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Telomere length, non-breeding habitat and return rate in male American redstarts

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Summary

1. Telomeres are long repetitive noncoding sequences of DNA located at the ends of chromosomes. Recently, the study of telomere dynamics has been increasingly used to investigate ecological questions. However, little is currently known about the relationships that link environmental conditions, telomere dynamics and fitness in wild vertebrates.
2. Using a small migratory bird (American redstart, *Setophaga ruticilla*), we investigated how telomere dynamics can be affected by non-breeding habitat quality and to what extent telomere length can predict the return rate of males.
3. We show that telomeres shorten in most individuals over a 1-year period and, importantly, that telomeres of individuals wintering in a low-quality habitat shorten more than those of individuals wintering in a high-quality habitat.
4. In addition, we found that longer telomeres are associated with a higher return rate than shorter telomeres, although the relationship between return rate and telomere length did not depend on habitat quality.
5. Our study suggests that telomere dynamics are affected by environmental conditions and are related to indices of fitness in a migratory bird species.

Key-words: American redstart, habitat, *Setophaga ruticilla*, survival, telomere dynamics

Introduction

During the recent decades, advances in biomedical science have demonstrated that telomeres – long repetitive non-coding sequences of DNA located at the ends of chromosomes – can play a major role in ageing processes and human diseases (Hastie *et al*. 1990; Blackburn 2000, 2005; Aviv 2002). Indeed, telomeres appear essential to genome integrity because they protect chromosomes from degradation and help to ensure proper replication processes (Aubert & Lansdorp 2008). Early telomere research showed that telomeres shorten each time a cell divides by meiosis or mitosis, and more recently, oxidative stress has been shown to accelerate this shortening process (Von Zglinicki 2002).

Biomedical research has focused on the causes and mechanisms of telomere length regulation because of the major role that telomere dynamics play in senescence and cancer formation (Blackburn 2005; Rodier *et al*. 2005; Shen *et al*. 2007). Recent advances in understanding telomere dynamics have been suggested as promising to investigate ecological and evolutionary questions (Monaghan & Haussmann 2006; Haussmann & Marchetto 2010; Horn, Robertson & Gemmell 2010; Monaghan 2010a). In this context, two major challenges that remain are to better understand how environmental factors affect telomere shortening and what variation in telomere length means for individual fitness of wild vertebrates (Monaghan & Haussmann 2006; Monaghan 2010b). Such investigations will help to determine how telomere length may be involved in life-history trade-offs and be used to investigate life-history strategies.

Little is known about how environmental factors affect the regulation of telomere length in wild animals. Several studies have reported that telomeres shorten with age in wild vertebrates (Haussmann & Vleck 2002; Haussmann *et al*. 2003; Juola *et al*. 2006; Pauliny *et al*. 2006; Bize *et al*. 2009; Salomons *et al*. 2009). However, Foote *et al*. (2011) showed that there is a large interindividual variability in the rate of telomere shortening in growing Lesser

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black-backed gulls (*Larus fuscus*). Similarly, Bize *et al.* (2009) reported that the rates of telomere shortening varied greatly from one individual to another over a 5-year period in Alpine swifts (*Apus melba*). In European shags (*Phalacrocorax Aristotelis),* Hall *et al.* (2004) found that greater telomere attrition was displayed by chicks laying down high tissue mass in relation to structural size and by chicks from eggs laid relatively late in the season, suggesting therefore that environmental factors may affect telomere dynamics. In humans and laboratory rodents, it has been suggested that lifestyle can affect telomere loss. For instance, repeated infection can cause telomere attrition in house mice (Ilmonen, Kotrschal & Penn 2008). In laboratory rodents and humans, repeated stressful events result in important and rapid telomere shortening (Epel *et al.* 2004; Kotrschal, Ilmonen & Penn 2007). However, such questions have rarely been examined in wild vertebrates (Monaghan & Haussmann 2006; Haussmann & Marchetto 2010; Monaghan 2010a; Heidinger *et al.* 2012). To date, environmental constraints and stressful or demanding events have been associated with telomere attrition (captive mice: Kotrschal, Ilmonen & Penn 2007; captive zebra finches: Heidinger *et al.* 2012; captive chicken: Haussmann *et al.* 2012) or no change in telomere length (wild Adélie penguins: Beaulieu *et al.* 2011). Therefore, it remains to be understood how telomere shortening can be affected by the environment in natural vertebrate populations (that we call 'the environmental determination of telomere length hypothesis' in the rest of this paper).

