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CAUSES AND CONSEQUENCES OF TELOMERE LENGTH VARIATION IN TWO WILD BIRD POPULATIONS

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Causes and consequences of telomere length variation in two wild bird populations

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GENERAL INTRODUCTION

Introduction

1.1. Telomeres

Telomeres are nucleoprotein complexes located at the termini of eukaryotic DNA (Meyne et al., 1989; Blackburn, 1991; Palm & De Lange, 2008), which play a pivotal role in genome integrity preventing chromosomal fusion during mitosis (Blackburn, 1991, 2005). In vertebrates, double stranded telomeric DNA consists of tandem repeats of the hexamer TTAGGG (Meyne et al., 1989) and ends with a G-rich 3’ overhang that, in humans, folds back into a T-loop structure (Griffith et al., 1999). Telomeric repeats are tightly bound to specific multiprotein complexes called ‘shelterin’, which form the protein subunit of telomeres (Palm & De Lange, 2008). These proteins, together with T-loop-like conformations, ensure proper functioning and protection to telomeres and, thus, to the whole genome (Blackburn, 1991; Palm & De Lange, 2008).

However, in somatic cells, DNA polymerases cannot convert the most distal RNA primer during the lagging strand replication. Consequently, the RNA primer is degraded, leading to telomere shortening at each cell division (Blackburn, 1991). This condition is known as ‘end replication problem’ (Harley et al., 1990; Blackburn, 2005). Telomerase, a nucleoprotein enzyme, tries to counteract this shortening by synthesizing new telomeric repeats (Greider & Blackburn, 1989). Briefly, its catalytic subunit TERT act as a reverse transcriptase, using the RNA subunit TERC as a template to add a telomeric sequence to the 3’ overhang (Greider & Blackburn, 1989; Lingner et al., 1997; Sandin & Rhodes, 2014). This step can be repeated several times via a translocation mechanism (i.e. realignment of the RNA template of TERC subunit with the extended 3’ overhang) and DNA polymerase α-primase can fill the complementary strand (Diede & Gottschling, 1999), leading to an iterative restoration of telomere length. However, the activity of this enzyme, which is not present across all life stages and somatic tissue types for most organisms (Lingner et al., 1997; Haussmann et al., 2004; Logeswaran et al., 2021), is insufficient to prevent progressive telomere shortening and when telomeric DNA reaches a critical threshold of length, telomeres lose functionality, leading to chromosomal fusion and the consequent cell senescence and/or apoptosis (Campisi et al., 2001; Deng et al., 2008).

Therefore, by controlling cellular turnover and, thus, renewal capacity and functioning of somatic tissues, telomeres are considered one of the main molecular drivers of organismal ageing and the associated tissues/performance degradation. By far the vast majority of studies on telomeres have been performed on humans and model organisms under laboratory conditions (López-Otín et al., 2023). However, over the last two decades, research has highlighted the possible role of telomere biology in ecology, conservation and evolutionary biology, as telomeres may have been shaped to meet species-specific needs across different environmental challenges and life-history (Monaghan et al., 2022; Monaghan, 2024).

1.2. Intrinsic and extrinsic sources of interindividual variability in telomere length

A central topic in the study of telomere biology is understanding why individuals differ so markedly in telomere length since early-life stages and, consequently, the origins of the variability observed across studies. A first possibility is that part of this variation may be inherited. Although a genetic basis in telomere length variation has been reported in birds (Asghar et al., 2015; Atema et al., 2015 - but see, Dugdale & Richardson, 2018), the heritability of telomere length is considered to operate through a non-Mendelian, epigenetic-like mechanism (Bauch et al., 2019). Indeed, the actual telomere length of parental gametes can be considered a phenotypic trait of the zygote, as it does not reflect a variation in a gene sequence. Because telomeres may shorten with age in somatic tissues (Aubert et al., 2012; Lapham et al., 2015; Remot et al., 2022), older individuals are expected to produce gametes, and consequently offspring, with shorter telomeres, highlighting parental age as a possible additional cause of telomere length variation. However, telomere dynamics in germ cells can substantially differ from those of somatic cells, and among sperms and ova. In species with promiscuous mating system, such as humans and chimpanzees, sperm telomeres interestingly lengthen with age, possibly due to the up-regulation of telomere maintenance genes under strong sexual selection, which requires meiosis to occur more frequently (Aston et al., 2012; Eisenberg & Kuzawa, 2018). Conversely, in seasonally breeding species, such as several birds, lower sperm production rates and, thus, the weaker selective pressures on germline maintenance may result in age-related telomere attrition, as in somatic tissues (Tricola et al., 2018; Criscuolo et al., 2021). In females, by contrast, ova derive from germline cells formed early in development that undergo few or no subsequent replications (Johnson, 2000), implying that maternal age-related effects on offspring telomere length are less likely to arise through gametic telomere attrition. At the same time, parental age may also influence offspring telomere length indirectly, through age-related differences in parental care. These two complementary pathways, commonly referred to as the ‘gamete effect’ and the ‘parental care effect’ (Heidinger & Young, 2020), may further jointly contribute to early-life telomere length variation. However, results are inconsistent across studies on wild animals, mainly birds, about the presence or absence of the effect (presence: Olsson et al., 2011;

Eisenberg et al., 2012, 2017; Asghar et al., 2015; Criscuolo et al., 2017; Bouwhuis et al., 2018; Bauch et al., 2019; Brown et al., 2021 - absence: Froy et al., 2017; Belmaker et al., 2019), the relative contribution of father and/or mother (father: Olsson et al., 2011; Eisenberg et al., 2012, 2017; Bouwhuis et al., 2018; Bauch et al., 2019 - mother: Asghar et al., 2015 - both: Criscuolo et al., 2017; Brown et al., 2021), and the direction of the effect (positive: Eisenberg et al., 2012, 2017; Asghar et al., 2015; Brown et al., 2021 - negative: Olsson et al., 2011; Criscuolo et al., 2017; Bouwhuis et al., 2018; Bauch et al., 2019). Such discrepancies likely arise from variation in study design and from the scarcity of research simultaneously accounting for pre- and postnatal influences in wild populations. Moreover, telomere dynamics may also depend on Mendelian-like genetic inheritance reflecting the potential heritability of somatic maintenance efficiency from parents to offspring. As assessing the inheritance of these possible molecular and physiological pathways is challenging (but see, Froy et al., 2021; Pepke et al., 2023), an alternative approach is to investigate relationships between proxies of parental quality and offspring telomere length and dynamics. For instance, in the European starlings (*Sturnus vulgaris*), a quality-related trait in this species (i.e. number of fledging in the 1st brood of the 1st year), was associated with telomere length of their offspring, with low-quality females producing nestlings with shorter telomeres than high-quality ones (Criscuolo et al., 2018). In this context, secondary sexual traits, which are widespread in birds, offer a promising tool for exploring possible heritable links between parental quality and offspring telomeres. However, the only study to my knowledge that has investigated this association, was conducted on the tawny owl (*Strix aluco*) and found no relationship between parental plumage coloration and offspring telomere length (Morosinotto et al., 2022)

Beyond the possible inherited components, particular interest also lies in understanding the additional sources of interindividual variation in telomere length that may operate during and after development. Since the very beginning of telomere research, evidence that telomeres consistently shorten with cell divisions in human fibroblasts (Harley et al., 1990) has led to the proposal of telomere length as a reliable biomarker of chronological age (Haussmann & Vleck, 2002). However, although telomere length has been shown to decline with age in both human (Aubert et al., 2012; Lapham et al., 2015) and non-human vertebrates (Remot et al., 2022), it became clear since the very beginning that the rate at which telomeres shorten is not always constant, suggesting that other factors than the ‘end replication problem’ may drive telomere length variation both in captivity and in the wild (Pepke, 2024). Indeed, in a meta-analysis including 98 species from all vertebrate classes, Remot et al., (2022) found an overall negative, yet weak, relationship between telomere length and age, highlighting the presence of telomere length variability both within- and between-species/populations (Chatelain et al., 2020; Remot et al., 2022). Across both mammals and birds, comparative studies indicate that species can differ in their rates of telomere shortening, with long-lived species generally exhibiting slower attrition rates than short-lived ones (Haussmann et al., 2003; Whittemore et al., 2019; Criscuolo et al., 2021). Therefore, whether, on the one hand, the 'end replication problem' provides a general insight into endogenous causes of telomere length variation, on the other hand, other extrinsic as well as intrinsic factors can also explain the high interindividual variability in telomere length and attrition rate observed across studies (Monaghan, 2014; Monaghan & Ozanne, 2018; Chatelain et al., 2020).

Several environmental biotic and abiotic conditions have been indeed associated with telomere length variation in wild populations (Haussmann & Marchetto, 2010; Monaghan, 2014; Bateson et al., 2015; Chatelain et al., 2020). Biotic conditions include, for instance, parasite burden (e.g., Asghar et al., 2016; Giraudeau et al., 2019; Tschirren et al., 2021), resource competition (e.g., Nettle et al., 2015; Costanzo et al., 2017; Pepke et al., 2022) and social isolation (e.g., Aydinonat et al., 2014), while examples of abiotic conditions encompass different types of pollution (e.g., Parolini et al., 2021; Hansen et al., 2022; Salmón & Burraco, 2022), human-induced disturbance (Herborn et al., 2014; Meillère et al., 2015; Caccavo et al., 2021) and weather conditions (Mizutani et al., 2013; Eastwood et al., 2022; Pepke et al., 2022). For instance, in birds, larger broods are considered stressful environments, with nestlings potentially facing lower food intake per capita, as well as increased parasite transmission and sibling competition (Dijkstra et al., 1990; Saino et al., 1997, 2000, 2002; Neuenschwander, 2003; Romano et al., 2013), potentially resulting in shorter telomeres and/or faster telomere shortening (Boonekamp et al., 2014; Nettle et al., 2015; Costanzo et al., 2017; Gil et al., 2019; Young et al., 2022; Voirin et al., 2023). In the Eurasian siskin (*Spinus spinus*), individuals experimentally infected with *Plasmodium ashfordi* showed shorter telomeres compared to uninfected conspecifics, highlighting parasite infection as another stressful condition potentially triggering telomere shortening (Asghar et al., 2016). Negative relationships between stressful abiotic, rather than biotic, factors and telomere loss were reported, for instance, by Meillère et al., (2015) in the house sparrow (*Passer domesticus*), where nestlings experimentally exposed to traffic noise showed reduced telomere length compared to their unexposed neighbours (Meillère et al., 2015), or by Eastwood et al., (2022) in the purple-crowned fairy-wrens (*Malurus coronatus*), where higher average maximum air temperatures were associated with reduced nestlings telomere length (Eastwood et al., 2022). Therefore, generally, individuals exposed to stressful environmental conditions are expected to exhibit shorter telomeres and/or faster telomere attrition rate (Chatelain et al., 2020). However, when telomerase operates in synergy with favourable environmental conditions, also telomere restoration and lengthening is possible. Notably, this pattern was observed in a non-negligible proportion of individuals in several wild bird populations, and thus increasingly recognised as biologically meaningful rather than a random or purely methodological artefact (Brown et al., 2022; Costanzo et al., 2022). For instance, in the Seychelles warbler (*Acrocephalus sechellensis*) Brown et al., (2022) found apparent telomere lengthening in both sexes, but sex-specific patterns emerged in response to environmental stressors. In females, telomere shortening occurred under conditions of low food availability and avian malaria infection, consistent with the expected negative effects of these conditions on telomere attrition. In contrast, such stress-related telomere loss was not observed in males. However, under favourable environmental conditions, both sexes exhibited instead telomere lengthening, indicating that reduced stress exposure may promote telomere restoration (Brown et al., 2022). Similar results emerged in the black-tailed gull (*Larus crassirostris*), where individuals showed stable or increased telomere length when exposed to El Niño conditions (Mizutani et al., 2013), or in the house sparrow, with optimal weather conditions resulting in longer telomeres (Pepke et al., 2022).

The possible influences of these factors on telomere length and dynamics are likely mediated through a set of shared physiological and molecular pathways. Mainly in vitro (Von Zglinicki, 2000, 2002), but also *in vivo* (Armstrong & Boonekamp, 2023), studies have identified oxidative stress as the one of the main mechanistic molecular link between telomeres and the environment. Oxidative stress arises when the production of reactive oxygen species (ROS), mainly generated by the mitochondrial electron transport chain, exceeds the capacity of intracellular antioxidant defences, leading to several damages at the macromolecular level (Costantini & Verhulst, 2009). Due to their high content in guanine base pairs, telomeres provide a preferential target for oxidation by ROS (Halliwell & Aruoma, 1991; Kohen & Nyska, 2002), potentially thus shortening when exposed to oxidative stress (Angelier et al., 2018; Armstrong & Boonekamp, 2023). One of the major physiological pathways through which environmental challenges may increase oxidative stress and, thus, telomere loss is represented by the hypothalamus-pituitary-adrenal (HPA) axis (Romero et al., 2009). Activation of the HPA-axis in response to stressful environmental conditions induces a rapid and reversible increase in circulating glucocorticoids (GCs), which occurs at different paces in animals and lead to energy reallocation towards activities necessary to cope with acute stressors (Romero, 2004). Although in the short-term this acute stress response is unknowledge to be adaptive (Wingfield et al., 1998), repeated and prolonged activation can boost metabolism and catabolic processes (Landys et al., 2006), increasing ROS production, or impair antioxidant capacity (Liu & Mori, 1999), overall leading to an increase in oxidative stress (Costantini et al., 2011; Costantini, 2014) and, ultimately, to telomere shortening (Angelier et al., 2018). Furthermore, prolonged exposure to GCs may further promote telomere attrition independently of oxidative stress by suppressing telomerase activity and, consequently, telomere restoration (Choi et al., 2008), although a moderate short-term increase in GCs levels may up-regulate telomerase activity (Epel et al., 2010).

In addition, life-history theory posits that, as resources are limited, organisms may prioritize immediate survival and reproduction at the expenses of somatic maintenance (i.e. the molecular and physiological processes that preserve tissue integrity and delay ageing) crucial for future Darwinian fitness gains (Stearns, 1989; Ricklefs & Wikelski, 2002). In practice, resources invested in a specific activity cannot be simultaneously allocated to other competing biological processes. In this context, GCs act as key mediators of the response to environmental stressors, promoting resources reallocation towards immediate survival and reproduction at the expense of somatic maintenance processes, such as antioxidant defence and telomere restoration (Wingfield et al., 1998; Haussmann & Marchetto, 2010). These life-history trade-offs represent another indirect mechanism through which the environmentally induced HPA-axis activation may affect telomere length variation (Angelier et al., 2018). However, in birds, only a limited number of studies have examined the relationship between basal glucocorticoid (mainly corticosterone) levels and telomere length/dynamics (Angelier et al., 2018). Results from adult birds are inconsistent, with negative (Bauch et al., 2016; Gangoso et al., 2016; Quirici et al., 2016; Young et al., 2016), positive (Young et al., 2016), or no associations (Ouyang et al., 2016; Young et al., 2016; Costanzo et al., 2022) reported, and the few studies conducted on developing birds have also yielded mixed results (Haussmann et al., 2012; Herborn et al., 2014). Furthermore, Young et al., (2016), in the thick-billed murre (*Uria lomvia*), found higher corticosterone levels associated with shorter telomeres when environmental conditions were stressful. However, when individuals were in good conditions, longer telomeres were associated with higher stress levels, indicating that individuals in these conditions may invest more in somatic maintenance while retaining high levels of stress. This suggests that environmental conditions may further shape and add complexity to the association between telomeres and HPA-axis activation (Young et al., 2016). Overall, research on avian taxa has mainly concentrated on baseline corticosterone levels, (Angelier et al., 2018 - but see, Costanzo et al., 2022), overlooking the potential influence of acute and reversible HPA-mediated responses on telomere length and dynamics.

Among the various stressors affecting telomere length/attrition, thermal stress has obtained increasing attention in recent years, not only because of the increasing frequency, intensity, and duration of extreme weather events (Rahmstorf & Coumou, 2011; Perkins-Kirkpatrick & Lewis, 2020; Rogers et al., 2022), but also because it exemplifies an environmental challenge in which stress-related and purely metabolic processes can converge, and not mutually exclusively, contribute to telomere shortening, highlighting the complexity of the scenario underlying the proximate causes of telomere length variation. Homeothermic animals, such as birds, passively dissipate heat only when environmental temperatures are below the upper limit of thermoneutral zone, the range within which individuals are able to maintain optimal body temperatures with minimum energetic costs (Diehl et al., 2023). However, when temperatures are above or below the thermoneutral zone limits, individuals experience thermal stress, which may lead to HPA-axis activation while simultaneously engaging in thermoregulatory behaviours, such as panting and water loss (McKechnie et al., 2021; Woodruff et al., 2025). Therefore, in the framework of life-history theory (Stearns, 1989; Ricklefs & Wikelski, 2002), given the high metabolic costs of both thermoregulation and HPA-axis activation (Landys et al., 2006; McKechnie et al., 2021; Woodruff et al., 2025), resources invested in the response to thermal stress cannot be allocated to somatic maintenance mechanisms, possibly leading to telomere attrition (Stier et al., 2021; Eastwood et al., 2022; Eastwood et al., 2023a; Rodriguez et al., 2025). However, increased temperatures may directly elevate metabolic rates and thus oxidative stress (Messina et al., 2025), leading to stress-unrelated telomeric damage (Armstrong & Boonekamp, 2023). Although growing awareness of the possible impact of recent climate change on health status of wild populations, especially in homeothermic taxa such as birds, relatively few studies have investigated the relationship between temperature and telomere length/dynamics (Stier et al., 2021; Eastwood et al., 2022; Eastwood et al., 2023a; Ton et al., 2023; Furic et al., 2026; Rodriguez et al., 2025). For instance, in nestling superb fairy wrens (*Malurus cyaneus*) in temperate south-eastern Australia, telomere length decreases with mean daily minimum temperature under low rainfall (Eastwood et al., 2023a). An experimental study on nestling great tits (*Parus major*) showed that an increase of ~2°C in nest boxes accelerated telomere attrition (Stier et al., 2021). However, experimental approaches remain scarce and largely confined to laboratory settings (Stier et al., 2020; Ton et al., 2023) or species from temperate regions (Stier et al., 2021; Furic et al., 2026), leaving a gap in the understanding of how thermal stress influence telomere length in birds adapted to warm-dry climates.

Finally, during their lifetime, organisms pass through different mandatory and predictable ontogenetic stages which, in the life-history theory scenario, may further trigger evolutionary trade-offs between competing life-history traits, potentially affecting somatic maintenance (Stearns, 1989; Ricklefs & Wikelski, 2002). Consequently, telomere length may be further influenced by physiological constraints imposed by these ontogenetic events (Monaghan, 2014). Early-life development may represent one of the possible main life-history traits shaping telomere length (Monaghan & Ozanne, 2018). Accordingly, several studies found indeed faster telomere shortening during early-life stages, when growth is faster, than in adulthood (Salomons et al., 2009; Heidinger et al., 2012; Parolini et al., 2015; Sheldon et al., 2022). Faster somatic growth entails a mandatory increase in cell cycles to attain higher body size/mass, thereby exacerbating telomeric attrition expected from the ‘end replication problem’ (Harley et al., 1990; Blackburn, 2005). Furthermore, intense metabolic activity during growth, which itself lead to an increase in oxidative stress (Costantini, 2014; Panda & Cherian, 2014), requires energetic resources that cannot be thus allocated to telomere maintenance and antioxidant defences (Haussmann & Marchetto, 2010; Chatelain et al., 2020; Armstrong & Boonekamp, 2023). These alternatives are not mutually exclusive, and can act synergistically in determining telomere attrition (Monaghan & Ozanne, 2018). For instance, correlative studies in birds reported shorter telomeres and/or faster telomere shortening associated with rapid somatic growth both in early-life and in adulthood (Monaghan & Ozanne, 2018). Adult house sparrows with longer tarsus displayed shorter telomeres (Pepke et al., 2022), and similar results were reported in nestlings of the same species (Ringsby et al., 2015). The possible detrimental effects of rapid early development on telomere dynamics may also emerge later in life, as shown by Salmón et al., (2021) in the zebra finch (*Taeniopygia guttata*). However, high quality individuals (i.e. those expected to be more efficient in somatic maintenance), may afford to retain faster growth rates while paying a lower cost in terms of telomere attrition. For instance, in king penguin chicks (*Aptenodytes patagonicus*), Geiger et al., (2012) found that initially smaller and lower-quality nestlings exhibited faster telomere shortening than initially larger and higher-quality ones at the end of the post-winter growth period (Geiger et al., 2012). These quality-related relationships likely extend to the metabolic and physiological trade-offs elicited also by stressful environmental conditions (Angelier et al., 2018, see above). Finally, the possible negative association between growth rate and telomere length variation may change based on the environmental context that can affect resources availability. In nestling zebra finches, for instance, individuals reared in smaller broods showed faster growth rates associated with longer telomeres, likely reflecting reduced sibling competition and greater resource availability for both somatic growth and maintenance (Reichert et al., 2014).

Given the potential links between telomere length or attrition and indicators of somatic condition and health, telomeres may thus represent a potential mechanistic link mediating the effects of environmental conditions, growth and stress on individual performance (Monaghan, 2014; Monaghan et al., 2022; Monaghan, 2024). This possibility is particularly relevant early in life, when organisms experience rapid somatic growth and developmental transitions that may profoundly shape later-life phenotype (Metcalfe & Monaghan, 2001; Tschirren et al., 2009). In this context, telomeres may capture the cumulative impact of these early-life sources of interindividual variation and, by possibly reflecting variation in somatic integrity, may help explaining how early-life experiences translate into differences in future survival and reproductive performance (Monaghan, 2024).

1.3. Consequences of interindividual variability in telomere length

Because telomeres usually progressively shorten with age (Remot et al., 2022) due to the ‘end replication problem’ (Harley et al., 1990; Blackburn, 2005) and the cumulative effects of environmental and physiological stress (Monaghan & Ozanne, 2018; Chatelain et al., 2020), they eventually reach a critical threshold at which they lose their protective function, triggering cellular senescence and tissue deterioration. Therefore, telomeres impose an intrinsic limit on cellular proliferative capacity (Olovnikov, 1973), and individuals that start life with longer telomeres or with more efficient telomere maintenance are expected to maintain somatic integrity for longer than those born with shorter and/or faster shortening telomeres, who may instead experience earlier the somatic degradation with senescence which ultimately leads to death.

For this reason, telomere length has been increasingly studied in association with survival and lifespan in wild animals, a major component of Darwinian fitness. Two recent meta-analyses reported shorter telomeres to be associated with reduced survival in non-model vertebrate species, especially in birds (Wilbourn et al., 2018; Eastwood et al., 2023b). However, the effect sizes of both studies were not strong enough to generalize this pattern across wild vertebrates, highlighting substantial complexity beyond the association between telomere length/dynamics and viability (Wilbourn et al., 2018; Eastwood et al., 2023b). One major source of variability is that wild species differ in life expectancy due to variation in stochastic mortality and this, together with species-specific environmental pressures, telomerase activity and somatic growth patterns, is likely to have shaped the variability in the associations between telomeres and survival observed across vertebrates (Sudyka et al., 2016; Tricola et al., 2018; Criscuolo et al., 2021). Indeed, consistent with this view, comparative studies across mammals and birds show that long-lived species generally exhibit slower telomere shortening rates than short-lived ones, suggesting that the pace of life, body size and longevity of the species may impose evolutionary constraints on telomere dynamics (Tricola et al., 2018; Whittemore et al., 2019; Criscuolo et al., 2021). Ectothermic vertebrates, such as reptiles, often retain telomerase activity in somatic tissues, probably due to their continuous somatic growth throughout life and the consequent increased selective pressure over telomere maintenance mechanisms (Gomes et al., 2010; Olsson et al., 2018). In contrast, endothermic vertebrates, such as birds for which we have most of data, typically exhibit suppressed telomerase activity in adult somatic tissues, resulting in different telomere dynamics with age compared to ectothermic organisms (Ujvari & Madsen, 2009; Ujvari et al., 2017; Remot et al., 2022), potentially affecting the link between telomere dynamics and survival/lifespan (Wilbourn et al., 2018).

However, substantial variation in results has been observed even within species (Stier et al., 2014; Eastwood et al., 2019; Heidinger et al., 2021; Sheldon et al., 2022). In the house sparrow, females with longer telomeres showed longer lifespan, while no associations were found in males, probably due to sex-specific stress sensitivity and life-history traits (Heidinger et al., 2021). Beyond sex, also environmental factors may produce variability in the association between telomeres and survival. Stier et al., (2014), in king penguin chicks, found that shorter early-life telomeres predicted higher mortality only under stressful developmental conditions, but not when conditions were favourable (Stier et al., 2014). Moreover, the relationship with mortality is more likely to arise when telomere length and dynamics are assessed early in life rather than in adulthood, as shown for instance in many bird species (e.g., Heidinger et al., 2012; Eastwood et al., 2019; Sheldon et al., 2022), although studies reporting the association in adult individuals also exist (e.g., Bichet et al., 2020; Chik et al., 2024). However, most studies on early-life telomere length have assessed mortality outcomes, shortly after measuring telomeres, potentially obscuring the long-term association between the two, especially considering the high stochastic mortality during early-life (Eastwood et al., 2023b). Early-life can be thus a critical period during which future survival prospects may be shaped by developmental condition and rapid somatic growth. This highlights the need for studies that track individuals across their entire lifespan to fully assess the long-term consequences of early-life telomere variation. However, the causal role of telomeres in mediating early-life environmental and rapid growth effects on survival still largely remain to be established, as telomeres may simply and non-mutually act as biomarkers of underlying physiological stress that causally drives mortality.

Within the life-history framework, telomere length and attrition may also be linked to reproductive success, the second major component of Darwinian fitness (Monaghan, 2010; Sudyka, 2019; Monaghan, 2024). Since reproduction, such as growth, requires substantial energetic investment, an increase in reproductive effort can reduce the resources available for somatic maintenance necessary for survival and future reproduction (Stearns, 1989; Ricklefs & Wikelski, 2002). As a result, associations between telomeres and reproduction may arise through more than one pathway, and their interpretation necessarily depends on how telomeres covary with survival.

A first possibility is that telomeres reflect intrinsic differences in individual quality. In this case, individuals having longer telomeres, and/or experiencing slower attrition, may be those better able to sustain both somatic maintenance and reproductive investment (Monaghan, 2010). In this scenario, a positive association between telomere length and reproductive success may arise, and because telomere length can be at least partly heritable (Asghar et al., 2015; Atema et al., 2015 - but see, Dugdale & Richardson, 2018), longer telomeres may also be favoured by natural selection. Studies on adult birds support this pattern, reporting that individuals with longer and/or slower shortening telomeres produce more offspring within a breeding season or offspring with higher reproductive value (e.g., Bauch et al., 2013; Le Vaillant et al., 2015; Parolini et al., 2017; Criscuolo et al., 2018). However, such a pattern may simply arise because individuals that experience more favourable environmental conditions may maintain longer telomeres (Mizutani et al., 2013; Brown et al., 2022; Pepke et al., 2022) and simultaneously achieve higher reproductive success, without incurring detectable somatic costs. In this case, telomere length and reproduction covary due to environmentally driven variation in condition rather than causally due to differences in intrinsic quality.

A second non-mutually exclusive possibility is that telomere length/dynamics directly reflect the physiological costs of reproduction (Sudyka, 2019). In this case, a negative association between reproductive effort and telomere length/dynamics can be expected. Several correlative and experimental studies also support this possibility, reporting shorter telomeres or faster telomere shortening in individuals investing heavily in reproduction (e.g., Heidinger et al., 2012; Bauch et al., 2016; Ouyang et al., 2016; Graham et al., 2019). In captive zebra finch (*Teaniopygia guttata*), individuals to which mating was experimentally prevented showed slower telomere loss than breeding individuals (Heidinger et al., 2012). Similar results emerged in also in the wild, with shorter telomeres associated with higher reproductive effort in common terns (*Sterna hirundo*) (Bauch et al., 2016). However, telomere dynamics may also reflect both reproductive costs and individual intrinsic quality in the same species, as shown by Bauch et al., (2013) again on common terns. Here, although individuals generally experienced faster telomere shortening associated with increased reproductive effort, the most successful individuals exhibited slower telomere loss than those of intermediate success, suggesting that high-quality birds can sustain substantial reproductive investment while partially buffering its somatic costs (Bauch et al., 2013). Therefore, depending on individual intrinsic quality and ecological context, reproductive effort may either positively covary with telomere length and/or attrition, or may directly trade-off with it. However, evaluating these patterns within single breeding attempts may be confounding, because short-term associations may not reveal whether telomeres are associated with fitness across the entire lifespan.

To better understand how telomere length is associated with fitness and, thus, how it may be shaped by natural selection, it is necessary to evaluate whether the somatic costs of reproduction ultimately translate into reduced long-term survival. Indeed, growing evidence in avian species shows that positive associations between telomere length and reproductive success often emerge only when analyses integrate long-term monitoring data, but not when analyses are focused on a single breeding season (Eastwood et al., 2019; Heidinger et al., 2021; Eastwood et al., 2023b; Chik et al., 2024). In the purple-crowned fairy-wren, early-life telomere length was not associated with early survival or annual reproductive success, yet individuals with longer telomeres lived longer and thus achieved higher lifetime reproductive success (Eastwood et al., 2019). These results suggest that longer telomeres early in life may reflect individual quality expressed as the ability to maintain somatic integrity despite repeated breeding attempts, rather than the ability to reproduce at higher rates, suggesting that fitness advantages conferred by longer telomeres may also lies in the duration of reproductive performance. However, these patterns do not hold in all studies. In the blue tit (*Cyanistes caeruleus*), early-life telomere length did not predict lifetime reproductive success, and individuals with higher reproductive success instead showed greater telomere attrition across life, although this increased attrition did not reduce lifespan, likely due to the high extrinsic mortality in this species (Sudyka et al., 2019). Therefore, in contexts where mortality is not strictly dependent on individual somatic state, telomere length/dynamics may primarily reflect the cumulative physiological costs of reproduction rather than intrinsic quality. Overall, these results suggest that the relationship between telomere and fitness depends on the evolutionary context in which each species has evolved, with species favouring high present reproductive investment showing higher telomere loss associated with higher lifetime fitness, dependently or independently of initial telomere length, whereas species relying more on maintaining somatic integrity across repeated breeding events may exhibit higher lifetime fitness associated with longer telomeres early in life.

