroalkylated
63 substances and mercury (Angelier et al., 2018). No relationship was found with some chemicals
64 including PCBs and other persistent organic pollutants (Blévin et al., 2016). This discrepancy led
65 Angelier et al. (2018) to conclude that "further experimental and correlative studies linking telomere
66 dynamics and contamination are necessary to better understand the impact of anthropogenic
67 pollution on wild vertebrates". Furthermore, telomere attrition is much higher in embryos and
68 nestlings compared to adults, when cell proliferation accompanies growth and development
69 (Angelier et al., 2018). Because stress experienced in early life will shape the phenotype of the future
70 adult, telomere loss during growth could be the mechanism linking stress and ageing across
71 generations (Haussmann and Heidinger, 2015). This also suggests that nestling exposure to chemicals
72 could impact life-history traits later in life through accelerated telomere attrition.

73 A common pathway that lead to various cellular and physiological changes and disorders, including
74 possibly telomere attrition, is corticosterone (CORT) release due to activation of the hypothalamic-
75 pituitary-adrenal axis (HPA) (Strong et al., 2015). Following exposure to endocrine disrupting
76 chemicals, CORT, the main hormonal mediator of allostasis in birds, may increase (Franceschini et al.,
77 2009; Herring et al., 2012; Meillère et al., 2016; Tartu et al., 2015) in order adapt life-trait expression
78 to environmental conditions. Chronic stress may generate an elevation in baseline CORT levels that
79 can entail important physiological consequences, such as suppression of somatic growth,
80 reproduction and, ultimately reduce long term survival (Quirici et al., 2016). In birds, high CORT level
81 in plasma have been related to shorter telomere, either experimentally (Hausmann et al., 2012) or
82 in wild populations (Quirici et al., 2016). Those findings have led to assume that CORT could mediate
83 the link between telomere dynamic and stressful environmental conditions like contamination
84 (Angelier et al., 2018).

85 To date, no study has focused on whether non-lethal exposure to chemicals may cause CORT release
86 in wild nestling birds and, whether CORT level may mediate the ageing cost of pollutants via
87 telomere shortening. In the present study, we investigated the relationship between exposure to
88 metals (both non-essentials: cadmium Cd, mercury Hg and lead Pb and essential: selenium Se) and
89 pesticides (anticoagulant rodenticides; ARs), and CORT levels in feathers and telomere length in
90 nestlings of Red Kites *Milvus milvus* in Eastern France. According to their feeding habits, the Red kite
91 is commonly exposed to ARs (Coourdassier et al., 2014) or metals (Berny et al., 2015) and poisoning
92 is deemed to be one of the main threats for this species (International Union for the Conservation of
93 Nature, 2018). Here, we hypothesize that high levels of toxicants in the blood will cause an increase
94 of CORT concentrations in feathers, and could lead to a decrease of telomere length.

95 **2. MATERIAL AND METHODS**

96 2.1. Study areas and sample collection

97 Fieldwork was carried out in 2015 from 4th to 25th June in Franche-Comté region, Eastern France.

98 Three breeding populations of Red Kite, from three areas were monitored: Sundgau Belfortain

99 (47°38'17"N, 6°51'46"E), Dugeon & Remoray basin (46°54'22"N, 6°21'17"E) and Besançon plateau
100 (47°14'35"N, 6°01'19"E). Forty-seven chicks (23 males and 24 females) corresponding to 24 nests
101 were retained for the study. The sampling was implemented ~15 days prior to the expected fledging
102 date, when the chicks were approximately 30-35 days old. The nestlings were removed from the
103 nests by an experienced arborist climber and carried to the ground for data collection. First, the body
104 mass (g) and the length of the primary wing feathers (mm) were measured. Between 4 and 8 breast
105 feathers were cut and blood samples (2 ml) were taken from the brachial vein using lithium-heparin
106 coated syringes. As recommended (Fair et al., 2010), the volume of blood collected was less than
107 0.5% of the chick's body mass. Blood samples were kept in the dark at 4°C and feathers were stored
108 at -20°C until processed in the laboratory. At the end of each sampling day, 300 µL of blood were
109 centrifuged at 500 g for 10 min. Red blood cells were stored at -80 °C to assess telomere length and
110 determine the chicks' sex according to a PCR method (Griffiths et al., 1998). The rest of the whole
111 blood was frozen at -20°C until analysis. All the manipulations were performed with governmental
112 authorizations no. 2015093-0007 and 2015093-0023 in the framework of the Red Kite national
113 conservation plan.