Several studies have also shown that telomere length can predict fitness components (e.g. reproductive performance or survival) in wild vertebrates (that we call 'the telomere-fitness hypothesis' in the rest of this paper). Pauliny *et al.* (2006) showed that telomere length is correlated with reproductive success in dunlins (*Calidris alpina*). Similarly, individuals with longer telomeres generally survive better than individuals with shorter telomeres in several vertebrate species (Haussmann, Winkler & Vleck 2005; Pauliny *et al.* 2006; Bize *et al.* 2009; Salomons *et al.* 2009; Foote *et al.* 2010; Olsson *et al.* 2011a,b). However, it is currently unclear whether environmental factors can affect the strength or the direction of this relationship. Recent studies have shown that the relationship between survival and absolute telomere length is not always similar between sexes, and this has been related to differences in selection pressure and environmental constraints between sexes (Foote *et al.* 2010; Olsson *et al.* 2010, 2011a; Barrett & Richardson 2011). These studies therefore suggest that the extent to which telomere length is related to survival may depend on the environmental context and may differ between individuals (Olsson *et al.* 2011a).

To test the 'environmental determination of telomere length' hypothesis, we examined how telomere length and the rate of telomere shortening varied between individual birds living in two contrasting environments. We focused on a small migratory bird, the American redstart (*Setophaga ruticilla*) over-wintering in two non-breeding habitat types that vary in food availability, which affects over-winter body condition and survival (Marra & Holmes 2001). We sampled male redstarts wintering in mangrove habitat known to have high food availability and in scrub habitat, known to have low food availability (Marra & Holberton 1998; Marra & Holmes 2001; Studds & Marra 2007; Angelier *et al.* 2011). Non-breeding habitat has also been shown to impose important constraints on the rest of the annual cycle of male redstarts (the concept of 'carry-over effect' including migration pattern, breeding success and survival, Marra, Hobson & Holmes 1998; Marra & Holmes 2001; Studds & Marra 2005; Reudink *et al.* 2009a), suggesting that wintering in a habitat of low quality may be associated with a constraining lifestyle. Because stressful events can induce a rapid shortening of telomere lengths in laboratory rodents and humans (Epel *et al.* 2004; Kotrschal, Ilmonen & Penn 2007), we predicted that telomeres of scrub-habitat redstarts should shorten faster than telomeres of mangrove-habitat redstarts (prediction 1). Consequently, we also predicted that scrub-habitat males should have on average shorter telomeres than mangrove-habitat males (prediction 2). Because telomeres tend to shorten through the life of individuals in several species (Bize *et al.* 2009; Foote *et al.* 2011; but see Haussmann *et al.* 2003; Hall *et al.* 2004; Ujvari & Madsen 2009), we also predicted that telomere length should be negatively correlated with age in redstarts (prediction 3) and, according to prediction 1, that this negative relationship should be steeper for the scrub-habitat males than for mangrove-habitat males.

To provide information on 'the telomere-fitness hypothesis', we examined whether telomere length predicted return rate (one component of fitness) to the wintering territory in the subsequent year in this species. According to previous studies conducted in wild vertebrates (Haussmann, Winkler & Vleck 2005; Pauliny *et al.* 2006; Bize *et al.* 2009; Salomons *et al.* 2009; Foote *et al.* 2010; Olsson *et al.* 2011a,b), we predicted that males with relatively long telomere should have a higher return probability than those with short telomeres (prediction 4). Because our previous studies demonstrate that scrub-habitat males must contend with stronger environmental constraints than mangrove-habitat birds during the annual cycle ('carry-over effect', Marra, Hobson & Holmes 1998; Marra & Holmes 2001; Studds & Marra 2005; Reudink *et al.* 2009a), selection on telomere length may be stronger for scrub-habitat males than for mangrove-habitat males. Thus, we predicted the relationship between the probability of return and telomere length would be more apparent for scrub-habitat birds than mangrove-habitat birds (prediction 5).

Materials and methods

STUDY AREA AND BIRDS

The American redstart breeds in wooded habitat throughout much of North America (Fig. 1). Redstarts begin arriving in

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Fig. 1. A HY male American redstart that was caught in the scrub habitat during the non-breeding season at the Font Hill Nature Preserve, Jamaica. Photo by Peter P. Marra ©.

Jamaica and establishing non-breeding territories in mid-September and remain on those territories for 6–8 months until they depart on spring migration in April/May. During the non-breeding period, birds establish their territories along a moisture gradient that also corresponds to a non-breeding habitat quality gradient, a range of which (high-quality black mangrove forest to low-quality second-growth scrub habitat) occurs within our long-term study site (see Marra & Holberton 1998; Marra & Holmes 2001; Studds & Marra 2007 for further details). Mangrove forest consisted mainly of black mangrove (*Avicennia germinans*), but also of white (*Languncularia racemosa*) and red mangrove (*Rhizophora mangle*). Second-growth scrub consisted mainly of logwood trees (*Haematoxylon campechianum*), but also had other species including *Bursera simarubra*, *Terminalia latifolia* and *Crescentia alata*. Unlike mangrove habitat that retains water and remains shady and cool, second-growth scrub never had standing water, and this habitat became increasingly arid as the non-breeding season progresses (Marra & Holmes 2001; Studds & Marra 2005).

