Telomere dynamics in parasitic great spotted cuckoos and their magpie hosts

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Abstract

Although little is known on the impact of environment on telomere length dynamics, it has been suggested to be affected by stress, lifestyle and/or life-history strategies of animals. We here compared telomere dynamics in erythrocytes of hatchlings and fledglings of the brood parasite great spotted cuckoos (*Clamator glandarius*) and of magpies (*Pica pica*), their main host in Europe. In magpie chicks, telomere length decreased from hatching to fledging, whereas no significant change in telomere length of great spotted cuckoo chicks was found. Moreover, we found interspecific differences in the association between laying date and telomere shortening. Interspecific differences in telomere shortening were interpreted as a consequence of differences in lifestyle and life-history characteristics of magpies and great spotted cuckoos. In comparison with magpies, cuckoos experience reduced sibling competition and higher access to resources and, consequently, lower stressful environmental conditions during the nestling phase. These characteristics also explain the associations between telomere attrition and environmental conditions (i.e. laying date) for magpies and the absence of association for great spotted cuckoos. These results therefore fit expectations on telomere dynamics derived from interspecific differences in lifestyle and life history of brood parasites and their bird hosts.

Introduction

Environmental conditions experienced during the first days of life may have important consequences later, during adulthood, in terms of survival prospects (Monaghan, 2008). Recently, it has been suggested that these delayed effects are at least partially driven by the influence of early environmental conditions on telomere dynamics (Beery *et al.*, 2012) and supporting evidences have been accumulated during the last few years (Hall *et al.*, 2004; Heidinger *et al.*, 2012; Boonekamp *et al.*, 2014; Herborn *et al.*, 2014; Nettle *et al.*, 2015). Telomeres are specialized structures at the end of the chromosome, which consist on shorts repeats of the noncoding DNA sequence TTAGGG that, among other functions,

protect wholeness of genetic information during cell division (Blackburn, 1991). Telomere attrition has traditionally been related with ageing (Sahin & DePinho, 2010) and is closely related to stress events of different nature that include those associated with cell division (i.e. growth) and with elevated concentrations of reactive oxygen species (Monaghan & Haussmann, 2006).

Ageing, lifestyle, lifespan and life-history characteristics of animals are closely related in nature (Monaghan & Haussmann, 2006); for example, animals with relative short lifespan grow faster and reproduce earlier at the cost of increasing metabolic and cellular degeneration rates (Ricklefs & Finch, 1995), which would affect telomere dynamics. Telomere length and dynamics are considered good indicators of fitness in terms of survival prospect of the adopted lifestyle and/or life-history strategies (Monaghan & Haussmann, 2006; Kotrschal *et al.*, 2007). Thus, detecting evidence linking lifestyle and/or lifespan with telomere length and shortening is of prime importance to understand the balance of costs

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and benefits associated with species-specific life histories (Salomons *et al.*, 2009b; Monaghan, 2014). Particularly interesting is the study of telomere dynamics during the first few days of life of altricial birds for several reasons. Natural selection and telomere shortening is particularly apparent at this stage due to very high cell division rate (Salomons *et al.*, 2009b). Moreover, the relatively down-regulation of telomerase activity (i.e. reduced telomere repair) during the exponential growth stage (Forsyth *et al.*, 2002; but see Haussmann *et al.*, 2007) makes easier the detection of changes in telomere length to explore the predicted associations with life-history characteristics or environmental conditions.

In addition, environmental conditions experienced by nestlings largely vary in association with laying date, with late hatchlings typically developing in worse, more stressful conditions than early hatchlings, due to poor parental quality of breeders and/or deteriorated resource availability (De Neve *et al.*, 2004; Verhulst & Nilsson, 2008). Further, the strength of the association between laying date and resource availability for developing nestlings depends on species-specific life-history traits (Svensson, 1995) and, thus, we should expect telomere dynamics to vary with laying date (Foote *et al.*, 2011), at least for some species. As far as we know, this predicted association has never been tested in the wild.