114 2.2. Metals and ARs analysis in blood

115 Blood concentrations of Cd, Hg, Pb and Se were measured by ICP-MS analysis (XSeries 2, Thermo
116 Scientific). 300 µL of whole blood were digested in a DigiPrep unit (MS SCP Science) after addition of
117 1 mL HNO₃ (Optima® quality, Fisher Chemical). Then, the volume was completed to 50 mL with ultra-
118 pure water. A certified reference material (TORT-2, NRC, Canada) was used to ensure the validity of
119 the obtained results. The limits of detection were $0.7 \cdot 10^{-3}$, $0.7 \cdot 10^{-2}$, $1.9 \cdot 10^{-3}$ and $3.0 \cdot 10^{-3} \mu\text{g L}^{-1}$ for Cd,
120 Hg, Pb and Se, respectively and the limits of quantification were $2.3 \cdot 10^{-3}$, $2.3 \cdot 10^{-2}$, $5.8 \cdot 10^{-3}$ and $9.0 \cdot 10^{-3}$
121 $\mu\text{g L}^{-1}$.

122 The concentrations of 8 ARs (bromadiolone, chlorophacinone, difenacoum, difethialone, warfarin,
123 coumatetralyl, brodifacoum and flocoumafen) were determined in whole blood by LC-ion trap
124 according to a method adapted from Fourel et al. (2010). The LOD and LOQ were respectively 0.1/0.2

125 $\mu\text{g L}^{-1}$ for flocoumafen, 0.2/0.5 $\mu\text{g L}^{-1}$ for chlorophacinone, bromadiolone, brodifacoum and warfarin,
126 0.5/1.0 $\mu\text{g L}^{-1}$ for difenacoum, difethialone and coumatetralyl.

127 2.3. Telomere length measurement

128 Telomere length was measured in DNA extracted from red blood cells using Quiagen Puregene®
129 Blood Core Kit B with a quantitative real-time amplification (qPCR) previously used in birds (Criscuolo
130 et al., 2009). Relative telomere length is expressed as the (T/S) ratio of telomere repeat copy number
131 (T) to a control single copy number gene (S), which was calculated using the exact amplification
132 efficiencies of each run (for T and S) and the Cq values corresponding to number of amplification
133 cycles allowing the detection of T or S amplification by fluorescence. We used the Zebra finch
134 *Taeniopygia guttata* Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence as a control
135 gene, with the primers GAPDH-F (5'-AACCAGCCAAGTACGATGACAT-3') and GAPDH-R (5'-
136 CCATCAGCAGCAGCCTTCA-3'). The primers used for the telomere sequence amplification were: Tel1b
137 (5'-CGGTTTGGTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3') and Tel2b (5'-
138 GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'). Both qPCR (control gene and telomere) was
139 performed in a total volume of 10 μL , including 0.8 \times Brilliant SYBR Green QPCR Master Mix
140 (Stratagene), 50 mM of GAPDH-F/GAPDH-R, 50 mM of Tel1b /Tel2b, and 2.5 ng μL^{-1} of DNA. Each
141 sample was run in duplicate. Samples were measured using two runs, each of them containing one
142 plate for S amplification and one plate of T amplification. In each plate, a serial dilution using an
143 identical DNA sample (identity 613) was carried out on triplicate to evaluate the efficiency of the
144 qPCR reaction. The control gene qPCR conditions were 10 min at 95°C followed by 40 cycles (1 min at
145 56°C and 1 min at 95°C), while the telomere sequence amplification was done following: 2 min at
146 95°C and 30 cycles (30 sec at 56°C, 30 sec at 72°C and 1 min at 95°C). All amplifications ended by a
147 dissociation curve analysis to check for unspecific signals. Amplification efficiencies were 101 and
148 102% (S), and 98 and 99% (T). Intra-run coefficients of variation were 0.47 ± 0.09 % (Cq S), $1.09 \pm$
149 0.09 % (Cq T) and 10.27 ± 1.38 % (T/S ratio). Inter-run coefficients of variation were 0.58 ± 0.17 % (Cq
150 S), 1.35 ± 0.16 % (Cq T) and 10.37 ± 0.93 % (T/S ratio).

151 2.4 Corticosterone analyses in feathers

152 Corticosterone was extracted from feathers as described by (Strong et al., 2015). Corticosterone
153 content was determined in 100 μ L aliquots of each extract using the radioimmunoassay protocol
154 described by (Pottinger and Carrick, 2001) for cortisol but substituting [1,2,6,7-3H]-corticosterone
155 (2.4 TBq mmol^{-1} ; GE Healthcare) as tracer with an anti-corticosterone antibody (Abcam ab1022,
156 1:2000 dilution; cross-reactivity: 11-dehydrocorticosterone 0.67%, Deoxycorticosterone 1.5%, 18-
157 OH-DOC <0.01%, Cortisone <0.01%, Cortisol <0.01% and Aldosterone 0.2%). Corticosterone
158 concentrations were expressed in pg cm^{-1} .