Our study was conducted at the Font Hill Nature Preserve (13 km west of Black River, St Elizabeth Parish, Jamaica, West Indies), and it includes both mangrove and scrub habitats, redstarts have been banded and aged (hatch-year HY or after hatch-year AHY) since 1993. At our study site, transition between mangrove and scrub habitats is very abrupt and can be accurately delineated. When individuals were located at the boundary between the mangrove and the scrub habitat, they were excluded from the analyses. Colour-banded birds were intensively followed and their location mapped during several weeks each year to define the boundaries of their territories. Importantly, AHY birds show a high site fidelity to their non-breeding territory allowing us to reliably monitor return rate of banded birds (Marra & Holmes 2001). We cannot totally exclude the possibility that a proportion of banded birds could have survived and wintered in locations outside of our study site that we did not survey (Studds & Marra 2005). Therefore, we measured apparent survival, and we refer to this as return rate rather than survival. However, we did search the vicinity of the study site on several occasions to check for short-distance dispersal and we did not find any evidence of such dispersal for our sampled male AHY redstarts. In addition, banding of HY birds has been conducted every year for the duration of this long-term study, allowing us to know the exact age of a large proportion (*c.* 63%) of the birds that were captured for this specific study (age of sampled birds varied from 1 to 7 years at the time of initial sampling, i.e. 2008).

CAPTURE AND BLOOD SAMPLING

Male American redstarts were captured during two consecutive years (2008 and 2009) using mist-nets (see Angelier *et al.* 2010 for details). A small blood sample (50–100 μL) was collected from the brachial vein into heparinized microcapillary tubes. All new birds were banded with a USFWS aluminium band and three-coloured plastic bands for individual identification from a distance. In addition, they were aged (HY or AHY) and sexed by plumage characteristics. Overall, 63 male redstarts (40 of known age) were captured in 2008 and 15 of them were captured again in 2009, allowing us to examine the yearly rate of telomere shortening (5 scrub habitat and 10 in mangrove habitat). In both years, birds were captured at the same period of the non-breeding season (from late February to early April). The time elapsed between the two sampling processes was very close for all birds (365 ± 30 days), and the rate of telomere shortening was not significantly affected by the time elapsed between the two sampling processes in any habitat (regressions: $P > 0{\cdot}500$). In 2009, the presence of several birds was recorded through observation of their coloured plastic bands. However, several of these birds were not captured in 2009 explaining that we were not able to monitor the rate of telomere shortening for all the returning birds. Blood samples were kept on ice for a few hours and were then centrifuged to separate plasma from red blood cells (10 min at 10·000 ***g***). Plasma was used for another study on stress hormones (Angelier *et al.* 2010), and red blood cells were stored at −20 °C (in Jamaica) and at −80 °C (after return to the USA) until DNA extraction.

Over the course of the study, sufficient numbers of known-aged males were present in both scrub and mangrove habitats allowing us to effectively test our hypotheses in males (scrubs, $n = 16$; mangroves, $n = 24$). However, we only captured and bled a limited number of known-aged females ($n = 14$). Although we could catch a reasonable number of scrub females ($n = 12$), only 2 known-aged mangrove females were caught in 2008 and only one of them was recaptured in 2009. Indeed, female redstarts are more difficult to catch than males because they are not as responsive to a playback song and to a simulated territorial intrusion, that is, presence of a decoy on their territory (Marra 2000; Marra & Holmes 2001; Angelier *et al.* 2010). This small and unbalanced sample size did not allow us to test our hypotheses in females. Therefore, we only focused on male American redstarts in this study.

TELOMERE ASSAY

Telomeres of male redstarts were measured using a real-time quantitative PCR technique validated for birds (Cawthon 2002; Criscuolo *et al.* 2009) with minor modifications at Iowa State University (Ames, IA, USA). Briefly, genomic DNA was extracted from previously frozen red blood cells using DNeasy Blood and Tissue kit (Qiagen) and following the manufacturer's protocol. A control single-copy gene (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) was amplified using the primers GAPDH-F (5′-TGACCACTGTCCATGCCATCAC-3′) and GAPDH-R (5′ TCCAGACGGCAGGTCAGGTC-3′). These primers have been optimized for passerine species. Amplification of GAPDH sequence in redstart DNA led to a single product. Telomeres primers were Tel1b (5′-CGGTTTGTTTGGGTTTGGGTTTGG GTTTGGGTTTGGGTT-3′) and Tel2b (5′-GGCTTGCCTTACC CTTACCCTTACCCTTACCCTTACCCT-3′). qPCR for both GAPDH and telomeres was performed using 7·5 ng of DNA per reaction. The telomere primers (Tel1b and Tel2b) were used at a concentration of 400 nM and 900 nM. The GAPDH primers (GAPH_F and GAPDH_R) were used at a concentration of 400 nM and 900 nM. These working concentrations were optimized to allow amplification of the GAPDH gene and telomeres without creating primer dimers. qPCR were performed following the

