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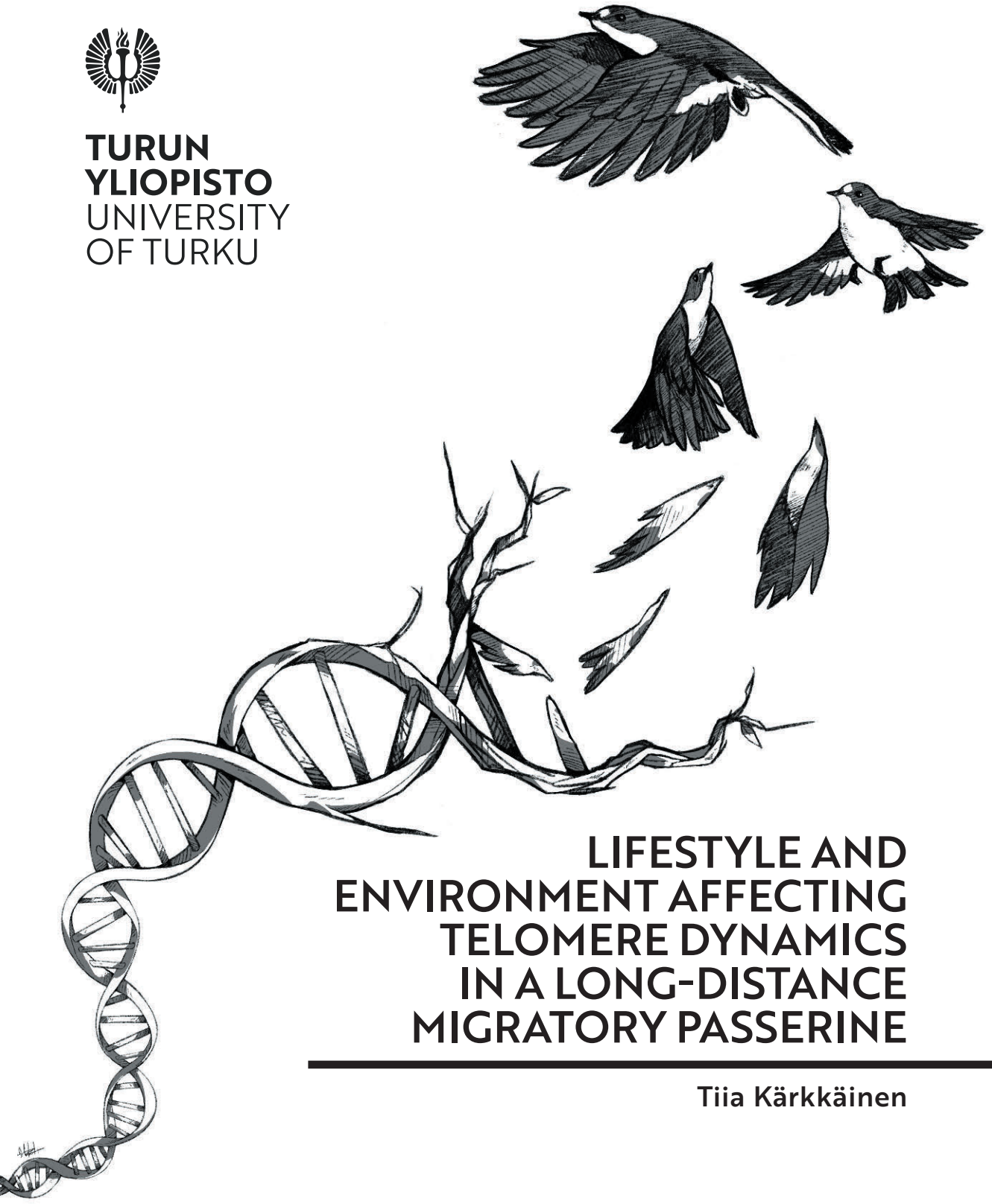

LIFESTYLE AND ENVIRONMENT AFFECTING TELOMERE DYNAMICS IN A LONG-DISTANCE MIGRATORY PASSERINE

Tiia Kärkkäinen

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To my dad,
who always took me with to his birding trips

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Section of Ecology
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ABSTRACT

Telomeres cap the true ends of linear chromosomes and thus have a key role maintaining genomic and cellular integrity. Telomeres shorten with each cell division which can be accentuated by intrinsic (*e.g.* metabolic activities) and extrinsic (*e.g.* stress) factors. Critically short telomeres are unable to maintain the genomic integrity and thus are associated with a range of age-related diseases and overall mortality risk in a wide range of vertebrates. Consequently, telomere length is starting to be widely used as biomarker for future fitness and survival in ecological and evolutionary contexts. In this thesis, I studied the general appropriateness of telomere length as an indicator of long-term costs using published data on non-mammalian vertebrates, within-individual repeatability, and meta-analytical methods. I also investigated the effects of different environmental factors, namely predation pressure and interplays between pre- and post-natal conditions, on telomere dynamics using different experimental designs, and examined differences in telomere length among populations at a large geographical scale using the pied flycatcher (*Ficedula hypoleuca*) as a model species. First, I found telomere length to be reasonably repeatable within individuals to be used to estimate long-term costs. However, the repeatability estimates ranged from almost 0 to almost 1 implying that particular attention to the repeatability must be paid when making such inferences. Second, my studies revealed that (i) predator presence influences the preys' telomere dynamics, (ii) a mismatch between pre-natal and realized post-natal conditions may impair future fitness, and (iii) telomere lengths can vary strikingly between populations of a same species. Predator presence seems to have a negative effect on adult, but positive effect on nestling telomere length. These differences may be explained by predator-induced stress, increased activity, and/or increased provisioning. A developmental mismatch resulted both in faster growth and telomere shortening in the early life in the first-hatched chicks, but in slow growth in the last-hatched chicks. Finally, there are marked differences in the telomere length of different pied flycatcher populations that could be due to the differences in genetics or in the breeding environment. These results add new dimensions to the knowledge of various factors that can deviate telomere length trajectories of closely related individuals and individuals within- and between-populations. Such knowledge could ultimately improve our understanding of life-history evolution and how organisms respond to environmental change.

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TIIVISTELMÄ

Telomeerit suojaavat kromosomien päitä, joten niillä on tärkeä osa geenien ja solujen tasapainon ylläpidossa. Ne kuitenkin lyhenevät jokaisen solunjakautumisen yhteydessä ja erilaiset sisäiset (esim. aineenvaihdunnan toiminnat) ja ulkoiset (esim. stressi) tekijät voivat nopeuttaa lyhenemistä. Kriittisen lyhyet telomeerit eivät enää kykene ylläpitämään solutasapainoa, ja näin ollen ne on yhdistetty moniin vanhuuteen liitettyihin sairauksiin ja yleiseen kuolleisuuden riskiin monilla selkärankaisilla. Telomeerien pituutta onkin aloitettu käyttämään tulevan kelpoisuuden ja selviytymisen mittarina ekologian ja evoluutiobiologian aloilla. Väitöskirjassani tutkin ensin telomeerien pituuden soveltuvuutta tulevaisuuden kustannusten ennustamiseen analysoimalla telomeerimittausten toistettavuutta käyttäen jo julkaistuja selkärankaistutkimuksia ja meta-analyyttisia menetelmiä. Tutkin myös eri ympäristötekijöiden, eli saalistuspaineen ja kehitysaikaisten olosuhteiden vuorovaikutusten vaikutuksia telomeeridynamiikkaan käyttäen erilaisia koeasetelmia, sekä telomeerien pituuden maantieteellistä vaihtelua eri populaatioiden välillä käyttäen mallilajina kirjosieppoa (*Ficedula hypoleuca*). Havaitsin, että yksilön peräkkäiset telomeerimittaukset olivat riittävän toistettavia, jotta telomeerien pituudesta voisi arvioida tulevaisuuden kustannuksia. Toistettavuuden arviot kuitenkin vaihtelivat laajalti nollan ja yhden välillä, joten toistettavuus tulisi ottaa huomioon tällaisia arvioita tehtäessä. Muut tutkimukseni esittävät, että (i) pedon läsnäolo vaikuttaa saaliin telomeeridynamiikkaan, (ii) kehitysolosuhteiden välinen epätasapaino voi vaikuttaa kelpoisuuteen, ja (iii) saman lajin populaatioilla voi olla merkittävän eripituiset telomeerit. Pedon läsnäolo oli yhteydessä lyhyempiin telomeereihin aikuisilla, mutta pidempiin telomeereihin poikasilla. Näitä eroja voidaan mahdollisesti selittää pedosta johtuvalla stressillä ja lisääntyneellä aktiivisuudella, sekä poikasten ruokinnalla. Kehitysolosuhteiden epätasapaino kiihdytti sekä kasvua, että telomeerien lyhenemistä ensimmäisenä kuoriutuneilla poikasilla, mutta hidasti viimeisten poikasten kasvua. Telomeerien pituuksissa oli merkittäviä eroja kirjosieppopopulaatioiden välillä, jotka todennäköisesti selittyvät lisääntymisalueen elinympäristöjen tai geneettisillä eroilla. Nämä tutkimukset antavat lisätietoa tekijöistä, jotka voivat eriyttää lähisukulaisten, saman populaation yksilöiden, tai populaatioiden välisten yksilöiden telomeeridynamiikkaa toisistaan. Tällainen tieto voi lopulta edesauttaa elinkaarten evoluution ja eliöiden ympäristönmuutosvasteen ymmärtämistä.

