Naturwissenschaften (2012) 99:661–664
DOI 10.1007/s00114-012-0941-1

SHORT COMMUNICATION

Predictors of telomere content in dragon lizards

Cissy Ballen · Mo Healey · Mark Wilson · Michael Tobler · Mats Olsson

Received: 28 May 2012 / Revised: 25 June 2012 / Accepted: 27 June 2012 / Published online: 8 July 2012

Abstract Telomeres shorten as a consequence of DNA replication, in particular in cells with low production of telomerase and perhaps in response to physiological stress from exposure to reactive oxygen species, such as superoxide. This process of telomere attrition is countered by innate antioxidation, such as via the production of superoxide dismutase. We studied the inheritance of telomere length in the Australian painted dragon lizard (*Ctenophorus pictus*) and the extent to which telomere length covaries with mass-corrected maternal reproductive investment, which reflects the level of circulating yolk precursor and antioxidant, vitellogenin. Our predictors of offspring telomere length explained 72 % of telomere variation (including interstitial telomeres if such are present). Maternal telomere length and reproductive investment were positively influencing offspring telomere length in our analyses, whereas flow cytometry-estimated superoxide level was negatively impacting offspring telomere length. We suggest that the effects of superoxide on hatchling telomere shortening may be partly balanced by transgenerational effects of vitellogenin antioxidation.

Keywords Lizard · Reactive oxygen species · Superoxide · Telomere · Vitellogenin

Communicated by: Sven Thatje

C. Ballen (✉) · M. Healey · M. Tobler · M. Olsson
School of Biological Sciences, University of Sydney, Camperdown,
Sydney, Australia
e-mail: cissy.ballen@sydney.edu.au

M. Wilson
School of Biological Sciences, University of Wollongong,
Wollongong, Australia

Introduction

Despite the recognition of telomeres as protective chromosome 'caps' in the first half of the twentieth century, telomeres attracted little attention from the wider scientific community until the early 1990s (von Zglinicki 2002). This changed abruptly when it was experimentally demonstrated that telomeres in human fibroblasts shortened progressively during in vitro culturing (Harley et al. 1990), triggered replicative senescence (von Zglinicki 2001) and are a biomarker of disease (von Zglinicki 2001). In addition, telomeric repeat sequences are found in other parts of the genome as a result of past chromosomal fusions and DNA breakage and repair (Bolzan and Bianchi 2006). Thus, we may expect telomeres to be associated with components of fitness and forces of evolution also in non-human taxa.

We show, for example, that there is higher heritability of telomere length between sons and fathers than between daughters and mothers in sand lizards, *Lacerta agilis*, possibly set by paternal telomere length in the sperm at conception (Olsson et al. 2011a). In this species, telomere length is also shorter in lizards that suffered predator attacks in which they lost their tails and were forced to a more stressful lifestyle (Olsson et al. 2010). Female sand lizards also showed significant positive telomere effects on survival and lifetime offspring production (Olsson et al. 2011b). Furthermore, our previous studies show relatively high heritability of compounds that may erode telomere length, such as superoxide, in another lizard species, the painted dragon, *Ctenophorus pictus* (Olsson et al. 2008). Since oxidative stress has been demonstrated to shorten telomeres (von Zglinicki 2002, 2001), it is not unlikely that maternal reactive oxygen species (ROS) not only impacts offspring ROS but also their telomeres. To understand ongoing telomere evolution in the wild, these components need to be teased apart to facilitate appropriate analyses of proximate moderators, inheritance genetics, and selection.

Here, we simultaneously measure superoxide and telomere length (including interstitial telomeres) in both dams and offspring using flow cytometry (using fluorescent probes to measure superoxide and the Dako products, Telomere PNA Probe/FITC kit, to measure telomere length, with the rationale that this kit avoids interaction with subtelomeric regions, unlike methods such as TRF). We also entered an estimate of female reproductive investment into our model (residuals from a clutch mass–female mass regression). The rationale for this was twofold: (1) In honeybees, Seehuus et al. (2006) showed that by interfering with vitellogenin production using RNA interference (RNAi), honeybees became more sensitive to paraquat exposure, a compound that increases ROS production. Increased reproductive investment reduced oxidative stress due to the antioxidation of ROS by the yolk precursor vitellogenin. A similar observation was made in nematodes (*Caenorhabditis elegans*), for which Nakamura et al. (1999) showed that despite the lack of reproduction at old age, both males and females upregulated their production of vitellogenin late in life, resulting in an increased life span. This predicts that when more vitellogenin is produced, this results in larger relative clutch size, which was demonstrated in zebra fish (*Taeniopygioa guttata*; Han et al. 2009). Here, we test whether elevated maternal reproductive investment may protect offspring telomeres and that there are positive maternal effects (genetic and environmental) on offspring telomere length.