Finally, sibling competition for parental care is one important cause of physiological stress (Martínez-Padilla *et al.*, 2004; Blanco *et al.*, 2006) likely affecting telomere dynamics during growth. Consequently, previous studies have found that the strength of intrabrood competition for food in jackdaw (*Corvus monedula*) (Salomons *et al.*, 2009a) and European starling (*Sturnus vulgaris*) (Nettle *et al.*, 2013) nestlings was positively related with telomere attrition during their first few days of life. Thus, because the strength of sibling competition widely varies within and among bird species (Mock & Parker, 1997), it can be expected that nestlings of species with intense sibling rivalry had better mechanisms to cope with stress and to reduce telomere shortening, than those of species with low sibling competition. At the intraspecific level, telomere length and body size of barn swallow (*Hirundo rustica*) nestlings positively covaried (Caprioli *et al.*, 2013), and telomere attrition due to sibling competition was negatively related with nestling hierarchy in European starlings (Nettle *et al.*, 2013, 2015); both results suggesting the possibility of physiological adjustment depending on nestling hierarchy (i.e. body mass) that could even be mediated by maternal effects. Moreover, experimentally increased brood size also resulted in larger telomere attrition in zebra finch (*Taeniopygia guttata*) parents (Reichert *et al.*, 2014). Thus, species differing in the strength of intrabrood competition for food may also differ in telomere dynamics during growth.

With the aim of contributing to understand factors explaining telomere dynamics of developing animals, we here explored the association between telomere length of hatchlings and fledglings of magpies (*Pica pica*), and of their brood parasitic great spotted cuckoos (*Clamator glandarius*), in relation to laying date. Exploring these associations in this brood parasite–host system has several advantages. First, magpies are the main European host of great spotted cuckoos, and therefore, nestlings of both species are reared by magpie adults (Soler *et al.*, 1999; Soler & Soler, 2000). Second, environmental conditions experienced by developing great spotted cuckoo and magpie nestlings usually differ, the latter developing in a more competitive environment than the former. Great spotted cuckoo nestlings usually outcompete foster siblings soon after hatching. Moreover, post-hatching growth rate of great spotted cuckoos is much higher than that of magpie hosts (Soler & Soler, 1991). Consequently, if telomere dynamics depend on cell division rates, that of great spotted cuckoos and magpies should differ. Finally, because great spotted cuckoo nestlings usually share the nests with fewer chicks than magpies do, it is likely that the expected effects of environmental conditions related to resource availability (i.e. laying date (De Neve *et al.*, 2004)) on telomere dynamics are weaker for nestlings of the brood parasitic species.

Materials and methods

Study sites and fieldwork

The study was performed in the Guadix (37°18′N, 3°11′W, southern Spain) magpie population, situated in a high-altitude plateau (approx. 1000 m a.s.l.). The vegetation of the area is sparse, including uncultivated areas and many groves of almond (*Prunus dulcis*) and pine (*Pinus halepensis* and *Pinus pinaster*) trees and oaks (*Quercus rotundifolia*) in which magpies prefer to build their nests. Brood parasitism by great spotted cuckoos is quite common in the area (Soler *et al.*, 2001, 2013) and, although great spotted cuckoos usually outcompete magpie foster siblings (Soler & De Neve, 2013), parasitic nestlings do not evict host nestlings after hatching and, therefore, both species may grow up together in the same nest (Martín-Gálvez *et al.*, 2011).

Fieldwork was conducted during the 2013 breeding season. At the beginning of spring in March–April, we intensely searched for nests of magpies that were revisited regularly to determine their laying date. Expected hatching date of magpies and great spotted cuckoos was estimated as 25 and 19 days, respectively, after laying date of the first magpie egg. Magpie nests were then visited during these and subsequent days up to sampling magpie hatchlings (i.e. 0–1 day old) and, in the case of parasitized nests, great spotted cuckoo hatchlings. Because maternal and genetic factors are known

to determine early life telomere length (Asghar *et al.*, 2015b), we collected a drop of blood from the brachial vein of two randomly selected magpie hatchlings and from all cuckoo hatchlings in a magpie nest. All magpies and great spotted cuckoo nestlings in the nests 18 and 16 days after hatching, respectively, were again blood-sampled.

