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# Determinants of telomere length and implications in life history trade-offs

Sophie Reichert

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*ECOLE DOCTORALE Sciences de la Vie et de la Santé*  
IPHC-DEPE

**Thèse** présentée par :  
**Sophie REICHERT**

soutenue le : **25 Octobre 2013**

pour obtenir le grade de : **Docteur de l'université de Strasbourg**

*Discipline: Sciences du Vivant*

*Spécialité: Physiologie et Biologie des organismes et des populations*

**Determinants of telomere length and implications in  
life history trade-offs**

**Facteurs déterminant la longueur des télomères et  
implications dans les compromis évolutifs**

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Par

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Soutenue le 25 Octobre 2013

Devant le jury composé de:

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*A mes grands-parents, Catherine et Alphonse Kraemer.*



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# Papers

## Studies presented in this manuscript

- **Study 1**

**Reichert S**, Zahn S, Verinaud E, Massemin S, Criscuolo F. Telomere length correlations among somatic tissues in adult zebra finches. Plos One DOI: 10.1371/journal.pone.0081496.

- **Study 2**

**Reichert S**, Rojas E, Zahn S, Robin JP, Criscuolo F, Massemin S. Maternal telomere length inheritance in the king penguin. Submitted (in revision) - Heredity.

- **Study 3**

Becker PJJ\*, **Reichert S\***, Zahn S, Hegelbach J, Massemin S, Keller L, Postma E, Criscuolo F. Mother-offspring resemblance but no additive genetic variation in telomere length in European dippers. \*shared first authors, equal contributions. Submitted - Proceedings of the Royal Society B.

- **Study 4**

**Reichert S**, Criscuolo F, Zahn S, Arrive M, , Bize P, Massemin S. Immediate and delayed effects of growth on ageing parameters in nestling zebra finches. Submitted – Ibis.

- **Study 5**

**Reichert S**, Stier A, Zahn S, Bize P, Massemin S, Criscuolo F. Increased brood size leads to persistent eroded telomeres. Submitted – Frontiers in Ecology and Evolution.

- **Study 6**

**Reichert S**, Bize P, Zahn S, Arrive M , Massemin S, Criscuolo F. Experimental increase in telomere length leads to faster feather regeneration. Submitted – Experimental Gerontology.

## Studies presented in appendices

- **Appendix 1**

Beaulieu M, **Reichert S**, Le Maho Y, Ancel A and F Criscuolo. (2011)

Oxidative status and telomere length in a long-lived bird facing a costly reproductive event. Functional Ecology 25(3): 577-585.

- **Appendix 2**

Geiger S, Le Vaillant M, Lebard T, **Reichert S**, Stier A, Le Maho Y and F Criscuolo. (2012)

Catching-up but telomere loss : half-opening the black box of growth and ageing trade-off in wild king penguin chicks. Molecular Ecology, 21(6): 1500-1506.

- **Appendix 3**

Tissier M, Zahn S, **Reichert S**, Stier A, Massemin S, Williams T<sup>†</sup> & Criscuolo F<sup>†</sup>. Maternal effects, mediated by maternally derived hormones, underlie ageing costs of growth in the zebra finch (*Taeniopygia guttata*). Submitted – Plos One.

## Study not presented in this manuscript

Stier A, **Reichert S**, Massemin S, Bize P and F Criscuolo. (2012)

Constraint and cost of oxidative stress on reproduction: correlative evidence in laboratory mice and review of the literature. Frontiers in Zoology, 26;9(1):37.

# Communications

## Oral presentations

- S. Reichert, F. Criscuolo, S. Massemin. Cost of reproduction in long-lived vs. short-lived bird species: is accelerated ageing through telomere erosion involved? **EGI Field Ornithology** – Evolution & Ecology Conference -, Oxford, UK, January 2012.
- S. Reichert, M. Beaulieu S. Zahn, P. Bize, S. Massemin, F. Criscuolo. Cost of reproduction in long-lived vs. short-lived bird species: is accelerated ageing through telomere erosion involved? **8th Annual Meeting (SERL)** – Ecology and Behaviour-, Chizé, France, April 2012.
- S. Reichert, P. Becker, L. Keller, S. Zahn, S. Massemin, F. Criscuolo .Telomere length and heredity in bird: indications of maternal inheritance. **16<sup>th</sup> Evolutionary Biology Meeting** – Marseille, France, September 2012.
- S. Reichert, E. Rojas, S. Zahn, JP. Robin, F. Criscuolo, S. Massemin. Maternal telomere length inheritance in the king penguin. **EMPSEB 19th** – Cornwall, UK, September 2013.

## Poster presentations

- S. Reichert, S. Zahn, F. Criscuolo, S. Massemin, M. Beaulieu. Oxidative stress and telomere length in a long-lived bird facing a costly reproductive event. **Changins meeting** - Aging and Longevity-, Nyon, Switzerland, November 2010.
- S. Reichert, P. Becker, L. Keller, S. Zahn, S. Massemin, F. Criscuolo .Telomere length and heredity in bird: indications of maternal inheritance. **16<sup>th</sup> Evolutionary Biology Meeting** – Marseille, France, September 2012.
- C. Bret, L. Pelletier, S. Reichert, F. Rudwill, A. Stier, A-M. Thierry. “Let’s fish krill, or how to present ecological concepts to young students in a playful way”. **Journée de l’Ecole Doctorale Vie et Santé**, Strasbourg, December 2012.
- S. Reichert, F. Criscuolo, S. Zahn, M. Arrivé, P. Bize, S. Massemin. How growth conditions influence ageing parameters? **9th EOU** – Norwich, UK, August 2013.

- S. Reichert, P. Bize, S. Zahn, M. Arrivé, P. Bize, S. Massemin, F. Criscuolo. Telomeres and telomerase activity in relation to self-maintenance **11th INTECOL** – London, UK, August 2013.

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## Foreword on the context and structure of this thesis

Life history theory is built on the idea of trade-offs between different traits. However, little is known about the processes mediating and underlying life histories across life stages. Indeed, evolutionary pathways through which life histories may have evolved are numerous, and as a consequence identifying the underlying mechanisms of those evolutionary processes is considered as crucial for our overall comprehension of the origin of life diversity. There is a need to combine studies on proximate mechanisms that give indications on constraints or trade-offs and studies of function that relate these mechanisms to fitness (principles and potential proximate mechanisms of life history theory are described in **Chapter I**).

In this context, there is clearly a great potential in the study of the repetitive DNA sequences that cap eukaryotic chromosomes, the telomeres. In vertebrate somatic cells, telomeres shorten at each cell division, and the rate at which they do so has been linked to cellular and organismal senescence (details on telomere structure and function are presented in **Chapter I**). The interest for telomeres in evolutionary ecology first arose as they were thought to be a good indicator of age structure in wild populations. As telomere length was found to vary greatly among age-matched individuals, this idea was more or less abandoned. However it opened different perspectives of research, and telomere dynamics was recently suggested as a molecular marker mediating life history trade-offs and reflecting individual quality. Indeed, telomere dynamics may reflect individual lifestyle and past-history (via the effects of environmental factors) and therefore offer a way to measure individual quality (state of art on factors influencing telomere dynamics and on indications of telomeres role in life history trade-offs can be found at the end of **Chapter I**).

In this thesis, I added my contribution to the evolutionary study of telomeres by examining the determinants of telomere dynamics, by determining whether telomeres were implicated in two life history trade-offs (growth and reproduction), and by collecting some information on the link between telomere length and fitness-related traits. A brief presentation of experimental designs and model species can be found in **Chapter II**.



Telomere length is determined by several parameters: genetic, environmental and lifestyle factors. An open question is what is the relative importance of these factors? In **Chapter III**, we checked the genetic component of early life telomere length in two wild birds, the king penguins (*Aptenodytes patagonicus*) (study 2) and the dipper (*Cinclus cinclus*) (study 3). Previous studies have shown that most telomere loss occurs in young individuals and it thus makes sense that early life conditions may greatly influence telomere dynamics. Therefore, we investigated the effects of growth conditions / trajectories on telomere dynamics (see study 4 in **Chapter IV**). As lifestyle factors are also suspected to affect telomere dynamics during adulthood, we also examined whether the level of reproductive effort could have some effects on telomere dynamics (see study 5 in **Chapter IV**). After investigating which factors influenced telomere dynamics and how they were implicated in life history trade-offs, we examined the potential link between telomere length and individual quality. To test this hypothesis, we experimentally manipulated telomere length and determined whether it could be linked to individual self-maintenance capacity measured as feather rate of growth (study 6 in **Chapter V**).

In the last chapter (**Chapter VI**), after a summary of the main results presented in this thesis, I discuss the novelty that my results bring to the telomere field research, in an evolutionary context. Finally, I provide a critical view of my work and propose possibilities for future research avenues.





## Chapter I

# General introduction



## I - Evolutionary biology and life-histories

### 1.1 General notions

Evolutionary biology aims to understand the diversity of forms of life observed *in natura* and to identify the processes and mechanisms that shaped this diversity over ages. Indeed, the purpose is to determine how the major features of a life cycle (i.e. birth and death rates, growth rates, reproductive rates, the so-called life history traits) have evolved among species. A second purpose equally important is to explain how the variation of these life history traits may have led to differences in fitness among individuals which belong to the same species. This is one of the main bases of the evolutionary theory. Life history traits encompass growth rates, reproductive investment, somatic protection and self-maintenance (i.e. mechanisms that allow the organism to survive at a given time), survival and lifespan. They are thus the principal components of fitness (Stearns 1989; Stearns 1992), which can be defined as the expected contribution of an allele, genotype or phenotype to future generations. Therefore, fitness is considered as the resultant of individual survival and reproduction and is often measured as the lifetime reproductive success (LRS) of an individual (the number of offspring that survive to reproduce) (Stearns 1992).

The cornerstone and key mechanism to the evolution of life diversity is natural selection. Natural selection is the driving force whereby those with greater reproductive success have higher expected representation in future generations. Therefore, a prerequisite for natural selection to function is the presence of heritable genetic variation of traits that results in fitness differences. It shapes life history traits to maximise fitness in a given environment (Fig I.1).

One fundamental concept to understand the evolution of life histories is the notion that life history traits (growth rates, reproductive investment, somatic protection and maintenance, survival rates) are often negatively associated with each other (Roff 1992; Stearns 1992). Indeed, the central idea of the life history theory is that traits are competing for the limited pool of resources (internal and external). Therefore if two life history traits share a common resource pool, an increment of resources to one of the trait will result in a decrease of resources to the other trait. Basically, allocation of limiting energy to one trait has negative consequences for other traits requiring the same resources, leading to trade-offs. This also

suggests that trade-offs are constrained by ecological factors specific to an environment such as the extrinsic food supply.

## 1.2 Key life history trade-offs and proximate mechanisms

### *Cost of development and growth*

Conditions experienced during development - the period from conception to developmental maturity - and growth are of crucial importance as they can have significant influence on the optimisation of life histories (Ricklefs 1979; Lindström 1999). Indeed, factors affecting early development and growth are known to impact directly growth patterns (De Kogel 1997), metabolism (Desai & Hales 1997), immune function (Saino, Calza & pape Moller 1997), and sexual attractiveness (Gustaffson, Qvarnstrom & Sheldon 1995). Moreover, the adult body size that organisms achieve is directly linked to their capacity to acquire the necessary resources for escaping predators, living and reproducing. This suggests that development and growth patterns are likely to determine self-maintenance, survival and reproductive performances.

Growth being a vulnerable stage for individuals, organisms would be expected to grow as quickly as they could in order to escape this risky stage of life (Arendt 1997). However, there is good evidence that organisms do not grow at their maximal rates and that growth rate is a flexible parameter (Dmitriew 2011). An explanation for that lies in the fact that growth rates seem to have long term effects on subsequent lifespan (Metcalf & Monaghan 2003). Indeed, individuals presenting a more rapid juvenile growth rate tend to have reduced adult lifespan (Metcalf & Monaghan 2001; Olsson & Shine 2002; Rollo 2002; Dmitriew 2011; Lee, Monaghan & Metcalfe 2013).

### *Cost of reproduction*

Historically, and due to its direct link to individual fitness, one of the most fundamental and most studied trade-off is the cost of reproduction (Williams 1966; Partridge & Harvey 1985; Reznick 1985; Linden & Møller 1989). In an evolutionary point of view, maintaining the germ line is a crucial goal, thus reproductive activity might be given

priority in terms of resources allocation at the cost of other activities. For instance, investment into current reproduction can come at a cost of future reproduction (Gustafsson & Pärt 1990), through a decrease in somatic protection and body maintenance processes (Kirkwood & Rose 1991) and individual survival rate (Partridge & Farquhar 1981). This is actually the most famous example of two traits – reproduction and longevity – that are usually negatively correlated (Partridge, Gems & Withers 2005), with increased reproductive investment resulting in reduced lifespan (Daan, Deerenberg & Dijkstra 1996; Partridge, Gems & Withers 2005; Santos & Nakagawa 2012).

### *Lifespan and ageing*

This observation drives us directly to the fact that a reduction in resources investment in self-maintenance therefore alters the individual biological state and potentially reduces its lifespan. Individual lifespan is determined by extrinsic and intrinsic mortality. Extrinsic mortality (i.e. the risk of environmentally caused mortality by predation or infection) is central to understand how growth and reproduction will be favoured over maintenance (Kirkwood & Holliday 1979). For instance, if environmental mortality is due to high predation risk, it is more worthwhile to invest heavily in rapid growth and reproduction and less in somatic maintenance, thus leading to rapid ageing (source of intrinsic mortality) and shorter lifespan.

Ageing is usually referred to as the processes leading to diminished homeostasis and increased organism vulnerability due to an irreversible accumulation of damage with age. I will also add to this definition that the decline in function results from unrepaired damage to molecules, cells, tissues that increased tissue and organism malfunctions, decreased physiological capacities and ability to repair molecular damage (i.e. decreased self-maintenance ability), changes in biochemical composition in the tissues, and increased susceptibility and vulnerability to disease, all phenomena participating to the increasing risk of death with age (Kirkwood 1977; Kirkwood & Holliday 1979). Therefore, as suggested by the disposable soma theory (Kirkwood 1977; Kirkwood & Holliday 1979), the evolution of lifespan can be viewed as a balance between selection to grow and maximise the number of reproductive events while fighting against the indirect or direct costs of those traits that



increase the intrinsic sources of mortality. The disposable soma theory can be viewed as a phenotypic version of antagonistic pleiotropy. It postulates that as the force of selection declines with age, there are antagonistically pleiotropic genes that are deleterious at old ages but are nonetheless favoured early in life due to their contribution to fecundity and survival of young individuals (Williams 1957).

Ageing and its consequences on lifespan are highly complex and involve multiple mechanisms at different levels. To distinguish between the evolutionary causes of ageing and understand the circumstances under which they apply, we need to know about the mechanistic processes responsible for organism ageing. Identifying these processes will help us understand the relationship between growth, reproduction investment, ageing rates, their consequences on lifespan and how these traits have been differently traded-off in response to environmental and historical constraints.

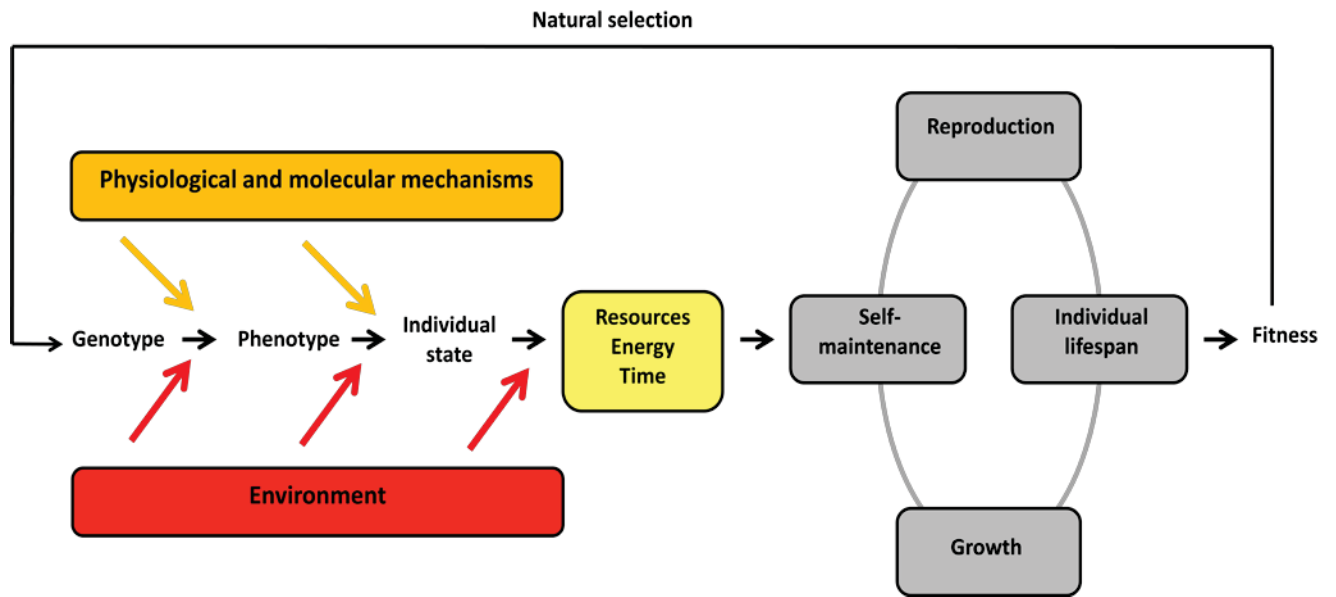
#### *Proximate mechanisms to explain life history trade-offs*

Trade-offs can be measured and analysed at different levels (Stearns 1989): the genotype, the phenotype and what lies in between (i.e. physiological and molecular mechanisms). Therefore, energy trade-offs might also be mediated by intrinsic individual properties (Ricklefs & Wikelski 2002; Speakman & Król 2005). Individuals have a set of systems that acquire and distribute energy and nutrients to tissues and organs that require them, suggesting that trade-offs could be mediated by energy and nutrients delivery and allocation to the demanding system. For instance, the constraint may be on the individual capacity to absorb and process resources into energy or on the capacity to allocate energy at the sites of utilisation (Speakman & Król 2005). Therefore, trade-offs are not only constrained by the availability of energy in the environment, but might also be constrained by physiological (such as hormones regulation) and molecular parameters (Fig I.1) (Zera & Harshman 2001; Speakman & Król 2005). Trade-offs might be considered as the result of physiological or molecular mechanisms generating negative consequences for other traits, thereby constraining the expression of multi-related traits and leading to a variability of life histories (Zera & Harshman 2001; Ricklefs & Wikelski 2002; Harshman & Zera 2007). Moreover, as the benefits of a given pattern of energy allocation might not be the same at

all stages of life (antagonistic pleiotropy), another constraint that needs to be taken into account when studying life history trade-offs is age. Complexity is thus added when traits or patterns of energy allocation are beneficial at one stage of life but are detrimental later on.

Consequently, to understand the physiological, energy and time constraints and costs shaping life-history trade-offs, and how life history traits are interlinked across life history stages, we need to acknowledge the crucial importance of the proximate mechanisms underlying the evolutionary trade-offs and how these underlying mechanisms might have influenced and shaped in return these trade-offs (Zera & Harshman 2001; Harshman & Zera 2007). Several physiological mechanisms have already been proposed as potential candidates such as hormonal regulation systems or immune function. However, to comprehend the global picture of the evolution of life history trade-offs, we need to span different biological levels and link cellular and molecular processes to these trade-offs. More particularly, to fully understand the relationships that link growth and lifespan, but also the reproductive investment to lifespan, we need to consider the proximate processes that determine lifespan via organisms ageing.

One of the processes believed to play an important part into ageing is the accumulation of oxidative damage (also known as the free radical theory of ageing, (Harman 1955; Beckman & Ames 1998; Finkel & Holbrook 2000)). The damage eventually accumulates to a level sufficient to result in the physiological changes associated with ageing. In accordance with this, Harman (1955) proposed that most ageing changes are due to molecular damage caused by free radicals produced during aerobic respiration. Free radicals will cause oxidative damage, which will accumulate over time resulting in ageing and eventually death. Another potentially important mechanism that might influence ageing which is intimately linked to oxidative damage (Von Zglinicki 2000), is telomere attrition. More recently these processes have been suggested to be related to individual life histories and involved in life history trade-offs (Monaghan & Haussmann 2006; Monaghan 2010), even in humans (Eisenberg 2011).



**Figure I.1:** Life-history theory.

The life history of an individual comprises a series of steps through which the genetic instructions unfold to produce the phenotype which will determine individual biological state and performances, which then acts within its environment to acquire resources and energy. Resources and energy will be dispatched between the main functions, with the ultimate goal of reproducing itself in future generations and maximising fitness. Different physiological and molecular mechanisms are suspected to shape and constrain these trade-offs. Natural selection will act as a feedback on this system based on the variation in fitness. Adapted from (Ricklefs & Wikelski 2002).

## II - Telomeres structure and function

### 2.1 Telomere structure

The importance of chromosome ends for chromosome stability was first suggested by McClintock (1939) working with maize and Muller (1938) working with fruit flies. They both noted that without these special structures chromosomes would fuse and often break upon mitosis, and they observed that the resulting chromosome instability was detrimental

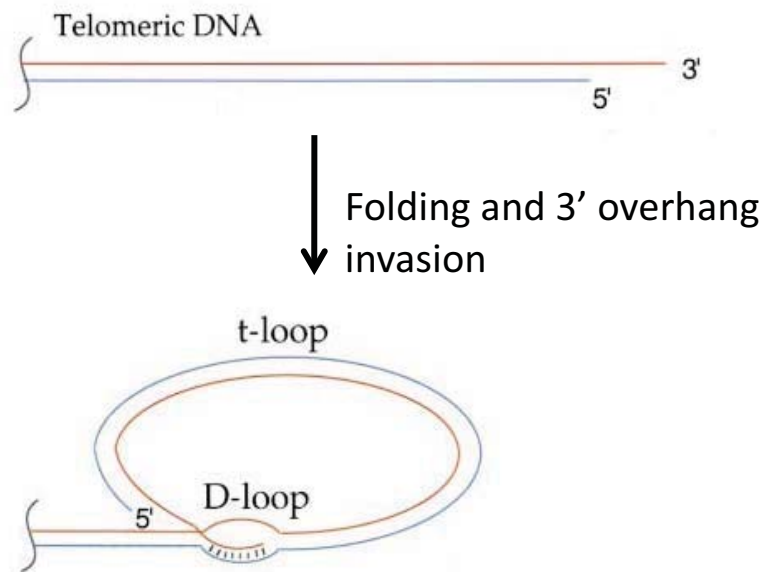
to cells. Muller was the one who named these chromosome ends “telomeres” meaning “end-part”.

Telomeres are the protective DNA-protein complexes found at the end of eukaryotic chromosomes (Blackburn 1991). They are made of non coding DNA sequences which consist of tandem repeats of a simple sequence of nucleotides that is rich in guanine (G). The number of repeats of the sequence determines telomere length. It varies greatly between species ranging from 5 to 25 kb in humans to 150 kb in laboratory mice (Louis & Vershinin 2005), but can also be of largely different sizes within an organism (e.g. from 10 kb to 1 Mb in the chicken (Delany, Krupkin & Miller 2000)). In vertebrates, the repeated sequence TTAGGG is highly conserved (Moyzis *et al.* 1988).

At the distal end of the telomere, only the guanine-rich single strand is present forming G strand overhang. This overhang can fold back into the double stranded telomere sequence, generating a looped structure known as the t-loop (Fig I.2) (Griffith *et al.* 1999). By hiding the terminal end of the chromosome, the t-loop structure creates a chromosome cap and protects telomeres end from degradation and from being mistaken for double stranded DNA breaks. Telomere sequences are associated with a number of proteins specific to the telomeric region which form a shelterin multiprotein complex (Fig I.3) (De Lange 2005). The t-loop structure and its associated proteins are conserved in vertebrates (De Lange 2005; Swanberg *et al.* 2010). This shelterin complex is formed of six proteins: TRF1, TRF2, POT1, TIN2, TPP1, and Rap1. Shelterin proteins are limited to the telomeric region as they do not accumulate elsewhere.

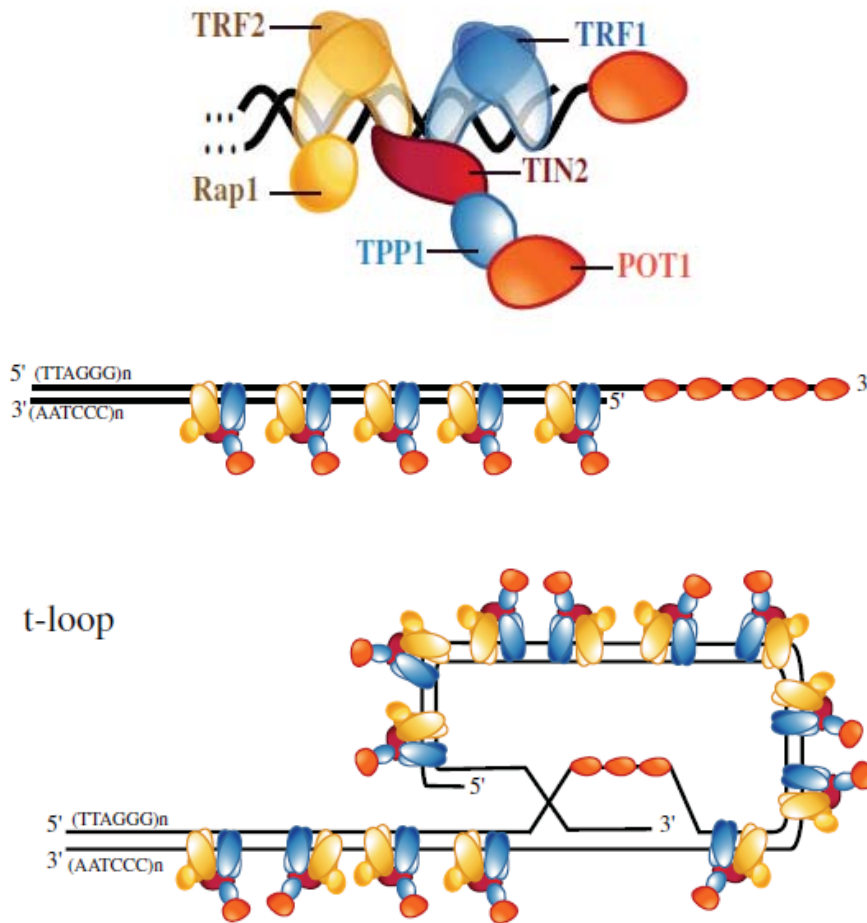
TRF1, TRF2 and POT1 directly bind to the DNA, with TRF1 and TRF2 binding the double stranded telomeric region as homodimer (Bianchi *et al.* 1999; Chapman, Fairall & Rhodes 2004), while POT1 bind the single stranded telomeric region. POT1, TRF1 and TRF2 are involved in the folding of the 3' overhang into the t-loop structure. TPP1, TIN2 and Rap1 do not interact directly with the DNA but are essential for bringing together the DNA-binding proteins. TIN 2 is the linchpin of shelterin. It tethers TPP1/POT1 to TRF1 and TRF2. TIN2 also connects TRF1 to TRF2 and this link contributes to the stabilisation of TRF2 on telomeres (Liu *et al.* 2004a).

The shelterin complex is essential for the t-loop structure formation and stabilisation (De Lange 2005).



**Figure I.2:** *Telomere structure.*

*The 3' overhang is folded and invades the main strand to form a t-loop structure. At the very end of the T-loop, the single-stranded telomere DNA is held onto a region of double-stranded DNA, this triple stranded structure is called the D-loop.*



**Figure I.3:** *The shelterin complex.*

*The shelterin complex is composed of TRF1, TRF2 and POT1 which directly bind to the DNA and of POT1, TIN2 and Rap1 which bring the complex together. (Hug & Lingner 2006)*

## 2.2 Telomere function

Telomeres main function is to ensure chromosome stability (Blackburn 2005). Indeed, telomeres provide a mechanism for the cells to distinguish between real chromosome ends and chromosome breaks that need to be repaired (Chan & Blackburn 2004). They therefore protect chromosome termini from degradation, recombination and end-joining reactions. Telomeres also play a role in the alignment and segregation of chromosomes during meiosis (Blackburn 2005).

Another major function of telomeres is the protection they provide to the encoding parts of the chromosomes from the loss of nucleotides. At each cell division chromosome ends are not replicated completely because of the chromosome end replication problem (Watson 1972), therefore chromosome erosion occurs during cell replication. This shortening of telomeres over time connects them to cell senescence.

In the early 1960's, Hayflick showed evidence that cells were not immortal and could undergo only a limited number of cell divisions. This phenomenon is referred to as the Hayflick limit (Hayflick & Moorhead 1961) and is due to a process now known as replicative senescence. Therefore, a senescent cell is a cell that has stopped to divide. Senescent cells arrest growth with a G1 DNA content and are irreversibly unable to enter phase S. The number of cell divisions a cell can undergo varies and depends on the species and the tissue type (Hayflick 2003). However, all cells are not subjected to cellular senescence, for instance, cancer cells can replicate for an indefinite number of times.

The mechanism whereby the cells autonomously count divisions involves chromosomal telomeres (Harley, Futcher & Greider 1990; Allsopp *et al.* 1992). Telomeres will shorten at each cell division due to the end replication problem (see section 2.3), leading to the hypothesis that a DNA damage signal from too short and uncapped telomeres causes cellular senescence (Blackburn 2000; Hemann *et al.* 2001; di Fagagna *et al.* 2003). Telomeres' shortening leads to telomere uncapping (disruption of the protective t-loop), thus exposing its end (Blackburn 2001). Uncapped telomeres are recognised as a double stranded DNA break, and when they reach a critical threshold length, a DNA damage checkpoint pathway is activated which triggers permanent cell-cycle arrest (Blackburn 2000; di Fagagna *et al.* 2003). This DNA damage response is signaled through ATM kinase to p53, upregulating p21 and causing G1 phase arrest (Herbig *et al.* 2004). Short telomeres and its consequence on cell senescence is thought to be a barrier against tumor development by preventing indefinite cell proliferation which might result in tumorigenesis by increased mutation accumulation (Blasco 2007). Since the replicative potential of cells is related to the longevity of the species from which they come (Röhme 1981), cellular senescence has been suggested to be an adequate marker for organismal ageing (Monaghan & Hausmann 2006).