In many bird species, individuals invest a conspicuous amount of resources in the expression of secondary sexual traits (ornaments), which can thereby contribute to overall reproductive costs (e.g., Huhta et al., 2003; Webster et al., 2018). According to sexual selection theory, these traits evolve as honest signals of individual quality through mate choice, because only high-quality individuals can afford to produce and maintain more elaborate ornaments without paying costs in terms of survival and/or future reproduction (Andersson, 1994; Nolazco et al., 2022). As for reproductive success, if telomere length/dynamics reflect variation in individual quality, they are expected to covary positively with ornament expression. For instance, in male common yellowthroats (*Geothlypis trichas*), individuals with longer telomeres and slower telomere attrition exhibited brighter colours which, in this species, is also associated with higher reproductive success and better survival (Taff

& Freeman-Gallant, 2017). Alternatively, if telomere length/dynamics primarily reflect the somatic costs of ornament expression and maintenance, individuals with more elaborate secondary sexual traits would be expected to show shorter telomeres and/or faster telomere attrition. In the tawny owl, indeed, telomere length negatively covaried with the degree of pheomelanin colouration, which is higher in high-quality individuals (Karell et al., 2017). Similarly, the expression of longer ornamental feathers has been associated with reduced telomere length in spotless starlings (*Sturnus unicolor*) (Azcárate-García et al., 2020). Furthermore, interestingly, both scenarios can coexist within the same species if different sexually selected traits are considered. In the barn swallow (*Hirundo rustica*), while darker and high-quality individuals showed longer telomeres (Parolini et al., 2017), the expression of longer outermost tail feathers was associated with shorter telomere length (Kauzálová et al., 2022). Beyond the possible differences between wild populations in conditions experienced, this discrepancy may potentially suggest different costs in producing and maintaining different ornaments.

1.4. Outline of the study

The present study is divided into two parts. The first part (**Chapters 1-2**) aims at investigating different potential sources of early-life telomere length variation in two wild bird species, the lesser kestrel (*Falco naumanni*) and the barn swallow (*Hirundo r. rustica*).

In lesser kestrel nestlings (**Chapter 1**), the thesis investigated how nest thermal conditions influence nestlings' telomere length and attrition during development. By experimentally shading a subset of nest boxes to reduce internal temperature during a natural heatwave, the chapter aimed at specifically testing whether nestlings in hotter, unshaded nests showed faster telomere attrition, and whether this effect was intensified under extreme heatwaves. The study also evaluated whether the impact of thermal stress varied with nestling rank, as sibling competition may be harsher for later-hatched, smaller chicks. Furthermore, as in the barn swallow, possible relationships between rapid growth during development and telomere length/dynamics have been investigated.

In the barn swallow (**Chapter 2**), the chapter examined how environmental conditions, growth and sex influenced HPA-axis activation and telomere length/dynamics of nestlings subjected to a standardized handling-restraint stress protocol. Specifically, the study aimed at investigating whether variation in corticosterone levels at the beginning, at the end and after the stress exposure were predicted by brood size, hatching date and body mass, and whether these responses differed between males and females. The chapter also assessed whether telomere length at 5 and 15 days of age, as well as its change during this period, were associated with corticosterone levels at different times of the HPA-mediated stress response, while accounting for environmental and developmental factors.

The second part of the thesis (**Chapter 3-4**) focused on the possible consequences on future survival and reproductive success of telomere length variation both in early-life and adulthood in the barn swallow.

Chapter 3 represents a conceptual link between the first and the second part of the study, because it investigated possible environmental and parental sources of variation in early-life telomere length, and then whether this variation translates into differential long-term survival. In detail, the chapter examined whether developmental conditions (i.e. brood size and hatching date), parental age and secondary sexual traits expression, potentially indicating parental quality, explain interindividual differences in nestling telomere length. Additionally, it also tested whether this early-life telomere variation predicts long-term survival after the first reproduction and lifespan in the study population.

Finally, **Chapter 4** investigated telomere length and dynamics in relation to lifespan, lifetime reproductive success and the expression of secondary sexual traits. Using longitudinal data on male and female barn swallows monitored throughout their entire lives, the study first examined how telomere length and its rate of change vary with age. It then tested whether telomere length at the onset of reproduction (i.e. 1-year old) predicts overall lifespan and lifetime reproductive success. Furthermore, it also assessed whether telomere length and attrition are associated with the expression of two secondary sexual traits, the length of the outermost tail feathers and ventral plumage coloration. Through this approach, the chapter evaluates the possible role of telomere length/attrition, as potential markers of individual quality, in understanding how natural and sexual selection may have shaped the evolution of species-specific life-history.

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PART 1

SOURCES OF INTERINDIVIDUAL VARIABILITY IN EARLY-LIFE TELOMERE LENGTH

Chapter 1

Nest cooling buffers telomere attrition during early growth in a cavity-nesting bird of prey, but not under heatwaves

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Nest cooling buffers telomere attrition during early growth in a cavity-nesting bird of prey, but not under heatwaves

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ABSTRACT

Climate change is projected to increase average temperatures as well as the frequency and intensity of extreme events such as heatwaves, exposing developing animals to novel thermal challenges. Cavity-nesting birds, whose offspring develop in enclosed and often poorly ventilated spaces, may be especially vulnerable to elevated temperatures during their growth. However, experimental investigations of how thermal stress affects nestling physiology in natural populations remain scarce. We manipulated the thermal nest environment of lesser kestrels (*Falco naumanni*) breeding in nest boxes by shading a group of nests, resulting in significant decreases in maximum temperature in shaded vs control nest boxes. We then tested whether this experimental manipulation, both under normal weather conditions and during naturally occurring heatwaves, affected telomere attrition, a biomarker of somatic maintenance, cellular aging and environmental stress, in growing nestlings. High-ranking (i.e. first-hatched) nestlings reared in control nests exhibited larger telomere attrition compared to their counterparts from shaded ones, while this was not the case for intermediate and low-ranking offspring. Yet, exposure to a natural heatwave increased telomere attrition, irrespective of shading and within-brood rank. Finally, faster-growing nestlings showed a greater telomere attrition, suggesting a trade-off between growth and somatic maintenance. These findings indicate that both elevated nest temperatures and heatwave events can compromise physiological state during development, inducing sublethal effects with potentially negative fitness effects in the long-term. As heatwaves are becoming more frequent and severe, the capacity of cavity-nesting bird species to withstand thermal stress may be increasingly challenged, which may potentially jeopardize their local persistence.

Keywords: climate change, heatwave, physiological costs, sibling competition, telomere, telomere attrition

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INTRODUCTION

Climate change is driving a rapid and profound reshaping of environmental conditions worldwide, with far-reaching consequences for biodiversity, species distributions, and ecological interactions (IPCC 2021). Among the most relevant effect of climate change is the increasing frequency, intensity, and duration of extreme weather events, including heatwaves, which are predicted to become more common in the coming decades (Rahmstorf & Coumou 2011; Perkins-Kirkpatrick & Lewis 2020; Rogers et al. 2022). These acute thermal anomalies are not only challenging for adult organisms, but can be especially threating to individuals during their early-life stages, particularly those confined to thermally constrained environments such as nests (McKechnie & Wolf 2010; Wingfield et al. 2017; Corregidor-Castro et al. 2023; Messina et al. 2025). In this context, understanding the effects of elevated and fluctuating temperatures during development is a key challenge in ecology, evolutionary biology, and conservation physiology.

Environmental temperatures play a major role in physiological processes, from cellular metabolism to whole-organism performance, across all taxa (Angilletta 2009). In birds, which are homeothermic but often reproduce in microclimates with limited thermoregulatory buffering, developing embryos and nestlings can experience considerable thermal stress. This is especially true for altricial cavity-nesting species, whose nesting sites may trap heat and amplify external thermal variability (Andreasson et al. 2018; Corregidor-Castro et al. 2023). Nest temperature can modulate developmental trajectories in avian embryos and chicks, influencing hatching success, growth rate, thermoregulatory ability, immune function, and survival (Nord & Nilsson 2019; Corregidor-Castro et al. 2023).

Despite growing awareness of the impact of thermal conditions on avian development, relatively few studies have adopted an integrative approach combining temperature experimental manipulations with physiological biomarkers to assess the effects of early-life thermal stress in natural populations. Among stress biomarkers, telomere dynamics seem particularly relevant for their long-term fitness implications. Telomeres are repetitive DNA-protein structures that cap the ends of linear eukaryotic chromosomes, protecting them from degradation and preventing harmful end-to-end fusions (Blackburn 1991; de Lange 2005). These structures are crucial for maintaining genomic stability during cell division (O'Sullivan & Karlseder 2010). However, telomeres shorten at each cellular replication cycle, the so-called end replication problem (Levy et al. 1992), a process that can be accelerated under harsh environmental conditions (Von Zglinicki 2002). Indeed, telomere length (TL) and dynamics are increasingly recognized as powerful biomarkers of somatic maintenance, reflecting the cumulative effects of intrinsic and extrinsic stressors experienced during early development (Haussmann & Marchetto 2010; Boonekamp et al. 2014; Monaghan & Ozanne 2018), and can have long-lasting consequences for survival and/or reproductive success (Heidinger et al. 2012; Boonekamp et al. 2014; Barrett et al. 2015; Parolini et al. 2017; Eastwood et al. 2019).

In birds, telomere attrition during the nestling period has been linked to a range of ecological variables, including brood size (Boonekamp et al. 2014; Costanzo et al. 2016; Young et al. 2017), parental care (Viblanc et al. 2020; Ton et al. 2023), parasite burden (Asghar et al. 2015; Bàdas et al. 2023) as well as social and physical environment (Mizutani et al. 2013; Salmòn et al. 2016). More recently, environmental temperature has emerged as a promising candidate shaping telomere dynamics during growth (Eastwood et al. 2022; 2023; Rodriguez et al. 2025). Indeed, homeothermic animals are unable to passively dissipate heat once temperatures rise above the upper threshold of the thermoneutral zone, the temperature range in which optimal body temperature is maintained with minimal expense of energy and water (Diehl et al. 2023). Under these conditions, heat loss depends primarily on metabolically costly processes, such as an increased water loss and panting (McKechnie et al. 2021). This additional energy expenditure for thermoregulation can, in turn, have negative physiological consequences, including telomere erosion.

Despite well-developed theoretical premises, experimental studies directly manipulating nest thermal environments are rare, and tend to be performed under laboratory conditions (Stier et al. 2020; Ton et al. 2023) or on species breeding in temperate climates (Stier et al. 2021; Furic et al. 2026). Thus, a general understanding of how nest temperature shapes telomere dynamics across avian species adapted to warm-dry climates is still lacking. Moreover, it remains unclear how these effects interact with other ecological variables such as laying date, brood size, or sibling competition, all of which can affect developmental stress. This is an important knowledge gap, considering that the nestling period represents a phase of intense growth and cellular proliferation, during which organisms are particularly vulnerable to environmental challenges and when telomere attrition is more intense (Sheldon et al. 2022).

We experimentally investigated the impact of nest thermal environment on nestling telomere dynamics in a cavity-nesting bird of prey, the lesser kestrel (*Falco naumanni*). This species readily breeds in nest boxes and provides valuable opportunities to study microclimatic effects on development due to the feasibility of manipulating nest (i.e. nest box) temperatures in the field (see Corregidor-Castro et al. 2023, 2025b). We assigned nest boxes to either a shading treatment aiming at reducing inner nest temperature or a control (unshaded) group, exposed to direct natural sunlight. We predicted that nestlings from control nest boxes will experience faster telomere attrition than those growing in shaded ones, because of higher thermoregulation costs of developing closer to (or higher than) the upper limit of their thermoneutral zone (around 42°C in other arid-adapted species; Wang et al. 2025). We also hypothesized that such an effect would have been exacerbated by a naturally occurring heatwave. Finally, we also evaluated how the effects of experimental manipulation and the occurrence of a natural heatwave may interact with nestling's rank, given that sibling competition may amplify physiological stress, especially for late-hatched, smaller nestlings.

MATERIALS AND METHODS

Study species, study area and general field procedures

The lesser kestrel is a small (approximately 140 g), long-distance migratory raptor of the Afro-Palearctic region. Its European populations arrive at the breeding sites between February and April (Sarà et al. 2019), with egg-laying typically occurring between late April to mid-May. As a secondary cavity-nesting species, it occupies existing natural or man-made cavities, such as rock crevices, old buildings, roof tiles, or nest boxes (Catry et al. 2011; Negro & Hiraldo 1993). Females lay a single 3-5 eggs clutch, with a 1-3-day interval between consecutive eggs. Both parents share incubation duties, which last about 30 days (Soravia et al. 2021; Corregidor-Castro et al. 2025b). Nestlings' hatching is asynchronous, causing a pronounced size hierarchy within the brood, often resulting in the death of the smallest, late-hatched individuals (Podofillini et al. 2018; Soravia et al. 2021). Both parents contribute prey provisioning (Soravia et al. 2021), mostly invertebrates, small mammals, and lizards (Morinay et al. 2023, Berlusconi et al. 2025). Nestlings fledge around 40 days after hatching.

The present study was conducted during the 2021 and 2022 breeding seasons in the city of Matera, located in southern Italy (40°66′N, 16°61′E). This city hosts one of the largest colonies of lesser kestrel in Europe, with approximately 1,000 breeding pairs (La Gioia et al. 2017), many of which breed in nest boxes (Podofillini et al. 2018; Corregidor-Castro et al. 2023). On June 21–24, 2021 and June 27–28, 2022, the area was hit by two intense heatwaves (details in Corregidor-Castro et al. 2023), with maximum temperatures rising as much as 3.5°C above the 1991–2020 average (ISAC 2024). Coherently with Catry et al. (2015), a heatwave was defined as a period when ambient temperatures surpassed 37°C for a minimum of two consecutive days (details in Corregidor-Castro et al. 2023; 2025b). These heatwaves highly impacted nestlings' survival, especially in control broods, where mortality reached 55% of the individuals compared to 10% of shaded ones, as previously documented (details in Corregidor-Castro et al. 2023).

The experiment was performed on concrete nest boxes (external size 30 cm height × 30 cm width × 37 cm length; entrance hole diameter 6.5 cm) with wooden front and rear panels, installed on roof terraces (for details on nest box characteristics, see Podofillini et al. 2018; Corregidor-Castro et al. 2023). Starting from the beginning of the breeding season, each nest box was monitored every 3-4 days to record the laying date of the first egg, and the hatching date of each nestling. Upon hatching, nestlings were temporarily identified using a unique pattern of black dots marked on their heads with a non-toxic permanent pen. Around the age of 10 days, each nestling was then individually ringed (Corregidor-Castro et al. 2023). Nestlings were ranked according to their hatching order, with rank 1 assigned to the first nestling to hatch (Podofillini et al. 2019). Whenever two or more nestlings hatched between consecutive monitoring visits, they were ranked based on their body mass, as during the initial days post-hatching, nestling size reliably reflects hatching sequence (Podofillini et al. 2018).

We regularly monitored nest boxes until nestlings reached approximately 15-20 days of age, when linear growth phase is completed (Romano et al. 2021), and nestling body size correlates with their size at fledging (Braziotis et al. 2017). At each monitoring session, we recorded brood size (number of nestlings alive) and measured each nestling's body mass using an electronic scale (accuracy 0.1 g). When a nestling was found for the first time in the nest (mean age 2.0 ± SE 0.1 days; range 0 – 9 days), we collected a small blood sample (~25 μL) by puncturing the brachial vein. Blood was collected in a capillary tube and kept at ~4°C before being stored at -20°C within two hours from collection. In order to study telomere shortening, we obtained a second blood sample on the final monitoring visit, when nestlings were on average 15.7 ± 0.1 days (range 12 – 20 days). Nestling sex was determined by polymerase chain reaction amplification of the sex-specific CHD-1 gene, following standard protocols (Griffiths et al. 1998). To minimize disturbance, no further morphological measurements or blood samples were taken after day 15-20, as the likelihood of premature nest or rooftop abandonment increases with handling at later stages (Podofillini et al. 2018).

Experimental manipulation of nest box temperature

Upon hatching of the first nestling, pairs of synchronously timed nests (i.e., those sharing hatching date; hereafter synchronous groups) with similar orientation and sun exposure, were randomly distributed between two experimental treatments (n = 62 nests). One nest box was equipped with a custom-made plywood shade (hereafter: shaded group) designed to lower the internal temperature, while the other unshaded nest box served as control group (hereafter: control group). The shading structure consisted of 5mm-thin plywood sheets that fully covered the roof and both lateral sides of the nest box, while allowing airflow between the panels and the box. This setup effectively blocked direct sunlight from reaching the concrete parts of the nest box, while the front wooden panel remained directly exposed to sunlight in both shaded and unshaded nest boxes. The shading panels were installed at the time of hatching and removed once fledging occurred (for further details on experimental nest cooling procedures, see Corregidor-Castro et al. 2023).

To recorded nest temperatures in the days between the two blood sampling events using temperature data loggers (Elitech RC-5+, Elitech, U.K.; with an accuracy of ± 0.5°C) that were installed in a subset of nest boxes. These loggers were affixed to the inner surface of the rear panel, close to the roof, to minimize contact with nestlings. We placed a tile (40 × 40 × 3 cm) against the outer side of the panel to prevent direct sunlight from biasing temperature recordings. Loggers were programmed to record temperature readings at 30-minute intervals (Corregidor-Castro et al. 2023). The maximum daily temperatures recorded in each nest box were used in the following analyses. Shading was effective in reducing nest temperature, in particular during the heatwave events, when the maximum temperatures registered were significantly lower in shaded (41.5 ± 0.17°C) than control nest boxes (44.6 ± 0.29°C; LMM with nest and synchronous group identities as random factors; estimate ± SE: -3.28 ± 0.39, $t = -3.26$, $df = 53.58$, $P < 0.001$; see also Corregidor-Castro et al. 2023 for further details on temperature differences between the experimental groups).

Telomere length analyses

We extracted genomic DNA from erythrocytes using the Qiagen DNeasy Blood and Tissue Kit. Extractions followed the manufacturer's protocol with a modification, where the final wash with buffer 2 was repeated twice. In addition, we conducted a second elution from the column with 30 µl of AE buffer provided in the kit. To assess DNA quality of each elution, we used a Nanodrop ND-2000 C spectrophotometer (Thermo Scientific, USA), considering acceptable 260/280 and 260/230 ratios greater than 1.8. DNA concentration was quantified using a Qubit fluorometer (Invitrogen) in conjunction with the AccuGreen Broad Range dsDNA Quantification Kit (Biotium). DNA samples were diluted to a working concentration of 3 ng/µl.

For each individual and visit, relative telomere length (RTL) was measured using quantitative real-time PCR (qPCR) (Cawthon, 2002). RTL was calculated as the ratio between telomeric DNA abundance and that of the single-copy reference gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Following Criscuolo et al. (2009), we used telomeric primers Tel1b and Tel2b, and GAPDH primers GAPDH-F and GAPDH-R. Amplifications were carried out using a BioRad CFX384 Touch Real-Time PCR System. Each reaction was performed in a 10 µl volume comprising 2 µl of 5× HOT FIREPol® EvaGreen® qPCR Mix Plus (without ROX; Solis BioDyne), 6 ng of genomic DNA, and 200 nM of each forward and reverse primer. The qPCR profile included an initial denaturation at 95°C for 12 minutes, followed by 40 cycles of 95°C for 20 seconds, 58°C for 18 seconds, and 72°C for 1 minute. At the end of each run, a melt curve analysis (from 65 to 95°C, with 0.5°C increments every 5 seconds) was conducted to verify amplification specificity.

Each plate included samples from nestlings at a mean age of 2- and 15-days post-hatching, two interpolated calibrators, and a negative control for both telomere and GAPDH assays. These were all run in triplicate. We adjusted baseline correction and quantification cycle (Cq) values using LinRegPCR software (version 2017.1; Ruijter et al., 2009), and corrected inter-run variation using the Factor qPCR method (Ruijter et al., 2015). We calculated relative telomere length following the equation proposed by Pfaffl (2001), similar to Morbiato et al. (2023) and in Monteforte et al. (2020). Amplification efficiency was considered acceptable within a threshold of 100 ± 20%. Inter-assay coefficients of variation (CV) were 3.7% for telomere and 1.3% for GAPDH, while intra-assay CVs were 1.3% and 1.4% for telomere and GAPDH, respectively.

We then calculated telomere erosion (ΔTL = TL at second measurement - TL at first measurement) for each nestling. Following Verhulst et al. (2013), ΔTL was regressed to the mean. This procedure is crucial whenever telomere erosion is strongly correlated with their initial length (i.e. telomere at first measure), as it is the case in our sample (Pearson's $r = -0.73$, $P < 0.001$). In addition, considering that the timespan between the two TL measurements differed among nestlings (mean: 14 days, range: 6-16 days), regressed-to-mean ΔTL was then divided by the number of days from the first to the second measurement. We therefore obtained an estimate of daily change in TL (negative values indicating an average daily decrease in TL; hereafter: daily-ΔTL). Consequently, the analyses were focused only on nestlings that survived at least until the second measurement (i.e. after the heatwaves). We measured TL of 148 nestlings (shaded: 102; control: 46) from 62 nests (shaded: 39; control: 23). The difference in the sample size of nest and nestlings between shaded and control nest boxes was due to the much higher mortality occurring in the latter compared to the former. This difference on nestling survival between shaded and control nest boxes during heatwave events was previously documented in the same years of the present study (i.e. 2021 and 2022) (all details in Corregidor-Castro et al. 2023). In addition, for 3 nestlings it was not possible to reliably estimate TL at the second measurement.

Statistical analyses

We firstly tested whether TL at birth (i.e. first visit after hatching) varied according to shading (0 = control, 1 = shaded) in a linear mixed model including nest and synchronous group identities as random factors to account for multiple nestlings sampled from the same broods and to consider paired nests, respectively, as well as year because data collected in 2021 and 2022 were not independent (e.g. same climate, same phenology, etc.). The model also included nestling's sex and rank (3-level factor: 1 = rank 1, 2 = rank 2, 3 = ranks 3 and 4) as well as Julian date (1 January = 1) of laying of the first egg of the clutch (hereafter: laying date) to account for the different phenology of different broods, that may be linked to environmental and parental quality. Finally, the age of the nestling when TL was measured (mean: 1.97 ± 1.38 SD; days; range: 0-5) was included as an additional fixed effect to account for possible age-related telomere erosion. A single nestling measured for the first time at age 9 days was excluded from this analysis because it was too old to provide a reliable estimate of its RTL at birth. An additional nestling was excluded because it was not possible to properly perform its molecular sexing. Therefore, this model was performed on 146 nestlings.

Secondly, we examined whether daily-ΔTL differed between shaded and control nestlings by means of a linear mixed model including shading as a dichotomic factor. Nestling's sex and rank, and brood size when the second blood sample was collected were included as fixed effects to account for the possible effect sibling competition, because it depends on sex/size-hierarchy within the brood as well as on the number of competitors, which are among the main drivers of telomere attrition in altricial offspring (e.g. Boonekamp et al. 2014; Costanzo et al. 2016; Young et al. 2017). Laying date was added to the model because telomere shortening may vary according to stage of the breeding season (i.e. different effects of environmental conditions and/or intensity of sibling competition). Moreover, because body growth may accelerate telomere shortening due to intense metabolic activity (Monaghan & Ozanne 2018), the model also included the daily increase in body mass recorded between the two measures of TL (i.e. delta body mass / days; hereafter: daily body mass change). Furthermore, because of the potential negative impact of extreme prolonged high temperature on nestlings' physiology, we also included a dichotomic factor indicating whether the nestlings experienced or not (1 = exposed; 0 = not exposed) heatwave conditions during the first 14 days of growing (i.e. during the phase of linear growth; Romano et al. 2021). Three two-way interactions were also added simultaneously to the model. Specifically, shading × heatwave, shading × nestling's rank and heatwave × nestling' rank were included to examine potential differences in the effects of extremely high ambient temperatures on nestlings reared in control or shaded nest boxes, and whether shading and/or heatwave effects may vary according to the nestling's rank. However, the one(s) not reaching statistical significance were then removed from the final model. This model was fitted on data from 141 nestlings because for three and one of them, respectively, we could not record body mass and determine the sex. The model provided very similar results when also including initial RTL as an additional predictor (details not shown for brevity).

All the models were fitted using the lmer function fitted within the 'lme4' package (Bates et al. 2015) in R (version 4.1.3). Post hoc tests were performed using the Tukey correction with the package 'emmeans' (Lenth 2023). The presence of collinearity among main effects and outliers were examined using the package 'performance' (Lüdecke et al. 2021), while residual diagnostics with the package 'DHARMa' (Hartig 2022).

Ethical note

Nest boxes used in this study were part of a long-term population monitoring program initiated in 2016 and were naturally exposed to sunlight and ambient temperatures. As such, mortality observed in unshaded nest boxes was assumed to reflect natural mortality rates. To mitigate thermal stress, shading covers were installed at hatching, and no noticeable disturbance to nestlings or attending adults was observed following their deployment (Corregidor-Castro et al. 2023). All capture and handling procedures were conducted by the Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA) under authorization in accordance with Law 157/1992 [Art. 4(1) and Art. 7(5)].

RESULTS

TL at hatching did not vary according to shading, laying date and nestling's rank, sex and age (Table 1). Overall, during the period of the TL measurements, nestlings experienced a significant attrition of their telomeres (-0.435 ± 0.771 SD; paired samples t-test, t144 = 6.79, P < 0.001). However, a non-negligible portion of them (42 out of 146; ~29%) increased their TL (Figure 1).

Daily-ΔTL was significantly predicted by the interaction between experimental manipulation and nestling rank (Table 1; Figure 2). Specifically, daily-ΔTL was more negative in high-ranked (i.e. rank 1) nestlings from control than shaded nest boxes (estimate difference ± SE: -0.038 ± 0.013, t = -2.99, P = 0.003), while such a difference did not emerge for intermediate (i.e. rank 2; estimate difference ± SE: -0.014 ± 0.013, t = -1.07, P = 0.29) and low-ranking nestlings (rank ≥ 3: estimate difference ± SE: 0.021 ± 0.012, t = 1.72, P = 0.09; Figure 2). In addition, daily-ΔTL was negatively predicted by both heatwave and daily body mass change (Table 1; Figures 3 and 4), irrespectively of nestling's rank and shading, indicating that individuals that experienced acute extremely warm temperatures and those growing faster displayed a more intense telomere erosion. The other two-way interactions did not attain statistically significance and were therefore not included in the final model (shading × heatwave: F = 1.94; df = 1.27; P = 0.17; heatwave × nestling's rank: F = 0.43; df = 2, 127; P = 0.65).

	Estimate	**CI**	**P**
RTL at birth			
Intercept	-2.38	-6.96 - 2.21	
Age	0.06	-0.03 - 0.15	0.21
Sex	-0.08	-0.30 - 0.15	0.50
Rank(2)	-0.19	-0.45 - 0.08	0.16
Rank(3)	-0.03	-0.29 - 0.23	0.80
Laying date	0.03	-0.00 - 0.06	0.08
Shading	-0.08	-0.34 - 0.18	0.55
Conditional R^2 = 0.274			
Daily-ΔTL			
Intercept	0.12	-0.12 - 0.36	
Sex	0.01	-0.01 - 0.02	0.39
Brood size	0.00	-0.00 - 0.01	0.47
Laying date	-0.00	-0.00 - 0.00	0.41
Daily body mass change	-0.01	-0.01 - 0.00	0.025
Heatwave	-0.02	-0.04 - 0.00	0.028
Rank(2)	0.02	-0.01 - 0.05	0.22
Rank(3)	0.04	0.01 - 0.07	0.009
Shading	0.04	0.01 - 0.06	0.003
Shading × Rank(2)	-0.02	-0.06 - 0.01	0.15
Shading × Rank(3)	-0.06	-0.09 - 0.03	0.001
R^2 = 0.167			

Table 1. Variation in RTL at birth and daily-ΔRTL (regressed to the mean difference between RTL measurements divided by the number of days between the two measurements) according to nestling traits and brood features in linear mixed models.

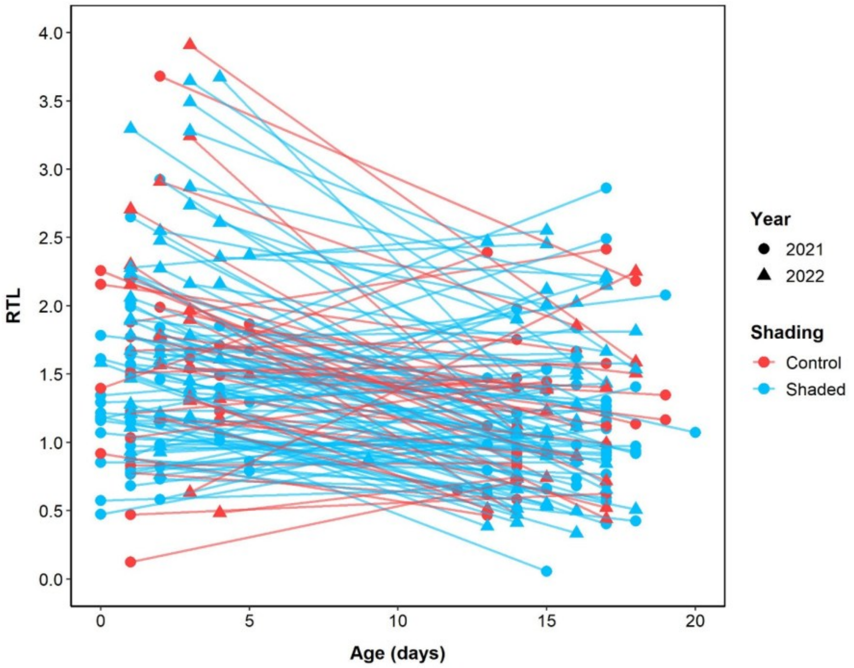

Figure 1. Nestlings' telomere length at first and second measurements. The lines link the two measurements of the same individual.