Materials and methods

Fourteen painted dragon (*C. pictus*) females were caught by noose or by hand at Yathong Nature Reserve, New South Wales (145° 35′, 32° 35′), a week before the onset of the experiments (October 2009) and were brought back to holding facilities at the University of Wollongong, Australia. All lizards were kept individually in cages (330×520×360 mm), on a 12:12 light regime, and were fed crickets and mealworms to satiation every second day. The lizards were weighed to the nearest 0.01 g and measured snout to vent to the nearest 1.0 mm. The females were checked for oviposition (evident from skin folds on their body sides) at least twice daily. The eggs were incubated in moist vermiculite (mixed with tap water in a 7:1 ratio) at 30 °C (±2.5 °C) within 4 h of laying until hatching (ca 60 days). All lizards were weighed to the nearest 0.1 g and measured to the nearest 1.0 mm. One offspring per female was sampled at random to assess cross-generational similarity in telomere length.

Quantifying superoxide

Blood was collected with a glass capillary after rupturing the vena angularis with the sharp tip of a syringe in the corner of the mouth at hatching (and on the same day for females) and diluted one in ten in phosphate-buffered saline (PBS). Fifty millilitres of diluted blood was used for juveniles and 20 ml for adults per test (blood cell density is less in the juveniles). After initial preparation of peripheral blood following Olsson et al. (2008), cells were resuspended in 100 ml of PBS containing 5 mM MitoSOX Red (Molecular Probes, Invitrogen, USA) and analysed by flow cytometry; 50,000 events were acquired for all samples. Flow cytometry was performed using a Becton Dickinson LSR II, with excitation at 488 nm, and emitted fluorescence collected using band pass filters of 575±13 nm. Data were acquired and analysed using FACSDiva v4.0.1 and FloJo v8.8.7 software. The arithmetic mean fluorescence for all 50,000 cells acquired was determined using FloJo software. The correlation coefficient between two samples from the same males has been verified to be 0.97 ($p<0.0001$, $n=14$) in a separate experiment (Olsson et al. 2008).

Quantifying telomere length

We used the Telomere PNA Kit/FITC for flow cytometry (Dako), which is recommended by the manufacturer as being suitable for use with nucleated cells from all vertebrates (http://www.dako.com/au/ar42/p107840/prod_products.htm). The kit is based upon the hybridization of a synthetic DNA/RNA analogue, conjugated with FITC, capable of binding to DNA/RNA in a sequence-specific manner obeying the Watson–Crick base pairing rules. The rationale for using this kit was primarily two reasons: (1) unlike methods such as TRF, this kit does not suffer from the interaction of subtelomeric sequences, and (2) the probe hybridizes with telomere repeat sequences (TTAGGG) typical of vertebrates including lizards, and the resulting fluorescence intensity of the cells is directly correlated with the length of the telomeres. This method therefore provides a relative indication of telomere length, including interstitial telomere sequences if such are present, between blood cells from different individuals. Tests of reproducibility of the Telomere PNA kit/FITC were performed at Dako's laboratories using human blood. Relative telomere length values showed a standard deviation of 8–13 % for single determinations and 6–9 % for duplicate determinations.

Blood was collected and washed in PBS by centrifugation (as described above), before subsequent processing within 4 h of sampling. Relative telomere length was compared between blood cells of individual lizards using the Dako kit, following the manufacturer's instructions. Cells were counterstained with propidium iodide (PI) to assess total nucleic acid content. Flow cytometry was performed using a Becton Dickinson LSR II, with excitation at 488 nm, and emitted fluorescence collected using band pass filters of 515±10 nm (fluorescein) and 695±10 nm (PI). More than 90 % of the cells had a similar level of PI staining, and this major population was

Table 1 Multiple regression analysis of predictors of offspring telomere length in painted dragon lizards, *C. pictus*