### 2.3 Telomere length is dynamic and results from a balance between loss and restoration

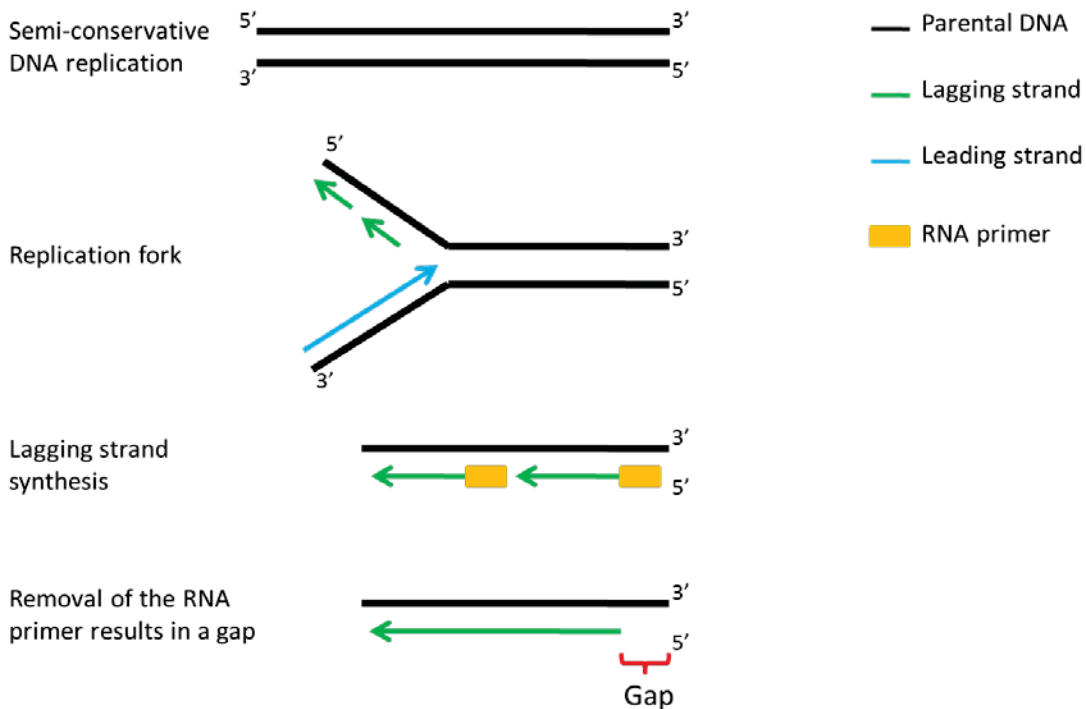
#### *Telomere loss*

- The end replication problem

Because DNA replication is a partially incomplete process, each time a cell divides telomeric DNA sequences of the chromosomes are lost (Harley, Futcher & Greider 1990; Blackburn 1991). DNA polymerase can only synthesise new DNA in the 5' → 3' direction and requires an RNA primer to bind and function properly. Whereas the DNA synthesis runs continuously for the leading strand, DNA replication for the lagging strand is discontinuous and incomplete: at the end of the replication process, the RNA primer dissociates and the most distal part where it was attached is not replicated, leading to a lagging strand slightly shorter (Fig 1.4).

The high rate of cell division that occurs during development and growth is partly responsible for the important telomere loss that is observed during these periods (Frenck, Blackburn & Shannon 1998; Rufer *et al.* 1999; Hall *et al.* 2004). This phenomenon is likely to be exacerbated by a potentially high energy metabolism during growth with its negative impact on body structures. In human cells, telomeres can shorten by 30 to 200 bp per division, but only 10 bp are thought to be due to the end replication problem (von Zglinicki, Bürkle & Kirkwood 2001). The rest of this loss is due to reactive oxygen species.





**Figure I.4:** The DNA end replication problem.

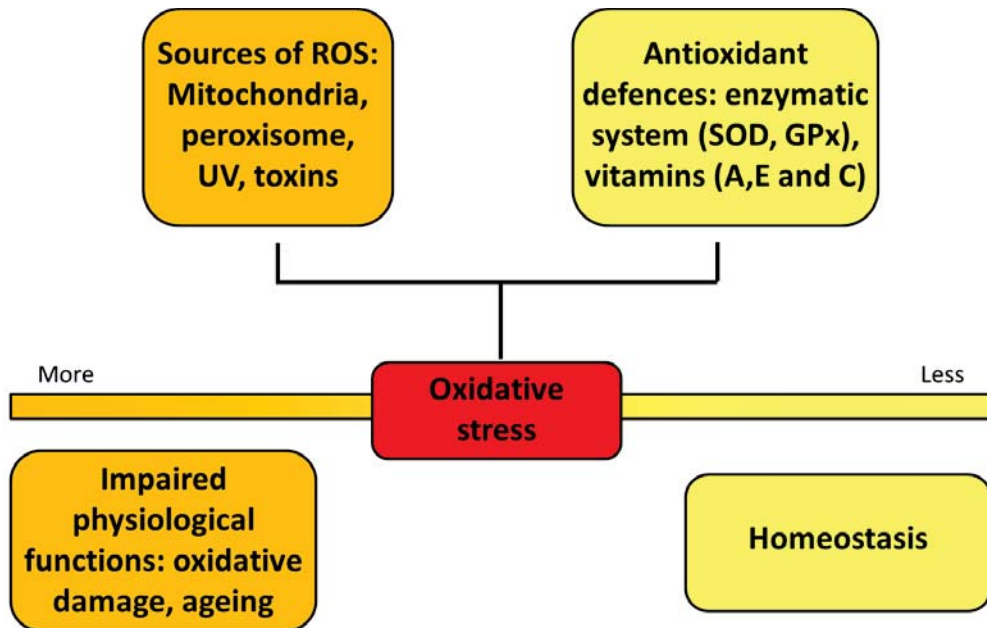
DNA replication is a semi-conservative process. The leading strand demonstrates continuous replication because DNA polymerase can only synthesise new DNA in the direction 5'→3'. Synthesis of the lagging strand is discontinuous and requires multiple RNA primers for the DNA polymerase to function. There is no DNA left at the distal end of the chromosome for the RNA primer to be attached, which results in a gap.

#### - Oxidative stress

Reactive oxygen species (ROS) are generated by normal intracellular metabolism in mitochondria and peroxisomes. Although ROS can also come from exogenous sources (UV radiation and pollutants), evidence indicates that the majority of ROS production is derived from the mitochondria (Turrens 1997). The normal oxidative production of ATP by the mitochondria produces reactive oxygen species as a by-product (Finkel & Holbrook 2000). ROS encompass free radical and non-radical oxidants. Free radicals are biochemical species, such as superoxide and hydroxyl, which contain one or more unpaired electrons which makes them very unstable and reactive. Non-radical oxidants, such as hydrogen peroxide, can persist for longer (Finkel & Holbrook 2000).

Oxidative stress is the result from an imbalance between enzymatic (catalase, superoxide dismutase - SOD, glutathione peroxidase - GPx) and non-enzymatic antioxidants (glutathione, vitamins A, C and E) and reactive oxygen species (ROS) (Fig I.5). When investigating effects on oxidative stress it is best to consider both aspects of the imbalance, antioxidant levels as well as oxidative damage which can be used as a proxy for ROS production. Independent measurements of these parameters can give a misleading picture (Monaghan, Metcalfe & Torres 2009). For this reason, in the present work, both antioxidant levels and oxidative damage were measured.

If the defences and/or the repair systems are not sufficient, the ROS produced will oxidise biomolecules, notably DNA, proteins and lipids, causing oxidative damage. Oxidative damage on DNA by ROS result in sugar and base modifications, single and double stranded breaks, and DNA-protein cross-links (Finkel & Holbrook 2000; Barja 2004). Due to their high content of guanines, telomeres are especially sensitive to oxidative damage (KAWANISHI & OIKAWA 2004). Accelerated telomere shortening due to oxidative damage can be avoided by increased levels of antioxidant defences and/or by repair DNA mechanisms. However, if not prevented, the oxidative damage on telomeric regions will lead to an accumulation of single breaks. As telomeric regions have a low efficiency of single break repair (Petersen, Saretzki & Zglinicki 1998), telomeres containing such single breaks will not be fully replicated and will shorten more rapidly as the sequence beyond the break will be lost (von Zglinicki, 2000). At the cellular level, it seems that oxidative stress triggers more telomere loss than the end replication problem (Von Zglinicki 2002). Therefore the rate of telomere shortening is not constant and do not only depends on the number of cell divisions, but is also determined by the oxidative damage inflicted on telomeres (Richter & Zglinicki 2007). It has thus been suggested that telomeres could act as sentinels for genomic damage and as warning indicator for the cells (Von Zglinicki 2002; Monaghan & Hausmann 2006).



**Figure I.5:** Oxidative stress is the result from an imbalance between production of ROS and antioxidant defences.

Oxidative stress also impacts telomerase (the enzyme responsible for telomere length maintenance, see below) activity by decreasing TERT activity (Borrás *et al.* 2004). Therefore, oxidative stress not only triggers telomere shortening by directly causing oxidative damage on the DNA, it also inhibits telomere restoration.

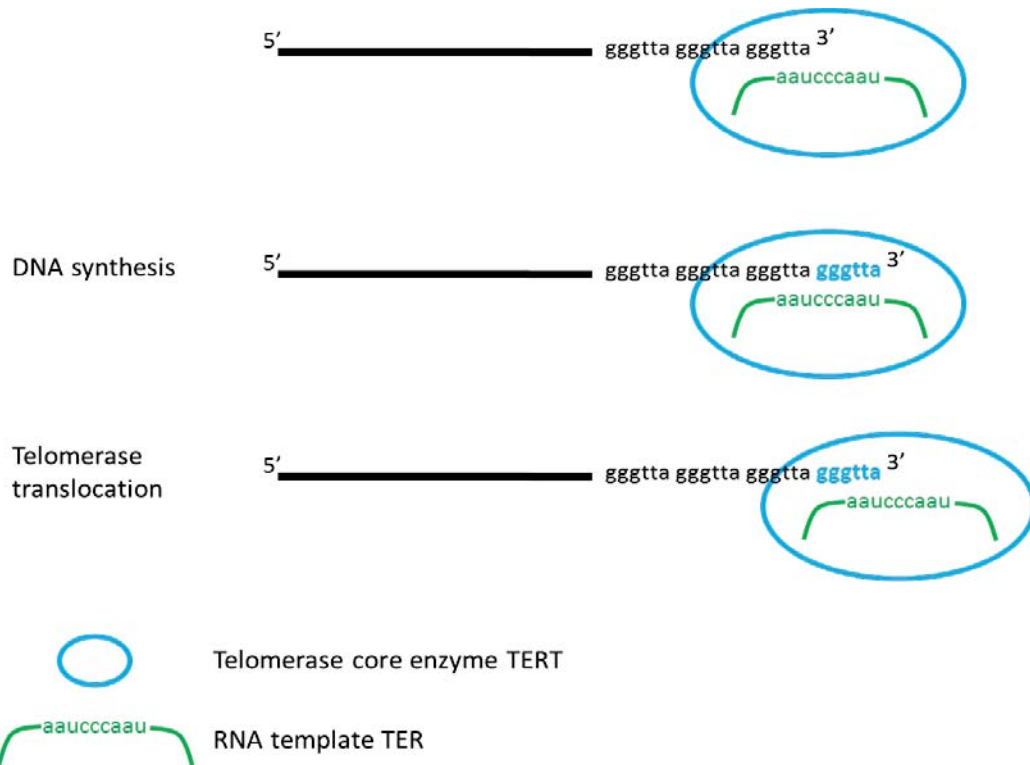
Because of its effects on telomere length, oxidative stress is thought to cause cellular senescence (Richter & Zglinicki 2007). Such an effect is in accordance with the predominant idea giving to oxidative stress a central role in organismal senescence and lifespan determination (Beckman & Ames 1998; Riklefs 2008; Monaghan, Metcalfe & Torres 2009). Both the rate of living hypothesis (Speakman *et al.* 2002) and the free radical theory of ageing (Beckman & Ames 1998) predict that a high rate of metabolism generates high levels of ROS, leading to greater oxidative stress and a faster rate of cellular damage and cellular senescence and thus shorter lifespan. Still, data collected *in vivo* are needed to test whether oxidative stress is of great importance for the determination of the rate of telomere loss in this situation. To have a clearer picture of how these two mechanisms are intimately linked

and to understand how they are involved in the growth or the reproduction evolutionary trade-offs, it is of great importance to consider both mechanisms at the same time and not to dissociate telomere dynamics from oxidative stress.

### *Telomere restoration*

However, different mechanisms exist to maintain or restore telomere length. In several eukaryotes, telomere length can be preserved through a recombination process called Alternative Lengthening of Telomeres (ALT). However this mechanism seems to be restricted to immortalised cell lines and tumors (Dunham *et al.* 2000).

One of the main mechanisms involved in telomere replication and restoration is telomerase activity. This enzyme was first discovered in a ciliate *Tetrahymena thermophila* by Greider and Blackburn (1985). The maintenance of telomere length by telomerase activity is conserved in most eukaryotes. Telomerase is a specialised cellular ribonucleoprotein reverse transcriptase complex that recognises chromosome ends as substrates. Telomerase consists of a core enzyme containing the TERT protein (a reverse transcriptase protein) and of a RNA template component (TER) with a sequence complementary to the telomeric repeats. The TERT protein copies the small template region of the RNA component to synthesise telomeric DNA repeats. Telomerase reaction sequentially adds nucleotides onto the 3' overhang at the end of the telomeric sequence (Fig I.6). Then the extended DNA terminus unpairs from its RNA template and is available for elongation by polymerase (Chan & Blackburn 2004).



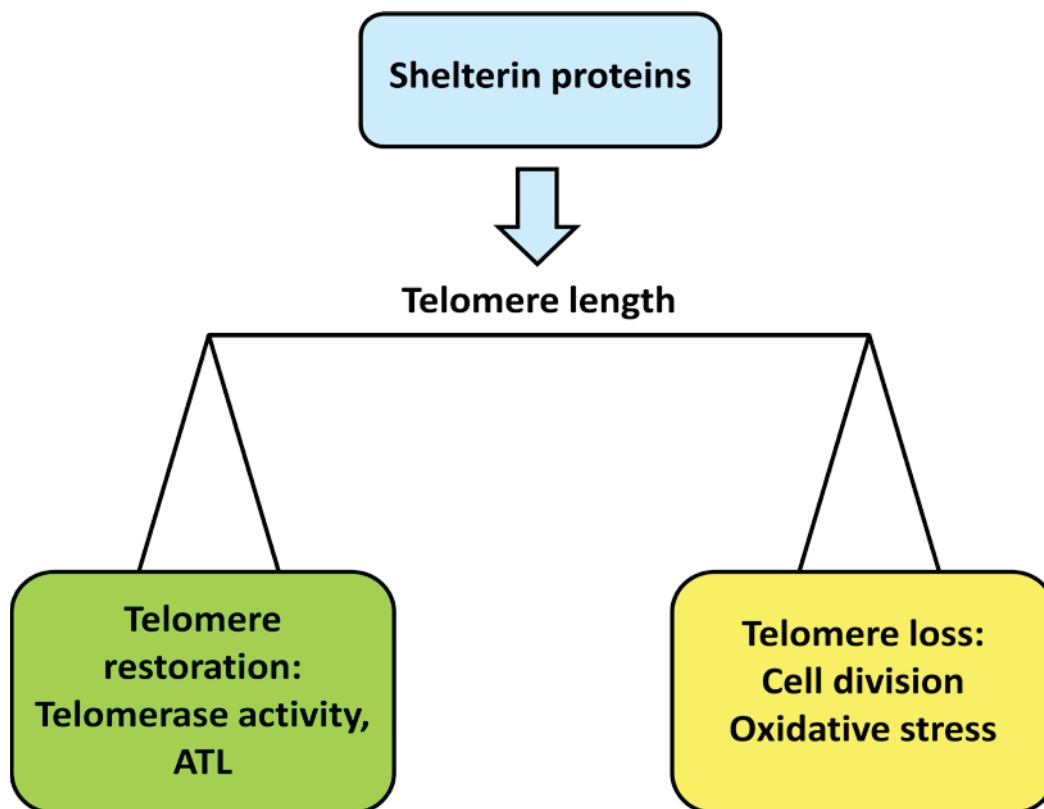
**Figure I.6:** *Telomerase activity and extension of the telomere 3' end.*

Telomerase activity at the chromosome ends is finely regulated by telomere binding proteins which may have pro- and anti-telomere erosion effects depending on their expression (de Lange 2002). Indeed, by promoting the formation of the t-loop, in which the 3' overhang is not accessible, the shelterin complex may inhibit telomerase activity. Telomerase activity is also determined by transcription factors such as p53 which represses telomerase activity through down regulation of TERT transcription (Kanaya *et al.* 2000).

Studies have shown that shorter telomeres are preferentially elongated compared to long telomeres (Liu *et al.* 2004b). This might be due to the action of POT1 which inhibits telomerase activity. Longer telomeres loading more shelterin complexes, and thus more POT1, telomerase activity might be inhibited. Load of shelterin complexes might provide a length sensing mechanism.

Telomerase activity varies between organisms, tissue, and cell types (Forsyth, Wright & Shay 2002). In mammals, telomerase is usually active in embryonic tissues and in the germ line

(Wright *et al.* 1996). The enzyme activity is extinguished during embryonic differentiation in most somatic cells but remains active in some tissues, such as male germ cells and ovaries (Wright *et al.* 1996), in activated lymphocytes, and stem cells populations such as the one in the bone marrow (Shay & Bacchetti 1997). These tissues displaying a high proliferative potential, need a certain level of telomerase activity to maintain telomere length and genetic stability. However, in most adult somatic tissues telomerase activity is repressed. In humans, this repressed activity is thought to be a mechanism to prevent tumour growth (Taylor *et al.* 2000; Shay & Wright, 2004). In any case, telomerase activity is essential for telomere length maintenance.



**Figure I.7:** Balance of cellular and molecular processes that determine telomere dynamics.

Therefore the state of telomere length is regulated by different cellular and molecular mechanisms and is determined by the equilibrium between telomere loss and telomere restorations (Fig I.7). How these two pans of the telomere balance are susceptible to

maintain a safely equilibrium depending on individual state, life stages or environmental challenges has focused the attention of recent research in evolutionary biology.

However, evidence shows that other parameters are of great importance in telomere length determination, which might explain the great inter-individual variability in changes of telomere length with age and the exact role of telomeres in the context of evolutionary processes.

### III - Indications of telomere dynamics implication in evolutionary trade-offs

#### 3.1 Great inter-individual variability in changes of telomere length with age

Given the direct link of telomere length to cellular senescence and its role in ageing (and relation in individual lifespan), one would expect to see telomere length decrease with age. In several taxa, cross-sectional studies comparing individuals from different age categories showed a trend for telomeres to shorten with age (Frenck, Blackburn & Shannon 1998; Haussmann, Vleck & Nisbet 2003; Hall *et al.* 2004). This trend is supported by data from longitudinal studies showing telomere loss within individuals with age in cats (Brümmendorf *et al.* 2002), humans (Zeichner *et al.* 1999) and tree swallows (Haussmann, Winkler & Vleck 2005). These studies indicated that telomere loss is fastest during embryonic development and early growth (Frenck, Blackburn & Shannon 1998; Haussmann, Vleck & Nisbet 2003; Hall *et al.* 2004). It was even initially suggested that telomere length could be used as to estimate individual age, for instance in wild population (Haussmann & Vleck 2002). However, even though several studies detected a link relating telomere length to age, there is a great variability in telomere length and in the rate of telomere loss between age-matched individuals (Hall *et al.* 2004). Therefore, telomere length can probably not be used as an indicator of individual chronological age. To avoid the variability of telomere length between age-matched individuals, a more powerful way to study telomere length variation is through longitudinal studies.

However, this great variability could represent the most interesting aspect of telomere dynamics. Indeed, it suggests that telomeres may be a marker of the biological age of individuals (Nakagawa, Gemmell & Burke 2004; Monaghan & Haussmann 2006), meaning

that if two individuals of the same age have different telomere lengths, the one with shorter telomeres is “older” biologically. The great variability in telomere length between individuals is to be expected given the various extrinsic and intrinsic factors that are susceptible to modulate telomere length. Hence, determining the factors causing telomere length variation between individuals might help understand the long term effects of life history strategies (Monaghan & Hausmann 2006).

### 3.2 Sources of variability between individuals

#### *Intrinsic factors*

- Genetic component

Some part of the differences observed is expected to be caused by the inherited telomere length. For any traits, the variation observed is partly due to genetic factors. Therefore, evaluating the part of the genetic component determining telomere length is of tremendous importance to understand the observed variation of telomere lengths. To determine whether telomeres are indeed involved in evolutionary processes, it is crucial to investigate telomere length inheritance and heritability patterns. (For a summary of present knowledge on telomere length inheritance patterns, telomere length heritability, see Table 1 in study 2). Studies on the subject have mainly been conducted on humans (Nawrot *et al.* 2004; Nordfjäll *et al.* 2005; Unryn, Cook & Riabowol 2005; Njajou *et al.* 2007). Reports of monozygotic and dizygotic twins’ studies indicate that mean telomere length is in part inherited (Slagboom, Droog & Boomsma 1994; Graakjaer *et al.* 2004). In humans, most studies support a paternal inheritance of telomere length (Nordfjäll *et al.* 2005; Njajou *et al.* 2007; Nordfjäll *et al.* 2009). These findings are comforted by several studies (Unryn, Cook & Riabowol 2005; De Meyer *et al.* 2007; Kimura *et al.* 2008; Eisenberg 2011) showing that paternal age is a strong determinant for offspring telomere length. However, in some cases, data also suggests a maternal inheritance of telomere length (Nawrot *et al.* 2004; Broer *et al.* 2013). Interestingly, it seems that telomere length determinants are also partly inherited from the parents. For instance, ROS production has been shown to be transmitted from mother to offspring in lizards (Olsson *et al.* 2008). In birds, very few studies focused on the telomere inheritance matter. A recent study conducted in the kakapo (*Strigops habroptila*)



(Horn *et al.* 2011), showed that telomere length was maternally inherited. Given the contrasting nature of the findings in humans and the scarcity of species in which the pattern of telomere length inheritance has been studied, this trend needs to be confirmed in wider range of organisms.

### *Extrinsic factors*

Differences in telomere length might also be explained by the intrinsic capacity of individuals to restore telomere length (telomerase activity levels), and also to their ability to deal with oxidative stress (antioxidant defence levels, repair mechanism levels) and extrinsic stressors.

- Environmental factors

There are a large number of studies, mainly in humans, suggesting that several environmental factors are prone to impact telomere dynamics (Lin, Epel & Blackburn 2012). Different types of pollution have been associated with shorter telomeres. For instance, airborne polychlorinated biphenyls, which are environmental pollutants, reduced telomerase activity and shortened telomeres in human skin cells (Senthilkumar *et al.* 2011). Similar patterns were also found in women living in polluted areas (waste landfill) in Italy (De Felice *et al.* 2012). Other environmental factors, such as smoking, bad diet and obesity also have been related to shorter telomeres (Valdes *et al.* 2005) and lower telomerase activity (Büchner *et al.* 2013). It is to be noted that the effects of these environmental factors do not necessarily have a direct impact on telomeres, but they might be mediated by determinants of telomere length such as oxidative stress or telomerase activity.

Stress is usually defined as a threat to homeostasis (McEwen & Wingfield 2003). Therefore it is used to describe *events* that are threatening to an individual and which elicit physiological and behavioural responses, as part of allostasis (which is the process of achieving stability) in addition to that imposed by the normal life cycle (McEwen & Wingfield 2003).

The work of Epel *et al.* (2004) established that chronic exposure to a certain level of environmental, and perceived as such, stress resulted in an increased rate of telomere loss and decreased telomerase activity. Later on they showed that elevated glucocorticoids levels

were related to the negative effects on telomeres (Epel *et al.* 2006; Blackburn & Epel 2012; Haussmann *et al.* 2012). This link between psychological stress and telomere length was also found in a study revealing shorter telomeres in maltreated children (Tyrka *et al.* 2010). To date, only two studies have explored the effects of physiological stress in non-human animals. In mice, individuals that were exposed to overcrowding (which can be assimilated to social stress) had shorter telomeres than mice that were not stressed (Kotrschal, Ilmonen & Penn 2007). In chickens, a recent study indicated that embryonic exposure to corticosterone resulted in higher levels of reactive oxygen metabolites and shorter telomeres (Haussmann *et al.* 2012).

Therefore it seems that stress is an important determinant of telomere length. The question is how could stress impact telomere length. In a recent review, (Haussmann & Marchetto 2010) suggested several pathways through which stress could affect telomere dynamics, the main one being through the hypothalamic-adrenal axis. Glucocorticoids may have diverse effects on oxidative stress. Indeed, they are known to exacerbate free radicals production (Liu & Mori 1999; Lin, Decuyper & Buyse 2004; Costantini, Fanfani & Dell'Omo 2008), to decrease enzymatic antioxidant capacity (Liu & Mori 1999) and to reduce telomerase activity (Choi, Fauce & Effros 2008; Haussmann & Marchetto 2010).

Over all, evidence shows that telomere dynamics is affected by several intrinsic, but also extrinsic factors, which is in accordance with the idea that telomeres might reflect individual lifestyle and life events. In this context, early life conditions and growth conditions might be particularly important as they are known to influence the optimisation of life histories (Lindström 1999).

#### - Early life conditions and growth

Most telomere loss occurs during the early life of an individual (Frenck, Blackburn & Shannon 1998; Zeichner *et al.* 1999; Brümmendorf *et al.* 2002; Forsyth, Wright & Shay 2002; Hall *et al.* 2004), which is likely to be due to the convergence of the high rate of cell division and of a potentially high energy metabolism. Consequently, conditions experienced during early life and growth are of prime importance in the determination of telomere length. It was therefore suggested that telomere dynamics could underlie the link between growth and lifespan (Metcalf & Monaghan 2003). Early environmental conditions have been linked

to telomere length and telomere loss in rats and in a couple of bird species (Foote *et al.* 2011b; Geiger *et al.* 2012 - appendix 2). In rats, growth acceleration following a period of poor nutrition triggered an acceleration of telomere shortening (Jennings *et al.* 1999; Tarry-Adkins *et al.* 2008; Tarry-Adkins *et al.* 2009; Tarry-Adkins *et al.* 2013). In European shags (Hall *et al.* 2004) and king penguins (Geiger *et al.* 2012), greater telomere loss was associated with fast growth rates. Such telomere loss seems actually to be related to a higher oxidative challenge, faster growth being linked to greater levels of cell oxidative stress (Merry 2002). However data that combine effects of brood size manipulation on both telomere dynamics and oxidative stress are lacking, and would be necessary to assess if this hypothesis is verified and if an *in vivo* link between these parameters can be actually verified. In addition, part of the deleterious effect of growth on the adult ageing rate may be due to delayed effect of early-stress (i.e. growth environmental conditions) rather than a consequence of growth rate *per se* as suggested by Voillemot *et al.* (2012). The remaining challenge is then to determine whether the effects on telomere dynamics are due to growth rates or to growth environmental conditions.

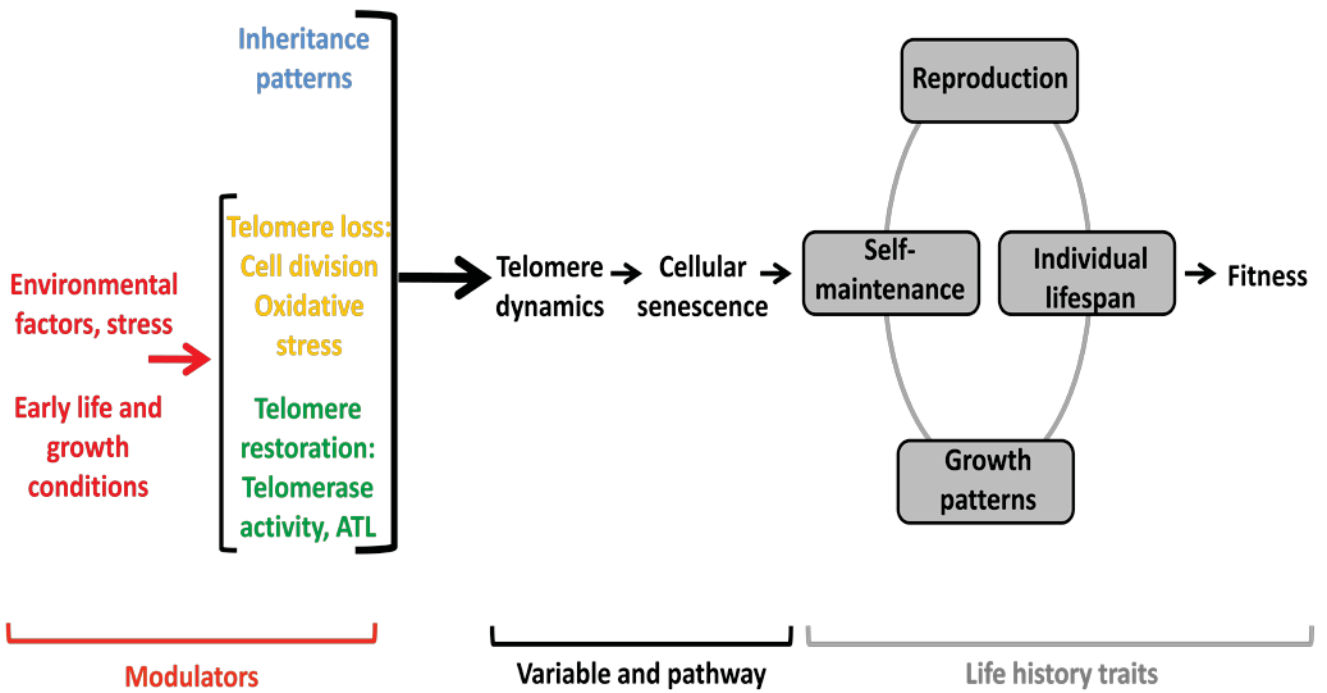
#### - Reproductive rates

Reproduction is a costly activity (Harshman & Zera 2007; Speakman 2008). The general interpretation is that body maintenance is neglected as investment in reproduction increases, leading to higher adult mortality rates. Whether this latter deleterious impact for the adult organism is potentially attained through accelerated senescence remains hypothetical. But, if so, reproductive effort would be expected to come at a cost of telomere erosion. Only a few studies have investigated this hypothesis in animals. Correlative data collected in a wild population of common terns indicate that birds that lost their broods (thus avoiding the cost of reproduction) had longer telomeres than those taking care of their chicks (Bauch, Becker & Verhulst 2013). More experimental approaches that manipulated reproductive effort displayed more contrasting results. In the long-lived Adélie penguin, increased reproductive effort was not associated with changes in telomere length, probably protected by increased maintenance mechanisms (i.e. high levels of antioxidants) (Beaulieu *et al.* 2011) (appendix 1). Alternatively, in the short-lived zebra finch, engaging in

reproduction was related to telomere shortening (Heidinger *et al.* 2012), even if accelerated shortening was independent of the number of reproductive attempts, suggesting that it might be costly to enter into reproductive life and that consecutive reproductive events are adjusted to the adult maintenance capacity (see study 5 for a critical discussion on this point).

Altogether these results indicate that accelerated senescence, via increased loss of telomere length, could be one of the costs of reproduction, thus suggesting that telomere dynamics might underlie the trade-off between reproduction and longevity. However, evidence of costs of reproduction on telomere dynamics remains controversial. To determine accurately how these costs may affect telomere dynamics, experimental approaches in which reproductive investment is manipulated by increasing and decreasing reproductive effort should be adopted. Moreover, if reproductive effort is achieved at the expense of longevity, other pro-ageing markers such as oxidative stress are also expected to be affected by the level of reproductive investment. Indeed, several studies have found that oxidative stress could be linked to the cost of reproduction (Alonso-Alvarez *et al.* 2004; Wiersma *et al.* 2004; Stier *et al.* 2012), with increased reproductive effort being associated with increased antioxidant capacity. In order to clarify how reproduction actually affects telomere dynamics, we will determine if the effects on telomere dynamics are concomitant to the state of oxidative stress.

