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Sex-specific effects of experimental ectoparasite infestation on telomere length in great tit nestlings

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Running title: Ectoparasites and telomere dynamics

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Abstract

Telomere length is a biomarker of biological ageing and lifespan in various vertebrate taxa. Evidence is accumulating that telomeres shorten more rapidly when an individual is exposed to environmental stressors. Parasites are potent selective agents that can cause physiological stress directly or indirectly through the activation of the host's immune system. Yet to date, empirical evidence for a role of parasites in telomere dynamics in natural populations is limited.

Here we show experimentally that exposure to ectoparasitic hen fleas (*Ceratophyllus gallinae*) during growth results in shorter telomeres in female, but not male, great tit (*Parus major*) nestlings. Females had longer telomeres than males when growing up in experimentally deparasitized nests but, likely because of the sex-specific effects of ectoparasitism on telomere length, this sexual dimorphism was absent in birds growing up in experimentally infested nests. Our results provide the first experimental evidence for a role of ectoparasitism in telomere dynamics in a natural vertebrate population, and suggest that the costs of infection manifest in sex-specific ways.

Keywords: biological ageing; life history evolution; host-parasite interactions; sex differences; senescence in wildlife populations; telomere shortening; costs of infection; parasitism; sexual dimorphism

Introduction

Evidence is accumulating that the length of telomeres, the protective caps at the end of chromosomes, is a predictor of ageing and lifespan across vertebrate taxa (Wilbourn et al. 2018). Telomeres have been found to shorten more rapidly when an individual is exposed to environmental stressors, such as harsh abiotic conditions or food limitation (Chatelain et al. 2020; Epel et al. 2004). Parasites are particularly potent selective agents that can induce physiological stress through direct damage (e.g. by consuming host resources (Tripet & Richner 1997)) or indirectly through the activation of the host's immune system (Costantini & Møller 2009). Yet to date, experimental evidence for an effect of parasitism on telomere attrition in natural populations is limited.

Accelerated telomere shortening in response to experimental pathogen infection has been demonstrated in captive house mice (*Mus musculus*) (Ilmonen et al. 2008), and a correlation between pathogen infection and subsequent telomere attrition is also observed in humans (Asghar et al. 2018). Correlative and experimental work in avian malaria host-pathogen systems has furthermore demonstrated accelerated telomere shortening in infected birds (Asghar et al. 2015; Asghar et al. 2016; Karell et al. 2017); and recent findings suggest that the costs of avian malaria infection in terms of accelerated telomere attrition might be host sex-specific (Sudyka et al. 2019). Yet, given the lack of experimental studies in other natural host-parasite systems, the generality of these findings is currently unclear.

Hematophagous hen fleas (*Ceratophyllus gallinae*) are common, nest-based ectoparasites of cavity nesting birds (Tripet & Richner 1997). We have previously shown that exposure to hen fleas during growth has sex-specific consequences for great tit (*Parus major*) nestlings (Tschirren et al. 2003). Male nestlings exposed to

hen fleas have a lower body mass and smaller body size compared to male nestlings growing up in an uninfested nest, whereas no negative effect of hen flea exposure on the growth of female nestlings is observed (Tschirren et al. 2003). Thus, at first sight, males appear to be more susceptible to hen fleas than females. However, individuals exposed to parasites (or other environmental stressors) are predicted to prioritise resource allocation to key fitness-related traits and functions, at the expense of traits and functions less related to fitness, to minimize the negative impact of parasitism on fitness (Boonekamp et al. 2018; Vedder et al. 2017; Waddington 1942). If selection is acting in a sex-specific way, these allocation priorities will differ between males and females (i.e. sex-specific canalization). As a consequence, the costs of parasitism might manifest differently in the two sexes. Here we experimentally quantify the sex-specific effect of ectoparasite infestation on telomere length in a natural bird population. We show that exposure to hen fleas during growth negatively affects telomere length of female, but not male, great tit nestlings, demonstrating a sex-specific role of ectoparasitism in telomere dynamics.