Table of Contents

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I **Kärkkäinen T.***, Briga M.*, Laaksonen T., Stier A. Within-individual repeatability in telomere length: a meta-analysis in non-mammalian vertebrates. Submitted manuscript.

II **Kärkkäinen T.,** Teerikorpi P., Panda B., Helle S., Stier A., Laaksonen T. 2019. Impact of continuous predator threat on telomere dynamics in parent and nestling pied flycatchers. *Oecologia*, 191: 757-766.

III **Kärkkäinen T.**, Teerikorpi P., Schuett W., Stier A., Laaksonen T. 2021. Interplays between pre- and post-natal environments affect early-life mortality, body mass and telomere dynamics in the wild. *Journal of Experimental Biology*, 224:1

IV **Kärkkäinen T.**, Laaksonen T., Burgess M., Cantarero A., Martínez-Padilla J., Moreno J., Potti J., Thomson R., Tilgar V., Stier A. Population differences in the length, early-life dynamics, and heritability of telomeres among European pied flycatchers. Submitted manuscript.

*These authors contributed equally to this work.

1 Introduction

1.1 Life-history theory and ageing in the wild

Diversity of organisms inhabiting the planet Earth is immense. Different organisms show enormous variation in traits such as birth rate, age at fecundity, reproductive effort, and lifespan. Various combinations of these traits are called life-history strategies, and the theory trying to explain the causes and consequences of these strategies is called life-history theory. Trade-offs are a key concept in life-history theory, and their basis is the idea that the more resources are invested in one trait, the less can be invested in another trait (Stearns, 1992). Different sets of trade-offs are assumed to have differing effects on fitness. Trade-offs maximizing fitness, that is often measured as reproductive success, are expected to become prevalent in a population (Roff, 1993). However, the array of potential trade-offs can be limited by genetic, physiological, mechanical, and ecological constraints. Thus, in a given situation and environment, natural selection works on the favor of the individuals exhibiting the most successful trade-offs in the framework of different constraints. The nowadays observed between- and within-species differences in different life-history traits are a result of this natural selection (Roff, 1993; Stearns, 1992).

But if natural selection maximizes fitness, why organisms have not evolved to live forever while reproducing at high rates (*i.e.* Darwinian demon) as this would certainly maximize fitness? Instead, almost all organisms age and finally die. Solution to this dilemma starts with the fact that individuals die somewhat arbitrarily due to extrinsic causes, *e.g.* accidents and predation. Hence, any individual that dies suddenly before reproduction has fitness of zero. Consequently, only those individuals that reproduce early enough, before the possible extrinsic death, will produce offspring and with time, individuals reproducing early will accumulate, *i.e.* natural selection favors early reproduction. Due to these conditions, extrinsic mortality and selection favoring early reproduction, the strength of natural selection is highest in younger age classes and weakens with age (Stearns, 1992), simply just because there are more young than old individuals that produce offspring. Thus, any trait that would benefit the survival and reproduction of young individuals is favored even if it has deleterious effects on older individuals. The early theories trying to explain aging are built on this foundation. Medawar (1952) hypothesized that aging

is caused by time-dependent accumulation of mutations, whose harmful effects will become apparent only later in life. Similarly, Williams (1957) theorized the occurrence of genes that are antagonistically pleiotropic, *i.e.* have beneficial effects in early life, but harmful effects on late life.

Nowadays, major theories explaining aging are based on the concepts of both life-history trade-offs and the early theories of aging (Kirkwood, 2005). Originally, Kirkwood (1977) suggested that aging is a result of greater investments in germline than somatic tissues. Thus, resources invested in reproduction are unavailable for self-maintenance, which eventually causes somatic damage to build up (Kirkwood et al., 1991). Accumulation of somatic damage and following cellular senescence ultimately impairs organismal performance, fertility and/or survival, that are characterizing ageing (Kirkwood, 2005; Kirkwood et al., 1991). Since then, this theory has received support, and the trade-off between reproduction and somatic maintenance and survival is well documented (Flatt and Heyland, 2011; Stearns, 1992; but see Lind et al., 2021). Despite initial doubts of ageing occurring in the wild due to high extrinsic mortality, nowadays, declines in reproductive capacity, performance, and survival with increasing age have been documented in variety of wild species (Gaillard and Lemaître, 2020; Nussey et al., 2013). Among other things, the rate of decline in performance can depend on the environment (Regan et al., 2020; Reznick et al., 2004; Robert and Bronikowski, 2010), and vary between individuals of a same species (Bouwhuis et al., 2010; Brommer et al., 2010). Although aging is by definition associated with old age, the underlying mechanism, molecular and cellular deterioration, can affect individuals independent of age resulting in lifespan differences between individuals of a same species (Récapet et al., 2016; Salmón et al., 2017). One of the key factors accounting for cellular deterioration is telomere attrition, which consequently is considered to be a hallmark of ageing (López-Otín et al., 2013)

1.2 Telomeres

Telomeres have a crucial role in the maintenance of cell viability and genomic stability as they protect genes from damage, ensure proper cell replication, and differentiate the natural chromosome ends from double-stranded DNA breaks (Griffith et al., 1999; Kim et al., 2002; Stewart et al., 2012). Telomeres are specialized, non-coding DNA structures with associated proteins at the ends of linear chromosomes. Their structure is remarkably similar among widely divergent species from several major eukaryotic lineages indicating them to be a very ancient feature (Gomes et al., 2010). Telomeres consist of short repeat sequences (5'-TTAGGG-3' in vertebrates) at the end tract of a chromosome, adjacent heterochromatic subtelomeric regions and a capping component (Blackburn et al., 2015; Schoeftner

and Blasco, 2009). The G-rich cluster is chemically oxidative-damage-prone, thus making telomeres highly susceptible for different kinds of damage (Blackburn et al., 2015; Petersen et al., 1998; von Zglinicki, 2002). Telomeres also tend to accumulate DNA damage as telomeric DNA repair can be restricted (Blackburn et al., 2015; von Zglinicki, 2002). Telomeres shorten with every cell division due to the end-replication problem *i.e.* the inability of DNA polymerase to copy terminal DNA (Levy et al., 1992; Olovnikov, 1973), but the amount of damage in the telomeres ultimately determines the rate of the shortening (von Zglinicki, 2002). Enzyme telomerase and some alternative telomere lengthening (ALT) mechanisms can elongate telomeres (Blackburn, 2005; Bryan et al., 1997), but in most cases telomeres shorten with time (Gomes et al., 2010). When telomeres get to a critically short length, they most often induce cell senescence and the cell stops further divisions (Campisi et al., 2001). Accumulation of senescent cells impairs tissue function and is strongly associated with aging phenotypes (Campisi, 2005; Campisi and Robert, 2014). If the senescence response fails, telomeres induce apoptosis, or genomic instability (Campisi et al., 2001). A genomically unstable cell may find a way, usually through mutations, to stabilize its telomeres, which can lead to formation of malignant tumor (Campisi et al., 2001). Consequently, short telomeres are associated with increased mortality risk both in humans and wild vertebrates (Arbeev et al., 2020; Boonekamp et al., 2013; Wilbourn et al., 2018). While causality between short telomeres and organismal ageing is equivocal (Simons, 2015; Young, 2018; but see Muñoz-Lorente et al., 2019), telomere length at the end of growth period predicts lifespan (Heidinger et al., 2012) and hence, telomere dynamics are suggested to link lifestyle and lifespan at the organismal scale (Monaghan and Haussmann, 2006).