159 2.5 Data analysis

160 First, relationships linking metal concentrations to individual relative variables (area, age, sex, rank of
161 the chicks in the brood) as fixed factors were checked using general linear models (LM) or mixed-
162 effect LM (LMER). Rodenticide blood concentrations were linked to individual relative variables (age,
163 sex, rank of the chicks in the brood) with non-parametric tests. Wing length was used as a proxy of
164 age according to Mougeot and Bretagnolle (2006). In addition, relationships between CORT in
165 feathers as response and chemical concentrations and individual parameters as fixed factors were
166 tested with LM or LMER. Then, telomere length was fitted against CORT, chemical concentrations
167 and individual parameters with LM or LMER. The cross-sectional rate of TL shortening was estimated
168 by comparing the difference between the TL lengths measured in chicks of two different ages
169 according to the CORT in feathers. Response variables were log-transformed when necessary to fit
170 residual normality and homoscedasticity. The variable named 'Nest' was introduced as random
171 factor when checking differences of concentrations between areas while Nest nested into Area
172 (Area/Nest) were used in others models (Zuur et al., 2009). For each response variable, the null
173 model was also tested. The most parsimonious model with the lowest Akaike information criterion
174 (AICc) was selected, a difference of AIC > 2 was retained to evidence differences between 2 models
175 (Burnham and Anderson, 2010). For each model selected, Cook distances were computed to
176 estimate the influence of the data points. When one (or several) data point exhibited Cook distance >

177 1, it was deleted from the dataset and the model was fitted again to check the robustness of the
178 relationship without any strongly influential data. All statistical analyses were performed using R
179 3.4.2. with the packages lme4 and lmerTest. The concentrations measured in kite blood for Hg and
180 Pb were compared to toxicity reference values (TRVs) previously published, *i.e.*, 200 $\mu\text{g L}^{-1}$ (Ackerman
181 et al., 2016) and 710 $\mu\text{g L}^{-1}$ in birds (Buekers et al., 2009), respectively.

182 3. RESULTS

183 3.1. Nestling exposure to chemicals

184 3.1.1 Metal concentrations in blood

185 For non-essential metals, Pb and Hg were detected in the blood of all chicks monitored while Cd was
186 > LOD in only 3 individuals (Table 1) and thus was not subsequently analysed. Blood [Pb] ranged from
187 2.3 to 59.0 $\mu\text{g L}^{-1}$ (Table 1) and remained at least 12 times lower than the Pb TRV (710 $\mu\text{g L}^{-1}$). No
188 differences in [Pb] were detected between areas (LM, $p = 0.39$). Moreover, [Hg] and [Pb] in blood
189 were not influenced by sex (LM, $p = 0.22$ for Pb and LMER, random factors: Area/Nest, $p = 0.28$ for
190 Hg), age (LMER, $p = 0.45$ for Pb and $p = 0.11$ for Hg, random factors: Area/Nest) or the rank of chicks
191 in the brood (LM, $p = 0.20$ for Pb and LMER, random factors: Area/Nest, $p = 0.15$ for Hg). Blood [Hg]
192 ranged from 1.4 to 115.7 $\mu\text{g L}^{-1}$ (Table 1) and were 3 times lower in nestlings from Besancon than in
193 Dugeon-Remoray and Belfort chicks (medians of 4.2, 13.4 and 11.8 $\mu\text{g L}^{-1}$, respectively). Among the
194 47 individuals sampled, none had blood [Hg] higher than the TRV (*i.e.* 200 $\mu\text{g L}^{-1}$). Concentrations of
195 Se, an essential element involved in Hg detoxification, ranged from 131.4 to 521.3 $\mu\text{g L}^{-1}$ (Table 1).
196 Nestlings from Dugeon-Remoray and Belfort exhibited higher concentrations than those from
197 Besancon (medians = 357.7, 328.3 and 248.5 $\mu\text{g L}^{-1}$, respectively). Blood [Se] also increased with the
198 age of the chicks only (LMER, random factors: Area/Nest, $p < 0.0001$) and were positively correlated
199 to [Hg] (LMER, random factors: Area/Nest, $p < 0.0001$; Fig. 2) with a mean Se/Hg molar ratio of 102.3
200 ± 62.9 (mean \pm SD)

201 3.1.2 Concentrations of ARs in blood

202 Nearly a third (30%) of the chicks ($N=14$) were exposed to 4 ARs, bromadiolone, difenacoum,
203 brodifacoum and/or difethialone. Bromadiolone was the most frequently detected and exhibited the
204 highest blood concentrations among ARs (Table 1). Brodifacoum residues were detected in 4 kites
205 and difethialone in 2 individuals, median concentrations of positive chicks being 10 and 2.4 times
206 lower than that of bromadiolone (Table 1). Four chicks were exposed to difenacoum at
207 concentrations up to $2.5 \mu\text{g L}^{-1}$ (Table 1) and three of them exhibited residues of 2 ARs (difenacoum +
208 brodifacoum or bromadiolone). As the number of nestlings with AR residues was low, we summed all
209 AR concentrations measured in a single chick (variable called ΣARs , Table 1) to check for further
210 relationships. No difference between areas was found for the proportion of exposed individuals
211 (binomial GLM, $p = 0.29$) or ΣARs concentrations (LM, $p = 0.29$). Moreover, any of the chick or brood
212 characteristics influenced the ΣARs concentrations in blood (Wilcoxon test for sex, $p = 0.10$;
213 Spearman correlation test for age, $p = 0.78$; Kruskal-Wallis test for rank, $p = 0.77$).