Over all, these studies suggest that the effects of these extrinsic factors can affect telomere dynamics either directly (through a higher cell turnover) or indirectly, being mediated by up-stream determinants of telomere length (oxidative stress and telomerase activity). Looking precisely to the cascade of physiological, cell and molecular events that follow growth or reproduction seems crucial to understand how telomere dynamics may vary, depending on the individual state or stage of life (Monaghan & Hausmann 2006; Lin, Epel & Blackburn 2012). A positive answer to this question would suggest that individual difference in telomere dynamics could be one of the mechanisms that underpin life history trade-offs (Fig 1.8).



**Figure I.8:** Intrinsic and extrinsic factors modulating telomere dynamics and involvement in evolutionary trade-offs.

### 3.3 What evidence for telomeres implication in evolutionary trade-offs and link to individual fitness?

#### *Telomeres, lifespan and survival*

As telomere dynamics is linked to cellular senescence, we would expect that telomeres might give some indications on organismal senescence, and therefore individual lifespan and survival. Studies exploring the link between telomeres dynamics and survival show that individuals over 60 years old with shorter telomeres had poorer survival (Cawthon *et al.* 2003). Moreover, for a given age, women have longer telomeres than men which concur with their longer lifespan (Kimura *et al.* 2008). Only a few studies have examined the inter-individual relationship between survival and telomere dynamics in animal populations in the wild. In tree swallows, telomere length was found to be associated with subsequent survival with individuals having short telomeres displaying lower survival (Hausmann, Winkler & Vleck 2005). In giant petrels (Foote *et al.* 2011a) and in sand lizards (Olsson *et al.*

2011), shorter telomeres were also associated with lower survival. In a wild population of alpine swift, having long telomeres and a slow rate of telomere loss was predictive of increased survival rates, emphasising the importance of both telomere length and telomere loss as indicators of individual survival (Bize *et al.* 2009). Similar results were found in corvids as faster telomere loss was indicative of lower survival rates (Salomons *et al.* 2009).

Some studies have also investigated the relationship between telomeres and lifespan. In the nematode *C. elegans*, telomere length has been linked to lifespan using a transgenic line with longer telomeres. Worms with longer telomeres lived longer (Joeng *et al.* 2004). However, interspecific comparisons in birds and mammals indicate no connection between telomere length and lifespan (Vleck, Hausmann & Vleck 2003; Seluanov *et al.* 2007). Although individual telomere length might not explain lifespan at adulthood, early life telomere length might. Recently, Heidinger *et al.* (2012) showed that early life telomere length was a good predictor of individual lifespan in zebra finches, thus supporting the importance of early life conditions on telomere dynamics. Besides telomere length, the rate of telomere shortening might be of crucial importance for individual lifespan. In birds and mammals, the rate of telomere loss was linked to lifespan as species with shorter lifespan lost more telomeric repeats with age than species with longer lifespan (Hausmann *et al.* 2003). For bird species, this could be the result of telomerase activity maintenance throughout lifespan in long-lived species (Hausmann *et al.* 2004; Hausmann *et al.* 2007). In mammals, even though no relationship between telomerase activity and lifespan was found (Seluanov *et al.* 2007) experimental treatment enhancing telomerase activity in mice triggered increased longevity (Bernardes de Jesus *et al.* 2012).

Even though the results are contrasted, these findings still suggest a link between telomere dynamics and subsequent survival, as well as with individual lifespan. The question that arises from these observations is whether telomere length and/or telomere dynamics are direct indicators of individual lifespan or if they reflect other upstream mechanisms, such as oxidative stress and telomerase activity. Importantly, we also need to determine how telomere length may be related to individual survival. An ultimate question is also whether survival is the key parameter or if it simply reflects overall individual performance level (i.e. individual quality and fitness). To fully understand how the patterns of telomere dynamics

and telomerase activity are related to lifespan, their effects need to be disentangled experimentally and more comparative work is needed.

*What link between telomeres, maintenance, individual state, and fitness?*

In humans, numerous studies suggest that short telomeres have been linked to a variety of diseases usually associated with telomerase deficiencies (Aubert & Lansdorp 2008), such as the inherited genetic disorder dyskeratosis congenital (Mitchell, Wood & Collins 1999) and progeroid syndromes (Hofer *et al.* 2005). Telomeres are also implicated in age related diseases, such as cancer (Donate & Blasco 2011). Telomere shortening and short telomere lengths have also been identified in type 1 (Uziel *et al.* 2007) and type 2 diabetes (Sampson *et al.* 2006), cardiovascular diseases (Fitzpatrick *et al.* 2007) and obesity (Valdes *et al.* 2005).

This supports the idea that short telomeres and accelerated telomere loss are indicative of high disease risks, poor individual biological state and thus low individual quality. On the other hand, individuals with long telomeres and/or limited telomere loss would be expected to be high quality individuals in good biological state. In order to understand the extent to which telomeres provide indications on individual biological state and individual quality, it is important to link them to individual performances and self-maintenance. Indeed, high quality individuals are expected to display good biological state, better performances and self-maintenance ability, but also greater fitness (Wilson & Nussey 2010).

Activation of telomerase activity in laboratory mice has demonstrated that this enzyme can rescue telomere length with concomitant positive impact on health, ageing and lifespan (Bernardes De Jesus & Blasco 2011; Bernardes de Jesus *et al.* 2012), thus experimentally supporting the link between telomeres, self-maintenance, biological state and individual quality. However, these studies are the only ones experimentally investigating this link, and we still know very little about the possible generality of telomerase-increased activity on the phenotype of non-mammalian species, especially on its potential positive effects on telomere lengths and individual quality. Further studies using comparable experimental design are needed to accurately assess this relationship in other taxa.

Over the past few years, studies have also tried to determine whether telomere dynamics could be a good indicator of individual fitness. Indeed, telomere dynamics have been linked to components of fitness, as they seem to be molecular indicators of individual lifespan and survival. This hypothesis is supported by studies relating telomere length to reproductive

Individual quality: an axis of among-individual heterogeneity that is positively correlated with fitness. High quality individuals have greater fitness than low quality ones due to their capacity to optimise and maximise investment in negatively correlated life history traits. (Wilson and Nusey, 2010)

success. Indeed, investment in reproduction accelerates senescence and telomeres may constitute a link between reproductive investment and its fitness consequences. In birds, Pauliny *et al.* (2006) were the first to show that in dunlins short telomeres were associated with reduced lifetime reproductive success. More recently, a study in common terns indicated that individuals with the highest reproductive success lower rates of telomere attrition (Bauch, Becker & Verhulst 2013). There are similar findings in other taxa, as in sand lizards (Olsson *et al.* 2011); a positive link was found between telomere length and reproductive success.

All in all, these findings support the idea that telomeres might reflect individual quality and be an indicator of individual fitness. Moreover, as seen in section 3.2, telomere dynamics is determined by a wide range of intrinsic and extrinsic factors, and thus reflects individual's life events and certain lifestyle habits (Monaghan & Haussmann 2006; Lin, Epel & Blackburn 2012). Altogether, this reinforces the hypothesis stating that telomeres might be more than a mitotic counter, but could be a molecular marker for individual history and current state and potentially of individual quality and fitness.

#### **IV - Aims and scope of this thesis**

There is now substantial evidence that telomeres might be implicated in different life history trade-offs and that telomere length as well as the rate of telomere loss might be used as a means to understand the effect of trait on the others. However more experimental work is needed to determine the exact nature of the role telomeres might have in shaping life-histories and understand how they have evolved.



Up until recently, the majority of studies on telomeres have been carried out on humans or captive mammalian species, such as rats or mice. Therefore, there is a need to broaden the range of organisms studied (in captivity as well as in wild populations) to cover a wide variety of life-histories.

Evidence suggests that telomere length and the rate of telomere loss are two parameters that give very different information. Given the crucial importance of the rate at which individuals lose their telomeres, longitudinal studies should be favoured when possible, as they allow getting information on the rate of telomere shortening which is more susceptible to indicate the influence of one trait on the other.

Furthermore, the influence of different environmental circumstances, which should involve experimental manipulations, on telomere dynamics should also be tested. Finally, telomere length being the result of a balance between pro and anti-erosion processes, there is also a need for studies that experimentally manipulate environmental factors and examine the effects on both telomere dynamics and its determinants.

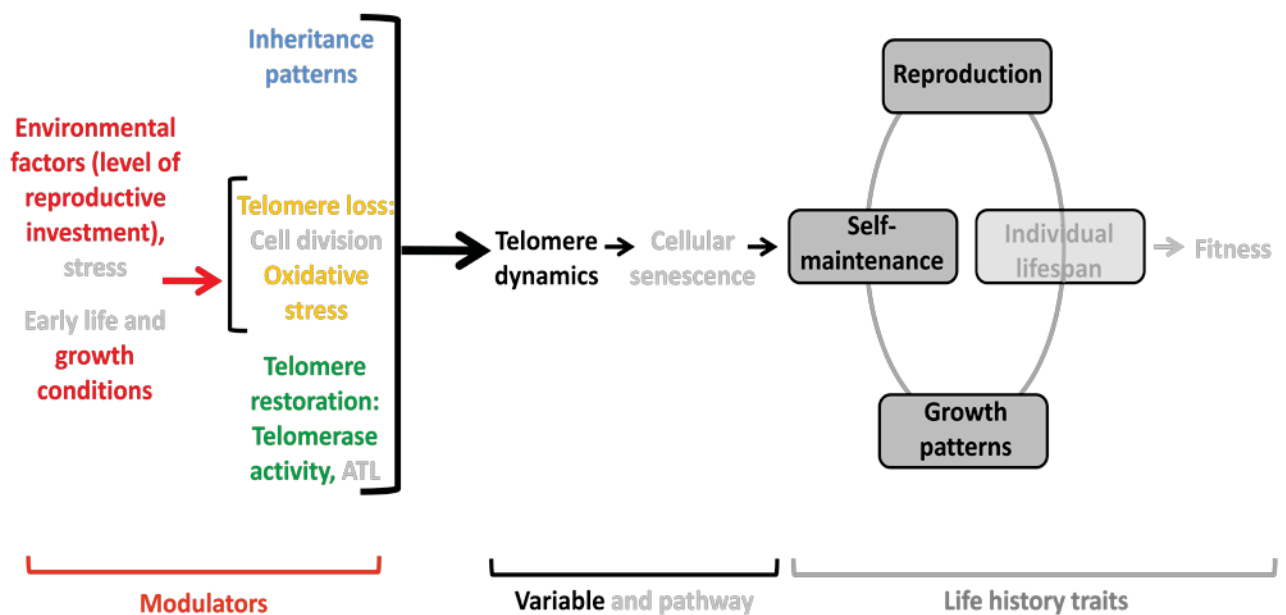
In this context, the present work proposes to investigate further which are the determinants of telomere dynamics and how these mechanisms are involved in key evolutionary trade-offs.

The first step is to estimate the genetic component determining telomere length (**Chapter III**). Indeed, only traits for which variation is transmitted are of importance in evolution, and transmission can be done through non-genetic and genetic mechanisms. Therefore, the estimation of telomere length inheritance is of tremendous importance to test whether telomere may be part of the processes that defined individual survival and lifespan over the evolutionary times. To tackle this question, we determined telomere length inheritance and heritability patterns in two wild populations of birds, the king penguin and the dipper.

In **Chapter IV**, we will try to identify which extrinsic factors are responsible for inter-individual variability of telomere dynamics and to assess how this gives us insights on the potential implication of telomere dynamics in two key life history trade-offs. By experimentally manipulating brood size in zebra finches, we studied the effects of growth

conditions and reproductive investment on telomere dynamics, thus giving insights on the role of telomere dynamics in the growth-lifespan and reproductive effort-lifespan trade-offs.

Finally, as a first step to clarify the nature of the link between telomere dynamics and individual quality, we investigated how telomere dynamics was related to self-maintenance. Using an experimental approach modifying telomere length via manipulation of telomerase activity in the zebra finch, we determined whether telomere length can be linked to self-maintenance via cell regeneration (**Chapter V**).



**Figure I.9:** Aims and scope of the thesis.

*Chapter III: inheritance and heritability patterns of telomere dynamics. Chapter IV: experimental manipulation of brood size to study the effects of growth conditions and reproductive investment on telomere dynamics, thus giving insights on the role of telomere dynamics in the growth-lifespan and reproduction-lifespan trade-offs. Chapter V: study of the link between telomerase activity, telomere dynamics and self-maintenance.*



## Chapter II

# Materials and methods



Sylvie Massemin, Philipp Becker, Antoine Stier



## I - Animal models and study sites

In this part, I will present the different species studied in the frame of this thesis, their biology and the field sites where studies were conducted. I will also describe briefly the different experimental designs (more complete versions of the experimental protocols can be found in the materials and methods section of each article).

### 1.1 Why study telomere dynamics in birds?

Most telomere studies are conducted on humans, mice and rats, especially in the context of cancer research, and to identify the lifestyle factors that accelerate ageing and morbidity (Aviv 2006; Baird 2006; Baird 2008). In order to understand the exact nature of the role that telomere dynamics might play in evolutionary trade-offs, we need to broaden the range of animal models to cover a wide range of life-histories. Therefore, assessing how telomere dynamics are implicated in trade-offs in different species is of significant importance (Monaghan & Haussmann 2006; Horn, Robertson & Gemmell 2010; Monaghan 2010). As such, the work presented here must be considered as a part of a multiple set, composed by the increasing number of studies devoted to telomeres in an evolutionary context (Monaghan & Haussmann 2006; Horn, Robertson & Gemmell 2010; Monaghan 2010).

Studying telomere dynamics in the context of evolutionary ecology in birds presents several advantages. First of all, laboratory mice and rodents generally do not exhibit age-related or division-dependent telomere shortening (Blasco *et al.* 1997; Wright & Shay 2000; Samper, Flores & Blasco 2001; Forsyth, Wright & Shay 2002) due to maintained telomerase activity in somatic tissues (Forsyth, Wright & Shay 2002). On the contrary, most birds display telomere loss with age (Venkatesan & Price 1998; Taylor & Delany 2000; Haussmann, Vleck & Nisbet 2003; Hall *et al.* 2004) and telomerase activity is absent from most somatic tissues (Haussmann *et al.* 2007). Therefore, as birds present telomere shortening, they seem to be a more suitable model to investigate how telomere dynamics might underpin the effects of one life history trait on another.

An additional advantage is that birds have nucleated red blood cells, which allows telomere length measurement in an easily accessible tissue that is representative of telomere lengths

exhibited by other somatic tissues (see paragraph III). Measuring telomere lengths in red blood cells is also very convenient as it allows longitudinal sampling therefore permitting to measure a rate of telomere shortening. Even though longitudinal studies limit the range of tissues that can be studied, they are a powerful tool to investigate multiple determinants of telomere dynamics and how the rate of telomere loss could underlie the link between life history traits.

### 1.2 Telomere heredity and inheritance patterns (Studies 2 and 3)

The first step to uncover the role of telomere dynamics in evolutionary trade-offs is to quantify the genetic component determining telomere length. Given the scarcity of bird species in which this issue has been examined, the pattern of telomere length inheritance needs to be confirmed in a wider range of species, particularly in wild populations. To tackle this question, we investigated telomere heredity and inheritance patterns in two wild populations of birds.

#### *The king penguin (*Aptenodytes patagonicus*) (Study 2)*



Sylvie Massemin

The king penguin is the second largest species of penguins. These long-lived semi-altricial pelagic seabirds reproduce in colonies in subantarctic islands and present a particular life cycle. Age at first reproduction is around six years old. Their breeding cycle is long and complex; it takes parents more than a year (14 to 16 months) to raise their single chick (Weimerskirch, Stahl & Jouventin 1992). Incubation lasts 54 days and both the male and the female take turn incubating. Chicks have to face in the middle of their growth period the 3 months of sub-Antarctic winter by their own, corresponding to a fast period with few food supply by the parents (Ancel, Beaulieu & Gilbert 2013). As a consequence, the total growth period of the unique chick lasts around 11 months, with two real phases of growth interrupted by a winter fast.

The study 2 was conducted in the colony called “La Grande Manchotière” (20 000 breeding pairs), Possession island, Crozet archipelago (Terres Australes Antarctiques Françaises) located 46° 25’S; 51° 52’E. Data were collected during two following field seasons (2009 and 2010) in the framework of the French Polar Institute IPEV (Institut Polaire français Paul Emile Victor) scientific programs (n° 119 and 137).

Due to its particular life cycle, the king penguin represents a unique model to understand how harsh environmental conditions have modelled and constrained early life history trajectories, and to study how telomere length might underlie these trajectories. In addition, it allowed studying telomere length inheritance in a wild population of birds and to determine whether the pattern of inheritance was influenced by environment and was maintained over growth (from day 10 to fledging 1 year later).



*The dipper (Cinclus cinclus) (Study 3)*

Philipp Becker

The dipper is an aquatic passerine bird found in Europe and Asia in natural conditions, with a life expectancy of eight years. It is well adapted to aquatic habitats as it lives and breeds along rivers and streams. Dippers build their nests by the water. Females lay from four to six eggs per clutch. Incubation lasts approximately sixteen days and is ensured only by the female. Both parents take care of chick rearing which lasts between 20 and 25 days (Tyler 2010).

The dipper study (study 3) was done in collaboration with Pr. Lukas Keller and his PhD student Philipp Becker from the University of Zurich. Samples were provided by Pr Keller's team, and came from an ongoing long term study initialised in 1987, conducted in eleven rivers spanning an area of approximately 400 km<sup>2</sup> in the proximity of Zurich, northern Switzerland (from 8°23'E / 47°25'N to 8°40'E / 47°10'N).

For the study 3, we used data collected during 10 field seasons (2002 to 2011) and from three different populations located along three rivers (Küsnacht K, Sihl S and Wehrenbach W) (Fig II.1) for which coverage was complete since then and population size exceeded five breeding pairs throughout. Fieldwork is year-round and consists in ringing the whole population. Nests are checked regularly during nest building, egg laying, incubation and / or nestling phase to allow for ringing and blood sampling of offspring preferably at the age of

ten to 14 days (min. eight, max. 17 days). Therefore, in study 3 parents' telomere lengths and their chicks' telomere lengths were both sampled early in life.



**Figure II.1:** *Field site for the study 3.*

Working on this long term data set allowed testing whether the same pattern of telomere inheritance was found in another wild population. Moreover, it allowed determining whether this pattern of inheritance was maintained over the years or if it varied due to environmental conditions. As we also had long term information on the animals' pedigree, it also gave the unique opportunity to test if there was a link between the level of inbreeding and telomere length. Thus this approach enabled us to test the impact of (i) genetic, and (ii) environmental (nest and year effects) factors, and of (iii) inbreeding coefficient (*via* pedigree data) on the pattern of telomere length inheritance.

### 1.3 Determinants of telomere length variability, implication in evolutionary trade-offs and individual state

*The zebra finch (Taeniopygia guttata) (Study 1, 4, 5 and 6)*



Céline Bret

The zebra finch is a small passerine bird most common in Central Australia and Indonesia, with a variable life expectancy. In natural conditions, they usually live up to five years, but when kept in an aviary, they have a life expectancy of five to seven years. Zebra finches present a sexual dimorphism; males are characterised by their red coloration on cheeks feathers, their black barring on the throat and their chestnut-coloured patch with white spots on the flanks.

In captivity, zebra finches can breed all year long. The number of eggs ranges from two to seven eggs per clutch. Briefly, after laying an egg per day, parents take turn at incubating the eggs for an average period of fourteen days, at which time eggs hatch according to their laying time. Chick rearing then takes approximately thirty days. Both the male and the female contribute to the chick rearing, they take turn sitting on the nest and bringing food to the young. Chicks usually fledge around fifteen days old, the growth period is usually over at thirty days old and the reddening of the beak and the sexual signals appears at two months (Zann & Bamford 1996).

The Australian zebra finch is a good model for laboratory research in a wide range of biological disciplines, and particularly in ecophysiology. Indeed, its biology is well known (Zann & Bamford 1996) and this relatively short-lived bird has the advantage to present a

short breeding cycle and a short growth period which allows experimental manipulations. Moreover its genome is entirely sequenced, which allows access to DNA sequences for gene expression studies.

Studies 1, 4, 5 and 6 were conducted on a captive population of zebra finches held at the IPHC-DEPE in Strasbourg. When not under experimental conditions, animals were kept in large aviaries, separated by sex, in a room maintained at a constant temperature of 24°C ( $\pm$  1°C) and light conditions were 13L : 11 D. Under experimental conditions, animals were housed in cages (0.57 x 0.31 x 0.39 m). At all times, they had access to food (a commercial mix of seeds for exotic birds enriched with vitamins and eggs) and water *ad libitum*.

### *Experimental designs*

- Brood size manipulation experiment in the zebra finch (Studies 4 and 5)

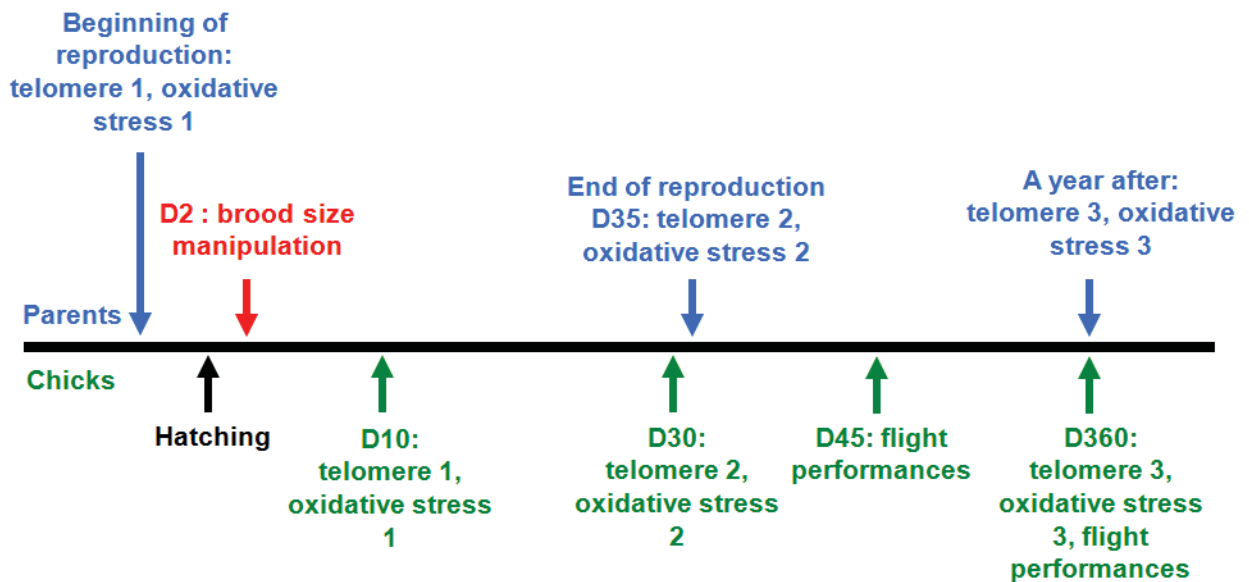
To test the effects of growth conditions and reproductive effort on telomere dynamics, we manipulated brood size. This type of experimental design has proven to be efficient and powerful to assess the costs of development and growth (Dijkstra *et al.* 1990; Alonso-Alvarez *et al.* 2006; Alonso-Alvarez *et al.* 2007) as well as the costs of reproductive investment (Daan, Deerenberg & Dijkstra 1996; Metcalfe & Monaghan 2013).

Twenty-five randomly formed pairs were placed in breeding cages. Nest boxes and straw were provided for breeding. Brood size was manipulated in order to create three groups: 9 enlarged broods, 9 reduced broods, and 7 control broods. Chicks, from 1 to 3 days old, were randomly cross-fostered to form enlarged (2 chicks added, mean clutch size 4.8,  $\pm$  0.75), reduced (2 chicks removed, 1.8  $\pm$  0.64) and control broods (number of chicks unchanged, 2.8  $\pm$  0.64). Brood size was constant in each of the treatment groups during the experiment. In total, there were 30 chicks in the enlarged group, 15 chicks in the reduced group and 25 chicks in the control group. Chicks were separated from their parents when the younger chick from the brood was 35 days old.

To determine the effects of growth conditions on telomere dynamics and oxidative stress, blood samples were collected from the brachial vein of the chicks during the growth period (i.e. 30 $\mu$ L at 10 days and 30 days), the same individuals were re-sampled when still alive one year after treatment (i.e. 50 $\mu$ L at 360 days). To assess the effects of the experimental

treatment on growth trajectories and individual performances, body mass growth trajectory was assessed by weighing the chicks every day from hatching until they attained 30 days old and flight performance measurements were performed at 45 days and 360 days (Fig II.2).

To assess the effects of manipulated reproductive effort on parents' body mass, oxidative stress and telomere dynamics, adults were weighed and small blood samples (50 $\mu$ L) were collected from the brachial vein of the parents at the beginning of the reproductive period (when the breeding pairs were formed), at the end of the reproductive period (when the chicks were 35 days old, which corresponds to the moment when they were separated from the parents) and a year after the experiment (Fig II.2).



**Figure II.2:** *Experimental design of brood size manipulation.*

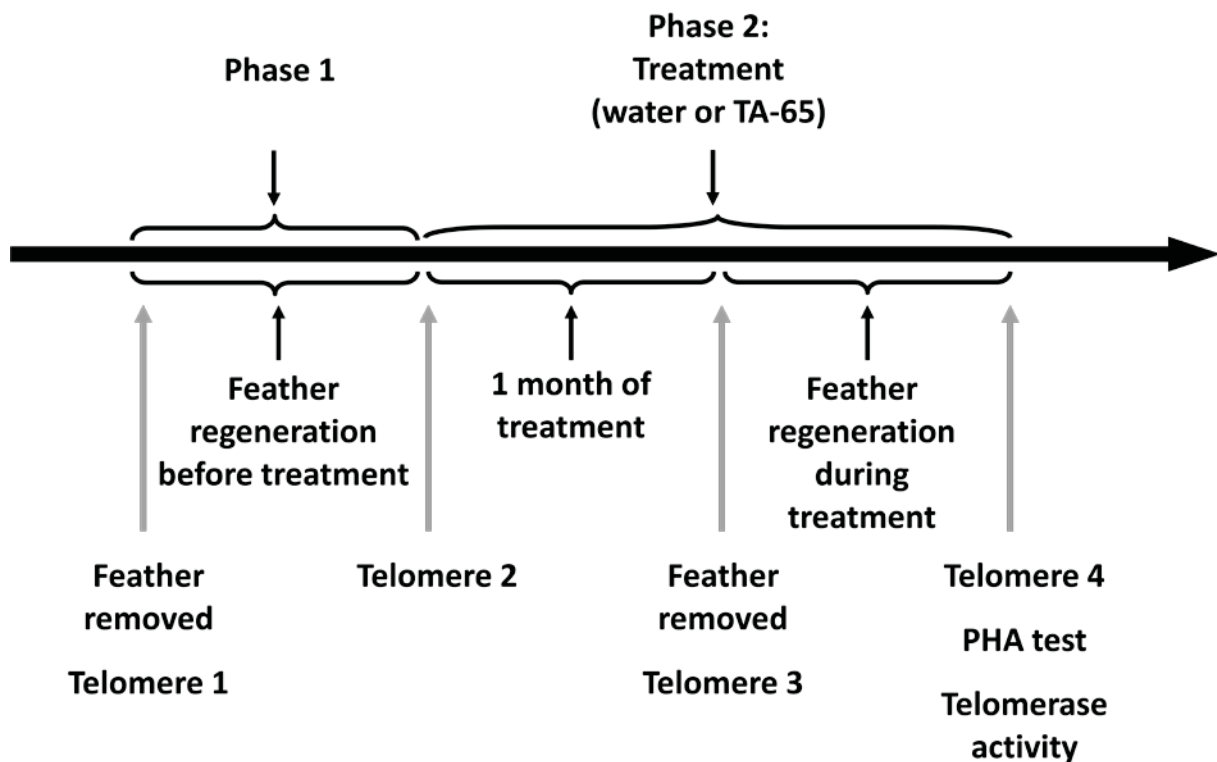
- Manipulation of telomerase activity in the zebra finch (Study 6 and Box 1)

To assess the potential link between telomere length and individual maintenance, we experimentally manipulated telomerase activity and determined the effects of this manipulation on telomere dynamics, feather regeneration capacity and immune response (used as proxy for individual maintenance).

Twenty-eight males were randomly assigned to two experimental groups: a control group treated with sterilised water (100µl) and a group treated with TA-65 (T.A. Sciences) (0.5 mg diluted in 100 µl of sterilised water per day) which is a small-molecule purified from the root of *Astragalus membranaceus*. It is an activator of the hTERT gene, thereby activating telomerase (de Jesus *et al.* 2011).

Feather regeneration capacity was determined by removing the 7<sup>th</sup> primary feather on the right wing of each bird and assessing the speed of feather growth by measuring feather length with an electronic calliper every two days until it attained its initial size. Feather growth capacity was assessed twice: before (phase 1) and during treatment (phase 2).

To evaluate telomere length, blood samples were collected (50µL) before the plucking of the feather (time 1), at the end of the first phase of feather regeneration (just before the start of treatment with water or TA-65) (time 2), a month after the beginning of the treatment (just before the second phase of feather regeneration started) (time 3), at the end of the second phase of feather growth (time 4). Immune function was determined using a PHA test which was performed at the end of the second experimental phase (time 4). Finally, at the end of the experimental treatment, we also measured telomerase activity (time 4) (Fig II.3).



**Figure II.3:** Experimental design of the manipulation of telomerase activity.

## II- Measuring telomere length: real time quantitative polymerase chain reaction (qPCR)

In this part I will describe the general principles of the qPCR assay. The specific qPCR protocols for each species can be found in the materials and methods section of each article.

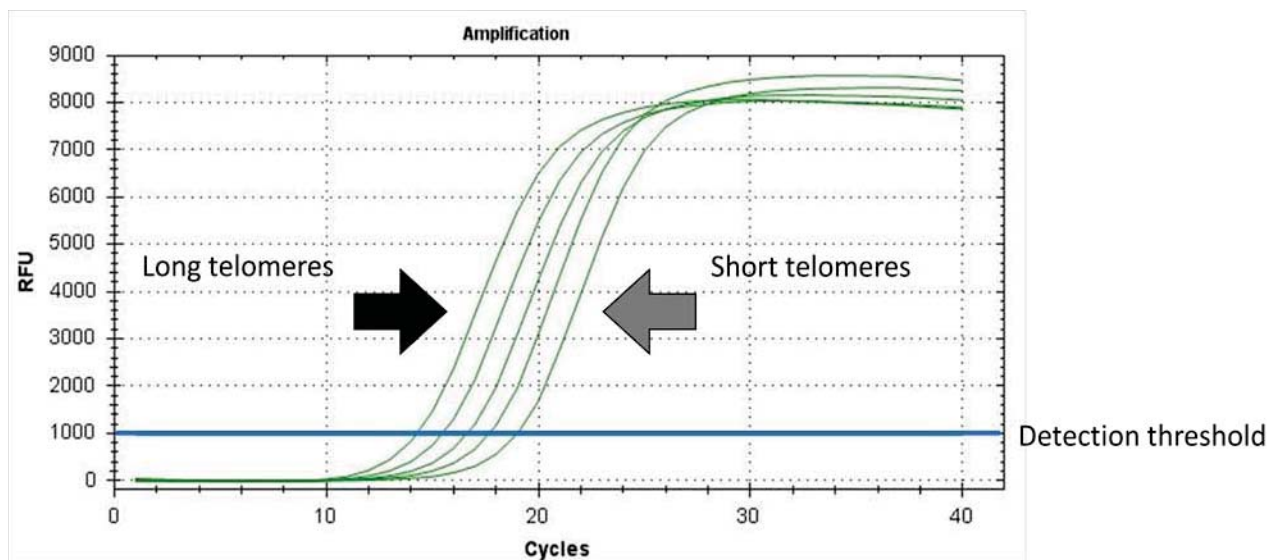
Several methods exist to measure telomere length. In the context of this work, telomere length was assessed by real time qPCR procedure first described for humans by (Cawthon 2002) and later adapted to birds by (Criscuolo *et al.* 2009). This method measures the relative amount of telomere repeats in a cell population; relative telomere length is then expressed as the ratio (T/S) of telomere repeat copy number (T) to a control single gene copy number (S). The control gene is chosen to avoid any variation in copy number among or within individuals over time to ensure that variation in T/S ratios is only due to variation in telomere size (Smith, Turbill & Penn 2011).

