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SHORT COMMUNICATION

Reduced telomere length in embryos exposed to predator cues

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ABSTRACT

It is often assumed that embryos are isolated from external influences, but recent studies indicate that environmental stressors during prenatal stages can exert long-term negative effects on fitness. A potential mechanism by which predation risk may lastingly shape life-history traits and phenotypes is via effects on telomeres. However, whether prenatal exposure to environmental stressors, such as cues of predator presence, affects postnatal telomere length has not hitherto been investigated. Using an experimental design in which we modified the exposure of yellow-legged gull (*Larus michahellis*) embryos to social cues of predator presence (i.e. alarm calls), we show that prenatally exposed chicks had shorter telomeres after hatching. As young birds with shorter telomere lengths have reduced fledging success, reproductive success and lifespan, the reduced telomere length in the exposed chicks is likely to have long-term fitness consequences. Moreover, our results provide a mechanistic link through which predators may negatively affect population dynamics.

KEY WORDS: Ageing, Predation risk, Stressors, Yellow-legged gull

INTRODUCTION

Across taxa, evidence indicates that conditions during embryo development can exert long-term effects on important life-history traits such as growth and lifespan (DuRant et al., 2013; Entringer et al., 2012; Thiéry et al., 2014; Vaiserman, 2014). An important mechanism through which early life development may lastingly shape life-history traits and phenotypes is via effects on telomeres (reviewed in Price et al., 2013). Telomeres are protective non-coding nucleoprotein structures located at the end of eukaryotic chromosomes that play a key role in preventing genome degradation (O'Sullivan and Karlseder, 2010). In the absence of restoration (e.g. via telomerase activity), average cellular telomere length decreases with age in many taxa, particularly during the growth period (Frenck et al., 1998; Noguera et al., 2015), and young individuals with shorter telomeres have increased risk of diseases and reduced longevity and reproductive success (Blackburn et al., 2015; Eastwood et al., 2019; Heidinger et al., 2012; van Lieshout et al., 2019). Thus, telomere shortening during prenatal stages may potentially have long-lasting consequences through life (Entringer et al., 2018; Noguera et al., 2016).

One important route through which telomere shortening may arise during embryo development is by exposure to environmental stressors. In humans and lab rodents, for instance, environmental and social stressors acting on mothers during pregnancy have been related to reduced postnatal telomere length in the offspring (e.g. Entringer et al., 2011; Tarry-Adkins et al., 2009). However, unlike most mammalian embryos, embryos of oviparous species develop outside the maternal influence and therefore it is often assumed that they are relatively more isolated from external stimuli during their development. However, recent empirical evidence indicates that embryos of different oviparous species can also be affected by a variety of environmental stressors (DuRant et al., 2013; Henriksen et al., 2011), including the presence of predators (e.g. Mathis et al., 2008; Noguera and Velando, 2019). Indeed, predator presence is one of the most important environmental stressors in animal populations (Clinchy et al., 2013). During postnatal life, the simple presence of predators (and their cues) induces stress responses in the prey, presumably leading to telomere shortening (Burraco et al., 2017; Olsson et al., 2010).

Although commonly overlooked, the exposure to predation risk is not limited to postnatal life as embryos are able to perceive chemical, tactile or acoustic cues of predator presence (Gilbert and Epel, 2009). For instance, it has recently been shown that bird embryos exposed to adult alarm calls show increased glucocorticoid levels (i.e. corticosterone; Noguera and Velando, 2019). While an increased secretion of glucocorticoids during embryo development may directly or indirectly lead to telomere shortening as a result of the negative effects that glucocorticoids exert on oxidative status (Haussmann et al., 2011; Reichert and Stier, 2017) and/or telomerase activity (Choi et al., 2008), whether the presence of predators during embryonic life affects telomere dynamics has not hitherto been investigated.