214 3.2. Influence of pollutant levels on corticosterone in feathers

215 Concentrations of corticosterone in feathers (CORT) ranged from 4.3 to 59.9 pg cm^{-1} (median: 9.3
216 pg/cm) with no differences between areas (LME, random factor: Nest, $p = 0.58$). Among factors
217 related to individual or brood, CORT levels was positively influenced by the age (LME, random
218 factors: Area/Nest, $p = 0.027$). CORT concentrations in feathers increased with chick exposure to Hg
219 in interaction with Pb while age was not retained in the model with the lowest AICc (LM, $R^2 = 0.17$, p
220 $= 0.013$, Fig. 1). Indeed, the effect of Hg on CORT concentrations was more pronounced when [Pb] in
221 blood increased. As one data point had a Cook distance > 1 , CORT was fitted against Hg and/or Pb
222 without this data and the best model remained that with Hg and Pb in interaction as fixed factors
223 (LM, $R^2 = 0.14$, $p = 0.025$).

224 3.3. Modulation of telomere length by CORT levels

225 Telomere lengths (T/S ratio) ranged from 0.509 to 1.170 and were higher in Dugeon-Remoray and
226 Besancon than in Belfort with means (\pm SD) of 0.92 ± 0.11 , 0.92 ± 0.13 and 0.71 ± 0.17 , respectively
227 (LM, $R^2 = 0.12$, $p = 0.025$). Telomere shortening was related to high CORT in feathers in interaction

228 with the age of individuals. Telomeres were shorter when CORT increased, this effect being
229 heightened in older chicks (LM, $R^2 = 0.16$, $p = 0.017$; Fig. 2). Using the relationship established here,
230 we present the telomere length predicted from the CORT levels in feathers for 2 ages of chicks (Fig.
231 2). We also evidence that the cross-sectional rate of TL shortening is modulated by CORT. Indeed, the
232 difference of TL length measured in chicks of 2 different ages is larger for elevated CORT level in
233 feathers compared to low ones (Fig. 2).

234

235 4. DISCUSSION

236 Nestling Red Kites (30-35 day-old) are exposed to non-essential metals and/or pesticides early in
237 their development. Blood Hg and Pb concentrations remain relatively low compared to those
238 reported in other studies on raptors (Ackerman et al., 2016; Gómez-Ramírez et al., 2010) and are
239 below the toxic reference values proposed for wildlife (Ackerman et al., 2016; Buekers et al., 2009).
240 However, we found a positive relationship between CORT in feathers and Hg and Pb concentrations
241 in Red Kite, despite Se/Hg ratios well above 1, which are commonly considered as protective against
242 Hg toxicity (Scheuhammer, 1987). Our findings suggest that these metals may impair chicks even at
243 low exposure level. In humans, it is even considered that there is no known safe level of exposure for
244 these elements as they provide no physiological benefit and thresholds of concern, notably for
245 children, and are far lower than those retained for wildlife (Brodkin et al., 2007). The high sensitivity
246 of foetuses and early stages of animals to Hg and Pb has been previously reported for mammals and
247 birds. The survival rates assessed for embryos of 26 bird species exposed to methyl-Hg show that
248 raptors could be among the avian groups most sensitive to Hg (Heinz et al., 2009). However, another
249 study suggests that Bald eagles *Haliaeetus leucocephalus* may be less vulnerable to Methyl-Hg
250 toxicity than Common loons *Gavia immer* because of higher ability to detoxify Hg in the brain
251 (Scheuhammer et al., 2007), making it challenging to draw any clear conclusion on the relative Hg
252 susceptibility between bird species. Furthermore, the behaviour of Golden eagles *Aquila chrysaetos*
253 (in terms of flight height and movement rate) was negatively affected in individuals exhibiting blood

254 [Pb] from 25 $\mu\text{g L}^{-1}$ (Ecke et al., 2017), which suggests that subtle effects may occur at low exposure,
255 close to those measured in the Red Kite and well below TRVs.