Parameter estimates

Variable	Estimate	Std Error	*t*	Pr>*t*
Intercept	1.84	0.64	2.87	0.0186
Telomere length, dam	0.67	0.18	3.71	0.0048
Offspring mass (g)	−0.029	0.012	−2.48	0.0350
Offspring superoxide	−0.034	0.012	−2.83	0.0199
Residual clutch mass	0.064	0.023	2.79	0.0210

Female mass and level of superoxide were backwards eliminated from the final model ($p>0.25$). Residual clutch mass is the residuals from a clutch mass–female mass regression. The final model explained no less than 72 % of the variation in the data (model $F_{3,\ 14}=5.79$, $p=0.0138$, $R^2=0.72$)

electronically gated to select them for analysis of the level of telomere probe hybridization. Between 5 and 9 % of blood cells had higher levels of PI staining, and these were electronically excluded from the analyses (the excluded lizard blood cells may have inherently higher levels of nucleic acid content or be undergoing cell division). Data were acquired and analysed as described above.

Statistical analyses

Full models with interactions were first analysed and thereafter were covariates or other non-significant predictors removed with a p to enter value of 0.25 (i.e. parameters were backwards eliminated when $p>0.25$). Telomere lengths were log transformed, which normalized the data (maternal telomere length, Shapiro–Wilk's statistics, $W=0.96$, $p=0.785$; offspring telomere length, Shapiro–Wilk's statistics, $W=0.94$, $p=0.485$).

Results

Female mass and superoxide were non-significant ($p>0.25$) and excluded from further analysis. Two of the predictors, dam telomere length and female reproductive investment, showed significant positive effects on offspring telomere length ($p=0.0048$ and $p=0.0210$, respectively; $R^2=0.72$, Table 1). The remaining two predictors, offspring mass and offspring superoxide level, both showed negative effects on offspring telomere length ($p=0.0350$ and $p=0.0199$, respectively; Table 1). Thus, telomere length appears to be heritable in painted dragons, and offspring telomeres are shorter at higher superoxide levels (Fig. 1).

Discussion

We set out to examine basic predictors of offspring telomere length based on observations in the current literature (von Zglinicki 2001, 2002; Harley et al. 1990). These observations suggest effects of parental telomere length on the telomere lengths of the offspring, effects of age (and mass), and attrition effects of free radicals or similar reactive molecules (von Zglinicki 2001). Here, however, we did not find any evidence that ROS levels in mothers influenced the telomere length of their offspring. Importantly, however, the telomere length of dams did positively predict the telomere length of the offspring. We note that this genetic effect (encompassing additive genetic variance) may be inflated by

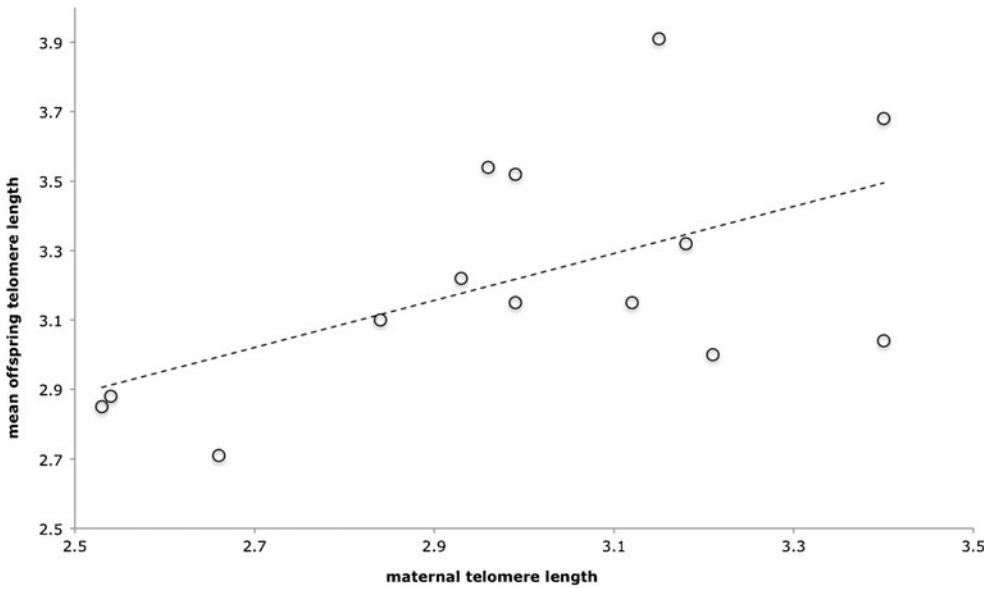

Fig. 1 The figure depicts the relationship between offspring and relative maternal telomere length as estimated with the Telomere PNA Kit/FITC. The units are mean fluorescence intensity and represent relative telomere length

maternal effects but, that said, including offspring mass in the model controls for some maternal effects (e.g. variation in egg size). We also note that our estimates of telomere length will be conservative if interstitial telomeric sequences exist and are part of our estimates.