manufacturer's protocol (Brilliant II SYBR Green QPCR Master Mix, Stratagene, La Jolla, CA, USA). Thus, the forward primer (GAPDH_F or Tel1b), the reverse primer (GAPDH_R or Tel2b) and a reference dye were added to the 2× Brilliant II SYBR Green master mix in this specific order. After gently mixing the reaction, the redstart DNA was added and the reaction was gently mixed. Telomere and GAPDH real-time amplifications were performed on two different plates using an M×300P qPCR system (Stratagene, USA). Optimized telomere PCR conditions were 10 min at 95 °C followed by 30 cycles of 1 min at 54 °C and 1 min at 95 °C. GAPDH PCR conditions were 10 min at 95 °C followed by 40 cycles of 1 min at 62 °C and 1 min at 95 °C. Each 96-well plate included a standard curve, which consisted of various concentrations of a redstart DNA pool (35–0·5 ng mL^{-1}). This standard curve was used to generate a reference curve to control for the amplifying efficiency of the qPCR (Cawthon 2002; Criscuolo *et al.* 2009). All standard curves and redstart DNA samples were run in triplicate. Moreover, a common sample was run on every plate to compare measurements between plates. Some redstart samples were run a second time when the triplicate values were too variable. Telomere length is expressed relative to the single-copy gene (GAPDH) measured on the same sample of DNA (Cawthon 2002; Criscuolo *et al.* 2009). According to recent recommendations (Horn, Robertson & Gemmell 2010), we checked several factors when running our qPCR. First, the efficiency of our qPCR was always within the acceptable range for both GAPDH and Telomere qPCR (Mean ± SD, GAPDH: 106·1 ± 2·7%, Telomere: 102·6 ± 4·0%). Second, we used a pool of many redstart samples as our reference sample rather than a purified commercial sample to avoid potential differences in amplification efficiency between the samples and the standard curve (Horn, Robertson & Gemmell 2010). Third, we selected the GAPDH gene as our reference gene ('single-copy gene') because it has already been successfully used for telomere measurements by qPCR in birds and, more specifically, in passerine species (Bize *et al.* 2009; Criscuolo *et al.* 2009; Beaulieu *et al.* 2011; Heidinger *et al.* 2012). This method has been successfully used in several vertebrate species and gives similar results to the Telomere restriction fragment method (TRF, Cawthon 2002; Criscuolo *et al.* 2009; Bize *et al.* 2009; Heidinger *et al.* 2012). Finally, we also performed a preliminary analysis by measuring telomere length of 12 samples by using the 'gold standard' method (TRF) to confirm that qPCR results were correlated with TRF method results (see Appendix S1, Supporting information). TRF assay was conducted at Iowa State University as previously described using DNA extracted in agarose plugs (Haussmann *et al.* 2003; Haussmann, Winkler & Vleck 2005).

STATISTICAL ANALYSES

All statistical analyses were performed with SAS statistical software (v. 9.2; SAS Institute, Chicago, IL, USA). First, we used repeated-measures GLMs to test whether telomeres shortened between 2008 and 2009 and to test whether this shortening was affected by the non-breeding habitat (prediction 1). Age was not included because only a few known-aged male redstarts were sampled in both 2008 and 2009, but age did not differ between scrub and mangrove birds in our data set ($F_{1,38}$ = 0·01, P = 0·911). For this analysis, we examined the influence of each observation on the estimated coefficients using Cook's distance and residuals analyses. No data points were found to be potential outliers (based on residuals analyses; all Cook's distances were < 0·55, Cook 1977). Second, we used GLMs with a normal error distribution and an identity link function to test whether telomere length was affected by age and non-breeding habitat. We only focused on the 2008 year to avoid pseudo-replication and because telomere length was available only for a few individuals in 2009 (15 birds were caught both in 2008 and 2009). Because absolute age is not known for male redstarts that were banded as AHY birds, two analyses were run. The first one included all the birds (n = 63) and aimed to test for an effect of 'non-breeding habitat' and 'age class' (HY vs. AHY) on telomere length. The second one included only male redstarts of known age (n = 40, age varying from 1 to 7 years old) and aimed to test for an effect of 'non-breeding habitat' and 'age' on telomere length (prediction 2 and 3). In these analyses, the 'age class x habitat' or the 'age x habitat' interactions allowed us to test whether the relationship between age and telomere length differed between scrub-habitat and mangrove-habitat males (according to prediction 1, the negative relationship between age and telomere length should be steeper for the scrub-habitat males than for mangrove-habitat males). Finally, GLMs with a binomial error distribution and a logit link function were used to test whether return probability was affected by 'telomere length' (measured in 2008), 'non-breeding habitat' (scrub vs. mangrove) and their interactions (n = 36, a few AHY birds were excluded from the analyses because their territories were located in an area that could not be adequately monitored for return rate in 2009). We only used AHY birds in this analysis because return rate of HY birds is difficult to assess (Marra 2000; Marra & Holmes 2001). Specifically, we tested whether telomere length in 2008 predicted return rate in 2009 (prediction 4) and whether the relationship between return rate and telomere length differed between scrub-habitat and mangrove-habitat males (prediction 5). Because only a few birds were of known age, we did not include age as an explanatory variable in this analysis. However, age does not affect return rate in redstarts (Marra & Holmes 2001) and preliminary analyses showed that return rate did not vary with age in any habitat (habitat, $F_{1,14}$ = 0·32 P = 0·578, age: $F_{1,15}$ = 0·28 P = 0·607, age × habitat interaction: $F_{1,13}$ = 0·01 P = 0·910). We performed all our model selection starting from the most general model that included all the variables/factors of interest and their interactions and we suppressed step by step the nonsignificant interactions, variables or factors.