1.2.1 Telomeres as dynamic structures

Telomeres tend to shorten with time as described previously. Shortening is usually fastest in juveniles during growth period (Spurgin et al., 2018) and fast rate of growth is associated with short telomeres (Monaghan and Ozanne, 2018; Stier et al., 2020; Vedder et al., 2018). Telomere shortening is more restricted in adult individuals, but different cellular stressors, such as oxidative stress can accentuate the rate of telomere loss (Reichert and Stier, 2017). Oxidative stress occurs when the antioxidant defenses cannot neutralize all the reactive oxygen species (ROS) generated by metabolic activities and external stressors, such as nutritional and environmental stress (Jennings et al., 2000; Monaghan et al., 2009). However, the role of oxidative stress in organismal telomere shortening in healthy individuals has been questioned to some extent (discussed in Boonekamp et al., 2017). Metabolic activities during substantial energetic challenges, *e.g.* during growth or increased

physical activity, might also increase telomere shortening independent of oxidative stress (Casagrande and Hau, 2019). Yet, some adult individuals in some species (*e.g.* Soay sheep, edible dormouse, mice, badger, and Seychelles warbler) have been observed to lengthen their telomeres with time (Fairlie et al., 2016; Hoelzl et al., 2016; Kotrschal et al., 2007; Spurgin et al., 2018; van Lieshout et al., 2019). Whether telomere lengthening is a real biological phenomenon or a result of measurement error has been highly discussed but remains to be properly proven (Bateson and Nettle, 2017; Steenstrup et al., 2013). Nevertheless, telomere length is somewhat heritable (Dugdale and Richardson, 2018), but the rate of telomere change is an individual trait. In addition to survival (Wilbourn et al., 2018), telomere length has been associated with stressful experiences (Chatelain et al., 2020; Pepper et al., 2018), future disease risk (Fasching, 2018), phenotypic quality (Angelier et al., 2019), and fitness prospects (Eastwood et al., 2019). Telomere length is consequently started to be widely used as an integrative biomarker for past stress exposure and future survival and fitness (Monaghan, 2014; Monaghan et al., 2018). Still, to make such inferences about the past or the future based on any given telomere length, successive telomere length measurements should be somewhat repeatable. Yet, extremely high within-individual repeatability (*i.e.* close to 1) would suggest that environment has only weak effect on the rate of telomere shortening, reducing the capacity of telomere length to work as a biomarker linking environmental conditions and fitness. Thus, the within-individual repeatability of telomere length is an important parameter for the interpretation of telomere data, while it is still rarely quantified and knowledge of factors potentially affecting such within-individual repeatability is scarce. Ultimately, understanding factors that can diverge telomere length trajectories of closely related individuals and individuals within and between populations could help in understanding life-history evolution and organismal responses to environmental change.

1.2.1.1 Stress exposure and predator effect

The end product of vertebrate stress response is the release of glucocorticoid hormones (Romero, 2004). Both chronic stress and extended glucocorticoid exposure can lead to oxidative stress (Costantini et al., 2011; Marasco et al., 2017), which can increase telomere shortening (Reichert and Stier, 2017). Additionally, glucocorticoids might inhibit telomerase expression (Choi et al., 2008) and contribute to telomere shortening through metabolic adjustments that reduce resources available for telomere maintenance (Casagrande and Hau, 2019). Chronic psychological stress, as well as increases in glucocorticoid levels, are indeed associated with accelerated telomere shortening, and short telomeres in different vertebrates from humans to laboratory mice and wild birds (Casagrande et al., 2020;

Epel et al., 2004; Herborn et al., 2014; Kotrschal et al., 2007; Meillère et al., 2015). Predator encounters are one of the most frequent stressors for wild animal populations. Continuous predation risk is known to rise the levels of glucocorticoids and certain stress protein levels in adult prey individuals (Clinchy et al., 2011, 2004; Scheuerlein et al., 2001; Sheriff et al., 2011; Thomson et al., 2010). It has also been shown that predator cues promoted oxidative stress in an amphibian species (Pinya et al., 2016). Predators are known to affect their prey indirectly in multiple ways, for example through reduced fecundity, survival, clutch size, provisioning rate, and impaired foraging (Boonstra, 2013; Brown et al., 1988; Clinchy et al., 2013; Tilgar et al., 2011; Travers et al., 2010; Zanette et al., 2011). Nevertheless, while physiology of stress is well studied, the molecular mechanisms that might connect predation risk to survival, via *e.g.* telomere dynamics, have remained uninvestigated (Angelier et al., 2018; Boonstra, 2013; Wilbourn et al., 2018) until very recently (Monteforte et al., 2020).

1.2.1.2 Prenatal maternal effects

Mothers can have an influence on the phenotypes of their offspring through maternal effects *in utero* or *in ovo*, possibly in order to produce offspring that is better adapted to the current/future environment (Groothuis et al., 2005; Sheriff et al., 2010). Female birds are known to deposit varying quantities of nutrients (Ramírez et al., 2015), immunoglobulins (Hargitai et al., 2006), antioxidants (Török et al., 2007), and hormones (Gil, 2008) in their eggs. One group of maternally transferred hormones is glucocorticoids (Saino et al., 2005). Despite of some recent evidence suggesting that maternal glucocorticoids could promote telomerase activity and longer telomeres in the offspring (Noguera et al., 2020), high glucocorticoid levels in the eggs have been observed to produce chicks with short telomeres (Haussmann et al., 2012). Furthermore, concentrations of different resources in the eggs can vary within the laying order, which would create differences in the physiological developmental conditions between different embryos (Mentesana et al., 2018) potentially resulting in different life-history trajectories between siblings (Groothuis et al., 2005). For example, androgen levels in the eggs can increase with laying order, possibly to compensate for the competitive disadvantage of the last-hatched chicks in asynchronous broods by enhancing growth (Morosinotto et al., 2016; Müller and Groothuis, 2013; Schwabl, 1996; Stier et al., 2015a). Fast postnatal growth can however have negative long-term effects in terms of increased telomere shortening (Monaghan and Ozanne, 2018; Stier et al., 2015a) but in the case of birds, maternal resource allocation seems still to be adaptive to match the pre-natal conditions to the post-natal conditions (Müller and Groothuis, 2013; Thomson and Hadfield, 2017). Although much is already known about prenatal maternal effects for individual

phenotype and fitness prospects, much remains unknown about their relative importance in the wild, as they are intrinsically linked with post-natal effects and separating these factors is often impossible.

1.2.1.3 Hatching asynchrony and sibling competition

Similarly to pre-natal maternal effects, also post-natal developmental conditions can affect individuals' later-life phenotype (Merkling et al., 2014; Trillmich and Wolf, 2008). In birds, starting the incubation before clutch completion results in hatching asynchrony, *i.e.* the first-laid eggs to hatch before the last-laid eggs, which creates unequal competitive conditions in the early-life between the first- and the last-hatched chicks (Magrath, 1990). Hatching asynchrony might have evolved as an adaptive strategy to distribute predation risk, to maximize energy efficiency, or to produce diverse offspring into variable environment (Glassey and Forbes, 2002; Laaksonen, 2004; Magrath, 1990). However, the inferior competitive position of the last-hatched, usually smaller chicks, can lead to reduced food intake, lowered growth and body mass at fledging, and increased early-life mortality (Hildebrandt and Schaub, 2018; Kilgas et al., 2010; Malacarne et al., 1994). Competitive disadvantage and high level of competition in general in the early-life has also been shown to increase telomere shortening during growth and even decrease survival in the adulthood (Boonekamp et al., 2014; Nettle et al., 2015). Therefore, in the absence of asynchrony all the chicks sharing a nest could be expected to perform equally poorly due to their equal competitive positions, at least in constraining environments. Yet, post-natal developmental conditions interact with pre-natal developmental conditions to shape individuals' phenotype. Mismatches between experienced pre-natal conditions and realized post-natal conditions likely impair subsequent fitness and health (Gluckman et al., 2019), but there is still much to learn about how the effects of post-natal conditions, *e.g.* level of sibling competition, are influenced by the pre-natal conditions on determining individuals' future prospects.

1.2.1.4 Large-scale geographical variation

Differences in the immediate surroundings, such as in food availability (Spurgin et al., 2018), anthropogenic noise (Injaian et al., 2019), pollution (Stauffer et al., 2017), and temperature (Simide et al., 2016) can modify telomere length patterns within populations. Many species inhabit vast geographical areas, hence different populations of those species experience distinct environmental conditions, for example in terms of temperature, seasonality, and elevation (Willig et al., 2003), which likely affect their telomere length trajectories (Angelier et al., 2018). For example, northern-living individuals of North American black bear exhibit shorter

telomeres than more southern-living individuals (Kirby et al., 2017). Similarly, it has been shown that individuals of two passerine species, the great tit and the coal tit, living at high altitudes show faster telomere attrition than individuals living at lower altitudes (Stier et al., 2016) and that distinct populations of a tree frog species had strikingly different telomere dynamics (Canestrelli et al., 2020). Telomere dynamics can also be associated with genetic polymorphism (Eisenberg, 2019; Karell et al., 2017). Despite of the emerging between-population studies, the among-population patterns of telomere length are still largely unknown. Such knowledge, however, is essential, because short telomeres can indicate local extinction risk (Dupoué et al., 2017) and telomere data could potentially be used to predict habitat suitability for the specific species (Burraco et al, in prep.).

1.3 Aims of the thesis

In this thesis, I studied various environmental and life-history factors that potentially contribute to within-individual changes, and between-individual differences in telomere length across different life stages and populations in order to understand how the environment and lifestyle can affect an individual's cellular state, and thus potentially survival. Moreover, I aimed to validate the general use of telomere length as a biomarker for past stress experiences and long-term future prospects across various species.