Materials & Methods

Experimental procedures

The study was performed in a great tit population breeding in nest boxes in a forest near Bern, Switzerland (46°54′N 7°17′E/46°57′N 7°21′E). Nest boxes were visited regularly from the beginning of the breeding season onwards to determine the start of nest building and egg laying. One day after hatching, nestlings from broods with the same hatching date and a similar brood size (max. difference: 2 nestlings; mean ± 1SD: 7.2 ± 1.8 nestlings) were partially exchanged (cross-fostering). Nestlings were marked individually by clipping down feathers. They were weighed (to the

nearest 0.01g) and ranked in their original nest according to body mass and then alternately assigned to stay in their original nest or to be moved to the foster nest (e.g. heaviest nestling stays, second heaviest nestling goes, etc). The decision if the heaviest nestling stays or goes was randomised across cross-fostering pairs. This approach ensured that body mass differences between nest pairs were minimised at the start of the infestation experiment.

The nesting material of all nests was heat-treated in a microwave oven during cross-fostering to kill hen fleas naturally present in the nest (Richner et al. 1993). One randomly chosen nest of each cross-fostering pair was then assigned to be experimentally re-infested with 40 female and 20 male hen fleas, whereas no fleas were added to the other nest (hereafter referred to as 'uninfested') (Richner et al. 1993). The hen fleas used for the experimental re-infestation were obtained from old nests collected in the study area at the start of the breeding season. Note that although fleas can naturally immigrate into 'uninfested' nests, these numbers are small compared to the flea load of experimentally infested nests (Heeb et al. 1996). This experimental design ensured that for each family, half of the siblings were exposed to ectoparasitic hen flea during growth, whereas the other half grew up in a nest with no (or very few) fleas present. Eight days post-hatching, nestling were weighed (to the nearest 0.01g) and a small blood sample (approx. 20µl) was taken for molecular sex determination (as described in Tschirren et al. (2003)) and telomere length analysis. Because blood was only sampled at one time point, we inferred telomere dynamics by comparing nest mates and biological siblings that were randomly assigned to the flea treatment groups, rather than based on within-individual telomere shortening.

All procedures comply with the current laws of Switzerland and were approved by the Animal Experimentation Board of the Cantonal Veterinary Office of Berne, Berne, Switzerland (license 16/02) and the Federal Office for the Environment, Berne, Switzerland (license 2200). The municipality of Berne approved field work in their forests.

Telomere length analysis

Telomere length was quantified in whole blood of a randomly selected subset of 159 nestlings from 62 nests (39 males and 43 females from uninfested nests and 42 males and 35 females from flea-infested nests) using real-time qPCR. In short, relative individual telomere length was calculated as the ratio (T/S ratio) of the quantity of telomere repeated sequences (T) relative to the DNA quantity of a reference gene (S) (see Stier et al. (2016) and the electronic supplementary materials ESM1 and ESM2 for a detailed description). Telomere measurements were standardised (z-score) for statistical analysis.

Statistical analysis

We used a linear mixed model to test for sex-specific effects of ectoparasite exposure during growth on nestling telomere length using the *R* package *lme4* (Bates et al. 2011). Nestling sex, flea treatment and their interaction were included as fixed effects. Nest of origin and nest of growth were included as random effects to account for the non-independence of biological siblings and nestlings growing up in the same nest, respectively. We also ran a second model, including the factors described above and body mass as an additional covariate. Significance of predictors was evaluated using

Satterthwaite's degree of freedom method in *lmerTest* (Kuznetsova et al. 2017). Pairwise posthoc comparisons were performed for significant outcomes in the main models using *lsmean*s (Lenth 2016). All statistical analyses were performed in *R* version 4.0.2. (R Core Team 2017). Normality of the residuals was confirmed by visual inspection and Shapiro-Wilk test.

