Exposure to oxychlordane is associated with shorter telomeres in arctic breeding kittiwakes

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HIGHLIGHTS

- Potential impacts of POPs on telomeres were studied in an arctic seabird.
- No relationship was found between PCBs and telomere length.
- Oxychlordane concentration was associated with shorter telomeres in females.
- This study highlights sex-related sensitivity to banned organochlorine pesticides.

GRAPHICAL ABSTRACT
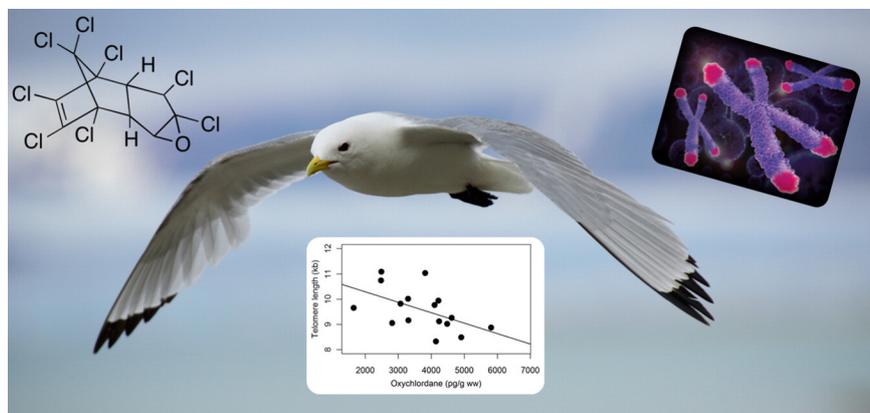
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ABSTRACT

Telomeres are DNA-protein complexes located at the end of chromosomes, which play an important role in maintaining the genomic integrity. Telomeres shorten at each cell division and previous studies have shown that telomere length is related to health and lifespan and can be affected by a wide range of environmental factors. Among them, some persistent organic pollutants (POPs) have the potential to damage DNA. However, the effect of POPs on telomeres is poorly known for wildlife. Here, we investigated the relationships between some legacy POPs (organochlorine pesticides and polychlorobiphenyls) and telomere length in breeding adult black-legged kittiwakes (*Rissa tridactyla*), an arctic seabird species. Our results show that among legacy POPs, only blood concentration of oxychlordane, the major metabolite of chlordane mixture, is associated with shorter telomere length in females but not in males. This suggests that female kittiwakes could be more sensitive to oxychlordane, potentially explaining the previously reported lower survival rate in most oxychlordane-contaminated kittiwakes from the same population. This study is the first to report a significant and negative

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relationship between POPs and telomere length in a free-living bird and highlights sex-related susceptibility to banned pesticides.

1. Introduction

Telomeres are DNA-protein complexes located at the end of linear chromosomes which play a critical role in maintaining the genomic integrity (Blackburn, 1991; Monaghan and Haussmann, 2006). The DNA polymerase protein complex cannot replicate the very ends of chromosomes during mitosis, and, consequently telomeres shorten at each cell division (Olovnikov, 1996). When telomeres reach a critical lower threshold, cell division can damage coding DNA leading to apoptosis or replicative senescence (Olovnikov, 1996; Campisi et al., 2001). It was originally thought that telomere loss occurred at a constant rate in individuals through their life, and telomere length could therefore act as an internal 'mitotic clock' to measure the chronological age of organisms into the wild (Haussmann and Vleck, 2002). However, recent studies have shown that telomere length predicts survival (Haussmann et al., 2005; Bize et al., 2009; Salomons et al., 2009; Heidinger et al., 2012; Angelier et al., 2013; Barrett et al., 2013) and is related to a wide range of environmental stressors (Mizutani et al., 2013; Meillère et al., 2015). Consequently, telomere length is considered as more related to biological age than chronological age *per se* (Monaghan and Haussmann, 2006; Barrett et al., 2013).