First, by using published longitudinal telomere data and meta-analytic methods, I investigated the within-individual repeatability of telomere length. Additionally, I addressed several biological and methodological factors that potentially create variation in the estimates of within-individual repeatability of telomere length to assess its usefulness as a biomarker (**I**). Next, I studied factors that might cause within- and between-individual alterations in the rate of telomere change within a same age group using a common and widely distributed passerine bird, the pied flycatcher (*Ficedula hypoleuca*) as a model species (**II**-**IV**). I first studied whether constant predator threat affects the telomere dynamics of a prey species (pied flycatcher) nesting near a predator. To this end, the offspring were cross-fostered between predator inhabited and control sites, and the telomeres of both the offspring and their parents were assessed **(II).** In the following study, I used the natural occurrence of hatching asynchrony (*i.e.* nestlings hatching at different days) in the pied flycatcher to study experimentally the potential interplays between laying- and hatching order to understand their effects on the chicks' phenotype and fitness prospects (**III**). Lastly, I examined the biogeographical variation of telomere length and early-life dynamics in the pied flycatchers by measuring telomeres from chick and adult individuals from six different European populations in four countries (**IV**).

2 Materials and Methods

2.1 Literature search

Literature searches were performed using Web of Science search engine to find suitable studies for chapter **I**. Additional studies were found by screening the reference list of a key paper (Olsson et al., 2018) and screening all the studies citing another key paper (Heidinger et al., 2012). While screening the studies, the inclusion criteria were (i) a non-mammalian (bird or ectotherm *i.e.* fish, reptile, amphibian) vertebrate study species, (ii) telomere length measured at least twice, (iii) at least one day between the telomere measurements, and (iv) the raw data available online/upon request. Mammal studies were excluded because (i) humans do not fit with the eco-evolutionary scope of this study since their contemporary lifestyle and improved living conditions have profoundly altered human life history traits (Corbett et al., 2018) making the ecological relevance of human studies rather questionable, and (ii) mammalian telomeres are almost exclusively measured from white blood cells. White blood cell composition can naturally vary, for example between seasons, which can bias telomere length estimates (Beaulieu et al., 2017), while non-mammalian vertebrate telomeres are mostly measured from nucleated red blood cells, which represent a more homogenous blood cell population (Stier et al., 2015b). A number of methodological and biological factors that were hypothesized to cause variation in between-individual telomere dynamics were recorded from each suitable study. These factors included (i) taxon (bird or ectotherm) since telomerase expression can differ between taxa (Gomes et al., 2010), (ii) species, (iii) sample size, (iv) number of telomere samples, (v) average time between the samples, (vi) telomere measurement method (qPCR or TRF) because of the fundamental differences in telomere quantification between the two most common methods (Aviv et al., 2011), (vii) age class of an individual (juvenile, from juvenile to adult, or adult) because of differential growth patterns (Spurgin et al., 2018), (viii) living environment (wild, captive, or wild held in captivity) due to the differences in environmental stability, and (ix) lifespan of the species as it might be linked with the rate of telomere shortening (Tricola et al., 2018). Altogether 74 eligible studies were found from which 82 study groups *i.e.* effect sizes were obtained based on above mentioned categorical factors.

2.2 Study species and basic field work

I used the pied flycatcher as a study species in chapters **II**, **III**, and **IV**. The pied flycatcher is a small, insectivorous, cavity-nesting, long-distance migratory passerine that is easy to handle and does not abandon the nest easily. Pied flycatchers breed in most of Europe and western Siberia inhabiting mountainous, deciduous, and coniferous forests (Lundberg and Alatalo, 1992). Distinct pied flycatcher populations show genetic differentiation to some extent (Lehtonen et al., 2009). Pied flycatchers winter in sub-Saharan Africa where they migrate through Iberian peninsula from all over the breeding range (Chernetsov et al., 2008; Lundberg and Alatalo, 1992; Ouwehand et al., 2016), thus populations experience marked differences in the length and duration of their migration. In Finland, pied flycatchers arrive to the breeding grounds in May (Velmala et al., 2015) when the nesting predators have already settled down (Thomson et al., 2006). Pygmy owl (*Glaucidium passerinum*) is one of the main predators of passerines in boreal forests (Kellomäki, 1977). As a diurnal, central-place forager, the pygmy owl poses a great risk for the pied flycatchers, which is seen for instance as a general avoidance of breeding sites near a pygmy owl (Kellomäki, 1977; Morosinotto et al., 2010).

Pied flycatchers breed readily in human-made nest boxes (nest box inner size used in Turku area 12.5 × 12.5 × 25 cm with entrance hole Ø of 3.2 cm). After nest construction, females lay typically one egg per day until the final clutch size of on average six or seven eggs and incubate them approximately 14 days. However, the exact timing of clutch initiation and clutch size varies among populations (Sanz, 1997). Female pied flycatchers are known to deposit to the eggs different hormones and antioxidants, amounts of which can vary across the laying order (Morosinotto et al., 2016) or among populations (Ruuskanen et al., 2011). The incubation often starts before the last egg is laid leading to hatching asynchrony with hatching times varying typically 0.5-1.5 days between the first- and the last-hatched chicks (Lundberg and Alatalo, 1992; Slagsvold, 1986). The chicks usually fledge 15-17 days after hatching.

2.2.1 Study sites

The data used to study the effects of predator presence on pied flycatcher telomere dynamics in chapter **II** were collected from Southwest Finland (Masku, Nousiainen, and Mynämäki area), 20-50 km north of the city of Turku (ca. 60°39'N, 22°09'E) in 2017. Study area consists of ca. 500 km^2 of forests areas and human inhabitation, that has been used for pygmy owl monitoring since 2013. At the time of the field work for this chapter, there were 195 nest boxes for the pygmy owls (one box approximately per one km^2 in the forested part of the area) within the study area. There were altogether 11 nest boxes with an active pygmy owl nest in the provided boxes in spring before the pied flycatchers' arrival. Accordingly, 11 sites that had a

pygmy owl nest box but no active nest in the year of the study were chosen as control sites from the same forested areas where the active pygmy owl nests were. In each study site eight pied flycatcher nest boxes per site were installed 30-40 m from the neighboring box surrounding the pygmy owl box with a radius of 60-90 m. The distance to the pygmy owl nest box was decided based on previous knowledge that pied flycatchers are aware of predator presence at this distance (Moks et al., 2016; Morosinotto et al., 2010).

The data used in chapter **III**, and part of the data in chapter **IV** were collected from the Island of Ruissalo, Turku, southern Finland (ca. 60°25'60"N, 22°10'0"E) in 2018 and 2019 when there were, respectively, 290 and 230 nest boxes available for the pied flycatchers. Study area consists of forests dominated by pine and oak and has been used for pied flycatcher monitoring since 2004. Data for chapter **IV** were additionally collected in 2019 by collaborators from five other, distinct study sites along the south-north axis of the breeding range of the pied flycatcher. In addition to Turku, another Finnish study site was in Oulu, northern Finland (65°0'0"N, 25°48′0"E). One site was in Kilingi-Nõmme, southern Estonia (58°7'0"N, 25°5'0"E), one in East Dartmoor, southern England (50°36'0"N, 3°43'0"W) and two in central Spain at the mountains of Guadarrama: one in Valsaín, Segovia (44°54'0"N, 4°01'0"W) and the other in La Hiruela, Madrid (41°04'0"N, 3°37'0"W). All the nest box areas at the sites had been established several years before this study (Fig. 1).

Figure 1. Locations of the study sites; breeding area of the pied flycatcher in Eurasia shown in orange. Map modified from: BirdLife International. 2018. *Ficedula hypoleuca*. *The IUCN Red List of Threatened Species* 2018: e.T22709308A131952521. Downloaded in January 2021.

2.2.2 Nestbox monitoring

Routine nest box monitoring in Finland started every year in May shortly after the pied flycatcher's arrival. All nest boxes were checked every four days and the state of the nest box was recorded. In the case if there were more than one egg in a nest, the laying date of the first egg was retraced based on the assumption that one egg was laid per day (**II** and **IV**). In chapter **III** it was crucial to know the exact laying date of all the eggs, and thus all nearly finished nests were checked daily until the clutch completion. After clutch completion the start of the incubation was estimated conservatively from the final clutch size. The incubation was estimated to start after laying the fifth egg if the clutch size was seven or less, and after the sixth egg if the clutch size was eight or more (**II** and **IV**). In chapter **III** the start of the incubation was experimentally controlled. The hatching date was estimated to be 14 days after the start of incubation (Lundberg and Alatalo, 1992). Nests were checked every day from one day before the estimated hatching date until hatching to determine the exact date of hatching (hatching day = day 0 in chick's life) (**II-IV**). The nests were visited 17 days after hatching to determine the nestling mortality and fledging success by counting the dead chicks in the nest (**II-IV**).

2.2.3 Blood sampling

Adult pied flycatchers were trapped for measurements and blood sampling from their nest boxes using a trap at the nest box entrance or a net in front of the nest box. The chicks were easily accessible in their nests. When adults were caught, their weight, wing length and tarsus were measured and a blood sample (50–75/10–30 µl) was taken from the wing vein and diluted in 125/65 µl of PBS (**II**/**IV**). Chicks' weight and wing length were measured, and a blood sample (about 35/10-30 µl) was taken twice during the nestling period (**II** and **III**/**IV**). The blood samples were taken similarly from the wing vein and diluted in 65 µl of PBS. All blood samples were taken with non-heparinized capillary tubes, kept chilled while in the field and stored at the end of the day at − 80 °C (**II** and **III**) or − 20 °C (**IV**).