The main advantages of the qPCR method are that it is quick and requires low concentration of DNA. This method also presents drawbacks, for instance relative measurement of telomere length with qPCR assays only provides a ratio in relation to the reference DNA sample. When comparing between individuals of the same species for a given study, this is not an issue. However, it makes it more difficult to compare between studies and between species. The other issue associated with qPCR assays is that interstitial telomeric repeats (which avian chromosomes contain near the centromeres (Venkatesan & Price 1998)) are measured as well as terminal telomeric repeats. This problem is only relevant when considering cross sectional analyses but not when measuring the rate of telomere change within individuals, as the amount of interstitial repeats does not change over time (Delany *et al.* 2003). Moreover, estimating telomere length using methods that incorporate interstitial telomeric repeat, such as qPCR analyses, reduces the power to detect differences between treatment groups as it increases the chance of making a type II error (Foote, Vleck & Vleck 2013).

The qPCR method could be viewed as conservative as it does not prevent to detect connections between telomere dynamics and life history traits. It is therefore still very useful for determining interindividual differences in telomere length within a species, and is a very

adequate method when comparing individuals in longitudinal studies (Nakagawa, Gemmell & Burke 2004).

Telomere and control gene real time amplification were performed on different plates (except in study 2). Each qPCR plate (telomere plate and control gene plate) included serial dilutions of DNA of the same reference bird (the internal control) which was used to generate a reference curve to control for the amplifying efficiency of the qPCR and to set the threshold Ct value. The Ct of a DNA sample is the number of PCR cycles to which the sample must be subjected in order to accumulate enough products to cross the set threshold of fluorescent signal (Fig II.4).



**Figure II.4:** Example of quantitative PCR assay.

Relative telomere length is proportional to the number of PCR cycles necessary to accumulate products to cross the detection threshold.

To take into account the slight variation of efficiencies between telomere and control gene amplifications, we calculated relative telomere length using the method suggested by (Pfaffl 2001). The efficiencies values were used to calculate the relative T/S ratios, reflecting the length differences in telomeric DNA, which is relative to the constant control gene amplicon and is calculated following the formula:  $((1 + E_{telomere})^{\Delta Ct_{telomere}} (internal\ control - sample) / (1 + E_{control\ gene})^{\Delta Ct_{control\ gene}} (internal\ control - sample))$ .



### III- Validation of telomere length measurement in red blood cells

## Study 1

# Telomere length correlations among somatic tissues in adult zebra finches

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## Introduction

Les télomères sont des structures d'ADN double brin non codant situées aux extrémités des chromosomes des eucaryotes. Leur rôle principal est de protéger l'intégrité du génome, en évitant que les extrémités des chromosomes ne soient reconnues comme des cassures. La taille des télomères varie selon les espèces, mais aussi au sein des individus en fonction de l'âge, des tissus et des chromosomes.

La longueur télomérique est dynamique, et résulte de multiples facteurs pro (taux de division cellulaire et stress oxydant) et anti-érosion (activité télomérase et expression des protéines shelterin). Au niveau de la cellule, une fois qu'une longueur télomérique critique est atteinte, la voie de signalisation de mort cellulaire ou de sénescence cellulaire est mise en route. De ce fait, de nombreuses études se sont intéressées au lien entre la dynamique d'érosion des télomères et la durée de vie des organismes.

De plus, l'importante variation interindividuelle de longueur de télomères observée entre individus du même âge, de même que l'influence de nombreux facteurs environnementaux sur la dynamique d'érosion des télomères, indiquent que les télomères sont susceptibles d'être un marqueur de l'âge biologique des organismes. En effet, les télomères, reflétant l'hygiène et le mode de vie des individus, pourraient être un bon indicateur de la fitness individuelle. Par conséquent, les télomères suscitent un intérêt tout particulier en biologie évolutive et en écologie, comme mécanismes susceptibles de sous-tendre les compromis évolutifs.

Dans le cadre de ces études, la longueur des télomères est mesurée sur des échantillons sanguins, et ce, pour des raisons méthodologiques. En effet, le sang est un tissu qui est aisément accessible, et qui permet le suivi longitudinal des individus. Cependant, la variation de la longueur des télomères entre les tissus, soulève la question de la validité de la mesure des télomères dans le sang.

La synchronie de la longueur des télomères entre les tissus a été testée chez différentes espèces de mammifères (homme, singe, chien). Toutefois, cette information manquait chez d'autres espèces de vertébrés tels que les oiseaux.

En mesurant et comparant la longueur des télomères dans différents tissus (globules rouges, moelle osseuse, cerveau, rate, muscle pectoral, cœur et foie) chez le diamant mandarin

(*Taeniopygia guttata*), nous avons cherché à déterminer si la longueur des télomères dans les globules rouges était représentative de celle des autres tissus.

## Principaux résultats

Quelque soit le tissu considéré, la longueur des télomères n'est pas corrélée à l'âge des individus. En revanche, le type de tissus a un effet significatif sur la longueur des télomères, la taille des télomères dans le muscle pectoral étant plus importante que celle dans le cœur, le foie, la moelle osseuse et la rate. La longueur des télomères dans les globules rouges n'est pas significativement différente de celle observée dans les autres tissus.

Au niveau individuel, la longueur des télomères dans les globules rouges est significativement corrélée à celle constatée dans les autres tissus. Ainsi, les individus présentant de longs télomères dans les globules rouges présentent également de longs télomères dans la rate, le muscle, le cœur, le foie et le cerveau. Le seul tissu dont la longueur télomérique ne corrèle pas avec celle observée dans les globules rouges, est la moelle osseuse.

## Discussion

Jusqu'à présent les études s'intéressant à la synchronie de la longueur télomérique entre les tissus se sont focalisées sur des espèces de mammifères. Au vue de l'utilisation grandissante de la mesure télomérique dans les globules rouges, dans le cadre de diverses études en écologie, nous avons testé la synchronie de la longueur des télomères entre différents tissus chez une espèce d'oiseau.

Plus spécifiquement, nous avons testé si la longueur des télomères dans les globules rouges différait de celle de différents tissus somatiques chez des individus jeunes et vieux.

Notre étude montre des liens significatifs entre la longueur des télomères dans les globules rouges et celle de plusieurs tissus indépendamment de leur capacité répliquative. Ceci confirme que la mesure télomérique effectuée dans les globules rouges est représentative de la longueur des télomères à l'échelle de l'organisme chez les oiseaux.

Cette synchronie est similaire à celle constatée dans les études effectuées sur les mammifères.

De plus, cette étude démontre que la synchronie observée entre les tissus, se maintient au cours du temps, suggérant ainsi qu'à l'âge adulte, le taux d'érosion des télomères n'est pas différent entre les tissus.

Ceci corrobore l'hypothèse suivante : la différence de la longueur des télomères entre les tissus est probablement fixée durant la période de croissance. Afin de confirmer ce postulat, il faudrait vérifier la synchronie entre les organes sur des individus en croissance.

En conclusion, il semble que dans le cadre d'études en écologie et en biologie évolutive, la mesure des télomères dans les globules rouges soit susceptible de refléter la longueur globale des télomères des individus.

## Abstract

Telomeres are repetitive non coding DNA sequences located at the end of eukaryotic chromosomes, which maintain the integrity of the genome by hiding the chromosome ends from being recognised as double stranded breaks. Telomeres are emerging as biomarkers for ageing and survival, and are susceptible to reflect different individual life history trajectories. In particular, the telomere length with which one starts in life has been shown to be linked with individual life-long survival, suggesting that telomere dynamics can be a proxy for individual fitness and thereby be implicated in evolutionary trade-offs. As a consequence, an increasing number of studies were conducted on telomeres in the fields of ecology and evolutionary biology, in which telomere length was almost exclusively measured from blood samples. However, not only do the number of repeats of the telomeric sequences vary among species, but also within species with great inter-individual telomere lengths variability with age, tissues, and chromosomes. This raises the issue of the exact biological meaning of telomere measurement in blood cells and stimulated the study of the correlation of telomere lengths among tissues over age. By measuring telomere length in adult zebra finches (*Taeniopygia guttata*) in different somatic tissues displaying variable cell turnovers (bone marrow, brain, spleen, pectoral muscle, heart, liver and in red blood cells), we checked that the measure of telomere length in red blood cells is related to telomere lengths in the other tissues. Here we show significant relationships between the telomere lengths of red blood cells and several somatic tissues at adulthood. As red blood cells are easily accessible and suitable for the longitudinal monitoring of the individual rate of telomere loss, our study confirms that telomere length measured in red blood cells could serve as a surrogate for telomere length in the whole avian organism.

## Introduction

Telomeres are repetitive non coding DNA sequences located at the end of eukaryotic chromosomes. They maintain the integrity of the genome by hiding the chromosome ends as being recognised as double stranded breaks by DNA repairing systems (Blackburn 1991). The number of repeats of the telomeric sequences not only varies among species, but also within species with great inter-individual telomere lengths variability with age, tissues, and chromosomes (Forsyth, Wright & Shay 2002).

Telomere length is dynamic and results from pro and anti-erosion factors. Telomere length is progressively lost because of the inability of DNA polymerase to completely replicate telomeres, progressive telomere shortening thus occurs over cell divisions (Blackburn 1991). This loss is thought to be further aggravated by oxidative stress (Von Zglinicki 2002) which comes from the imbalance between the production of reactive oxygen species (ROS) and the antioxidant capacity. Telomere length is also regulated by restoration factors such as telomerase activity and the shelterin proteins complex (Blackburn 2001; De Lange 2005). In the absence of telomere restoration, telomeres get shorter each time a cell divides. Eventually, a lower critical length is reached and telomere signalisation pathway induces cell division arrest and/or cell senescence (Harley, Futcher & Greider 1990; Blackburn 1991).

Given these characteristics, telomere length and the rates of telomere loss have been widely studied in the context of species or individual variability in lifespan (Cawthon *et al.* 2003; Hausmann *et al.* 2003; Bize *et al.* 2009; Hartmann *et al.* 2009; Salomons *et al.* 2009; Heidinger *et al.* 2012). The substantial variation observed in the relationship between telomere length and age between individuals (Hall *et al.* 2004), and the influence of several environmental factors (Monaghan & Hausmann 2006), such as stress (Epel *et al.* 2004; Hausmann & Marchetto 2010), on telomere dynamics, hint that telomeres could be more than just an indicator of chronological age (Hausmann, Winkler & Vleck 2005; Monaghan & Hausmann 2006; Pauliny *et al.* 2006; Olsson *et al.* 2011). Indeed, telomeres could be a marker of individual lifestyle (Monaghan & Hausmann 2006; Monaghan 2010) and therefore be a good proxy for individual fitness (Bauch, Becker & Verhulst 2013). Consequently, telomeres prove to be of great interest for evolutionary biologists and

ecologists as a mechanism potentially involved in the evolution of life histories (Monaghan & Haussmann 2006; Haussmann & Marchetto 2010; Monaghan 2010).

In most of the studies conducted on telomeres in the fields of ecology and evolutionary biology, telomere length was measured from blood samples. The main reason is methodological, as blood samples can be easily obtained and repeated during an experiment in most animals, allowing an individual recording of telomere length over time. Indeed, nucleated erythrocytes in birds, reptiles, fish and amphibians, and leukocytes in mammals can be used to provide DNA to measure telomere length from blood samples. As most of the blood cells do not divide once in circulation, telomere lengths in peripheral blood cells are thus susceptible to reflect instantaneous telomere lengths in hematopoietic stem cells (Vaziri *et al.* 1994). However, the determinants of the rate of telomere dynamics vary greatly between tissue types. For instance, some tissues such as intestinal mucosa and peripheral blood cells have cell rapid turnovers that require high cell proliferation, while other tissues are predominantly mitotically inactive, such as skeletal muscle and the brain. Eroded telomere length can be restored by telomerase activity (Greider & Blackburn 1985). Even though telomerase activity is repressed in most normal adult somatic tissues (Forsyth, Wright & Shay 2002), a number of exceptions exist and telomerase activity has been detected in proliferative cells of tissues and organs having rapid cell turnovers (Forsyth, Wright & Shay 2002; Haussmann *et al.* 2007). Telomerase is generally active in germ cell lines, in embryonic tissue and in tissues with a high cell turnover such as bone marrow, intestine and gonads (Forsyth, Wright & Shay 2002; Haussmann *et al.* 2007). Such inter-tissue differences in the rate of cell division and of telomere anti-erosion activities should lead to large differences in telomere shortening rates (Forsyth, Wright & Shay 2002; Takubo *et al.* 2002), with shorter telomeres in highly replicative tissues (Gardner *et al.* 2007; Granick *et al.* 2011).

This raised the issue of the exact biological meaning of telomere measurement in blood cells and had stimulated the study of the correlation of telomere lengths among tissues over age (i.e. synchrony in telomere length). Data in human studies highlight strong correlations in telomere length across somatic tissues (Friedrich *et al.* 2000; Takubo *et al.* 2002; Daniali *et al.* 2013). This synchrony in telomere lengths across somatic tissues was found in other mammals (monkeys (Gardner *et al.* 2007) or dogs (Benetos *et al.* 2011)) where leukocytes



telomere length was similar to the one measured in other somatic tissues such as muscle, skin and fat. A comparable pattern was found in the zebrafish (*Danio rerio*), characterised by little difference in telomere lengths among organs (Lund *et al.* 2009).

However, corresponding data are lacking in other vertebrates species, such as birds. By measuring telomere length in adult zebra finches (*Taeniopygia guttata*) in different somatic tissues with variable cell turnovers such as the bone marrow, the brain, the spleen, the pectoral muscle, the heart, the liver and in red blood cells (RBC), we propose to determine if the measure of telomere length in red blood cells is related to the telomere lengths in these other tissues. We also aim to verify if the rate of telomere loss differ among tissues in different adult age classes.

## Methods

### *Ethics statement*

During this experiment, animal care was in accordance with institutional guidelines. The study complied with the 'Principles of Animal Care' publication no.86-23, revised 1985 of the National Institute of Health, and with current legislation (L87-848) on animal experimentation in France and with the European Instruction 2010/63/UE of the 22nd September 2010. The DEPE holds a license from the French Department of Veterinary Service (license number G67-482-18). Dr François Criscuolo, team leader of this project, holds a license to experiment on animals delivered by the French prefecture (license number 67-78). Animals were originally obtained from a pet shop (Nilufar Strasbourg Lampertheim, Lampertheim, France).

The euthanasia method complies with the CNRS instructions regarding animal experimentation.

Prior to euthanasia, animals were in cages (0.57 x 0.31 x 0.39 m) with food (a commercial mix of seeds for exotic) and water *ad libitum*. The cages were put in a room with constant temperature of 24°C ( $\pm 1^\circ\text{C}$ ) and light conditions were 13L : 11 D.

### ***Experimental design***

The present study was conducted on twenty captive adult zebra finches (*Taeniopygia guttata*) (10 males and 10 females). Before euthanasia, a blood sample was taken (50  $\mu$ L) and snap-frozen into liquid nitrogen, and birds were then sacrificed by cervical dislocation. The brain, the spleen, the heart, the liver, the right pectoral muscle and the bone marrow were collected just after euthanasia and snap-frozen into liquid nitrogen. Blood and tissue samples were stored at -80°C until analysis. Mean animal age was 3.7 years and ranged from one to seven years old.

### ***Telomere length measurement***

Telomere length was measured on DNA extracted from each tissue, using DNeasy Blood and Tissue kit (Qiagen). Telomere length was assessed by quantitative real-time amplification (qPCR) procedure (Cawthon 2002) adapted to zebra finches and described by Criscuolo (Criscuolo *et al.* 2009). Cycle number is proportional to the sample telomere length (T), or to the number of copies of a non-variable copy number gene (or control gene S). Relative telomere length for each sample is expressed as the ratio (T/S). We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a single control gene. Forward and reverse primers for the GAPDH gene were 5'-AACCAGCCAAGTACGATGACAT-3' and 5'-CCATCAGCAGCAGCCTTCA-3' respectively. Telomere primers were: Tel1b (5'-CGGTTTGGTTGGGTTTGGGTTTGGGTTTGGGTT-3') and Tel2b (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'). qPCR for both telomere and GAPDH were performed using 5 ng of DNA with sets of primers Tel1b/Tel2b (or GAPDH-F/GAPDH-R), each used at a concentration of 200 nM/200 nM, in a final volume of 10  $\mu$ l containing 5  $\mu$ l of Power SYBR Green PCR Master Mix (Applied biosystems). Telomere and GAPDH real time amplifications were performed on separate plates. Samples were run in duplicate on each plate. Samples were randomly assigned on a total of four plates with samples of one or two tissues source per plate. Each plate (telomere and GAPDH) included serial dilutions (40 ng, 20 ng, 10 ng, 5 ng, 2.5 ng, 1.25 ng) of DNA of the same reference bird which were run in duplicate. This was used to generate a reference curve to control for the amplifying efficiency of the qPCR. Mean  $r^2$  of the qPCR runs were 0.95 for telomere and 0.99 for the control gene. Mean amplification efficiency of the qPCR runs ranged between 105 and 110

for telomere and between 104 and 110 for the control gene. qPCR conditions for telomeres were 10 min at 95°C followed by 50 cycles of 30s at 94°C, 34s at 62°C and 30s at 74°C. qPCR conditions for the GAPDH were 10 min at 95°C followed by 50 cycles of 1 min at 95°C and 1 min at 60°C.

Intra-plate mean coefficients of variation for Ct values were 1.77 % for the telomere assay and 0.65 % for the control gene assay. Inter-plate coefficients of variation based on repeated samples were 2.8% for the telomere assay and 2.4% for the control gene assay (Ct values again). To take into account the slight variation of efficiencies between telomere and control gene amplifications, we calculate relative telomere length using the method suggested by Pfaffl (2001). The mean values were used to calculate the relative T/S ratios using the formula:  $((1 + E_{\text{telomere}})^{\Delta C_{t \text{ telomere}} (\text{control} - \text{sample})} / (1 + E_{\text{control gene}})^{\Delta C_{t \text{ control gene}} (\text{control} - \text{sample})})$ . Both a negative control (water) and melting curves were run for each plate to control for the absence of (i) non-specific amplification and of (ii) primer-dimer artefact.

### ***Statistical analysis***

Statistical analyses were performed in three steps. First of all, to test whether telomere length differed between tissues, telomere length was analysed using a generalized estimating equations (GEE) model. To account for the fact that tissues were coming from the same individual, the type of tissue (RBC, the brain, the spleen, the heart, the liver, the right pectoral muscle and the bone marrow) was included as a repeated variable (tissues being repeated within individuals) in the model structure. Sex, as well as the interaction between sex and tissue type were added as fixed factors. The potential effect of age on telomere length was controlled for by using individuals' age as a covariate. For the GEE analysis, post hoc comparisons among tissues were conducted using Bonferroni tests.

Secondly, Pearson's correlations were used to test the significance of the relationships between (a) telomere length and age within the different tissues and (b) telomere lengths among the different tissues. Because of an extreme outlier individual presenting long telomere lengths which may drive most of the linear relationship, analyses were also performed without this individual (see results).

Finally, similarly to the analysis conducted in (Daniali *et al.* 2013), we assessed if the differences in telomere length among RBC and other tissues remained stable over age. To do so, ANOVA was conducted on telomere length differences (i.e. RBC – tissue X), in which Sex, Age and the interaction Sex x Age were used as fixed factors.

Normal distribution (Shapiro-Wilk tests) and equality of variances (Levene tests) were respected for telomere lengths (all  $p > 0.05$ ).

All statistical analyses were performed using SPSS v. 18.0.

## Results

No significant progressive erosion of telomere length with age was detected in any of the tissues studied (Tables 1, 2, 3).

Variable	Wald chi-squared	P
<b>Telomere length</b>		
Sex	,958	,328
Tissue type	36,951	<b>&lt;,001</b>
Age	,163	,687
Sex*Tissue	10,966	,089

**Table 1:** *Determinants of telomere length evaluated in different tissues of adult zebra finches (GEE analysis). Data were analysed to determine the impact of the type of tissue (repeated variable within individual), sex, and the interaction between sex and the type of tissue (fixed factors). Individuals' age potential impact on telomere length variability was controlled for (covariate). Bold value indicates a significant effect ( $P \leq 0.05$ ).*

		red blood cells	bone marrow		spleen		muscle		heart		liver		brain		age	
red blood cells	r	1	,325	<i>-,120</i>	0,789	<i>0,79</i>	0,57	<i>,172</i>	0,479	<i>,133</i>	0,646	<i>0,67</i>	0,724	<i>0,79</i>	<i>-,144</i>	<i>-,208</i>
	p		,174	<i>,635</i>	<b>,000</b>	<b>,000</b>	<b>,014</b>	<i>,509</i>	<b>,044</b>	<i>,611</i>	<b>,032</b>	<b>,034</b>	<b>,003</b>	<b>,001</b>	<i>,546</i>	<i>,392</i>
bone marrow	r		1	1	,281	<i>,281</i>	0,723	<i>,184</i>	,322	<i>-,418</i>	0,861	<i>,250</i>	0,896	<i>,166</i>	<i>,053</i>	<i>,020</i>
	p				,275	<i>,275</i>	<b>,000</b>	<i>,464</i>	,179	<i>,084</i>	<b>,000</b>	<i>,458</i>	<b>,000</b>	<i>,571</i>	<i>,825</i>	<i>,935</i>
spleen	r				1	1	,259	<i>,259</i>	,272	<i>,272</i>	0,791	<i>0,79</i>	0,638	<i>0,64</i>	<i>-,281</i>	<i>-,281</i>
	p						,333	<i>,333</i>	,308	<i>,308</i>	<b>,006</b>	<b>,006</b>	<b>,014</b>	<b>,014</b>	<i>,274</i>	<i>,274</i>
muscle	r						1	1	0,709	<i>,105</i>	0,943	<i>,027</i>	0,932	<i>,027</i>	<i>,165</i>	<i>,325</i>
	p								<b>,001</b>	<i>,678</i>	<b>,000</b>	<i>,938</i>	<b>,000</b>	<i>,929</i>	<i>,499</i>	<i>,188</i>
heart	r								1	1	0,796	<i>-,027</i>	0,73	<i>,099</i>	<i>,151</i>	<i>,152</i>
	p										<b>,002</b>	<i>,937</i>	<b>,003</b>	<i>,747</i>	<i>,537</i>	<i>,547</i>
liver	r										1	1	0,979	<i>,616</i>	<i>,005</i>	<i>-,361</i>
	p												<b>,000</b>	<i>,141</i>	<i>,988</i>	<i>,276</i>
brain	r												1	1	<i>-,044</i>	<i>-,452</i>
	p														<i>,876</i>	<i>,105</i>
age	r														1	1
	p															

**Table 2:** Results of the Pearson’s correlations testing the linear relationships between telomere lengths in different tissues, as well as the relationships between telomere length and age in the different tissues (right column).

Results of the same Pearson’s correlation analysis after removing the outlier point (the individual presenting the longest telomeres) from the analysis are presented in italics.

<b>Univariate analysis</b>		
<i>RBC - Muscle</i>	<i>F</i>	<i>p</i>
Age	,952	,345
Sex	,714	,411
Age x Sex	1,355	,263
<i>RBC – Bone marrow</i>		
Age	,282	,603
Sex	3,123	,098
Age x Sex	2,571	,130
<i>RBC - Spleen</i>		
Age	,070	,795
Sex	5,422	,354
Age x Sex	2,945	,108
<i>RBC – Heart</i>		
Age	,724	,408
Sex	1,050	,322
Age x Sex	,791	,388
<i>RBC – Liver</i>		
Age	,765	,407
Sex	1,596	,242
Age x Sex	2,673	,141
<i>RBC - Brain</i>		
Age	,106	,750
Sex	,025	,878
Age x Sex	,128	,727

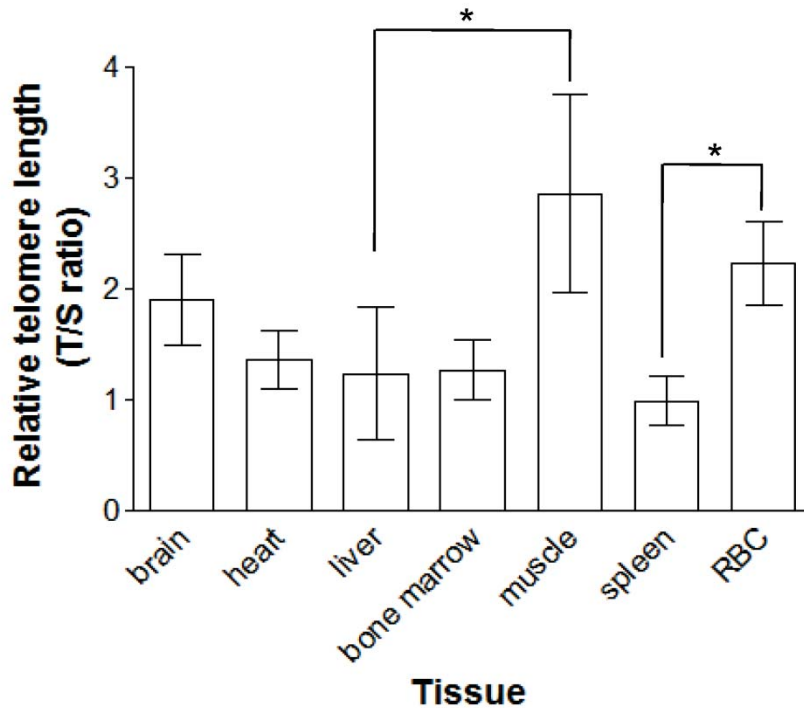
**Table 3:** Result of separate analyses of variance for the difference in red blood cells (RBC) and somatic tissues' telomere length in relation to Sex, Age and the interaction Sex x Age.

***Tissue differences in telomere lengths***

The type of tissue had a significant effect on telomere lengths ( $p < 0.001$ ; Table 1). As the sex effect, the interaction between sex and tissue type, and age were not significant (Table 1). Post-hoc analyses revealed that the pectoral muscle was characterised by significantly longer telomere length than liver telomere length (Bonferroni posthoc comparisons  $p = 0.015$ ), but not significantly so from the other tissues (Bonferroni posthoc comparisons, brain  $p = 1$ ; heart  $p = 0.884$ ; bone marrow  $p = 0.631$ ; spleen  $p = 0.841$  and RBC  $p = 1$ ) (Figure 1, Table 4). RBC telomere length was not significantly different from telomere lengths in the other tissues (brain  $p = 1$ , heart  $p = 0.303$ , liver  $p = 0.922$ , bone marrow  $p = 0.260$ , pectoral muscle  $p = 1$ ) except for the spleen ( $p < 0.001$ ). Telomere lengths in the other tissues (brain, heart, liver, bone marrow, and spleen), were not significantly different from the others (Figure 1, Table 4).

	brain	heart	liver	bone marrow	muscle	spleen	red blood cells
Telomere length	1,905 (,403)	1,368 (,263)	1,235 (,597)	1,269 (,267)	2,859 (,897)	,991 (,217)	2,229 (,377)

**Table 4:** Mean telomere lengths measured by qPCR in different tissues of adult zebra finches. Values are mean  $\pm$  SE.

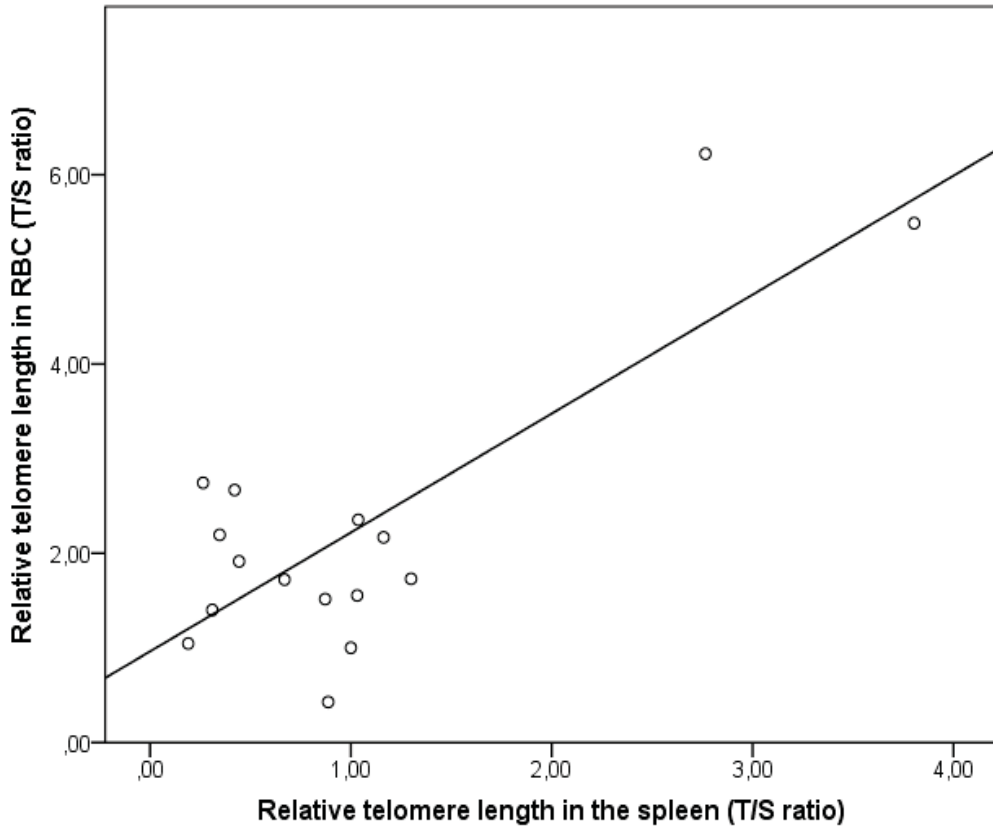


**Figure 1:** Mean relative telomere lengths ( $\pm$  SE) in the different somatic tissues (brain, heart, liver, bone marrow, muscle, spleen, RBC), tissues presenting significantly different telomere lengths are marked with an asterisk.