In humans, telomere erosion can be accelerated by different environmental factors such as exposure to pollutants (Zhang et al., 2013). For instance, it has been reported that outdoor workers exposed to traffic pollution have shorter telomeres than indoor office workers (Hoxha et al., 2009). Similarly, women telomere length decreases as exposure to pollution caused by hazardous wastes increases (De Felice et al., 2012). One underlying mechanism that could potentially explain accelerated telomere shortening is oxidative stress (Von Zglinicki et al., 2000; Zhang et al., 2013). This corresponds to the imbalance between the production of reactive oxygen species (ROS) and the antioxidant capacity of an organism (Finkel and Holbrook, 2000). When metabolic by-products, such as ROS, are not fully neutralized by anti-oxidant defenses, they may oxidize cellular macromolecules such as DNA (Houben et al., 2008). Hence, telomere length may partly reflect oxidative stress history of an individual (Houben et al., 2008). Among contaminants, some persistent organic pollutants (POPs) have the potential to damage DNA by triggering oxidative stress and even decrease survival (Fernie et al., 2005; Isaksson, 2010; Letcher et al., 2010; Erikstad et al., 2013; Costantini et al., 2014; Sletten et al., 2016). However, the effect of POPs on telomere length is poorly known for wildlife. To the best of our knowledge, only one study has addressed this topic in a free-living animal with low contamination levels but failed to find any significant relationships (Sletten et al., 2016).

Due to their high volatility and persistence in time, POPs reach remote areas such as the Arctic (Gabrielsen and Henriksen, 2001). Once deposited in marine ecosystems, living organisms assimilate the POPs *via* food intake. The POP concentrations then increase from the marine environment into the organisms and throughout food webs due to bioaccumulation and biomagnification (Letcher et al., 2010). Seabirds are top predators; consequently, they are particularly exposed to POPs contamination. They therefore appear as highly relevant biological models to investigate the influence of POPs on telomere length. A previous study in a Svalbard population of black-legged kittiwakes *Rissa tridactyla* ("kittiwakes" hereafter) has reported high oxidative stress levels in most POPs contaminated individuals (POPs included polychlorobiphenyls: PCBs and one organochlorine pesticide: OCP; Lindsøe, 2012). Additionally, adult survival rate in the same population of kittiwakes was negatively linked to some OCPs (Goutte et al., 2015). In this study, we investigated the relationships between some legacy POPs (OCPs and PCBs) and telomere length in Svalbard kittiwakes. Because telomere length is classically reduced in response to oxidative stress (Von Zglinicki et al., 2000; Zhang et al., 2013) and often tightly linked to survival (Haussmann et al., 2005; Bize et al., 2009; Salomons et al., 2009; Heidinger et al., 2012; Angelier et al., 2013; Barrett et al., 2013), we predicted that POP levels would be negatively related to telomere length.

2. Materials and methods

2.1. Study area and sampling collection

Fieldwork was carried out in 2012 from July 12th to July 27th in Krykkjefjellet colony of Kongsfjorden, Svalbard (78°54′N, 12°13′E). A total of 38 individuals (22 males and 16 females) were caught on their nest with a noose at the end of a 5 m fishing rod during the chick rearing period. At capture, a 2 mL blood sample was collected from the alar vein using a heparinized syringe and a 25G needle to determine legacy POP levels, telomere length and the sex of individuals. Blood samples were stored at −20 °C until subsequent analyses. The sex of individuals was determined from red blood cells by polymerase chain reaction (PCR) at the Centre d'Etudes Biologiques de Chizé (CEBC) as previously described (Weimerskirch et al., 2005).

2.2. Telomere assay

Telomere length was determined at the CEBC by Southern blot using the TeloTAGGG Telomere Length Assay (Roche, Mannheim, Germany) as previously described and with minor modifications (Foote et al., 2010; Kimura et al., 2010). Telomere length analysis has already been successfully achieved on the same population of Svalbard kittiwakes (Schultner et al., 2014a). Briefly, samples were digested with proteinase K, and DNA was extracted from red blood cells by using the DNeasy blood and tissue kit (Qiagen). DNA quality was checked by gel electrophoresis and optical density spectrophotometry. Preliminary tests have been conducted to determine the optimal amount of DNA to be used and, for each sample, 0.7 µg of DNA was digested with the restriction enzymes *HinfI* and *RsaI* for 16 h at 37 °C. Digested DNA samples were then separated using a pulse-field gel electrophoresis (Bio-Rad) on a 0.8% agarose gel. All samples were run in four gels. Samples were randomly assigned to a gel. Internal controls were run on each gel to measure inter-gel variations. The gels were run at 3.0 V/cm with an initial switch time of 0.5 s to a final switch time of 7 s for 14 h. Following that step, the gel was depurined and denatured in an alkaline solution. The gel was then neutralized and DNA was transferred onto a nitrocellulose membrane by Southern blot (Hybond N+, Amersham Life Science, Amersham, UK). The membrane was incubated at 120 °C for 20 min in order to fix the DNA. The DNA was then hybridized with a digoxigenin-labeled probe specific for telomeric sequences and incubated with antidigoxigenin-specific antibody before visualization with a Chemidoc (Bio Rad). Telomere length was then analyzed using ImageJ to extract telomere smear densities. Lane-specific background was subtracted from each density value and telomere length (mean value) was then calculated using a window of 5–30 kb that includes the whole smear (Nussey et al., 2014). Inter-gel CV was 1.40%.