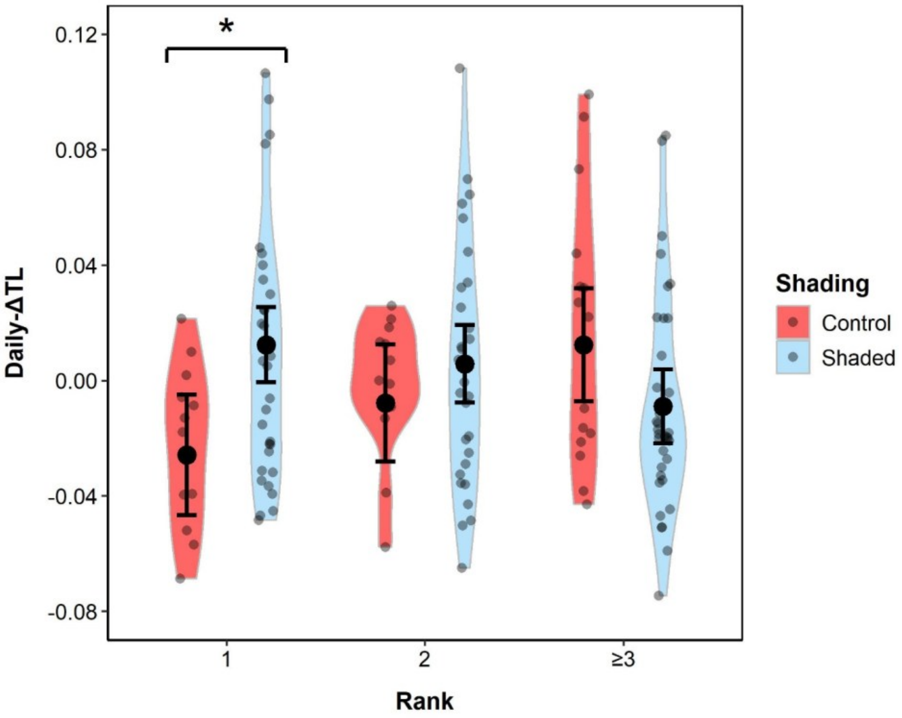

Figure 2. Violin plot showing the effect of shading and nestlings rank on daily telomere shortening. The asterisk indicates a statistically significant difference after post-hoc tests. Small dots show original datapoints, while large dots and whiskers represent the estimates of the model and their standard errors from Table 1. Negative values of daily-ΔTL indicate an average daily decrease in TL.

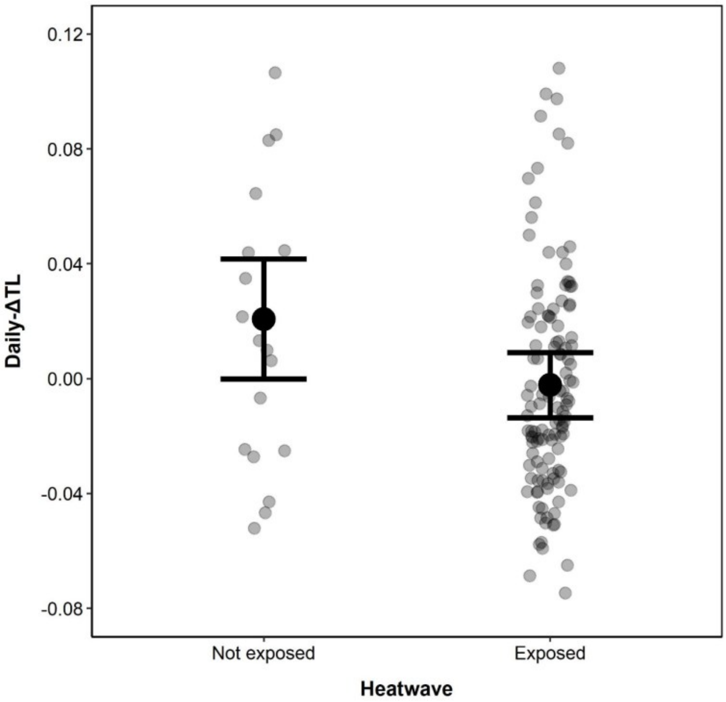

Figure 3. Variation in daily telomere shortening between nestlings exposed or not to the heatwave during the phase of linear growth (i.e. within 14 days of age). Small dots show original datapoints, while large dots and whiskers represent the estimates of the model and their standard errors from Table 1. Darker symbols represent overlapping datapoints. Negative values of daily-ΔTL indicate an average daily decrease in TL.

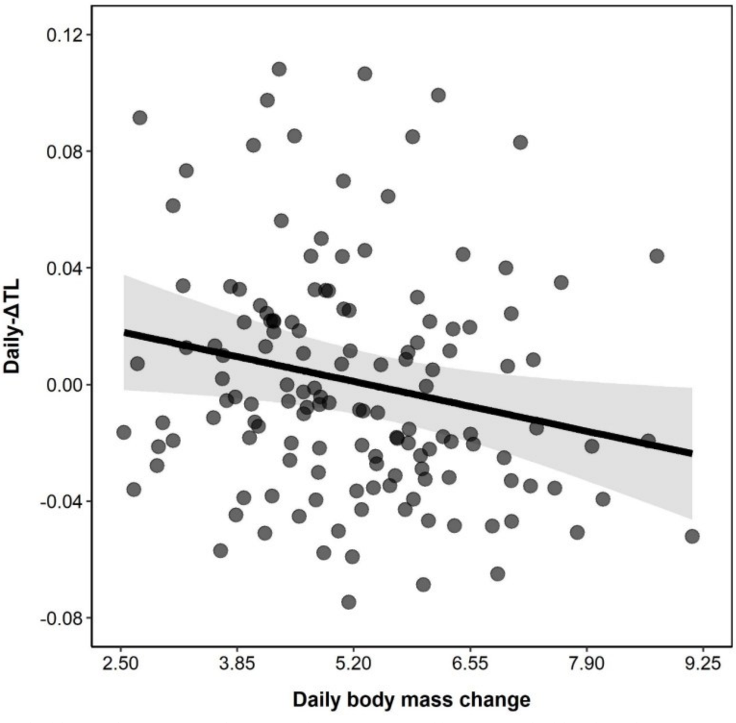

Figure 4. Variation in daily telomere shortening according to daily body mass increase. Dots show original datapoints, while the line represent the estimate of the relationship between the variables calculated from the model reported in Table 1. Shades represent the 95% confidence intervals of the estimate. Darker symbols represent overlapping datapoints. Negative values of daily-ΔTL indicate an average daily decrease in TL.

DISCUSSION

Our study showed that telomere shortening was more intense in within brood first-hatched nestlings reared in control, warmer nest boxes compared to their counterparts from shaded nests, and in those nestlings experiencing a heatwave, irrespective of the experimental treatment and rank. These findings suggest thermal stress can have a strong impact on telomere dynamics of growing nestlings, even in a species well adapted to warm and arid climates.

These results therefore showed that nestlings' telomeres can be negatively affected not only by exposure to chronically elevated temperatures during growth, coherently with the few previous experimental studies demonstrating that an increase in nest temperatures during development led to significantly greater telomere shortening compared to broods exposed to natural thermal environment (Stier et al. 2020, 2021; Ton et al. 2023; Furic et al. 2026), but also by acute thermal stress, like the natural occurrence of heatwaves. The proximate mechanisms involved in such patterns of telomere shortening may have been multiple and non-mutually exclusive. First, increased temperatures may elevate metabolic rates and oxidative stress (Messina et al. 2025), leading to direct telomeric damage (Reichert & Stier 2017; Young et al. 2017; Casagrande & Hau 2019). Indeed, the release of reactive oxygen species after intense metabolic activities, due to a wide variety of environmental and social stresses, has been repeatedly shown to be a major driver of telomere shortening (see Introduction). Second, thermal stress is well-known to alter developmental timing and hormone profiles, particularly glucocorticoids such as corticosterone, which have been associated with accelerated telomere erosion (Haussmann et al. 2012; Angelier et al. 2018). These two mechanisms may be involved not only in the difference in telomere shortening observed in first-hatched nestlings between shaded and control broods, but also in the effect of heatwaves irrespective of the experimental treatment. This is because during the heatwave the maximum temperature was very high also in shaded nest boxes, exceeding 42°C in most of them. This temperature has been shown to negatively impact nestlings' condition, strongly affecting their survival (Corregidor-Castro et al. 2023). Third, extreme temperatures may have affected parental provisioning behaviour. This is because the sustained rate of energy expenditure in costly activities, such as foraging, is limited by the individual capacity to dissipate the heat surplus generated by metabolic processes (Speakman & Król 2010), and may be further reduced under extreme warming ambient temperatures (Nilsson & Nord 2018; Nord & Nilsson 2019). The energetic balance and stress exposure of nestlings may therefore be indirectly influenced by a reduced parental provisioning during the heatwaves (duPlessis et al. 2012; Wiley and Ridley 2016; Wingfield et al. 2017; Wheeler et al. 2023). In our experiment foraging parents were exposed to the same ambient temperatures irrespectively of the nest box treatment. Thus, indirect effects (i.e. mediated by the parents) do not explain the differences between treatments in telomere erosion that we observed, but may be among the reasons why nestlings experiencing a heatwave displayed a more rapid telomere attrition.

In other species, the potential for heat-induced telomere shortening has been shown to be buffered by the greater availability of water (i.e. higher humidity; Eastwood et al. 2022; 2023; Rodriguez et al. 2025), linked with a decreased risk of dehydration (Song & Beissinger 2020). In the lesser kestrel, water is supplied to the nestlings only through the food. While food supplementation promoted a similar growth rate in shaded and control nest boxes, it did not reduce survival under heatwaves (Corregidor-Castro et al. 2025a). Testing whether an increased food provisioning is associated with a reduced telomere attrition under high temperatures may provide an indication of the potential for parents to attenuate telomere attrition though differential parental investment.

The effect of shading on telomere dynamics was evident only for high-ranked, larger nestlings, while such a pattern did not emerge for low-ranked individuals. We can only speculate about the mechanisms underlying this result. One possible explanation relates to the ontogenetic transition in thermoregulatory capacity that altricial birds undergo during early development from complete ectothermy at hatching, when nestlings rely entirely on parental and environmental heat, to partial endothermy during feather growth, and finally to full endothermy at near-fledging (Sirsat et al. 2016). During this transition, the energetic costs associated with coping with temperature fluctuations are expected to vary substantially according to individual age (Sirsat et al. 2016), and thus within-brood rank. Given the hatching asynchrony in the lesser kestrel, higher ranking individuals were more likely to have been impacted by the heatwave when they were already endotherms (i.e. older). Under such circumstance, very high ambient temperatures (but below the lethal threshold) may impose significant energetic and physiological stress (Farag & Alagawany 2018; Ton et al. 2021), potentially accelerating telomere attrition. Conversely, younger individuals (i.e. lower ranks) may have been impacted when they were still ectothermic or in the transitional phase, potentially being better at tolerating elevated temperatures, as these may be partly buffered by reduced self-regulatory demands. However, this interpretation is not supported by our data, as telomere attrition did not change according to nestling's age when heatwave occurred (LMM: estimate difference ± SE: 0.002 ± 0.001, t = 1.66; p = 0.10), suggesting that other mechanisms may be involved. On the other hand, differences (or lack thereof) between the two groups may therefore be due to differential survival of high quality/condition nestlings among low-ranking, control nestlings, as nestlings had a much higher mortality rate in control nest boxes compared to shaded ones (Corregidor-Castro et al. 2023, 2025a,b). Indeed, given that the assessment of telomere attrition required two measures of TL within a given time frame, we could not estimate TL of all those nestlings that died before the second blood sample, effectively retaining a subset of higher-quality individuals. This may have been especially true for control broods upon exposure to the heatwave when a mortality spike was observed (Corregidor-Castro et al. 2023, 2025a,b). Such a survival bias excluded individuals that did not survive early developmental stages due to environmental stress, that may have also suffered from a stronger telomere erosion (Von Zglinicki 2002), thus also potentially explaining why the effect of heatwaves on telomere shortening was not stronger in control than shaded broods as expected. In addition, late-hatched nestlings from control broods may have benefitted from less harsh sib-sib competition because of the much higher mortality (Corregidor-Castro et al. 2023; 2025b). Indeed, the mean brood size when the second telomere measurement was recorded was considerably smaller in control than shaded nests (2.69 ± 0.142 SE vs. 1.87 ± 0.188 SE; t = 3.54; df = 60; P <0.001). Therefore, in control broods, competition among offspring was strongly reduced or even null (in case of the mortality of all but one sibling) for the surviving nestlings. This may have been particularly beneficial for smaller, less competitive, nestlings, that in larger (i.e. shaded) broods had to confront with superior siblings. This suggests that benefits in terms of telomere attrition from being reared at lower temperatures can be at least partly offset by the costs of competing against a larger number of siblings surviving because of the upper limit of the thermoneutral zone was not exceed. This interpretation is coherent with the observation that in shaded broods telomere length tended to decrease progressively with nestling's rank, while the opposite was the case in control broods (see Figure 2). In addition, considering that aggressive interactions among siblings are more frequent under lower temperatures (our unpub. results), it is possible that low-ranked nestlings from control broods may have also benefitted from a smaller stress due to sib-sib competition, even in the presence of larger competitors, with possible positive effects on telomere attrition. Additionally, we also note that the analysed sample included nestlings from control nests with naturally cooler microclimates, as higher mortality occurred in control nests reaching higher average maximum temperatures (binomial GLM of the proportion of dead nestlings per brood, effect of maximum daily nest temperature: 0.407 ± 0.069 SE, $Z = 5.87$, $P < 0.001$), thus again limiting the possible detectable differences in telomere attrition between the experimental groups.

Finally, nestlings growing at a faster rate, irrespective of shading heatwave exposure, showed greater telomere attrition, in line with current evidence (reviewed in Monaghan & Ozanne 2018). Proximately, such a relationship may be mediated by two distinct but non-mutually exclusive mechanisms. On the one hand, a larger body size can be achieved by an increased cell division rate, thus provoking a faster telomere erosion due to the end-replication problem (Levy et al. 1992; Von Zglinicki 2002). On the other hand, a faster body growth should be sustained by a higher metabolic activity with a raise of oxidative stress (Alonso-Alvarez et al. 2007; Smith et al. 2016), potentially impacting on telomere structures (Monaghan & Ozanne 2018). Overall, individual growth rate and environmental conditions to which nestlings are exposed are likely important drivers of telomere dynamics.

Because telomere length at the nestling stage has been shown to be a reliable biomarker of lifespan and lifetime reproductive success in several species (Heidinger et al. 2012; Boonekamp et al. 2014; Barrett et al. 2015; Parolini et al. 2017; Eastwood et al. 2019), our results suggest that an exposure to high temperatures in early-life can have negative long-lasting fitness effects. The effect of thermal stress on telomere dynamics implies that nestlings experiencing those conditions may have to balance the investment in self-maintenance against lifespan (Monaghan & Haussman 2006). However, the proximate mechanisms linking telomere dynamics in early-life and subsequent survival is unclear (Monaghan & Haussman 2006, Young et al. 2018). A possible candidate mechanism relies on the direct role of telomeres in the process of aging. Because telomeres shorten until a certain threshold beyond which the cell enters a senescent state or apoptosis, they play a major role in organismal aging. In addition, telomere dynamics could be linked to others physiological processes associated with mortality, thus potentially affecting lifespan indirectly. The sublethal effects of thermal stress on telomere shortening, with their possible long-term consequences on survival, combined with the direct strong impact on nestling pre-fledging mortality (Corregidor-Castro et al., 2023, 2025a,b), could have alarming implications at the population level, as already observed in other species (Rodriguez et al. 2025). This is especially the case for the regions characterised by increasing average temperatures and exposed to more frequent and intense extreme heat events over time, like the Mediterranean area (Tejedor et al. 2024).

In conclusion, by incorporating multiple ecological predictors and focusing on a molecular marker of somatic integrity, our experimental approach allowed for a nuanced assessment of the consequences that heatwave events associated with climate warming may have on avian development and, possibly, population dynamics. As climate change continues altering developmental conditions in wild populations, other studies are essential to anticipate potential demographic and evolutionary consequences for species exposed to extreme thermal regimes during reproduction.

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Chapter 2

Variation in corticosterone and telomere dynamics according to sex and rearing environment in barn swallow nestlings

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ABSTRACT

Telomeres are the chromosome ends that shorten at each cell division and are widely recognized as biomarkers of individual quality and environmental stress exposure. By controlling the stress response through corticosterone release, which in turn can damage telomeres, the hypothalamus–pituitary–adrenal (HPA) axis may mediate environmental influence on telomere dynamics. However, these links remain poorly investigated in developing birds. Here, in barn swallow (*Hirundo rustica*) nestlings, we investigated environmental, growth and sex influences on baseline corticosterone level, after acute handling-induced stress, and during recovery of homeostasis, as well as on telomere length (TL) and attrition (ΔTL) during growth. Moreover, we tested whether corticosterone levels/changes predicted TL/ΔTL. Baseline corticosterone decreased with body mass, suggesting that nestlings in better condition experienced reduced chronic stress. In addition, corticosterone after acute stress increased with brood size, showing that more competitive family environments elicited stronger stress responses. Recovery was faster in heavier and faster-growing nestlings, indicating that individuals in better condition returned to homeostasis more efficiently. Heavier and faster-growing males, but not females, had shorter TL and faster ΔTL, suggesting male-specific growth costs. Moreover, among males, the relationship between corticosterone variation during acute stress response and ΔTL shifted from negative in smaller broods to positive in larger broods, suggesting increased telomere maintenance in competitive family environments. Overall, we revealed that early-life TL/ΔTL are jointly shaped by rearing conditions, HPA-axis activity, and growth in different ways among male and female offspring, possibly contributing to shape sex-specific life trajectories during adulthood through carry-over effects.

Key words: Developmental conditions; corticosterone; *Hirundo rustica*; sex; stress response; telomere

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INTRODUCTION

Telomeres are the conserved, non-coding and repetitive DNA sequences that cap the termini of eukaryotic chromosomes and contribute to genome stability (Blackburn, 1991, 2005). In somatic cells, telomeres shorten at each cell division ('end replication problem' - Harley et al., 1990; Blackburn, 2005), until they reach a threshold length under which cells enter reproductive senescence or apoptosis (Palm & De Lange, 2008). Due to these shortening dynamics, telomere length and its attrition rate are recognized as hallmarks of aging and organismal loss of functions with age (Campisi et al., 2001; Deng et al., 2008). Therefore, over the past two decades, there has been a marked increase in studies highlighting the possible role of telomere dynamics in shaping life-history trade-offs and selection processes in wild vertebrates (Haussmann & Marchetto, 2010; Monaghan, 2010, 2024).

In birds, there is general, though not unequivocal, evidence for a covariation between short or rapidly shortening telomeres and reduced survival/lifespan (Wilbourn et al., 2018; Eastwood et al., 2019; Sheldon et al., 2022; Vedder et al., 2022; but see, Pepke et al., 2022), and/or reproductive success (Parolini et al., 2017; Eastwood et al., 2019; Heidinger et al., 2021; Chik et al., 2024 - but see, Sudyka et al., 2019). Together, these findings suggest that longer telomeres and/or slower attrition may reflect higher individual somatic integrity and reflect high overall physiological quality (Haussmann & Marchetto, 2010; Monaghan, 2010, 2024).

Although vertebrate telomere length decreases over lifetime (Remot et al., 2022), it cannot be considered an accurate biomarker of chronological age, because telomere length and its attrition rate can broadly differ among individuals (Monaghan, 2010; Boonekamp et al., 2013; Monaghan, 2014). Such variation have both genetic, although heritability of telomere length broadly vary across species (Asghar et al., 2015; Atema et al., 2015; Chatelain et al., 2020; Bauch et al., 2022; Vedder et al., 2022), and environmental components (Reichert et al., 2015; Dugdale & Richardson, 2018). Telomere length and attrition are susceptible to a suite of environmental stress factors such as parasite infections (Asghar et al., 2016; Badás et al., 2023), sibling competition (Boonekamp et al., 2014; Costanzo et al., 2017; R. C. Young et al., 2017), social isolation (Aydinonat et al., 2014), human disturbance (Herborn et al., 2014; Meillère et al., 2015), environmental temperature (Eastwood et al., 2022, 2023; Rodriguez et al., 2025) and pollution (Blévin et al., 2016; Stauffer et al., 2017). One of the main proximate causes possibly mediating these relationships is oxidative stress (Angelier et al. 2018), as telomeres are particularly vulnerable to reactive oxygen species (Haussmann & Marchetto, 2010; Chatelain et al., 2020; Armstrong & Boonekamp, 2023). Variation in telomerase activity, which counteracts telomere loss by adding telomeric sequences to chromosome ends (Greider & Blackburn, 1989), can further modulate telomere dynamics, maintaining or even elongating telomeres when environmental and physiological conditions promote its up-regulation (Mizutani et al., 2013; Badás et al., 2015; Brown et al., 2022).

Moreover, different ontogenetic stages, such as development, growth and reproduction, show specific physiological and metabolic processes and constraints that may influence telomere dynamics (Monaghan, 2014). Among these, early development represents a particularly sensitive period, thus usually resulting in a faster telomere shortening early in life than in adulthood (Salomons et al., 2009; Foote et al., 2011; Parolini et al., 2015, 2018; Reichert et al., 2015). This accelerated shortening may arise because telomere dynamics depends on the rate of cellular division, which is higher during rapid somatic growth (Monaghan & Ozanne, 2018), and because intense aerobic metabolism associated with growth causes an increase in oxidative damage and stress (Costantini, 2014; K. Panda & Cherian, 2014; Haussmann & Marchetto, 2010; Chatelain et al., 2020; Armstrong & Boonekamp, 2023). Therefore, telomere length and attrition may be biomarkers of organismal allostatic load, which is defined as the cumulative cost of maintaining physiological stability (homeostasis) across predictable (e.g. growth) and unpredictable (e.g. environmental stressors) events (McEwen & Wingfield, 2003).

By mediating organismal response to allostatic load (Romero et al., 2009), the hypothalamus-pituitary-adrenal (HPA) axis is considered one of the main physiological pathways that translate the experience of environmental conditions into the observed individual variation in telomere length and attrition (Angelier et al., 2018). Activation of the HPA-axis causes secretion of glucocorticoids (GC) which can boost the mechanisms ultimately resulting in telomere shortening via multiple processes (Angelier et al. 2018). Prolonged exposure to high GC levels can cause increased production of reactive oxygen species, by increasing metabolism and catabolic processes (Landys et al., 2006), and impair antioxidant defences, ultimately resulting in oxidative stress and damage (Costantini et al., 2011; Costantini, 2014). In the short-term, elevated GC can boost both innate and acquired immune function, again fostering pro-oxidant production (Ilmonen et al., 2008; Martin, 2009). The effects of GC on the generation of pro-oxidants would thus lead to expect a negative relationship between telomere length and basal GC levels (Angelier et al., 2018). In addition, prolonged exposure to GC can down-regulate telomerase activity, thus contrasting telomere restoration and further promoting telomere attrition (Choi et al., 2008), although a moderate short-term increase in GC levels may up-regulate telomerase (Epel et al., 2010). Yet relatively few studies have investigated the relationships between basal GC (mainly corticosterone) levels and telomere length in birds, and most have focused on adult individuals (Angelier et al., 2018). The results of these studies are mixed, as negative (Bauch et al., 2016; Gangoso et al., 2016; Quirici et al., 2016; Young et al., 2016), and positive (Young et al., 2016) or no (Ouyang et al., 2016; Young et al., 2016; Costanzo et al., 2022) covariation between basal plasma concentrations of corticosterone and telomere length have been documented, and the few studies on developing birds have also provided contrasting evidence (Haussmann et al., 2012; Herborn et al., 2014). This discrepancy is likely due to differences in environmental conditions experienced (Young et al., 2016), as well as to the different life-stages analysed (Angelier et al., 2018). Moreover, sex-specific covariation between glucocorticoids and telomere length has been reported in birds, potentially because males and females differ in their responses and susceptibility to environmental stressors (e.g., Bauch et al., 2016).

Importantly, almost all the studies focused on the relationships between corticosterone and telomeres in birds have concentrated on baseline corticosterone levels (Angelier et al., 2018 - but see, Costanzo et al., 2022). However, vertebrates cope with a diverse array of stressors via an activation of the HPA-axis, resulting in a rapid-acute, reversible increase in circulating GC levels (Romero, 2004), which occurs at different pace in animals (Angelier et al., 2018). This 'acute stress' HPA-mediated response is acknowledged as adaptive, as it temporarily shifts physiological and behavioural priority in favour of functions crucial to immediate performance and survival to the expense of allocation to competing functions that can be suspended or delayed (Wingfield et al., 1998). Because elevated GC levels may increase oxidative stress (Costantini et al., 2011) and reduce telomerase activity (Choi et al., 2008), mounting a stronger acute stress response may be expected to lead to accelerated telomere shortening. However, high quality individuals, like those with better antioxidant protection and/or with reduced physiological susceptibility to stressors, may afford to retain relatively high GC levels and/or engage in adaptive acute stress responses without incurring the costs that they induce, causing no or even a positive relationship between GC levels and telomere length (Angelier et al., 2018). In practice, GC levels and telomere dynamics may positively covary in cases when higher GC levels correspond to higher individual quality, which is potentially reflected by telomere dynamics (the CORT adaptation hypothesis - Bonier et al., 2009).

We assessed both growth and environmental influences on the activation of the HPA axis and telomere length/attrition, as well as their association, in male and female barn swallow (*Hirundo rustica*) nestlings subjected to a standard handling-restraint protocol simulating predation stress (Balm, 1999; Jones et al., 2016; Costanzo et al., 2018). Specifically, we tested if corticosterone concentrations at the basal level, at the end of prolonged handling (acute stress response) and after handling (return to homeostasis), as well as the two changes between these time points, were predicted by environmental (i.e. brood size and hatching date) and body conditions (i.e. body mass), as well as whether they differ between the sexes. Moreover, we investigated if telomere length measured at 5 and 15 days of age, together with its temporal variation, were predicted by corticosterone levels and their variation during handling, while controlling also the possible influence of environmental conditions, growth and sex. Male and female barn swallow nestlings differ in several physiological and behavioural traits related to stress susceptibility and competitive ability (Boncoraglio et al., 2008; Bonisoli-Alquati et al., 2008; Saino et al., 2008a; Romano et al., 2011), with male nestlings attaining lower body mass in experimentally enlarged broods (Saino et al., 2008c) and being more susceptible to food deprivation than females (Bonisoli-Alquati et al., 2008). Accordingly, we expected that stressful rearing conditions and rapid growth may shape both HPA-axis activity and telomere length/attrition during development, and that these relationships could be sex- and/or context-specific.

METHODS

Study species

The barn swallow is a socially monogamous, semi-colonial passerine bird with biparental care of the progeny (Turner, 2006). In Europe, females lay 1 up to 3 clutches per breeding season (April-August) and, alone, incubate the 2-7 eggs per clutch (Turner, 2006). Both social parents provision the dependent offspring for ca. 20 days from hatching to fledging and for approximately 10 days afterwards (Turner, 2006). While breeding adults show moderate sexual dimorphism (Romano et al., 2017), male and female nestlings cannot be distinguished at visual inspection by human observers, but only through molecular sexing (see below). However, begging displays include sex-specific components, either visual or acoustic, that may mediate differential parental investment (Boncoraglio et al., 2008; Saino et al., 2008a).

Sampling protocols for telomere length and corticosterone measurements

Data were collected during April-June 2018 at 5 barn swallow breeding colonies located near Milan (Northern Italy; average coordinates: 45.30° N, 9.50° E). Nests were inspected every 2 days from the beginning of April to determine the presence of eggs, as well as their laying and hatching dates (day 0 = day of hatching of the first eggs), together with the number of nestlings survived until 10 days after hatching, which reliably reflect the final brood size (hereafter, brood size; Romano et al., 2025). Brood size was included as a key environmental variable in both corticosterone and telomere analyses, because larger broods are more stressful rearing environment compared to smaller ones due to increasing sibling competition for access to parentally delivered food (Saino et al., 1997, 2000; Romano et al., 2013), potentially leading to increased corticosterone and faster telomere shortening (Costanzo et al., 2017; Novelli et al. 2026 - submitted). Hatching date was also considered because adults reproductive success and offspring future quality are known to progressively decrease throughout the breeding season in the study population (Ambrosini et al., 2006; Saino et al., 2012).

Data were collected from 26 nests, from which we selected 3 nestlings at random (N =78 nestlings) that were marked with an aluminium ring, weighted and blood sampled (ca. 30 µl), for telomere length quantification and molecular sexing, when 5 days old. At 15 days, they were subjected to the handling-restraint protocol (Balm, 1999; Jones et al., 2016; Costanzo et al., 2018). Briefly, they were removed from their nests and a blood sample (T_0) (ca. 60 µl) was collected from the brachial vein in heparinized capillary tubes within less than 3 min to determine baseline corticosterone (CORT) levels (Romero & Reed, 2005). Nestlings were then placed into a cloth bag until a second blood sample (ca. 60 µl) was collected 20 min after capture (T_{20}) to measure acute stress-induced CORT levels (Romero & Reed, 2005) and telomere length to determine telomere attrition between 5 and 15 days. Furthermore, nestlings were weighed to determine their increase in body mass. Both body mass at 5 and 15 days, as well as their change, were then considered in the analyses because impaired growth may be associated with higher levels of corticosterone (Wingfield et al., 1998; Haussmann & Marchetto, 2010), and because rapid somatic growth entails elevated cellular proliferation rates and increased metabolic activity, both of which can promote faster telomere attrition (Haussmann & Marchetto, 2010; Monaghan & Ozanne, 2018; Armstrong & Boonekamp, 2023). Finally, from a subset of randomly selected nests, one nestling (N = 17) was chosen at random and again blood sampled 30 min after capture (T_{30}) to assess how rapidly CORT levels return to baseline after the acute stress response. This measures individual ability to recover homeostasis, which is crucial as the acute stress response requires CORT levels to rise rapidly during stressful events and then decline quickly once stress ends to be adaptive. Blood samples were kept cool until centrifugation (within 6 h of collection) to separate red blood cells (used for telomere length analyses) from plasma (used for CORT analyses), after which they were stored at -80 °C. Blood samples were also processed for molecular sexing (Griffiths et al., 1998, Saino et al., 2008b), resulting in 34 males and 44 females.

Ages for blood samplings were chosen so to allow assessing telomere length variation along the longest possible timespan. Age 5 days is indeed the earliest age when blood sampling by puncturing of the brachial vein of barn swallow nestlings is feasible with minimal risk of harmful consequences for them. Age 15 days, in turn, is the maximum age when nestlings can be approached by observers at the nest without incurring the risk of them leaving the nest prematurely and inducing brood failure (Costanzo et al., 2023).