Results

We observed a significant interaction effect between nestling sex and experimental flea infestation on nestling telomere length (Table 1a). Posthoc contrasts revealed that flea-exposed female nestlings had shorter telomeres than female nestlings growing up in an uninfested nest (mean ± 1SE: 0.039 ± 0.155 and 0.520 ± 0.164, respectively; $t = 2.103$, $df = 79.8$, $P = 0.039$; Fig. 1), whereas the telomere length of male nestlings in flea-infested and uninfested nests did not differ significantly (mean ± 1SE: -0.207 ± 0.146 and -0.385 ± 0.130, respectively; $t = -0.686$, $df = 67.5$, $P = 0.495$; Fig. 1). Consequently, a sex difference in telomere length was observed in uninfested nests, with female nestlings having longer telomeres than male nestlings ($t = 4.194$, $df = 152$, $P < 0.001$; Fig. 1), whereas in flea-infested nests no sex difference in telomere length was observed ($t = 1.332$, $df = 152$, $P = 0.185$; Fig. 1). No association between body mass and telomere length was found (Table 1b). Furthermore, including body mass as a covariate in the model did not change the significant sex x flea infestation effect on telomere length (Table 1b).

Discussion

This study provides the first experimental evidence for a sex-specific effect of ectoparasite infestation on telomere length, adding to the growing evidence that

environmental stressors accelerate telomere attrition in natural populations (Chatelain et al. 2020).

Thus far, evidence for parasite-induced telomere attrition in wild vertebrates was limited to avian malaria host-pathogen systems, where accelerated telomere shortening in response to chronic infection was observed (Asghar et al. 2015; Asghar et al. 2016; Karell et al. 2017). In contrast, the effect of ectoparasites on nestling telomere length presented here was acute, occurring after only eight days of exposure. This finding is in line with previous studies that demonstrated particularly rapid telomere shortening in response to environmental stressors during the juvenile period (Salomons et al. 2009). Importantly, early life telomere length (Heidinger et al. 2012; Eastwood et al. 2019; van Lieshout et al. 2019) and rate of telomere attrition (Boonekamp et al. 2014) appear to be a particularly strong fitness predictors. Currently it is unclear if the observed effect of hen flea exposure on host telomere length is caused by direct blood loss and subsequently enhanced blood cell division (Tripet & Richner 1997), the transfer of flea-borne pathogens (Aivelo & Tschirren 2020), or the activation of the nestlings' immune system (Costantini & Møller 2009). The effect of ectoparasite exposure on nestling telomere length was sex-specific with females having shorter telomeres when exposed to fleas, whereas telomere length of males in flea-infested and uninfested nests did not differ. Interestingly, the opposite pattern was found in captive house mice (*Mus musculus*) where telomere shortening in response experimental *Salmonella enterica* infection was much more pronounced in males than in females (Ilmonen et al. 2008). It is currently unclear why infection has opposite sex-specific effects on telomere length in the two systems (see also below).

Previously we have shown that male nestlings exposed to hen fleas are smaller and lighter, whereas no difference in body size or mass is found in female nestlings from flea-infested and uninfested nests (Tschirren et al. 2003). It suggests that costs of infection manifest in sex-specific ways, with males prioritising telomere maintenance at the expense of early life growth when exposed to ectoparasites, and vice versa in females. We would expect such sex-specific resource allocation priorities if early life telomere attrition and growth, respectively, are differentially associated with fitness in males and females. No sex-specific effect of fledging body mass or size on first year survival is found in the study population (Tschirren 2015). However, juvenile body mass or size might affect other fitness components in a sex-specific way, such as reproductive success. Indeed, juvenile mass (but not adult mass) is a predictor of clutch size in female great tits (Tilgar et al. 2010).