Females were sampled during incubation (**II**) and at the end of chick rearing (**II** and **IV**). Males were sampled only at the end of chick rearing (**II** and **IV**). If an adult had not been previously ringed, they were ringed with a unique metal ring before any measurements were taken. The chicks were sampled once soon after hatching (day 5) and once shortly before fledging (day 12) (**II-IV**). The latter sampling point was decided to be few days before normal fledging age since if the nestlings are disturbed too close to fledging, they may become restless and fledge prematurely. All the chicks were also ringed for identification when they were five days old.

Data used in chapter **IV** consists of the taken measurements and blood samples. These data were collected from six distinct European pied flycatcher populations by

me and the collaborators. The collaborators were provided with above described protocol to standardize field practices, as differences in sample collection and storage might affect the subsequent telomere measurements (Reichert et al., 2017). Their samples were shipped on dry ice to the University of Turku, where I performed all the laboratory work. Data for chapters **II** and **III** were obtained following different experimental procedures that are described in the following sections.

2.3 Experimental designs

2.3.1 Cross-fostering (**II**)

To study the effects of growing under predator threat on telomere dynamics, the pied flycatcher nestlings were partially cross-fostered between owl inhabited and control sites. Cross-fostering procedure was used because it enables differentiation of environmental trait effects from genetic/prenatal effects by rearing full siblings in different environments (Merilä, 1996), and chicks rather than eggs were cross-fostered, as separating cross-fostered chicks from the control chicks after hatching would have been almost impossible. Cross-fostering exchanges were done when the chicks were three days old, and all the exchanges happened within five days. Cross-fostering pairs were matched by age and clutch size. Matching the chicks by age equalizes the conditions for the parents during chick rearing and reduces differences in the environmental conditions experienced by the chicks. Depending on the brood size two or three chicks were selected by randomly picking a chick from the nest and swapped between the cross-fostering pairs (Fig. 2). Rest of the chicks stayed in the original nest. Chicks were made identifiable from each other by gently removing the feather tufts either from the back or the head.

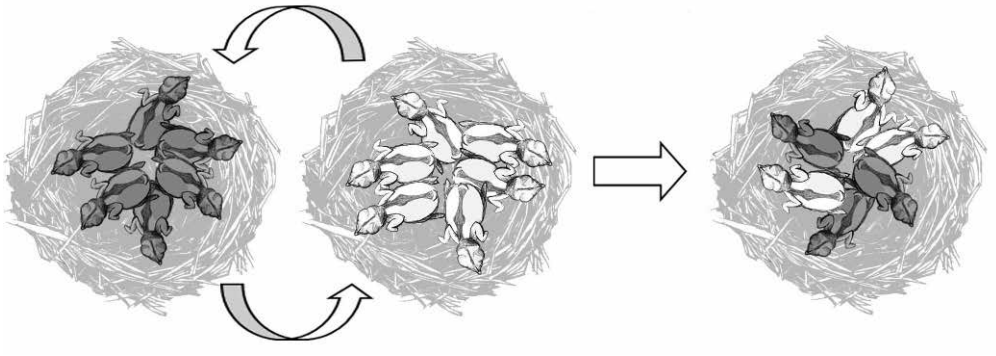

Figure 2. An example of the partial cross-fostering procedure for a nest pair with brood size of six. Three chicks per each nest are swapped resulting in two nests with mixed chicks. Figure by J. Murillo.

At owl inhabited sites nest construction and egg laying were slightly delayed, which resulted chicks at owl inhabited sites to hatch on average one day later than the chicks at control sites. Consequently, five nests at owl sites could not be matched with a control nest from the original control sites. Thus, five pied flycatcher nests from other nest box site outside of our study area had to be included to complete the cross-fostering, but only chicks from these sites were used in the study. In case of unhatched eggs, same age extra chicks were brought to the nest to match the original clutch size, but the extra chicks were not sampled.

2.3.2 Hatching order manipulation (**III**)

The aim of the hatching order manipulation was to experimentally control for the start of incubation for each egg, and consequently determine the hatching order of each egg. To achieve this, each egg was marked with corresponding number to its laying order using a permanent marker. After the third egg was laid, the eggs were replaced by matching number of dummy eggs and transferred into a little wooden holding box fitted with a fake nest. The holding box was attached underneath the actual nest box to keep the ambient temperature similar to what it would be inside the nest box (Ouwehand et al., 2017). All the subsequent eggs were moved to the holding box and replaced with a dummy egg on the same morning as they were laid. All the females had started incubation on the second day without a new egg, when the real eggs were swapped back to the nest according to the experimental design (Fig. 3). Corresponding number of dummy eggs was removed from the nest when the real eggs were returned.

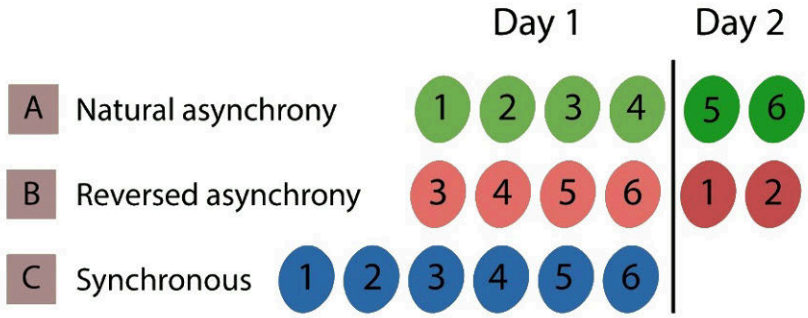

Figure 3. Schematic representation of the hatching order manipulation and experimental groups (A-C) with a clutch size of six (number inside the egg represents the rank in the laying order). Real eggs were returned to the nests on days 1 and 2. Day 1 refers to the second day without a new egg and Day 2 to the following day. Figure modified from: Kärkkäinen, T., Teerikorpi, P., Schuett, W., Stier, A., Laaksonen, T., 2021. Interplays between pre- and post-natal environments affect early-life mortality, body mass and telomere dynamics in the wild. J. Exp. Biol. 224.

The nests were divided into three experimental groups (Fig. 3). In the 'Natural asynchrony group' the two last laid eggs were left in the holding box while the other eggs were put back to the nest. The two last eggs were swapped back to the nest on the next day (Fig. 3A). In the 'Reversed asynchrony group', all but the two first laid eggs were returned to the nest on the first day, and the first and second laid eggs the next day (Fig. 3B). In 'Synchronous group', all eggs were returned to the nest on the first day, and the nests were visited the next day to standardize human disturbance (Fig. 3C). A temperature logger was placed inside a subset of nests to verify the occurrence of hatching asynchrony in the study population.

After hatching, the first-hatched chicks were marked by gently removing the feather tufts from their backs. If there were unhatched eggs at the time of first hatchings, the nests were visited again the next day. In three asynchronous nests all the chicks had hatched on the same day, but the last-hatched nestlings were easily identified by wetness of their feather tufts and body size. Rank in the laying sequence of the synchronous chicks could not be determined as they hatched at the same time and could not be differentiated from each other.

2.4 Laboratory methods

I extracted genomic DNA from whole blood samples using salt extraction alcohol precipitation method (Aljanabi and Martinez, 1997) within 2-4 months after data collection (**II**-**IV**). Extracted DNA was diluted in elution buffer BE for DNA storage. DNA concentration and purity were checked with ND-1000- Spectrophotometer (**II**-**IV**) and DNA integrity was assessed using gel electrophoresis (50ng of DNA, 0.8% agarose gel at 100mV for 60min) and DNA staining with Midori Green for a subset of samples (**III** and **IV**). Samples were then diluted to a final concentration of 2.5 ng/µl for subsequent qPCR analysis.

I quantified the telomere length by using quantitative PCR method (qPCR) that measures the amount of telomeric sequences (T) relative to the amount of non-variable, single copy gene sequences (S) producing an estimate of relative telomere length (T/S ratio). This method was first developed to measure telomere length in humans (Cawthon, 2002) but was later applied to birds (Criscuolo et al., 2009). One drawback of the qPCR method is that it measures both terminal and non-terminal, *i.e.* interstitial telomeric sequences (ITS) which do not shorten with age. When studying the terminal telomeres, between-individual differences in the ITS amount can considerably decrease the statistical power (Foote et al., 2013). Therefore, the use of qPCR for pied flycatcher samples was validated by analyzing 13 samples (10 from chicks and 3 from adults) with both qPCR and in-gel TRF, that measures only the terminal telomeres ($r=0.74$, $p=0.004$) (**II**).

qPCR analyses were performed using two different machines. Most of the analyses (**II**, **III**, and 71% of **IV**) were performed on a QuantStudio™ 12 K Flex Real-Time PCR System using 384-well qPCR plates. Due to the closedown of many laboratory service providers in spring 2020 after the worldwide Covid-19 pandemic, 29 % of the data used in chapter **IV** were analyzed on MicPCR (Magnetic Induction Cycler PCR Machine) using 48-well plates. Before the formal analyses, a test analysis using MicPCR was performed with a subset of previously analyzed samples (n = 21). The technical repeatability between the two measurements was 0.768 (95% Cl [0.54, 0.893], P<0.001), leading to the approval of the method.