256 Although the impact of metals has been widely studied in birds (Kekkonen, 2017), only a dozen
257 studies have checked whether CORT concentrations were related to exposure levels. When
258 considered individually, effects of Hg on CORT remain inconsistent in many avian species and depend
259 strongly on whether baseline or stress-induced CORT is considered (Franceschini et al., 2017). The
260 trends reported between Hg exposure and baseline CORT, as measured here in Kites, were either
261 positive (Meillère et al., 2016; Herring et al., 2012), or negative (Franceschini et al., 2009), or null
262 (Tartu et al., 2015). For Pb, biomedical studies have shown that long-term exposure to low doses can
263 affect the HPA axis in mammals (Virgolini et al., 2005). Although Pb exposure has been associated
264 with alteration of behaviour and physiological functions in wild vertebrates (Burger and Gochfeld,
265 2005; Geens et al., 2009), few studies have examined its impact on CORT level in birds. In white stork
266 (*Ciconia ciconia*) and black birds (*Turdus merula*), Pb exposure was associated with elevated CORT
267 levels (Baos et al. 2006; Meillère et al. 2016), supporting the hypothesis that Pb may affect the HPA
268 axis and CORT regulation and/or secretion. However, CORT did not change in nestling Great tits
269 (*Parus major*) experimentally fed with different doses of Pb spiked food (Eeva et al., 2014) or in tits
270 living in Pb-contaminated or unpolluted sites (Eeva et al., 2003). These discrepancies could be partly
271 due to differences in sampling methods measurements. Indeed, most studies have focused on
272 instantaneous measures of baseline and/or stress-induced CORT in blood that represent short-term
273 measures. Here, we analysed CORT in feathers, which provides a more integrated measure of its
274 secretion (Bortolotti et al., 2008) and thus probably better represents the stressful events
275 experienced by the chicks at least during the period of feather growth. Other factors such as inter-
276 species differences in gastro-intestinal metal absorption level (Scheuhammer, 1987), interspecies
277 variations of sensitivity to toxicants (Heinz et al., 2009), or the specific constraints of a given habitat
278 (Bonier, 2012) may influence the relationship between metals and CORT. Finally, the concomitant
279 presence of several toxic compounds within an organism may scramble the link between CORT and a

280 single metal because of co-variation and/or interactions between chemicals. Despite their relatively
281 high occurrence in Kites, ARs did not influence CORT concentration in feathers. This supports
282 previous studies that did not identify any potential for endocrine disruption by compounds of the
283 hydroxycoumarin family (Biocidal Products Committee, 2016).

284 Ecosystems are often contaminated by mixtures of chemicals, which may interact in almost
285 unpredictable ways on exposed individuals. Reported interactions include additivity (*i.e.*, mixture
286 toxicity = \sum toxicity of each chemical), antagonism (*i.e.*, mixture < \sum each chemical) or synergy (*i.e.*,
287 mixture > \sum each chemical) (European Commission & Directorate General for Health & Consumers,
288 2012). Thus, chemicals may have different effects, depending if they act individually or combined.

289 Our findings suggest that Hg and Pb interact synergistically and at low concentrations on CORT
290 synthesis and/or regulation in Red Kite chicks. According to Andrade et al. (2017), Hg and Pb are
291 relevant candidates to produce synergic effects because they share similar target organs such as
292 brain and also similar modes (*e.g.* neurotoxicity), and mechanisms of actions (*i.e.*, ROS production,
293 antioxidant decrease, interactions with essential metals) (Karri et al., 2016). Furthermore, in
294 mammals, Pb concentrations in the brain increased in the presence of other metals including Hg
295 (Andrade et al., 2017). Such synergistic interaction between Pb and Hg was noticed in mammals (Lin
296 et al., 2016) and invertebrates (Fernandez and Beiras, 2001) but, to our knowledge, metals
297 interaction related to CORT concentration has rarely been studied in birds. In black bird, CORT in
298 feathers were positively related to feather concentrations of Hg, Pb or Cd along an urbanization
299 gradient but possible interactions between metals on CORT were not explored (Meillère et al., 2016).

300 Pollock and Machin (2009) showed that the relationship between Hg and CORT was modulated by Cd
301 concentrations in greater and lesser scaup (*Aythya marila* and *A. affinis*). Both the Pollock and
302 Machin study and ours show that metal mixtures may also impair CORT metabolism in birds.

303 Interactions occurring in organisms following exposure to complex mixtures may lead to potentiate
304 the effects of metals even at concentrations far below commonly accepted toxicity thresholds for
305 single metal. Because these findings are correlative, it is obviously challenging to assess the exact

306 impact of metals on CORT regulation. Such interactions operate through cellular and physiological
307 mechanisms that currently remain under question. Hg and Pb could act as endocrine disruptors
308 (Bergman et al., 2013) and affect the HPA axis in the Red Kite, explaining therefore the higher feather
309 CORT levels. Such questions should motivate future experimental studies to understand to what
310 extent Hg, Pb and other contaminants may disrupt CORT secretion, and more generally, the
311 physiological mechanisms involved notably on sensitive early life stages of birds (Jackson et al.,
312 2015).