Similarly, the associations between telomere length or attrition and fitness might be sex-specific (Barrett & Richardson 2011; Olsson et al. 2011; Wilbourn et al. 2018). In birds, males typically live longer than females (Che-Castaldo et al. 2019), and accelerated ageing and / or a shorter lifespan might thus have more severe fitness consequences for males than for females (Barrett & Richardson 2011). In great tits, telomere length predicts first year survival (Salmon et al. 2017), but it is currently unknown if this association is sex-specific.

Our results demonstrate sex differences in telomere length in response to ectoparasite exposure, but at the same time we also found a sex difference in early life telomere length in the absence of infection, with females having longer telomeres than males. Interestingly, sex differences in telomere length and attrition are found in various taxa (e.g. Barrett & Richardson 2011; Gardner et al. 2014; Watson et al. 2017), but we currently do not have a good understanding of the causes and

consequences of sexual dimorphism in telomere length or attrition across species (Barrett & Richardson 2011).

In conclusion, our study provides the first experimental evidence for a role of ectoparasitism in telomere dynamics in a natural vertebrate population. Importantly, the effect of ectoparasite exposure on telomere length was sex-specific, with telomere length of females being more strongly affected than telomere length of males. This finding contrasts previous observations of ectoparasite-induced growth reduction in male nestlings and suggests that males and females are prioritising different traits and functions when exposed to environmental stressors, such as ectoparasites (i.e. sex-specific canalization, Boonekamp et al. 2018; Vedder et al. 2017; Waddington 1942). Understanding sex-specific associations between telomere length and fitness within and across taxa will help to elucidate the causes and consequences of such sexual dimorphism in telomere dynamics.

Data Accessibility

Data are available from the Dryad Digital Repository: doi: 10.5061/dryad.3j9kd51gd

Authors' Contributions

BT conceived and designed the study, performed sample collection and analysed the data. SZ and FC performed telomere length analysis. BT and AARH prepared the manuscript. All authors revised the manuscript, gave final approval for publication and agreed to be accountable for all aspects of the work.

Competing interests

We declare we have no competing interests.

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References

Aivelo, T., & Tschirren, B. (2020). Bacterial microbiota composition of a common ectoparasite of cavity-breeding birds, the Hen Flea *Ceratophyllus gallinae*. *Ibis, 162*, 1088-1092, doi:10.1111/ibi.12811.

Asghar, M., Hasselquist, D., Hansson, B., Zehtindjiev, P., Westerdahl, H., & Bensch, S. (2015). Hidden costs of infection: Chronic malaria accelerates telomere degradation and senescence in wild birds. *Science, 347*(6220), 436-438, doi:10.1126/science.1261121.

Asghar, M., Palinauskas, V., Zaghdoudi-Allan, N., Valkiunas, G., Mukhin, A., Platonova, E., et al. (2016). Parallel telomere shortening in multiple body tissues owing to malaria infection. *Proceedings of the Royal Society B-Biological Sciences, 283*(1836), 7, doi:10.1098/rspb.2016.1184.

Asghar, M., Yman, V., Homann, M. V., Sonden, K., Hammar, U., Hasselquist, D., et al. (2018). Cellular aging dynamics after acute malaria infection: A 12-month longitudinal study. *Aging Cell, 17*(1), 8, doi:10.1111/acel.12702.

Barrett, E. L. B., & Richardson, D. S. (2011). Sex differences in telomeres and lifespan. *Aging Cell, 10*(6), 913-921, doi:10.1111/j.1474-9726.2011.00741.x.

Bates, D., Maechler, M., & Bolker, B. (2011). lme4: Linear mixed-effects models using S4 classes. R package version 0.999375-42.

Boonekamp, J. J., Mulder, E., & Verhulst, S. (2018). Canalisation in the wild: effects of developmental conditions on physiological traits are inversely linked to their association with fitness. *Ecology Letters, 21*(6), 857-864, doi:10.1111/ele.12953.