Tel 1b was used as forward telomere primer and Tel 2b as a reverse telomere primer in all studies. Two different single copy genes were used in the analyses: GAPDH (**II**) and RAG1 (**III** and **IV**). Both GAPDH and RAG1 were verified as single copy genes using BLAST analyses on a close relative of the pied flycatcher, the collared flycatcher (*Ficedula albicollis*) genome. Additionally, a single narrow peak in the qPCR melting curves confirmed the specificity of the primers. Both primers were used at a final concentration of 200nM.

Final reaction volume in all the analyses was 10µl per reaction consisting of 2µl of DNA, 0.1µl of each primer, 2.8µl of water, and 5µl of SensiFAST SYBR Lo-ROX master mix. In QuantStudio the samples were analyzed in triplicates, telomere and SCG reactions next to each other on the same plate, while in MicPCR the samples were run in duplicates, telomere and SCG reactions on separate plates. Each plate included three internal standards, and QuantStudio plates additionally included one negative control.

Baseline fluorescence, the qPCR efficiencies, and the quantification cycle (Cq) values were determined using LinRegPCR (Ruijter et al., 2009). Telomere length was calculated based on the plate-specific efficiencies following the mathematical model presented in Pfaffl (2001). More detailed descriptions of the qPCR assays can be found in the original publications (**II**-**IV**).

2.5 Data analyses

General linear, general linear mixed, and generalized linear mixed models were used to study within-individual repeatability of telomere length (**I**), effects of continuous predator presence on prey telomere length and nestling growth (**II**), effects of laying and hatching order rank on nestling mortality, telomere dynamics and growth (**III**), and telomere length and chick growth patterns in European pied flycatcher populations (**IV**). All the change variables were calculated by subtracting the first value form the second. Therefore, negative telomere change values indicate telomere loss, and positive body mass change values indicate growth. Regression to the mean phenomenon (*i.e.* non-independence of the change-value from the initial value) was

addressed by correcting the telomere change values following the equations in Verhulst et al. (2013).

In chapter **I**, within-individual repeatability estimates for each study were calculated first. Then, mixed-model meta-analyses were performed to examine the magnitudes of measurement method, time between samples, taxon, living environment, age class, and species lifespan in explaining the variation in the repeatability estimates. Laboratory ID and species were used as random intercepts to control for measurements coming from the same laboratory or species. To control for species relatedness, phylogeny was used as a random effect. Phylogenetic signal was also estimated in order to detect if the repeatability estimates were phylogenetically distributed. All the analyses were executed using (i) both Fisher's Z-transformed *i.e.* standardized, and untransformed repeatability estimates, (ii) with and without phylogeny, and (iii) using frequentist (*i.e.* mixed-model) and Bayesian approaches. These different statistical analyses gave consistent results.

In chapter **II**, whether predator presence affected telomere length in the parent birds at the end of chick rearing on sex-dependent manner was tested while using nest ID as random factor to control for the shared environment experienced by the breeding pair. The effects of predator presence on telomere length change in females were examined while controlling for the females' initial telomere length. Repeated measures models were used to study effects of predator presence on female body mass (predator presence, breeding stage, and their interaction as fixed effects), and nestling telomere dynamics and growth (in both models nestling age, predator presence at original nest site, predator presence at rearing nest site, and interactions between (i) predator presence at original and rearing nest sites, (ii) nestling age and predator presence at original nest site, and (iii) nestling age and predator presence at rearing nest site were used as fixed effects, and cross-fostering pair, and both original and rearing nest boxes nested within the cross-fostering pair as random effects).

In chapter **III**, first the two asynchrony groups were divided into two subgroups based on the laying and hatching order making a five-level treatment variable: (i) first eggs hatching first, (ii) last eggs hatching last, (iii) last eggs hatching first, (iv) first eggs hatching last, and (v) all the eggs hatching at once. Nestling mortality among treatment groups both before day 5 and before fledging was investigated with logistic regression models with binary distribution (alive or dead) while taking individual hatching date into account as mortality may increase with advancing season. The effects of treatment on nestling telomere length (at days 5 and 12), telomere change, body mass (at days 5 and 12), and growth were analyzed separately while also taking the individual hatching date into account. Nest ID was included as random factor in all the models to control for the effects of shared environment.

In chapter **IV**, first the potential differences in telomere length among populations, life stages and their interactions were examined while including nest

ID, bird ID, and qPCR plate as random effects to control for the potential similarities in telomere length arising from shared environment, genetics, or telomere length quantification batch. Associations between both (i) adult telomere length, and (ii) chick telomere length at day 12 and migration distance were assessed with correlation analyses. The pattern in chick body mass at days 5 and 12, and growth between days 5 and 12 among populations were examined separately. Differences in telomere change among populations were examined with a similar model but including qPCR plate as a random effect. Relationships between growth and telomere length at days 5 and 12, as well as telomere change among populations were examined with six models (corresponding body mass first as fixed effect and then also its interaction with population) with qPCR plate as a random factor. Parent-offspring regressions were used to roughly estimate heritability of telomere length. First only population and parental (mid-parent/maternal/paternal) telomere length were included as fixed factors, then also including their interactions, while keeping qPCR plate as a random factor in all the models.

The models in chapter **II**, **III**, and **IV** were estimated using restricted maximum-likelihood (REML). Degrees of freedom of fixed factors were calculated and parameter estimates, and their standard errors were assessed with Kenward-Roger method. Least square means and Tukey–Kramer adjustment for multiple comparisons were used to evaluate the specific differences between groups (**III** and **IV**). Normality and heteroscedasticity assumptions were checked visually from the model residuals and deemed satisfactory. Statistical analyses were conducted with R (R Core Team) (**I**) and SAS statistical software (SAS Institute) (**II**-**IV**).

3 Main results and discussion

3.1 Within-individual repeatability of telomere length (**I**)

In this study, the within-individual repeatability of telomere length was assessed with a meta-analysis on published non-mammalian telomere data in order to evaluate the capacity of telomere length to indicate long-term costs. The study revealed that the overall within-individual repeatability of telomere length was moderately high (R = 0.55), but the individual repeatability estimates ranged from almost 0 to almost 1. The repeatability tended to decrease with increasing species lifespan, and phylogeny, *i.e.* the relatedness between species, had a minor, but significant effect on the within-individual repeatability estimates. Similarly, the repeatability decreased with increasing sampling interval. Most of the variation in the repeatability estimates was however explained by telomere measurement method, TRF method being markedly more repeatable than qPCR method (R = 0.80 vs. R = 0.46). Long-lived species of birds might maintain telomerase activity through life (Haussmann et al., 2007) which could explain the tendency of within-individual repeatability to decline with lifespan. However, the overall minor effect of phylogeny together with non-significant effect of other biological factors (taxon, life stage, environment) suggests that biology does not have a major role influencing the repeatability of telomere length. However, some studies have found telomere length to be highly heritable (*e.g.* Bauch et al., 2020), and high heritability would imply some degree of biological component in the within-individual repeatability. Alternatively, the existing longitudinal telomere data might not be able to identify a biological component in the within-individual repeatability of telomere length potentially due to a lack of statistical power, and it could be worthwhile to rerun the analyses in some years when the quantity of studies have increased.

Consequently, most of the variation in the repeatability estimates was explained by methodological factors. As previously reported with repeatability of metabolic rate (Auer et al., 2016; Briga and Verhulst, 2017), the within-individual repeatability of telomere length also declined with increasing time between the measurements, possibly due methodological factors (*e.g.* differences in sample storage time or processing) or increasing variation in telomere lengths with increasing time.

Nevertheless, the repeatability of telomere length was mostly affected by the telomere measurement method. The marked difference between TRF and qPCR most likely stems from the distinct ways these methods quantify telomere length: TRF utilizes gel-electrophoresis and estimates the length of terminal telomeres while qPCR relies on PCR amplification of the target sequences and estimates relative telomere length as a ratio of telomeric sequence (T) and non-variable single-copy gene sequence (S) in the sample. Measurement errors both in telomere and single-copy gene amplifications are magnified in the resulting T/S ratio and consequently, measurement error alone can decrease within-individual repeatability of telomere length (Nettle et al., 2019). Both methods might however inadvertently over-estimate the within-individual repeatability. qPCR measures also the non-terminal telomeric regions (ITS), and TRF measure includes some amount of subtelomeric regions (Baird, 2005; Foote et al., 2013). The sizes of these regions can vary between individuals but is not expected to vary in time within individuals, thus their inclusion in the telomere measure could inflate the repeatability estimates and partially explain the observed importance of measurement method.

