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1 Fish from urban rivers and with high pollutant levels have  
2 shorter telomeres

3  
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28 **Abstract:**

29 Environmental pressures, such as urbanization and exposure to pollutants may jeopardize  
30 survival of free-living animals. Yet, much remains to be known about such an effect on wild  
31 vertebrate ectotherms, since most studies still failed to explore the relationship between levels  
32 of currently-released pollutants and physiological responses. This study aims at testing the  
33 effect of urbanization and pollution (phthalate, pesticides: organochlorine [OCP] and  
34 pyrethroid pesticides, polychlorobiphenyles: PCB, polybromodiphenylethers: PBDE,  
35 polycyclic aromatic hydrocarbons: PAH and some of their metabolites) on telomere length, a  
36 suggested biomarker of life expectancy, in the European chub *Squalius cephalus* from urban  
37 and agricultural rivers of the Marne hydrographic network, France. Our results show that  
38 telomere length was reduced in chub from urban rivers. Moreover, among the wide range of  
39 anthropogenic contaminants investigated, high levels of PCBs in muscle and metabolites of  
40 phthalates, PAH and pyrethroids in liver were associated with shorter telomeres in chub. This  
41 study suggests that urbanization and chemical pollution may compromise survival of wild  
42 fish, by accelerating telomere attrition.

43

44 **Keywords:** chub, telomere, metabolites, phthalate, pesticides

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53 **1. Introduction**

54 Chronic exposure to complex mixtures of environmental toxicants may have severe  
55 consequences in free-living animals by reducing reproductive outputs and survival [1],  
56 thereby leading to population collapse [2]. However, we currently lack robust data to link  
57 contaminant burden and survival, probably because studying demographic responses to  
58 chemical exposure requires long-term (years to decades) monitoring surveys of numerous  
59 marked individuals, which are often difficult to achieve in the wild. Aquatic organisms in  
60 urban areas are exposed to a wide array of environmental pollutants, because of sewage and  
61 runoff from artificialized surfaces.

62 In that context, the measurement of telomere length has been recognized as a robust  
63 molecular tool to predict life expectancy in endotherms [3,4] and to some extent in ectotherms  
64 [5] and even population vulnerability in wild lizards [6]. Located at the end of eukaryote  
65 chromosomes, telomeres shorten through successive cell division. Beyond a critical telomere  
66 length, the cell starts to senesce, leading to apoptosis and a decline in tissue function [7].  
67 Importantly, this natural process can be accelerated under stressful environmental conditions  
68 reviewed in [8,9]. In particular, oxidative stress has been recognized as a mechanistic pathway  
69 linking environmental stress and telomere erosion in vertebrates [10,11].

70 Chemical pollution is part of multiple stress factors generating or enhancing oxidative  
71 stress [12], yet the effect of chemical exposure on telomere length is poorly known for  
72 wildlife, especially for vertebrate ectotherms [13]. To date, studies mostly focused on birds  
73 exposed to trace metals [14], chlorinated [15,16] and halogenated compounds [16,17], which  
74 are classes of organic chemicals either banned or phased-out from use and production.  
75 Currently-release pollutants that are ubiquitous in the environment, such as polycyclic  
76 aromatic hydrocarbons (PAHs), phthalate plasticizers and pesticides [18], are known to  
77 impair telomere length in human cohorts [19,20]. Although exposure pathways and

78 concentrations might differ, similar effects are expected on aquatic vertebrates. Indeed,  
79 vertebrates share the ability to regulate the level of internal organic pollutant exposure *via*  
80 metabolic processes [21] that have the potential to produce reactive oxygen species through  
81 redox cycling or yield more toxic intermediates than the parent pollutant [22].

82 In addition, many vertebrates are particularly vulnerable to habitat alterations [23],  
83 other than chemical pollution. With the expanding urbanization, urban river systems have  
84 suffered from profound changes through damming, banking and channelization exerting  
85 additional stress on aquatic organisms. For instance, environmental harshness, disease  
86 prevalence and thermal stress have been associated with disturbed oxidative balance and  
87 shorter telomeres in fishes [24-28]. Non-chemical alterations of river systems, combined to  
88 environmental pollution, could therefore lead to a cumulative effect on telomere shortening.  
89 In this study, we examined the effect of organic pollutant burden and habitat alteration (urban  
90 and agricultural) on relative telomere length of wild populations of European chub, *Squalius*  
91 *cephalus*. We expected higher chemical pollution on urban rivers, as previously reported  
92 [29,30] and predicted that telomeres would be shorter in fish from urban rivers compared to  
93 agricultural ones and would decline with pollutant burden.