CORT levels were measured on day 15 because the stress protocol has been designed for adult birds, whose cognitive abilities enable them to respond to handling by humans with fearful behaviour likely associated with a physiological stress response mediated by the HPA-axis (Balm, 1999). It is unclear whether cognitive abilities of 5-days-old barn swallow nestlings allow them to perceive handling as a stressful stimulus to react to as adults do. Conversely, handling at 15 days of age elicits the same behavioural reactions that are elicited in adults, including screaming, attempting to escape or tonic immobility (Costanzo et al., 2018). This evidence strongly suggests that handling is stressful to nestlings at 15 days of age in a way similar to that experienced by adults, therefore probably eliciting a similar HPA-axis response. In addition, we refrained from collecting two blood samples on the same day from small 5-days-old nestlings, as required for the assessment of both basal and acute CORT levels, because this procedure may have been too harmful for so little individuals (body mass approximately: 10g). Throughout the study we assumed that CORT levels at T_0, T_{20} and T_{30} recorded on day 15 were representative of CORT levels of individual nestlings relative to other nestlings also at previous (and subsequent) stages during the nestling period. Such consistency in relative CORT levels is an implicit assumption in virtually all studies of birds in the wild, where corticosterone levels at one point in time may be assumed to reflect corticosterone levels also at other times (Cockrem et al., 2017).

Corticosterone assay

We quantified total plasma CORT concentration (ng/ml) using a commercially available CORT ^{125}I radioimmunoassay kit (catalog no. 07-120102; MP Biomedicals, Solon, OH, USA). Procedures were the same adopted in Costanzo et al., (2022) and followed the instructions of manufacturer with modifications after Washburn & Millspaugh (2002). Briefly, all reagent volumes were reduced by half and samples were diluted 1:50 vol/vol rather than 1:200 vol/vol. In addition, two lower-concentration points were added to the standard curve to enhance assay sensitivity. This assay has been validated and widely applied in avian species (Soldatini et al., 2015; Huber et al., 2017). All samples were run in duplicate across 10 assay plates. The inter- and intra-assay coefficients of variation were below 6% and 10%, respectively.

Due to laboratory issues, one sample was excluded, resulting in basal corticosterone levels (CORT at T_0) available for 77 individuals (34 males and 43 females). Acute stress CORT levels (CORT at T_{20}) were instead available only for 76 individuals (34 males and 42 females) out of these 77. CORT levels at T_{30} were measured only in a subset of 17 individuals to reduce the possible impact of three consecutive blood samplings on nestlings. However, no effect of multiple (either two or three) blood samplings was observed on subsequent nestling survival until fledging.

Telomere length analyses

Genomic DNA was extracted from 10-15 µl of red blood cells using the Wizard DNA extraction kit (Promega, WI, USA). The concentration and purity of DNA were measured with a Nanophotometer (IMPLEN) and we excluded samples with absorbance ratios OD260/280 = 1.8–2.0 and OD260/230 = 2.0–2.2. Telomere length was determined via monochrome multiplex quantitative PCR method (MMQPCR - Cawthon, 2009), following the method developed on the barn swallow by Parolini et al., (2015). In brief, amplifications were performed on a iQ5 thermal cycler (BioRad) and each well contained 20 ng of template DNA (genomic DNA), 2X Quantitative Master Mix SYBR Green (Genespin) and primers pairs to simultaneously amplify telomere and single copy gene CTCF, at the final concentration of 1,000 nM and 500 nM, respectively. Telomere forward and reverse primer sequences were: telg (5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3)' and telc (5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3'). The CTCF primers were: forward (5'-CCCGCGGCGGGCGGCGCGGGCTGGGCGGCTCCCAATGGAGACCTCAC-3') and reverse (5'-CGCCGCGGCCCGCCGCGCCCGTCCCGCCCATCACCGGTCCATCATGC-3'). The amplification of both target sequences in the same reaction well is allowed by the presence of the GC-clamp (underlined part) on CTCF primers, which delays the amplification start of CTCF compared to telomeres. Additionally, a four-point standard curve (5 ng, 20 ng, 50 ng and 100 ng of a reference DNA) was included in each plate. All the samples and the four dilutions of the standard curve were run in triplicate. According to Cawthon (2009), the standard curve was used to measure telomere length as the T/S ratio, representing the abundance of telomeric repeats (T) normalized to that of the single-copy reference gene CTCF (S), and expressed relative to the T/S ratio of the 20ng standard. The mean intra- and inter-plate coefficients of variation (±SD) were 2.8 ± 2.3% and 2.3 ± 1.6%, respectively.

We included in the analyses (see below) RTL at 5 and 15 days only for nestlings with at least one CORT measurement, excluding one with missing RTL at both ages but available CORT levels (N = 76; males = 34; females = 42).

Statistical analyses

Variation in baseline, acute, and recovery levels of corticosterone

We performed a paired t-test to investigate whether CORT levels at T_0 differed from those at T_{20} within individuals, to assess whether handling protocol induced an acute stress HPA axis-mediated response. A further paired t-test between CORT levels at T_{20} and T_{30} tested if CORT levels significantly decreased after acute stress. We used this approach instead of a single three-level repeated-measures analysis because corticosterone at T_{30} was available only for a subset of individuals, which would have reduced sample size for the T_0-T_{20} comparison.

We relied on linear mixed models (LMMs) to investigate environmental, morphological and sex-related influence on nestlings' baseline stress levels, acute stress HPA axis-mediated response and ability to recover after stress. To improve residuals, CORT and changes in CORT levels were mean-centered and scaled to standard deviation (z-transformed) in all these models. Brood identity was added to all the models as a random intercept.

The first LMM included CORT at T_0 as dependent variable, while brood size, hatching date and body mass at 15 days of age as fixed effects together with sex. In addition, two LMMs including CORT at T_{20} and change in CORT levels between T_0 and T_{20} (CORT at T_{20} – CORT at T_0; hereafter, $\Delta CORT_{0\text{-}20}$) as dependent variable respectively, were fitted to test the reactivity of individuals to the handling-induced acute stress response. Fixed effects were identical to the previous model, except for the LMM testing variation in $\Delta CORT_{0\text{-}20}$ where we included the increase in body mass between 5 (hereafter, body $mass_5$) and 15 days (hereafter, body $mass_{15}$) instead of body mass (hereafter, Δbody mass).

Similarly, we fitted two additional linear models (LM) to test for variation in CORT at T_{30} and in change between T_{20} and T_{30} (CORT at T_{30} – CORT at T_{20}; hereafter, $\Delta CORT_{20\text{-}30}$). Including all predictors from the previous models in a single model could have led to overparameterization due to the small sample size. Therefore, we tested the effects of brood size, hatching date, and body mass/Δbody mass in separate models, while keeping sex as fixed factor in all of them.

Variation in telomere length and attrition

To analyse RTL variation, we first applied a paired t-test to assess whether RTL changed between 5 and 15 days of age within individuals. Furthermore, we investigated inter-individual variation in RTL at 5 days of age (hereafter, RTL_5), at 15 days of age (hereafter, RTL_{15}) and change in RTL between 5 and 15 days (RTL_{15} – RTL_5; hereafter, ΔRTL) using LMMs. In all the models, RTL values were standardized (z-transformed) to improve comparability of results with studies adopting the quantitative PCR method (Verhulst, 2020), while ΔRTL was corrected for the regression to the mean, the statistical artifact that emerges when changes between two consecutive measurements are intrinsically correlated with their baseline values (this study: Pearson's $r = -0.7$, $p < 0.001$). Therefore, to account for this bias, we adjusted each ΔRTL following the formula proposed by Verhulst et al. (2013), which removes the observed change expected solely from the regression to the mean, estimated based on the correlation between the two consecutive measurements. Again, we included brood identity as random intercepts in all models.

In detail, RTL_5 and RTL_{15} were analysed using LMMs including CORT at T_0 and T_{20}, brood size, hatching date, sex and body mass at the corresponding age (i.e. body $mass_5$ with RTL_5 and body $mass_{15}$ with RTL_{15}). To assess sex-specific effects, we tested, in separate models, the interaction between sex and brood size and the interaction between sex and body mass. For RTL_{15}, we also fitted two alternative models including, separately, the three-way interaction among sex, brood size and CORT at T_{20}, or the three-way interaction among sex, body $mass_{15}$ and CORT at T_{20}.

ΔRTL was analysed with a LMM including $\Delta CORT_{0–20}$, brood size, hatching date, sex and Δbody mass as fixed effects. As above, we tested, in separate models, the interaction between sex and brood size as well as between sex and Δbody mass. Again, we fitted two alternative models including, separately, the three-way interaction among sex, brood size and $\Delta CORT_{0–20}$, or the three-way interaction among sex, Δbody mass and $\Delta CORT_{0–20}$.

Finally, on the subset including individual for which blood samples at T_{30} were available, we could not include the same fixed effects structure of the previous analyses to test whether the ability to recover after the acute stress response was associated with telomere dynamics, given the small sample size and the consequent risk of overparameterization. Furthermore, as individuals in these analyses were one per nest, we removed brood identity from random factors. Therefore, we ran two LMs: one including RTL_{15} as a dependent variable and sex and CORT at T_{30} as fixed effects, the second with ΔRTL as a dependent variable and both sex and $\Delta CORT_{20-30}$ as fixed factors.

Statistical assumptions and model validation

All statistical analyses were performed with the statistical software R version 4.5.1 (R Core Team 2025). LMMs were fitted with the *lmer* functions of the 'lme4' package (Bates et al., 2015). Residual diagnostics were explored using the 'DHARMa' package (Hartig, 2016) confirming that all model assumptions were met. Additionally, we did not detect influential outliers nor collinearity (package 'performance', Lüdecke et al. 2021), respectively. Non-significant interactions were removed from the final models starting with the three-way interactions and then the two-way terms in a single step.

Ethical note

Nestling capture, ringing and blood sampling were performed under license from the local wildlife management authorities (Regione Lombardia, Direzione Generale Agricoltura, Decreto N° 11316 issued on 1st December 2014; Decreto N° 599 issued on 19th January 2018). The handling restraint protocol did cause any obvious harm to nestlings. During restraint, birds were kept warm in dark cloth bag as it is the case in standard ringing operations. All nestlings fledged successfully together with their non-handled nestmates within one week after handling.

RESULTS

Variation in baseline, acute, and recovery levels of corticosterone

CORT significantly increased between T_0 and T_{20} ($t_{75} = 20.19$, $p < 0.001$; Figure 1), while no significant differences between T_{20} and T_{30} were detected ($t_{16} = -0.80$, $p = 0.43$), although a large interindividual variability was observed for this endpoint (Figure 1).

Basal CORT negatively covaried with body mass_{15} (Figure 2a), while it did not vary according to sex, brood size, and hatching date (Table 1). CORT at T_{20} was not significantly associated with body mass_{15}, but showed a positive and significant association with brood size (Table 1; Figure 2b). However, it did not differ between the sexes or varied with hatching date (Table 1). Results for $\Delta\text{CORT}_{0\text{-}20}$ were qualitatively similar to those CORT at T_{20} (Table 1).

Finally, CORT at T_{30} and $\Delta\text{CORT}_{20\text{–}30}$ were negatively associated with body mass_{15} and Δbody mass, respectively (Table 1, Figure 3), indicating that heavier individuals at the end of skeletal growth, and those that gained more weight, showed a higher capacity to recover after the acute stress response. However, recovery capacity did not vary between the sexes (Table 1). Brood size and hatching date did not significantly predict neither CORT at T_{30} nor $\Delta\text{CORT}_{20\text{–}30}$ (Table S1).

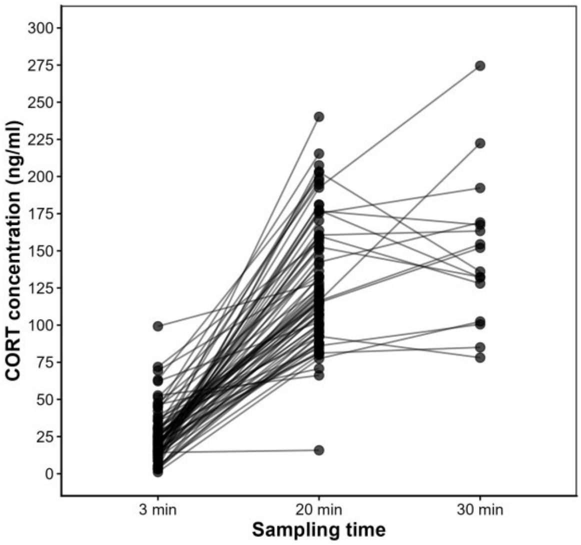

Figure 1. Variation in corticosterone concentration (ng/ml) within individual between T_0 (3 min), T_{20} (20 min), and T_{30} (30 min).

Variation in telomere length and attrition

RTL significantly declined between 5 and 15 days ($t_{75} = -3.88$, $p < 0.001$; Figure 4). In addition, variability in RTL_5 was not significantly explained by CORT at T_0, CORT at T_{20}, body $mass_5$, sex, brood size, and hatching date (Table 2).

RTL_{15} was significantly predicted by the interaction between sex and body $mass_{15}$, thus indicating that the association between body mass and RTL is differentially expressed in the two sexes (Table 2; Figure 5a). Specifically, the interaction resulted from a significant and negative association between body $mass_{15}$ and RTL_{15} in males ($\beta \pm SE = -0.45 \pm 0.13$, $t_{45.1} = -3.35$, $p = 0.001$), while a no significant relationship was found in females ($\beta \pm SE = 0.17 \pm 0.13$, $t_{55.8} = 1.31$, $p = 0.19$). In addition, RTL_{15} was not predicted by

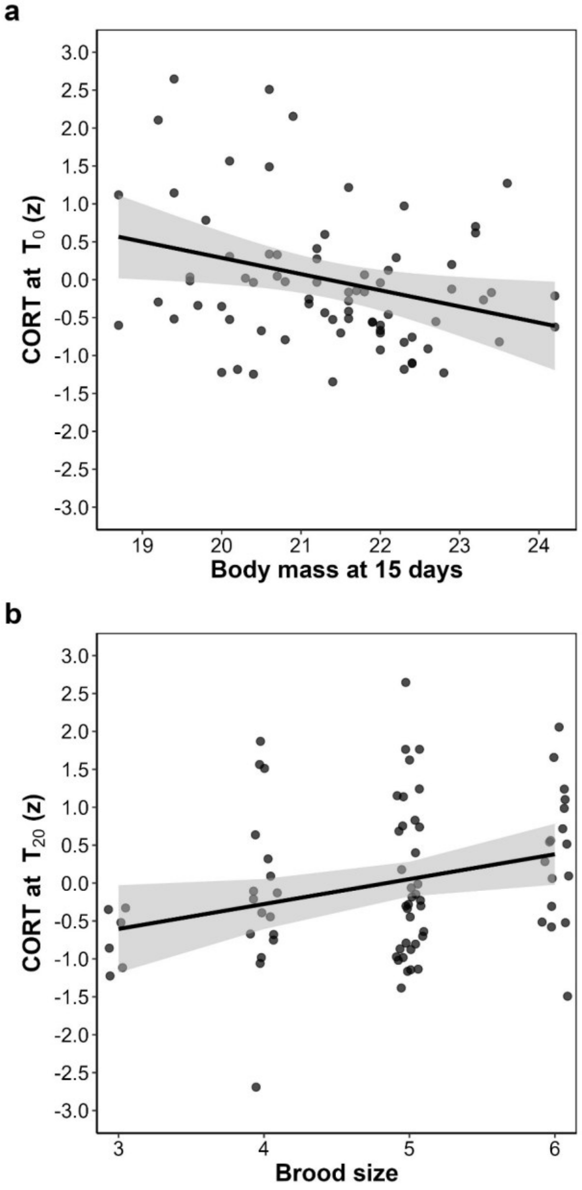

Figure 2. a) Effect of body mass at age 15 days on basal corticosterone levels (CORT at T_0); **b)** Effect of brood size on acute corticosterone levels (CORT at T_{20}). Lines represent the model predictions from the linear mixed-effect models, shaded areas the limits of the 95% confidence interval of the model estimates and dots the observed datapoints.

CORT levels (T_0 and T_{20}), brood size and hatching date (Table 2).

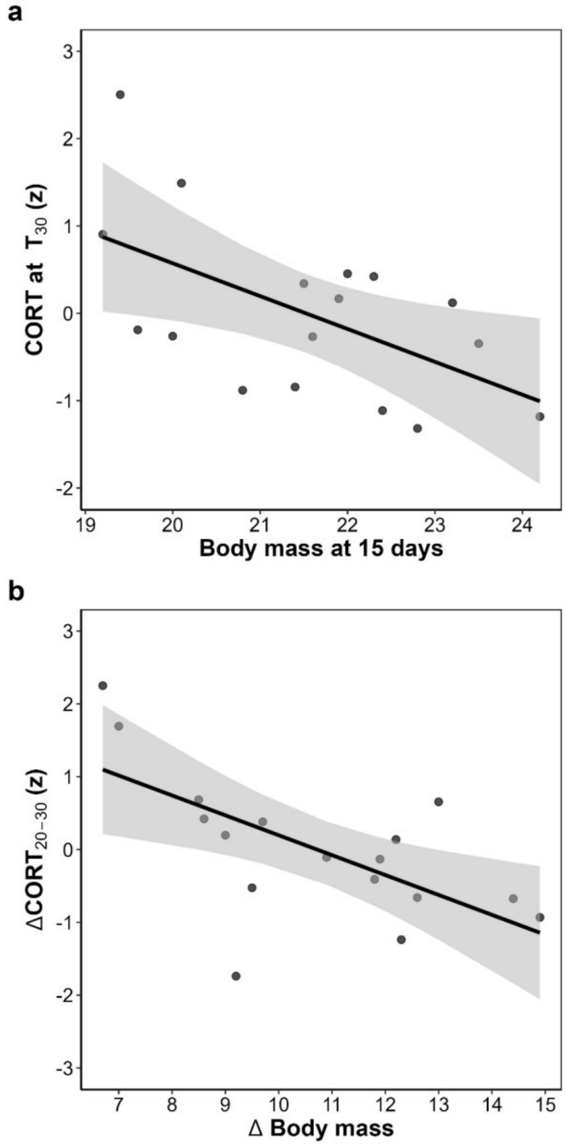

Figure 3. a) Effect of body mass at age 15 days on corticosterone levels during recovery of homeostasis after acute stress (CORT at T_{30}); **b)** Effect of body mass change (Δbody mass) on change in CORT levels between T_{20} and T_{30} ($\Delta CORT_{20-30}$). Lines represent the predictions of the linear mixed-effect models including also sex as fixed effect. Shaded areas represent the limits of the 95% confidence interval of the model estimates, while dots the observed datapoints.

Similarly, ΔRTL was significantly predicted by the two-way interaction between sex and Δbody mass (Table 2; Figure 5b), with the association being again negative and significant in males ($\beta \pm SE = -0.23 \pm 0.07$, $t_{51} = -3.03$, $p = 0.004$), and non-significant in females ($\beta \pm SE = 0.12 \pm 0.06$, $t_{35.9} = 1.98$, $p = 0.06$). Furthermore, we found a significant effect of the three-way interaction between sex, brood size and $\Delta CORT_{0-20}$ (Table 2). Specifically, among males, an increase in $\Delta CORT_{0-20}$ in smaller broods was associated with a decrease in ΔRTL, while the trend changed to the opposite when brood size increased (Figure 6). No patterns were instead observed in females ($\beta \pm SE = 0.002 \pm 0.004$, $t_{35} = 0.53$, $p = 0.59$).

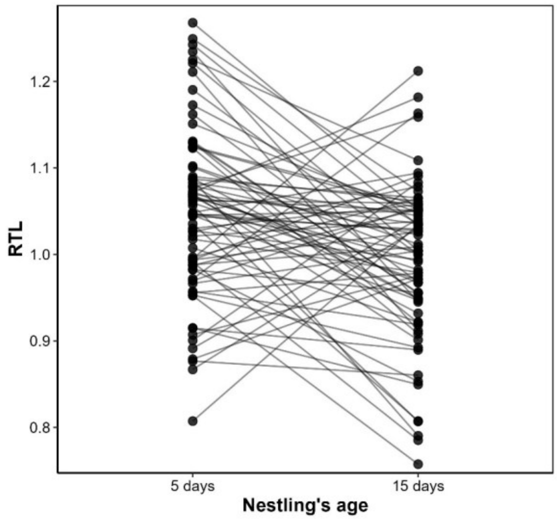

Figure 4. Intraindividual variation in relative telomere length (RTL) between 5 and 15 days of age.

Neither CORT at T_{30} nor $\Delta CORT_{20-30}$ predicted RTL_{15} and ΔRTL, respectively (Table S2). Furthermore, both models showed differences in RTL_{15} and ΔRTL between the sexes, with males displaying longer RTL and slower attrition than females (Table S2).

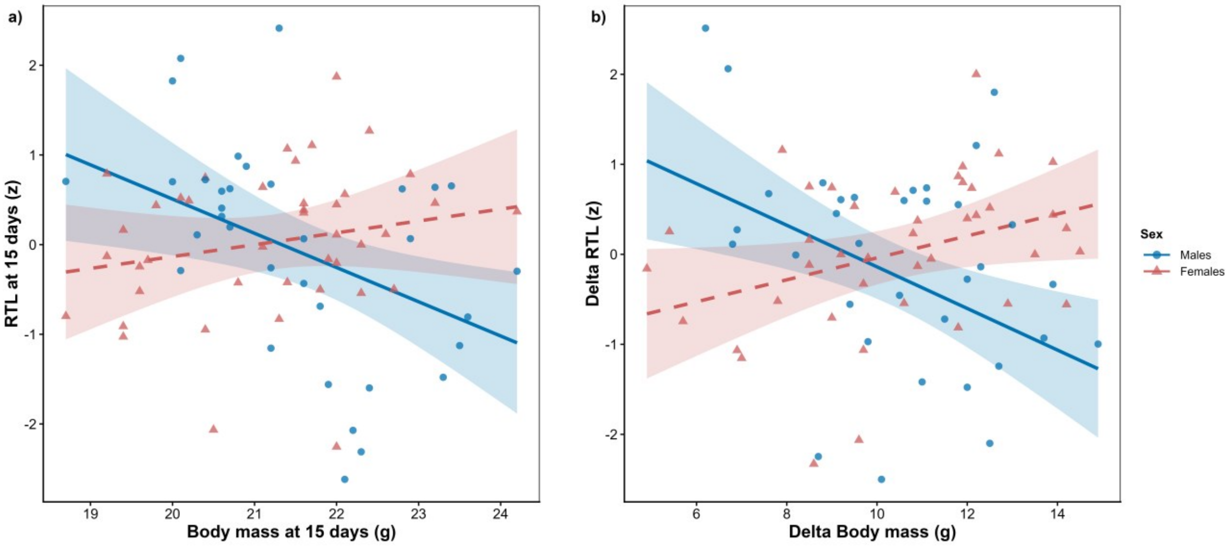

Figure 5. a) Effect of the interaction between body mass at age 15 days and sex on relative telomere length (RTL) at 15 days. **b)** Effect of the interaction between body mass change (Δbody mass) and sex on change in RTL (ΔRTL). Dots represent observed datapoints of the relationships for males (blue circles) and females (red tringles), while lines the prediction of the linear regressions within-males (blue) and females (red) between body mass at age 15 days and Δbody mass with RTL at 15 days and ΔRTL, respectively. Solid lines display the statistically significant effects, while dashed lines the non-significant ones. Shaded areas display the upper and lower limits of the 95% confidence interval of the model estimates for males (blue) and females (red).

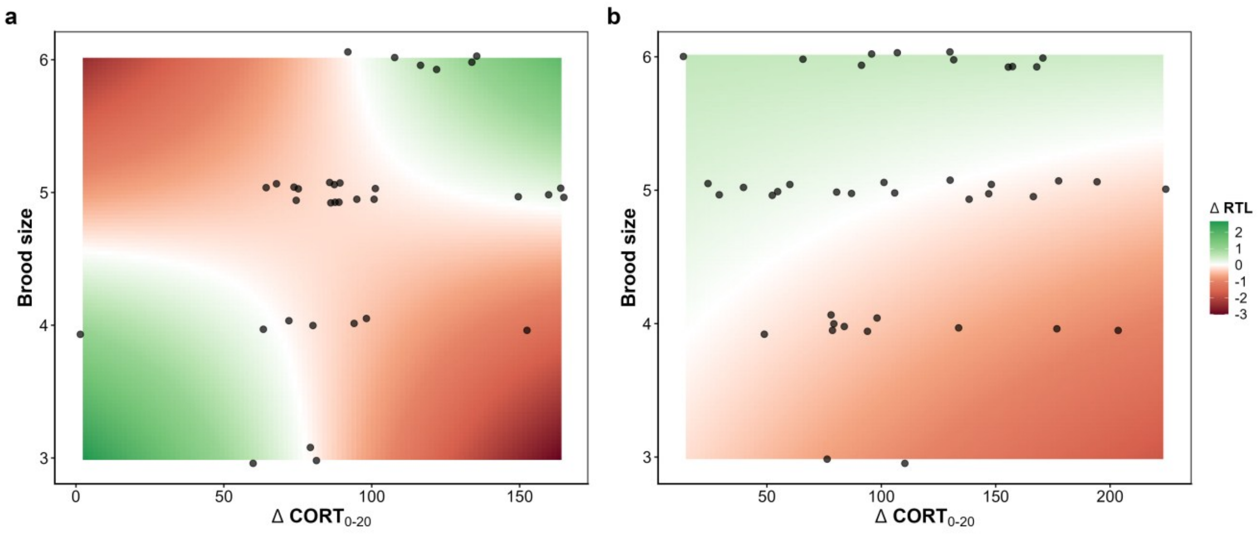

Figure 6. Heatmaps illustrating the effect of the interaction between ΔCORT$_{0-20}$ (z) and brood size on change in relative telomere length (ΔRTL, z) in **a)** male and **b)** female nestlings. Colours represent model prediction for ΔRTL, with green indicating predicted telomere lengthening and red indicating predicted telomere shortening. Black dots represent observed ΔCORT$_{0-20}$ × brood-size combinations for each sex.

CORT at T_0				**N = 77**
Predictors	**Estimate**	**CI**	**df**	**P**
(Intercept)	7.13	-2.24 – 16.49	70	0.13
Body mass at age 15 days	**-0.21**	**-0.40 – -0.03**	**70**	**0.025**
Sex	-0.17	-0.63 – 0.28	70	0.44
Brood size	0.04	-0.27 – 0.34	70	0.81
Hatching date	-0.02	-0.08 – 0.04	70	0.51
Random effects	**τ00 (SE)**			
Brood identity	0.08 (0.28)			
CORT at T_{20}				**N = 76**
Predictors	**Estimate**	**CI**	**df**	**P**
(Intercept)	-2.79	-11.76 – 6.16	69	0.53
Brood size	**0.33**	**0.04 – 0.62**	**69**	**0.026**
Sex	0.20	-0.25 – 0.66	69	0.38
Body mass at age 15 days	-0.07	-0.24 – 0.11	69	0.46
Hatching date	0.02	-0.04 – 0.07	69	0.53
Random effects	**τ00 (SE)**			
Brood identity	<0.001 (<0.001)			
$\Delta CORT_{0-20}$				**N = 76**
Predictors	**Estimate**	**CI**	**df**	**P**
(Intercept)	-5.06	-13.25 – 3.12	69	0.22
Brood size	0.29	-0.00 – 0.58	69	0.051
Sex	0.24	-0.22 – 0.70	69	0.30
ΔBody mass	0.01	-0.09 – 0.11	69	0.83
Hatching date	0.02	-0.03 – 0.08	69	0.39
Random factors	**τ00 (SE)**			
Brood identity	<0.001 (<0.001)			
CORT at T_{30}				**N = 17**
Predictors	**Estimate**	**CI**	**df**	**P**
(Intercept)	**7.72**	0.68 – 14.77	14	**0.033**
Body mass at age 15 days	**-0.37**	**-0.69 – -0.06**	**14**	**0.022**
Sex	0.23	-0.69 – 1.15	14	0.59
$\Delta CORT_{20-30}$				**N = 17**
Predictors	**Estimate**	**CI**	**df**	**P**
(Intercept)	**3.46**	0.54 – 6.38	14	**0.023**
Δbody mass	**-0.27**	**-0.47 – -0.08**	**14**	**0.008**
Sex	-0.33	-1.26 – 0.59	14	0.45

Table 1. Summaries of the linear and linear mixed-effects models evaluating environmental, morphological and sex-related influence on nestlings' stress response to the handling-restraint protocol. The dependent variables were baseline corticosterone (CORT at T_0), corticosterone levels and change during acute stress response (CORT at T_{20} and $\Delta CORT_{0-20}$), and corticosterone levels and change after stress (CORT at T_{30} and $\Delta CORT_{20-30}$). Fixed effects differed slightly among models, as body mass at age 15 days was used to test CORT at T_0, CORT at T_{20} and CORT at T_{30}, whereas body mass change (Δbody mass) was used as predictor for models describing $\Delta CORT_{0-20}$ and $\Delta CORT_{20-30}$. Brood size, hatching date, and sex were included as fixed effects in all models, while brood identity in all linear mixed-effects models for which we have more than one measure per brood. Significant fixed effects are reported in bold.