2.3. POPs analyses

POPs were analyzed from whole blood at the Norwegian Institute for Air Research (NILU) in Tromsø, Norway. Only compounds that

potentially affect survival of kittiwakes were considered in this study (Goutte et al., 2015). Thus, we selected the $\sum$PCBs (CB-99, -118, -138, -153, -180, -183 and -187), and the OCPs (HCB, *p,p′*-DDE, oxychlordane, *trans*- and *cis*-nonachlor). To a blood total sample of 0.5 to 1.5 mL, a 100 μL internal standard solution was added (13C-labeled compounds from Cambridge Isotope Laboratories: Woburn, MA, USA). The sample was extracted twice with 6 mL of *n*-hexane, after denaturation with ethanol and a saturated solution of ammonium sulphate in water. Matrix removal on florisil columns, separation on an Agilent Technology 7890 GC and detection on an Agilent Technology 5975C MSD were performed as previously described (Herzke et al., 2009). For validation of the results, blanks (clean and empty glass tubes treated like a sample, 3 in total) were run for every 10 samples, while standard reference material (3 in total, 1589a human serum from NIST) was run for every 10 samples. The accuracy of the method was within the 70 and 108% range.

2.4. Statistical analyses

Statistical tests were performed using R 2.13.1 (R Development Core Team, 2011). We first checked if telomere length and POP levels differed between sexes using the parametrical test of Welch. The influence of POPs' contamination on telomere length was investigated with Linear Models. Thus, $\sum$POPs, $\sum$PCBs, HCB, *p,p′*-DDE, oxychlordane, *cis*- and *trans*-nonachlor were defined as independent variables and telomere length as the dependent variable. Because blood contaminant concentrations differed between males and females (see Results), including the factor "sex" and the variable "$\sum$POPs" in the same model could lead to multicollinearity problems and biased results (Graham, 2003). Additionally, it is now well established that males and females can react in different way to environmental stressors such as POPs contamination. Specifically, previous studies conducted on kittiwakes from Krykkjefjellet colony have reported sex differences regarding body condition, hormones levels, endocrine disruptions, phenology, breeding decision and even survival rate (Goutte et al., 2010, 2015; Schultner et al., 2014b; Tartu et al., 2013, 2014, 2015a). Therefore, male and female kittiwakes were separated in statistical analyses. Diagnostic plots were assessed and Shapiro normality tests were performed on residuals to test whether data sufficiently met the assumption of the linear model. Multiple testing can potentially lead to misleading results, indicating statistical significance in situations where there is none. Consequently, we performed bootstrapping (*i.e.* resampling method) from the data sets of significant relationship and then assessed diagnostic plot to corroborate the results (see Supplementary materials; Chernick and Labudde, 2014). A significance level of α < 0.05 was used for all tests.

3. Results

Telomere length was not related to sex (t = − 1.438, P-value = 0.160) and $\sum$POPs tended to be higher in male than in female kittiwakes (t = − 1.976, P-value = 0.056). $\sum$POPs, $\sum$PCBs, HCB, *p,p′*-DDE, *cis*- and *trans*-nonachlor were not related to telomere length in male nor in female kittiwakes (All P-values ≥ 0.259; Table 1). In females, we found a significant and negative association between oxychlordane and telomere length (Table 1; Fig. 1a). However, we found no relationship between oxychlordane and telomere length in males (Table 1; Fig. 1b).