Results

RATE OF TELOMERE SHORTENING FROM 2008 TO 2009

Telomeres significantly shortened between 2008 and 2009 (repeated-measures GLM, within subjects, $F_{1,13}$ = 41·73, P < 0·001; Fig. 2). Among the 15 male redstarts that were sampled in both 2008 and 2009, all but one showed a shortening of their telomeres (Fig. 2). Telomere length did not vary between non-breeding habitats (repeated-measures GLM, between subjects, $F_{1,13}$ = 1·56, P = 0·233). However, the 'change in telomere length' × 'non-breeding habitat' interaction was significant demonstrating that the 'rate of telomere shortening' significantly differed between scrub and mangrove redstarts (repeated-measures GLM, within subjects, $F_{1,13}$ = 12·25, P = 0·004). Specifically, the 'rate of telomere shortening' of scrub-habitat redstarts was greater than that of mangrove-habitat redstarts (Fig. 2).

HABITAT, AGE AND TELOMERE LENGTH

When considering all the individuals (n = 63), model selection indicated that 'telomere length' was not significantly affected by the 'non-breeding habitat' ($F_{1,60}$ = 0·70, P = 0·406, Fig. 3) and the 'age class × non-breeding habitat' interaction ($F_{1,59}$ = 0·03, P = 0·860). However, telomere

Fig. 2. Influence of non-breeding habitat (black: scrub, white: mangrove) on the yearly rate of telomere shortening (from 2008 to 2009) of male American redstarts (n = 15, Mean ± SE). All individuals but one showed a shortening of their telomeres over this period. One individual showed a slight increase in telomere length (+ 0·015). The effect of non-breeding habitat on the yearly rate of telomere shortening is still significant if the scrub redstart with the greatest rate of telomere shortening is removed from the analysis (P = 0·014).

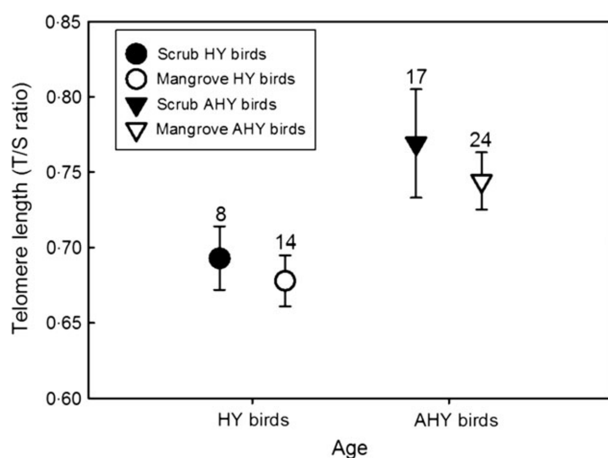

Fig. 3. Influence of age (circle: HY, triangle: AHY) and non-breeding habitat (black: scrub, white: mangrove) on telomere length of male American redstarts (n = 63, Mean ± SE). Numbers above bars denote sample size.

length was significantly affected by the 'age class': telomeres of HY males were shorter than those of AHY males ($F_{1,61}$ = 6·96, P = 0·011, Fig. 3). In addition, the variance in 'telomere length' of AHY redstarts was greater than that of HY redstarts (n = 66, F = 3·80, P = 0·002).

When considering only known-aged individuals (n = 40), model selection indicated that 'telomere length' was not significantly affected by the 'non-breeding habitat' ($F_{1,38}$ = 1·94, P = 0·171, Fig. 4) and the age of individuals ($F_{1,37}$ = 0·85, P = 0·363, Fig. 4). Moreover, there was no significant effect of the 'age × non-breeding habitat' interaction ($F_{1,36}$ = 1·40, P = 0·244) demonstrating therefore that the influence of age on telomere length did not significantly differ between non-breeding habitats.

