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Physiology

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

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Environmental pressures, such as urbanization and exposure to pollutants may jeopardize survival of free-living animals. Yet, much remains to be known about physiological and ecological responses to currently-released pollutants, especially in wild vertebrate ectotherms. We tested the effect of urbanization and pollution (phthalates, organochlorine and pyrethroid pesticides, polychlorobiphenyls, polybromodiphenylethers, polycyclic aromatic hydrocarbons, and some of their metabolites) on telomere length, a suggested biomarker of life expectancy, in the European chub, *Squalius cephalus*, from urban and agricultural rivers of the Marne hydrographic network, France. We showed that telomere length was reduced in chub from urban rivers. Moreover, among the wide range of anthropogenic contaminants investigated, high levels of phthalate metabolites in liver were associated with shorter telomeres. This study suggests that urbanization and chemical pollution may compromise survival of wild fish, by accelerating telomere attrition.

1. Introduction

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

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

Exposure to chemical pollution is part of multiple stress factors generating or enhancing oxidative stress [12], yet its effect on telomere length is poorly known for wildlife, especially for vertebrate ectotherms [13]. To date, studies have mostly focused on birds exposed to trace metals [14], and chlorinated

[15,16] and halogenated compounds [16,17], which are classes of organic chemicals either banned or being phased-out from use and production. Currently-released pollutants that are ubiquitous in the environment, such as polycyclic aromatic hydrocarbons (PAHs), phthalate plasticizers and pesticides [18], are known to impair telomere length in human cohorts [19,20]. Although exposure pathways and concentrations differ, one may predict similar effects on aquatic vertebrates. Indeed, vertebrates share the ability to metabolize organic pollutant in the liver [21]. Importantly, metabolic processes may produce reactive oxygen species through redox cycling or yield more toxic intermediates than the parent pollutant [22].

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

2. Material and methods

(a) Sample collection and chemical analyses

A total of 118 chub, *S. cephalus*, were caught by electrofishing within a period of 12 days in September 2016 from the Marne River and its tributaries, France, in urban and agricultural areas, representing differently-contaminated riverine habitats (electronic supplementary material, appendix A, table S1 and figures S1 and S2). Physico-chemical and hydrological parameters of each sampling site are presented in the electronic supplementary material (electronic supplementary material, appendix A, table S2). Left pelvic fins were consistently sampled for DNA extraction and scales were removed for age determination (see [31], for details). Muscle samples ($n = 118$) were used for the quantification of parent organic contaminants and metabolites of pollutants were analysed in the liver ($n = 93$), as the primary organ of xenobiotic biotransformation. Samples were stored in polycarbonate tubes to limit phthalate contamination and frozen at −20°C until subsequent analyses. Metabolites were quantified in fewer individuals ($n = 66$; electronic supplementary material, appendix B, table S3) since some of them did not yield sufficient biological material to carry out chemical analyses. Analyses of organic pollutants (16 polycyclic aromatic hydrocarbons (PAHs); 7 phthalate esters; 7 pyrethroids; 4 organochlorine pesticides (OCPs); 7 polychlorinated biphenyls (PCBs); 6 polybrominated diphenyl ethers (PBDEs) and their metabolites (11 hydroxylated PAHs; 9 phthalate monoesters; 4 metabolites of pyrethroids) were performed in muscle and liver respectively, following previously published protocols [32].

(b) Telomere analysis

Telomere length was determined by quantitative PCR (qPCR; BioRad CFX 96, Bio-Rad USA) according to [33], adapted for the European chub. Briefly, fin samples were digested with proteinase K and DNA was extracted using the Nucleospin Tissue Kit (Macherey-Nagel), following the manufacturer's instructions. DNA concentration and purity were assessed with a Nanodrop ND1000 spectrophotometer (Thermo Scientific). The telomere primers were similar to those previously used [34]. The control single-copy gene Recombination Activating Gene 1 (RAG1) was selected and amplified using specific primers [35] designed for the European chub. Reverse and forward primer sequences for both telomeric and RAG1 genes were, respectively: Tel1b: 5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′; Tel2b: 5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCT-TACCCT-3′; RAG1-F: 5′-AGAGAGAGGGGGCTAGATGA-3′ and RAG1-R: 5′-CCATGCTTCTCGCTGACAT-3′. The efficiency of the telomere and RAG1 assays ranged from 98.8 to 100% and 88.9 to 92.6%, respectively. All qPCR runs were performed using 2.5 ng of DNA per reaction and using the BioRad SYBR Green Supermix. The universal telomere primers were used at a concentration of 800 nM, and RAG1-F/RAG1-R at 300 nM. To generate a six-point standard curve (from 10.0 ng to 0.31 ng) for controlling the amplifying efficiency of the reactions, serial dilutions of DNA from a pooled sampled of 10 chub were included on the plate. All samples were randomly distributed across the PCR plates. The reference sample ('golden sample') was run in triplicate on all plates to account for inter-plate variation, and each sample was run in duplicate on every plate. Intra-plate variation (coefficient of variation: $100 \times$ s.d./mean value) for telomere and RAG1 were 2.07 and 2.80%, respectively. The relative telomere length (expressed as T/S ratio) was calculated as the telomere copy number (T) relative to single-copy gene (S; RAG1), according to [36]. Overall, 20 samples were excluded owing to DNA degradation. As a result, telomere length (T/S ratio) was determined for 98 individuals. Additional information about the method is presented in the electronic supplementary material (electronic supplementary material, appendix C).