4. Discussion

Our results on adult kittiwakes showed that among legacy POPs, only blood concentrations of oxychlordane were negatively associated with telomere length in females but not in males. Oxychlordane and chlordane mixture have already been associated with lower survival in both males and females from the same kittiwake population (Goutte et al., 2015). Our study reports a negative relationship between telomere length only in females, thus providing a possible mechanism

Table 1
Relationships between whole blood $\sum$POPs, $\sum$PCBs, OCPs and telomere length in female and male chick-rearing black-legged kittiwakes, *Rissa tridactyla* from Kongsfjorden, Svalbard.

Dependent variables		Independent variables	df	F	P-value
Females	Telomere length	$\sum$POPs[*]	1,14	0.802	0.386
		$\sum$PCBs[**]	1,14	0.833	0.377
		HCB	1,14	0.534	0.477
		p,p′-DDE	1,14	0.066	0.801
		Oxychlordane	1,14	5.343	**0.037**
		cis-Nonachlor	1,14	1.246	0.283
		trans-Nonachlor	1,14	1.301	0.273
Males	Telomere length	$\sum$POPs[*]	1,20	0.394	0.537
		$\sum$PCBs[**]	1.20	0.114	0.740
		HCB	1.20	0.002	0.967
		p,p′-DDE	1.20	1.351	0.259
		Oxychlordane	1.20	0.025	0.876
		cis-Nonachlor	1.20	0.077	0.785
		trans-Nonachlor	1.20	0.922	0.348

Significant variables are in bold.
[*] $\sum$POPs: CB-99, -118, -138, -153, -180, -183, -187, HCB, *p,p′*-DDE, oxychlordane, *trans*- and *cis*-nonachlor.
[**] $\sum$PCBs: CB-99, -118, -138, -153, -180, -183, -187.

linking oxychlordane exposure and female mortality. Regarding males, it is thus likely that oxychlordane exposure may lead to a lower survival rate in another way, independent of telomere attrition. Effects of contaminants on telomere length are still poorly known; most of the studies have focused on humans (Zhang et al., 2013) and to the best of our knowledge, only one study has investigated the relationships between POPs and telomere length in wildlife (Sletten et al., 2016).

So far, 11 of the 14 studies investigating the relationship between telomere length and environmental and occupational chemical exposure in humans, have reported significant negative associations (Rigolin et al., 2004; Hoxha et al., 2009; Bin et al., 2010; McCracken et al., 2010; Pavanello et al., 2010; Li et al., 2011; Rollison et al., 2011; De Felice et al., 2012; Eshkoor et al., 2012; Hou et al., 2012; Wu et al.,
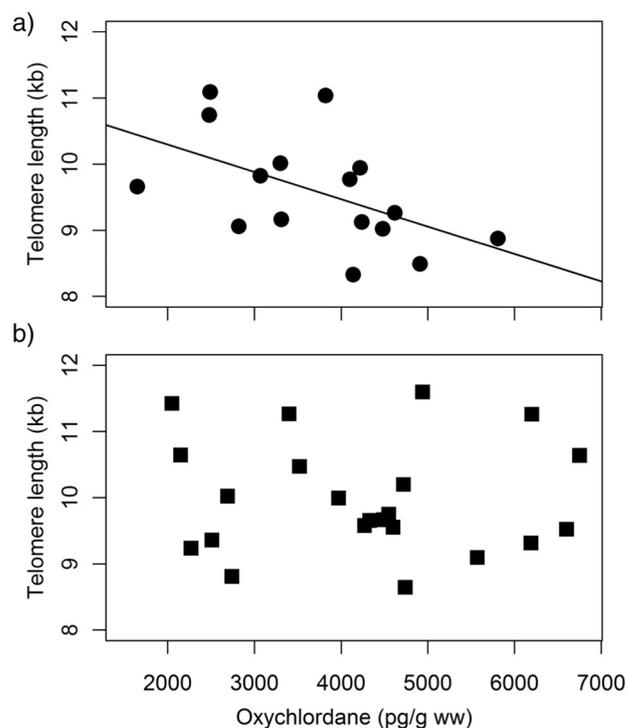

Fig. 1. Relationships between telomere length and whole blood oxychlordane concentrations in female (a) and male (b) chick-rearing black-legged kittiwakes, *Rissa tridactyla* from Kongsfjorden, Svalbard.