Our previous work shows that free radical levels may be age dependent (Olsson et al. 2008) and that unspecified ROS rather than superoxide was correlated with mass (and age) in painted dragons (Olsson et al. 2008). In the current analysis, however, the effect of female mass fell short of significance (we do not know maternal age), whereas that of offspring mass proved significant (larger offspring had shorter telomeres, Table 1). This would agree with the notion of higher attrition from increased embryonic growth rate.

In summary, our results on telomeres largely agree with the oxidative stress data for this species that we have previously reported on. Here, we show that what the theories of von Zglinicki (2002) and colleagues have advocated, that oxidative stress may be a strong determinant of telomere attrition and subsequent telomere length, may apply also in painted dragon lizards. This effect does, however, not seem to be transgenerational but acts as a telomere modifier in offspring that have inherited their telomeres with high predictability from their mothers. Furthermore, the positive effect of a large relative clutch size on offspring telomere length suggests that, in some taxa, higher, not lower, investment into reproduction may increase antioxidation and protect against telomere attrition. To test this idea in a wide range of taxa with varying levels of yolk provisioning to the developing offspring appears to be an interesting future challenge.

Acknowledgments The Australian Research Council is acknowledged for funding support (MO). These experiments were carried out under ethics permit AE10/11–13.

References

Bolzan AD, Bianchi MS (2006) Telomere, interstitial telomeric repeat sequences, and chromosomal aberrations. Mutat Res 612:189–214. doi:10.1016/j.mrrev.2005.12.003

Han D, Haunerland NH, Williams TD (2009) Variation in yolk precursor receptor mRNA expression is a key determinant of reproductive phenotype in the zebra finch (*Taeniopygia guttata*). J Exp Biol 212:1277–1283. doi:10.1242/jeb.026906

Harley CB, Futcher AB, Greider CW (1990) Telomeres shorten during ageing in human fibroblasts. Nature 345:458–460. doi:10.1038/345458a0

Nakamura A, Yasuda K, Adachi H, Sakurai Y, Ishii N, Goto S (1999) Vitellogenin-6 is a major carbonylated protein in aged nematode, *Caenorhabditis elegans*. Biochem Biophys Res Commun 264:580–583. doi:10.1006/bbrc.1999.1549

Olsson M, Wilson M, Uller T, Mott B, Isaksson C, Healey M, Wanger T (2008) Free radicals run in lizard families. Biol Lett 42:186–188. doi:10.1098/rsbl.2007.0611

Olsson M, Pauliny A, Wapstra E, Blomqvist D (2010) Proximate determinants of telomere length in sand lizards (*Lacerta agilis*). Biol Lett 6:651–653. doi:10.1098/rsbl.2010.0126

Olsson M, Pauliny A, Wapstra E, Uller T, Schwartz T, Blomqvist D (2011a) Sex differences in sand lizard telomere inheritance: paternal epigenetic effects increases telomere heritability and offspring survival. PLoS One 6(4):e17473. doi:10.1371/journal.pone.0017473

Olsson M, Pauliny A, Wapstra E, Uller T, Schwartz T, Miller E, Blomqvist D (2011b) Sexual differences in telomere selection in the wild. Mol Ecol 20:2085–2099. doi:10.1111/j.1365-294X.2011.05085.x

Seehuus SC, Norberg K, Gimsa U, Krekling T, Amdam GV (2006) Reproductive protein protects sterile honey bee workers from oxidative stress. Proc Natl Acad Sci USA 103:962–967. doi:10.1073/pnas.0502681103

von Zglinicki T (2001) Telomeres and replicative senescence: is it only length that counts? Cancer Lett 168:111–116. doi:10.1016/S0304-3835(01)00546-8

von Zglinicki T (2002) Oxidative stress shortens telomeres. Trends Biochem Sci 27:339–344. doi:10.1016/S0968-0004(02)02110-2