94

## 95 **2. Materials and Methods**

### 96 (a) Samples collection and chemical analyses

97 A total of 118 chub, *S. cephalus*, were caught by electrofishing within twelve days in  
98 September 2016 from the Marne River and its tributaries, France, on urban and agricultural  
99 areas, representing differently-contaminated riverine habitats (Supplementary material,  
100 Appendix A, table S1 and figures S1 and S2). Physico-chemical and hydrological parameters  
101 of each sampling sites are presented in the supplemental material (Appendix A, table S2). Left  
102 pelvic fins were consistently sampled for DNA extraction and scales were removed for age

103 determination (see [31], for details). Muscle samples (n = 118) were used for the  
104 quantification of parent organic contaminants and metabolites of pollutants were analyzed in  
105 the liver (n = 93), as the primary organ of xenobiotic biotransformation. Samples were stored  
106 in polycarbonate tubes to limit phthalate contamination and frozen at -20 °C until subsequent  
107 analyses. Metabolites were quantified in fewer individuals (n = 66; Appendix B, table S3)  
108 since some of them did not yield sufficient biological material to carry out chemical analyses.  
109 Analyses of organic pollutants (16 polycyclic aromatic hydrocarbons: PAH; 7 phthalate  
110 esters; 7 pyrethroids; 4 organochlorine pesticides: OCP; 7 polychlorinated biphenyls: PCB; 6  
111 polybrominated diphenyl ethers: PBDE) and their metabolites (11 hydroxylated PAHs; 9  
112 phthalate monoesters; 4 metabolites of pyrethroids) were performed in muscle and liver  
113 respectively, following previously published protocols [32].

114

#### 115 (b) Telomere analysis

116 Telomere length was determined by quantitative PCR (qPCR; BioRad CFX 96, Bio-Rad  
117 USA) according to [33], adapted for the European chub. Briefly, fin samples were digested  
118 with proteinase K and DNA was extracted using the Nucleospin Tissue Kit (Macherey-  
119 Nagel), following the manufacturer's instructions. DNA concentration and purity were  
120 assessed with a Nanodrop ND1000 spectrophotometer (Thermo Scientific). The telomere  
121 primers were similar to those previously used [34]. The control single-copy gene  
122 Recombination Activating Gene 1 (RAG-1) was selected and amplified using specific primers  
123 [35] designed for the European chub. Reverse and forward primer sequences for both  
124 telomeric and RAG1 genes were, respectively: Tel1b: 5'-  
125 CGGTTTGTGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3'; Tel2b: 5'-  
126 GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'; RAG1-F: 5'-  
127 AGAGAGAGGGGGCTAGATGA-3' and RAG1-R: 5'-CCATGCTTCTCGCTGACAT-3'.

128 The efficiency of the telomere and RAG- 1 assays ranged from 98.8% to 100% and 88.9% to  
129 92.6%, respectively. All qPCR runs were performed using 2.5 ng of DNA per reaction and  
130 using the BioRad SYBR Green Supermix. The universal telomere primers were used at a  
131 concentration of 800 nM, and RAG1-F/RAG1-R at 300 nM. To generate a six-point standard  
132 curve (from 10.0 ng to 0.31 ng) for controlling the amplifying efficiency of the reactions,  
133 serial dilutions of DNA from a pooled sampled of 10 chub were included on the plate. All  
134 samples were randomly distributed across the PCR plates. The reference sample (“golden  
135 sample”) was run in triplicate in all plates to account for inter-plate variation and each sample  
136 was run in duplicate on every plate. Intra-plate variation (coefficient of variation:  
137  $100 \times \text{SD}/\text{mean value}$ ) for telomere and RAG-1 were 2.07% and 2.80%, respectively. The  
138 relative telomere length (expressed as T/S ratio) was calculated as the telomere copy number  
139 (T) relative to single-copy gene (S; RAG1), according to [36]. Overall, 20 samples were  
140 excluded due to DNA degradation. As a result, telomere length (T/S ratio) was determined for  
141 98 individuals and was relative to the internal single gene control (RAG-1). Additional  
142 information about the method are presented in supplemental material (Appendix C).