Here, we studied whether prenatal exposition to social cues of predator presence affects early postnatal telomere length and corticosterone levels. To answer this question, we carried out a field experiment where we experimentally modified the exposure of yellow-legged gull (*Larus michahellis*) embryos to cues of predator presence (i.e. adult alarm calls) and then we assessed the effects on telomere length at hatching and after 5 days of postnatal life. We predicted that gull chicks hatched from eggs exposed to predator cues would show reduced telomere length. Additionally, we also measured hatchlings' basal corticosterone levels. Corticosterone is the main stress hormone in birds, and we have recently shown that prenatal exposure to adult alarm calls promotes important physiological and molecular changes in hatchlings, including a higher secretion of corticosterone (Noguera and Velando, 2019). Hence, we expected increased basal corticosterone levels in hatchlings from eggs exposed to predator presence.

MATERIALS AND METHODS

Study area and general procedures

We performed the field experiment from April to June 2018 and 2019 at a colony of yellow-legged gulls (*Larus michahellis* Naumann) in Salvora Island, Spain. We used in total 87 nests (N=54 in 2018 and N=33 in 2019) containing a clutch of three eggs with known laying date and egg order. We visited each study nest beginning when the second-laid egg in each clutch had had 20 days of incubation. Every morning we monitored the nests, collected the second eggs and

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immediately transported them in a thermal container to our field station. We selected second-laid eggs because previous data suggest that they may be more susceptible to external stimuli because of their more advanced sensory systems (Noguera and Velando, 2019). Once at the field station, we measured the eggs (±0.001 mm) to calculate their volume and randomly assigned them to two experimental groups: exposed or unexposed to social cues of predator presence (hereafter, 'exposed' and 'unexposed' groups, respectively). We placed the eggs into artificial incubators where they were kept at 38°C and 55% relative humidity and turned once per hour (Noguera and Velando, 2019). Eggs collected on the same day (range: 10–20) and assigned to the same experimental treatment were placed together in the same incubator so that each experimental treatment was replicated in five different incubators (three in 2018 and two in 2019) to avoid possible incubator effects.

To experimentally modify the exposure of gull embryos to social cues of predator presence, we followed the methodology previously described for yellow-legged gulls (Noguera and Velando, 2019). Briefly, every day between day 21 and day 27 of incubation, we removed the eggs assigned to the exposed group from their incubators and placed them inside a sound-proof box for 3 min (see Noguera and Velando, 2019, for details on box dimension). During that time, we exposed the eggs to continuous playback stimuli of adult alarm calls that had been previously recorded in the same colony. Adult alarm call audio files were broadcast with a speaker (BSP60, Sony) placed in the lid of the box, at 30 cm from the eggs and with a standard sound intensity of ca. 80 dB. After the payback stimuli, the eggs were immediately returned to the incubator. We exposed the eggs to the playback stimuli 4 times a day between 09:00 h and 20:00 h on a random time schedule to avoid habituation but using each time a different audio file from a subset of four files. In the unexposed group, the eggs were subjected to the same experimental procedure during the 3 min playback trials but audio files only contained a control acoustic stimulus, i.e. stonechat (*Saxicola torquata*) breeding calls previously recorded in the same breeding colony. On day 27 of incubation (i.e. 24 h before the expected time of hatching), once the experimental eggs (exposed and unexposed) received their last playback stimuli, we returned the eggs to the colony. To disrupt any potential antipredator covariation between parental and offspring phenotype, we cross-fostered the eggs between pairs of nests that had the same laying date (±1 day). The duration of the playback stimuli, sound intensity and the daily frequency of exposure to the playback were within the natural range of variation in the study colony (see Noguera and Velando, 2019, for further details).