To increase the number of parasitized nests with magpies and great spotted cuckoo fledglings growing up together, we experimentally parasitized 11 magpie nests with a single cuckoo egg close to hatching time from multiparasitized magpie nests. In three of these nests, only the cuckoo fledged [cuckoos hatched 3.33 (SE = 0.67) days before magpies]; in one, only magpies (four magpie fledglings that hatched 2 days in advance of cuckoos); and in seven nests, both the great spotted cuckoo and magpies (average number of fledglings per nest = 2.6, SD = 0.98) fledged [cuckoos hatched 2.14 (SE = 0.86) days before magpies]. In addition, we sampled 27 natural magpie nests. From eight of these nests, only cuckoos fledged (mean = 1.13, SD = 0.35), from 18 only magpies fledged (mean = 4.5, SD = 1.04), and from the last nest, one great spotted cuckoo and one magpie successfully reached the fledging stage. However, duplicated estimates of telomere length of some samples were not consistently similar (i.e. CV > 5%, see below) and were not considered in the analyses. We thus collected information with adequate estimates of telomere attrition for 36 magpie nests. In eight of these nests, magpies and great spotted cuckoos grew together, but information for magpies and cuckoo nestlings was only available for four of them. For the other four nests with both species, only information for magpie nestlings was available for the analyses. In addition, we collected information on telomere attrition for 18 and 10 nests, respectively, where only chicks of one species, that is either magpies or great spotted cuckoos, grew up. For some additional nests, information of telomere length was only available for hatchling or for fledglings. Thus, for cuckoo nestlings, final sample size was 18 for telomere length of hatchling and 14 for telomere attrition. For magpie nestlings, final sample size used in the analyses was 26 and 27 for telomere length of hatchlings and fledglings, respectively, and 26 for telomere attrition.

Laboratory work

DNA was extracted from blood samples using a standard chloroform–isoamyl alcohol-based protocol (see Ferraguti *et al.*, 2013). DNA concentration was adjusted to 20 ng μL^{-1} using distilled water and conserved frozen until further analyses. Relative telomere length (hereafter telomere length) was estimated by q-PCR following the protocol and primers of Criscuolo *et al.* (2009). As control single-copy gene we used the glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

GAPDH was used to normalize the quantity of telomere sequence to the amount of DNA in the q-PCR. The final PCR volume was 20 μL containing 10 μL of LightCycler 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) and 1 μL of DNA at 20 ng μL^{-1} of DNA. The reactions for telomeres or GAPDH were done in different plates due to the differential PCR conditions. Telomere PCR conditions were 10 min at 95 °C followed by 30 cycles of 1 min at 56 °C and 1 min at 95 °C. GAPDH PCR started with 10 min at 95 °C followed by 40 cycles of 1 min at 60 °C and 1 min at 95 °C, both performed in a LightCycler 480 RT-PCR System (Roche). Each sample was run in duplicate, and samples with a coefficient of variation higher to 5% were removed from the analyses. Each 96-well plate included serial dilutions of DNA (40 ng, 10 ng, 2.5 ng, 0.66 ng of DNA per well) from a reference pool (the internal control) run in triplicate, which were used to generate the standard curves, and a blank control with no DNA. Quantification cycle values (Ct) were transformed into normalized relative quantities (NRQs) following Hellemans *et al.* (2007) procedure, which controls for the amplifying efficiency of each qPCR. Amplification efficiency for telomere products ranged between 1.85 and 2.01 and for the GAPDH product between 1.88 and 2.14. The slope of the calibration curve ranged between -3.792 and -3.277 for the telomere product and -3.647 and -3.031 for the GAPDH product. The melting curves of the control gene cycles confirmed no evidence of primer dimer or non-specific amplification. Different techniques are available for measuring telomere length in wildlife (reviewed in Nussey *et al.*, 2014). Each of these techniques has advantages and limitations that may affect the reliability of the results. In the case of q-PCR, an important limitation is that interstitial telomeric sequences may occur on the genome and consequently may bias estimates of relative telomere length. Consequently, q-PCR estimated telomere lengths may be not adequate to compare telomere length among species (Nussey *et al.*, 2014). However, it is adequate to compare patterns of variation across species based on repeated measures of the same individuals across time, as we have done in this study (for a similar approach, see Asghar *et al.*, 2015a). Consequently, we will not deal with interspecific differences in telomere length but compare the temporal and developmental patterns of variation in telomere length between magpies and cuckoos.

