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

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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.

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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-(Σ -) 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 Σ 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 (Σ Phthalate metabolites, figure 1B;
180 LMM: $F_{1,49.9} = 5.57, p = 0.022$; Σ 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>

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Authors' contributions

A.G. conceived the idea, designed the methodology and acquired the funding. A.G. and F.Alliot contributed to field work. F.Alliot performed chromatographic acquisition. F.Angelier and C.R. performed telomere assays. N.M. performed chemical and data analyses, scales reading and drafted the manuscript with suggestions and comments from F.Angelier, C.R. and A.G. All authors approved the final version of the manuscript.

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Supplementary material

Appendix A – Habitat determination and sampling sites, Appendix B – Descriptive statistics, Appendix C –Telomere analysis, Appendix D – Methods: statistical results, and Appendix E – Model outputs

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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 ⁽¹⁾ (Σ 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