At hatching, we marked all experimental chicks (exposed and unexposed) with numbered leg flags for their identification. Nine eggs failed to hatch but hatching success did not differ among experimental groups (see Table S1). We blood sampled and measured all experimental chicks at two time points: day 1 and day 5 of age. Although near in time, these two samples allowed us to assess whether or not any effect of our experimental treatment on early postnatal telomere length and body size remained after hatching. We collected blood samples (~90 µl) from the brachial vein with heparinized capillary tubes and weighed them on a Pesola spring balance (±1 g). We always collected blood samples within 3 min of capture to avoid any increase of baseline corticosterone levels as a consequence of handling (Romero and Reed, 2005).

Laboratory analyses

Quantification of plasma corticosterone

We measured corticosterone concentration in plasma sampled at day 1 of age using a commercially available enzyme-linked immunosorbent assay (ELISA Kit EIA-4164 from DRG Diagnostics, Marburg, Germany), and following the manufacturer's instructions. Briefly, plasma samples (20 µl) were incubated with a corticosterone–HRP conjugate for 60 min in a microtitre plate. Afterwards, the microtitre plate was washed 3 times and samples were allowed to react with a substrate solution leading to a blue–green complex. The change in absorbance at 450 nm (Synergy™ 2 Multi-Mode Microplate Reader, Bio-Tek Instruments, Inc.) of the blue–green complex was reverse proportional to the concentration of corticosterone. Plasma samples were analysed in duplicated and the assay showed high repeatability (intra-class correlation coefficient ICC=0.86, $P<0.001$, $N=71$). The cross-reactivity of the polyclonal corticosterone antisera with related substances was negligible in this species (see Noguera et al., 2017, for further details).

Quantification of red blood cell telomere length

Telomere length was measured in red blood cell (RBC) DNA samples using real-time PCR (qPCR) on a StepOnePlus system (Applied Biosystems) and following a previously established protocol for bird samples (Criscuolo et al., 2009) but with some minor modifications described for yellow-legged gull samples (Kim and Velando, 2015). Prior to the analyses, DNA was extracted from RBC samples taken on day 1 and day 5 of age with a DNeasy Blood and Tissue kit (Qiagen), following the manufacturer's protocol. The qPCR method 'normalizes' the quantity of telomere product (T) to a single-copy gene (S) to provide a mean telomere length for the cell population (T/S ratio). Briefly, the DNA samples were assayed using the Absolute blue qPCR SYBR green Low Rox master mix (Thermo Scientific) with telomere (Tel1b and Tel2b) and GADPH primers (GapF and GapR) and following the assay conditions described in Kim and Velando (2015). Each plate also included a reference (gold) sample and a negative control sample. The efficiency of each amplicon was estimated from the slope of the amplification curve for each qPCR reaction using LinRegPCR software (Ruijter et al., 2009) (Telomere: range 1.83–1.84; GAPDH: range 1.89–1.91). All DNA samples were run in triplicate, and average values were used to calculate the relative T/S ratios, controlling for plate efficiency as described in Pfaffl (2001). T/S values were repeatable (ICC=0.87, $P<0.001$, $N=137$).

Molecular sexing

Gull chicks were sexed following the methodology and primer sequences described by Fridolfsson and Ellegren (1999). The method is based on PCR to amplify part of the W-linked avian CHD gene (CHD-W) in females, and its non-W-linked homologue (CHD-Z) in both sexes. The DNA products were run on a 2% agarose gel and stained with Greensafe Premium (NZYtech).

Statistical analyses

We used linear mixed-effects models (LMMs) to test the effect of prenatal exposition to predator cues on incubation time and basal corticosterone levels at day 1 of age. The model included the experimental treatment (exposed and unexposed), year and their two-way interaction as fixed terms, and the incubator identity (ID) as a random term. We assessed the effect of the experimental treatment on postnatal telomere length and growth rate during the first 5 days of age using LMMs. These models included the experimental treatment, chick age (day 1 and 5 of age), year and their three- and two-way interactions as fixed factors, and incubator ID and chick ID as random terms. Sex and egg volume were also included in all the above models. As any effect of experimental treatment on telomere length may be related to an effect on

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experimental treatment on basal corticosterone levels (e.g. Herborn et al., 2014), we re-ran the model on telomere length but including basal corticosterone level and its interaction with age as covariates.