Statistical analyses

Frequency distribution of NRQ values estimated for nestlings and fledglings of cuckoos and magpies did not differ from normal distributions (Kolmogorov–Smirnov test for continuous variables, $P > 0.05$). Moreover, between-nest variation in telomere length of magpie fledglings was significantly larger than within-nest vari-

ation ($R = 0.67$, $F_{26,77} = 4.99$, $P < 0.0001$); thus, we used within-nest mean values for subsequent analyses. Telomere attrition was thus estimated as the difference between average values at the time of hatching and at the time of fledging. Laying date did not differ from normal distribution (Kolmogorov–Smirnov test for continuous variables, $P > 0.05$), and thus, we used parametric statistical tests for comparing telomere length and attrition of great spotted cuckoos and magpies and its relationship with their respective laying date.

Whether or not cuckoo or magpie chicks grew up together with nestlings of the other species did not affect telomere length and telomere attrition of fledgling cuckoos [sample size: N (only cuckoos) = 10, N (cuckoos and magpies) = 4, $t < 1.02$, $P > 0.33$] or of magpie nestlings [sample size: N (only magpies) = 19, N (cuckoos and magpies) = 8, $t < 0.35$, $P > 0.73$]. Thus, this factor was not considered in further analyses. Some nestlings of both species died later during the nestling phase before the second blood sampling, and thus, sample sizes for great spotted cuckoos and magpies in parasitized nests rearing nestlings of both species do not coincide.
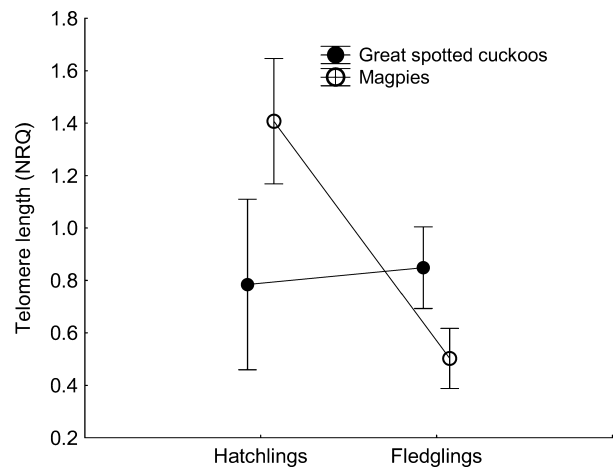
Telomere dynamics of great spotted cuckoos and magpies were first explored separately for each species by means of repeated-measures ANOVAs with estimates from the same nest at different times as within factor and brood size and laying date as covariables. Interspecific differences in the association between laying date and either telomere length at different nest stage (i.e. hatching and fledging) or telomere shortening were explored with ANCOVAs with species identity as the categorical independent factor, laying date of the first magpie egg as the covariable and the interaction between laying date and species identity as the factor informing on the interspecific differences. Relationships between laying date and either telomere length or telomere shortening were explored using Pearson correlations.

All statistical tests were performed in Statistica 10.0 (Statsoft Inc., 2011).

Results

As magpie nestlings experienced a relatively drastic telomere shortening during the nesting period (repeated-measures ANOVA, $F_{1,25} = 45.65$, $P < 0.0001$, Fig. 1), this was not the case for cuckoo nestlings (repeated-measures ANOVA, $F_{1,13} = 0.19$, $P = 0.67$, Fig. 1; interspecific comparisons of telomere shortening, interaction between developing time and species identity, $F_{1,38} = 16.68$, $P = 0.0002$, Fig 1). Overall, telomere length of magpies declined by 64.3% from hatching to fledging. Including the nonsignificant effects of laying date and brood size ($P > 0.28$) in the statistical models exploring interspecific differences did not change any conclusions.