313 We did not find evidence to support any direct link between exposure to pollutants and telomere
314 length in nestling Red Kites. Only a few correlative studies have investigated whether telomere
315 length is influenced by toxicants in birds and most of them have dealt with organic chemicals
316 (Angelier et al., 2018). Overall, no obvious pattern was observed with metals. Stauffer et al. (2017)
317 reported shorter telomeres in nestling Great tits living in metal polluted sites while no difference was
318 measured in adult tits or in nestlings or adults of Pied flycatchers. In sea birds, telomere length was
319 positively correlated to Hg exposure in adult females of Black-legged kittiwake whereas no similar
320 relationships were found for males in the same population (Angelier et al., 2018). In their recent
321 review, Angelier et al. (2018) questioned whether CORT could be the link between environmental
322 constraints, including pollutants, and telomere dynamics in wild vertebrates. If they emphasized the
323 scarcity of empirical data for strong conclusions, they raised a hypothetical pathway by which CORT
324 secretion would contribute to telomere attrition by inducing oxidative stress and/or modulating
325 telomerase activity (Choi et al., 2008). Here, we show that telomere length is shorter in chicks with
326 the highest CORT levels in feathers. This suggests two different possibilities. Firstly, the existence of a
327 possible link between CORT and telomere length that has been previously examined in birds, mainly
328 in adults, in both field and experimental studies (Angelier et al., 2018). Field correlative studies
329 disclosed inconsistent trends even if 50% of them (5/10) reported negative correlations, including
330 the only study on raptor. Interestingly, negative relationships were found in all experimental studies
331 available, notably in those where CORT was administrated to nestlings (Angelier et al., 2018; Pegan

332 et al., 2018). Secondly, CORT and telomere dynamics may covary in response to metals on
333 metabolism. If exposure to pollutants drives the organisms to shift into an emergency physiological
334 status (as it is suggested by increased CORT), it is likely that cellular pathways sensitive to the energy
335 balance are activated, in particular during growth at juvenile stages. Cell signalling such as the
336 “Target Of Rapamycin” pathway (TOR) or changes in mitochondrial functioning may be triggered,
337 with potential consequences on telomere erosion (Sahin and DePinho, 2012). In Kite, the relationship
338 between CORT and telomere length is modulated by the age, telomere being shorter in older chicks.
339 The growth-aging trade-off on telomere attrition is well-documented and it is commonly considered
340 that telomeres shorten naturally and faster early in life in most wild vertebrates (Angelier et al.,
341 2018). This higher rate of telomere erosion is either attributed to the higher rate of cell division
342 enabling somatic growth or to the higher metabolism and concomitant oxidative stress of growing
343 chicks (Monaghan and Ozanne, 2018). We also point out that the rate of telomeres shortening is
344 accelerated by CORT as the differences in telomere length for two given ages of Kites is higher for
345 high CORT concentrations. Thus, consistent with previous studies, our finding suggests the
346 hypothesis that CORT could mediate telomeres attrition under stressful situations like chemical
347 exposure during growth. It moreover underlines important implications for fitness since the rate of
348 telomere loss may have a more accurate value as a proxy of future survival than telomere length
349 (Boonekamp et al., 2014).

350

351 **5. CONCLUSION**

352 For more than 50 years, scientists have highlighted evidence that chemicals threaten wildlife. From
353 the first statements showing direct toxic effects on life-history traits such as survival or reproduction,
354 we know that low levels of exposure to chemicals may have subtle impact on animals and sometimes
355 delayed consequences later in the life-cycle. Mixture effects are often discussed but rarely
356 demonstrated. The findings of the present study suggest that Hg and Pb interact synergistically to
357 raise baseline CORT in the feather of Red Kite. Such interaction could result in a stress response

358 being induced in nestlings even when blood concentrations of the metals remain below the toxicity
359 threshold benchmarks proposed for wildlife. As telomere length decreased with high CORT level, we
360 can assume that telomere erosion is an indirect consequence of metal exposure mediated by CORT
361 but further experimental studies will be needed to confirm such a link and identify both the cellular
362 and physiological processes involved in this potential adverse outcome pathway. What the
363 consequences of an early shortening of telomeres might be remains under question but this may
364 impair the bird during their whole life by increasing disease occurrence, and reducing individual
365 fitness and/or survival (Angelier et al., 2018). Moreover, increased CORT levels can have other effects
366 that persist after the stressful event has ceased such as changes in nestling behaviour, immune
367 function or an altered corticosterone response to future stressors with possible impact on the ability
368 of the bird to adapt its life-traits to changing environmental conditions.

369

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378

379 **Data Availability**

380 The data supporting this manuscript are referenced in the dat@OSU platform (https://dataosu.obs-besancon.fr/FR-18008901306731-2016-03-11_Red-kite-monitoring.html) and are available upon
381 request from Michael Coeurdassier.
382

383

384 **Ethical approval**

385 All procedures performed in studies involving human participants were in accordance
386 with the ethical standards of the institutional and/or national research committee and with the 1964
387 Helsinki declaration and its later amendments or comparable ethical standards. All applicable
388 institutional and/or national guidelines for the care and use of animals were followed.