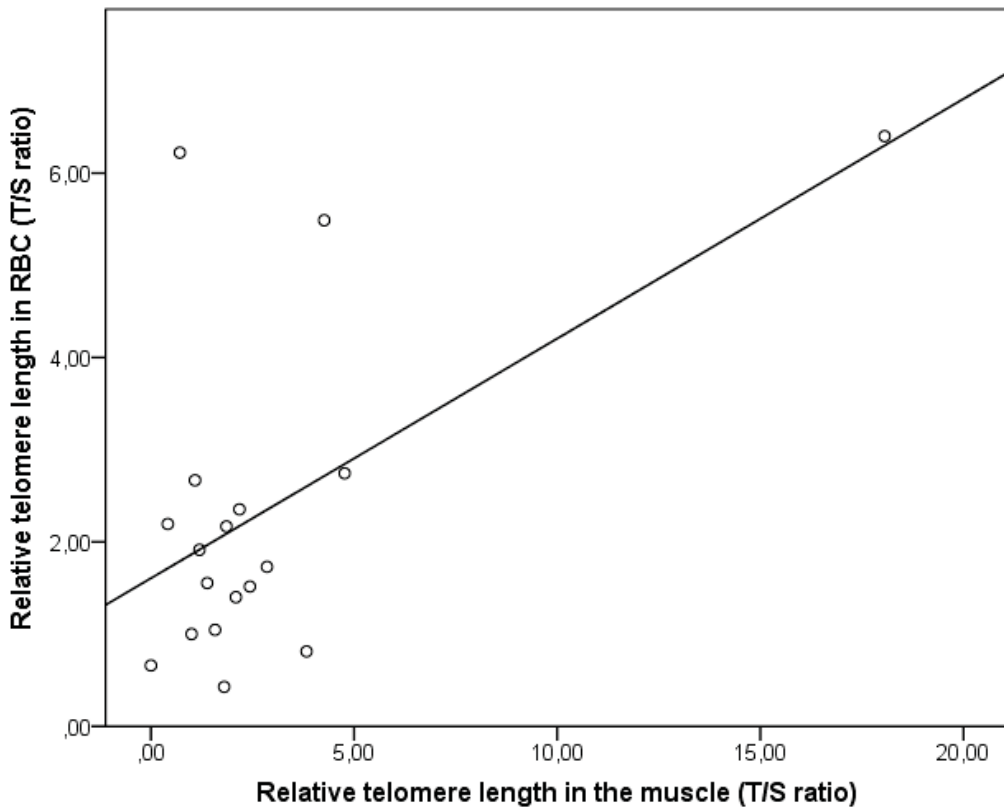
Individual zebra finches showed significant correlations of their telomere lengths measured in RBC and those obtained in the additional tissues studied. Consequently, individuals presenting long RBC telomere lengths were also presenting long telomeres in the spleen (Figure 2a), the muscle (Figure 2b), the heart (Figure 2c), the liver (Figure 2d) and the brain (Figure 2e) (respectively  $p < 0.001$ ,  $p = 0.014$ ,  $p = 0.044$ ,  $p = 0.032$ ,  $p = 0.003$ ). Surprisingly, the only tissue for which there was a lack of relationship with red blood cells was the bone marrow ( $p = 0.174$ ) (Table 2). Telomere lengths in the brain, the liver and the muscle were also correlated to telomere lengths in several other somatic tissues (Table 2).

**Figure 2:** Correlations between red blood cells telomere length and telomere lengths in different somatic tissues.

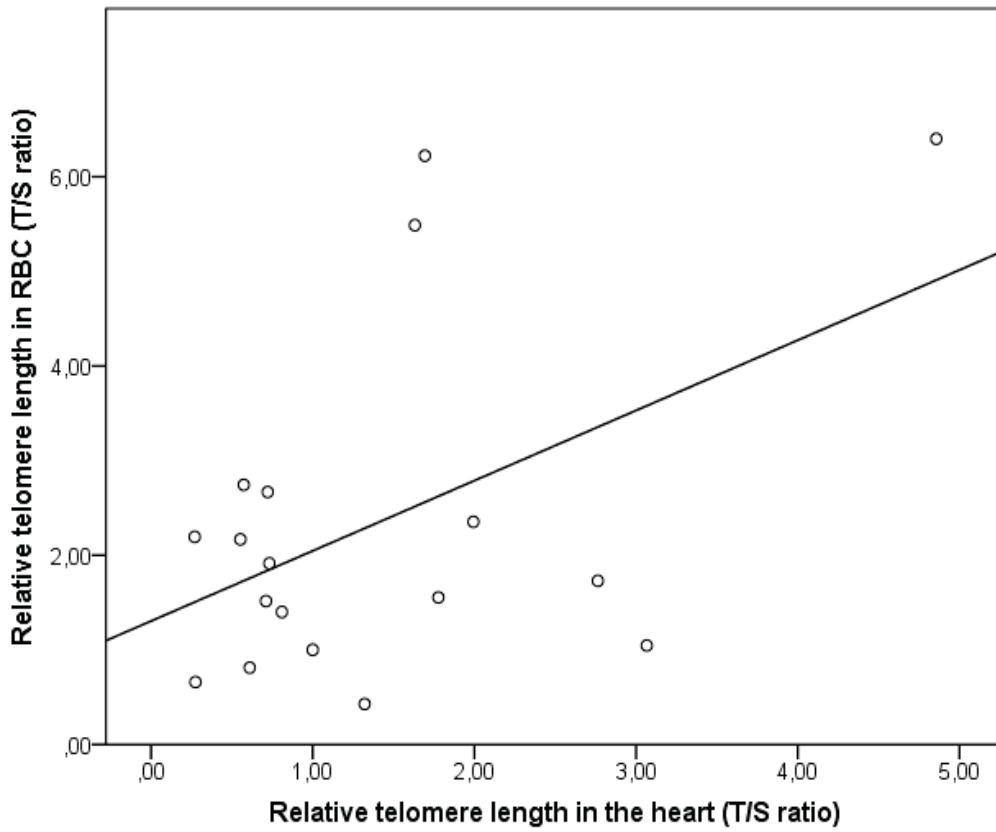




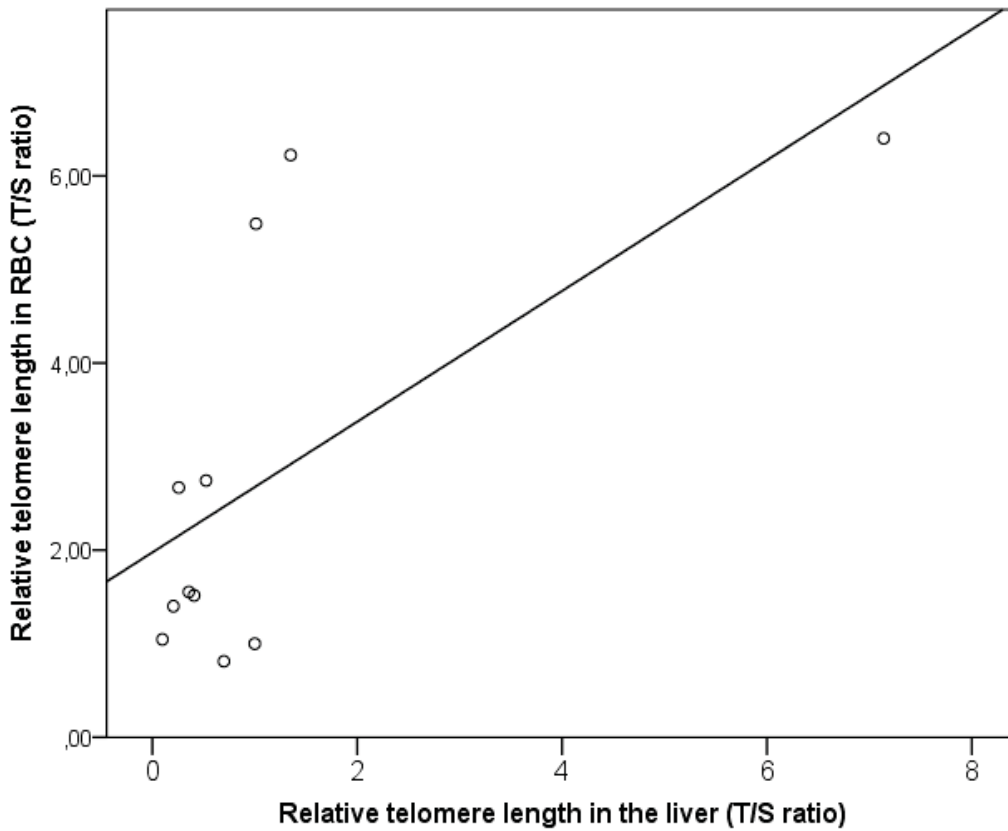
a. Correlation between red blood cells telomere length and spleen telomere length.



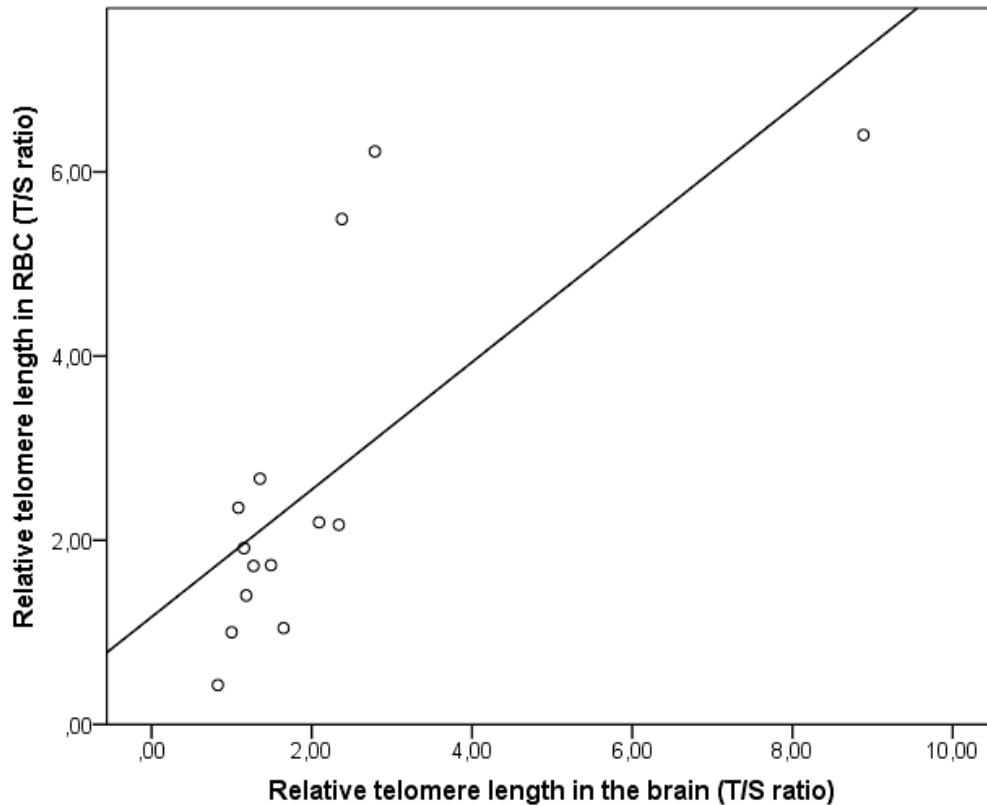
b. Correlation between red blood cells telomere length and muscle telomere length.



c. Correlation between red blood cells telomere length and heart telomere length.



d. Correlation between red blood cells telomere length and liver telomere length.



*e. Correlation between red blood cells telomere length and brain telomere length.*

In our data, there was an individual carrying particularly long telomeres. A potential bias may have been induced by the extreme telomere values of this individual. Accordingly, we performed an additional Pearson's correlation analysis without this individual (Table 2, values in italics). Again, no significant link was detected between telomere lengths in the different tissues and age. In addition, RBC telomere lengths were still correlated to telomere lengths in the spleen, the liver and the brain (respectively  $p < 0.001$ ,  $p = 0.034$ ,  $p = 0.001$ ) but not to those in the muscle and the heart (respectively  $p = 0.509$ ,  $p = 0.611$ ). Telomere lengths in the liver and the brain were still correlated with telomere lengths in other tissues, but it was no longer the case for the muscle any more (Table 2, values in italics).

***Relative rate of telomere loss among tissues***

Differences between RBC and other tissues' telomere length were constant with individual age (Table 3), indicating that ageing does not change the relationship among tissue telomeres (other comparisons NS, data not shown).

## Discussion

Telomeres, through their impact on cell senescence (Allsopp *et al.* 1992), are supposed to be part of the multifactor process of ageing in most eukaryotes (Harley, Futcher & Greider 1990; Monaghan & Haussmann 2006; Aubert & Lansdorp 2008). Telomere length measured in blood cells has been well correlated with individual lifespan or survival rate in humans or captive and free-ranging animals (Cawthon *et al.* 2003; Haussmann *et al.* 2003; Haussmann, Winkler & Vleck 2005; Bize *et al.* 2009; Heidinger *et al.* 2012). For instance, telomere length measured from RBC in captive zebra finches at the end of growth (25 days) was positively related to individual lifespan (Heidinger *et al.* 2012), suggesting that our understanding of ageing processes will pass through our understanding of the telomere-driven ageing process. In this context, determining accurately how telomere dynamics in peripheral blood cells relates to telomere lengths in the whole organism is an important starting question.

By finding significant relationships between telomere length of RBC and telomere lengths of several other somatic tissues independently of age, our study confirms that telomere length measured in RBC could serve as a surrogate for telomere length in the whole avian organism.

However, we ought to be cautious with these findings. Indeed, when the individual carrying the longest telomeres was removed from the analysis, the relationships between RBC and other somatic tissues telomere lengths remained true only for the spleen, the liver and the brain. This actually reduces the weight of the conclusions that may be driven from our data, because of the relative small sample size. However, rather than limiting the interest in using RBC telomeres as an ageing proxy for the whole organism, it suggests that further data needs to be collected in the future to assess why certain tissues, such as the skeletal muscle or the heart, may present particular telomere erosion rates.

*In vitro* experiments have actually demonstrated that telomere length is decreasing while cells divide (Allsopp *et al.* 1992). As a consequence, highly replicative tissues should present shorter telomeres on average than post-mitotic ones. However, since the discovery of the enzyme telomerase and of its “immortalization activity” *via* telomere length maintenance

(Greider & Blackburn 1985), we know that erosion of telomeres, with time, may be counter-balanced (Wright *et al.* 1996). This is actually the case early in development (i.e. when telomerase is highly active in a large number of tissues, for birds see (Hausmann *et al.* 2007)) and thereafter in specific tissues, particularly those containing a large pool of stem cells ((Forsyth, Wright & Shay 2002; Hausmann *et al.* 2007)). As a consequence, telomere loss is likely to vary among tissues depending on their replicative potential and/or their capacity to retain telomerase activity (Youngren *et al.* 1998) with telomere length resulting from the impact of these two factors. Our data seem to fit with those predictions. The spleen, a highly replicative tissue, which displays a high telomerase activity at adulthood ((Forsyth, Wright & Shay 2002; Hausmann *et al.* 2007)), was characterised by shorter telomeres compared to the RBC. Interestingly, the shorter telomeres in the liver compared to muscle telomere length, suggested in our case that this organ of prime metabolic role may face particular pro-ageing challenges (see also long muscle telomeres in mammals; (Benetos *et al.* 2011; Daniali *et al.* 2013)).

Altogether, these results suggest that replicative tissues, with known telomerase activity, may have short telomeres compared to non-replicative tissues. This supports the hypothesis that telomerase *per se* is not the guarantee of an absolute long telomere length. Rather, telomerase should be viewed as a factor that helps to maintain telomere lengths above a threshold value (e.g. in particular short telomeres, see (Vera & Blasco 2012)), preserving the renewal capacities of stem cells (Hao *et al.* 2005). Other mechanisms may also be implicated such as particular shelterin protein expression (Sfeir & De Lange 2012). It also confirms that maintaining a proliferative capacity in a given tissue is done at a potential cost in terms of telomere erosion, probably early in development to set-up a large progenitor cell pool, as recently suggested (Morrison & Kimble 2006; Daniali *et al.* 2013).

Our study shows that in adult zebra finches, RBC telomere length is correlated to telomere lengths in other somatic tissues independently of their replicative status. Similar relationships have been found in humans (using leucocytes, (Takubo *et al.* 2002; Daniali *et al.* 2013)). Telomere lengths in peripheral blood cells are susceptible to reflect telomere lengths in hematopoietic stem cells as was indicated by previous studies (Vaziri *et al.* 1994). However, in our case there was no correlation of RBC telomere length with telomere length in bone marrow cells. A first explanation may be that we extracted different cell types in our

bone marrow samples, potentially blurring our telomere measurement with osteoclasts / osteoblasts family types. Additionally, given our relatively small sample size (n=20), confounding factors such as age or sex that, even if they were taken into account in our model, may have led to the non-significant relationship. The study of the telomerase activity in avian RBC or particular expression of shelterin proteins may be of prime interest to tackle this question.

Importantly, the differences among RBC and the different somatic tissues studied were kept stable with age in our bird model, indicating that rates of telomere loss may not be significantly different among tissues at adulthood. This supports the idea that differences among tissues in telomere length are probably set-up during the growth period (pre- and post-hatch/birth;(Youngren *et al.* 1998; Hall *et al.* 2004; Sidorov, Gee & Dimitrov 2004; Heidinger *et al.* 2012)) and that it is fixed thereafter during adult life (Daniali *et al.* 2013). However, we still lack information on the nature of the cellular mechanisms that may explain why proliferative and non-proliferative tissues are losing telomeres at the same rate in adult zebra finches. It may be valuable to look at tissues' telomere attrition in species known to keep telomerase activity at adulthood (Hausmann *et al.* 2007). In addition, we were not able to establish that telomere length was a function of age, probably because of the survival of the best individuals at old ages (i.e. those having the longest telomeres). Such an absence of significant telomere shortening in non-replicative tissue has been previously described, probably for similar reasons as in (Benetos *et al.* 2011).

Our study gives support of telomere length synchrony in adults only. Telomere dynamics has been found to vary between chicks and adults in different bird species (Hall *et al.* 2004; Pauliny, Larsson & Blomqvist 2012). For this reason, relationships between telomere lengths of somatic tissues might differ in the young age class than the one we observed. The possibility of accelerated telomere shortening in tissues of rapid expansion during growth compared to late-developing ones has indeed been proposed in a recent study conducted on humans (Daniali *et al.* 2013). Consequently we still need to look at telomere synchrony among organs in early life in zebra finches (i.e. during the first 30 days of life). Furthermore, in birds, telomere dynamics and telomere length maintenance mechanisms differ between short-lived and long-lives species (Hausmann *et al.* 2003; Pauliny, Larsson & Blomqvist 2012), thus the links between telomere lengths in different somatic tissues might also

depend on the longevity of the species considered. Telomere length synchrony between organs should then also be tested in long-lived species.

It is still the case that for evolutionary biologists, using RBC's telomere measurement seems to be representative of the whole organism telomere picture, explaining why peripheral blood telomeres has been found as a proxy for adult individual health status (Cawthon *et al.* 2003; Vera *et al.* 2012) and survival (Bize *et al.* 2009; Salomons *et al.* 2009; Heidinger *et al.* 2012).

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# Inheritance and heritability patterns of telomere length



Sylvie Massemin



## Introduction

Récemment, un intérêt considérable a été porté au rôle des télomères en biologie évolutive, en écologie évolutive et plus particulièrement à leur implication potentielle comme mécanisme sous-tendant les compromis évolutifs. En effet, de nombreuses études suggèrent que les télomères sont susceptibles de refléter le mode de vie des individus, mais aussi leur capacité à se protéger contre le stress, ainsi que leur durée de vie. De ce fait, la dynamique d'érosion des télomères pourrait nous permettre d'avoir des indications sur la fitness des organismes.

Par conséquent, pour identifier exactement quel rôle les télomères peuvent avoir dans les processus évolutifs, il est essentiel de déterminer dans quelle mesure la longueur des télomères est régulée par la composante génétique.

Jusqu'à présent les études s'intéressant à cette question, ont été principalement effectuées chez l'homme. Celles-ci indiquent que la longueur des télomères chez l'homme serait héritée majoritairement du père. L'unique étude s'intéressant au pattern d'hérédité de la longueur des télomères chez les oiseaux, montre, elle, en revanche, que ce paramètre semble hérité principalement de la mère.

Afin de déterminer si ce pattern d'hérédité de la longueur des télomères est une règle générale, il est nécessaire de le tester dans grand nombre d'espèces et en milieu naturel.

Par conséquent, nous nous proposons d'examiner ce pattern dans deux populations d'oiseaux sauvages : chez le manchot royal (*Aptenodytes patagonicus*) et chez le cincle plongeur (*Cinclus cinclus*), en examinant le lien entre la longueur des télomères des jeunes et la longueur des télomères des parents. Chez le manchot royal, nous allons également vérifier si lien se maintient au cours du temps, ce qui nous permettra de différencier l'effet de la composante génétique, de celui de l'environnement, sur la régulation de la longueur des télomères. Chez le cincle plongeur, nous allons déterminer la part relative des effets de la composante génétique de celle des effets des facteurs environnementaux.

## Principaux résultats

Chez le manchot royal, nous n'observons aucun lien entre la longueur des télomères des jeunes et celle des pères. Cependant, les résultats de cette étude indiquent que la longueur des télomères des jeunes (à 10 jours) est positivement corrélée à celle des mères. Il

est important de noter que ce lien n'est observé qu'à dix jours et qu'il disparaît au cours de la croissance des poussins.

De même, chez le cincle plongeur, nous observons une corrélation positive entre la longueur des télomères des jeunes et celle de la mère. De plus, la longueur des télomères chez les jeunes semble significativement affectée par les effets de l'environnement (effet de l'année d'échantillonnage et effet du nid).

## Discussion

Dans l'ensemble, les résultats de ces études établissent un lien entre la longueur des télomères des jeunes, et la longueur des télomères des mères. Ces résultats sont en accord avec ceux de la précédente étude, portant sur le pattern de transmission de la longueur des télomères chez les oiseaux.

Chez l'homme, on observe principalement une hérédité de la longueur des télomères par le père. De ce fait, le pattern trouvé chez les oiseaux semble refléter celui trouvé chez l'homme, - ce qui suggère donc que le sexe hétérogamétique (mâle XY chez les mammifères, femelle ZW chez les oiseaux) aurait potentiellement un rôle dans le pattern de transmission de la longueur des télomères. D'un point de vue mécanistique, ce rôle du sexe hétérogamétique pourrait s'expliquer par l'implication de gènes soumis à l'empreinte parentale (ou « imprinting »). L'empreinte parentale est le mécanisme par lequel l'un des allèles parentaux du gène considéré, est réprimé alors que l'autre est exprimé. Il est possible que ces mécanismes d'empreinte parentale (passant par la modification des histones et la méthylation de l'ADN) agissent non pas directement sur les télomères, mais plutôt sur les déterminants moléculaires régulant la longueur des télomères (expression de la télomérase, expression des protéines associées aux télomères).

Cependant, il est important de noter que dans les deux cas, les facteurs environnementaux semble jouer un rôle crucial dans la régulation de la longueur des télomères chez les jeunes.

## Study 2

### Maternal telomere length inheritance in the king penguin

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## Abstract

Telomeres are emerging as a biomarker for ageing and survival, and are susceptible to be implicated in different life history trade-offs. In particular, telomere length with which one starts in life has been shown to be linked with life-long survival at the individual level, suggesting that early telomere dynamics is somehow associated with individual life history trajectories. This result highlights the importance of the estimation of telomere length inheritance as one of the factors that will determine early life telomere length. Given the scarcity of species for which telomere length inheritance has been studied, it is still necessary to assess the general pattern of telomere length inheritance. It will be also informative to evaluate how this pattern is changing over the course of growth in individuals living under natural conditions.

To partly fill this gap, we followed telomere inheritance in a population of king penguins (*Aptenodytes patagonicus*), where both parents and the only chick can be sampled early in life and followed over the 11 months of their growth period. We checked paternal and maternal influence on chick initial telomere length (at day 10 after hatching), and how these relationships changed with chick age. Based on a correlative approach, offspring telomere length was positively correlated with maternal telomere length. However, this relationship was found non-significant at older ages until chick moulting. These data suggest that telomere length in birds is maternally inherited. Still, the influence of environment during growth remained important as the maternal link disappeared while chicks aged, reinforcing the idea that early growth conditions are key determinants of an individual telomere length.

## Introduction

Among transmitted traits, only those with interindividual variation are of importance in evolution and are shaped by natural selection. Transmission and heritability (i.e. disentangling additive and non-additive genetic, and environmental variability in inheritance, estimated as the genetically based variation of inheritance expressed by  $h^2$ ) of morphological (Voillemot *et al.* 2012), physiological and behavioural traits, as well as ultimate fitness, have been widely studied (Kruuk *et al.* 2000). The logic assertion that traits strongly related to fitness, such as fecundity and lifespan, are inherited without great genetic variation does not mean that no variability exists but might mean that environmental and non-additive genetic variation may be high.

It remains then important to check inheritance of fitness related traits, lifespan in our case, and of the mechanisms susceptible to shape them. Among these ageing mechanisms, oxidative stress and telomere erosion have focused the attention of several previous studies (Beckman & Ames 1998; Monaghan & Haussmann 2006). These two processes are linked since oxidative stress, which is the imbalance between the production of reactive oxygen species (ROS) and the antioxidant capacity (Finkel & Holbrook 2000), accelerates telomere loss (Von Zglinicki 2000). Telomeres are highly conserved non coding repetitive DNA sequences hiding the chromosome ends from being recognised as double stranded breaks. Once telomeres reach a lower critical length, it induces cell division arrest and/or cell senescence (Blackburn 2001).

The rate at which telomeres are lost is dependent on the balancing of pro- (oxidative stress, rate of cell division) and anti-erosion factors like the telomerase activity (mainly active in germinal and stem cells) (Blackburn 2001), which activity is partly regulated by the shelterin protein complex associated to telomere (De Lange 2005). Consequently, telomere length has been previously defined as a polygenic trait (Andrew *et al.* 2006; Gatbonton *et al.* 2006), a characteristic likely to increase non-additive genetic variance. It is interesting to note that ROS production has been shown to be transmitted from mother to offspring in lizards (Olsson *et al.* 2008), showing that ageing processes are somehow at least partly inherited.



Present knowledge on telomere length inheritance patterns, telomere length heritability and the effects of parental age on telomere length, is summarised in Table 1. Interestingly, a sex-biased inheritance pattern has been pointed out (Olsson *et al.* 2011). To date, studies on the subject have mainly been conducted on humans (Nawrot *et al.* 2004; Nordfjäll *et al.* 2005; Unryn, Cook & Riabowol 2005; Njajou *et al.* 2007; Nordfjäll *et al.* 2009), with reports of monozygotic and dizygotic twins' studies (Slagboom, Droog & Boomsma 1994; Graakjaer *et al.* 2004). Most of these studies support a paternal inheritance of telomere length in human (Nordfjäll *et al.* 2005; Njajou *et al.* 2007; Nordfjäll *et al.* 2009). In addition, paternal age is a strong determinant of offspring telomere length (Unryn, Cook & Riabowol 2005; De Meyer *et al.* 2007; Kimura *et al.* 2008; Eisenberg 2011). In non-human species, contrasting results exist, showing either a bi-parental inheritance pattern (predominantly male-sire in lizards (Olsson *et al.* 2011)) or a maternal inheritance in birds (females being ZW, (Horn *et al.* 2011)). All these studies presented highly variable heritability estimations (from >1 to 0.18), the lowest (but significant) value being based on a linear mixed model analysis (Voillemot *et al.* 2012).

Telomeres not only control cell replication capacity, but are also somehow indicators of individual life expectancy in captivity (Heidinger *et al.* 2012) as well as *in natura* (Bize *et al.* 2009). The great variation in telomere length among age-matched individuals has been suggested to have an early-life origin (Hall *et al.* 2004). In addition, the telomere length at the end of growth was found to be a proxy of individual lifespan as a future adult (Heidinger *et al.* 2012). This underlines that our understanding of the causes of telomere dynamics variability during early growth is crucial to assess the role of telomeres in lifespan variability. Recently, interest in characterizing the extent to which variation in telomere loss might constrain life history trade-offs and be involved in the evolution of life histories has been growing (Monaghan & Hausmann 2006). Indeed, telomeres are to be important life history markers that determine how much to invest in maintenance efforts over the life course (Eisenberg 2011) and to “powerfully quantify life’s insults” (Blackburn & Epel 2012). This makes telomeres a potential proxy of future individual fitness in a large variety of species, from humans to birds (Cawthon *et al.* 2003; Heidinger *et al.* 2012). If telomere length reflects intrinsic trade-offs, the capacity to preserve telomere length stable may indicate the organism capacity to repair time and stress insults, and thus its fitness. Therefore, telomere

dynamics might be potential indicators of individual quality and could provide an insight into ecology and evolutionary biology.

The determination of telomere length inheritance pattern is one of the steps that will lead us to understand the putative role of telomeres in lifespan variability. Given the scarcity of species for which telomere length inheritance has been studied, one way to tackle this question is to assess the general pattern of telomere length inheritance in a broader range of species, in particular in wild populations. In addition, there are no data available testing whether the pattern of telomere inheritance is maintained over growth, which is a mean to evaluate how far environmental constraints may blur our perception of telomere length inheritance.

We studied telomere inheritance in wild king penguins (*Aptenodytes patagonicus*), a species where both parents and the single chick can be sampled early in life and the chick followed over the 11 months-long growth period during which environmental challenges, such as a long winter fast, may have a tremendous impact on individual telomere loss (Geiger *et al.* 2012). We were then able to check paternal and maternal influence on chick starting telomere length, and follow how these relationships change with time over growth which will allow distinguishing the genetic versus the environmental influence.

## Materials and Methods

### *Study site and animal model*

The study was conducted on king penguins (*Aptenodytes patagonicus*), in the colony called “La Grande Manchotière” (20 000 breeding pairs), Possession island, Crozet archipelago (Terres Australes Antarctiques Françaises) located 46° 25’S; 51° 52’E. Data were collected during two following field seasons (2009 and 2010).

The king penguin is a long-lived pelagic bird that presents a particular life cycle. The entire annual breeding cycle of adults lasts 14 to 16 months (including moult, (Weimerskirch, Stahl & Jouventin 1992)) during which the parents alternate attendance to the unique chick and foraging at sea. Chicks have to face in the middle of their growth period the 3 months of sub-

Antarctic winter by their own, with a reduced food supply by the parents. The consequence is that the total growth period of the unique chick spreads over 11 months before the final moult to acquire the sub-adult plumage and departure at sea (Weimerskirch, Stahl & Jouventin 1992).

### **General procedure**

Fifty-three breeding pairs were followed and chick monitoring started as close as possible after hatching (i.e. 10 days to avoid breeding failure). Chicks were individually identified with fishtags and followed from hatching to the beginning of the final moult, which takes place before their first at-sea departure.

In order to measure telomere length and to follow the potential link with parent telomere length over time, blood samples from the chicks were taken from the flipper vein at different times during the growth period: 10 (number of chicks =53), 70 (n=37), 200 (n=34) and 300 days-old (n=30). The sample size decreased over time due to the fact that not all chicks survived the growth period (causes of death were not determined but death was not provoked by chick handling). Chick's head was always covered by a hood to reduce handling stress.