143

#### 144 (c) Statistical analysis

145 Data were first checked for normality and homogeneity of variances. Differences in age-  
146 corrected relative telomere length (RTLc; residuals of RTL against age) between habitats and  
147 sampling sites were tested using Student's t-test and analysis of variances (ANOVA),  
148 respectively. Prior to analyses, all contaminant concentrations were log-transformed to attain  
149 a normal distribution and relationships between contaminant families were assessed using a  
150 Pearson's correlation matrix (Appendix D, table S4). To test whether relative telomere length  
151 (RTL) was affected by organic pollutant burdens, linear mixed model (LMMs) analyses were  
152 conducted using the *lme4* and *lmerTest* packages [37,38] in R v. 3.3.2 software [39] using the

153 restricted maximum likelihood (REML) estimation method. Given that telomeres shortened  
154 with age in this study ( $F_{1,92} = 17.8$ ,  $p \leq 0.001$ ;  $\beta \pm \text{s.e.} = -0.038 \pm 0.009$ ; Appendix D, figure  
155 S3) and that telomere attrition is linked to normal aging in fish [40, but see 5], RTL was first  
156 corrected with age (RTLc) to account for different age profiles between individuals. Then,  
157 differences in RTLc were assessed with sum-( $\Sigma$ -) contaminant concentrations (log-  
158 transformed) of each pollutant family and habitat (urban and agricultural) as fixed effects and  
159 sites as random effects to account for potential genetic variation between chub population.  
160 Indeed, fish from different sampling sites were considered distinct populations given the  
161 relatively short-range movements of chubs [41]. Chemicals being highly correlated with each  
162 other and being characterized by different chemical properties, we used the sum of each  
163 family of organic contaminants to take into account potential mixture effects. Denominator  
164 degrees of freedom for fixed effects were calculated using the Satterthwaite approximation.  
165 The significance of random effect was assessed using likelihood ratio tests (Appendix D,  
166 tables S5 and S6). We performed diagnostic plots and Shapiro normality tests on residuals to  
167 check model assumptions. A significance level of  $\alpha < 0.05$  was used for all tests.

### 168 **3. Results**

169 RTL was  $\sim 9.82\%$  longer in fish near agricultural areas than those closest to Paris, at  
170 proximity to urban habitats. Age-corrected relative telomere length (RTLc) of chub  
171 significantly differed between habitats (t-test:  $t = 2.82$ ,  $p = 0.006$ , figure 1A) and among  
172 sampling sites (ANOVA:  $F_{5,88} = 4.34$ ,  $p = 0.001$ ; Appendix D, figure S4B). Organic pollutant  
173 mean levels  $\pm$  standard deviation in chub tissues are listed in table S3 (Supplementary  
174 material, Appendix B). Fish from urban habitats had higher levels of OCPs ( $p < 0.001$ ),  
175 phthalates ( $p = 0.045$ ) and pyrethroid pesticides ( $p = 0.010$ ) relative to agricultural areas,  
176 representing a contamination increase of 48.6%, 20.8% and 15.4%, respectively, but not for  
177 PAHs, PBDEs, PCBs and  $\Sigma$ metabolites (all  $p \geq 0.126$ ).



178 Age-corrected telomere length (RTLc) significantly decreased with increasing levels  
179 of pollutants, especially for pollutants' by-products ( $\Sigma$  Phthalate metabolites, figure 1B;  
180 LMM:  $F_{1,49.9} = 5.57, p = 0.022$ ;  $\Sigma$ metabolites of phthalates, LMM:  $F_{1,71.0} = 9.34, p = 0.003$  ).  
181 The other chemical families did not show any significant relationships with RTLc (all  $F \leq$   
182  $2.86, p \geq 0.101$ ; Appendix E, tables S5 and S6).