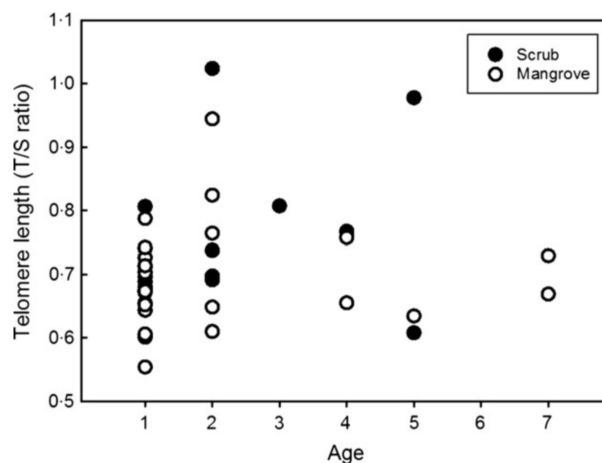

Fig. 4. Influence of exact age and non-breeding habitat (black: scrub, white: mangrove) on telomere length of male American redstarts (n = 40, Mean ± SE).

RETURN RATE AND TELOMERE LENGTH

Model selection indicated that 'return rate' was significantly affected by 'telomere length' ($F_{1,34}$ = 5·22, P = 0·028) but not by the 'non-breeding habitat' ($F_{1,33}$ = 0·31, P = 0·581). Independently of their 'non-breeding habitat', redstarts with longer telomeres were more likely to return to their non-breeding territory than birds with shorter telomeres (Fig. 5). Moreover, there was no significant effect of the 'telomere length × non-breeding habitat' interaction on return rate ($F_{1,32}$ = 0·56, P = 0·458), demonstrating therefore that the influence of telomere length on return rate did not significantly differ between non-breeding habitats. When the 'telomere length' variable was not included in the analyses, we did not find any difference in return rate between non-breeding habitats ($F_{1,34}$ = 0·05, P = 0·827), suggesting that return rate did not differ between scrub and mangrove males from 2008 to 2009.

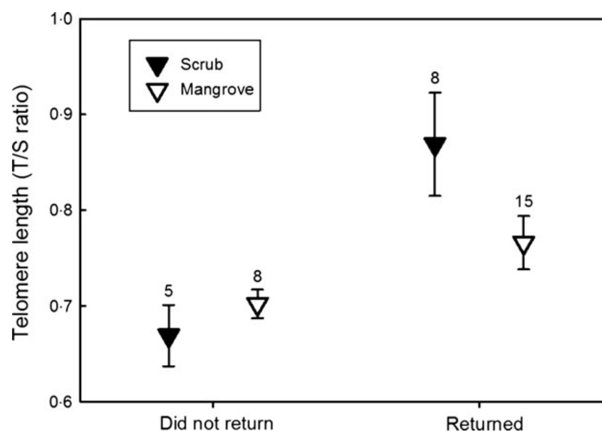

Fig. 5. Telomere length of male American redstarts sampled in 2008 (black: scrub, white: mangrove) that returned or not to their non-breeding territory in 2009 (n = 36, Mean ± SE). Numbers above bars denote sample size.

Discussion

Consistent with other studies, we showed that longer telomeres are associated with a higher return rate than shorter telomeres (i.e. one component related to fitness, 'the telomere-fitness hypothesis'). However and contrary to our prediction, the relationship between return rate and telomere length did not vary with the quality of the non-breeding habitat. Moreover, we showed that telomere shortening may be affected by the environment in a wild vertebrate species ('the environmental determination of telomere length hypothesis'). Specifically, we report that telomeres of male American redstarts wintering in a low-quality non-breeding habitat shorten faster than those of individuals wintering in a high-quality non-breeding habitat.

NON-BREEDING HABITAT QUALITY: A MAJOR DETERMINANT OF TELOMERE DYNAMICS?

In this study, we found that telomeres shortened with advancing age in almost all male redstarts sampled in two consecutive years (14 of 15 individuals). Interestingly, we also found that telomeres seem to shorten significantly more over the course of a year for scrub-habitat males than for mangrove-habitat males, suggesting that non-breeding habitat plays a major role in determining the rate of telomere shortening (prediction 1). Therefore, we provide for the first time some clues suggesting that environmental conditions may underlie telomere dynamics in a wild vertebrate species. Previous work has shown that non-breeding habitat sets up important constraints as it determines individual performances during the subsequent year (carry-over effects, Marra, Hobson & Holmes 1998). Mangrove-habitat males perform better than scrub-habitat males over the winter and during the following year in term of over-winter condition, survival, reproduction and timing of departure for spring migration (Studds & Marra 2005, 2007; Reudink *et al.* 2009a,b; Angelier *et al.* 2011). All these results suggest that scrub-habitat individuals face significant energetic and environmental constraints during their entire annual cycle. These constraints could induce greater oxidative stress and, thus, explain why telomeres shorten at a greater rate in scrub-habitat birds than mangrove-habitat birds as oxidative stress is a major determinant of telomere shortening (Von Zglinicki 2002). In addition, wintering in a non-breeding habitat of low quality may also induce a stressful way of life (as previously found in American redstarts, Marra & Holberton 1998), which has been associated with accelerated telomere shortening in laboratory rodents and humans (Epel *et al.* 2004; Kotrschal, Ilmonen & Penn 2007; Ilmonen, Kotrschal & Penn 2008; Haussmann & Marchetto 2010).