Boonekamp, J. J., Mulder, G. A., Salomons, H. M., Dijkstra, C., & Verhulst, S. (2014). Nestling telomere shortening, but not telomere length, reflects

developmental stress and predicts survival in wild birds. *Proceedings of the Royal Society B-Biological Sciences, 281*(1785), 7, doi:10.1098/rspb.2013.3287.

Chatelain, M., Drobniak, S. M., & Szulkin, M. (2020). The association between stressors and telomeres in non-human vertebrates: a meta-analysis. *Ecology Letters, 23*(2), 381-398, doi:10.1111/ele.13426.

Che-Castaldo, J. P., Byrne, A., Perisin, K., & Faust, L. J. (2019). Sex-specific median life expectancies from ex situ populations for 330 animal species. *Scientific Data, 6*, 7, doi:10.1038/sdata.2019.19.

Costantini, D., & Møller, A. P. (2009). Does immune response cause oxidative stress in birds? A meta-analysis. *Comparative Biochemistry and Physiology A-Molecular & Integrative Physiology, 153*(3), 339-344, doi:10.1016/j.cbpa.2009.03.010.

Eastwood, J. R., Hall, M. L., Teunissen, N., Kingma, S. A., Aranzamendi, N. H., Fan, M., et al. (2019). Early-life telomere length predicts lifespan and lifetime reproductive success in a wild bird. *Molecular Ecology, 28*(5), 1127-1137, doi:10.1111/mec.15002.

Epel, E. S., Blackburn, E. H., Lin, J., Dhabhar, F. S., Adler, N. E., Morrow, J. D., et al. (2004). Accelerated telomere shortening in response to life stress. *Proceedings of the National Academy of Sciences of the United States of America, 101*(49), 17312-17315, doi:10.1073/pnas.0407162101.

Gardner, M., Bann, D., Wiley, L., Cooper, R., Hardy, R., Nitsch, D., et al. (2014). Gender and telomere length: Systematic review and meta-analysis. *Experimental Gerontology, 51*, 15-27, doi:10.1016/j.exger.2013.12.004.

Heeb, P., Werner, I., Richner, H., & Kolliker, M. (1996). Horizontal transmission and reproductive rates of hen fleas in great tit nests. *Journal of Animal Ecology*, *65(4)*, 474-484.

Heidinger B.J., Blount J.D., Boner W., Griffiths K., Metcalfe N.B. & Monaghan P. (2012). Telomere length in early life predicts lifespan. *Proceedings of the National Academy of Sciences of the United States of America* 109, 1743-1748. doi:10.1073/pnas.1113306109.

Ilmonen, P., Kotrschal, A., & Penn, D. J. (2008). Telomere attrition due to infection. *PLoS One, 3*(5), 6, doi:10.1371/journal.pone.0002143.

Karell, P., Bensch, S., Ahola, K., & Asghar, M. (2017). Pale and dark morphs of tawny owls show different patterns of telomere dynamics in relation to disease status. *Proceedings of the Royal Society B-Biological Sciences, 284*(1859), 8, doi:10.1098/rspb.2017.1127.

Kuznetsova, A., Brockhoff, P. B., & Christensen, R. H. B. (2017). lmerTest Package: Tests in Linear Mixed Effects Models. *Journal of Statistical Software, 82*(13), 1-26. doi:10.18637/jss.v082.i13.

Lenth, R. V. (2016). Least-squares means: the R package lsmeans. *Journal of Statistical Software, 69*(1), 1-33.

Olsson, M., Pauliny, A., Wapstra, E., Uller, T., Schwartz, T., Miller, E., et al. (2011). Sexual differences in telomere selection in the wild. *Molecular Ecology, 20*(10), 2085-2099, doi:10.1111/j.1365-294X.2011.05085.x.

R Core Team (2017). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Richner, H., Oppliger, A., & Christe, P. (1993). Effect of an ectoparasite on reproduction in great tits. *Journal of Animal Ecology, 62*, 703-710.