Blood samples were taken from the first parent (the male) when the chicks were 10 days old. Blood sample of the second parent (the female) was taken at the first breeding shift (around day 15 after hatching), when the bird came back to the colony to relieve its partner (Descamps *et al.* 2002).

### **Telomere measurements**

Telomere length measurements were done when the chicks were 10, 70, 200 and 300 days-. Telomere length was measured on DNA extracted from red blood cells (stored at – 20°C until analysis), which are nucleated in birds, using Nucleospin® Blood QuickPure kit (Macherey-Nagel, Düren Germany). Telomere length was assessed by the quantitative real-time amplification (qPCR) (Cawthon 2002), a procedure previously used in birds (Criscuolo *et al.* 2009). Relative telomere length is expressed as the ratio (T/S) of telomere repeat copy number (T) to a control single copy number gene (S). We used the *Aptenodytes patagonicus* zinc finger protein as a single control gene. Forward and reverse primers for the zinc finger

protein gene were 5'-TACATGTGCCATGGTTTTGC-3' and 5'-AAGTGCTGCTCCCAAAGAAG-3' respectively. Telomere primers were: Tel1b (5'-CGTTTTGTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3') and Tel2b (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'). qPCR for both telomere and the zinc finger protein were performed using 2.5 ng of DNA with sets of primers Tel1b/Tel2b (or zinc finger protein -F/ zinc finger protein -R), in a final volume of 10 µl containing 5 µl of BRYT Green<sup>®</sup> fluorescent dye (GoTaq<sup>®</sup>qPCR Master Mix, Promega, France). Primer concentrations in the final mix were 200 nM for telomere length determination and 300 nM for the control gene. Telomere and control gene PCR conditions were: 2min at 95°C followed by 40 cycles of 15s at 95°C, 30s at 56°C, 30s at 72°C and 60s at 95°C. Telomere and control (i.e. non-variable in copy number) gene amplification of each sample were done on the same plate. Each plate included serial dilutions (telomere and control gene, 5 ng, 2.5 ng, 1.25 ng, 0.625 ng) of DNA of the same reference bird which were run in triplicate. These serial dilutions were used to generate a reference curve on each plate, in order to control for the amplifying efficiency of the qPCR (both telomere and control gene). Mean amplification efficiency calculated from the reference curves of the qPCR runs were comprised between 100.9 and 103.1 for telomere and between 100.6 and 102.9 for the control gene. R<sup>2</sup> calculated from the reference curves of the qPCR runs were comprised between 0.96 and 0.99 for telomere and between 0.97 and 0.99 for the control gene. Intra-plate mean coefficients of variation for Ct values were 1.35 ± 0.06 % for the telomere assay and 0.79 ± 0.04 % for the control gene assay and inter-plate coefficients of variation based on repeated samples were 1.56% for the telomere assay and 1.35% for the control gene assay (Ct values again). Mean coefficient of variation for the relative T/S ratios was 16%. To take into account the slight variation of efficiencies between telomere and control gene amplifications, we calculate relative telomere length using the method suggested by (Pfaffl 2001). The relative T/S ratios were calculated using the formula:  $((1 + E_{telomere})^{\Delta Ct_{telomere}} / (1 + E_{control\ gene})^{\Delta Ct_{control\ gene}})$  (control – sample). Both a negative control (water) and melting curves were run for each plate to check for non-specific amplification and primer-dimer artefacts.

Sex determination was done using DNA extracted from red blood cells. The method followed was adapted from (Griffiths *et al.* 1998): 80 ng of DNA in a final volume of 20µl, the PCR conditions were 2min at 94°C, followed by 10 cycles of a touchdown protocol: 30s at 94°C,

30s at 60°C (reducing the temperature of this step by 1°C at each cycle), 35s at 72°C, followed by 30 cycles of 30s at 50°C and 35s at 72°C, finished by 5min at 72°C.

### ***Statistical analysis***

Telomere lengths were log transformed to achieve a normal distribution and homoscedasticity. To test the time effect on telomere length, we used a General estimating equation (GEE) model with time period (which corresponds to the different times of sampling during the growth period: 10, 70, 200 and 300 days) as a repeated variable, chick identity as subject (random factor), chick sex and year of sampling as fixed factors in the model, and maternal and paternal telomere lengths as covariates. All interactions between the different factors and covariates were tested. When found to be non-significant, terms were removed sequentially from the analysis, starting with the least significant ones. The most parsimonious models are presented.

Then, to assess whether the mother-offspring link was significantly different from the father-offspring one, we used a General estimating equation (GEE) model with offspring telomere length as a dependent variable, time period (10, 70, 200 and 300 days) as a repeated variable, chick identity as subject, parents' sex as a fixed factor, parents' telomere length as a covariate and the interaction between parents' sex and parents' telomere length.

Finally, even though the interactions between parent's telomere lengths and the time period were not significant, to further investigate the potential link between chicks' telomere length and parents' telomere lengths over time, we conducted a more exploratory analysis using Pearson's correlations. This link was tested at the four different chick ages.

The slopes of the regressions between both mid-parental and offspring telomere lengths, and between maternal and offspring telomere lengths were used to estimate  $h^2$ .

All statistical analyses were performed using SPSS v. 18.0. Significance level was set at 5%.

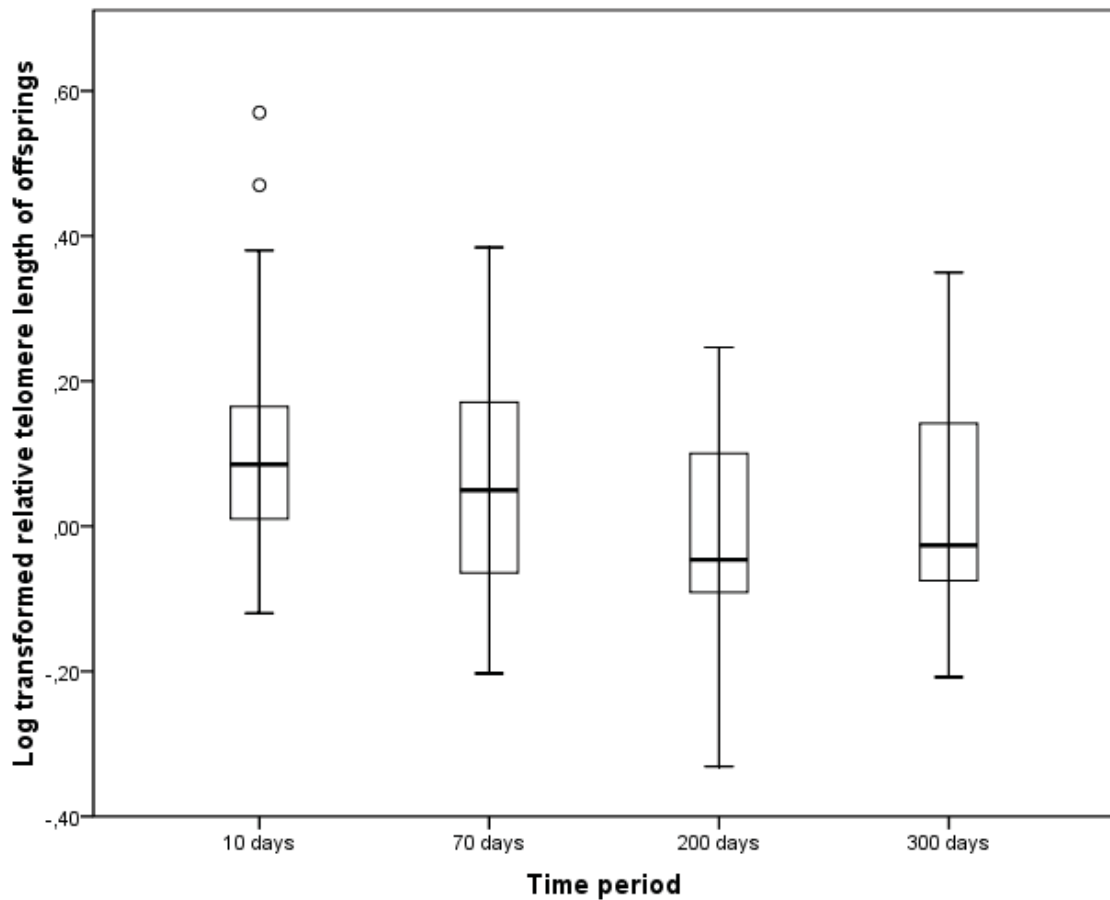
## **Results**

There was no effect of sex or year on offspring telomere length (Table 2,  $p=0.573$  and  $p=0.823$  respectively). However, there was a significant effect of the time period on offspring telomere length (Fig. 1, Table 2,  $p<0.001$ ), suggesting that telomeres are progressively

eroded over the growth period (means values  $\pm$  SE: 10 days (0.107 $\pm$ 0.02); 70 days (0.064 $\pm$ 0.024); 200 days (-0.06 $\pm$ 0.025); 300 days (0.012 $\pm$ 0.026). In contrast to paternal telomere length, maternal telomere length had a significant effect on offspring telomere length (Table 2, p=0.028).

Variable	Wald chi-squared	P
Telomere length		
Year	,050	,823
Sex	,318	,573
Time period	29,317	,001
Paternal telomere length	,009	,923
Maternal telomere length	4,838	,028

**Table 2:** Results of the GEE model on offspring telomere length with time period as a repeated variable, and chick as subject, sex, year, as well as the time period as fixed factors in the model, and maternal and paternal telomere lengths as covariates.



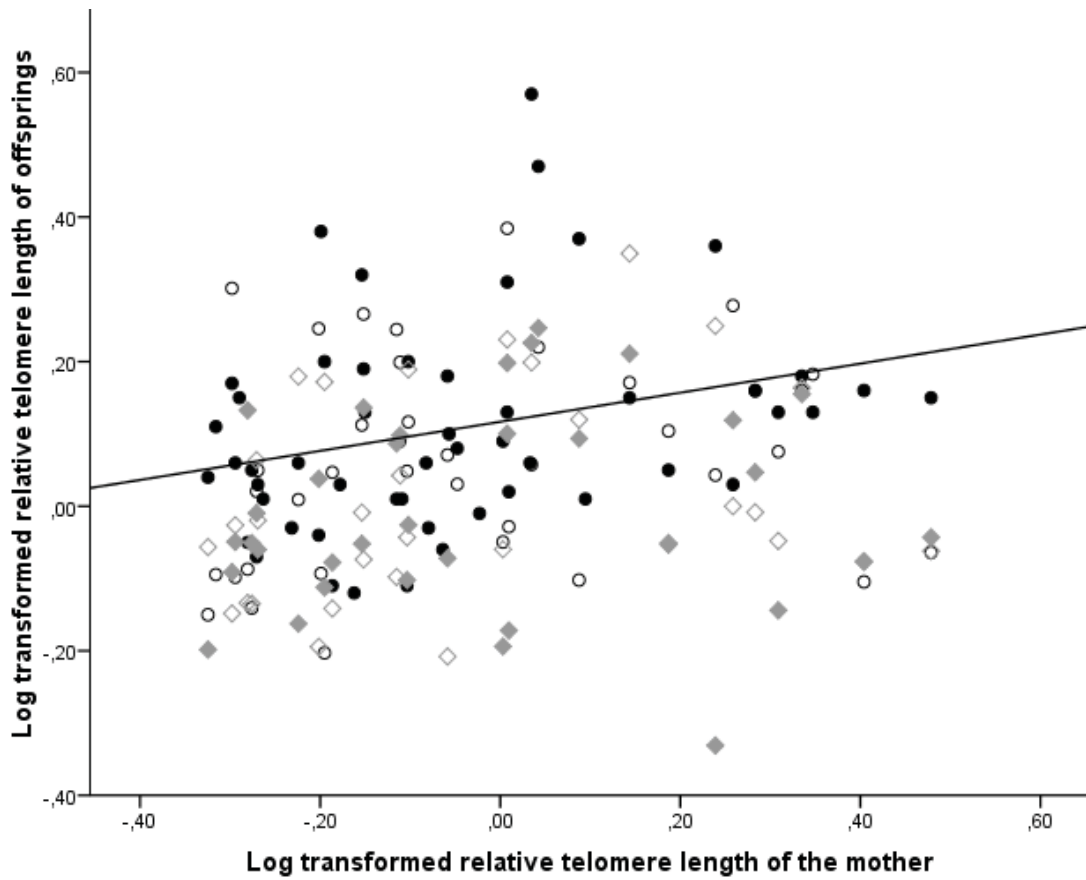
**Figure 1:** Log transformed offspring telomere lengths over the growth period (10 days, 70 days, 200 days, 300 days) (means values  $\pm$  SE).

Offspring telomere lengths at 10 days were significantly correlated to maternal telomere length (Fig. 2, Table 3,  $p=0.042$ ), contrary to paternal telomere length (Fig. 3, Table 3,  $p = 0.388$ ). However, the homogeneity of slope test shows no significant effect of the interaction between parents' sex and parents' telomere length (Table 4,  $p=0.513$ ), indicating that the mother-offspring and the father-offspring slopes were not significantly different.

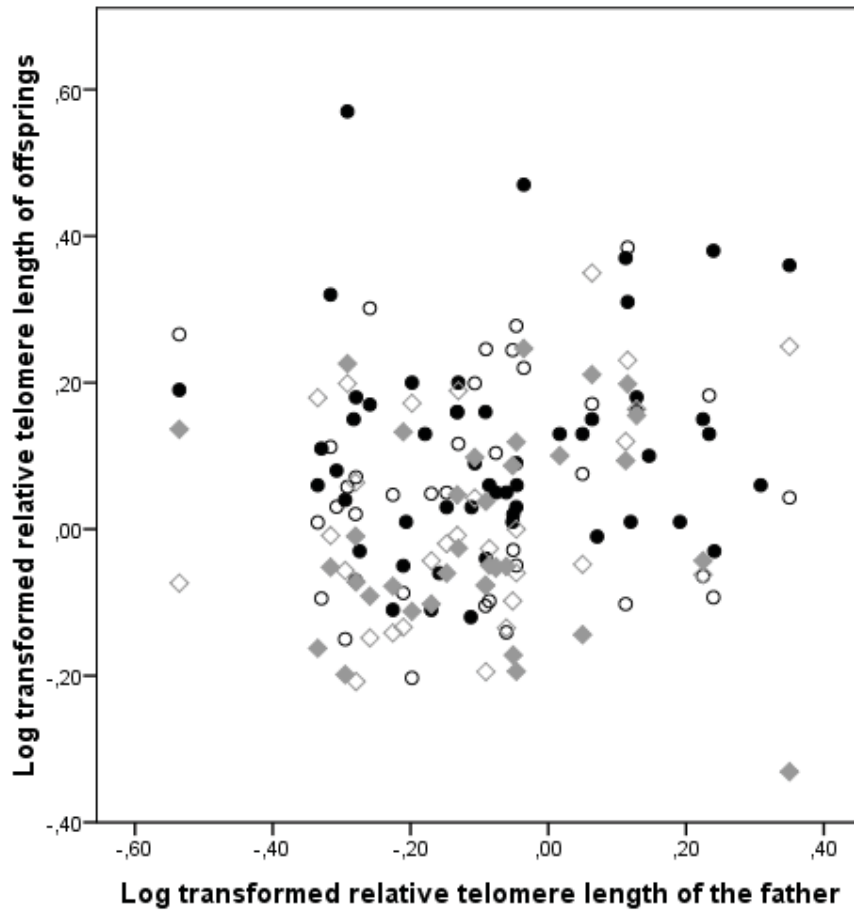
		Paternal telomere length	Maternal telomere length
Telomere length	r	,122	,284
10 days (number of chicks=53)	p	,388	,042
Telomere length	r	,027	,198
70 days (n=37)	p	,873	,240
Telomere length	r	-,011	,111
200 days (n=34)	p	,950	,532
Telomere length	r	,346	,252
300 days (n=30)	p	,061	,180

**Table 3:** *Pearson’s correlations between offspring telomere length over the growth period and parental telomere length.*





**Figure 2:** *Mother-offspring correlations of log transformed telomere length at 10 days (black filled circles), 70 days (black open circles), 200 days (grey filled diamonds), 300 days (grey open diamonds). The black slope represents the significant mother-offspring correlation at 10 days.*



**Figure 3:** *Father-offspring correlations of log transformed telomere length at 10 days (black filled circles), 70 days (black open circles), 200 days (grey filled diamonds), 300 days (grey open diamonds).*

Variable	Wald chi-squared	P
<b>Telomere length</b>		
Time period	29,671	,000
Parents' sex	,232	,630
Parents' telomere length	3,661	,056
Parents' sex * Parents' telomere length	,428	,513

**Table 4:** Results of the homogeneity of slopes test using a GEE model on offspring telomere length with time period as a repeated variable, chick identity as subject, parents' sex as a fixed factor, parents' telomere length as a covariate and the interaction between parents' sex and parents' telomere length.

Heritability calculated using the slope of the regression between offspring and parents' telomere lengths was of 0.2 (SE 0.110) and was of 0.2 (SE 0.096) for the regression between offspring and maternal telomere lengths. Offspring telomere lengths at 70 days, 200 days, and 300 days, were not significantly correlated to maternal or paternal telomere length (Table 3).

## Discussion

Our study indicates that early life (10 days) offspring telomere length is linked to maternal telomere length in the king penguin. However, this link disappeared as chicks grew and became older. These findings are in accordance with previous data collected on the kakapo (Horn *et al.* 2011), showing maternal inheritance of telomere length in birds. This pattern of telomere length inheritance mirrors the one found in humans (Nordfjäll *et al.*

2005) and suggests that the heterogametic sex plays a key role in offspring telomere length determination.

Gene imprinting may be one of the mechanisms explaining why telomere inheritance is done through heterogamy. Imprinting occurs when both maternal and paternal alleles are present and one allele is expressed while the other remains inactive (Pfeifer 2000). To date, very few studies have identified imprinting of telomere length regulating genes. Recently, (Gao *et al.* 2011) identified a paternal imprinting on sperm chromatin that is essential for the inheritance of telomere identity in *Drosophila melanogaster*. Other studies determined that nucleotide polymorphisms in a large number of loci that code for proteins regulating DNA and histone methylation are associated with telomere length variability in humans (Gatbonton *et al.* 2006; Blasco 2007a). Usually, the imprint marks distinguishing the parental alleles are epigenetic and generally due to DNA methylation and histone modifications which can alter transcription patterns, suggesting that the genes of importance would be inactivated (Pfeifer 2000). A possibility would be that the imprinting mechanisms regulate expression levels of genes modulating telomere length such as telomerase, telomere capping proteins (De Lange 2005) or DNA helicase (Vasa-Nicotera *et al.* 2005). Overall, it underlines that different pathways (from DNA and histone state to telomere maintenance protein expression) are likely to regulate telomere length inheritance.

Besides telomere length inheritance, some studies mentioned in Table 1 looked more deeply in telomere length heritability (i.e. disentangling additive and non-additive genetic, and environmental variability in inheritance). Studies in humans reported heritability estimates of telomere length between 0.44 and 0.78 (Slagboom, Droog & Boomsma 1994; Njajou *et al.* 2007) and of 0.52-1.23 in other vertebrates such as the sand lizards (*Lacerta agilis*), with an interesting difference in sex-specific heritability, ((Olsson *et al.* 2011), Table 1). However, these estimations were done mainly using regressions between parents and offspring (or comparisons between relatives) telomere lengths, which are known to result in higher heritabilities (which may lead in our case to an  $h^2$  of 0.2, a higher value than what was previously found in birds, (Voillemot *et al.* 2012)). These data on humans have led to the conclusion that individual variation in telomere length mainly originates from differences in the zygote and that epigenetic / environmental influences are relatively minor (Graakjaer *et*

*al.* 2004). A powerful approach to untangle genetic and non-genetic variation of the transmitted variation necessitates a cross-fostering design (Voillemot *et al.* 2012). Chick exchange between nests was recently used in a wild population of collared flycatchers (Voillemot *et al.* 2012). The additive-genetic component (heritability) of telomere length inheritance was found to be 0.09, a low value which implies alternative explanations, the first one being that environmental factors are important determinants of early-life telomere length (Monaghan & Haussmann 2006). In fact, we found that the link between offspring and maternal telomere length early in life (10 days) disappears over the growth period. This supports that environmental factors have a strong influence on telomere loss in king penguin chicks (see (Voillemot *et al.* 2012)). (Geiger *et al.* 2012) showed that king penguin chicks with faster growth display faster rates of telomere loss, illustrating the potential importance of the impact of the growth conditions on telomere dynamics, which might have hidden the link between offspring and maternal telomere lengths as chicks aged. Secondly, because telomere length is related to individual fitness (Bauch, Becker & Verhulst 2013), it is possible that natural selection has tended to deplete genetic variation of the locus regulating telomere length in early life (Mirabello *et al.* 2012). As telomeres have been defined as a polygenic trait (Andrew *et al.* 2006; Gatbonton *et al.* 2006), a third possibility would be that the non-additive genetic component of the residual variance variability is large (thereby decreasing  $H^2$  but not because of additive-genetic variability depletion, (Merilä & Sheldon 1999)). We could hypothesise that the pattern of telomere length heredity is dependant of the inheritance pattern of multiple telomere length restoration / maintenance factors (Blackburn 2001; Blasco 2007b). A resetting mechanism of early-life telomere length has been pointed-out in mammals, in which both telomerase activity and parent telomere length are interacting to set-up the embryo starting telomere length (Chiang *et al.* 2010). Focusing on these processes in the future may enable us to better understand how progressive telomere shortening over generations is avoided and how inter-specific variability in telomere dynamics may have evolved.

Due to the absence of siblings in our study, testing these alternatives was not possible. Still, a longitudinal following of the same breeders over years might enable us to estimate the additive and non-additive genetic components of transmitted telomere length (i.e. relative telomeres comparisons). Even more interesting is the fact that, due to the long development

duration of their chick, king penguin alternatively starts as early or late breeders, depending on the fact that they were successful the preceding season (Weimerskirch, Stahl & Jouventin 1992). Late-breeders reproduce in more stressful ecological environment, due to the higher density of couples (Viblanç *et al.* 2011). Comparisons of the same pairs, during successive early and late breeding attempts, may enable us to estimate some components of the residual variance such as ecological inheritance.

In conclusion, our study confirms that telomere length is maternally inherited in birds and supports the hypothesis of inheritance from the heterogametic sex and shows that environment have strong effects on telomere dynamics as this link disappeared over time. However, in the view of the present knowledge (Table 1), the results on telomere length inheritance and heritability patterns are still contrasted. To have a more straightforward idea of the transmission of telomere length, future works should focus on the whole balance (i.e. pro- and anti-erosion factors and their inheritance) that is going to shape the offspring telomere length, in order to accurately understand how telomeres are transmitted over generations. State may be as important as length in this case.

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All procedures were approved by an independent ethics committee commissioned by the French Polar Institute. Working in the colony, handling chicks and sampling was allowed by Terres Australes et Antarctiques Françaises (TAAF). The experiments comply with the current laws of France.

We declare no conflict of interest.



Species	Father-offspring correlation	Mother-offspring correlation	Heritability	Outcome	Study
<b>Telomere length inheritance pattern</b>					
Human ( <i>Homo sapiens</i> )	Not measured	Not measured	$h^2=0,78$	Telomere length is genetically determined	Slagboom et al. 1994
Human ( <i>Homo sapiens</i> )	Not measured	Not measured	Not measured	Telomere length is genetically determined	Graakjaer et al. 2004
Human ( <i>Homo sapiens</i> )	Yes	Yes	Not measured	Telomere length inheritance linked to X chromosome	Nawrot et al. 2004
Human ( <i>Homo sapiens</i> )	Yes	No	Not measured	Paternal telomere inheritance	Nordfjall et al. 2005
Human ( <i>Homo sapiens</i> )	Yes	Yes	$h^2=0,44$	Paternal telomere inheritance	Njajou et al. 2007
Human ( <i>Homo sapiens</i> )	Yes	No	Not measured	Paternal telomere inheritance	Nordfjall et al. 2009
Human ( <i>Homo sapiens</i> )	Yes	Yes	$h^2=0,70$	Paternal and maternal inheritance, effect of paternal age	Broer et al. 2013
Human ( <i>Homo sapiens</i> ) and Mouse ( <i>Mus musculus</i> )	Not measured	Not measured	Not measured	Telomere length is inherited	Chiang et al. 2010
Kakapo ( <i>Strigops habroptila</i> )	No	Yes	Not measured	Maternal inheritance of telomere length in a bird	Horn et al. 2011
<b>Telomere length heritability</b>					
Human ( <i>Homo sapiens</i> )	Not measured	Not measured	$h^2=0,82$		Jeanclous et al. 2000
Human ( <i>Homo sapiens</i> )	Not measured	Not measured	$h^2=0,34$		Bischoff et al. 2005
Human ( <i>Homo sapiens</i> )	Not measured	Not measured	$h^2=0,82$		Vasa-Nicotera et al. 2005
Human ( <i>Homo sapiens</i> )	Not measured	Not measured	$h^2=0,36$		Andrew et al. 2006
Human ( <i>Homo sapiens</i> )	Not measured	Not measured	$h^2=0,64$		Atl-Attas et al. 2012
Collared flycatcher ( <i>Ficedula albicollis</i> )	Not measured	Not measured	$h^2=0,18$	Weak but significant telomere length heritability	Voillemot et al. 2012
<b>Parental age linked to telomere length</b>					
Human ( <i>Homo sapiens</i> )	Not measured	Not measured	Not measured	Offspring telomere length linked to paternal age	Unryn et al. 2005
Human ( <i>Homo sapiens</i> )	Not measured	Not measured	Not measured	Telomere length at birth linked to paternal age	De Meyer et al. 2007
Human ( <i>Homo sapiens</i> )	Not measured	Not measured	Not measured	Paternal age effects on offspring's telomere length	Kimura et al. 2008
Human ( <i>Homo sapiens</i> )	Not measured	Not measured	Not measured	Offspring telomere length linked to paternal age	Eisenberg et al. 2012



Sand lizard ( <i>Lacerta agilis</i> )	Not measured	Not measured	$h^2=0,52 / h^2=1,23$	Paternal age correlated to offspring's telomere length	Olsson et al. 2011
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**Table 1:** Summary of the studies on telomere length inheritance patterns, telomere length heritability and link between parental age and telomere length.





## Study 3

### Mother-offspring resemblance but no additive genetic variation in telomere length in European dippers

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## Abstract

Telomeres are highly conserved protective DNA-protein complexes located at the ends of eukaryotic chromosomes. Their length has been shown to significantly predict life-history parameters in a number of organisms. Although this suggests that telomere length is subject to natural selection, the evolutionary dynamics crucially depend on the heritability of telomere length. Using pedigree data from a wild population of European dippers (*Cinclus cinclus*), we here test whether and how variation in telomere length is transmitted across generations. More specifically, we disentangle the relative effects of genes and the environment on early-life telomere length, and test for sex-specific patterns of inheritance. Despite strong and significant resemblance between mother and offspring (and between mothers and their sons in particular), and a positive effect of inbreeding in some models, we find very low levels of additive genetic variance and heritability. We show that neither maternal imprinting nor Z-linked inheritance can explain these patterns, suggesting that this resemblance is due to a non-genetic maternal effect. Environmental factors appear to be the main drivers of variation in early-life telomere length in dippers. While it is crucial to model them to obtain unbiased estimates of heritability, their accurate identification remains a major challenge in natural populations.

## Introduction

Telomeres are highly conserved protective DNA-protein complexes based on tandem repeats of a simple sequence of nucleotides. They are located at the ends of eukaryotic chromosomes, where they prevent deterioration of chromosome ends and fusion among chromosomes (Blackburn 2000). Changes in their length depend on the interplay of pro- and anti-erosion factors (von Zglinicki 2000; Blackburn 2001). Telomere length has been shown to significantly predict life-history parameters in a number of organisms, both when telomeres are measured early in life (Lindström 1999; Metcalfe & Monaghan 2001; Heidinger *et al.* 2012), and during adulthood (Bize *et al.* 2009). For example, telomere length or the rate of loss have been linked to lifespan in humans (Cawthon *et al.* 2003) and in natural populations of several bird species (Hausmann, Winkler & Vleck 2005; Bize *et al.* 2009; Salomons *et al.* 2009). Furthermore, Heidinger *et al.* (Heidinger *et al.* 2012) showed that in captive zebra finches (*Taenopygia guttata*), individuals with the longest telomeres at the end of the growth period live longest, highlighting the crucial importance of telomere length at an early life stage. More recently, it has been found that telomere length may not only be positively related to individual fitness through its link with lifespan, but also as a mediator of reproductive trade-offs (Bauch, Becker & Verhulst 2013). This further reinforces the idea that telomere length could be subject to directional natural selection (Fulnečková *et al.* 2013). However, whether this results also in an evolutionary response depends on the levels of additive genetic variation in telomere length.

Telomere length shows substantial amounts of variability, not only among eukaryote species (Forsyth, Wright & Shay 2002; Gomes, Shay & Wright 2010), but also among individuals of the same species and population (Bize *et al.* 2009). Although it is key to obtaining a better understanding of the origin of individual variability in early life telomere length, an answer to the questions whether variation in telomere length is transmitted from one generation to the next, and if it is, by what mechanism, remains elusive.

To date, various studies on the mode of transmission and narrow-sense heritability (i.e. the proportion of the phenotypic variance that is attributable to additive genetic effects,  $h^2$ ) of telomere length have been conducted in humans (Slagboom, Droog & Boomsma 1994; Graakjaer *et al.* 2004). Estimates from natural populations of other species, however, remain

scarce (but see Horn *et al.* 2011; Olsson *et al.* 2011; Voillemot *et al.* 2012). Ranging from 0.18 to 1.23 (Slagboom, Droog & Boomsma 1994; Njajou *et al.* 2007; Olsson *et al.* 2011; Voillemot *et al.* 2012), the majority of heritability estimates is relatively high, especially considering that heritabilities of traits that are closely related to fitness are often low (Price & Schluter 1991). These high heritabilities may at least partly be attributable to common environment and parental effects (Andrew *et al.* 2006), which – if unaccounted for – result in an overestimate of the heritability (Kruuk 2004). Indeed, telomere dynamics are known to be largely modulated by environmental factors both during development (Jennings *et al.* 1999; Tarry-Adkins *et al.* 2008; Geiger *et al.* 2012) and adult life (Epel *et al.* 2004; Monaghan & Hausmann 2006; Blackburn & Epel 2012). For example, early exposure to steroid hormones triggers an accelerated telomere loss in zebra finches (Hausmann *et al.* 2012).

Interestingly, many studies have found support for sex-specific patterns of inheritance of telomere length (Nawrot *et al.* 2004; Bauch, Becker & Verhulst 2013). For example, the few studies investigating telomere length inheritance in non-human animals (birds and lizards) have found either maternal inheritance (Horn *et al.* 2011) or a much higher son-father than daughter-mother resemblance (Olsson *et al.* 2011). Indeed, sex-specific patterns appear to be the rule rather than the exception in the literature on telomere inheritance, and suggested mechanisms include parent-specific imprinting, hormonal regulation and sex-chromosome linkage, either acting independently or jointly (Nordfjäll *et al.* 2005; Horn *et al.* 2011; Olsson *et al.* 2011; Broer *et al.* 2013)

Here, we investigate patterns of inheritance of telomere length in a wild population of European dippers (*Cinclus cinclus*). We use nestlings from an individual-based long-term study, enabling us to separate phenotypic variation in telomere length into variance components attributable to additive genetic, common environment (i.e. nest) and other environmental effects. In addition, we explicitly test for parental and imprinting effects, as well as sex-linked inheritance (Z-linkage).