The association between telomere length and laying date differed for great spotted cuckoo and magpie


Fig. 1 Mean ± confidence intervals (95%) of telomere length (NRQ values) of great spotted cuckoo and magpie hatchlings and fledglings. Lines connecting values of the same species indicate telomere dynamics during the nesting phase.
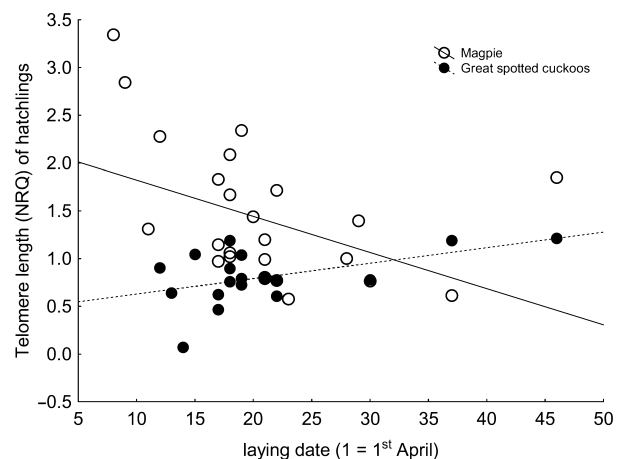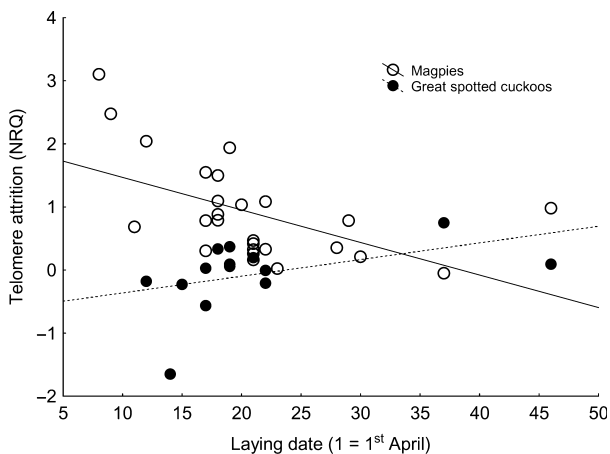

Fig. 2 Relationships between telomere length at hatching time and laying date of magpies (open circles, continuous lines) and great spotted cuckoos (full circles, dotted line). Values are mean nest values and lines are regression lines.

hatchlings (ANCOVA, interaction between species identity and laying date, $F_{1,40} = 7.48$, $P = 0.009$, Fig. 2). Telomere length of magpie hatchlings showed a decrease in relation to laying date ($R = -0.44$, $t = 2.37$, $N = 26$, $P = 0.026$, Fig. 2), whereas telomere length of cuckoo hatchlings was positively related to laying date ($R = 0.49$, $t = 2.28$, $N = 18$, $P = 0.037$, Fig. 2).

At the time of fledging, the association between telomere length and laying date for great spotted cuckoos and magpies also differed significantly (ANCOVA, interaction between species identity and laying date, $F_{1,41} = 6.40$, $P = 0.015$, Fig. 3). Telomere length of fledgling magpies increased as the season progressed ($R = 0.49$, $t = 2.83$, $N = 27$, $P = 0.009$, Fig. 3), even after controlling for the effect of telomere length at hatching (laying date: partial
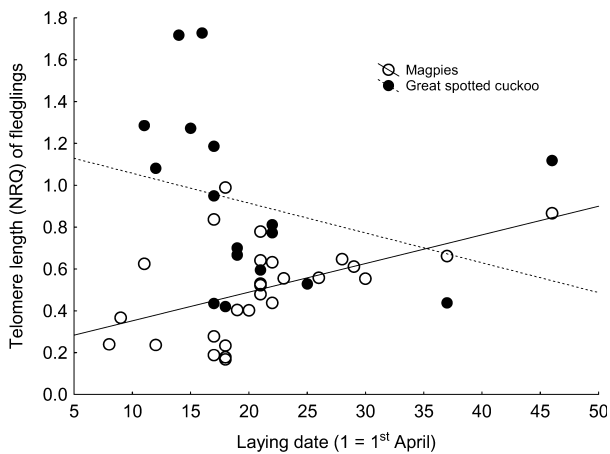
Fig. 3 Relationships between telomere length at fledging time and laying date of magpies (open circles, continuous lines) and great spotted cuckoos (full circles, dotted line). Values are mean nest values and lines are regression lines.

Fig. 4 Relationships between telomere attrition during development in magpies (open circles, continuous lines) and great spotted cuckoos (full circles, dotted line) and laying date. Values are mean nest values and lines are regression lines.