RTL at 5 days				**N = 75**
Predictors	**Estimate**	**CI**	**df**	**P**
(Intercept)	-1.61	-10.16 – 6.93	66	0.71
CORT at T_0	0.004	-0.01 – 0.02	66	0.51
CORT at T_{20}	0.004	<0.001 – 0.01	66	0.19
Body mass at age 5 days	-0.07	-0.21 – 0.06	66	0.30
Sex	-0.06	-0.53 – 0.40	66	0.78
Brood size	-0.007	-0.33 – 0.32	66	0.96
Hatching date	0.01	-0.05 – 0.08	66	0.62
Random effects	**τ00 (SE)**			
Brood identity	0.07 (0.26)			

RTL at 15 days				**N = 75**
Predictors	**Estimate**	**CI**	**df**	**P**
(Intercept)	20.87	6.89 – 34.85	65	**0.004**
Body mass at age 15 days	-1.08	-1.66 – -0.50	65	<0.001
CORT at T_0	<0.001	-0.02 – 0.01	65	0.77
CORT at T_{20}	<0.001	-0.01 – 0.01	65	0.87
Sex	-13.42	-21.17 – -5.67	65	0.001
Brood size	0.23	-0.08 – 0.54	65	0.15
Hatching date	0.01	-0.05 – 0.07	65	0.75
Body mass at age 15 days*sex	**0.63**	**0.27 – 0.99**	65	**0.001**
Random effects	**τ00 (SE)**			
Brood identity	0.05 (0.22)			

ΔRTL				**N = 75**
Predictors	**Estimate**	**CI**	**df**	**P**
(Intercept)	22.98	6.50 – 39.47	62	**0.007**
ΔCORT$_{0\text{-}20}$	-0.21	-0.35 – -0.06	62	0.006
ΔBody mass	-0.58	-0.90 – -0.27	62	0.001
Sex	-12.69	-21.12 – -4.25	62	0.004
Brood size	-4.01	-7.02 – -1.01	62	0.011
Hatching date	0.01	-0.05 – 0.06	62	0.80
ΔBody mass*sex	**0.35**	**0.16 – 0.54**	62	**<0.001**
ΔCORT$_{0\text{-}20}$*sex	0.10	0.02 – 0.18	62	0.017
ΔCORT$_{0\text{-}20}$*brood size	0.05	0.01 – 0.08	62	0.004
Brood size*sex	2.04	0.33 – 3.76	62	0.021
ΔCORT$_{0\text{-}20}$*brood size*sex	**-0.02**	**-0.04 – -0.01**	62	**0.012**
Random factors	**τ00 (SE)**			
Brood identity	<0.001 (<0.001)			

Table 2. Linear mixed models testing whether telomere length and attrition were predicted by CORT levels and changes during the handling-restraint protocol. The dependent variables were relative telomere length (RTL) at 5 and 15 days, as well as telomere attrition between these days (ΔRTL). Fixed effects differed among models, as baseline corticosterone (CORT at T0) and acute corticosterone (CORT at T_{20}) were used to test both RTL at 5 and 15 days, whereas change in CORT between T_0 and T_{20} (ΔCORT$_{0\text{-}20}$) was included to test ΔRTL variation. Furthermore, body mass at age 5 days, 15 days and change in body mass (Δbody mass) were used to investigate variation in RTL at 5 days, 15 days and ΔRTL, respectively. Brood size, hatching date, and sex were included as fixed effects in all models. Significant two-way and three-way interactions are shown when retained in the final models. Brood identity was included as a random intercept in all models. Significant fixed effects are reported in bold.

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DISCUSSION

Allostasis, defined as “stability through change”, consists of behavioural and physiological adjustments organisms make in response to predictable and unpredictable challenges to maintain physiological stability throughout life (McEwen & Wingfield, 2003). These adjustments are regulated by the HPA-axis, and may incur in physiological costs (allostatic load) that can trigger telomere attrition (McEwen & Wingfield, 2003). In our study, besides showing associations between developmental conditions and both corticosterone levels and telomere length/attrition, we found also possible evidence of a relationship between magnitude in HPA-axis acute stress response and telomere attrition limited to male nestlings and based on varying environmental pressures.

Nestlings attaining higher body mass at the end of skeletal growth (i.e. 15 days) showed reduced levels of baseline corticosterone. This pattern aligns with findings of other studies showing negative covariation between circulating corticosterone levels and individual body condition in birds (Criscuolo et al., 2006; Strong et al., 2015). Reduced circulating baseline corticosterone in heavier individuals is consistent with the role of glucocorticoids in reallocating resources and suppressing other physiological activities, such as growth, in order to preserve resources for stress response and immediate survival (Wingfield et al., 1998; Haussmann & Marchetto, 2010). Furthermore, as individuals with higher body mass are assumed to have developed under favourable environmental conditions and be thus of higher quality and/or better physical condition, they were expected to be less stressed and show lower circulating corticosterone (Boersma & Wingfield, 1998; Breuner & Hahn, 2003).

In addition, we found the magnitude of acute stress response (i.e. CORT at T_{20} and $\Delta CORT_{0-20}$) to positively correlate with brood size. Larger broods represent stressful and competitive environments, including in the study population (Saino et al., 1997, 2000; Romano et al., 2013), and nestlings exposed to higher sibling competition are expected to experience more frequent stressors and to reallocate greater resources into stress responses, thereby potentially resulting in elevated corticosterone levels at the end of the acute stress phase. Furthermore, as we did not find any association between baseline corticosterone and brood size, the association between acute corticosterone and brood size potentially suggests that individuals in larger broods may be better in responding to acute stress without incurring an increase in circulating corticosterone.

Individual body conditions, expressed in terms of body mass and growth rate, predicted also the ability of an individual to recover homeostasis after acute stress, as heavier individuals showed lower corticosterone and a steeper decrease in corticosterone 10 minutes after the end of the handling-induced stress. However, as shown in adults (Costanzo et al., 2022), corticosterone levels did not significantly decrease overall after the stress exposure, suggesting that 10 minutes may be too short timespan for nestlings of this age and size to recover homeostasis after acute stress. The fact that individuals gaining more weight between 5 and 15 days showed a greater ability to recover homeostasis supports this interpretation, indicating that only individuals in better condition may start recovering within this short period.

In male nestlings only, RTL_{15} and ΔRTL (but not RTL_5) decreased with increasing body mass and with higher growth rate, respectively. Our results are consistent with current literature, showing faster telomere attrition associated with faster somatic growth (Monaghan & Ozanne, 2018). This association is likely to arise from two complementary mechanisms. First, rapid somatic growth entails an increase in cell division rates, which in turn may lead to accelerated telomere loss due to the 'end replication problem' (Harley et al., 1990; Blackburn, 2005). Second, higher growth rates require an increase in metabolic activity, enhancing the production of reactive oxygen species and oxidative stress (Costantini, 2014; K. Panda & Cherian, 2014), potentially triggering telomere loss (Monaghan & Ozanne, 2018). Moreover, the fact that the relationships were significant in males but not females may stem from the well-documented differences in competitive ability and sensitivity to stressors between nestlings of the two sexes (Saino et al., 1998; Boncoraglio et al., 2008; Bonisoli-Alquati et al., 2008; Saino et al., 2008c). Therefore, male nestlings could have experienced higher allostatic load from multiple environmental stressors (e.g. sibling competition), particularly in conjunction with increasing growth rates. These results, together with the significant telomere shortening observed between 5 and 15 days of age highlight the crucial role of the early-life period in shaping telomere dynamics and, possibly, performance and fitness in adulthood (Eastwood et al., 2019; Sheldon et al., 2022; Eastwood et al., 2023).

Furthermore, we found a male-specific association between the magnitude of acute stress response ($\Delta CORT_{0\text{-}20}$) and telomere attrition, which varied according to brood size, while no other relationships emerged between corticosterone and telomere length/attrition. Specifically, the relationship between telomere attrition and acute stress response changed progressively with brood size in male nestlings, with stronger stress responses predicting greater telomere loss in smaller broods, but a surprisingly reduced loss as brood size increased. This pattern may suggest that brood size alters developmental resource allocation and, possibly, the physiological pathways linking corticosterone to telomere attrition. Although corticosterone is generally thought to impair telomere maintenance by increasing oxidative stress (Costantini et al., 2011) and down-regulate telomerase activity (Choi et al., 2008), transient elevation of corticosterone levels within physiological limits may unexpectedly promote telomerase up-regulation (Epel et al., 2010). A study on yellow-legged gull (*Larus michahellis*) has indeed shown that embryos exposed to increased egg corticosterone experienced enhanced telomerase activity which resulted in longer telomere after hatching (Noguera et al., 2022). However, interpretation of the male-specific association remains speculative due to the lack of similar evidence in previous studies. A possible explanation may lie again in the differences in stress-related competitive quality and sensitivity to stressors between barn swallow nestlings of the two sexes (Saino et al., 1998; Boncoraglio et al., 2008; Bonisoli-Alquati et al., 2008; Saino et al., 2008c; Romano et al., 2011), which may lead to sex-specific stress perception and corticosterone release. In this context, if a threshold over which corticosterone may up-regulate telomerase activity exists (Epel et al., 2010; Noguera et al., 2022), males, being generally more susceptible to stressors during development than females (Bonisoli-Alquati et al., 2008; Saino et al., 2008c), may reach this threshold during acute stress response, whereas females do not. This may therefore potentially result in reduced telomere attrition, via telomerase up-regulation, in males reared in larger broods compared to females.

Overall, our results are largely consistent with previous findings on breeding adults of the same population (Costanzo et al., 2022), suggesting comparable patterns linking stress physiology and telomere length/attrition across life stages in this species. However, we further provided possible evidence for sex and context influence on the associations between telomere length/attrition and acute stress response. Such differential associations suggest that functional links, either direct or mediated by common underlying physiological pathways, exist between the HPA-axis activation and the dynamics of telomeres and that these links operate differently in male and female nestlings. Such links may provide a mechanistic basis for sex-dependent translation of experience, as mediated by the HPA axis, into variation in telomere dynamics and, possibly, individual fitness (Monaghan, 2014). Future studies should therefore aim at addressing sex-dependency in these associations, both from mechanistic and functional perspectives, and its possible long-term consequences on fitness.

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SUPPLEMENTARY MATERIALS

CORT at T_{30}				
Predictors	**Estimate**	**CI**	**df**	**P**
(Intercept)	-0.85	-4.74 – 3.05	14	0.65
Sex	0.37	-0.75 – 1.49	14	0.49
Brood size	0.06	-0.62 – 0.73	14	0.86
(Intercept)	-3.06	-27.30 – 21.17	14	0.79
Sex	0.31	-0.88 – 1.50	14	0.58
Hatching date	0.02	-0.17 – 0.21	14	0.83
$\Delta \text{CORT}_{20-30}$				
Predictors	**Estimate**	**CI**	**df**	**P**
(Intercept)	1.65	-2.15 – 5.44	14	0.37
Sex	-0.01	-1.11 – 1.08	14	0.98
Brood size	-0.35	-1.00 – 0.31	14	0.27
(Intercept)	4.29	-20.24 – 28.82	14	0.71
Sex	0.15	-1.05 – 1.36	14	0.79
Hatching date	-0.03	-0.22 – 0.15	14	0.71

Table S1. Summaries of the linear models evaluating whether corticosterone levels and change after stress (CORT at T_{30} and $\Delta \text{CORT}_{20-30}$) were predicted by brood size and hatching date, in separate models, while controlling for sex in both cases. Significant fixed effects are reported in bold.

RTL_{15}				
Predictors	**Estimate**	**CI**	**df**	**P**
(Intercept)	1.31	-0.68 – 3.29	14	0.18
CORT at T_{30}	0.002	-0.01 – 0.01	14	0.65
Sex	**-1.01**	-2.00 – -0.02	14	**0.045**
ΔRTL				
Predictors	**Estimate**	**CI**	**df**	**P**
(Intercept)	1.59	-0.09 – 3.12	14	0.06
$\Delta \text{CORT}_{20-30}$	0.004	-0.01 – 0.02	14	0.43
Sex	**-0.98**	-1.94 – -0.01	14	**0.047**

Table S2. Summaries of the linear models evaluating whether relative telomere length at 15 (RTL_{15}) and change between 5 and 15 days were predicted by corticosterone levels and change after stress (CORT at T_{30} and $\Delta \text{CORT}_{20-30}$), respectively, while controlling for sex in both cases. Significant fixed effects are reported in bold.

PART 2

CONSEQUENCES OF INTERINDIVIDUAL VARIABILITY IN TELOMERE LENGTH ON FITNESS

Chapter 3

Early-life telomere length negatively covaries with brood size and father age, and predicts mortality later in life in male barn swallows (*Hirundo rustica*)

Running head: Telomeres link environment to long-term survival

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Early-life telomere length negatively covaries with brood size and father age, and predicts mortality later in life in male barn swallows (*Hirundo rustica*)

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ABSTRACT

Parental decisions on breeding, such as brood size and timing of reproduction, influence the early-life environment of the offspring, thus exposing them to a variable level of stressors. In this context, telomeres, the conserved DNA sequences at chromosome termini that shorten at each cell division, are particularly relevant, because telomere length (TL) may integrate environmental (e.g. exposure to stressors) and inherited sources of variation in somatic state, and may predict survival, potentially linking early-life conditions to future fitness. However, studies examining whether the effects of early-life environment translate into different survival prospects through TL remain limited and provided contrasting results. Here, we examined the effects of parental decisions (brood size and hatching date), as well as parental age and quality-related traits, on TL of male barn swallow (*Hirundo rustica*) nestlings that recruited in their natal site as breeding adults in the following years. We also investigated whether TL before fledging predicted long-term survival. Nestling TL decreased with increasing brood size, suggesting a detrimental effect of a stressful developmental environment on TL. In addition, nestlings with older fathers (but not mothers) had shorter telomeres, indicating a possible father aging effect on TL. Furthermore, individuals with longer telomeres at the nestling stage showed a higher likelihood of surviving to at least their second breeding season (≥ 2 years of age). Overall, our results suggest that early-life TL may link parental decisions and aging to survival prospects later in life, and may thus be target of selection even in short-lived species.

Keywords: Telomere, Lifespan, Paternal age, Early-life environment, Barn swallow, Sibling competition

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INTRODUCTION

Detrimental environmental conditions experienced during early life have been associated with reduced somatic maintenance and compromised health in later life stages (Metcalfe & Monaghan 2001; Tschirren et al. 2009; Balbontín & Møller 2015). Consequently, the developmental environment, together with the genetic component inherited from the parents, could influence the life-history trajectory of organisms, affecting their performance, survival and overall fitness in adulthood (Metcalfe & Monaghan 2001; Ricklefs & Wikelski 2002; Eyck et al. 2019). The developmental environment is partly shaped by parental decisions regarding the allocation of time and resources selected to achieve the optimal balance between parental viability and their offspring quantity and quality, ultimately impacting the future fitness outcomes of the progeny (Trivers 1972; Roff 1993; Royle et al. 2012). Parental decisions are driven by endogenous factors, reflecting parental quality and their capacity for reproductive investment (van Noordwijk & De Jong 1986), as well as by environmental biotic (e.g. predation; Lima 1987; Fontaine & Martin 2006) and abiotic constraints (e.g. climate; Lehikoinen et al. 2011; Kentie et al. 2018). Among endogenous factors, parental age can also influence these decisions affecting parental quality through physiological senescence and increased experience in parental care (Vleck et al. 2011; Moorad & Nussey 2016; Lemaître & Gaillard 2017). While senescence can reduce the quality of the developmental environment and offspring future survival probability and fitness (Lansing effect; Beamonte-Barrientos et al. 2010; Reid et al. 2010; Cooper et al. 2020), older parents may also benefit from their greater experience mitigating this negative effect (Daan et al. 1990; Hasselquist 1998; Sheldon 2000). As a result, the influence of parental age on developmental conditions and subsequent survival may be negative, positive, or non-linear, depending on how aging and increasing parental experience interact based on species-specific traits (e.g. Geslin et al. 2004; Catry et al. 2006; Beamonte-Barrientos et al. 2010).

In birds, one of the most crucial parental decisions is brood size. Larger broods result in reduced parental investment per offspring (Dijkstra et al. 1990; Saino et al. 1997a), as parents face a trade-off between allocating more resources per capita and producing a greater number of offspring in a context of limited resources availability (e.g. Smith et al. 1989). The resulting intensified sibling competition (Saino et al. 2000; Neuenschwander et al. 2003; Romano et al. 2013) further amplifies the challenge, possibly leading to long-lasting effects on offspring physiology, morphology and survival (de Kogel et al. 1997; Tschirren et al. 2009). Similarly, the timing of reproduction is critical, as delayed timing in breeding correlates with reduced offspring fitness both in the short- and long-term (Cooch et al. 1991; Moreno et al. 2005; Saino et al. 2012a). However, breeding too early can also entail fitness costs, because early-season conditions may be harsher and less predictable (e.g., Shipley et al. 2020):

Identifying molecular mechanisms that mediate the effects of parental conditions and decisions on offspring fitness in wild animals is a topic of growing interest, as it is essential for understanding the evolution of life-history strategies via natural selection. Telomeres are conserved, repetitive, non-coding DNA sequences (TTAGGG in vertebrates) that cap the ends of eukaryotic chromosomes, safeguarding genome stability and integrity by forming protective nucleoprotein complexes with shelterin proteins (Blackburn 1991, 2005). Telomeres shorten at each cell division in somatic tissues due to the inability of DNA polymerase to fully replicate the ends of linear chromosomes (‘end-replication problem’; Harley et al. 1990; Blackburn 2005). The enzyme telomerase counteracts this shortening by adding new telomeric repeats (Greider & Blackburn, 1989; Lingner et al. 1997), but its activity is often insufficient to prevent telomere attrition over time, resulting in progressive telomere shortening with age, a pattern observed primarily across endothermic vertebrate species (Remot et al. 2022). When telomere length reaches a critical threshold, cells lose functionality facing senescence or apoptosis, with negative consequences on somatic tissues maintenance (Campisi et al. 2001; Deng et al. 2008). For this reason, telomere length has been thus linked to survival probability at different life stages and lifespan in animals (Wilbourn et al. 2018; Eastwood et al. 2019, 2023, Heidinger et al. 2021). Given this possible link (Wilbourn et al. 2018; Eastwood et al. 2019, 2023), telomere length may contribute to interindividual fitness differences, although this relationship is expected to vary across ecological contexts and among species with contrasting life-history strategies. For instance, telomere dynamics covary with pace of life, with short-lived species typically showing faster telomere attrition than long-lived ones in concomitance with higher mortality rates, and this may affect the association between early-life somatic state, potentially captured by telomere length, and survival measured later in life (Haussmann et al. 2003; Tricola et al. 2018; Whittemore et al. 2019; Criscuolo et al. 2021). In addition, the relationship between telomere length and survival is not consistent across studies, even among species sharing similar life-history traits (Caprioli et al. 2013; Boonekamp et al. 2014; Eastwood et al. 2019; Heidinger et al. 2021; Le Pepke et al. 2022), because of high variability in the timing of both telomere length measurement and survival assessment (Eastwood et al. 2019).

In addition to the ‘end replication problem’, environmental factors have been observed to induce telomere length variation across various vertebrate taxa, especially birds (Monaghan 2010; Olsson et al. 2018; Chatelain et al. 2020), by directly influencing cell division rates and/or indirectly altering intra-cellular conditions associated with oxidative stress, which is considered the primary mechanism behind environmentally-induced telomere attrition (Haussmann & Marchetto 2010; Chatelain et al. 2020; Armstrong & Boonekamp 2023). Harsh environmental conditions, potentially resulting from certain parental decisions (e.g. larger broods), such as parasite infections, limited food availability and competition, have been linked to reduced telomere length

(Chatelain et al. 2020). On the contrary, favorable environmental conditions might not only limit telomere attrition but also trigger telomere elongation, allowing resources to be allocated for telomere restoration mechanisms (e.g. telomerase; Mizutani et al. 2013; Brown et al. 2022).

Telomere length has also a genetic background (Asghar et al. 2015a; Atema et al. 2015; but see Dugdale & Richardson 2018), but its heritability resembles epigenetic rather than genetic inheritance (Bauch et al. 2019). Instead of inheriting a variation in a gene sequence, the zygote inherits the telomere length of parental gametes as a phenotypic trait, meaning that differences in parental telomere length may persist in the offspring over their lifespan (Hjelmborg et al. 2015; Brown et al. 2021). Therefore, the possible association between parental age and offspring telomere length (Heidinger & Young 2020) may depend on both postnatal environmental conditions linked to parental age (the 'parental care effect') and the inheritance of gametes telomere length (the 'gamete effect'). Since telomere length and developmental environmental quality are expected to decline with age, a negative relationship between parental age and offspring telomere length is likely. However, previous findings are inconsistent regarding the presence or absence of the effect (presence: Olsson et al. 2011; Eisenberg et al. 2012, 2017; Asghar et al. 2015a; Criscuolo et al. 2017; Bouwhuis et al. 2018; Bauch et al. 2019; Brown et al. 2021 – absence: Froy et al. 2017; Belmaker et al. 2019), the relative contribution of father and/or mother (father: Olsson et al. 2011; Eisenberg et al. 2012, 2017; Bouwhuis et al. 2018; Bauch et al. 2019 – mother: Asghar et al. 2015a – both: Criscuolo et al. 2017; Brown et al. 2021), and the direction of the effect (positive: Eisenberg et al. 2012, 2017; Asghar et al. 2015a; Brown et al. 2021 - negative: Olsson et al. 2011; Criscuolo et al. 2017; Bouwhuis et al. 2018; Bauch et al. 2019). The absence of a clear generalizable pattern may stem from species-specific differences in how parental age affects the quality of the developmental environment (e.g. Geslin et al. 2004; Catry et al. 2006; Beamonte-Barrientos et al. 2010). Furthermore, the studies investigating parental and environmental effects on offspring telomere length simultaneously in wild populations are limited, complicating the evaluation of their relative contributions (Belmaker et al. 2019).

Telomere length is thus recognized as a possible mediator between early-life environmental, parental quality and survival later in life, since it can reflect the somatic state of an individual during development, thereby linking early-life conditions to future fitness (Monaghan & Haussmann 2006). However, despite the impact of early-life stages on telomere length due to intense metabolic activity during growth (Boonekamp et al. 2014; Parolini et al. 2015; Vedder et al. 2018; Stier et al. 2020), the factors shaping early-life telomere length and its consequences on long-term survival have not yet been extensively studied together in wild animals. In addition, most studies to date have assessed mortality outcomes shortly after measuring telomeres, which may obscure the long-term effects of telomere length on survival, especially considering the stochastic nature of early-life mortality (Eastwood et al. 2023). Investigating the relationship between early-life telomere length and total lifespan is a more effective approach to understand telomere length-associated mortality. However, studies on wild animals under natural selection regimes remain limited due to the practical challenges of conducting research that span the entire longevity of organisms in the wild. Therefore, the present study aims at investigating possible sources of variation in telomere length among male barn swallow (*Hirundo rustica*) nestlings, assessing whether the inter-individual variation translates into differential long-term survival. Specifically, we first evaluated the potential effects of developmental environment, parental age at conception and quality in the study species on nestlings' telomere length. Secondly, we tested whether the variation in telomere length among nestlings was significantly associated with a variation in survival after recruitment as breeding adults in the population. We predict that nestlings exposed to a stressful developmental environment and/or born from older and/or lower-quality parents should exhibit shorter early-life telomeres, which may be associated with reduced long-term survival.

MATERIAL AND METHODS

Study species

The barn swallow is a socially monogamous, short-lived, semicolonial migratory passerine bird (Møller 1994a). The breeding season of the European populations begins in March, as individuals return from their wintering sites in sub-Saharan Africa (Bazzi et al. 2015), with timing varying according to latitude (Møller 1994a), and lasts until August. Breeding adults exhibit strong breeding site fidelity by returning every year to the same breeding colony (Møller 1994a; Saino et al. 2002a, 2012a). In contrast, the barn swallow shows high natal dispersal, with most yearling breeders (i.e. 1-year-old birds in their first breeding season after hatching) nesting in different colonies from their natal ones (Møller 1994a; Balbontín et al. 2009). Barn swallows predominantly nest in rural buildings, favoring sites with presence of livestock and higher quality of crops (Møller 1994a; Ambrosini et al. 2012). Females lay 1-3 clutches per breeding season, with a modal size of 5 eggs (1 egg laid per day), which hatch after ca. 14 days of incubation. Both the male and female provide parental care until the offspring fledge, approximately 20 days after hatching.

While breeding adults (≥ 1 years-old) exhibit a moderate sexual dimorphism in size and some plumage ornaments, such as ventral coloration and outermost tail feathers length (Møller 1994a; Romano et al. 2017a), the nestlings of the two sexes are phenotypically indistinguishable until they moult after their first winter migration and reach sexual maturity (Romano et al. 2011, 2016).

Field procedures

In the 2010-2021 breeding seasons, as part of a long-term ongoing project started in 1993, we studied five colonies (= farms) of barn swallows breeding in Northern Italy near Milan. Nests were regularly inspected to collect breeding data (e.g. hatching dates and brood sizes). In addition, during each breeding season, breeding adults were captured and marked with unique metal and colored plastic rings for individual identification as well as breeding pair and nest assignment. Almost all the breeding adults within each colony were captured and marked with unique metal and colored plastic rings for individual identification, as well as breeding pair and nest assignment (see protocol details: Saino et al. 2011, 2012a). Capture efficiency was extremely high, as demonstrated by the negligible proportion of unmarked breeding adults within colonies at the end of each breeding season (~5%; Romano et al. 2025). Consistently, capture–recapture analyses in the same long-term monitoring project and colonies showed a high recapture probability between consecutive years (92.3%; Romano et al. 2025). Furthermore, individuals were subjected to standard measurement of various morphometric traits (e.g. outermost tail feathers length), following standard procedures (Saino et al. 2011, 2012a), and sexed according to morphological and behavioral traits, with molecular sexing used only in particularly rare uncertain cases (see protocol details: Saino et al. 2008a). Because of the high breeding philopatry of the species (Møller 1994a; Saino et al. 2002a, 2012a), as also supported by the virtually absent breeding dispersal in the study population (0.02%, Romano et al. 2025), combined with the high capture efficiency, individuals captured in the year-i, but not recaptured in the year-$i+1$, were considered dead. Furthermore, breeding adults captured for the first time in year-$i+1$ in a colony where we performed capture sessions the year-i were considered yearlings (i.e. 1-year-old birds in their first breeding season after hatching). Therefore, every captured individual was followed throughout its life and lifespan was estimated as the time in years between the year of hatching (i.e. the year before the first capture as breeding adult) and the last year in which an individual was recaptured as a breeding adult (e.g., an individual ringed as a nestling in 2012 and recaptured from 2013 to 2015, but not from 2016 onward, was assigned a lifespan of 3 years) . Because capture sessions were also conducted in the years following the last capture of each individual included in the study, lifespan was known for all individuals.

In addition, during nest monitoring, each nestling within colony was handled directly at the nest and marked with a unique metal ring at 6-16 days-old to enable long-term identification. Despite the extremely high natal dispersal (Møller 1994a; Balbontín et al. 2009), a little proportion of individuals, almost exclusively males (ca. 5%), return to their natal colonies as breeding adults and are referred as local recruits (Saino et al. 2012a). Due to the extensive marking of a huge number of nestlings, we were able to track many of these individuals throughout the study period and obtain complete and detailed life-history data, from the nestling stage to death. Specifically, throughout the study period (cohorts = 2010-2017), we ringed a total of 3,222 nestlings. Among these, 120 individuals (males = 111, females = 9) were recaptured as breeding adults the following years. For these individuals, information about their rearing environment were recorded, including hatching date (i.e., Julian date when the first egg in the clutch hatched), brood size (i.e. the number of nestlings present in the brood 10 days after hatching, reliably reflecting the final brood size; Møller 1994a), as well as the identities of their parents. Since extra-pair fertilizations may occur in the study species (Møller 1994a; Costanzo et al., 2017a), and paternity analyses were unfeasible because of the large number of years and colonies included in the study, thus implying molecular analyses of thousands of adult individuals, we refer to parents and fathers as “social parents” and “social fathers”, although the social father is typically also the genetic sire (Costanzo et al., 2017a). By contrast, obviously, social mothers can be assumed to be also the biological ones in all cases, given the negligible intra-specific brood parasitism (Møller 1994a, Costanzo et al. 2017a).

During the study period, 100 (95 males and 5 females) out of the 120 recruits, were also blood sampled at nestlings (age range: 8-13 days old; but for several individuals this information was missing), by puncturing their brachial vein and collecting the blood in heparinized capillary tubes. Blood samples were transported in cool bags from the field to the lab where they were centrifuged at 11500 x rpm for 15 minutes to separate red blood cells and plasma. Subsequently, they were kept at -20 °C until telomere length measurements were performed. However, when the quantification of telomere length was performed, blood sample was available for 79 individuals (males = 74, females = 5), as the others had been previously used for other research purposes. Given that the very small sample of available females would have prevented any analyses of sex-related variation, the analyses were performed on a total of 74 male recruits, thus representing the 67% of the total population of the local recruits of the study cohorts (2010-2017). For these individuals we therefore collected blood samples as nestlings, together with information on their rearing conditions and parental phenotype, as well as they were followed until death thanks to extensive capture sessions across their lives (see above). Overall, the 74 male recruits originated from 51 mothers and 47 fathers, with 13 mothers and 14 fathers contributing to more than one recruit in the dataset.

Telomere length analysis

Genomic DNA was isolated from 10-15 μL of red blood cells, reliable surrogates for telomere length of the whole organism in birds (Daniali et al. 2013; Reichert et al. 2013), using the Wizard DNA extraction kit (Promega, WI, USA). The DNA concentration and purity were assessed with a Nanophotometer (IMPLEN). Telomere length was measured following the monochrome multiplex quantitative PCR method (MMQPCR), according to the method developed by Cawthon et al. (2009), and using an iQ5 thermal cycler (BioRad). The reaction solution in each well was composed of 20 ng of template DNA (genomic DNA), 2X Quantitative Master Mix SYBR Green (Genespin) and primers pairs to amplify both telomere and the single copy gene CTCF, at the final concentration respectively of 1,000 nM and 500 nM each. The sequences of telomeric primers were: telg (5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3)' and telc (5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3'). The CTCF primers used were: forward (5'-CCCGCGGCGGGCGGCGCGGGCTGGGCGGCTCCCAATGGAGACCTCAC-3') and reverse (5'-CGCCGCGGCCCGCCGCGCCCGTCCCGCCCATCACCGGTCCATCATGC-3'). CTCF primers contain the GC-clamp (underlined part), which allow the amplification of both the primer pairs simultaneously in the same reaction. Each sample was run in triplicate as well as the four dilutions (5 ng, 20 ng, 50 ng and 100 ng) of a reference sample loaded to generate the standard curve. This standard curve was essential for calculating the reaction efficiency, determined by the formula $E = [10^{(-1/a)} - 1] \times 100$, where 'a' represents the slope of the linear function $y = ax + b$ that describes the curve, and the amount of telomeric repeats as well as CTCF in each sample. Because amplification efficiencies may deviate from 100% and differ between telomere and the single-copy gene across plates, RTL was calculated using the Pfaffl method (Pfaffl 2001), which accounts for differences in amplification efficiency between target sequences and plates. The mean efficiency for telomere amplification reaction was (±SD) 98.4% ± 10.4% while for the single-copy gene 97.4% ± 10%. The mean intra-plate coefficient of variation is 2.8%, while the inter-plate coefficient is 9.1%. The age when blood samples were collected (hereafter, age at sampling) did not predict nestling RTL, as suggested by the very weak correlation between age at sampling and nestling RTL (Pearson's correlation coefficient = -0.13; P = 0.27; n = 68). Hence, age at sampling was excluded from all the models (see "Statistical analyses") to preserve a larger sample size.