Salmon, P., Nilsson, J. F., Watson, H., Bensch, S., & Isaksson, C. (2017). Selective disappearance of great tits with short telomeres in urban areas. *Proceedings of the Royal Society B-Biological Sciences, 284*(1862), 8, doi:10.1098/rspb.2017.1349.

Salomons, H. M., Mulder, G. A., van de Zande, L., Haussmann, M. F., Linskens, M. H. K., & Verhulst, S. (2009). Telomere shortening and survival in free-living corvids. *Proceedings of the Royal Society B-Biological Sciences, 276*(1670), 3157-3165, doi:10.1098/rspb.2009.0517.

Stier, A., Delestrade, A., Bize, P., Zahn, S., Criscuolo, F., & Massemin, S. (2016). Investigating how telomere dynamics, growth and life history covary along an elevation gradient in two passerine species. *Journal of Avian Biology, 47*(1), 134-140, doi:10.1111/jav.00714.

Sudyka, J., Podmokla, E., Drobniak, S. M., Dubiec, A., Arct, A., Gustafsson, L., et al. (2019). Sex-specific effects of parasites on telomere dynamics in a short-lived passerinethe blue tit. *Science of Nature, 106*(1-2), 8, doi:10.1007/s00114-019-1601-5.

Tilgar, V., Mänd, R., Kilgas, P., & Mägi, M. (2010). Long-term consequences of early ontogeny in free-living Great Tits *Parus major*. *Journal of Ornithology, 151*(1), 61-68, doi:10.1007/s10336-009-0426-y.

Tripet, F., & Richner, H. (1997). The coevolutionary potential of a 'generalist' parasite, the hen flea *Ceratophyllus gallinae*. *Parasitology, 115*, 419-427.

Tschirren, B. (2015). Differential effects of maternal yolk androgens on male and female offspring: a role for sex-specific selection? *PLoS One, 10*(7), e0133673. doi:0133610.0131371/journal.pone.0133673.

Tschirren, B., Fitze, P. S., & Richner, H. (2003). Sexual dimorphism in susceptibility to parasites and cell-mediated immunity in great tit nestlings. *Journal of Animal Ecology, 72*(5), 839-845.

van Lieshout, S. H. J., Bretman, A., Newman, C., Buesching, C. D., Macdonald, D. W., & Dugdale, H. L. (2019). Individual variation in early-life telomere length and survival in a wild mammal. *Molecular Ecology, 28*(18), 4152-4165, doi:10.1111/mec.15212.

Vedder, O., Verhulst, S., Bauch, C., & Bouwhuis, S. (2017). Telomere attrition and growth: a life-history framework and case study in common terns. *Journal of Evolutionary Biology, 30*(7), 1409-1419, doi:10.1111/jeb.13119.

Waddington, C. H. (1942). Canalization of development and the inheritance of acquired characters. *Nature, 150*, 563-565, doi:10.1038/150563a0.

Watson, R. L., Bird, E. J., Underwood, S., Wilbourn, R. V., Fairlie, J., Watt, K., et al. (2017). Sex differences in leucocyte telomere length in a free-living mammal. *Molecular Ecology, 26*(12), 3230-3240, doi:10.1111/mec.13992.

Wilbourn, R. V., Moatt, J. P., Froy, H., Walling, C. A., Nussey, D. H., & Boonekamp, J. J. (2018). The relationship between telomere length and mortality risk in non-model vertebrate systems: a meta-analysis. *Philosophical Transactions of the Royal Society B-Biological Sciences, 373*(1741), 9, doi:10.1098/rstb.2016.0447.

Fig. 1. Experimental hen flea exposure during growth results is shorter telomeres (z-transformed T/S ratio) in female but not male great tit nestlings. Means ± 1SE are shown.