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556

557 **TABLES and FIGURES Legends**

558 **Figure 1:** Relationship between corticosterone concentrations (pg cm^{-1}) in feathers and
559 concentrations of Hg in blood ($\mu\text{g L}^{-1}$) of nestling Red kite modulated by the blood concentrations of
560 Pb. The predicted values of CORT as a function of [Hg] in blood were presented for 3 concentrations
561 of Pb (3.7 (dashed line), 5.8 (thick solid line) and 25.4 (dotted line) $\mu\text{g L}^{-1}$ corresponding to the 5th,
562 median and 95th percentiles of [Pb] measured in chicks.

563

564 **Figure 2:** Relationship between telomere length (T/S ratio) and corticosterone concentrations (CORT
565 in pg cm^{-1}) in feathers of nestling Red kite modulated by the age of the chicks. Two ages of chicks,
566 *i.e.*, 32 (solid line) and 36 (dashed line) days old (corresponding to wing lengths of 245.3 and 285.0
567 mm, respectively) were retained to predict telomere length from CORT. The cross-sectional rate of TL
568 shortening in chicks between the ages of 32 to 35 days old is figured by a double-headed arrow for 2
569 CORT, 20 and 40 pg cm^{-1} (ΔTL_{20} and ΔTL_{40} , respectively).

570

571 **Table 1:** Occurrence frequency and concentrations of metals and anticoagulant rodenticides (ARs) in
572 the whole blood of Red kite chicks (in $\mu\text{g L}^{-1}$).

573

574 TABLES and FIGURES

575 Table 1

Chemicals	% detected*	Median (min-max)**
<i>Metals</i>		
Pb	100%	5.8 (2.3 – 59.0)
Hg	100%	6.1 (1.4 - 115.7)
Se	100%	298.3 (131.4 – 521.3)
Cd	6%	1.4 (0.7 – 3.0)
<i>ARs</i>		
Bromadiolone	15%	16.9 (0.2 - 29.4)
Difenacoum	9%	0.7 (0.5 - 2.5)
Difethialone	4%	6.9 (4.3 - 9.5)
Brodifacoum	9%	1.6 (0.6 - 3.0)
∑ARs	30%	6.1 (0.2 - 29.4)

576 *corresponds to the percentage of individuals with blood residues higher than the limits of detection (LOD).

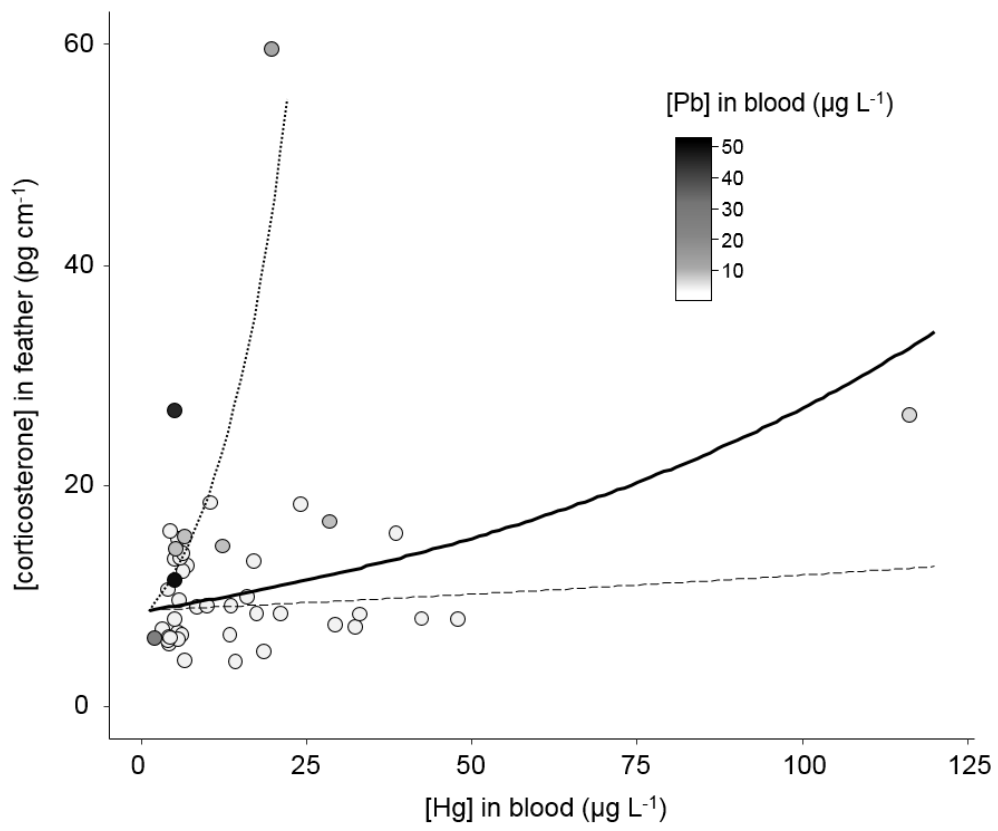
577 **Median, minimum and maximum concentrations reported for individuals with blood concentrations >LOD

578 only.