Statistical analyses

To test the hypothesis that early-life RTL may mediate the effect of the environmental conditions and social parents' traits on offspring lifespan, the analyses were structured in two parts. Sample size differed among models because not all individuals had complete information for all predictors (e.g. the identity of a parent was missing). In all models, either being the dependent or independent variable, nestling RTL was z-transformed (mean centered and scaled to standard deviation) to express effects in standard-deviation units and thus facilitate comparison with effect sizes reported in other studies adopting quantitative PCR (qPCR; Verhulst 2020).

A linear (mixed) model was used to examine whether and how nestling RTL variability within population was influenced by environmental factors and social parents' features. In this model, fixed effects included brood size (mean ± SD = 4.43 ± 0.81; range: 3 – 6) and hatching date, mean centered within cohort (hereafter, hatching date) to control for inter-annual variation in the onset of the breeding season, both as continuous covariates. Brood size was included due to its known association with nestling RTL in many bird species, including the barn swallow (Costanzo et al. 2017b; Voirin et al. 2023), while hatching date because reproductive success and offspring quality are known to progressively decline with the progression of the breeding season in the study population as a result of the gradual degradation of habitat quality (Ambrosini et al. 2006; Saino et al. 2012a). Social parents' traits, such as age and tail length, were also included among the covariates of the model to explore their possible effect on nestling RTL. The latter predictor was included because the length of the outermost tail feathers is a well-established secondary sexual trait, reflecting individual quality, in the barn swallow (Møller 1994a; Saino et al. 1997b; Romano et al. 2017a, 2017b). Furthermore, the correlation between paternal and maternal age within pairs was rather weak (Pearson's r =

0.38), thus justifying the inclusion in the same model. Initially, cohort (2010-2017), colony (n = 5) and family identity (n = 47), a variable identifying nestlings from the same or different broods that shared at least one of the social parents, were included as random factors to account for the non-independence of the data. However, they were later removed from the final model in favor of a simpler linear model as they explained a negligible amount of variance (< 0.01% of the total variance of the model, as indicated by the identical coefficients provided by both the linear and the linear mixed-effects models). Data from 10 individuals were excluded from this analysis due to missing social parents' information, including 8 individuals lacking assignment of both parents and 2 individuals only lacking father identity (sample size after removal of these individuals: n = 64). However, since the large number of predictors relative to sample size included in this analysis may have somehow affected the results, we checked the model by adopting a model selection approach. This was done by comparing AICc calculated for all the possible combinations of the previously described fixed effects, using *dredge* function from the MuMIn package (Barton 2009) in R version 4.4.1 (R Core Team 2024). Furthermore, to maximize sample size while reducing the number of predictors and random factors, the same model was re-run on the full dataset (n = 74) including only environmental variables (brood size and hatching date) as fixed effects and excluding family ID from the random effects, as eight individuals lacked assignment of both mother and social father. Also in this case, we removed all random effects as they explained a negligible amount of variance (< 0.01% of the total variance of the model), finally relying on a linear model.

Given the skewed distribution of lifespans in our dataset, reflecting the typical age distribution of our population, with a strong predominance of 1- and 2 year-old individuals (1 year-old: n = 50; 2 years-old: n = 17; 3 years-old: n = 3; 4 years-old: n = 2; 5 years-old: n = 2), the association between nestling RTL and long-term survival was assessed using two complementary analyses. First, we relied on a mixed-effects Cox proportional hazard model including nestling RTL, brood size and hatching date as fixed effects to test whether nestling RTL predict realized lifespan (n = 66; after exclusion of the 8 individuals lacking assignment of both parents due to the inclusion of family ID as a random effect). The latter two predictors were included since they have been shown to predict lifespan in the study population (Saino et al. 2012a, 2018). To account for potential variability linked to inter-annual variation in conditions, differences between colonies and shared social parentage, cohort, colony and family ID were modeled as random factors. Furthermore, we also included the two-way interaction between RTL and hatching date to test whether a possible effect of RTL on long-term survival varied along the breeding season (Stier et al. 2014), potentially intensifying under harsh developmental conditions. Again, to fully retain the largest possible sample size (n = 74), we re-run the same model excluding family ID from the random structure of the model.

Second, we investigated the relationship between nestling RTL and long-term survival probability, running a binomial mixed-effects model assuming a logit link function (n = 66), where long-term survival was analyzed as a binary variable, indicating whether an individual survived beyond the first year or not (0 = died at 1 year-old; 1 = survived 2 or more years). This categorization was justified because of the biology of the study species, where many differences in life-history (e.g. seasonal breeding output, timing of migration and breeding) and phenotypic traits (e.g. body size and tail length) between yearlings and older adult breeders have been well-documented (e.g. Møller 1994a; Saino et al. 2004, 2017). Such a categorization has been indeed used in dozens of previous studies on the same population (e.g. Saino et al. 2004, 2012b, 2013, 2017, 2019). The same fixed and random effect structure was applied as in the Cox proportional hazard model. This binomial model was likewise re-run on the total sample (n = 74) by excluding family ID from the random effects (to include the entire sample size).

All the analyses were performed with the statistical software R version 4.4.1 (R Core Team 2024). Linear mixed and binomial models were respectively performed by the *lmer* and *glmer* functions of the ‘lme4’ package (Bates et al. 2015). For the mixed-effects Cox proportional hazard model, we relied on the *coxme* function of the “coxme” package (Therneau 2024). Residual diagnostics for both linear and linear mixed-effects models were performed using *testResiduals* function of the ‘DHARMa’ package (Hartig 2019), confirming that all model assumptions were met. In addition, neither influential outliers nor collinearity were detected, as assessed using *check_outliers* and *check_collinearity* functions of the package ‘performance’ (Lüdecke et al. 2021), including in the binomial model. The proportional hazards assumption of the mixed-effects Cox model was inspected visually, as diagnostics are not available for this class of models.

RESULTS

Environmental and parental sources of variation in nestling RTL

Nestling RTL was negatively predicted by brood size, with nestling RTL progressively decreasing with increasing brood size, while hatching date did not have a significant effect (Table 1). In addition, social father age had a significant negative effect on nestling RTL, indicating that increasing age in social fathers is associated with reduced RTL in the offspring (Table 1; Figure 1). On the contrary, maternal age, along with both parents' tail length, did not predict nestling RTL (Table 1). These findings were confirmed by the model selection approach, as the 3 best-supported models (ΔAICc < 2) always retained both brood size and social father age (Table S1). Specifically, the best supported one included only father age and brood size both of which showed negative and significant relationships with nestling RTL (brood size: β ± SE = -0.41 ± 0.15, t_{61} = -2.77, p = 0.007; social father age: β ± SE = -0.32 ± 0.14, t_{61} = -2.32, p = 0.024).

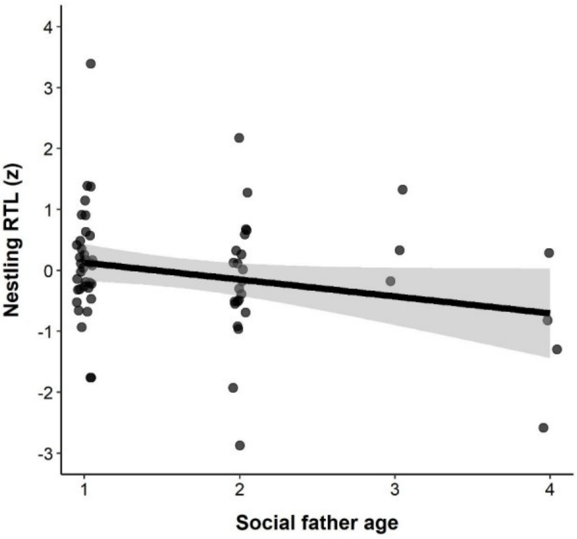

Figure 1. Relationship between social father age and nestling relative telomere length (RTL) z-transformed. The line represents the predictions of the linear model (n = 64) which included both environmental factors (brood size and hatching date) and social parents characteristics (age and tail length) as fixed effects. Shaded area represents lower and upper limits of the 95% confidence interval for the model estimates.

The linear model, applied to the larger sample and including only environmental variables as fixed effects, confirmed that brood size still had a significant negative effect on nestling RTL (β ± SE = -0.37 ± 0.14, t_{71} = -2.63, p = 0.01; Figure 2), even without controlling for social parents age and tail length. Hatching date again showed no significant effect (β ± SE = 0.004 ± 0.005, t_{71} = 0.77, p = 0.44).

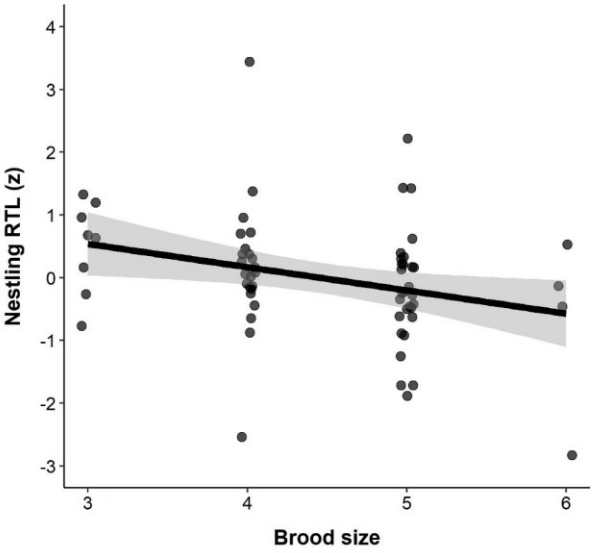

Figure 2. Association between brood size and nestling relative telomere length (RTL) z-transformed. The line represents the predictions of the linear model (n = 74), which included brood size and hatching date as fixed effects. Shaded area indicates the 95% confidence interval of the model predictions.

Relationship between nestling RTL and long-term survival

A positive, although non-statistically significant, trend emerged between nestling RTL and realized lifespan in the mixed-effects Cox proportional hazards model (Table 2). Brood size and hatching date also showed no significant effects (Table 2). Furthermore, the two-way interaction between RTL and hatching date did not significantly affect mortality risk (HR ± SE = 1.0002 ± 0.0002, z = 0.03, p = 0.98), suggesting that telomere-associated mortality remains consistent along the breeding season, and it was thus removed from the final model (Table 2).

However, in the binomial model assessing whether nestling RTL predicted the probability of surviving beyond the first year of life, we observed that individuals with longer RTL were more likely to become long-lived (age ≥ 2 years) adults that will attend more than one breeding season (Table 2; Figure 3). Again, the two-way interaction between RTL and hatching date was not significant (β ± SE = –0.009 ± 0.024, z = –0.39, p = 0.69) and thus removed from the final model (Table 2).

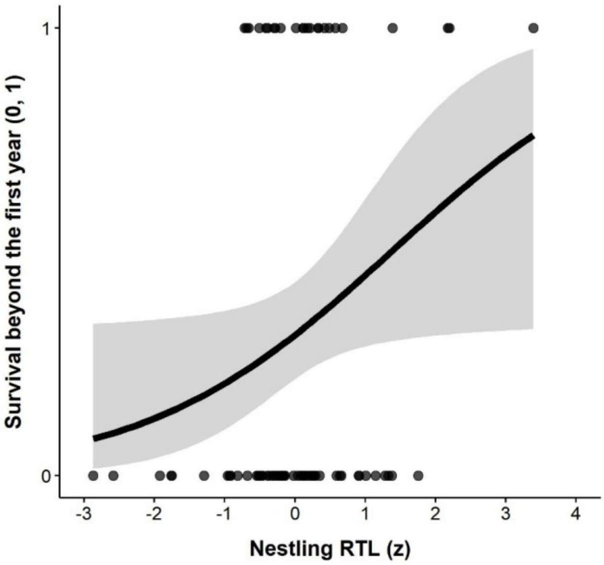

Figure 3. Relationship between nestling relative telomere length (RTL) z-transformed and survival beyond the first year (0 = died at 1 year-old; 1 = survived 2 or more years). The line is the predicted probability of surviving beyond the first year of life, estimated by using a binomial generalized linear mixed model (n=66). The model included nestling RTL (z-transformed), brood size and hatching date as fixed effects, while cohort, colony and family identity as random factors. Shaded area represents the 95% confidence interval for the model estimates.

Predictors	**Estimate (SE)**	***df***	**t**	**P**
(Intercept)	1.993 (5.982)	57	0.33	0.74
Brood size	**-0.404 (0.154)**	57	-2.63	**0.011**
Father age	**-0.345 (0.168)**	57	-2.05	**0.045**
Hatching date†	0.005 (0.006)	57	0.95	0.34
Mother age	-0.070 (0.163)	57	0.43	0.66
Father tail length	-0.011 (0.014)	57	-0.81	0.41
Mother tail length	0.023 (0.025)	57	0.93	0.35

† *mean centered within year*

Table 1. Summary of the linear model (n=64) assessing the effects of developmental environment, as well as social parents' age and tail length on nestling relative telomere length (RTL) z-transformed. Fixed effects of the model were brood size, hatching date, social parents age and social parents tail length. Significant fixed effects are reported in bold

a)

Predictors	**Coefficient**	**Exp (coef.)**	**SE**	**z**	**P**
Nestling RTL	-0.20	0.82	0.15	-1.38	0.17
Hatching date†	-0.001	1.001	0.006	0.23	0.82
Brood size	-0.26	0.77	0.176	-1.50	0.13
Random effects	**τ00 (SE)**				
Cohort	0.35 (0.12)				
Colony	<0.001 (0.010)				
Family identity	<0.001 (0.008)				

b)

Predictors	**Estimate (SE)**	**z**	**P**
(Intercept)	**-6.14 (2.71)**	-2.26	**0.023**
Nestling RTL	**0.86 (0.41)**	2.08	**0.037**
Hatching date†	-0.001 (0.017)	-0.09	0.92
Brood size	1.10 (0.56)	1.95	0.051
Random effects	**τ00 (SE)**		
Cohort	0.35 (0.12)		
Colony	0.016 (<0.001)		
Family identity	0.018 (<0.001)		

† *mean centered within year*

Table 2. a) Results of the mixed-effects Cox proportional hazard model (n=66) evaluating how survival probability changes over the entire lifespan based on nestling relative telomere length (RTL) z-transformed, hatching date and brood size; **b)** Results of the binomial linear mixed-effects model evaluating the association between nestling RTL (z-transformed) and survival beyond the first year. Nestling RTL, hatching date and brood size were again included as fixed effects. In both models, random effects included were cohort, colony and family identity. Significant fixed effects are reported in bold

All the results were further confirmed by removing the random factor family identity and thus applying both approaches to the entire sample (n = 74; Table S2).

DISCUSSION

The present study identified brood size and social father age as significant sources of variation in in telomere length among male barn swallow nestlings. Moreover, telomere length at the nestling stage positively the probability of surviving beyond the first year.

Although telomere length in relation to brood size has been extensively studied in recent years, with coherent findings across species (Nettle et al. 2015; Gil et al. 2020; Young et al. 2022), our analysis provided a valuable insight by evaluating this relation across a wide range of cohorts. We found a progressive reduction in telomere length at nestling with increasing brood size, a pattern consistent with previous studies on birds (Nettle et al. 2015; Gil et al. 2020; Young et al. 2022), including the barn swallow (Costanzo et al. 2017b; Voirin et al. 2023). This pattern can be interpreted in the framework of the life-history theory. In this context, parental decision regarding brood size involves balancing offspring quantity against individual quality, with parents of larger broods prioritizing the number of nestlings but taking the risk of not providing optimal investment to each individual, thus potentially reducing the per capita allocation of essential resources such as food (Dijkstra et al. 1990; Saino et al. 1997a, 2000). A reduced food intake may then impair offspring development, affecting growth and survival prospects (e.g. Saino et al. 1997a; Brzęk & Konarzewski 2007). Under such circumstance, nestlings in larger broods also face larger sibling competition (Saino et al. 2000; Neuenschwander et al. 2003; Romano et al. 2013), and may therefore experience a stressful developmental environment that may activate the hypothalamic-pituitary-adrenal axis and induce oxidative stress (Liu & Mori 1999; Costantini et al. 2011), which have been proposed among the main proximate mechanisms linking stressful environmental condition to shorter telomeres (Haussmann & Marchetto 2010; Chatelain et al. 2020; Armstrong & Boonekamp 2023).The higher density of individuals in larger broods also facilitates the transmission of ectoparasite (Saino et al. 2002b), which is another external factor known to exacerbate telomere attrition, thus possibly resulting in shorter telomeres (Asghar et al. 2016, Badás et al. 2023). Since both brood size (e.g. Saino et al. 1997a; Brzęk & Konarzewski 2007) and telomere length (Wilbourn et al. 2018; Eastwood et al. 2019, 2023) have been linked to reduced survival prospects, our results suggest that variation in early-life telomere length may mediate the trade-off between offspring quantity and quality.

In addition, nestling telomere length declined with increasing social father age, providing possible evidence of a sort of epigenetic inheritance of telomere length. Assuming an age-dependent decrease in sperms telomere length in adult barn swallows, as it occurs in somatic cells of other vertebrates (Remot et al. 2022), including the barn swallow (Kauzálová et al. 2022), this negative trend with paternal age might arise because of the ‘gamete effect’ (inheritance of gametes telomere length). In a socially monogamous species with extra-pair fertilizations, such as the barn swallow (Costanzo et al. 2017a), the social father may not always correspond to the biological sire, and thus genetic interpretation of this association applies only in cases where social father also represents the genetic sire. However, the percentage of extra-pair nestlings during the study period was shown to be only 12.7% (Costanzo et al. 2017a), thus likely providing only marginal contribution to the results. In addition, and non-mutually exclusive, as the barn swallow is a species with biparental care of the offspring, it is possible that a decline in social father performances due to physiological senescence might be detrimental for parental care quality, compromising the developmental environment (Vleck et al. 2011; Moorad & Nussey 2016; Lemaître & Gaillard 2017). In that context, although the barn swallow does not show visible phenotypic signs of senescence (Møller et al. 2005), social father aging could have still affected offspring development (Saino et al. 2002a) and, thus, the pattern observed might be also consistent to the 'parental care effect' (effect of parental care quality on developmental conditions). The reduction of telomere length at increasing social father age is in contrast with the pattern observed in humans and chimpanzees (Eisenberg et al. 2012, 2017), which are the most comprehensively studied species, but it is consistent with studies conducted on other birds (Bouwhuis et al. 2018; Bauch et al. 2019). In humans and chimpanzees, sperm telomeres usually elongate over time (Aston et al. 2012), possibly because of an overexpression of telomere maintenance genes that might have been driven by their promiscuous mating system. Frequent sperm production, which accelerates telomere attrition through the 'end replication problem', may have intensified the selective pressure on telomere length maintenance. In contrast, seasonally breeding species such as the barn swallow, with lower sperm production rates, may face lightened selective pressure over telomere restoration mechanisms (Eisenberg & Kuzawa 2018). This may be even more pronounced in short-lived species such as the barn swallow, where the selective pressures over molecular mechanisms involved in the maintenance of germinal and somatic tissues may be weaker than in long-lived species. This is supported by studies showing a lower rate of telomere attrition in long-lived species compared to short-lived ones (Tricola et al. 2018; Criscuolo et al. 2021).

On the contrary, maternal age did not predict telomere length of the nestlings. Differently from social father age, maternal age is less often associated with offspring telomere length in wild animals, but few examples exist (positive relationship: Asghar et al. 2015a; negative relationship: Criscuolo et al. 2017; Brown et al. 2021). This sex-specific influence of parental age for offspring telomeres may reflect the different replicative history of sperms and ova. The formers are produced continuously throughout most of life (Kirby & Froman 2000), while the latter originate from germline cells established early in development (Johnson 2000), possibly hiding maternal aging effect on offspring telomeres. Speculating, since both the mother and father provide parental care in the barn swallow, the observed association with paternal, but not maternal, age may suggest that the social father age influence on nestling telomere length may arise more likely because of the ‘gamete effect’ rather than the ‘parental care effect’.

An additional, though indirect, evidence of this interpretation is provided by the lack of any relationship between parents’ tail length (condition/quality-dependent parental traits; Møller 1994a; Saino et al. 1997b; Romano et al. 2017a, 2017b) and telomere length of their nestlings. Given that in the barn swallow longer-tailed individuals have been observed to provide less food to their broods compared to low-quality ones (e.g. Møller 1994b; Møller & Thornhill 1998; Hasegawa & Arai 2015), consistent with a trade-off where more ornamented males allocate relatively more effort into mating activities (e.g., extra-pair fertilizations) at the expense of parental care (e.g. Møller 1994a ; Møller 1994b; Møller & Thornhill 1998; Hasegawa & Arai 2015), the lack of any relationship between paternal tail and nestling telomere length suggests that parental care after hatching may not be primarily involved in affecting offspring telomere length. However, as the study is correlational and lacks paternity data and parental telomere length, we cannot directly disentangle the ‘gamete effect’ and the ‘parental care effect’.

Nestling telomere length significantly predicted survival beyond the first year of life, but not the realized lifespan. Our results are therefore partly consistent with previous studies reporting positive association between early-life telomere length and long-term survival (reviewed in Wilbourn et al. 2018; Eastwood et al. 2019, 2023). This association may reflect a causal pathway, whereby individuals with longer RTL take longer to reach the critical threshold, beyond which cells lose functionality, thus impairing somatic functions (Campisi et al., 2001; Deng et al., 2008). Alternatively, it may be mainly driven by shared underlying determinants (e.g., early-life conditions) that jointly influence both RTL and survival (Monaghan, 2010, 2024; Young, 2018). Because our study is correlational, we cannot disentangle these alternative mechanisms. The reason why such a relationship emerged only on survival between the first and the second years of life, and not thereafter, may stem from the specific life-history of the study species. In our population, annual mortality is very high (~65%; Romano et al. 2025), and the vast majority of adult breeders are thus 1-year old individuals. Consequently, after the likely strong selection occurring from nestling to recruitment, which cannot be tested because the extremely high natal dispersal makes impossible to distinguish between death vs. dispersal events (Møller 1994a), the transition from the first to the second year of life does likely represent another main selective phase, making any association between proxies of somatic state and survival most detectable at this stage. This interpretation is consistent with the marked differences between yearlings and older breeders in many life-history (e.g. seasonal breeding output, timing of migration and breeding) and phenotypic traits (e.g. body size and tail length), while differences among older age classes are negligible (Møller 1994a; Saino et al. 2004, 2017). By contrast, older age classes comprise only a small fraction of the population, and survival later in life is therefore expected to be more weakly shaped by selection on survival-related traits. In addition, because only few individuals in our sample reached older ages, lifespan analyzed as a continuous variable may provide limited power to detect an association with telomere length.

In many species, lifespan is a major determinant of lifetime reproductive success, predicting the number of reproductive events (Newton 1989), including in the study population (Saino et al. 2012a). Considering our results, along with previous findings showing that telomere length at sexual maturity positively covaried with seasonal reproductive success in the study population (Parolini et al. 2017), it is therefore possible that male barn swallow nestlings with longer telomeres may have ultimately achieved higher lifetime fitness, coherently with other recent avian studies (Eastwood et al. 2019; Heidinger et al. 2021), suggesting that longer telomeres at nestling may be favored by natural selection. However, we could not test this hypothesis on our sample due to the lack of complete reproduction data across the entire life of several individuals, thus preventing any formal statistical analysis.

Overall, our findings suggest that nestlings exposed to competitive rearing conditions and raised by older fathers exhibit shorter telomeres. This could have reduced individuals' somatic maintenance potential and thereby impaired their long-term survival. More specifically, the present study supports the idea of telomere length as mediator of the effect of early-life conditions, which are partly determined by parental decisions, on mortality later in life. However, notably, the association between early-life telomere length and survival emerged in our study likely reflects species- and population-specific traits, where surviving to the second reproduction may be particularly challenging. In addition, our analyses are restricted to males (local recruits) only and therefore the conclusions drawn may not extend to females (and/or dispersers), especially considering that sex-specific competitive behavior and stress responses have been reported in barn swallow nestlings (Boncoraglio et al. 2008; Bonisoli-Alquati et al. 2008; Saino et al. 2008b; Romano et al. 2011), and such differences could modulate the relationship between early-life conditions, telomere length and later-life survival. Therefore, more empirical research investigating sources of variation in telomere length in wild populations is needed, especially to disentangle environmental influences and heritable components. Furthermore, future studies assessing the possible relationship between telomere length at nestling and lifetime reproductive success are necessary to better understand the role of telomere length in driving the evolution of life-history traits through natural selection.

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SUPPLEMENTARY MATERIALS

model	(Intercept)	brood size	social father age	mother age	tail father	tail mother	hatching date	df	logLik	AICc	delta AICc	weight
1	2.36074	-0.41176	-0.32438					4	-84.7022	178.0823	0	0.18813069
2	0.46019	-0.41365	-0.36976			0.02224		5	-84.2809	179.5962	1.5139	0.08824965
3	2.27108	-0.39246	-0.32131				0.00481	5	-84.3372	179.7090	1.6267	0.08341301
4	3.10938	-0.41432	-0.29132		-0.00742			5	-84.5446	180.1236	2.0413	0.06779482
5	2.34847	-0.41735	-0.33929	0.03776				5	-84.6713	180.3771	2.2948	0.05972319
6	1.64789	-0.37005						3	-87.4081	181.2163	3.1339	0.03925828
7	0.39214	-0.39456	-0.36624			0.02200	0.00475	6	-83.9203	181.3144	3.2321	0.03737868
8	3.60716	-0.38629			-0.01769			4	-86.4173	181.5126	3.4303	0.03385136
9	1.19617	-0.41699	-0.33342		-0.00916	0.02445		6	-84.0410	181.5556	3.4733	0.03313151
10	3.14914	-0.39381	-0.28192		-0.00878		0.00523	6	-84.1166	181.7069	3.6246	0.03071824
11	2.24489	-0.39991	-0.34480	0.06009			0.00517	6	-84.2602	181.9941	3.9118	0.02660816
12	0.48243	-0.41745	-0.37925	0.02590		0.02188		6	-84.2663	182.0063	3.9240	0.02644733
13	3.16524	-0.42234	-0.30884	0.05256	-0.00814			6	-84.4860	182.4457	4.3634	0.02123071
14	1.55893	-0.34977					0.00517	4	-87.0217	182.7213	4.6390	0.01849715
15	3.63420	-0.36393			-0.01887		0.00597	5	-85.8886	182.8116	4.7293	0.01768068
16	1.76891	-0.36018		-0.10152				4	-87.1629	183.0037	4.9214	0.0160616
17	1.23093	-0.39643	-0.32410		-0.01053	0.02452	0.00525	7	-83.6034	183.2068	5.1245	0.01451077
18	0.45092		-0.27749					3	-88.4979	183.3958	5.3135	0.01320254
19	1.54859	-0.36986				0.00110		4	-87.4071	183.4921	5.4098	0.01258181
20	2.85377	-0.38573			-0.01902	0.01001		5	-86.3320	183.6984	5.6161	0.01134857
21	0.42922	-0.40045	-0.38360	0.04797		0.02132	0.00504	7	-83.8711	183.7421	5.6598	0.01110328
22	3.53184	-0.38073		-0.04613	-0.01652			5	-86.3697	183.7739	5.6916	0.01092814
23	3.23925	-0.40404	-0.30792	0.08091	-0.01003		0.00577	7	-83.9804	183.9608	5.8785	0.00995321
24	1.27681	-0.42342	-0.34677	0.04241	-0.00972	0.02400		7	-84.0024	184.0048	5.9225	0.00973647
25	0.45072		-0.27619				0.00715	4	-87.7621	184.2021	6.1198	0.008822
26	1.66773	-0.34374		-0.08356			0.00463	5	-86.8579	184.7503	6.6680	0.00670681
27	0.00000							2	-90.3081	184.8130	6.7306	0.00650006
28	-1.37385		-0.32065			0.02126		4	-88.1565	184.9909	6.9086	0.00594673
29	2.84053	-0.36313			-0.02027	0.01054	0.00602	6	-85.7922	185.0581	6.9758	0.00575017
30	1.46340	-0.34958				0.00106	0.00517	5	-87.0207	185.0759	6.9935	0.00569942
31	3.60560	-0.36234		-0.01716	-0.01840		0.00584	6	-85.8821	185.2380	7.1556	0.00525571
32	1.33977	-0.35871		-0.10780		0.00486		5	-87.1429	185.3203	7.2380	0.00504371
33	1.06989		-0.24946		-0.00623			4	-88.3991	185.4761	7.3938	0.00466565
34	0.00193						0.00723	3	-89.5963	185.5927	7.5104	0.00440154
35	1.36830	-0.40528	-0.34550	0.07060	-0.01157	0.02377	0.00572	8	-83.4986	185.6153	7.5330	0.00435201
36	0.47977		-0.26671	-0.02854				4	-88.4819	185.6417	7.5594	0.00429497
37	1.61964				-0.01519			3	-89.6408	185.6816	7.5992	0.00421022
38	-1.34912		-0.31877			0.02097	0.00710	5	-87.4222	185.8789	7.7966	0.00381458
39	2.65720	-0.37881		-0.05670	-0.01775	0.01139		6	-86.2614	185.9966	7.9143	0.00359667
40	1.81078				-0.01696		0.00803	4	-88.7519	186.1818	8.0995	0.00327846
41	1.27287		-0.23890		-0.00828		0.00755	5	-87.5858	186.2061	8.1238	0.0032389
42	0.21772			-0.13398				3	-89.9136	186.2272	8.1449	0.00320487
43	0.44430		-0.27858	0.00634			0.00719	5	-87.7613	186.5570	8.4747	0.00271767
44	-0.24578					0.00276		3	-90.3020	187.0040	8.9217	0.00217339
45	-0.75391		-0.28907		-0.00788	0.02315		5	-87.9994	187.0333	8.9510	0.00214177
46	1.30024	-0.34261		-0.08908		0.00417	0.00459	6	-86.8431	187.1599	9.0776	0.00201044
47	-1.38224		-0.30656	-0.04039		0.02183		5	-88.1243	187.2830	9.2007	0.00189036
48	0.17507			-0.10667			0.00651	4	-89.3486	187.3751	9.2928	0.00180535
49	2.74509	-0.36053		-0.02766	-0.01961	0.01120	0.00582	7	-85.7758	187.5516	9.4693	0.00165283
50	1.52641			-0.09214	-0.01291			4	-89.4661	187.6103	9.5279	0.00160506
51	0.82571				-0.01659	0.01059		4	-89.5544	187.7867	9.7044	0.0014695
52	-0.56488		-0.27878		-0.00995	0.02334	0.00758	6	-87.1691	187.8119	9.7296	0.00145113
53	1.06422		-0.24367	-0.01824	-0.00599			5	-88.3927	187.8198	9.7375	0.00144539
54	-0.22733					0.00257	0.00723	4	-89.5909	187.8598	9.7774	0.00141682
55	-1.35044		-0.31680	-0.00570		0.02105	0.00706	6	-87.4216	188.3169	10.2345	0.00112734
56	0.96736				-0.01846	0.01126	0.00808	5	-88.6514	188.3373	10.2550	0.00111589
57	-0.45207			-0.14371		0.00769		4	-89.8675	188.4130	10.3307	0.00107445
58	1.74884			-0.05146	-0.01560		0.00761	5	-88.6984	188.4314	10.3491	0.00106461
59	1.28504		-0.24621	0.02380	-0.00864		0.00772	6	-87.5751	188.6238	10.5415	0.00096694
60	-0.78771		-0.28060	-0.02834	-0.00752	0.02347		6	-87.9838	189.4413	11.3590	0.00064251
61	-0.39477			-0.11524		0.00655	0.00644	5	-89.3146	189.6637	11.5814	0.00057491
62	0.53179			-0.10403	-0.01435	0.01310		5	-89.3359	189.7063	11.6240	0.00056278
63	-0.54617		-0.28270	0.01357	-0.01014	0.02319	0.00768	7	-87.1656	190.3312	12.2489	0.00041177
64	0.77995			-0.06321	-0.01699	0.01275	0.00758	6	-88.5721	190.6180	12.5357	0.00035676

Table S1. Results of the model selection applied on the linear model including brood size, hatching date and social parents’ traits (age and tail length) as fixed effects.

a)

Predictors	**Coeffiecient**	**Exp (coef.)**	**SE**	z	**P**
Nestling RTL	-0.20	0.82	0.14	-1.41	0.16
Hatching date†	0.002	1.002	0.006	0.37	0.71
Brood size	-0.27	0.76	0.17	-1.62	0.11
Random effects	**τ00 (SE)**				
Cohort	0.49 (0.24)				
Colony	0.02 (<0.001)				

b)

Predictors	**Estimate (SE)**	z	**P**
(Intercept)	**4.83 (2.19)**	-2.20	**0.020**
Nestling RTL	**0.76 (0.36)**	2.08	**0.037**
Hatching date†	-0.004 (0.015)	-0.29	0.77
Brood size	0.84 (0.46)	1.83	0.067
Random effects	**τ00 (SE)**		
Cohort	1.70 (1.30)		
Colony	<0.001 (<0.001)		

† *mean centered within year*

Table S2 – a) Results of the mixed-effects Cox proportional hazard model applied to the entire sample (n=74) and evaluating how survival probability changes over the entire lifespan based on nestling relative telomere length (RTL) z-transformed, hatching date and brood size; **b)** Results of the binomial linear mixed-effects model applied to the entire dataset (n=74) and evaluating the association between nestling RTL (z-transformed) and survival beyond the first year. Nestling RTL, hatching date and brood size were included as fixed effects. In both models, random effects included were cohort and colony. Significant fixed effects are reported in bold.