## Materials & Methods

### *Study system*

The European dipper is a medium-sized passerine living along streams and rivers. Since 1987, dippers have been studied at eleven rivers spanning an area of approximately 400 km<sup>2</sup> in the proximity of Zurich, northern Switzerland (8°23'E / 47°25'N to 8°40'E / 47°10'N). Here, we use data from the Küsnacht (*K*), Wehrenbach (*W*), and Sihl (*S*) rivers. Every year, monitoring starts in early February in order to map territories and to find nests. Territories are checked regularly between the nest building and nestling phase. When nestlings are 10-14 days old, they are ringed and a small blood sample (max. 30 µl) is collected by puncturing the tarsal vein. Unringed adults (i.e. immigrants) are captured using mist nets and ringed, usually before the breeding season, but at the latest before their offspring are ringed.

### *Pedigree reconstruction and inbreeding estimation*

Parentage of each brood was determined from behavioural observations, assuming that the social parents are also the genetic parents of all nestling. This is a reasonable assumption given the low incidence of extra-pair paternity (less than 1% of nestlings; unpublished data). The identity of the territorial breeding male and female were recorded during territory establishment, incubation and/or feeding of the offspring.

We were able to construct a pedigree spanning 15 generations, covering the cohorts from 1987 to 2012. We calculated Wright's coefficients of inbreeding (*f*; Wright 1969) for each individual using *Pedigree Viewer* (available at <http://www.personal.une.edu.au/~bkinghor/pedigree.htm>). Inbreeding coefficients are relative to the base population, i.e. relative to all birds with unknown parents. These birds, i.e. founders and immigrants, are assigned an inbreeding coefficient of zero, which carries no information. Therefore, in the following analyses we use only birds that hatched in the study area.

We selected a total of 177 individuals for telomere length measurement (see below) consisting of groups of four individuals, made up by a sire, a dam and – whenever possible – a female and a male offspring. We also ensured that there were numerous relatedness links among these groups to allow analyses of the patterns of inheritance. Pruning the complete



pedigree used for the calculation of inbreeding coefficients (see above) to include only individuals with known telomere length, or that provide a pedigree link between two individuals with known telomere length (R package "pedantics", Morrissey & Wilson 2010), resulted in a pedigree containing 315 individuals, 255 maternities, 263 paternities, 221 full sibs, 103 maternal half sibs and 147 paternal half sibs. Mean pedigree depth was 5.9 generations (max. 14 generations).

### ***DNA extraction and sexing***

Blood samples were preserved in APS buffer (Arctander 1988), DNA was extracted using the QIAmp DNA mini kit (BioSprint 96, Quiagen), and then stored at -80°C (see Bucher *et al.* 2009 for more details). Nestling sex was determined by amplifying the CHD-W and CHD-Z genes using modified versions of the P2 and P8 primers (Griffiths *et al.* 1998; Hoeck *et al.* 2009).

### ***Telomere length measurements***

Telomere length was assessed using a quantitative real-time amplification (qPCR) procedure (Cawthon 2002) adjusted for bird species (Crisuolo *et al.* 2009). All samples were analysed in duplicates. The number of amplification cycles needed to reach the threshold fluorescent signal ( $C_t$  value) is proportional to the sample telomere length (T), or to the number of copies of a control gene that is non-variable in copy number (control gene S). Relative telomere length is expressed as T/S (Smith, Turbill & Penn 2011). Here we used glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Genbank Accession No: AF255390) as the control gene. Forward and reverse primers for the GAPDH gene were 5'-AACCAGCCAAGTACGATGACAT-3' and 5'-CCATCAGCAGCAGCCTTCA-3' respectively. Telomere primers were 5'-CGGTTTGGTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3' and 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3' (Tel1b and Tel2b, see Crisuolo *et al.* 2009). qPCRs for both telomere and GAPDH were performed using 5 ng of DNA with both sets of primers, in a final volume of 10 µl containing 5 µl of Power SYBR<sup>®</sup> Green PCR Master Mix (Appliedbiosystems, UK). Primer concentrations in the final mix were 100 nM for telomere length determination and 200 nM for the control gene. Telomere and GAPDH real time amplification were performed on separate plates. qPCR conditions for telomeres were

10 min at 95°C, followed by 30 cycles of 1 min at 56°C and 1 min at 95°C. qPCR conditions for GAPDH were 10 min at 95°C, followed by 40 cycles of 1 min at 60°C and 1 min at 95°C. Serial dilutions of a reference sample were used to generate a reference curve on each plate, in order to control for variation in the amplification efficiency of the qPCR among plates. Both a negative control (water) and melting curves were run for each plate to check for non-specific amplification and primer-dimer artefacts. Samples were randomly assigned on a total of six plates.

Intra-plate mean coefficients of variation for  $C_t$  values were  $1.35 \pm 0.06\%$  for the telomere assay and  $0.79 \pm 0.04\%$  for the control gene assay (based on duplicates), and inter-plate coefficients of variation based on repeated samples were 1.56% for the telomere assay and 1.35% for the control gene assay. Amplification efficiencies of the qPCR runs were between 98% and 100% for telomere and between 99% and 100% for the control gene. To take into account both this slight difference in amplification efficiency (E), as well as the non-zero intra- and inter-plate coefficients of variation, we calculated relative telomere length applying the method suggested by Pfaffl (Pfaffl 2001) as the following ratio:

$$\text{relative telomere length} = \frac{1 + E \cdot T^{\Delta C_t T(\text{control} - \text{sample})}}{1 + E \cdot S^{\Delta C_t S(\text{control} - \text{sample})}}$$

### **Quantitative genetic analyses**

We fitted a series of animal models (Kruuk 2004) to estimate the absolute and relative amount of additive genetic variance ( $V_A$  and  $h^2$ , respectively) underlying telomere length, and to test for parental effects, sex-specific inheritance and imprinting. Animal models were fitted using restricted maximum likelihood (REML) in ASReml version 3.0 (Gilmour *et al.* 2009), except for models including imprinting effects, which were implemented in WOMBAT (Meyer 2007).

Telomere length was best described by a normal distribution and residuals of the final model did not show any deviations from normality (Shapiro-Wilk test of normality,  $W=0.99$ ,  $p=0.25$ ). Nestling telomere length was modelled as a function of sex and natal population (*i.e.* river), age at sampling (in days), and hatching date (as Julian day). Additionally, all models included inbreeding coefficient as a covariate, which in the presence of inbreeding

depression in telomere length ensures unbiased estimates of additive genetic variance (Hoeschele & van Raden 1991; de Boer & van Arendonk 1992; reviewed in Wolak & Keller in press). Fixed effects were removed in a stepwise manner, starting with the least significant, as inferred from a Wald test. All effects with  $p < 0.15$  were retained in the model.

In addition to the fixed effects listed above, we fitted a random additive genetic effect (animal effect), as well as a random nest and year of birth effect. The animal effect ( $V_A$ ) estimates the variance in the trait that is due to additive genetic effects, using information on the relatedness and resemblance in telomere length among all individuals in the pedigree. The nest effect ( $V_{NEST}$ ) estimates the variance among nests that can be attributed to the shared environment of full sibs, over and above the variance that is attributable to additive genetic effects. Finally, variation that can be attributed to random environmental variability among years ( $V_{YEAR}$ ) is accounted for by the year of birth effect. Heritability, the proportion of the phenotypic variance that is explained by additive genetic variance, was calculated as:

$$h^2 = V_A / (V_A + V_{NEST} + V_{YEAR} + V_R),$$

where  $V_R$  is the residual variance. Statistical significance of random effects was assessed using likelihood ratio tests, comparing log-likelihoods of models with and without the specific random effect.

To test whether there are general features of the mother or father that affect offspring telomere length over and above any additive genetic effects that she or he passed on (sensu Willham 1972), initial models included maternal and paternal identity as additional random effects. However, both explained little to no variation ( $2.6 \times 10^{-5} \%$  and  $3.1 \times 10^{-5} \%$  of phenotypic variation, respectively). Hence, parental identities were excluded from any further models.

Although the structure of the data did not allow us to unequivocally attribute the increased resemblance among full sibs to properties of the nest or the mother, we were able to directly test for an effect of maternal telomere length (as a nestling) on the telomere length of her offspring, again over and above the effect of the genes that she passes on to them. To do so, we extended the animal model arrived at above with maternal telomere length ( $m$ , after correcting for the effects outlined above) as an additional covariate (page 706 in Lynch

& Walsh 1998). Similarly, we included residual paternal telomere length, and also both maternal and paternal telomere length simultaneously.

In birds, females are the heterogametic and males the homogametic sex (ZW and ZZ, respectively). Animal models allow for the explicit estimation of sex-linked effects as these follow a different pattern of inheritance than autosomal traits (Gilmour *et al.* 2009; Husby *et al.* 2012). In order to quantify Z-linked genetic variance, we used a relatedness matrix that accounts for the specific inheritance of Z-linked genes (e.g. the relatedness between mothers and their daughters is zero) (Husby *et al.* 2012) instead of the usual autosomal relatedness matrix.

Finally, we tested for imprinting effects, which may provide a further source of sex specific resemblance, using WOMBAT (Meyer 2007). We externally calculated the inverse of a gametic relationship matrix (code written by Bruce Tier, provided on <http://didgeridoo.une.edu.au/womwiki>) and used this to fit either a random maternal or a paternal imprinting effect, in addition to the animal's additive genetic effect.

In addition to the animal models above, we performed a number of parent-offspring regressions, in which we regressed mean offspring, son or daughter telomere length against maternal or paternal telomere length. Values that were used for these regressions were residuals taken from a mixed model that accounts for the effects of inbreeding coefficient and year of birth. Not only do these help to visualise our main findings, they also make it possible to directly compare them to other studies on parent-offspring resemblance in telomere length.

## Results

### *Animal model analysis*

Telomere length was not affected by hatching date ( $b=2.5 \times 10^{-4} \pm 1.5 \times 10^{-3}$ ,  $F_{1,102.6}=0.03$ ,  $p=0.9$ ), age at sampling ( $b=1.4 \times 10^{-3} \pm 1.7 \times 10^{-2}$ ,  $F_{1,95.4}=0.01$ ,  $p=0.9$ ), sex (male-female length:  $1.4 \times 10^{-2} \pm 4.1 \times 10^{-2}$ ,  $F_{1,102.8}=0.11$ ,  $p=0.7$ ), or natal population ( $F_{2,93.1}=1.74$ ,  $p=0.2$ ). Although it did not reach statistical significance, inbreeding had a positive effect on telomere length ( $b=0.53 \pm 0.35$ ,  $F_{1,64.0}=2.29$ ,  $p=0.14$ ), and was retained in all models to

obtain unbiased estimates of the random variance components (Hoeschele & van Raden 1991; de Boer & van Arendonk 1992).

Nest identity and year of birth explained large and significant proportions of the phenotypic variation ( $\pm$  approximate standard error) (nest identity:  $19.6 \pm 8.3\%$ ,  $\chi^2=8.59$ , d.f.=1,  $p=0.002$ ; and year of birth:  $45.7 \pm 13.2\%$ ,  $\chi^2=52.06$ , d.f.=1,  $p<0.001$ ). However, the estimate of the variance component for additive genetic variance was not significantly different from zero ( $0.007 \pm 0.013$ ,  $\chi^2=0.45$ , d.f.=1,  $p=0.25$ ), and heritability of telomere length was estimated to be  $3.8 \pm 6.9\%$ . For all statistical details, see Table 1.

covariate	model A				model B			
	slope $\pm$ s.e		test statistic	p-value	slope $\pm$ s.e		test statistic	p-value
f	$0.53 \pm 0.35$		F=2.29	0.14	$0.87 \pm 0.43$		F=4.14	0.05
maternal TL	–		–	–	$0.22 \pm 0.11$		F=4.06	0.048
random effect	variance $\pm$ s.e.	proportion $\pm$ s.e.	test statistic	p-value	variance $\pm$ s.e.	proportion $\pm$ s.e.	test statistic	p-value
animal ( $V_A$ )	$0.007 \pm 0.013$	$0.038 \pm 0.069$	$\chi^2=0.45$	0.25	$1 \times 10^{-7}$	$6 \times 10^{-7}$	$\chi^2=0$	0.5
nest ( $V_{NEST}$ )	$0.037 \pm 0.014$	$0.196 \pm 0.083$	$\chi^2=8.59$	0.002	$0.031 \pm 0.015$	$0.182 \pm 0.092$	$\chi^2=5.77$	0.008
year of birth ( $V_{YEAR}$ )	$0.086 \pm 0.044$	$0.457 \pm 0.132$	$\chi^2=52.0$	<0.001	$0.075 \pm 0.045$	$0.438 \pm 0.152$	$\chi^2=23.7$	<0.001
residual ( $V_R$ )	$0.058 \pm 0.012$	$0.310 \pm 0.096$	6	1	$0.066 \pm 0.012$	$0.038 \pm 0.118$	1	1

**Table 1:** Animal model analysis, explaining variation in nestling telomere length with an individual's inbreeding coefficient ( $f$ ) as well as an additive genetic effect (animal effect), a nest and a year of birth effect (model A,  $n=177$ ). Model B ( $n=114$ ) additionally includes maternal residual telomere length (TL) as a covariate. Slopes for covariates, variance components for random effects as well as their proportions are given including approximate standard errors (s.e.) and test statistics (conditional F-test and  $\chi^2$ -test, respectively).

Including residual maternal telomere length resulted in a smaller sample size ( $n=114$ ) and in a further reduction of  $V_A$  ( $1 \times 10^{-7}$ ,  $\chi^2=0$ , d.f.=1,  $p=0.5$ ) and  $h^2$  ( $6 \times 10^{-5}\%$ ), but did not affect estimates of the other variance components (Table 1). Again, the effect of inbreeding was positive and this time did reach statistical significance ( $b=0.87 \pm 0.43$ ,  $F_{1,46.8}=4.14$ ,  $p=0.05$ ). Most importantly, maternal telomere length significantly explained variation in offspring telomere length ( $b=0.22 \pm 0.11$ ,  $F_{1,47.1}=4.06$ ,  $p=0.048$ ; Table 1). Although the additional inclusion of paternal telomere length further reduced the sample size ( $n=101$ ), this did not alter the positive point estimate of the mother's phenotype on offspring phenotype ( $b=0.23 \pm 0.12$ ,  $F_{1,39.4}=3.35$ ,  $p=0.075$ ), whereas there was no effect of the father's phenotype ( $b=0.02 \pm 0.13$ ,  $F_{1,37.9}=0.04$ ,  $p=0.85$ ).

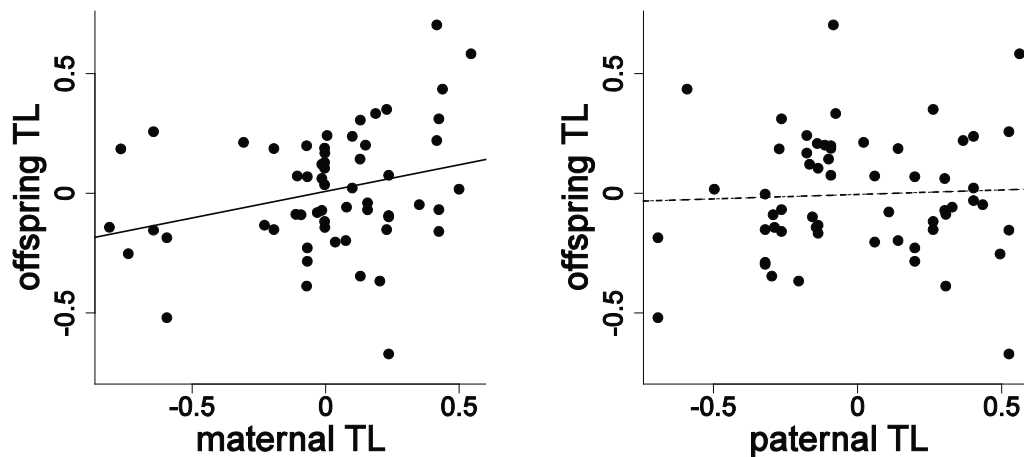
Additional analyses attempting to explain these patterns of resemblance by means of sex-linked inheritance revealed a random variance component for Z chromosome-linked variance of  $5.3 \times 10^{-8}$  ( $\chi^2=0$ , d.f.=1,  $p=0.5$ ), with all other variance component estimates remaining unchanged. Similarly, neither maternal nor paternal imprinting explained much variance (maternal imprinting  $1.6 \times 10^{-5}$ ,  $\chi^2=0$ , d.f.=1,  $p=0.5$ ; paternal imprinting:  $1.2 \times 10^{-5}$ ,  $\chi^2=0$ , d.f.=1,  $p=0.5$ ).

### ***Parent-offspring regressions***

The slope of the regression of mid-offspring on maternal telomere length was significantly positive ( $b=0.22 \pm 0.10$ ,  $p=0.03$ ;  $n=59$ ), whereas the slope of the regression of mid-offspring on paternal telomere length was small and non-significant ( $b = 0.04 \pm 0.11$ ,  $p=0.73$ ;  $n=59$ ) (Fig. 1, Table 2). Similarly, single sex offspring – parent regressions revealed that father-daughter and father-son regressions were not significantly different from zero, with estimates close to zero ( $b=0.09 \pm 0.14$ ,  $p=0.50$ , and  $b=-0.03 \pm 0.12$ ,  $p=0.79$ , respectively). However, mother - single sex offspring regressions showed that resemblance between mothers and their sons ( $b=0.27 \pm 0.04$ ,  $p=0.01$ ) was considerably higher than the resemblance between mothers and their daughters ( $b=0.11 \pm 0.14$ ,  $p=0.43$ ).

parent – offspring combination	n	b ± s.e	p-value
mother – offspring	5	0.22 ±	0.03
	9	0.10	
mother – son	5	0.27 ±	0.01
	8	0.04	
mother – daughter	5	0.11 ±	0.43
	6	0.14	
father – offspring	5	0.04 ±	0.73
	9	0.11	
father – son	6	-0.03 ±	0.79
	1	0.12	
father – daughter	5	0.09 ±	0.50
	7	0.14	

**Table 2:** Parent-offspring regressions of telomere length. Slope including standard error ( $b \pm s.e.$ ), sample size ( $n$ ) and  $p$ -value are given for different regressions of offspring (mid-offspring, son, or daughter) on parental telomere length. Values of telomere length are residuals from a mixed model that accounts for an individual's inbreeding coefficient and its year of birth. All telomere measurements were done on samples taken at the nestling stage.



**Figure 1:** Linear regressions of mid-offspring telomere length (TL; mean of nestlings of one nest) against their mother's (left panel,  $n=59$ ) and father's (right panel,  $n=59$ ) telomere length. TL values are residuals from a mixed model accounting for an individual's inbreeding coefficient and its year of birth. Whereas offspring resemble their mother ( $b=0.22 \pm 0.10$ ,  $p=0.03$ ), there is no correlation between fathers and their offspring ( $b=0.04 \pm 0.11$ ,  $p=0.73$ ).

## Discussion

Here we tested whether and how variation in telomere length is transmitted across generations. By using pedigree data from a wild population of European dippers, we were able to disentangle the relative effects of genes and the environment on early life telomere length, and to test for sex-specific patterns of inheritance.

Previously reported heritability estimates of telomere length range between 0.44 and 0.78 for humans (Slagboom, Droog & Boomsma 1994; Njajou *et al.* 2007; Broer *et al.* 2013) and between 0.18-1.23 for other vertebrates (Horn *et al.* 2011; Olsson *et al.* 2011; Voillemot *et al.* 2012). However, several of these estimates are based on the resemblance between parents and offspring or among full sibs, which may result in an overestimate of heritability



if environmental sources of resemblance are unaccounted for (Kruuk 2004). Indeed, based on a mother-offspring regression, we would have obtained a statistically significant and relatively high heritability of 44% (twice the slope of the mother-offspring regression), even after accounting for year of birth and inbreeding coefficient. Similarly, not accounting for nest effects in our animal model would have resulted in a heritability of 9.2%, whereas accounting for nest effects reduces heritability to 3.8% ( $\pm 6.9\%$ ). This low heritability is in line with a cross-fostering experiment in a wild population of collared flycatchers (*Ficedula albicollis*), which in principle allows for the separation of additive genetic and common environment effects, and which found a heritability of 18% (and not 9% as reported previously (Bize, pers. comm., Voillemot *et al.* 2012)). Although this value is higher than our estimate in dippers, it still is substantially lower than what has been found in other studies. Furthermore, it should be noted that the nature of their experimental design and statistical analyses still allows for a substantial inflation of the heritability (Voillemot *et al.* 2012).

Although we find no evidence for autosomal additive genetic variance underlying variation in telomere length, we do find a significant link between offspring and maternal telomere length, and in particular between mothers and their sons, whereas we find no such link between offspring and paternal telomere length. Such parental effects have been found previously in humans and birds. For example, Horn *et al.* (Horn *et al.* 2011) found patterns of parent-offspring resemblance in kakapos (*Strigops habroptilus*) that are strikingly similar to our results: whereas mothers had similar telomere lengths as their offspring, in particular when compared with their sons, offspring did not show any resemblance to their father (Horn *et al.* 2011). Sand lizard males on the other hand, show a higher resemblance to their father than to their mother.

One mechanism that could explain such sex-specific patterns is sex-specific gene imprinting. Imprinting occurs when both maternal and paternal alleles are present, but only one allele is expressed (Pfeifer 2000). The imprint marks distinguishing the parental alleles are epigenetic and generally due to DNA methylation and histone modifications which alter transcription patterns (Pfeifer 2000). While earlier papers have challenged its presence in oviparous species (Nolan *et al.* 2001), recent studies have provided some support for its existence in birds, particularly in the context of parental conflict (Frésard *et al.* 2013). However, we found

no evidence of either maternal or paternal imprinting. Similarly, we found no evidence for sex-linkage, specifically Z-linkage, as an explanation for the sex-specific patterns of resemblance. Furthermore, as the W-chromosome is only transmitted to daughters, the strong resemblance between mothers and their sons rules out W-linkage. In conclusion, we therefore propose that the mother-offspring resemblance observed here is the result of a non-genetic maternal effect. However, we cannot explain the difference in resemblance between mothers and sons or daughters, respectively.

It is worth emphasising that the mother-offspring resemblance observed here is based on measurements of telomere length at nestling stage for both mothers and offspring. Although the rate of telomere loss over an individual's life is expected to vary in response to environmental challenges (i.e. stress, Epel *et al.* 2004), our findings are in accordance with a previous study showing that captive zebra finches with initial long/short telomeres (i.e. at the end of growth) consistently showed long/short telomeres over their life (Heidinger *et al.* 2012).

In accordance with previous studies, we find a major role for environmental effects in shaping variation in telomere length (Jennings *et al.* 1999; Hall *et al.* 2004; Tarry-Adkins *et al.* 2008; Foote *et al.* 2011; Geiger *et al.* 2012). For example, a substantial part of the phenotypic variation is attributable to environmental differences among birth years (cohorts). Although increased telomere loss in response to suboptimal environmental conditions has been observed in mammals, lizards and birds (Hausmann & Marchetto 2010; Ballen *et al.* 2012), it remains unclear in our case which environmental variables are responsible for the observed annual variation in early-life telomere length.

In addition to population-level environmental year effects, the micro-environment of the nest is not only of crucial importance for nestling development and growth (Deeming 2002), but as we show here, it is also an important determinant of early life telomere length. This suggests that the main parameters that determine nest microclimate, temperature, humidity, and gas composition (Deeming 2002) may modulate telomere shortening. Indeed, early development is a period characterised by rapid telomere shortening (Hall *et al.* 2004). For example in zebra finches, telomere shortening is six to 30 times faster during their first year compared to the rest of their life, and six times faster during the first 30 days of growth

when compared to the following 550 days of life (F. Criscuolo unpublished data). An additional factor which may contribute to nest micro-environment is parental quality. Parents might differ in features that are related to condition or quality, such as foraging skills (Wendeln & Becker 1999), body mass (Weimerskirch, Stahl & Jouventin 1992) and territory quality (Daan, Dijkstra & Tinbergen 1990). These differences will influence reproductive decisions, including parental care and parental investment in reproduction (McNamara & Houston 1996; Wilson & Nussey 2010). High quality parental investment may buffer stressful events during early development and preserve telomeres from adverse stress-related weakening, causing significant nest effects. Similarly, Andrew *et al.* (Andrew *et al.* 2006) showed a strong common environment effect (family) in a human twin-study.

We found telomere length to be positively related to an individual's inbreeding coefficient, which is in line with findings in laboratory mouse strains (Manning *et al.* 2002) and domesticated chicken lines (O'Hare & Delany 2009). This positive relationship is at first sight surprising, as longer telomeres have been associated with increased survival rate and lifespan (Bize *et al.* 2009; Heidinger *et al.* 2012), whereas survival and lifespan are typically lower in inbred individuals (for review see Keller & Waller 2002). Hence, one would intuitively expect telomeres to be shorter in inbred individuals. However, inbred dippers in our population do not have decreased survival rates and lifespan (Becker *et al.*, unpublished data), and in general little is known about the relationship between telomere length and other fitness-related traits such as fecundity (Bauch, Becker & Verhulst 2013). Indeed, the longer telomeres of inbred nestlings may be a by-product of inbreeding effects on other traits. For example, inbred dipper nestlings are smaller at the age of ringing (Becker *et al.*, unpublished data). Slower growth rates might entail lower cell division rates, causing less telomere loss due to cell division. Further work focusing on the interplay between telomere length, inbreeding and fitness is warranted.

In summary, we here demonstrate significant mother-offspring resemblance and mother-son resemblance in particular, in early life telomere length in a bird species, but at the same time show that its heritability is very low. As we show that neither maternal imprinting nor Z-linked inheritance contribute to this pattern, we conclude that this resemblance is due to some non-genetic maternal effect. In line with its very low heritability, non-genetic

environmental factors are the main drivers influencing early life telomere length in dippers, a result that contrasts with the findings in many other species. Yet, to our knowledge this study is the first to explicitly separate additive genetic from non-genetic environmental sources of variation in telomere length in a non-human species, providing an unbiased estimate of its heritability.

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## **Authors' contribution**

SR, PB, FC, SM and LK planned and designed the study. JH and PB collected samples, SZ measured telomere length, PB and EP did the statistical analysis, and PB, SR, EP, FC, SM and LK interpreted the results and wrote the manuscript.



## Chapter IV

# Extrinsic determinants of telomere length: indications of telomere dynamics role in life- history trade-offs.



Cédric Sueur



## Study 4

### Immediate and delayed effects of growth on ageing parameters in nestling zebra finches

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## Introduction

La capacité d'un organisme à grandir et à avoir une grande taille, à l'âge adulte, confère des bénéfices en termes de fitness individuelle. En effet, les individus les plus petits et les plus faibles sont souvent plus vulnérables, notamment en termes de prédation. Par ailleurs, le fait de grandir et d'atteindre la taille adulte le plus vite possible, est une stratégie avantageuse pour les organismes, dans la mesure où cela leur permet d'échapper aux risques qui sont associés à la période de croissance (vulnérabilité, risques de prédation plus importants). Cependant, malgré les avantages d'une croissance rapide, les organismes ne grandissent pas à leur vitesse maximale. Ceci semble s'expliquer par le fait qu'une vitesse de croissance rapide est associée à différents coûts, notamment en termes de longévité. Par conséquent, le taux de croissance d'un individu semble résulter d'un compromis entre les avantages que peut apporter une croissance rapide et les coûts à courts et longs termes qu'elle est susceptible d'entraîner.

Afin d'identifier les coûts qui sont associés à la croissance, il est nécessaire de manipuler les conditions environnementales lors de la croissance (modification de la qualité et/ ou de la quantité de nourriture, variation de la température, manipulation de la taille de la nichée). De plus, pour comprendre la nature du compromis entre croissance et longévité, il est essentiel de déterminer quels sont les mécanismes sous-jacents de ce compromis. Afin d'identifier les acteurs potentiels de ce compromis entre croissance et longévité, nous avons modifié les conditions de croissance chez des poussins de diamants mandarins en manipulant la taille de la nichée (en créant trois groupes expérimentaux : nichées contrôle, nichées de taille augmentée, nichées de taille réduite). Nous nous sommes ensuite intéressés à l'effet de cette manipulation de la taille des nichées sur la dynamique d'érosion des télomères et sur le stress oxydant, deux mécanismes impliqués dans le vieillissement des organismes.

## Principaux résultats

Les résultats indiquent que les poussins des groupes contrôle et augmenté, grandissent moins vite que ceux du groupe réduit. En revanche, comparés aux poussins des groupes contrôle et réduit, ceux du groupe augmenté présentent une accélération de

l'érosion des télomères et une augmentation du stress oxydant (qui se traduit par une augmentation des dommages oxydatifs sur l'ADN) pendant la croissance. L'effet sur le stress oxydant semble transitoire, dans la mesure où un an après l'expérience, les niveaux de stress oxydant sont similaires pour tous les groupes expérimentaux. Cependant, en ce qui concerne l'effet sur les télomères, celui-ci semble persister, puisqu'un an après l'expérience, les individus du groupe augmenté présentent des télomères plus courts que les individus des groupes contrôle et réduit.

## Discussion

Contrairement à ce que l'on aurait pu attendre, les individus ayant grandi dans des nichées réduites et présentant un taux de croissance plus élevé, ne montrent pas d'accélération de l'érosion des télomères. En effet, une des causes de l'érosion des télomères est la division cellulaire. De ce fait, on pourrait supposer que lors d'une croissance rapide, le taux de division cellulaire est augmenté et, de ce fait, l'érosion des télomères accélérée.

En revanche, il semblerait que l'augmentation de la taille de la nichée ait induit des effets délétères sur les marqueurs du vieillissement (érosion des télomères et stress oxydant). Ces effets peuvent être attribués soit à une altération des conditions nutritionnelles soit à une élévation des niveaux de corticostérone. En effet, l'exposition chronique à des niveaux de stress élevés peut induire une augmentation des dommages oxydatifs et une accélération de l'érosion des télomères. Les poussins ayant grandi dans les nichées de taille augmentée, ont pu être confrontés à des niveaux de stress plus élevés (augmentation du stress social du fait de nombre de poussins par nichée, accompagnée d'une augmentation de la compétition entre les poussins), - ce qui a pu entraîner une élévation du stress oxydant et une accélération de l'érosion des télomères.

Par conséquent, nos résultats semblent indiquer que l'érosion des télomères pendant la croissance ne dépend pas seulement de la vitesse de croissance en tant que telle, mais pourrait être sous le contrôle direct des conditions environnementales.

## Abstract

1. Conditions experienced during development and growth are of crucial importance as they can have significant influence on the optimisation of life histories. Indeed, the ability of an organism to grow fast and achieve a large body size often confers short and long term fitness benefits. However, there is good evidence that organisms do not grow at their maximal rates as growth rates seem to have long term effects on subsequent lifespan. Several proximate causes of such a reduced lifespan might be involved. Among them, one emerging hypothesis is that growth impacts adult survival and/or longevity through a shared, endpoint, ageing mechanism: telomere erosion.