(c) Statistical analysis

Data were first checked for normality and homogeneity of variances. Given that telomeres shortened with age in this study ($F_{1,92} = 17.8$, $p \leq 0.001$; $\beta \pm$ s.e. $= -0.038 \pm 0.009$; electronic supplementary material, appendix D, figure S3) and that telomere attrition is linked to normal ageing in fish ([37], but see [5]), relative telomere length (RTL) was first corrected with age (RTLc) to account for different age profiles between individuals. Differences in RTLc (residuals of RTL against age) between habitats and sampling sites were tested using Student's *t*-test and analysis of variance (ANOVA), respectively. Prior to analyses, all contaminant concentrations were log-transformed to attain a normal distribution and relationships between contaminant families were assessed using a Pearson's correlation matrix (electronic supplementary material, appendix D, table S4). To test whether telomere length was affected by organic pollutant burdens, linear mixed model (LMM) analyses were conducted using the *lme4* and *lmerTest* packages [38,39] in R v. 3.3.2 software [40] using the restricted maximum likelihood (REML) estimation method. Differences in RTLc were assessed with summed (Σ) contaminant concentrations (log-transformed) of each pollutant family and habitat (urban and agricultural) as fixed effects and sites as random effect to account for potential genetic variation between chub populations. Indeed, fish from different sampling sites were considered distinct populations given the relatively short-range movement of chubs [41]. Chemicals being highly correlated with each other, the sum of each family of organic contaminants was individually tested on

RTLc. Denominator degrees of freedom for fixed effects were calculated using the Satterthwaite approximation. The significance of random effect was assessed using likelihood ratio tests (electronic supplementary material, appendix E, tables S5 and S6). We performed diagnostic plots and Shapiro normality tests on residuals to check model assumptions. A significance level of $\alpha < 0.05$ was used for all tests.

3. Results

RTL was approximately 9.82% longer in fish near agricultural areas than those closest to Paris, near urban habitats. Age-corrected relative telomere length (RTLc) was significantly shorter in chubs from urban rivers than in agricultural areas (*t*-test: $t = 2.82$, $p = 0.006$, figure 1*a*) and differed among sampling sites (ANOVA: $F_{5,88} = 4.34$, $p = 0.001$; electronic supplementary material, appendix D, figure S4B). Electronic supplementary material, table S3 provides the levels of organic pollutant in chub tissues (mean levels ± s.d.; electronic supplementary material, appendix B). Fish from urban habitats had higher levels of OCPs ($p < 0.001$), phthalates ($p = 0.045$) and pyrethroid pesticides ($p = 0.010$) relative to agricultural areas, representing a contamination increase of 48.6, 20.8 and 15.4%, respectively. No difference between habitats was observed for PAHs, PBDEs, PCBs and metabolites (all $p \geq 0.126$).

Age-corrected telomere length (RTLc) significantly decreased with increasing levels of Σphthalate metabolites (figure 1*b*; LMM: $F_{1,49.9} = 5.57$, $p = 0.022$). The other chemical families did not show any significant relationships with RTLc (all $F \leq 2.86$, $p \geq 0.101$; electronic supplementary material, appendix E, tables S5 and S6).