183

#### 184 **4. Discussion**

185 As previously found in birds [41-44], telomeres were shorter in urban habitats  
186 compared to agricultural ones, suggesting higher life-threatening situations for fish in urban  
187 rivers. In fact, fish from urban and agricultural rivers did not differ in their pollutant load,  
188 except for slightly higher plasticizer and pesticide levels in urban watercourses. Urban river  
189 systems have however undergone profound changes, such as damming, banking and  
190 channelization that have led to the disruption of longitudinal connectivity, loss of wetlands  
191 and spawning grounds, but also increased water temperature, pathogens and boat noise  
192 [45,46]. Our study suggest that the diverse and profound degradation of urban streams induce  
193 deleterious effects in fish by accelerating telomere attrition and probably jeopardizing their  
194 survival. Those results are in line with previous findings, stating that environmental stressors  
195 accelerate telomere shortening in avian and fish species [8,25,28].

196 To the best of our knowledge this is the first evidence that exposure to organic  
197 pollutants negatively impacts telomere length in fish. In different species of birds, exposure to  
198 environmental contaminants (OCPs, perfluoroalkyl substances: PFAS and trace metals) was  
199 associated with a general reduction in telomere length [13,14-16, but see 17]. The originality  
200 of this study is to investigate currently-released pollutants and their metabolites in a common  
201 freshwater fish species. Among the wide range of analyzed contaminants, the levels of the  
202 sum of metabolites (phthalates, PAHs and pyrethroids) were more prone to explain

203 differences in our data than parent pollutants, except for PCBs. In a previous study using the  
204 same data set, metabolites of organic pollutants were negatively correlated to antioxidant  
205 capacity and peroxidase activity in chub plasma [31]. Organic pollutants may therefore  
206 produce oxidative stress by disrupting the pro-oxidant/antioxidant balance, which is a  
207 potential pathway of telomere shortening [11]. Even though the underlying mechanisms are  
208 still poorly understood, we hypothesize that electrophilic intermediates generated through the  
209 metabolization of parent compounds (PAHs, phthalates and pyrethroid pesticides) could  
210 increase oxidative attacks by depleting or weakening defense mechanisms (*i.e.*, antioxidants),  
211 ultimately shortening telomeres. Still, some caution is needed to interpret these findings as  
212 other factors may mask the effects of environmental contaminants when using a cross-  
213 sectional approach. Further work is thus advised to support the correlation between levels of  
214 organic contaminants and age-corrected telomere length, by using an experimental approach  
215 and testing other tissues for telomere length (e.g., liver).

216 Our results reveal physiological costs to fish living in polluted urban habitats, which  
217 may ultimately jeopardize their survival. Moreover, they highlight the importance of  
218 considering metabolites of environmental pollutants to better assess the impacts of currently-  
219 released chemicals on wildlife.

220

## 221 **Ethics**

222 Fish sampling has been conducted according to relevant national and European guidelines  
223 (L436-9, EN14011). The authorization for the scientific fish capture was delivered by local  
224 administration authorities (Departmental Direction of Territories of Seine-et-Marne).

225

## 226 **Data accessibility**

227 Data related to this article are available on Dryad Digital Repository. Dataset,  
228 <https://doi.org/10.5061/dryad.bcc2fqz9d>

229

230 **Authors' contributions**

231 A.G. conceived the idea, designed the methodology and acquired the funding. A.G. and

232 F.Alliot contributed to field work. F.Alliot performed chromatographic acquisition.

233 F.Angelier and C.R. performed telomere assays. N.M. performed chemical and data analyses,

234 scales reading and drafted the manuscript with suggestions and comments from F.Angelier,

235 C.R. and A.G. All authors approved the final version of the manuscript.

236

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241

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244

245 **Supplementary material**

246 Appendix A – Habitat determination and sampling sites, Appendix B – Descriptive statistics,

247 Appendix C –Telomere analysis, Appendix D – Methods: statistical results, and Appendix E –

248 Model outputs

249

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416 **FIGURE CAPTIONS**

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418 **FIGURE 1.** Age-corrected telomere length (RTLc, residuals  $RTL \sim age$ ) of chub depending  
419 on A) each habitat (urban: gray and agricultural: orange) and B) on the levels of phthalate  
420 metabolites <sup>(1)</sup> ( $\Sigma$  phthalates metabolites,  $ng\ g^{-1}$  dry wt, Liver). Filled diamonds represent the  
421 arithmetic mean and \*\* indicates a significant difference ( $p < 0.001$ ). Numbers represent  
422 sample size. Dashed lines represent the 95% confidence interval