579

580 Fig. 1

581



582

583

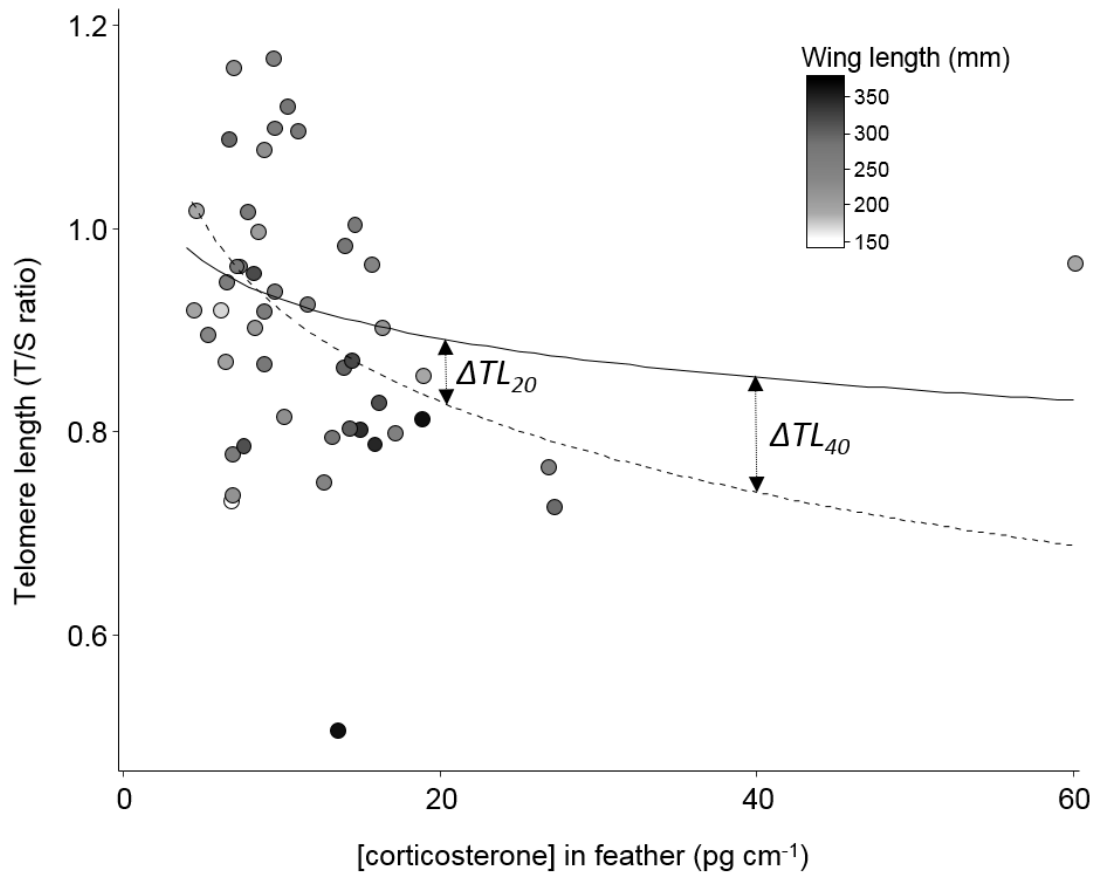
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587 Fig. 2

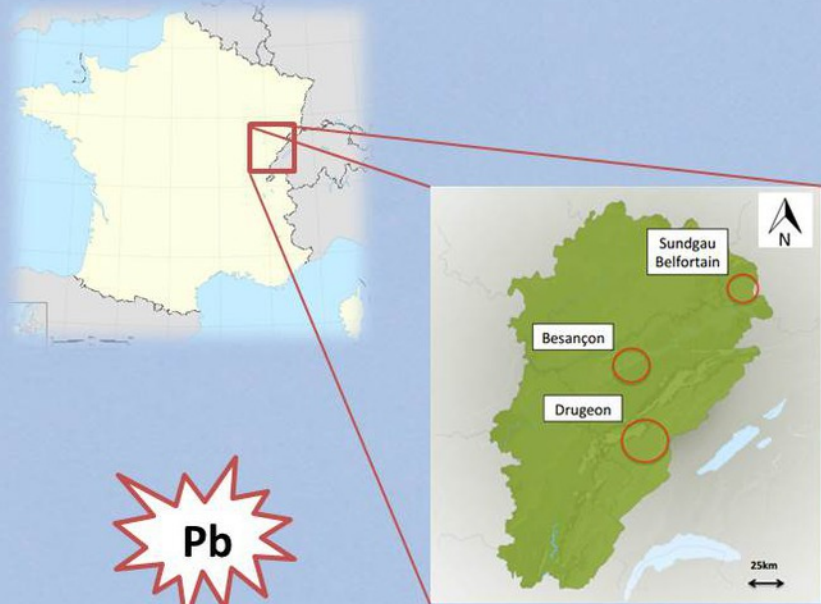
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Pb

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