$R = 0.50$, $P = 0.021$; telomere length at hatching: partial $R = 0.02$, $P = 0.93$). This trend was not detected in cuckoos for which late broods had nonsignificant smaller telomeres than those of early broods ($R = -0.30$, $t = 1.25$, $N = 18$, $P = 0.23$, Fig. 3) even after controlling for the effect of telomere length at hatching (laying date: partial $R = 0.01$, $P = 0.96$; telomere length at hatching: partial $R = -0.27$, $P = 0.36$).

The above reported associations between laying date and telomere length of hatchlings and fledglings of great spotted cuckoos and magpies suggest that the associations between telomere attrition and laying date should also differ for magpie and great spotted cuckoo nestlings (ANCOVA, interaction between species identity and laying date, $F_{1,36} = 10.15$, $P = 0.003$, Fig. 4).

Telomere attrition during the nesting phase was negatively associated with laying date for magpie nestlings ($R = -0.53$, $t = 3.10$, $N = 26$, $P = 0.005$, Fig. 4) even after controlling for the effect of telomere length at hatching (laying date: partial $R = -0.46$, $P = 0.021$; telomere length at hatching: partial $R = 0.95$, $P < 0.0001$). The positive association detected for great spotted cuckoos ($R = 0.44$, $t = 1.69$, $N = 14$, $P = 0.12$, Fig. 4) was far from statistical significance after controlling for telomere length at hatching (laying date: partial $R = -0.01$, $P = 0.96$; telomere length at hatching: partial $R = 0.69$, $P = 0.008$).

Discussion

Our main finding is that telomere length of magpie nestlings is drastically reduced during development at the nest, whereas no changes occur in cuckoo nestlings. This suggests that magpies are suffering higher levels of stress than cuckoos or that cuckoos can deal with such stress better than magpies. Moreover, we found interspecific differences in the association between laying date and telomere length and shortening. The stronger associations were detected in magpies for which late hatchlings had the shorter telomeres, whereas telomere attrition was stronger for magpie nestlings of early broods. Below we discuss these results in the contexts of interspecific differences in developmental strategies of great spotted cuckoos and magpies that may affect telomere length and environmental influences on the dynamics of telomere change.

Interspecific differences in telomere length along the nestling period

When great spotted cuckoos successfully parasitize magpie nests before the onset of incubation, the accelerated embryonic developmental rates of parasites relative to those of their hosts confer parasitic offspring with huge advantages when competing for food with host nestlings (Soler & Soler, 1991). In these parasitized nests, host nestlings hatch five or six days after cuckoos hatch, at a time when parasitic foster siblings weigh more than 60 g, which is ten times more than the weight of magpie hatchlings (J.J. Soler, unpublished information). Because magpie adults prefer to feed the largest nestling in the nest (Soler et al., 1995), magpie offspring has no option to survive in these nests where successful parasitism occurs before incubation started (Soler et al., 1998). Thus, it is likely that after paying the costs of accelerated rate of cell division during their short embryonic stage, cuckoos will enjoy a peaceful nestling period with no apparent resource limitation for growing (Soler & Soler, 2000). In contrast, magpie nestlings should always compete for food with siblings and might therefore suffer increased costs in terms of telomere attrition (see Introduction). In agreement with this

scenario, we found that telomeres of magpies, but not of great spotted cuckoos, decreased significantly over the nesting period, which is in accordance with differential costs associated with intense intrabrood competition in terms of telomere attrition detected in some other species (Salomons et al., 2009b; Nettle et al., 2013).

Particularly intriguing is the lack of evidence of consistent telomere attrition of cuckoo nestlings over the nesting period. Even more when considering that for one-third of the studied nests with great spotted cuckoos, telomere length increased rather than decreased (Fig. 4), although some of these cases may be explained by measurement errors. Telomere enlargement over the nestling period has been previously detected in starlings, but because telomerase activity is considered to be down-regulated in juveniles as a tumour-protecting mechanism (Wright & Shay, 2001), and it was only detected in two individuals, these were considered as outliers due to measurement errors (Nettle et al., 2013). However, contrary to the idea that telomerase activity is mainly suppressed in most somatic tissues of developing animals, Haussmann et al. (2007) detected that it is maintained throughout the lifespan of birds, including the hatchling age class. Thus, it is possible that telomerase activity was especially high in great spotted cuckoo nestlings, counteracting degenerative effects of the rapid growth experienced during the nestling stage, possibly even allowing some recovery of telomere length after their extremely speedy growth during the embryonic phase (and the presumably very high telomere attrition rate during this phase, see above). However, it is also possible that the interspecific differences in telomere trajectories were due to differences in the capacity to control oxidative stress (i.e. higher access to antioxidants given a higher access to food or more intense antioxidant enzymatic activity) (Badás et al., 2015).