2012). For example, shorter telomeres were associated with pesticide exposure in patients with myelodysplastic syndrome (Rigolin et al., 2004; Rollison et al., 2011). Similarly, a study conducted in an apparent healthy Korean population reported a negative association between telomere length and exposure to high levels of POPs, including OCPs (p,p′-DDE, trans-nonachlor and oxychlordane), PCBs and polybrominated diphenylethers (Shin et al., 2010). The only study investigating relationships between telomere length and contamination in wildlife has been performed on white-tailed eagle (Haliaeetus albicilla) chicks in northern Norway (Sletten et al., 2016). In this study, the authors did not find any relationship between telomere length and POPs, including OCPs, PCBs and perfluorinated compounds. However, the relatively low levels of contaminants in white-tailed eagle chicks compared to those measured in adult kittiwakes may explain the discrepancy in the results. Oxychlordane concentrations in kittiwakes measured in the present study (4019 ± 1315 pg/g ww) were on average, around 3 times higher than those reported for eagle chicks (1483 ± 197 pg/g ww). With this exception, our results are thus consistent with previous works and suggest a negative effect of oxychlordane on telomere length in kittiwakes.

Surprisingly, the relationships between oxychlordane and telomere length were sex dependent and a significant relationship was found in females, but not in males. How to explain such a difference? There are some indications that female seabirds may be more sensitive to high levels of OCPs than males. In glaucous gulls (Larus hyperboreus) and in kittiwakes, OCPs exposure mostly affected survival rate in females (Erikstad et al., 2013; Goutte et al., 2015). Furthermore, in wandering albatrosses (Diomedea exulans), another long-lived seabird species, the relationships between POP levels and oxidative stress were dependent of reproductive effort and breeding females had the highest levels of haptoglobin, a well-known acute phase protein that indicates an ongoing inflammatory response which can limit the spread of oxidative damages, compared to breeding males (Costantini et al., 2014). Thus, the influence of contaminants on oxidative stress may be exacerbated by reproductive effort. Although kittiwakes are considered to share equally incubation and chick rearing duties (Coulson, 2011), egg production represents a significant and important cost for females (Monaghan and Nager, 1997). It is thus possible that the cost of egg production increases the sensitivity of female kittiwakes to oxychlordane. This may therefore explain the sex-dependent relationship between telomere length and oxychlordane burden. Furthermore, although female birds transfer a significant part of their PCBs and DDT burden to their eggs, oxychlordane on the other hand, seemed to be more selectively retained by the female, at least in female glaucous gulls (Verboven et al., 2009). In female kittiwakes, it is thus possible that the energetic cost of clutch production added to the maintenance of significant oxychlordane levels would exacerbate the toxic effects of this chlorinated pesticide.

Our study was conducted on breeding adults (i.e. at least 3–4 years old; Coulson, 2011). However, the birds' age was unknown in our study, thus a possible confounding factor could be that birds with high levels of OCPs are the oldest birds with the shortest telomeres. If so, the negative relationship between oxychlordane and telomere length would be induced by the age of individuals rather by a direct effect of oxychlordane on telomere attrition. Although several studies suggested a negative effect of age on telomere length (Haussmann and Vleck, 2002; Haussmann et al., 2003), other reported that telomere loss mainly occurs early in life of long-lived seabird (i.e. between chick and adult stage) rather than during adulthood (Hall et al., 2004; Foote et al., 2010), as is the case in other vertebrates (Frenck et al., 1998; Rufer et al., 1998; Zeichner et al., 1999; Friedrich et al., 2001). Additionally, Haussmann et al. (2003) showed a significant and positive relationship between telomere length and age in another long-lived seabird species, the Letch's storm petrel (Oceanodroma leucorhoa). Consequently, effect of age on telomere length appears to be more complex rather than a simple negative and constant decrease of telomere length with age. Furthermore, it has been reported that in several seabird species, blood level of POPs is unrelated to age in adult birds (Bustnes et al., 2003; Carravieri et al., 2014; Tartu et al., 2015b), and rather reaches a steady state of equilibrium once adult (Newton et al., 1981; Henriksen, 1995; Drouillard, 2001; Bustnes et al., 2003). Therefore, it is unlikely that the negative relationship between oxychlordane and telomere length originates from birds with high levels of oxychlordane being the oldest birds with the shortest telomeres.