Although we discovered a potential effect of non-breeding habitat quality on telomere dynamics, we cannot totally disentangle the effect of non-breeding habitat from a potential effect of individual quality on the rate of telomere shortening. Our study is only correlational and experimental manipulation of the non-breeding habitat would be necessary to demonstrate the causality of the correlations we reported. Thus, low-quality males may winter in the scrub habitat and, concomitantly, show higher rates of telomere shortening without any causal effect of the non-breeding habitat on telomere shortening. Moreover, we only followed the birds during approximately half of the year (during the non-breeding period) and we do not have any information on what they have done during the rest of the year. Therefore, most of telomere shortening may have been caused by breeding and/or migration and the non-breeding habitat might have played only a minor role in determining telomere shortening. However, all these steps of the annual life cycle (wintering, migration and breeding) are very likely to be tightly linked in this species because previous studies found that non-breeding habitat is the main determinant of individual performances (Marra, Hobson & Holmes 1998; Marra & Holberton 1998; Marra & Holmes 2001; Studds & Marra 2007; Reudink *et al.* 2009a,b; Angelier *et al.* 2011). In an experimental manipulation, wintering redstarts that were upgraded from a scrub habitat to a mangrove habitat performed better than control scrub-habitat birds: they maintained mass across the winter season, departed earlier on spring migration, had a higher breeding success on the breeding grounds (Reudink *et al.* 2009a) and returned at a higher rate in the subsequent year than control redstarts (Studds & Marra 2005). These experimental findings suggest that differences in food availability in the two non-breeding habitats is the main determinant of individual performance over the whole annual life cycle (Studds & Marra 2007, 2011) and thus may also cause either directly or indirectly (through its influence on migration on breeding effort) an accelerated telomere shortening for the scrub-habitat redstarts.

Although our study only focused on male American redstarts, it is important to note that there are often important sex differences in telomere dynamics in vertebrates (reviewed in Barrett & Richardson 2011). Moreover, there is also growing evidence that the relationship between fitness and absolute telomere length can differ between sexes (Foote *et al.* 2010; Olsson *et al.* 2010, 2011a; Barrett & Richardson 2011). Because male and female redstarts differ in some components of their life-history cycle (timing of migration, Studds & Marra 2011; reproductive effort, Reudink *et al.* 2009a,b), it is possible that the relationship between non-breeding habitat, performances and telomere dynamics varies between sexes in this species. Although it was not the primary goal of our study, future investigations should focus on these potential sex differences to improve our understanding of telomere selection in the wild (Barrett & Richardson 2011; Olsson *et al.* 2011a).

AGE, HABITAT, SURVIVAL AND TELOMERE LENGTH: A SELECTION PROCESS?

We did not find strong support for a difference in telomere length between non-breeding habitats in our data set

(prediction 2). This is surprising because we found that telomeres of scrub-habitat redstarts shorten faster than telomere of mangrove-habitat redstarts. Similarly, in our cross-sectional sample, we did not find much support for a relationship between age and telomere length in either non-breeding habitat (prediction 3), although telomeres did shorten from 1 year to the next in 14 of 15 redstarts we sampled twice. Most surprisingly, we also found that young, HY males had overall shorter telomeres than older, AHY male redstarts. However, many studies have reported that telomeres are negatively correlated with age in several vertebrate species (Haussmann & Vleck 2002; Haussmann *et al.* 2003; Hall *et al.* 2004; Juola *et al.* 2006; Pauliny *et al.* 2006; Bize *et al.* 2009; Salomons *et al.* 2009). How can we then explain these results?

In this study, we showed that telomere length is a reliable predictor of return rate in American redstarts. Birds with shorter telomeres are less likely to return to their non-breeding territory the following year than those with longer telomeres (prediction 4). This study therefore confirms that telomere length is related to survival in vertebrates (Haussmann *et al.* 2003; Haussmann, Winkler & Vleck 2005; Pauliny *et al.* 2006; Bize *et al.* 2009; Salomons *et al.* 2009; Foote *et al.* 2010; Olsson *et al.* 2011a,b; Heidinger *et al.* 2012; but see Ujvari & Madsen 2009). This means that redstarts are likely to disappear from the population when their telomeres become shorter (see also Heidinger *et al.* 2012 for a longitudinal study of telomere dynamics in captive zebra finches). Therefore, scrub-habitat birds with small telomeres are unlikely to be found in the population. In conjunction with high inter-individual variability in telomere length, this selective process probably precludes us to find (i) a significant difference in telomere length between scrub-habitat and mangrove-habitat redstarts (prediction 2) and (ii) a negative relationship between age and telomere length in redstarts (prediction 4). In addition, telomere length of birds probably varies from one cohort to another. Recently, Foote *et al.* (2011) suggested that early environmental conditions can affect telomere dynamics. Similarly, Hall *et al.* (2004) reported that natal environmental conditions – hatching date and chick's condition – are correlated with telomere dynamics in European shags. Therefore, the average telomere length of different cohorts is likely to vary with the environmental conditions that these cohorts faced during their development so comparing single samples between unknown cohorts may mask biologically relevant relationships between telomere length and ecological variables. In our study, this cohort effect may be the major factor explaining why we unexpectedly discovered that HY male redstarts had shorter telomeres than AHY individuals. Indeed, we found that the variance in telomere length of HY birds was less than that of AHY birds, suggesting that the progressive disappearance of birds with short telomeres cannot alone explain the difference that we report.