Table 1. Telomere length (z-transformed T/S ratio) of male and female great tit nestlings growing up in experimentally deparasitized and experimentally flea infested nests, respectively.

a) Effects of experimental flea infestation, sex and their interaction on nestling telomere length. b) Same model with body mass included as an additional covariate. The factor level of comparison is indicated in brackets. Nest of origin and nest of rearing were included as random effects in the models. Degrees of freedom were obtained via Satterthwaite's degrees of freedom method using *lmerTest* (Kuznetsova et al. 2017).

a)

Fixed effects					
Predictor	*Estimate*	*SE*	*t*	*df*	*P*
Intercept	0.512	0.148			
Flea treatment (fleas)	-0.454	0.212	-2.14	88.6	0.036
Sex (males)	-0.889	0.207	-4.29	151.6	<0.001
Flea treatment (fleas) x sex (males)	0.598	0.291	2.05	130.1	0.042
Random effects					
Predictor	*Variance*				
Nest of origin	0.117				
Nest of rearing	0.039				
Residuals	0.745				

b)

Fixed effects					
Predictor	*Estimate*	*SE*	*t*	*df*	*P*
Intercept	0.158	0.586			
Flea treatment (fleas)	-0.437	0.214	-2.04	87.45	0.044
Sex (males)	-0.887	0.207	-4.28	149.5	<0.001
Body mass (g)	0.029	0.047	0.62	131.0	0.535
Flea treatment (fleas) x sex (males)	0.581	0.291	2.00	127.1	0.048
Random effects					
Predictor	*Variance*				
Nest of origin	0.134				
Nest of rearing	0.049				
Residuals	0.728				

Electronic Supplementary Material

ESM1. Quantification of great tit telomere length using qPCR

Genomic DNA was extracted from frozen red blood cells (approximately 5 µL), using the Nucleospin Blood Quickpure kit (Macherey-Nagel, Düren, Germany). The concentration and purity (presence of residuals proteins or solvants) of the extracted DNA were checked with a Nano-Drop ND-1000 spectrophotometer (Thermo Fisher Scientific, Massachussets, USA). The quality and the integrity of DNA were confirmed by gel electrophoresis on 1% agarose gels stained with ethidium bromide (checking for the absence of DNA smears).

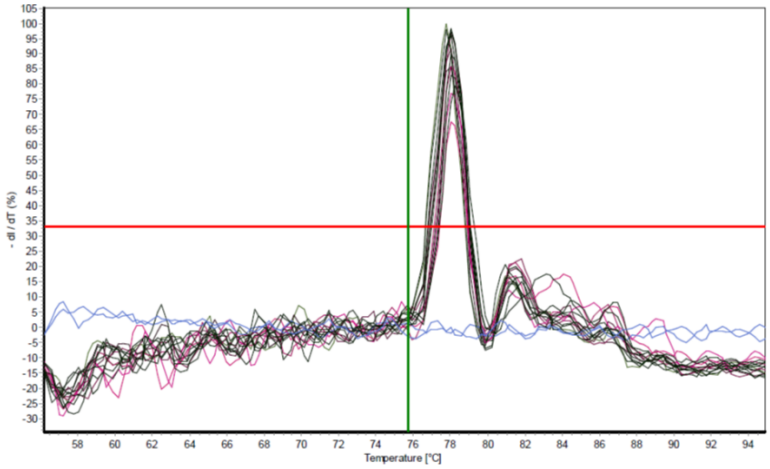
Telomere length measurement was done using a real-time qPCR protocol first set-up for human telomeres (Cawthon 2002) and extended to birds (Criscuolo et al. 2009). qPCR telomere measurement produces a relative telomere length expressed as the T/S ratio between telomere (T) and control genomic sequence (S) amplifications. T and S values were calculated following (Pfaffl 2001), based on the number of amplification cycles of each sequence, controlled for the efficiencies of each reaction. Individual T/S ratios are expressed relatively to a randomly chosen individual, for which the value is set at 1 (golden sample). All samples were run in duplicates (allowing intra-plate repeatability evaluation), and randomly distributed over runs (i.e. each run encompassed 2 plates of 96 wells, one for telomere and one for control gene amplification). In each plate, a negative control (ultra-pure water instead of DNA), the golden sample, four additional control samples and one dilution curve obtained from a serially diluted sample (from 4 to 0.25 ng) were included. This allowed to evaluate (i) the absence of DNA contamination of your materials and solutions, (ii) the non-variability of amplification efficiencies between plates, and (iii) the inter-plate