Previous studies have shown decreased antioxidant enzyme activity in relation to contaminants, such as in herring gulls (Larus argentatus), where chicks exposed to PCBs combined with dietary restrictions showed negative relationships between catalase, glutathione peroxidase and contaminant levels (Hegseth et al., 2011). Similarly, significant negative associations between oxychlordane, p,p′-DDE, PCB-153 and superoxide dismutase enzyme in white-tailed eagle chicks have been reported (Sletten et al., 2016). Finally, a significant positive relationship between oxychlordane, PCBs and oxidative stress was found in a Svalbard population of adult kittiwakes (Lindsøe, 2012). Consequently, oxychlordane could be possibly involved in the generation of oxidative stress through an increase of ROS which are known to reduce telomere length (Von Zglinicki, 2002). As the nucleobase guanine is a major oxidation target for ROS, the (TTAGGG)n repeats, that constitute vertebrate telomeres, are particularly vulnerable to oxidative attacks (Wang et al., 2010). In our study, in vivo biomarkers of oxidative stress were not measured. Thus, further studies measuring at the same time POP levels, proxies of oxidative stress and telomere length are thus needed to test if oxidative stress induced by oxychlordane exposure could be linked to telomere attrition. Among other potential mechanisms, oxychlordane could induce a down-regulation of telomerase activity. Indeed, telomere integrity is largely maintained by a telomerase-based mechanism, in which the enzyme telomerase plays a key role by adding hexameric (TTAGGG) repeats to chromosome ends, partially compensating telomere shortening (Greider and Blackburn, 1989; Xin et al., 2008). However, this hypothesis seems unlikely since telomerase is generally inactivated in adult somatic cells of most studied species so far (Monaghan and Haussmann, 2006; Vleck et al., 2007; but see Hatakeyama et al., 2008). Consequently, telomerase down-regulation seems to be more a common feature of large and/or long-lived species (Gomes et al., 2011) rather than being specifically related to contamination levels.

Extensively used during > 35 years as a pesticide, usage of chlordane, of which oxychlordane is a major metabolite, tended to decrease in the 80s (U.S. Department of Health and Human Services, 1994). Banned from use and listed as a legacy POP by the Stockholm convention since 2004, oxychlordane provided a clear diagnostic criterion related to the lethal poisoning in several bird species (Blus et al., 1983, 1985; Stickel et al., 1983; Okoniewski and Novesky, 1993; Stansley and Roscoe, 1999; Wiemeyer, 1996). Furthermore, in an experimental study where female rats were gavage with oxychlordane, high administrated doses (10 mg·kg^{-1}) revealed acute toxicity, characterized by feed refusal, rapid weight loss and thymic atrophy. At lower doses (2.5 mg·kg^{-1}), female rats showed signs of hepatic changes indicative of microsomal enzyme induction (Bondy et al., 2003). Finally, as previously mentioned, in glaucous gulls and in kittiwakes from Svalbard, oxychlordane has been associated with lower survival rates, especially in females (Erikstad et al., 2013; Goutte et al., 2015). In the present study, among legacy POPs, only oxychlordane was negatively associated with telomere length in females. Our results are thus consistent with the idea that oxychlordane is one of the most toxic POPs (Erikstad et al., 2013). Consequently, female kittiwakes' sensitivity to oxychlordane could affect telomere length, explaining the previously reported lower survival rate in highly contaminated female kittiwakes. On the other side, the lack of relationships between oxychlordane and telomere length in males suggest that the lowest survival rate of most oxychlordane contaminated birds is thus probably not mediated by telomere shortening in male kittiwakes.

This study is the first to report a significant and negative relationship between POPs and telomere length in wildlife and therefore partly fills the gap of knowledge about contaminants effect on telomere attrition. However, the present work has some limitations. First, we did not measure oxidative stress or other potential mechanisms. Thus, further studies measuring at the same time POP levels, proxies of oxidative stress and telomere length are thus needed to test if oxidative stress induced by oxychlordane exposure could be linked to telomere attrition. Secondly, among all tested contaminants only blood oxychlordane concentration was negatively associated with telomere length and even though several studies have reported that oxychlordane is highly toxic for birds, an experimental approach would enable to confirm the acute toxicity of this compound on telomere length through oxidative stress. Finally, our study was conducted on a limited number of individuals and to fully validate our finding, future studies investigating effects of POPs on telomere length should be conducted on a larger sample size and other species.

Conflict of interest

The authors declare no competing financial interest.

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