Finally, because fledgling cuckoos were sampled when they were two days younger than magpies, difference in age might explain the interspecific differences detected in telomere attrition. This last possibility is, however, unlikely as cuckoos develop faster than magpies and nestlings of both species are at similar developmental stage at the age of sampling (Soler & Soler, 1991).

Telomere length and shortening, and laying date

Laying date is one of the most employed variables reflecting territory quality and availability of resources and/or parental quality (Brinkhof et al., 1993; Moreno, 1998; De Neve et al., 2004; Verhulst & Nilsson, 2008). Increased nestling competition reduces resource availability for growing nestlings, and deterioration of quality of parental care is post-natal stressors typically associated with laying date (Perrins, 1970; Brinkhof et al., 1993). Therefore, an increase in endogenous glucocorticoids is predicted in nestlings from late breeding attempts (Banerjee et al., 2012), which consequently should affect telomere length dynamics (Monaghan, 2014). In accordance with this scenario, we found that late magpie hatchlings had shorter telomere length than early hatchlings. However, some of our results were not in accordance with this hypothesis as telomere length of great spotted cuckoo hatchlings was positively associated with laying date, and telomere attrition experienced by magpie nestlings decreased over the breeding season; a tendency not detected in cuckoos. The detected associations between laying date and either telomere length or telomere attrition did not depend on telomere length at hatching, and therefore, these associations cannot be explained by the commonly described higher telomere attrition in individuals with initially longer telomeres (Marcand et al., 1999; Karlseder et al., 2002; Hall et al., 2004). Thus, interspecific differences in the association between telomere length and laying date strongly suggest that related environmental characteristics do influence telomere dynamics of magpie and of great spotted cuckoo nestlings differently. It is difficult, however, to explain contradictory results to the proposed scenario, and we can only speculate about possible reasons. It is, for instance, possible that the negative association between telomere length of magpie hatchlings and laying date was due to variation in resource availability for laying females (Perrins, 1970; Drent, 2006). Late magpie nestlings have poorer immune system (Sorci et al., 1997) and lower developmental rate than early nestlings (De Neve et al., 2004), which may thus explain the detected negative correlation between laying date and telomere attrition of magpies during development. However, this possibility predicts a positive relationship between telomere attrition and laying date that we did not find. Another possibility explaining the detected relatively larger telomere attrition rates of early magpie hatchlings is that later hatchlings experience a relatively low within-brood competition. However, resource availability decreases as the season progresses in magpie nests (De Neve et al., 2004), and it is likely negatively related with level of within-brood competition. Additionally, telomere length has a maternal and a hereditary component in birds (Monaghan, 2014; Asghar et al., 2015b), and consequently, relationships between hatchling telomere length and laying date may reflect an underlying relationship between the genetic basis of telomere length of parents (mainly females) and laying date.

Taken together, all these results for the first time point out a relationship between environmental conditions (laying date) and telomere length and shortening that vary between species experiencing similar ecological conditions during growth. As telomere length and telomere shortening are variables reflecting survival prospect and costs associated with stress responses

(Asghar *et al.*, 2015a; Nettle *et al.*, 2015), these results may suggest that species varying in developmental strategies (i.e. nestling growth and/or embryonic growth rates) also vary in suffered costs in terms of telomere shortening and/or dynamics. Therefore, our results fit expectations according to interspecific differences in life-style and life history. Exploring possible causes of the detected interspecific differences and associations may help to understand the evolutionary relationship between telomere dynamics and life-history traits of animals.

Acknowledgments

Statement of authorship

JJS and JF designed the study with considerable assistance from JMP and CRC. CRC performed all molecular analyses with considerable assistance JMP. JJS, MRR and GT performed most of the field work with assistance by CRC. JJS performed all the statistical analyses and wrote the manuscript with substantial contribution from all authors

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