Before running the analyses, corticosterone levels were log-transformed to improve data distribution. We confirmed there was no initial bias in laying date, egg volume, sex ratio or hatching success between experimental groups (see Table S1). Note that differences in sample sizes in some analyses reflect missing values owing to the death or loss of chicks and/or insufficient volume sample (see Table S2 for further details). We report results for full models after removing non-significant interactions (Engqvist, 2005). All analyses were conducted in IBM SPSS statistics v.24 using Satterthwaite's degrees of freedom, the significance level was set at α=0.05 and all statistical tests were two-tailed.

Ethics

The study complied with the standards of animal experimentation and animal welfare established under current Spanish law (RD53/2013), and permissions were granted by the authorities of Parque Nacional de las Islas Atlánticas and approved by the Xunta de Galicia review board (45/RX97704 and 263/RX583146).

RESULTS AND DISCUSSION

Our experimental treatment did not affect incubation time (treatment: $F_{1,72}$=2.517, P=0.117; treatment×year: $F_{1,71}$=3.091, P=0.083) but, on average, exposure to alarm calls during embryo development increased basal corticosterone levels in gull hatchlings, although differences were only marginally significant (treatment: $F_{1,66}$=3.542, P=0.064; year: $F_{1,66}$=0.921, P=0.341; treatment×year: $F_{1,65}$=0.210, P=0.648; Fig. 1A). On average, the incubation time was longer in 2018 than in 2019 ($F_{1,72}$=23.866, $P\leq$0.001) but neither egg volume nor sex was significant in the models (P>0.190 in both cases). From a mechanistic point of view, the repeated exposure to alarm calls probably exposed the embryos to increased corticosterone levels during their last week of development (e.g. Noguera and Velando, 2019), although in our study the difference at hatching was marginally significant. Alternatively, it might also be possible that prenatal stress exposure had a remodelling effect on the hypothalamic–pituitary–adrenal (HPA) axis in the gull embryos that resulted in a higher HPA axis activity after hatching (e.g. increased basal or stress-induced levels of glucocorticoids; see Haussmann et al., 2011). To elucidate the precise mechanism through which prenatal stressors affect basal glucocorticoid levels early on after hatching, future studies in which glucocorticoid levels are measured at the time of stress exposure (i.e. embryonic development) would be particularly helpful.

Interestingly, adult alarm calls during the embryo stage affected early postnatal telomere length ($F_{1,11.77}$=5.534, P=0.037; see Table S3); 1 day old chicks in the prenatally exposed group had shorter telomeres than chicks in the unexposed group, and this difference was still evident at 5 days of age (Fig. 1B). Chick body mass increased with age and was positively related to egg volume (P<0.001 in both cases; see Table S3), but did not differ between experimental groups or sexes (P>0.103 in all cases; see Table S3). An early (prenatal) exposure to increased corticosterone levels may have contributed to increased oxidative damage in the embryos (Costantini et al., 2011), which, in turn, may be responsible for telomere shortening observed in the gull chicks (Reichert and Stier, 2017). Similarly, predator-induced stress may also have contributed to a reduction in telomerase activity (Choi et al., 2008) and therefore the capacity of the embryos to restore their telomeres before hatching (Schaetzlein et al., 2004). However, differences in