Chapter 4

Telomere length at recruitment predicts lifespan and lifetime reproductive success in the barn swallow (*Hirundo rustica*)

Andrea Novelli, Manuela Caprioli, Roberto Ambrosini, Alessandra Costanzo, Diego Rubolini, Marco Parolini, Andrea Romano

Telomere length at recruitment predicts lifespan and lifetime reproductive success in the barn swallow (*Hirundo rustica*)

Andrea Novelli[1*], Manuela Caprioli[1], Roberto Ambrosini[1], Alessandra Costanzo[1], Diego Rubolini[1], Marco Parolini[1], Andrea Romano[1]

ABSTRACT

Identifying molecular mechanisms linked to variation in lifespan, reproductive output, and sexually selected traits is key to understanding life-history evolution in wild populations. Telomere length (TL) and dynamics (ΔTL) have been proposed as biomarkers of somatic state and maintenance (survival). Yet evidence linking telomeres to lifetime fitness and ornament expression remains limited. We quantified age-related variation in TL and annual ΔTL between the year of recruitment (i.e. 1-year old) and death, as well as tested whether TL at recruitment predicts realized lifespan and lifetime reproductive success, in a short-lived migratory passerine, the barn swallow (*Hirundo rustica*). We also examined associations between TL/ΔTL and two sexual ornaments (outermost tail feathers length and ventral plumage colouration). TL constantly declined with age, and longer TL as yearling was linked to increased lifespan and higher number of fledglings during the entire life, indicating that TL captures meaningful variation in individual quality. Moreover, longer tail feathers and a larger increase in tail length between consecutive years were associated, respectively, with shorter TL and faster ΔTL among females, whereas plumage darkening between consecutive years was associated with reduced ΔTL in both sexes, indicating that telomeres covary with secondary sexual traits in a sex- and trait-specific manner, and may be involved in sexual selection processes. Overall, even in a species with high annual mortality, TL positively predicted major fitness components, supporting TL as a biomarker of individual quality and suggesting that longer telomeres at the onset of adulthood may be favoured by natural selection in wild populations.

Key words: Telomere length, Telomere attrition, Lifespan, Lifetime reproductive success, Sexual ornaments, Barn swallow

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INTRODUCTION

Explaining the mechanisms shaping interindividual differences in Darwinian fitness is a central goal in evolutionary ecology. Although fitness components are expressed at the organismal level as the combination of survival and reproductive success, understanding why they differ among individuals necessarily requires considering the physiological processes linked to individual somatic quality and, ultimately, performance. Identifying molecular mechanisms that capture this physiological variation is therefore crucial for understanding how natural and/or sexual selection may have driven the evolution of species-specific life-history traits. In this context, telomeres, the nucleoprotein complexes at the ends of eukaryotic chromosomes known to preserve genome stability and integrity (Blackburn, 1991, 2005), may play a pivotal role (Monaghan, 2010; Monaghan et al., 2022; Monaghan, 2024).

In somatic cells, telomere length progressively decreases with each cell division due to the ‘end-replication problem’, which occurs because DNA polymerases cannot fully replicate linear chromosomes (Harley et al., 1990; Blackburn, 2005). In addition to the replicative history, the rate at which telomeres shorten also depends on the exposure to various forms of stressful environmental conditions (e.g., harsh climatic conditions, competition, parasite burden - Monaghan, 2014; Costanzo, Parolini, et al., 2017; Eastwood et al., 2022; Badás et al., 2023; Eastwood et al., 2023a) , as well as on metabolic rates (e.g., Monaghan & Ozanne, 2018; Criscuolo et al., 2021), both of which can increase cell division rates and/or reactive oxygen species (ROS) production, thereby resulting in higher oxidative stress to which telomeres can be particularly vulnerable (Haussmann & Marchetto, 2010; Chatelain et al., 2020; Armstrong & Boonekamp, 2023). Although the activity of the enzyme telomerase partially counteract telomere attrition by adding new telomeric repeats (Greider & Blackburn, 1989; Lingner et al., 1997), the above-mentioned factors generally lead to telomere shortening with age, as shown across multiple vertebrate species using both cross-sectional and longitudinal approaches (Remot et al., 2022). However, evidence of telomere elongation under favourable physiological or environmental conditions suggests that telomere restoration may occur, although such cases are rare and context-dependent (Mizutani et al., 2013; Noguera et al., 2015; Brown et al., 2022).

Critically shortened telomeres trigger cell senescence or apoptosis, thereby impairing tissue renewal capacity and potentially leading to a progressive deterioration of individual phenotype and performance (Campisi et al., 2001; Deng et al., 2008). This suggests that telomere length and its attrition rate may be used as biomarkers of individual somatic state and investment in self-maintenance. They have been indeed increasingly investigated as predictors of survival in many taxa, with longer telomeres and/or slower telomere attrition associated with reduced mortality risk (Salomons et al., 2009; Boonekamp et al., 2014; Wilbourn et al., 2018). Moreover, this pattern is more likely to emerge when telomere length is investigated in association with entire lifespan, because repeated survival outcomes across years may better reflect persistent differences in somatic state among individuals (Eastwood et al., 2019; Bichet et al., 2020; Sheldon et al., 2022; Vedder et al., 2022;

Eastwood et al., 2023b). However, the relationship between telomere length and lifespan is not consistently observed across studies in wild populations (Caprioli et al., 2013; Sudyka et al., 2019; Kauzálová et al., 2022; Pepke et al., 2022; Chik et al., 2024), likely because stochastic extrinsic mortality can weaken the link between physiological condition and survival (Monaghan, 2010; Stier et al., 2014; Criscuolo et al., 2021; Pepke et al., 2023; Monaghan, 2024).

In iteroparous species increased longevity may provide a higher number of reproductive opportunities, thus lifespan often translates into higher lifetime reproductive success (Newton, 1989). Therefore, if longer telomeres are associated with higher somatic quality, and this translates into increased longevity, they may also predict higher lifetime reproductive success, and thus be favoured by natural selection (Monaghan, 2010; Monaghan et al., 2022; Monaghan, 2024). Accordingly, long-term studies reported positive associations between telomere length and lifetime reproductive success (Pauliny et al., 2006; Eastwood et al., 2019; Heidinger et al., 2021; Chik et al., 2024). These studies are therefore particularly informative for understanding whether, and under which conditions, telomeres are relevant to life-history evolution. However, they remain scarce mainly due to the logistical challenges of monitoring individuals throughout their entire lifespan in the wild (e.g., Pauliny et al., 2006; Eastwood et al., 2019; Sudyka et al., 2019; Heidinger et al., 2021; Morland et al., 2023; Chik et al., 2024). Furthermore, findings remain mixed across species because pace-of-life differences and ecological conditions can modulate the association between telomere length and lifespan (e.g., Tricola et al., 2018; Criscuolo et al., 2021), while also influencing the metabolic costs of reproduction (Bauch et al., 2016; Merkling et al., 2017; Noguera, 2017; Costantini, 2024), which are more likely to be captured by telomere dynamics rather than by telomere length measured at a single time point (Sudyka et al., 2019; Sudyka, 2019).

The production and maintenance of secondary sexual traits to attract potential mates can represent a substantial component of reproductive costs (e.g., Huhta et al., 2003; Webster et al., 2018). However, sexual selection theory also suggests that secondary sexual traits function as honest signals of individuals' genetic/phenotypic quality and are used by the sex investing more in reproduction to select high-quality mates (Andersson, 1994; Nolazco et al., 2022). Under such a scenario, again, if telomere length and/or dynamics are a major component in variation in individual quality, they are expected to covary with the expression of secondary sexual traits. Alternatively, if telomere length and/or dynamics reflect the physiological cost of ornament expression, more elaborate traits would be associated with shorter telomeres and/or faster telomere attrition. Given these contrasting predictions, investigating the association between telomeres and secondary sexual traits becomes crucial to understanding the molecular mechanisms underlying sexual selection processes. However, previous studies are limited and provided conflicting evidence, from positive (Parolini et al., 2017; Taff & Freeman-Gallant, 2017), to negative (Azcárate-García et al., 2020; Kauzálová et al., 2022) or no association (Morosinotto et al., 2022) between telomere and sexually selected traits (Azcárate-García et al., 2020; Kauzálová et al., 2022).

Here, we conducted a longitudinal study of telomere length and dynamics in a short-lived long-distance migratory passerine bird, the barn swallow (*Hirundo rustica*). The aim of the study was to evaluate their relationships with lifespan, lifetime reproductive success, and the expression of secondary sexual traits, and thus assess whether and how telomeres are associated with fitness in a short-lived species where stochastic mortality is expected to be high. Specifically, we focused on breeding adults (≥ 1-year old) monitored throughout their entire lives, and we aimed to: 1) examine variation in telomere length and its annual change according to age; 2) assess whether telomere length at recruitment (yearlings = 1-year old individuals) predicts lifespan and lifetime reproductive success; and 3) investigate the association between telomere length/annual change in telomere length and the expression of secondary sexual traits, specifically the length of outermost tail feathers (hereafter, tail length) and ventral plumage colouration, that are well-established sexually selected traits in the barn swallow (Costanzo et al., 2017a; Romano et al., 2017a; Romano et al., 2017b).

MATERIAL AND METHODS

Study organism

The barn swallow is a short-lived, long-distance migratory and socially monogamous passerine bird (Turner, 2006). European populations breed semi-colonially in rural buildings from April to August and spend the non-breeding season south of the Sahara (Turner, 2006). Breeding adults (≥ 1-year old) show strong breeding philopatry, returning to the same colony in consecutive years (Turner, 2006; Romano et al., 2025). Females lay 1-3 clutches per season with 1-7 eggs each (modal size: 5 eggs). They lay one egg per day and incubate for approximately 14 days. After hatching, both parents provide parental care to the offspring, which fledge after ca. 20 days (Turner, 2006). Adults show moderate sexual dimorphism, with males typically displaying darker ventral plumage colouration and longer outermost tail feathers than females (Turner, 2006; Saino et al., 2013a). Both traits are under sexual selection, with longer tail feathers and darker ventral plumage associated with higher viability and fitness (Costanzo et al., 2017a; Romano et al., 2017a; Romano et al., 2017b). Additionally, these traits show age-dependent variation in both sexes, with individuals becoming generally darker and developing longer tail feathers as they age (Møller, 1991; Romano et al., 2017b). Independently of these age-related patterns, ventral plumage colouration and outermost tail feathers length can also vary between years within the same individual, as they are both renewed annually during the complete plumage moult performed in African non-breeding grounds (Moller et al., 1995; Saino et al., 2013b).

Field procedures

We studied three cohorts (2012 to 2014) of breeding barn swallows from recruitment as adult breeders (i.e. yearlings: age = 1-year old) to death at five colonies (= farms) located south-east of Milan (average coordinates: 45.30° N, 9.50° E), in northern Italy, over nine years. In all study years (2012-2020), between April and June, we performed at least two capture sessions of breeding adults, during which they were marked with univocally numbered metal and plastic colour rings (details in Saino et al., 2012; Romano et al. 2025). As we exhaustively captured almost all adults within colonies (proportion of unmarked individuals at the end of the breeding seasons over 30 years of captures: ~5%; Romano et al., 2025) and barn swallows of our population exhibit strong breeding site fidelity, as shown by the virtually nihil breeding dispersal (0.02%; Romano et al., 2025), newly captured individuals in any year_i were considered yearlings that were born the year_{i-1} in other colonies, except for the rare cases of local recruits (individuals ringed as nestlings and returned to their natal colony to breed: ~ 5% mostly males). Thus, age could reliably be assigned based on the year of the first capture (details in Saino et al., 2012; Romano et al., 2025). Along the same way of reasoning, individuals captured in year_i, but not recaptured in year_{i+1}, were assumed to be dead (details in Saino et al., 2012; Romano et al., 2025). Lifespan was therefore accurately estimated as the number of years elapsed between the year preceding the first capture (i.e. the hatching year) and the last year the individual was observed in the colony (e.g. an individual first captured in year 2013, hatched in 2012 and last recaptured in 2015, was assigned a lifespan of 3 years).

Upon capture, standard measurements were performed on different morphometric traits (i.e. tail length) and adults were sexed according to morphological and behavioural traits (incubation behaviour, performed by females only in the study population; Turner, 2006). We also collected contour feathers from the ventral region and blood samples from almost all individuals in each year of life for later spectrometric colour analysis and telomere length assessment, respectively (Saino et al., 2013c). Blood was collected from the brachial vein using heparinized capillary tubes that were immediately placed in cool bags in the field. Blood samples were then transported to the lab for centrifugation at 11,500 × rpm for 15 minutes to separate red blood cells from plasma, and finally stored at -20 °C within 3-4 hours from collection.

In all the study years, except for 2018, along with capture sessions, we regularly (at least twice a week) inspected all the nests to record breeding events within season. In addition, we assigned most adult breeders to their clutches by observing their univocal colour ring markings. We were thus able to quantify seasonal reproductive success for most individuals, expressed as the sum of the number of nestlings fledged in each brood of the individual within a breeding season. For a large number of individuals, we were able to collect seasonal reproductive success across all breeding seasons of their lives, thereby allowing us to estimate lifetime reproductive success (i.e. number of fledglings produced during the entire lifespan; hereafter, LRS). However, since extra-pair fertilizations are known to occur in this species (Turner, 2006), including in the study population (12.7%; Costanzo et al., 2017a), the estimated LRS for males correspond to social instead of genetic offspring. For females, by contrast, LRS can be assumed to accurately reflect genetic reproductive output, as intra-specific brood parasitism in this species is negligible (Turner, 2006; Costanzo et al., 2017a).

Individuals included in the study were selected according to their sex, realized lifespan and cohort. Specifically, we included breeding adults recruited between 2013 and 2015 (i.e. cohorts between 2012 and 2014) that reached different lifespan, trying to balance the number of individuals between sexes, lifespan classes and years. In total, we relied on data from 106 individuals (59 males and 47 females): 14 males and 13 females with lifespan of 1 year, 14 males and 11 females with lifespan of 2 years, 14 males and 9 females with lifespan of 3 years, 9 males and 10 females with lifespan of 4 years, 6 males and 2 females with lifespan of 5 years, 1 male and 2 females with lifespan of 6 years and another male of 7 years. While lifespan was known for all individuals, LRS was available for a subset of 76 individuals (35 males and 41 females), because LRS can be estimated only when seasonal reproductive success is recorded for every breeding season. Therefore, for instance, in a 4-years old breeder, even a single unassigned clutch in one breeding season prevents us from distinguishing between true non-breeding and missing observation, and the individual was therefore excluded. Minor changes in sample size of different analyses (see below) is due to missing or uncollectable data (e.g. broken tail).

Spectrometric ventral plumage colour analysis

Ventral plumage colouration was assessed measuring the reflectance spectrum of the distal portion of a single feather, as performed by Saino et al., (2013a). We relied on the tetrahedral colour space model developed for avian species to analyse reflectance data (Stoddard & Prum, 2008). The model allowed colour to be described in terms of three components: θ, which represents the chromatic variation within human visible range, φ, which account for the ultraviolet component, and rA which account for colour saturation. Among these variables, only θ was retained in the analyses, because φ and rA are correlated, colours in the visible spectrum are easier to interpret, and θ has been previously associated with telomere length in the barn swallow (Romano et al., 2015; Costanzo et al., 2017a; Parolini et al., 2017). In this species, higher θ values correspond to paler, more whitish ventral plumage (Saino et al., 2013a). This method provides highly repeatable measures both between successive measures of the same feather and between measures of different feathers collected from the same ventral region (Saino et al., 2013a; Romano et al., 2015). Moreover, colouration measured according to this method correlates with measurements performed on live birds, supporting its accuracy as a proxy of *in vivo* colouration (Romano et al., 2015).

Telomere length analysis

We extracted genomic DNA from 10-15 μl of red blood cells, with the Wizard DNA extraction kit (Promega, WI, USA). DNA concentration and purity were assessed using a Nanophotometer (NanoDrop One – thermo scientific) and we considered as adequate samples with absorbance ratios OD260/280 = 1.8–2.0 and OD260/230 = 2.0–2.2. Telomere length was quantified via monochrome multiplex quantitative PCR method (MMQPCR; Cawthon, 2009), according to the protocol developed on the barn swallow by Parolini et al., (2015). Briefly, amplifications were performed on a CFX Opus 96 thermocycler (Bio-Rad) using a reaction solution composed of 20 ng of template DNA (genomic DNA), 2X Quantitative Master Mix SYBR Green (Genespin) and primers pairs targeting telomere and single copy gene CTCF, at the final concentration of 1,000 nM and 500 nM, respectively. The telomere primers were: telg (5’-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3)’ and telc (5’-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3’). The CTCF primers were: forward (5’-CCCGCGGCGGGCGGCGCGGGCTGGGCGGCTCCCAATGGAGACCTCAC-3’) and reverse (5’-CGCCGCGGCCCGCCGCGCCCGTCCCGCCCATCACCGGTCCATCATGC-3’). CTCF primers include a GC-clamp (underlined part) to allow the amplification of both target sequences in the same reaction well. A four points standard curve (5 ng, 10 ng, 20 ng and 50 ng of a reference DNA sample) was included in each plate to calculate reaction efficiency, using the formula $E = [10^{(-1/a)} - 1] \times 100$ with 'a' representing the slope of the standard curve, and the amount of telomeric and CTCF repeats in each sample. All the samples and the four dilutions of the standard curve were run in triplicate. Due to mismatches in the primer sequences relative to a TTAGGG repeat (Parolini et al., 2018), MMQPCR usually yields lower efficiencies for telomere amplification than conventional qPCR. Therefore, relative telomere length (hereafter, RTL) was estimated according to the Pfaffl formula (Pfaffl, 2001), which accounts for differences in amplification efficiency between telomere and CTCF reactions.

Mean (± s.d.) amplification efficiencies were 132 ± 22 % for telomeres and 99 ± 16% for CTCF. Mean intra- and inter-plate coefficient of variation were 4.61% and 3.45% respectively.

Among the 106 individuals included in the analyses, RTL was measured in all years of life (from recruitment to death) for 89 individuals (27 with lifespan of 1 year, i.e. not surviving to the second year), while 17 individuals lacks of RTL measures in at least one year of life (23 RTL measures in total), because of missing/unsuitable DNA samples (e.g. not collected, missing or used previously for other purposes) or problems during RTL quantification. Consequently, 106 individuals resulted in 264 RTL measures in total collected between 2013 and 2018, with the maximum age at which RTL was available corresponding to 5-years old.

RTL was log-transformed ($\log_e$) prior to analyses to improve the normality of its distribution and reduce right-skewness of qPCR estimates (Nettle et al., 2019), following the same procedure used by several previous studies on other species (e.g., Zee et al., 2010; Huzen et al., 2014; Lynch et al., 2016), including birds (e.g., Reichert et al., 2017; Bauer et al., 2018; Xiong et al., 2025). Furthermore, as a measure of RTL attrition rate, we calculated annual variation in RTL ($\text{RTL year}_i - \text{RTL year}_{i-1}$; hereafter, ΔRTL). To control for the regression to the mean effect, the statistical artifact arising from the correlation between the change in two successive measurements and the baseline measure (here: $r = -0.63$, $t_{154} = -10.16$, $p<0.001$), we corrected all ΔRTL values included in the analyses following the method proposed by Verhulst et al., (2013). This approach adjusts each observed ΔRTL by subtracting the deviation expected from the regression to the mean, estimated from the correlation between two successive measurements.

Statistical analyses

To investigate whether RTL shortens with age over individual lifespan, we fitted a linear mixed-effects model (LMM), including RTL as a dependent variable (N = 264 datapoints, 106 individuals). Fixed effects were age and sex, while individual identity (ID) was modelled as random intercept to account for repeated measures within individuals. Moreover, we also tested the interaction between sex and age to investigate whether the potential effect of age on RTL differ between males and females. Furthermore, to disentangle between- and within-individual effects of age, we fitted the same LMM while partitioning age into age_{mean} (mean of available age of an individual in the sample) and age_{dev} (actual age of that individual at a given measure – age_{mean}). All these LMMs were also refitted using annual ΔRTL, z-transformed to improve model residuals distribution, as a dependent variable (N = 156 datapoints, 78 individuals).

To investigate the association between telomere length and lifespan, we relied on a mixed-effects Cox proportional-hazard model (N = 106). Lifespan was included as a dependent variable, while RTL at recruitment, sex, and both tail length and ventral plumage colouration (hereafter, θ) at recruitment (both mean centred within sex) as fixed effects. Furthermore, we also tested the interaction between RTL at recruitment and sex. Cohort (2012-2014) was included as a random intercept to account for potential interannual variability in survival.

Additionally, we investigated the possible association between RTL at recruitment and LRS fitting a generalized linear mixed model (GLMM) assuming a negative binomial distribution to account for data overdispersion (N = 76). LRS was included as a dependent variable with RTL at recruitment, sex, and both tail length and θ at recruitment (both mean centred within sex) as fixed effects. Cohort was included as a random intercept. Also in this case, we tested the effect of the interaction between RTL at recruitment and sex.

To test whether and how RTL is associated with secondary sexual traits expression, we fitted a LMM (N = 254 datapoints, 104 individuals) including RTL as a dependent variable, while tail length, θ and sex, together with age, as fixed effects. Tail length and θ were mean centred within age class (0 = yearling’s measures; 1 = 2 or more year-old’s measures) and sex, in order to account for the known variation in these traits between yearlings and older adults as well as between males and females (e.g. Saino et al., 2004; Turner, 2006; Saino et al., 2013a; Saino et al., 2017; Romano et al., 2017b). Random intercept accounted for year (2013-2018) and individual ID to control for interannual and interindividual variability. Moreover, we fitted another LMM with annual ΔRTL as a response variable (N = 148 datapoints, 76 individuals). Predictors included annual variation in tail length (tail length $year_i$ – tail length $year_{i-1}$) and θ (θ $year_i$ – θ $year_{i-1}$), together with sex and age. Again, year and individual ID were included as random intercepts. To test for sex-specific patterns, we also included the interaction terms between each term, excluding age, and sex in both models.

All statistical analyses were implemented in R version 4.5.1 (R Core Team, 2024). LMMs were fitted using the *lmer* function from the "lme4" package (Bates et al., 2015), while mixed-effects Cox proportional hazard model using the *coxme* function from the "coxme" package (Therneau, 2024). The GLMM was instead fitted using the *glmmTMB* function of the package “glmmTMB” (Brooks et al., 2017). We assessed model assumptions for LMMs using the *testResiduals* function of the ‘DHARMa’ package (Hartig, 2016), confirming that all LMMs met the assumptions. Additionally, we also found no evidence of influential outliers or multicollinearity, as indicated by the functions *check_outliers* and *check_collinearity* from ‘performance’ package (Lüdecke et al. 2021), respectively. Overdispersion in the GLMM was assessed using *check_overdispersion* from the package ‘performance’ (Lüdecke et al. 2021). Interaction terms were retained in the final models only when statistically significant.

Results

Age-related change in telomere length

Telomeres progressively shortened with age (Table 1; Figure 1), and did not differ between the sexes (Table 1). This age-related shortening occurred in a similar way between males and females, as the interaction between age and sex did not reach statistical significance (χ^2 = 0.86, df = 1, p = 0.35) Furthermore, this pattern is mainly driven by the within individual variation component of age (age_{dev}: $\beta = -0.03 \pm 0.01$, $t_{145.38} = -2.82$, p = 0.005; age_{mean}: $\beta = 0.03 \pm 0.02$, $t_{92.95} = 1.44$, p = 0.15).

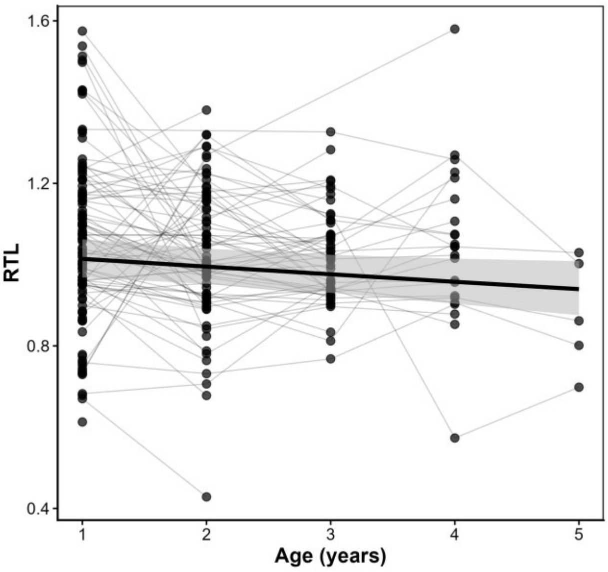

Figure 1. Variation in relative telomere length (RTL) according to individual age. The black line represents the effect of age on RTL log-transformed ($\log_e$) estimated by the linear mixed model reported in Table 1, and grey shaded band represents lower and upper limits of the 95% confidence interval for the model estimates. Points represent instead the observed values of the relationship between age and RTL, non-log-transformed, for easier interpretation. Light grey lines connect each RTL value within-individual at different ages.

Additionally, the rate of annual telomere attrition did not vary during lifespan and between sexes, because age (Table 1), as well as both age_{mean} and age_{dev} (age_{dev}: $\beta = 0.007 \pm 0.117$, $t_{152} = 0.06$, $p = 0.95$; age_{mean}: $\beta = 0.12 \pm 0.18$, $t_{152} = 0.66$, $p = 0.51$), and sex (Table 1) did not significantly predict ΔRTL. The absence of age-related pattern held in both males and females as interaction between age and sex was not statistically significant ($\chi^2 = 0.07$, df = 1, $p = 0.79$).

Telomere length, lifespan and lifetime reproductive success

Lifespan was positively predicted by RTL at recruitment (Table 2, Figure 2), indicating that individuals with longer telomeres at first reproduction lived significantly longer. No statistically significant effects emerged for any other predictors (Table 2). The interaction between RTL at recruitment and sex was also not statistically significant ($\chi^2 = 0.06$, df = 1, $p = 0.80$), meaning that the positive association between RTL and lifespan was similar in males and females.

RTL at recruitment was also positively associated with LRS (Table 2; Figure 3). Sex, tail length, and θ at recruitment, did not reach statistical significance (Table 2). Similarly, the interaction between sex and RTL at recruitment was not statistically significant ($\chi^2 = 0.30$, df = 1, $p = 0.58$).