There are some factors, both biological and methodological, that could not be taken into account in this study but might affect the repeatability of repeated telomere measurements. For example, species with indeterminate growth might require a distinctive solution to the problem of telomere shortening that could affect the within-individual repeatability. This could not be tested here as there are only very few species with longitudinal telomere measurements studied to date that exhibit indeterminate growth, and this would have also strongly confounded with taxon in the current analyses. Methodological factors include sample storage (Reichert et al., 2017), DNA extraction method (Dagnall et al., 2017; Seeker et al., 2016), DNA integrity (Ropio et al., 2020; Tolios et al., 2015), qPCR master mix (Morinha et al., 2020a), and even the stability of power supply (Hastings et al., 2020), which are especially important when using qPCR. Additionally, careless sample structure when analyzing longitudinal data (van Lieshout et al., 2020) can unintentionally decrease or inflate the following within-individual repeatability estimates both when using TRF and qPCR. These results highlight the importance of both the careful methodology (see Morinha et al., 2020b for specific guidelines), and the estimation of within-individual repeatability in longitudinal telomere studies for reliable interpretation of the data.

3.2 Telomere dynamics under constant predator threat (**II**)

The effects of continuous predator threat on telomeres of adult and nestling pied flycatchers were investigated in this study. The results reveal that female pied

flycatchers breeding in owl inhabited sites showed more telomere shortening than the females breeding in control sites. During incubation, the female telomere length did not differ between the sites, whereas close to the end of the chick rearing period, owl site females had shorter telomeres than the control site females. Also pied flycatcher males breeding in the owl sites had shorter telomeres than males in control sites at the end of chick rearing. However, since male telomeres were measured only once, it is impossible to say if this difference in telomere length is due to different rates of telomere shortening. Telomere length could be reflective of quality differences between individuals (Angelier et al., 2019; Le Vaillant et al., 2015). As it has been previously shown that the pied flycatchers often avoid breeding near the pygmy owls (Morosinotto et al., 2010), which could imply low quality of the individuals settling at owl inhabited sites, possible quality differences between adults at owl and control sites were examined. No significant differences in breeding parameters (clutch size, brood size, number of fledglings) or in wing length between owl and control site adults were found. Owl site adults were slightly lighter than control site adults, but there was no difference in the body mass change in the females between the sites during the study period. Indeed, the body mass of the pied flycatchers in the owl sites might be adjusted according to predator presence since the time of settling to improve flight performance and thus escaping capabilities (Kullberg et al., 1996; Lilliendahl, 1997). Therefore, although an original quality difference between owl and control site adults cannot be completely ruled out, it seems fairly unlikely as pied flycatchers at owl sites performed equally good to the control site adults.

Instead, the shorter telomeres in owl site adults might be caused by the stress instigated by the constant predation risk. Previous studies have associated a variety of different stressors with increased telomere shortening (Chatelain et al., 2020). Indeed, stress, that likely arises from the fear of being predated, produces glucocorticoids, which could increase telomere shortening via oxidative stress (Angelier et al., 2018; Morosinotto et al., 2018; Thomson et al., 2010) or via metabolic processes (Casagrande and Hau, 2019). Additionally, glucocorticoids are upregulated also during times of substantially increased energy demands (Casagrande and Hau, 2019). Breeding near a predator possibly increases prey's activity levels, and thus energy demands, due to avoidance flight. Parent pied flycatchers also visit their nests more often under predation risk (Hakkarainen et al., 2002; Thomson et al., 2010) which also undoubtedly increases parents' activity levels. Furthermore, lower body mass of the owl-site adults observed in this study might also indicate increased activity. The results in this study could therefore reflect differences in glucocorticoid levels between owl and control site adults. Shorter telomeres in the owl site adults might also reflect personality differences, especially in males, as bold individuals, that could be more likely to settle near a predator, are

previously reported to have shorter telomeres than more cautious individuals (Adriaenssens et al., 2016; Kim and Velando, 2015).

Chicks' telomere length was reduced from day 5 to day 12, similarly to previous studies as telomere shortening is usually fastest during growth (Spurgin et al., 2018) when cell proliferation is pronounced (Monaghan and Ozanne, 2018). In contrast with the adults, regardless of the site of origin, the chicks that were reared at the owl inhabited sites had longer telomeres throughout the study period than chicks that were reared at control sites. Thus, pre-natal, *e.g.* maternal transfer of glucocorticoids to the eggs (Haussmann et al., 2012; Noguera et al., 2020; Saino et al., 2005), or early post-natal effects had little or no importance on chick telomere length, but later post-natal conditions were more critical. This might be explained by the possibility of parent birds responding behaviorally to predator presence. As mentioned above, parent birds visit their nests more often under predation risk and they also feed their nestlings more often and resume feeding quicker after frequent predator encounters (Hakkarainen et al., 2002; Thomson et al., 2011, 2010; but see Tilgar et al., 2011). In this study there was no difference in the growth rate between chicks reared in owl and control sites, but it is possible that owl site chicks used the potential additional energy to telomere maintenance processes rather than excess growth. Alternatively, despite of increased provisioning, the parents might have reduced load size (Martindale, 1982) to diminish the begging behavior of the chicks to reduce nest conspicuousness to a potential nest predator, like the pygmy owl (Hakkarainen et al., 2002; Thomson et al., 2010). Additionally, exposure to nest predator calls can get the chicks to lower their baseline glucocorticoid levels (Ibáñez-Álamo et al., 2011) which in turn can reduce begging rate (Loiseau et al., 2008). Any reduced begging activity might lower the oxidative stress of the chicks (Moreno-Rueda et al., 2012), and thus protect their telomeres. However, to what extent this surprising positive effect of predator proximity on chick telomere length affects post-fledging survival and fitness remains to be investigated.

3.3 The importance of pre- and post-natal conditions (**III**)

In this study, a hatching order manipulation was used to study the effects of potential interplays between pre- and postnatal conditions on early-life mortality, growth, and telomere dynamics. Nestling mortality was mainly determined by the hatching order, while growth and telomere dynamics were influenced by the combined effects of laying and hatching order in a particularly harsh breeding season. This study was conducted in 2018 when the breeding season was notably drier compared to the previous and following seasons in 2017 and 2019. Dry weather likely affects the availability of insect prey, and, accordingly, the fledging success was markedly

lower in this study (51 %) than in 2017 (92 %) and 2019 (89 %), reflecting a particularly poor breeding year.

The last-hatched chicks were more likely to die before fledging than the first-hatched chicks regardless of the rank in the laying order, similarly to a recent study with common terns (Braasch and Becker, 2019). This result is also in accordance with the 'adaptive brood reduction hypothesis' suggesting that under poor environmental conditions the last-hatched chicks would starve to death (Lack, 1954; Magrath, 1990) due to their competitive disadvantage (Gottlander, 1987; Malacarne et al., 1994) or parental food distribution favoring the bigger, first-hatched chicks (Cotton et al., 1999; Smiseth et al., 2003). Indeed, the surviving last-hatched chicks in this study gained body mass slower during the study period than the first-hatched chicks. The hypothesis could be further tested by repeating the experiment in benign conditions and monitoring the survival of the last-hatched chicks. In addition to the brood reduction, Lack (1954) also suggests that equally competitive chicks in a synchronous brood would suffer from impaired growth when the conditions are poor. Contrastingly, in this study the synchronous chicks grew as fast and showed similar fledgling mass and telomere dynamics as the chicks that were naturally asynchronous. In fact, a previous study showed that pied flycatcher parents fed their chicks with bigger prey items and more often if they had hatched synchronously rather than asynchronously (Slagsvold and Wiebe, 2007). Thus, it seems that synchronous hatching could be more costly to the parents than to the offspring, but this hypothesis remains to be tested.

Competitive disadvantage has been shown to accelerate telomere shortening (Nettle et al., 2015). However, in this study, despite their competitive disadvantage, the last-hatched chicks in naturally asynchronous broods were able to reach similar body size than their older siblings without particularly increased telomere shortening. Contrary to some previous results (Stier et al., 2015a), this might indicate that the developmental match regarding laying and hatching order optimizes the chick's phenotype to reduces potential fitness costs of sibling competition. However, as nestling mortality was substantially higher among the last- than the first-hatched chicks (70% vs. 38%), selective disappearance of the chicks in poorest condition might bias the result. Thus, the potential delayed fitness costs induced by asynchronous hatching could be more evident in more favorable conditions when the direct fitness cost, *i.e.* mortality is lower (*e.g.* 8% mortality in Stier et al. (2015a)).