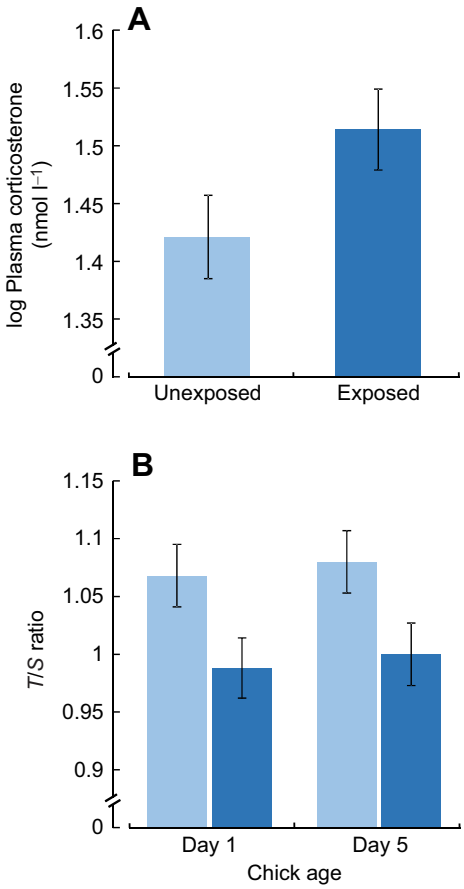

Fig. 1. Basal corticosterone level and telomere length in yellow-legged gull chicks. (A) Plasma corticosterone level on day 1 of age (N=71) and (B) telomere length (T/S ratio) on day 1 (N=73) and day 5 (N=64) of age in gull chicks prenatally exposed (dark blue bars) or not (unexposed; light blue bars) to adult alarm calls. Data are estimated marginal means±s.e.m.

telomere length seemed to be not directly related to the observed variation in basal corticosterone level as neither basal corticosterone level nor its interaction with age was significant when added into the model (basal corticosterone: $F_{1,60.64}$=0.108, P=0.744; basal corticosterone×age: $F_{1,63.55}$=2.284, P=0.136) This interesting result suggests that the observed reduction of telomere length may not be directly mediated by an increase in corticosterone secretion. It might be plausible that predator risk induced oxidative damage in the embryos as a result of corticosterone-independent mechanisms. For instance, embryos exposed to predator cues may have increased their motor activity and therefore skeletal muscle contraction (Noguera and Velando, 2019), resulting in an overproduction of oxidizing free radical species (McArdle et al., 2001). Additionally, adult alarm calls may have induced epigenetic alterations during embryo development (see Noguera and Velando, 2019), with negative consequences on other important telomere regulatory pathways rather than telomerase activity (reviewed in Blasco, 2007). Regardless of the mechanism, our results indicate that exposure to predator cues, even during embryo development, may shape early postnatal telomere length.

In conclusion, our findings indicate that bird embryos are not passive agents but rather are sensitive to environmental stressors. We have provided clear evidence that prenatal exposure to predator cues can negatively affect early postnatal telomere length in gull chicks. Moreover, the reduced telomere length in gull hatchlings

prenatally exposed to adult alarm calls lasts for several days after hatching (day 5 of age), suggesting that predator-induced effects were not transient and may potentially last throughout the postnatal growth period (e.g. Entringer et al., 2018; Noguera et al., 2016). As young birds with shorter telomere length have reduced fledging success and lifespan (Heidinger et al., 2012; Watson et al., 2015), the reduction of telomere length in the exposed chicks is likely to have long-term fitness consequences. Our results further provide an explanation of, for instance, why predation risk may itself reduce future survival in wild populations (MacLeod et al., 2018; Zanette et al., 2011) and influence life-history decisions (Taborsky, 2017, and references therein). Future studies should investigate the mechanisms underlying the predator-induced telomere shorting in embryos and whether these prenatal effects affect life-history trajectories and modulate population trends and dynamics.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.C.N., A.V.; Methodology: J.C.N.; Software: J.C.N.; Validation: J.C.N.; Formal analysis: J.C.N.; Investigation: J.C.N.; Resources: J.C.N.; Data curation: J.C.N.; Writing - original draft: J.C.N., A.V.; Writing - review & editing: J.C.N.; Visualization: J.C.N.; Supervision: J.C.N.; Project administration: J.C.N.; Funding acquisition: A.V.

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Data availability
Raw data are available from the figshare digital repository: 10.6084/m9.figshare.10560218.v1.

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.216176.supplemental

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