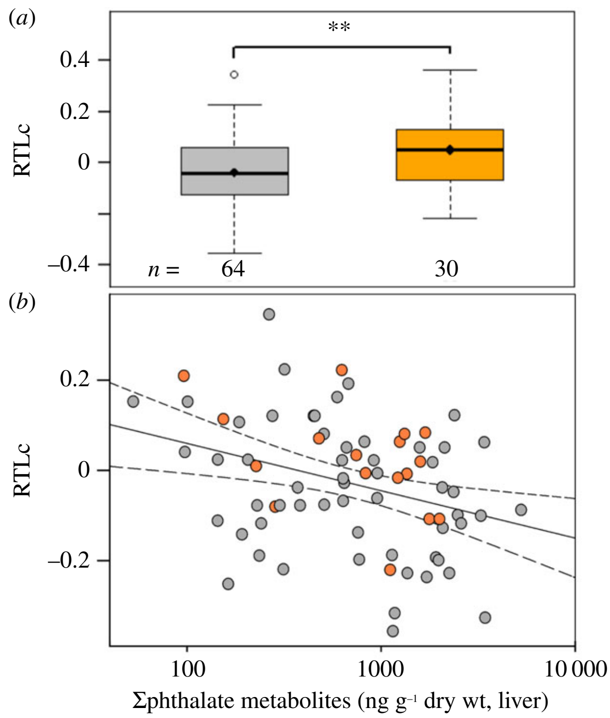

Figure 1. Age-corrected telomere length (RTLc, residuals RTL ∼ age) of chub depending on (*a*) each habitat (urban: grey, and agricultural: orange) and (*b*) the levels of phthalate metabolites (Σphthalate metabolites, ng g^{-1} dry wt, liver). In (*a*) filled diamonds represent the arithmetic mean and ** indicates a significant difference ($p < 0.01$). Numbers represent sample size. Dashed lines represent the 95% confidence interval. ΣPhthalate metabolites: mono-methyl phthalate, mono-ethyl phthalate, mono-*iso*-butyl phthalate, mono-*n*-butyl phthalate, mono-benzyl phthalate, mono-*n*-octyl phthalate, mono-2-ethylhexyl phthalate, mono-2-ethyl-5-oxohexyl phthalate, mono-2-ethyl-5-hydroxyhexyl phthalate. (Online version in colour.)

4. Discussion

As previously found in birds [41–44], telomeres were shorter in urban habitats compared with agricultural ones, suggesting higher life-threatening conditions for fish in urban rivers. In fact, fish from urban and agricultural rivers did not differ in their pollutant load, except for slightly higher plasticizer and pesticide levels in urban watercourses. Urban river systems have however undergone profound changes, such as damming, banking and channelization that have led to the disruption of longitudinal connectivity, and loss of wetlands and spawning grounds, but also increased water temperature, pathogens and boat noise [45,46,47]. Our study suggests that the diverse and profound degradation of urban streams induces deleterious effects in fish by accelerating telomere attrition and probably jeopardizing their survival. Those results are in line with previous findings, stating that environmental stressors accelerate telomere shortening in avian and fish species [8,25,28].

To the best of our knowledge this is the first evidence that exposure to organic pollutants negatively impacts telomere length in fish. In different species of birds, exposure to environmental contaminants (OCPs, perfluoroalkyl substances (PFAS) and trace metals) was associated with a general reduction in telomere length ([13,14–16], but see [17]). The originality of this study is to investigate currently-released pollutants and their metabolites in a common freshwater fish species. Among the wide range of analysed contaminants, the levels of phthalate metabolites were more prone to explain differences in our data than parent pollutants. In a previous study using the same dataset, metabolites of organic pollutants were negatively correlated to antioxidant capacity and peroxidase activity in chub plasma [31]. Organic pollutants may therefore produce oxidative stress by disrupting the pro-oxidant/antioxidant balance, which is a potential pathway of telomere shortening [11]. We thus hypothesize that electrophilic intermediates generated through the metabolization of parent compounds could increase oxidative attacks by depleting or weakening defence mechanisms (i.e. antioxidants), ultimately shortening telomeres. Still, some caution is needed to interpret these findings as other factors may mask the effects of environmental contaminants when using a cross-sectional approach. Further work is thus required to understand the underlying mechanisms linking organic contaminants and telomere attrition, through an experimental approach and the use of liver tissues for telomere length.

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

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

Data accessibility. Data related to this article are available in the Dryad Digital Repository: https://dx.doi.org/10.5061/dryad.bcc2fqz9d [48].

Authors' contributions. A.G. conceived the idea, designed the methodology and acquired the funding. A.G. and F. Alliot contributed to fieldwork. 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., F. Alliot and A.G. All authors agree to be held accountable for and approve the final version of the manuscript.

Competing interests. We declare we have no competing interests.

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