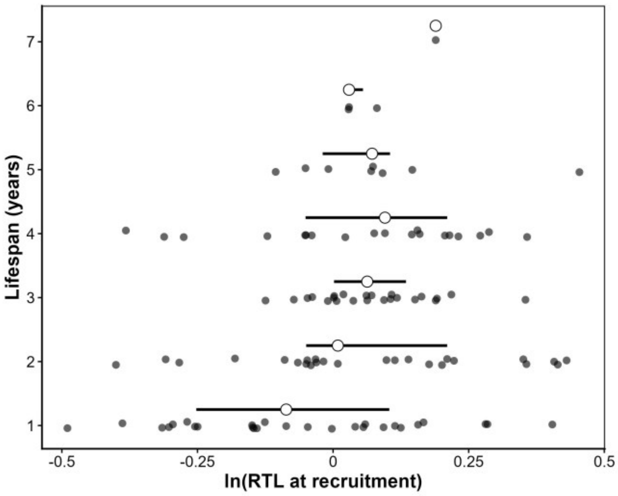

Figure 2. Relationship between relative telomere length (RTL) at recruitment (1-year old), log-transformed ($\log_e$), and realized lifespan. Black points represent observed individual values of the relationship. Open circles and horizontal bars represent the median and the interquartile range (Q1-Q3) of RTL ($\log_e$) within each lifespan class.

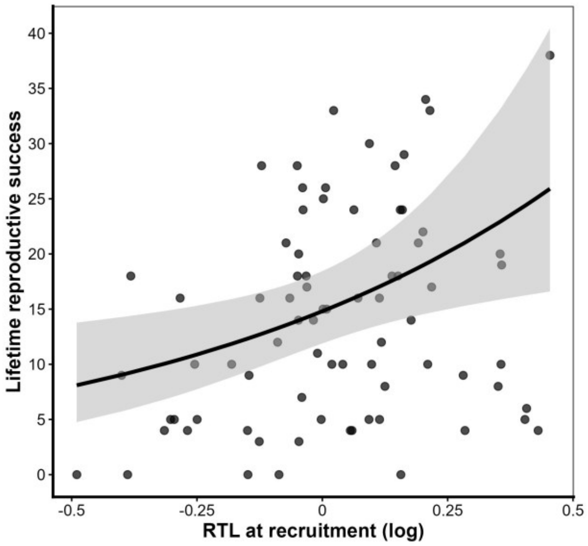

Figure 3. Relationships between RTL at recruitment, log-transformed ($\log_e$), and lifetime reproductive success. Points represent the observed values of the association, while the line the prediction of the negative binomial mixed model reported in Table 2. Shaded area represents the upper and lower limits of the 95% confidence interval.

RTL

Predictors	**Estimate**	**CI**	***df***	**P**
(Intercept)	0.03	-0.02 – 0.09	259	0.23
Age	**-0.02**	**-0.04 – -0.01**	259	**0.036**
Sex	0.03	-0.03 – 0.09	259	0.33
Random factors	**τ00 (SE)**			
Individual ID	0.01 (0.11)			

ΔRTL

Predictors	**Estimate**	**CI**	***df***	**P**
(Intercept)	-0.19	-0.77 – 0.39	151	0.51
Age	0.04	-0.15 – 0.23	151	0.67
Sex	0.14	-0.18 – 0.46	151	0.38
Random factors	**τ00 (SE)**			
Individual ID	<0.001 (<0.001)			

Table 1. Summaries of the linear mixed-effects models assessing the variation in telomere length and annual changes in RTL (ΔRTL) according to age and sex. In both models, individual ID was included as random intercept. Statistically significant fixed effects are reported in bold.

Lifespan

Predictors	**Coeffiecient**	**Exp (coef.)**	**SE**	**z**	**P**
RTL at recruitment	**-1.24**	**0.29**	0.60	-2.05	**0.041**
Sex	-0.29	0.74	0.21	-1.40	0.16
Tail length at recruitment ‡	0.02	1.02	0.01	1.60	0.11
Ventral plumage colouration(θ) at recruitment‡	0.32	1.38	1.84	0.18	0.86
Random effects	**τ00 (SE)**				
Cohort	0.05 (0.22)				

Lifetime reproductive success

Predictors	**Estimate**	**CI**	**z**	**P**
(Intercept)	**2.70**	**2.48 – 2.91**	24.14	**<0.001**
RTL at recruitment	**1.23**	**0.31 – 2.15**	2.61	**0.009**
Sex	-0.31	-0.63 – 0.02	-1.85	0.07
Tail length at recruitment‡	-0.01	-0.04 – 0.01	-0.94	0.35
Ventral plumage colouration(θ) at recruitment‡	-0.76	-3.5 – 1.98	-0.55	0.58
Random factors	**τ00 (SE)**			
Cohort	<0.001 (<0.001)			

‡ mean centred within sex to account for well-known differences between males and females

Table 2. Summaries of the models assessing: whether RTL at recruitment predicted realized lifespan and lifetime reproductive success, while controlling for sex, tail length and ventral plumage colouration at recruitment. To test lifespan, we relied on a mixed-effects Cox proportional hazard model, whereas for lifetime reproductive success on negative binomial mixed model. Cohort was included as a random intercept in both models. Statistically significant effects are reported in bold in all models.

Telomere length and expression of plumage ornaments

The interaction between sex and tail length showed a statistically significant effect on RTL (Table 3, Figure 4), indicating that the relationship between tail length and RTL differed between males and females. Specifically, in females, longer tails were associated with shorter RTL (β = -0.014 ± 0.004, t_{151} = -3.47, p < 0.001), whereas no significant association emerged in males (β = 0.001 ± 0.002, t_{106} = 0.65, p = 0.52). No other predictors reached statistical significance (Table 3), and the same was for the interaction between sex and θ (χ^2 = 0.66, df = 1, p = 0.41).

Consistent with the pattern shown for RTL, the LMM on ΔRTL showed a statistically significant effect of the interaction between sex and annual change in tail length (Table 3; Figure 4). In females, a larger increase in tail length between consecutive years was associated with faster telomere shortening over the same timespan (β = -0.12 ± 0.05, t_{133} = -2.25, p = 0.026), whereas this relationship was not significant in males (β = 0.01 ± 0.04, t_{133} = 0.27, p = 0.78). Furthermore, ΔRTL was predicted by annual change in θ, with plumage darkening between consecutive years corresponding to reduced telomere shortening over the same interval (Table 3; Figure 5). Notably, this pattern held in both sexes, as the interaction between annual changes in θ and sex was statistically non-significant (χ^2 = 0.02, df = 1, p = 0.89). Other predictors did not show statistically significant association with ΔRTL (Table 3).

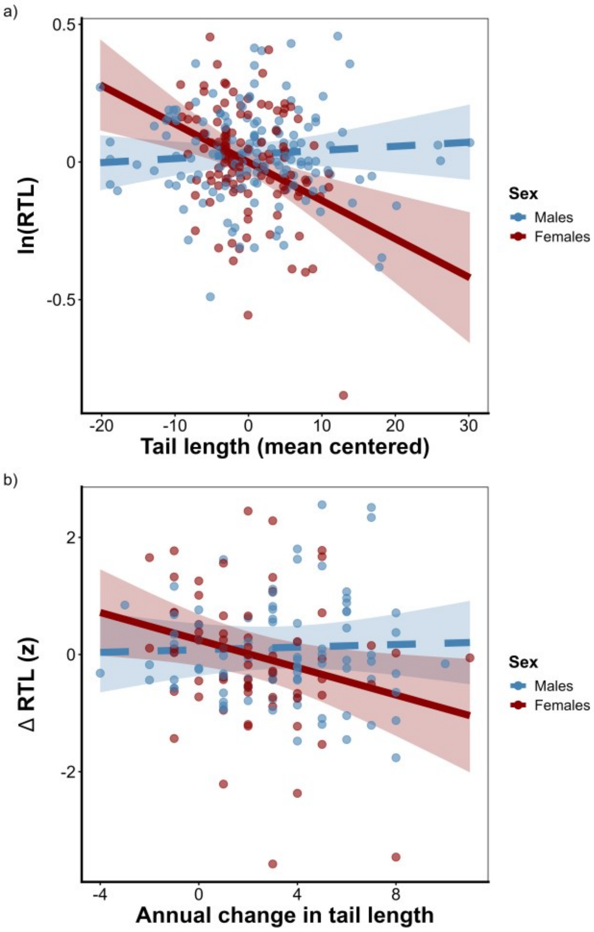

Figure 4. a) Relationships between outermost tail feathers length (mean centered within sex and age class: 0 = 1-year old; 1 = ≥ 2-years old) and relative telomere length (RTL), log-transformed ($\log_e$), in the two sexes; **b)** associations between annual change in tail length and annual variation in RTL (ΔRTL), z transformed, in the two sexes. In both plots, solid and dashed lines represent statistically significant and non-statistically significant effects within each sex estimated by the models in Table 3, respectively, while shaded bands illustrate the relative 95% confidence interval.

RTL

Predictors	Estimate	CI	df	P
(Intercept)	0.04	-0.02 – 0.10	245	0.22
Tail length†	-0.01	-0.02 – -0.01	245	<0.001
Ventral plumage colouration (θ) †	-0.26	-0.66 – 0.14	245	0.21
Age	-0.02	-0.040 – -0.001	245	0.07
Sex	0.03	-0.03 – 0.08	245	0.33
Sex*Tail length†	**0.02**	**0.01 – 0.02**	245	**0.001**
Random effects	**τ00 (SE)**			
Individual ID	0.01 (0.11)			
Year	<0.001 (0.02)			

ΔRTL

Predictors	Estimate	CI	df	P
(Intercept)	0.17	-0.70 – 1.03	139	0.70
Annual change in ventral plumage colouration (θ)	**-3.53**	**-6.53 – -0.53**	139	**0.021**
Annual change in tail length	-0.12	-0.22 - -0.02	139	0.024
Age	-0.02	-0.27 – 0.23	139	0.88
Sex	-0.17	-0.62 – 0.28	139	0.47
Sex* Annual change in tail length	**0.13**	**0.01 – 0.25**	139	**0.037**
Random effects	**τ00 (SE)**			
Individual ID	<0.001 (<0.001)			
Year	0.09 (0.29)			

† mean-centered within age class (0 = measures at recruitment; 1 = measures in ages ≥ 2 years) and sex due to the known differences between both age classes and sexes

Table 3. Summary of the two linear mixed-effects models investigating, the first, whether and how RTL is associated with seasonal reproductive success, tail length and ventral plumage colouration, while controlling for age and sex, and the second, whether and how ΔRTL is related to reproductive success of the previous season, as well as to annual changes in both tail length and ventral plumage colouration, while controlling again for sex and age. Individual ID and year were included as random intercepts in both models. Significant fixed effects are reported in bold.

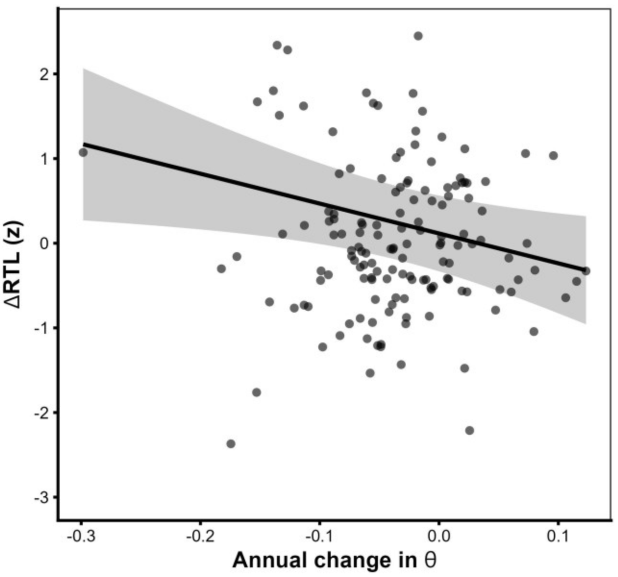

Figure 5. Relationship between annual change in ventral plumage colouration (θ) and annual change in RTL (ΔRTL), z transformed. Points represent individual observations, while the line the fitted values from the linear mixed model reported in Table 3. The shaded band indicates the 95% confidence interval around the model prediction.

DISCUSSION

Telomere length and dynamics have been suggested as key predictors of inter-individual variation in life-history traits, with telomere length potentially reflecting individual somatic state and telomere attrition rate the efficiency of mechanisms underlying somatic maintenance (Haussmann & Marchetto, 2010). In this longitudinal study of a short-lived migratory passerine, we confirmed the age-related decline in telomere length reported across vertebrates, and we found that telomeres are linked to major fitness components, as individuals with longer telomeres at recruitment lived longer and achieved higher LRS. In addition, telomere length and dynamics covaried with sexually selected traits. In females, longer tails and stronger increases in tail length between consecutive years were associated with shorter telomeres and faster telomere attrition, respectively, whereas in both sexes ventral plumage darkening between consecutive years related to reduced telomere attrition over the same time span.

RTL at recruitment (i.e. 1-year old) positively predicted realized lifespan, meaning that individuals with longer telomeres at the beginning of adulthood lived longer. Our result aligns with studies on other wild bird populations reporting positive association between telomere length measured early in life (or at the beginning of adulthood) and long-term survival/longevity (Eastwood et al., 2019; Bichet et al., 2020; Vedder et al., 2022; Eastwood et al., 2023b; Chik et al., 2024), and is therefore consistent with the idea that telomere length can reflect inter-individual variation in somatic state (Monaghan, 2010, 2024). Notably, in the same study population, RTL measured at the nestling stage predicts survival beyond the first year of life (Novelli et al., - submitted), and together with our finding suggest that differences in somatic state may be established early in life and may persist into recruitment. Two non-mutually exclusive scenarios may explain this relationship. First, telomeres may contribute causally to survival because critically short telomeres can trigger cellular senescence and apoptosis, thereby impairing tissue renewal capacity and organismal performance (Campisi et al., 2001; Deng et al., 2008). Under this scenario, individuals entering adulthood with longer RTL may require more time to reach the critical thresholds associated with functional decline and increased mortality risk (Monaghan, 2010, 2024). This may be further supported by our finding showing RTL to shorten with age at a constant rate, as reported across vertebrate species (Remot et al., 2022). Second, RTL at recruitment may correlate with lifespan simply because both are influenced by shared underlying determinants (e.g., early-life environment), rather than because telomeres directly cause differences in longevity (Monaghan, 2010; Young, 2018; Monaghan, 2024). Because our study is correlational, it cannot disentangle these possible pathways. Nonetheless, our result suggests that, even in a short-lived migratory passerine species where stochastic mortality is expected to be extremely high, individual somatic state may still contribute measurably to inter-individual variation in longevity.

RTL at recruitment also positively predicted LRS, expressed as the total number of fledglings produced over the entire individual life. In iteroparous species, lifespan is a major determinant of LRS because it predicts the number of reproductive events (Newton, 1989), and this is also the case for the barn swallow (Saino et al., 2012). Accordingly, as reported on other bird species (e.g., Eastwood et al., 2019; Heidinger et al., 2021), the positive association between RTL at recruitment and LRS is most likely mediated by lifespan, because individuals with longer telomeres at the onset of reproduction lived longer and thus had more opportunities to breed. However, a second and non-mutually exclusive pathway remains plausible. Specifically, telomere length at the beginning of adulthood may predict LRS even when association with lifespan is not detected (Chik et al., 2024). This suggests that individuals starting adulthood with longer telomeres, and maintaining relatively long telomeres thereafter, may also sustain higher seasonal reproductive performance, as reported in other bird species (e.g., Le Vaillant et al., 2015; Angelier et al., 2019; Benowitz-Fredericks et al., 2022). Notably, also a previous study on our population showed a positive relationship between telomere length and seasonal reproductive success (Parolini et al., 2017), and it is therefore possible that the effect of RTL at recruitment on LRS in barn swallows may reflect the combined contribution of increased longevity (i.e. increased number of reproductive opportunities) and higher reproductive output per breeding attempt. Regardless of the relative importance of these pathways, our results generally indicate that longer telomeres at the onset of reproduction are associated with higher individual quality, expressed in terms both of higher lifespan and LRS, and may therefore be favoured by natural selection. Because extra-pair fertilization occurs in this species and in our study population (12.7%; Costanzo et al., 2017a), male LRS reflects social rather than strictly genetic offspring in a minority of cases, whereas female LRS can be assumed to represent overall genetic reproductive output. However, notably, since LRS and lifespan are positively correlated, any bias introduced by extra-pair paternity is unlikely to qualitatively affect the interpretation of the observed patterns.

In addition, telomere length and dynamics across adulthood were associated with the expression of a sexually selected trait in the barn swallow, the outermost tail feathers length (Costanzo et al., 2017a; Romano et al., 2017a; Romano et al., 2017b), although the relationships were sex-specific. Specifically, females with longer tails had shorter RTL, and females that increased tail length more strongly between consecutive years, experienced faster telomere shortening over the same interval. These parallel patterns for RTL and ΔRTL highlight the possibility that investment in tail ornamentation can entail somatic costs in females of this species. Similar negative associations between ornamentation and telomere dynamics have been reported in other vertebrates (Azcárate-García et al., 2020; Kauzálová et al., 2022), including another barn swallow population where tail length covaried negatively with telomere length (Kauzálová et al., 2022). Notably, the sex specificity of our results suggests that the costs and/or benefits of tail feathers production may differ between males and females. In barn swallows, tail length is under strong sexual selection in males, and longer tails are more consistently associated with male quality and mating advantages (Saino et al., 1997; Costanzo et al., 2017a; Romano et al., 2017a; Romano et al., 2017b). Females, by contrast, may express relatively elongated streamers partly due to genetic correlation with tail length of the father, even though the trait is not the primary target of sexual selection in females (Cuervo & Møller, 2000). When females express more male-like tail length, they may incur disproportionate physiological costs associated with this male-typical trait expression (Cuervo, 2003; Vitousek et al., 2016), that may lead to faster telomere shortening (e.g., flight costs - Schultner et al., 2014; Bauer et al., 2016; Atema et al., 2022). At the same time, females also bear additional reproductive costs associated with egg production and exclusive incubation (Turner, 2006). The combined burden of reproduction and ornament expression could therefore constrain somatic maintenance more strongly in females, leading to detectable telomere costs associated with tail investment.

Interestingly, we also found that annual changes in ventral plumage colouration (θ) predicted ΔRTL, with individuals that darkened more between consecutive years showing reduced telomere shortening over the same interval. This relationship held in both sexes, suggesting that the processes linking changes in plumage colouration to telomere dynamics are not sex-limited, as occur for tail length. This finding complements previous evidence from the same barn swallow population reporting that darker ventral colouration covaries with longer telomeres both in breeding adults (Parolini et al., 2017) and nestlings (Costanzo et al., 2017b). Taken together, these results suggest a coherent scenario whereby darker ventral plumage colouration is associated not only with longer telomeres at a given time, but also with improved telomere maintenance across years, reinforcing the view that telomere dynamics capture biologically meaningful variation in individual quality.

By linking telomere length and dynamics to lifespan, LRS, and sexually selected traits expression within the same longitudinal study, our results show that telomere biology can be informative of inter-individual variation in fitness related traits, even in short-lived migratory species where very high annual mortality may reduce the strength of the association between somatic state, and thus telomere length, and lifespan. Specifically, the fact that RTL at recruitment predicts lifespan and LRS implies that differences in somatic state at the onset of reproduction can translate into considerable fitness differences, making telomere length a plausible target of selection in this population. At the same time, the sex-specific association between RTL/ΔRTL and tail ornamentation, together with the covariation between annual changes in ventral plumage colouration and ΔRTL, highlights that telomere maintenance can also reveal how investment in secondary sexual traits relates to possible sex- and trait-specific physiological constraints in the study species. Overall, these findings support the idea that telomeres are linked to natural and sexual selection processes in the wild, and highlight the need for experimental studies manipulating reproductive and ornament costs, as well as comparative longitudinal work across species and ecological contexts, to test whether relationships between telomere and fitness mirror species-specific life histories, and may therefore have contributed to their evolution.

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DISCUSSION AND CONCLUSIONS

General discussion and concluding remarks

The present dissertation has examined possible sources of interindividual variation in early-life telomere length and attrition. Furthermore, it has also investigated whether telomere length and dynamics, both during early life stages and in adulthood, may have long-term consequences on fitness, thus possibly driving life-history evolution in wild animals.

To address these aims, the thesis used a combination of experimental and longitudinal approaches in two migratory bird species, the lesser kestrel and the barn swallow, characterized by rapid life-histories and high extrinsic mortality. Across four chapters, it provides insights into possible influence of parental traits, environmental stressors and rapid somatic growth on telomere length and dynamics during early development. Additionally, results show that telomere length variability, both early in life and in adulthood, may translate into differences in future fitness, suggesting that longer telomeres may be favoured by natural selection even in species experiencing high annual mortality. Moreover, telomere dynamics may also be involved in sexual selection processes through their associations with secondary sexual traits, including sex- and trait-specific somatic costs of ornament expression.

The main findings emerged in each chapter are presented and summarized below.

Sources of interindividual variability in early-life telomere length

The first part of the thesis investigates several possible causes of interindividual variation in telomere dynamics during early development. This was achieved through an experimental approach in lesser kestrel nestlings (**Chapter 1**), aimed at testing the impact of thermal stress on telomere loss, and through a correlative study in barn swallow nestlings (**Chapter 2**), which explored a potential physiological mechanism linking developmental environment to telomere length/dynamics.

In **Chapter 1,** the dissertation reveals that early-life environmental conditions experienced during growth may shape telomere dynamics. Experimental exposure to extreme temperatures, as well as the natural occurrence of heatwaves, showed that thermal stress imposes possible costs in terms of somatic maintenance on growing nestlings, accelerating telomere attrition even in a species adapted to warm and arid climates. However, the effect of the experiment depended on nestlings' rank, suggesting that the association between environmental stressors and telomere dynamics may vary based on a complex interplay between different ecological factors. Furthermore, such chapter indicates that rapid somatic growth during development may further shape telomere dynamics, with nestlings growing at faster rates, irrespective of shading heatwave exposure, showing faster telomere attrition. Together, these results highlight the extent to which developmental intrinsic and extrinsic factors contribute to shaping interindividual variation in telomere length early in life and underscore the sensitivity of telomere dynamics to the thermal stress under highly competitive nest environment. Furthermore, given this possible sensitivity to temperature-related stress, these findings align with the idea suggesting that telomere dynamics may offer a useful biomarker of the negative impact of increasing and unpredictable heatwave events, associated with recent climate warming, on developing birds.

Chapter 2 further shows in barn swallow nestlings, but only in males, that rapid growth during development may contribute to accelerated telomere attrition, consistent with the pattern observed in lesser kestrels and with the previous observations that male barn swallow nestlings are more susceptible to stressors compared to females. Furthermore, the chapter adds also a possible physiological pathway that may act alongside growth in shaping early-life telomere dynamics. Eliciting an acute HPA-axis mediated stress response through a handling-restraint protocol, the study shows that the magnitude of stress response, but not the circulating levels of corticosterone, may be associated with nestlings' telomere dynamics. Yet, the association was dependent on brood size, with stronger acute responses to stress linked to faster telomere shortening in smaller broods, but to slower attrition in larger broods, highlighting again the importance of considering the environmental context in which telomere dynamics are investigated. Although correlative, these results suggest that mounting a stronger stress response in some developmental context may be advantageous (e.g., higher food availability), and nestlings may benefit from better somatic state, potentially reflected in slower telomere attrition or even telomere lengthening. More broadly, the substantial context-dependency of these associations, together with additional variability introduced by sex-related differences in developmental constraints, highlights the complexity of the links between early-life growth, stress physiology and telomere dynamics, underscoring in particular the need of investigating acute stress responses, an aspect rarely examined early in life, that may reveal different associations with telomere dynamics compared to those with baseline measures of stress.

Consequences of interindividual variability in telomere length on fitness

The second part of the thesis focuses on the associations between telomere dynamics, during development (**Chapter 3**) and adulthood (**Chapter 4**), with survival/lifespan and lifetime reproductive success in the barn swallow. Importantly, **Chapter 3** represents a conceptual bridge between causes and consequences of telomere length interindividual variation, as it not only evaluates associations between early-life telomere length and long-term fitness correlates, but also analyses possible sources of early-life variation that may contribute to shaping telomere dynamics at fledging. This chapter therefore complements the first two by showing the possible role of telomere length in mediating possible carry-over effects of developmental conditions into later life performance.

Results of **Chapter 3** reveal that nestlings in larger broods, in which individuals are known to experience stronger competition and less food availability, exhibit shorter telomeres. Moreover, paternal age emerges as an additional predictor of early-life telomere length, with offspring of older males showing shorter telomeres. Although correlative, this pattern is consistent with the hypothesis that paternal ageing, potentially mediated by age-related deterioration in sperm quality and/or by differential paternal care investment, may contribute to determining offspring telomere length, suggesting also possible epigenetic-like inheritance influences. Taken together, the effect of environmental and parental factors highlighted in this chapter, as well as those identified in **Chapter 1** and **Chapter 2**, suggest that multiple early-life influences may shape telomere length at fledging, which in turn may affect individual condition during development. Indeed, nestlings with longer telomeres showed a higher likelihood of surviving beyond their first year of life, highlighting that early-life telomere length may capture aspects of somatic state that become particularly relevant for fitness in adulthood. More broadly, although these associations are correlative, the results of the chapter hint at the possible role of early-life telomere dynamics in mediating the effect of early-life conditions on differences in later-life viability.

Chapter 4 shows that telomere length in adult barn swallows, while decreasing with age within individual as expected, is positively associated with realized lifespan. This pattern aligns with what was reported in **Chapter 3** and, taken together, these results indicate that telomere length may either contribute causally to individual viability, such that individuals starting life with longer telomeres may take longer to reach a critical threshold associated with functional decline and ultimately death, or instead reflect underlying developmental and physiological conditions that jointly shape both telomeres and survival. Importantly, although **Chapter 3** and **4** measured telomere length at two different life stages (nestlings and early adulthood), both show that its association with long-term survival is particularly strong across the transition from the first to the second year of life, suggesting that this may represent an important selective episode for telomere length. Furthermore, as expected in iteroparous species where longevity is a major determinant of lifetime fitness because it increases the number of breeding opportunities, telomere length at recruitment also positively predicts lifetime reproductive success. Therefore, **Chapters 3** and **Chapter 4** highlight the possibility that differences in somatic quality established early in life, and potentially captured by telomere length, may persist into early adulthood and translate into divergent lifespans and lifetime fitness outcomes. Under such circumstances, longer telomeres at the beginning of life, and/or the processes that promote their maintenance, may be favoured by natural selection, even in species where high stochastic mortality and a fast pace of life could affect associations between somatic state and fitness. Furthermore, the evidence that interannual changes in plumage colouration covary with telomere dynamics strengthens the interpretation that telomeres and their maintenance capture interindividual variation in somatic quality that can translate into fitness differences, including via links with the expression of secondary sexual traits. At the same time, associations with tail ornamentation point to a different, sex-specific scenario, suggesting that the expression of longer tail feathers in females may entail measurable somatic costs. Such sex-specific costs may arise through paternal inheritance of a trait that primarily evolves under male-specific sexual selection. When this male-typical ornament is expressed by females, its physiological cost may become evident, leading to faster telomere attrition and suggesting that, together with results on paternal age presented in **Chapter 3**, paternal inherited factors may introduce additional complexity to the multifactorial nature of telomere dynamics. In contrast, in male barn swallows, tail length is under directional sexual selection which may tightly link the expression of this ornament to individual quality. Consequently, males expressing and maintaining longer tail feathers may be less susceptible to paying large physiological costs for bearing such ornaments, resulting in better telomere maintenance than females. Moreover, in females, somatic costs associated with longer tail feathers could add to their generally higher reproductive investment (e.g., egg production and exclusive incubation), such that the observed relationship may reflect the cumulative burden of both processes. Overall, the sex-specific patterns emerging here, as well as in **Chapter 2**, suggest thus that telomeres may also capture, at the molecular level, key aspects of the species' biology and life-history differences between males and females at different life stages. More broadly, these findings highlight the potential role of telomeres in sexual selection processes and in the evolution of sexually selected traits.

Conclusion

To conclude, the results of the present thesis highlight the substantial complexity underlying the possible sources of interindividual variation in telomere length and attrition. Importantly, these results also show that telomere length variability can be associated with key fitness proxies, including survival across major life-stage transitions, realized lifespan and lifetime reproductive success, as well as sexually selected traits expression, supporting the idea that telomere length and attrition capture biologically meaningful variation in somatic state and maintenance and may therefore contribute to evolutionarily relevant variation in life-history strategies in natural populations. Overall, these findings emphasize the value of integrative approaches and, in particular, long-term longitudinal studies that follow individuals across life stages, to better understand whether and how telomeres act as mediators linking early-life experiences to performance and fitness in adulthood.

Building on these results, the thesis provides several avenues for future research on telomeres of wild birds. First, to fully understand how early-life conditions shape telomere dynamics is necessary to integrate other possible proximate causes (e.g., oxidative stress) that may underlie telomere attrition to disentangle their relative contributions and better understand the physiological pathways behind telomere dynamics. Moreover, the results on the barn swallow, emphasise the importance of investigating also acute, rather than only baseline, HPA-axis activity during development. Additional work is also needed to clarify the main contribution in the origins of early-life interindividual telomere variation, especially the relative roles of environmental conditions and genetic factors, ideally performing studies that jointly consider parent–offspring telomere covariation and paternity analyses, together with the contribution of nest environment. Furthermore, a crucial next step could be to test experimentally whether telomere length relates to lifetime reproductive success primarily through association with longevity or also through reproductive performance per breeding attempt. This could be achieved by manipulating reproductive effort (e.g., brood size manipulation and/or cross-fostering) and then tracking telomere attrition during and after the breeding season, together with subsequent reproductive success and return rate/survival. Finally, experimental and comparative approaches are essential to assess the possible somatic costs of plumage traits expression in terms of telomere attrition, particularly focusing on the causes of their sex-specific nature, and, more broadly, to determine how telomeres may act in mediating possible life-history trade-offs in wild animals.

Appendix

List of publications in ISI-ranked journals by Andrea Novelli

1. Romano, A., Florent, G., **Novelli, A.,** Séchaud, R., & Roulin, A. (2024). Spatio-temporal shift in body size and plumage coloration is associated with the magnitude of climate change in a cosmopolitan owl. *Journal of Biogeography*, *51*(10), 1921–1932. https://doi.org/10.1111/jbi.14863

2. Romano, A., Ambrosini, R., Caprioli, M., Costanzo, A., **Novelli, A.,** & Rubolini, D. (2025). Shrinking body size under climate warming is not associated with selection for smaller individuals in a migratory bird. *Journal of Animal Ecology*, *94*(5), 958–970. https://doi.org/10.1111/1365-2656.70027

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