repeatability. Repeatabilities of T/S ratio were quantified using Intraclass Correlation Coefficient (ICC) (Eisenberg 2016).

For telomere sequence amplification, we used the primers Tel1b: 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3' and Tel2b: 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'. The EGR1 (ZENK) early growth response 1 gene (Gene ID: 107210829) was used as the control gene (S) The used primers were ZENK1: 5'-TACATGTGCCATGGTTTTGC-3' and ZENK2: 5'-AAGTGCTGCTCCCAAAGAAG-3'. These primer sets have previously been validated and used for the great tit (Stier et al. 2015).

Primer concentrations in the final mix were 100 nM for telomere and 500 nM for the control gene. We used 2 ng DNA per reaction and GoTaq qPCR mix (Promega, Madison, WI, USA).

qPCR runs were conducted using Realplex Master-cycler Eppendorf (Hamburg, Germany), using the same amplification procedures for telomere and control. The reaction conditions were: 95°C for 2 min – followed by 30 (telomere) or 40 (control) cycles of 95°C for 15 sec, 56°C for 30 sec, 72°C for 90 sec. qPCR runs encompassed 1 plate of telomere amplification and 1 plate of control gene amplification, using the same sample distribution over the 96 wells of both plates. Each qPCR amplification program ended with a melting curve, allowing for the detection of non-specific amplification events (see ESM2 below). Amplicon size of the control gene was controlled using electrophoresis on 10 samples randomly chosen, to check for non-variability in copy number. Mean amplification efficiencies were of 94.1 ± 0.8% (telomere) and 94.9 ± 1.2% (control) and r^2 of dilution curves of 0.979-0.995 (telomere) and 0.913-0.987 (control). Intra-plate ICC was 0.754 and inter-plate ICC was 0.713.



ESM2. Dynamics of the amplification signal (fluorescence) of the control gene in great tit samples during the melting program. The bold red line is the threshold of fluorescence at which the measure is done. The thin blue lines are fluorescent signals of negative controls (ultra-pure water). The amplification of the control gene in great tits produces one single peak, denoting the absence of non-specific amplifications.

References

Cawthon, R. M. (2002). Telomere measurement by quantitative PCR. *Nucleic Acids Research, 30*(10), 6, doi:10.1093/nar/30.10.e47.

Criscuolo, F., Bize, P., Nasir, L., Metcalfe, N. B., Foote, C. G., Griffiths, K., et al. (2009). Real-time quantitative PCR assay for measurement of avian telomeres. *Journal of Avian Biology, 40*(3), 342-347, doi:10.1111/j.1600-048X.2008.04623.x.

Eisenberg, D. T. A. (2016). Telomere length measurement validity: the coefficient of variation is invalid and cannot be used to compare quantitative polymerase chain reaction and Southern blot telomere length measurement techniques. *International Journal of Epidemiology, 45*(4), 1295-1298, doi:10.1093/ije/dyw191.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research, 29*(9), 6, doi:10.1093/nar/29.9.e45.

Stier, A., Massemin, S., Zahn, S., Tissier, M. L., & Criscuolo, F. (2015). Starting with a handicap: effects of asynchronous hatching on growth rate, oxidative stress and telomere dynamics in free-living great tits. *Oecologia, 179*(4), 999-1010, doi:10.1007/s00442-015-3429-9.