All these results imply that the influence of ecological factors (such as age or habitat) on telomere length may be masked by selection (Haussmann & Mauck 2008; Olsson *et al.* 2011a) and high inter-individual variability in telomere length. This can explain why many cross-sectional studies have reported that telomere length does not always appear as an appropriate marker of chronological age in several species (Horn, Robertson & Gemmell 2010; Monaghan 2010a; Dunshea *et al.* 2011). For example, Ujvari & Madsen (2009) recently reported that telomere length is not correlated with age in adults of a wild reptile species, the water python *Liasis fuscus*. In contrast, our study emphasizes that examining telomere dynamics in longitudinal studies rather than absolute telomere length in single samples is crucial to better understand the influence of variables on telomere length (see also Bize *et al.* 2009; Salomons *et al.* 2009).

On the other hand, it is crucial to note that the measurement error of telomere length is higher for qPCR relatively to TRF (the 'gold standard' method, Aviv *et al.* 2011). Moreover, qPCR measures telomere length, but also interstitial telomeric sequences in the interior of the chromosomes (Nakagawa, Gemmell & Burke 2004). This may also be problematic because these interstitial telomeric sequences do not have the same functions as true telomeres, cannot be shortened by age or oxidative stress and may vary between individuals (Blackburn 2005). Therefore, the use of qPCR may have created additional noise in our data set. In addition with other factors such as the progressive disappearance of birds with short telomeres (Heidinger *et al.* 2012) or the lack of statistical power (small sample size), this may have precluded us to find a significant effect of non-breeding habitat on telomere length (type II error) and this might also explain why we reported that telomere length increased over a year in one individual. Despite these drawbacks, the use of qPCR is still very useful for interindividual comparison of telomere length within a species (Cawthon 2002; Nakagawa, Gemmell & Burke 2004; Criscuolo *et al.* 2009; Monaghan 2010a,b; Aviv *et al.* 2011), and it has been proven to be useful to investigate ecological questions (Bize *et al.* 2009; Criscuolo *et al.* 2009; Heidinger *et al.* 2012).

Contrary to our prediction, the relationship between return rate and telomere length did not differ between non-breeding habitats (prediction 5). This result suggests that telomere length is related to return rate independently of the non-breeding habitat of male redstarts and, therefore, that the effect of the non-breeding habitat on return rate may be primarily mediated by its effect on telomere shortening. Surprisingly, and contrary to previous studies, we also did not report any difference in return rate between non-breeding habitats (Marra & Holmes 2001; Studds & Marra 2007, 2011). These results may be related to a lack of statistical power, because return rate – a binary data – is associated with a high degree of stochasticity and, therefore, very large sample sizes may be required to detect the subtle effect of a specific variable. This may also result from the particular environmental conditions of 2008 where rainfall was abundant during the January–March

period (Angelier *et al.* 2011; Studds & Marra 2011). Therefore, it is possible that the relationship between return rate and telomere length does differ between non-breeding habitats when environmental conditions are more constraining (i.e. dry years). Supporting the idea of complex relationships between fitness and telomere length, it has been shown that the correlations between fitness and telomere length may vary because of different selection pressure on telomere length (Haussmann & Mauck 2008; Olsson *et al.* 2011a). Moreover, two recent studies also showed that the relationship between telomere length and return rate can be also affected by individual characteristics, such as the sex of individuals (Foote *et al.* 2010; Olsson *et al.* 2011a). Therefore, the relationship between telomere length and fitness appears complex and may be context dependent. However, the proximate and ultimate causes for these inconsistent and context-dependent relationships between telomere length and fitness remain unclear and need to be further investigated.

Future studies should also investigate the physiological mechanisms that lead to telomere attrition in natural populations of vertebrates (Haussmann & Marchetto 2010). In that context, it appears crucial to explore the role of allostasis and stress mechanisms (e.g. adrenal activity and glucocorticoid production) in determining oxidative damage and telomere attrition. Indeed, allostasis and stress mechanisms are activated to allow an organism to cope with perturbations that disrupt homoeostasis (Wingfield, Kelley & Angelier 2011). These mechanisms are therefore essential for immediate survival but they are also very well known to induce physiological costs that may lead to telomere attrition (see Haussmann *et al.* 2012).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Estimation of telomere length by qPCR and TRF.