Contrastingly, the potential fitness costs of asynchronous hatching were more evident when the positions in the laying and hatching rank did not match, indicating that both positions are adaptively matched potentially through maternal effects (Müller and Groothuis, 2013). Last-hatched chicks (that originated from the first-laid eggs in this group) could not keep up with the growth of the first-hatched chicks (originating from the last-laid eggs), which were the heaviest chicks of all already at

day 5. Compared to the natural order asynchrony group, the sibling competition seems to be more unbalanced and in the favor of the first-hatched chicks. Surprisingly, these chicks in the more favorable competitive position exhibited the shortest telomeres at day 5. The competitive advantage and the potential higher levels of growth-inducing testosterone in the last-laid eggs (Morosinotto et al., 2016) likely resulted in the fast growth in early developmental state as indicated by these chicks being the heaviest at day 5. Similarly to a previous study with yellow-legged gulls (Noguera and Velando, 2020), this non-adaptive growth likely accelerated telomere shortening of these chicks as both pre- and post-natal growth are known to affect the telomeres (Monaghan and Ozanne, 2018; Stier et al., 2020). Interestingly, while all other chicks reduced telomere length between days 5 and 12, these chicks that hatched first from the last-laid eggs did not show any telomere shortening during this time although they kept growing faster than their last-hatched siblings. It is possible that due to their competitive superiority they were capable of obtaining enough food from their parents in order to invest both in telomere-maintenance processes and growth (Pinto et al., 2011).

3.4 Population differences in telomere length (**IV**)

Potential among-population differences in telomere length in large geographical scale were examined in this study and indeed there were marked differences in telomere length among populations: English birds had the longest telomeres, followed by the birds from the two Spanish populations while Estonian and both Finnish populations had similar, short telomere length. These differences in telomere length persisted across life stages, *i.e.* the telomere length pattern from nestling stage to adulthood was parallel in all the populations. As the birds breeding in the central area of the breeding range (England) had the longest telomeres, there was no clear correlation between telomere length and migration distance. Therefore, there is no indication that the observed populations differences in telomere length would be a by-product of the differences in migration distance. Previously reported associations between migration and both telomere length and survival might be indeed more attributable to differences between subspecies and wintering site quality than migration flight and distance *per se* (Bauer et al., 2016; Reneerkens et al., 2020). Potential subspecies differences in telomere length based on migratory lifestyle (Bauer et al., 2016) suggests that the length of telomeres and migration might be genetically correlated also in the pied flycatcher, as there is genetic differentiation among populations (Lehtonen et al., 2009). However, environmental factors potentially contribute to the longest telomeres observed in the English pied flycatchers. Central European deciduous forests might be more favorable breeding sites than northern coniferous, and southern mountainous forests, as indicated by

bigger clutches, and higher high-quality prey and egg yolk carotenoid levels in central European populations compared to northern and southern populations (Burger et al., 2012; Eeva et al., 2011; Sanz, 1997; Török et al., 2007). Carotenoids work as antioxidants protecting tissues and molecules from oxidative stress (Surai et al., 2016), thus potentially protecting telomeres from extensive shortening.

In general, heavier chicks had shorter telomeres than lighter chicks. This is not surprising as fast growth has previously been associated with accelerated telomere shortening due to intense metabolic activity and cellular proliferation (Monaghan and Ozanne, 2018). Interestingly, this cost of growth was better seen at northern (Estonia and Finland) than southern latitudes (England and Spain). Cellular stress caused by somatic growth together with generally lower food quality in the northern parts of Europe might be overwhelming combination for the telomere-maintenance processes. Additionally, the higher levels of carotenoids found in the central European, but also to some extent in Spanish eggs (Eeva et al., 2011) might better protect the chicks' telomeres during growth (Pineda-Pampliega et al., 2020).

The heritability of telomere length was moderately high in this study (h^2 = 0.32 – 0.40). The heritability was stronger at chick day 5 than day 12, and paternal heritability was stronger than maternal heritability. While several bird studies have observed mainly maternal heritability of telomere length (Asghar et al., 2015; Bauch et al., 2020; Horn et al., 2011; Reichert et al., 2015), some recent studies have reported results similar to this study (Bennett et al., 2021; Bouwhuis et al., 2018). However, the method to estimate heritability in this study cannot separate the true genetic effects from the early-life effects. Contrastingly, a study using cross-fostering design in the collared flycatcher, a close relative of the pied flycatcher, found the heritability of telomere length to be much lower than in this study (Voillemot et al., 2012). Another study on king penguins found that associations between foster parent and offspring telomere length were stronger than associations between biological parent and offspring telomere length (Viblanc et al., 2020), indicating the importance of parental care on offspring telomere length. Interestingly, a study on pied flycatchers showed that males modified their feeding rates according to the demand of the brood, while feeding effort of females remained constant (Moreno et al., 1995), which could explain the strong association between paternal and offspring telomere lengths observed in this study. Therefore, it is possible that the reported heritability estimates here reflect more the parental care and shared environmental conditions than genetic inheritance (Dugdale and Richardson, 2018). Indeed, the associations between early-life telomere length and paternal telomere length were stronger in northern Finland, Estonia, and England than in other populations, possibly indicating differences in the parental care and/or environmental conditions.

4 Conclusions

Telomere length is widely used as a biomarker for past stress exposure and future fitness and survival. As was found in chapter **I**, within-individual repeatability of telomere length across studies was moderately high, supporting the use of telomere length as such a biomarker. However, as the estimates varied from almost 0 to almost 1, particular attention should be paid to the within-individual repeatability of longitudinal telomere measurements when interpreting telomere length as a biomarker. Given the importance of methodology in explaining the variation observed in the within-individual repeatability of telomere length, minimizing the factors that might create such variability when designing studies and refining methodology seems of utmost importance. Although TRF-based studies showed consistently higher repeatability estimates, it is noteworthy to remember that with careful planning and execution it is also possible to achieve high estimates using qPCR as shown by D. Nettle with European starlings. There was little indication that biological factors would explain the variation in repeatability estimates. Yet, as most of the studies used qPCR methodology, the propensity of qPCR to measurement error might have masked any associations between biology and repeatability of telomere length. In the future it would be important to both design the research to aim at sufficiently high within-individual repeatability, and to report the repeatability estimates for different study systems in order to improve the understanding of the variety in telomere dynamics. It would also be of interest to examine how deviations from full repeatability of (1.0) reflect ecological and environmental conditions.

In this thesis I also studied different early-life conditions and later-life stressors that might affect the telomere length and dynamics of the pied flycatcher, and found indeed that environment can affect individual telomere length trajectories at different life stages. Based on different experimental designs, results of my studies demonstrated the importance of immediate post-hatching conditions to individuals' telomeres as there were differences in telomere length between experimental groups already seen at day 5 after hatching (**II** and **III**). Furthermore, these results suggest that pied flycatcher chicks rather invest any potential extra food to self-maintenance, *e.g.* telomere maintenance processes than excess growth. The two groups of chicks potentially receiving extra food (chicks at owl sites in chapter **II** and first-hatched

chicks from the last-laid eggs in chapter **III**) were similar in size to other chicks just before fledging at day 12, but had either longer telomeres (**II**) or reduced telomere shortening between days 5 and 12 (**III**) than the other chicks. Additionally, the chapter **III** highlighted the adaptive importance of the rank in the laying sequence and realized hatching rank in terms of growth and telomere dynamics. The chapter **IV** showed how growing can be more costly in terms of telomere shortening in northern, potentially more constraining, environments than in southern environments. My results also suggest the potential importance of parental care in determining offspring telomere length in the early life. This is indicated by the potentially increased provisioning in predator presence (**II**) and with synchronous broods (**III**), and by the strong associations between parental and offspring early-life telomere length (**IV**), although these hypothesis remain to be tested.

Although most telomere shortening happens during growth, my results suggest that environmental stressors can affect telomere length also later in life. This is shown as the adult pied flycatchers breeding near pygmy owls had shorter telomeres and impaired telomere maintenance compared to birds breeding in areas without a breeding pygmy owl nearby (**II**). However, as an original quality difference between birds breeding in owl vs. control sites cannot be completely excluded, further, experimental studies are needed to confirm the relationship between predator presence and prey telomere dynamics. Finally, I also found marked differences in telomere length of different European pied flycatcher populations that are likely explained by genetics or different environmental conditions at the breeding sites (**IV**). These differences among populations emerged already at nestling stage indicating that the adult birds from different populations do not experience stressors during the adulthood that would affect their telomeres differently, but still underline the need for more studies on among-population differences in telomere length and factors driving these differences.

Overall, this thesis adds new knowledge linking environmental stress to a driver of cellular, and potentially organismal, senescence. Understanding why individuals differ in the rates of aging can improve our understanding of life-history evolution. Furthermore, knowledge of factors driving individual telomere length trajectories could help in predicting organismal responses to environmental change, for example possible expanding distributions of different predators due to the warming climate. These results emphasize the complexity of telomere biology and question the common use of ‘species’ as a unit in comparative and meta-analyses involving telomeres, as different populations of a same species can show markedly different telomere lengths. As it has been shown before that short telomeres may precede population extinction, and that telomere length might be indicative of habitat suitability, this thesis also provide further evidence for the potential importance of telomere data in conservation efforts, both locally and globally.

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When I finally had the general topic for my PhD, telomeres, and one study idea, the rest of my research plan started to take shape, while being quite a shapeshifter, but was finally finalized in spring 2017, just a couple of weeks before my first field season. Special thanks at this point goes to the people in Vertebrate Ecology Research group (VERG) who patiently listened to my presentation about my half-baked research plan in February 2017 and later provided me with feedback and some ideas.

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