2. In this study, we manipulated brood size in order to investigate if rapid growth (chicks in reduced broods) is effectively done at the cost of a short (end of growth) and long term (at adulthood) increase of oxidative damage and telomere loss.

3. Contrary to what we expected, chicks from the enlarged broods displayed more oxidative damage and had shorter telomeres at the end of the growth period and at adulthood.

4. Our study extends the understanding of the proximate mechanisms involved in the trade-off between growth and longevity. It highlights that adverse environmental conditions during growth can come at a cost *via* transient increased oxidative stress and pervasive eroded telomeres. Indeed, it suggests that telomeres are not only controlled by intrinsic growth rates *per se* but may also be under the control of some extrinsic environmental factors that may get our understanding of the growth ageing interaction more complicated.

## Introduction

The ability of an organism to grow fast and achieve a large body size often confers short and long term fitness benefits (Richner 1992; Reeve 2000; Bonduriansky 2001). In early life stages, organisms are often more vulnerable to predation because of their smaller size and restricted mobility, the latter one being especially true in altricial species. Hence, a fast growth is advantageous if it allows growing organisms to escape as quickly as possible this risky life history stage (Arendt 1997). Because growing fast is often associated with a large 'final' body size, especially in species with a determinate growth, a fast growth can also greatly influence fitness at adulthood. Indeed, adult body size is frequently under strong natural and sexual selection, and larger individuals are often better 'equipped' at defending their food resources, territories or mates against conspecifics. In addition, larger individuals are often the favourites when it comes to choose a mate (Shine 1988; Arendt 1997).

However, accumulation of counterintuitive data underline that individuals presenting a more rapid juvenile growth rate tend to have a reduced adult lifespan (Metcalf & Monaghan 2003; Dmitriew 2011; Lee, Monaghan & Metcalfe 2013). Consequently, one can stipulate that, if fastest growth rate should be the norm and individual fitness is a decreasing function of developmental time (Roff 1980; Ricklefs 2010), growth remains a flexible life history trait that can change quickly both up and down in response to environmental variations (i.e. such as resources availability and foraging risks) with potential costs (Dmitriew 2011).

Free access to food allows organisms to grow bigger and faster. When resources decline in quantity and/or quality, individuals grow slower and achieve smaller size at maturity (Morey & Reznick 2000; Day & Rowe 2002). However, even if animals have unlimited access to resources their growth rates is lower than what is physiologically possible. This suggests that intrinsic factors also constrain growth rate, suggesting that rapid growth carries inevitable physiological and ultimately life-history costs (Metcalf & Monaghan 2001; Metcalfe & Monaghan 2003). Ultimately, the realised growth rate will be the result of a compromise between the advantages that rapid growth procures and the short and long term costs it entails.

In order to identify the nature of the costs associated to growth rate plasticity, previous studies have been conducted using experimental approaches, usually through the

modification of early developmental conditions. Experimental designs that have been used either manipulate the quantity and/or quality of nutrition, the environmental growth conditions through temperature (Lee, Monaghan & Metcalfe 2013) or brood/pup size manipulation (Alonso-Alvarez *et al.* 2007). For instance, if food quality or availability is decreased during growth, animals tend to grow slower, to reach maturity later and to display a smaller size at adulthood, which might lead to short term costs such as those related to young increased predation risks (Dmitriew 2011). On the other hand, sustaining rapid growth rates may lead to the same kind of ecological risks due to the high adult feeding activity (predation), but more importantly also to hidden intrinsic deleterious effects that may appear on a more long term scale (Dmitriew 2011). Those adverse impacts on young phenotype have particularly been highlighted by the study of growth compensation (Metcalfe & Monaghan 2001; Langley-Evans 2006) showing that a faster-than-normal growth rate that takes place after an initial growth stunt allows the young organism to catch-up with standard adult body size (Bize, Metcalfe & Roulin 2006), but at the expense of a wide array of metabolic disfunctionings. For instance, glucose regulation (Ozanne & Hales 2002; Bateson *et al.* 2004), locomotive performances (Álvarez & Metcalfe 2005; Criscuolo *et al.* 2011) or decreased resistance to fasting (Gotthard, Nylin & Wiklund 1994), have been found to be impaired by high growth rates in early life, all of them being suspected to participate to the decrease of individual fitness (Metcalfe & Monaghan 2003; Dmitriew 2011). These short and long terms costs are the main reason invoked for the ubiquitous observation that growth rate is naturally constrained to sub-maximal levels in most of the species despite evident benefits to reach adult size as fast as possible.

An important step has been just crossed by Lee and colleagues (Lee, Monaghan & Metcalfe 2013), which demonstrated that the alteration of the growth patterns of juvenile three-spined sticklebacks *Gasterosteus aculeatus* led to an increased life expectancy when growth was previously slowed-down, while inducing a reduction of lifespan when growth was accelerated. This clear demonstration of the growth-longevity trade-off completed previous correlative data that suggested a link between early life conditions and senescence (Nussey *et al.* 2009) and it re-launches our interest in the mechanisms by which growth may alter longevity. Besides the ultimate consequences of fast growth presented above, several proximate causes of such a reduced-lifespan might be involved (Metcalfe & Monaghan

2003). Among them, one emerging hypothesis is that growth impacts adult survival and/or longevity through a shared, endpoint, ageing mechanism: telomere erosion (Allsopp *et al.* 1992; Jennings *et al.* 1999). Telomeres are highly conserved non coding repetitive DNA sequences located at the end of linear chromosomes which hide the chromosome ends as being recognised as double stranded breaks (Blackburn 1991). Telomeric DNA is progressively lost because of the inability of telomeres to be completely replicated by DNA polymerase, progressive telomere shortening occurs over cell divisions. Once reaching a lower critical size, telomere signalisation pathway induces cell division arrest and/or cell senescence (Harley, Futcher & Greider 1990; Blackburn 1991). Telomere loss is particularly important during development (Jennings *et al.* 1999; Hall *et al.* 2004), a phenomenon likely to be due to the convergence of the high rate of cell division and of a potentially high energy metabolism. In fact, telomere dynamics can be viewed as a balance of pro- and anti-erosion factors, and telomere loss is further exacerbated by oxidative stress (Von Zglinicki 2000) which comes from the imbalance between the production of reactive oxygen species (ROS) and the antioxidant capacity (Finkel & Holbrook 2000). In a simplified view, production of ROS is considered as a positive function of energy expenditure of the organism (Beckman & Ames 1998) and potentially of growth rate as suggested by several studies (Alonso-Alvarez *et al.* 2006; De Block & Stoks 2008; Nussey *et al.* 2009). However, there are anti-erosion factors, such as telomerase activity (mainly active in germinal and stem cells) and shelterin protein complex stability that favour telomere maintenance (Blackburn 2000; De Lange 2009) and may explain partly the high variability in telomere length among aged-match individuals (Hall *et al.* 2004).

So far, experimental studies yielded data indicating that rapid growth rates have a negative influence on telomere length. Manipulation of maternal nutrition in rats showed that accelerated postnatal growth of pups was associated with shorter telomeres at adulthood (Jennings *et al.* 1999; Tarry-Adkins *et al.* 2008; Tarry-Adkins *et al.* 2009; Tarry-Adkins *et al.* 2013). In wild king penguin chicks (Geiger *et al.* 2012), telomere shortening was related to growth trajectories, with small chicks that grew faster displaying accelerated telomere loss. Such telomere loss seems actually to be related to a higher oxidative challenge, faster growth being linked to greater levels of cell oxidative stress (Merry 2002). At the individual level, increased susceptibility to oxidative damage revealed also to be a potential cost of

accelerated somatic growth in zebra finches (Alonso-Alvarez *et al.* 2007). Even more recently, oxidative stress that derives from stressful event in early life was also found to accelerate telomere loss (Hausmann *et al.* 2011; Blackburn & Epel 2012). This latter report actually rises the point that part of the deleterious effect of growth on the adult organism ageing rate may be due to early-stress (i.e. growth environmental conditions) rather than a consequence of growth rate *per se*. The question that remains is to determine whether the effects on telomere dynamics are due to growth rates (i.e. intrinsic factor) or to growth environmental conditions (i.e extrinsic factors).

Although the trade-off between growth and longevity has been well documented, links between growth and telomere loss were mainly studied in the case of growth compensation. We still need an experimental test on whether rapid growth rate actually modify telomere loss during the growth period, and whether such a modification is likely to be persistent with age. In fact, the effects of a bad start and of the following growth acceleration have rarely been disentangled (Metcalf & Monaghan 2001). To clarify this point, we experimentally modify growth conditions by manipulating brood size in zebra finches (*Taeniopygia guttata*), thereby creating control broods (non-manipulated number of nestlings), enlarged broods by adding two nestlings (stressful growth conditions due to altered individual nutrition and/or to a stressful social environment) and reduced broods by retiring two nestlings (improved growing conditions).

Using telomere dynamic comparisons between clutches, we investigated if rapid growth (chicks in reduced broods) is effectively done at the cost of a short- (end of growth) and long-term increase (at adulthood) of oxidative damage and telomere loss. By investigating in parallel how the body maintenance capacity of chicks (i.e. antioxidant capacity and flying performances) is affected by the manipulation, we checked also at different time-scales whether initial stressful growth conditions (enlarged clutches) trigger an enhanced telomere shortening while chicks are not expected to grow at a fast rate, and whether this is concomitant or not of a worse body maintenance status.

## Material and methods

### *Experimental design*

The present study was conducted on a captive population of zebra finches. Twenty-five pairs (randomly formed) were placed in breeding cages (0.57 x 0.31 x 0.39 m) with food (a commercial mix of seeds for exotic birds enriched with vitamins and eggs) and water *ad libitum*. Nest boxes and straw were provided for breeding. The cages were put in a room with a constant temperature of 24°C ( $\pm 1^\circ\text{C}$ ) and light conditions were 13L : 11 D. The brood size was manipulated in order to create three groups: 9 enlarged broods (30 chicks), 9 reduced broods (15 chicks), and 7 control broods (25 chicks). Chicks, from 1 to 3 days old, were randomly cross-fostered to form enlarged (2 chicks added, mean clutch size 4.8,  $\pm 0.75$ ), reduced (2 chicks removed, 1.8  $\pm 0.64$ ) and control broods (number of chicks unchanged, 2.8  $\pm 0.64$ ). Pre-manipulated clutch size did not differ between groups ( $p=0.078$ ). Birds were separated from their parents when the younger chick from the brood was 35 days old. Afterwards, chicks were released into a large aviary, and they were followed individually up to one year after treatment to evaluate the long term effects of manipulated growth conditions on chick health, flight performance and survival.

Blood samples were collected from the brachial vein of the chicks during the growth period (i.e. 30 $\mu\text{L}$  at 10 days and 30 days), the same individuals being re-sampled when still alive one year after treatment (i.e. 50 $\mu\text{L}$  at 360 days) (14 individuals in the enlarged group, 20 individuals in the control group, 13 individuals in the reduced group). Roughly, one third of the birds disappeared within a year, which is a mortality rate comparable to what was previously observed in the same species (Heidinger *et al.* 2012).

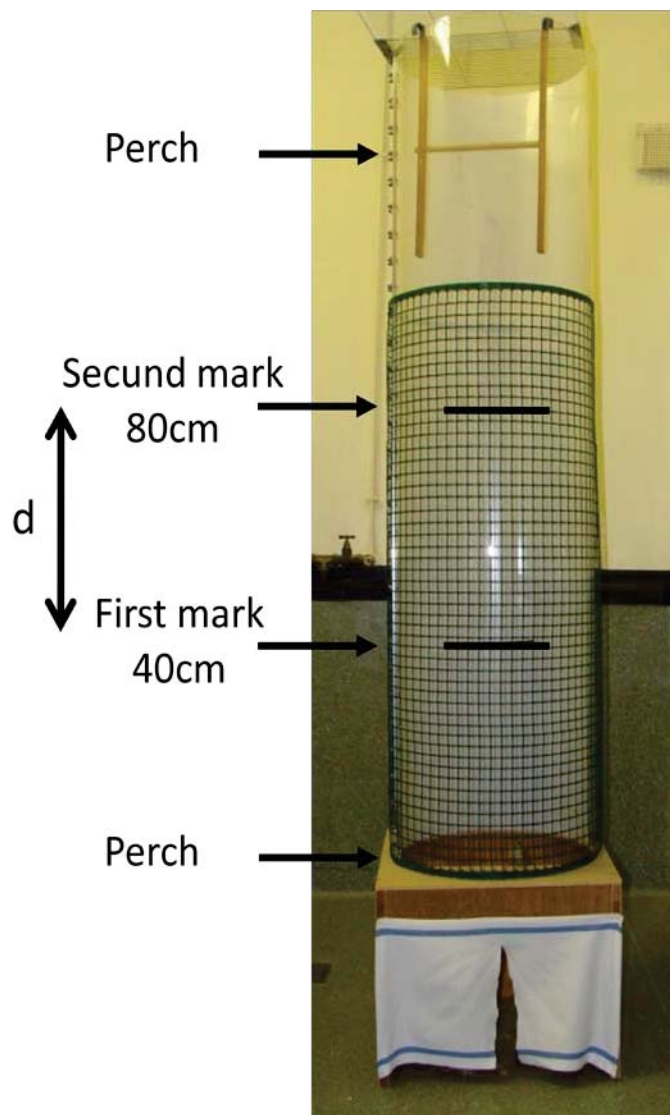
Body mass growth trajectory was assessed by weighting the chicks every day from hatching until they attained 30 days old (end of the growth period). Birds were also weighed at the time of the flight performance measurements (at 45 days and 360 days).

### *Flight performance measurements*

Flight performances, which are used as a proxy for individual maintenance (Lindhe Norberg 2002), were first measured when birds were 45 and 360 days old. Flight performance was measured using vertical take-off when alarmed. To test flight ability we



used a similar set up than the one which was used by two studies (Kullberg, Metcalfe and Houston (2002); Criscuolo *et al.* (2011)) (Fig 1). Measurements were made in a vertical plastic tube. The week before the final experimental trial, birds were trained twice in the same manner as during the experimental trial. Each bird was released on a perch situated at 20 cm from the ground, at the base of a transparent vertical plastic tube. At the top of the flight tube (120 cm from the first perch), there was a perch where the bird could be collected after each flight. For the training trial as well as for the experimental trial, birds were released five times in the tube and were allowed to rest for 30 seconds between each flight. During the experimental trial, all flights were video recorded with a video camera (Casio Highspeed EX-H100, 420 frames per second). To determine flight speed, we analysed the videos and calculated the time it took the bird to cover a distance of 40 cm. This measure was made by counting the number of video frames (each frame covering 0.002 s) between two marks on the tube at 40cm and 80cm height. Flight speed was calculated with this formula:  $V = d / ((F - I) / 420)$  where **d** is the distance between the two marks (40cm), **I** is the number of frames when the bird has crossed the first mark, and **F** the number of frames when the bird has crossed the second mark. All videos were analysed with Avidemux 2.5<sup>®</sup>. We used the fastest of the five flights as a measurement of the bird's escape flight ability (Kullberg, Metcalfe & Houston 2002).



**Figure 1:** *Experimental set up to assess flight performances.*

### ***Oxidative stress measurements***

Oxidative stress measurements were done during and at the end of the growth period (10 and 30 days), as well as a year after the experiment (360 days) and evaluated in two steps: antioxidant levels and oxidative damage. Antioxidant levels were assessed in plasma samples (stored at  $-20^{\circ}\text{C}$  until analysis), with the OXY-ADSORBENT tests (Diacron International, Grosseto, Italy), as previously described in birds (Costantini, Cardinale & Carere 2007; Costantini, Dell'Araccia & Lipp 2008; Beaulieu *et al.* 2010; Beaulieu *et al.* 2011) and mammals (Stier *et al.* 2012). The OXY-ADSORBENT test measures the total antioxidant capacity of the plasma using a colorimetric determination to quantify the ability of the

antioxidant barrier to cope with the oxidant action of hypochlorous acid (HOCl). Measurements are expressed as  $\mu\text{M HOCl/mL}$  neutralised. For the OXY- ADSORBENT assay mean intraplate coefficient of variation was of 2.2% and mean interplate coefficient of variation was of 7%.

We determined oxidative damage by measuring 8-hydroxy-2-deoxy Guanosine (8-OHdG), using the 8-OHdG EIA kit (StressMarq Biosciences Inc., Victoria, BC Canada). DNA was first extracted from blood cells (with DNeasy Blood and Tissue kit Qiagen) and then genomic DNA was totally digested following the instructions described in (Quinlivan & Gregory III 2008).

8-OHdG is the by-product of oxidative damage on DNA due to the deleterious effects of reactive oxygen species (ROS) and increased levels of this marker have been associated with the ageing process (Shen & Abate-Shen 2007). DNA damage measurements are expressed in  $\text{pg/mL}$ . Mean coefficient of intraplate variation was of 3.4%.

### ***Telomere length measurement***

Telomere length was measured at 10 days, 30 days, and a year after the experiment. Telomere length was measured on DNA extracted from red blood cells (stored three months at  $-20^{\circ}\text{C}$  until analysis), which are nucleated in birds, using DNeasy Blood and Tissue kit (Qiagen). Telomere length was assessed by the quantitative real-time amplification (qPCR) procedure (Cawthon 2002) adapted to zebra finches (Crisuolo *et al.* 2009). Relative telomere length is expressed as the ratio (T/S) of telomere repeat copy number (T) to a control single gene copy number (S). We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a non-variable control gene (Smith, Turbill & Penn 2011). Forward and reverse primers for the GAPDH gene were 5'-AACCAGCCAAGTACGATGACAT-3' and 5'-CCATCAGCAGCAGCCTTCA-3' respectively. Telomere primers were: Tel1b (5'-CGTTTTGTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3') and Tel2b (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'). qPCR for both telomere and GAPDH were performed using 5 ng of DNA with sets of primers Tel1b/Tel2b (or GAPDH-F/GAPDH-R), each used at a concentration of 200 nM/200 nM, in a final volume of 10  $\mu\text{l}$  containing 5  $\mu\text{l}$  of Power SYBR Green PCR Master Mix (Applied biosystems). Telomere and GAPDH real time amplification were performed on two different plates. qPCR conditions for telomeres were 10 min at  $95^{\circ}\text{C}$  followed by 30 cycles of 1 min at  $56^{\circ}\text{C}$  and 1 min at  $95^{\circ}\text{C}$ . PCR conditions for

the GAPDH were 10 min at 95°C followed by 40 cycles of 1 min at 60°C and 1 min at 95°C. Each plate (telomere and GAPDH) included serial dilutions (10 ng, 5 ng, 2.5 ng, 1.25 ng) of DNA of the same reference bird. This was used to generate a reference curve to control for the amplifying efficiency of the qPCR (mean efficiencies for GAPDH and telomere plates were between 95 % and 105 %). Samples were run in duplicate on each plate. Samples were run on a total of 4 plates. To take into account the slight variation of efficiencies between telomere and GAPDH amplifications, we calculate relative telomere length using the method suggested by (Pfaffl 2001). The mean values were used to calculate the relative T/S ratios using the formula:  $((1 + E_{\text{telomere}})^{\Delta C_{t \text{ telomere}} (\text{control} - \text{sample})} / (1 + E_{\text{GAPDH}})^{\Delta C_{t \text{ GAPDH}} (\text{control} - \text{sample})})$ .

Mean intraplate coefficient of variation was 1.1% for the Ct values of the GAPDH assays and 3.0% for the Ct values of the telomere assays. Interplate coefficient of variation (calculated on four samples repeated on the different plates) was of 1.6% for the Ct values the GAPDH assays, 2.9% for the Ct values of the telomere assays and 8.5 % for the relative T/S ratios. On each plate, a negative control (water) and a melting curve (primer-dimer) were run to check for the absence of non-specific amplification.

### **Statistical analysis**

#### *Effects on nestlings' development, fledging age and flight performances*

General linear mixed models with time (day 10, day 20, day 30, day 40, day 60, and day 360) as a repeated variable, nest identity as a random factor, and brood manipulation, sex as well as the interaction between the brood manipulation and the time as fixed factors, were used to analyse body mass changes during growth.

Body mass gain was also analysed using this same linear mixed models, with time period (0-20 days vs. 20-40 days vs. 40-60 days) as a repeated variable, nest identity as a random factor, and sex, brood group, as well as the interaction between the brood group and the time period as fixed factors body mass for each period of time as a covariate. Initial body mass of each time period was added to the model to control for any gap of growth stages between individuals (i.e. effect of tissue maturation on growth rate (Ricklefs 1979)). The first time point corresponds to the period when nestlings are most dependent on the parents. The second time point corresponds to the period when they are less dependent since they

fledged at approximately 20 days but are still in the parental cage and partially fed by their parents. The last time point corresponds to the period when the nestlings are totally independent as they were separated from their parents around 35 days old.

Fledging dates (in days) were analysed using linear mixed models with nest identity as a random factor, and sex, brood group as fixed factors. For flight performances analysis, we used linear mixed models with time period (45 days vs. 360 days) as a repeated variable, nest identity as a random factor, body mass as covariate, and sex, brood group, as well as the interaction between the brood group and the time period as factors in the model. Post hoc analyses were conducted using LSD tests.

#### *Effects on oxidative stress, telomere length and survival*

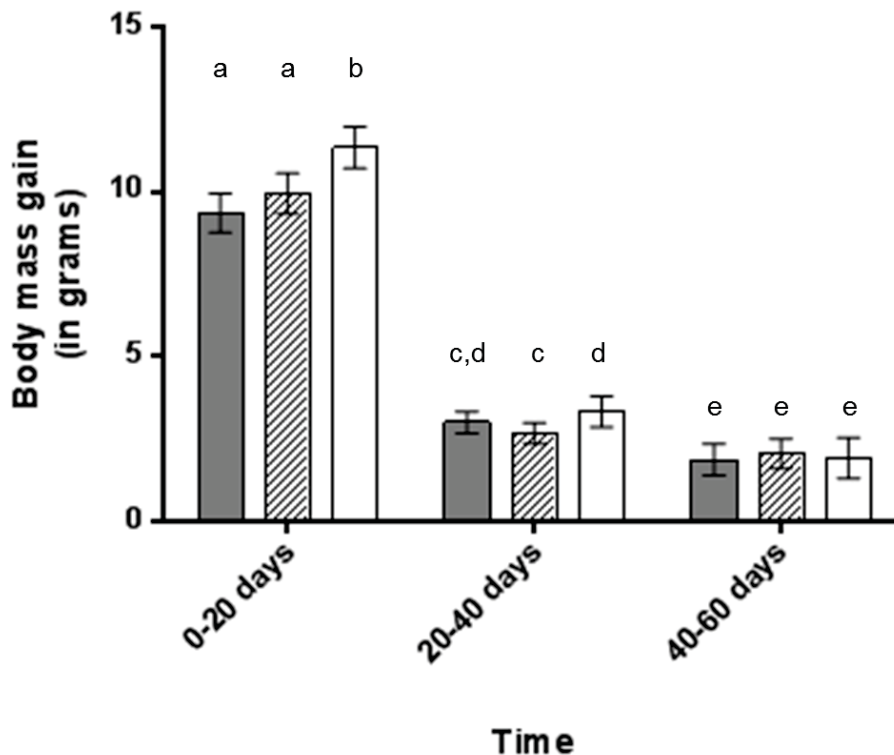
Antioxidant levels, oxidative damage and telomere length were analysed with linear mixed models with time period (10 days vs. 30 days vs. 360 days) as a repeated variable, nest identity as a random factor, and sex, brood group as well as the interaction between the brood group and the time period as fixed factors. Given the relationships that exist among these variables, when testing for differences in oxidative damage among treatment groups, antioxidant levels were used as a covariate, while when testing for the impact on the telomere length, DNA oxidative damage levels were used as a covariate. Post hoc analyses were conducted using LSD tests. Determinants of chick survival a year after the experiment between groups (control vs. enlarged vs. reduced) were evaluated using a Generalized Linear Modelling procedure (GzLM) with a logistic binary distribution of the dependent variable (survival or not). The experimental group and the sex were entered as fixed factors and telomere length, DNA oxidative damage and antioxidant levels at 30 days were entered as covariates. All statistical analyses were performed using SPSS v. 18.0. Means are indicated  $\pm$  SE.

## Results

### *Effects on nestlings' development, fledging age and flight performances*

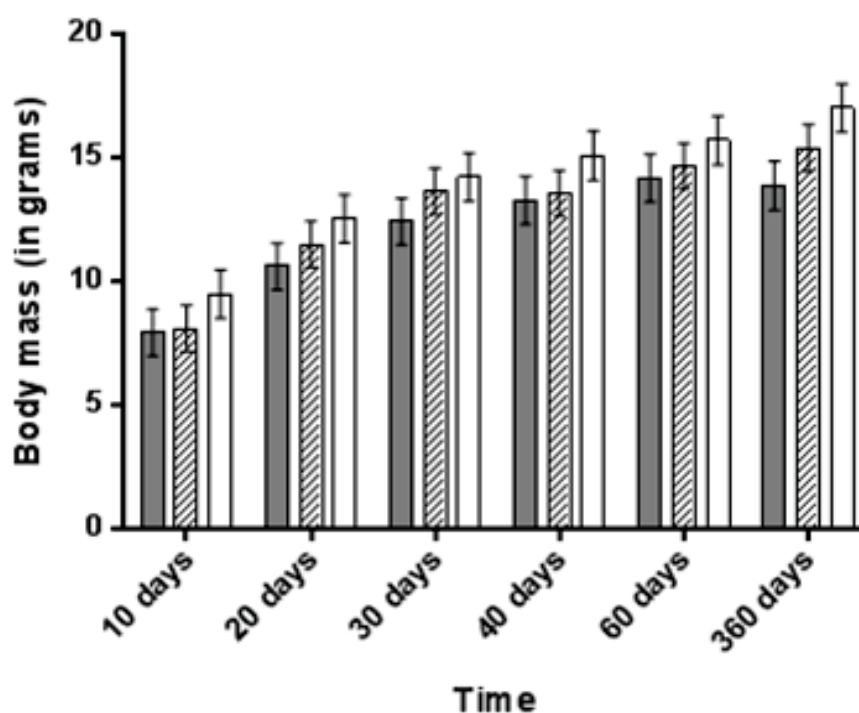
Body masses at cross fostering were not different among experimental groups or sex ( $p=0.203$  and  $p=0.279$ , respectively).

The interaction between the time period and brood size manipulation significantly affected body mass gain for both males and females (Fig 2, Table1). During the first period of growth (0 to 20 days), nestlings in enlarged broods grew more slowly than those in the reduced group ( $p<0.001$ ), but were not significantly different in terms of growth rates than the nestlings from the control group ( $p=0.289$ ). During the 20 to 40 days, nestlings from the enlarged group did not differ in their rate of growth from those from the reduced and the control groups. During the 40 to 60 days time period, growth rates were not significantly different for all experimental groups.



**Figure 2:** Body mass gain for both sexes in the three experimentally enlarged (grey), reduced (white) and control broods (hatched) during different time intervals (0-20 days vs. 20-40 days vs. 40-60 days). Sample size up until 60 days: enlarged ( $n=30$ ), reduced ( $n=15$ ) and control broods ( $n=25$ ). Bars represent  $\pm$  SE.

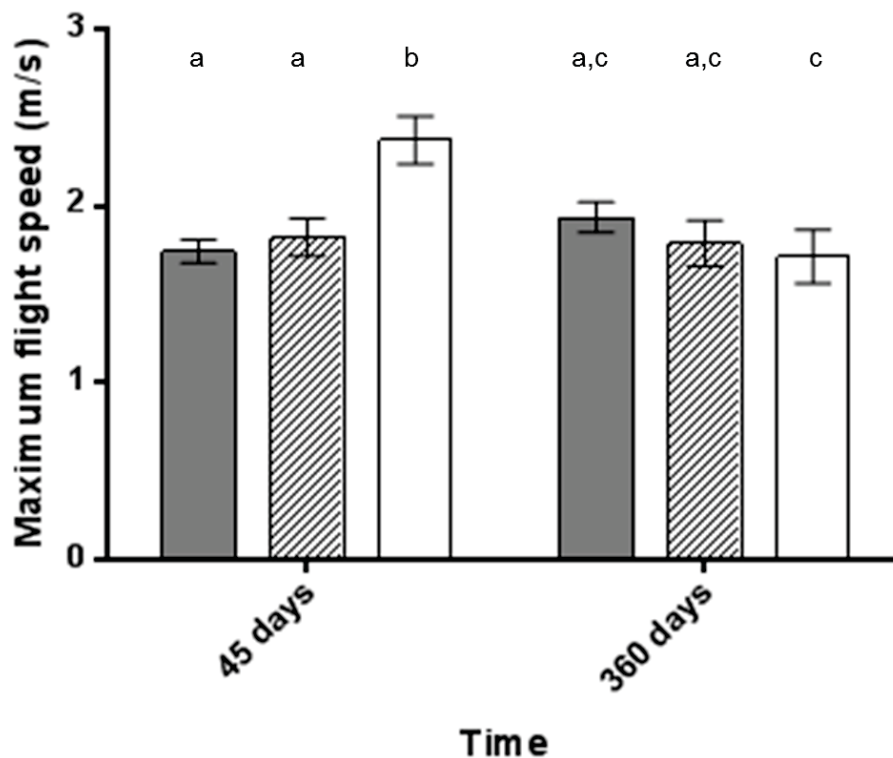
During the growth period (day 10, day 20 and day 30) and afterwards (day 40, day 60 and day 360), brood size manipulation significantly affected body mass for both males and females (Table 1, Fig 3). Nestlings in enlarged group and control group were not significantly different in terms of body mass ( $p=0.101$ ) but were lighter than those reared in the reduced group ( $p<0.001$ ).



**Figure 3:** Body mass for both sexes in the three experimental groups: enlarged broods (grey), reduced broods (white), and control broods (hatched) during different time periods (day 10, day 20, day 30, day 40, day 60, and day 360). Sample size up until 60 days: enlarged ( $n=30$ ), reduced ( $n=15$ ) and control broods ( $n=25$ ); sample size at day 360: enlarged ( $n=14$ ), reduced ( $n=13$ ) and control broods ( $n=20$ ). Bars represent  $\pm$  SE.

Brood size manipulation had significant effects on chick fledging dates (mean age in days at fledging: enlarged broods:  $21.3 \pm 0.4$ , reduced broods:  $17.0 \pm 0.6$ , control broods:  $18.4 \pm 0.7$ ) (Table 1). Chicks from the enlarged group fledged later than those from the control and the reduced groups ( $p=0.001$  and  $p<0.001$  respectively). Similarly, chicks from the control group fledged later than those from the reduced group ( $p=0.003$ ).

Variations in flight performances of males and females were significantly explained by the interaction between brood size manipulation and the time period (Fig 4, Table 1), Just after the fledging time (45 days), birds from the enlarged group exhibited lower flight performances than the ones from the reduced group ( $p < 0.001$ , no significant differences were found with the control group,  $p = 0.501$ ). When birds were one year old, this difference disappeared, as all groups exhibit the same flight performances (i.e. LSD pairwise comparisons, all  $p > 0.1$ ).

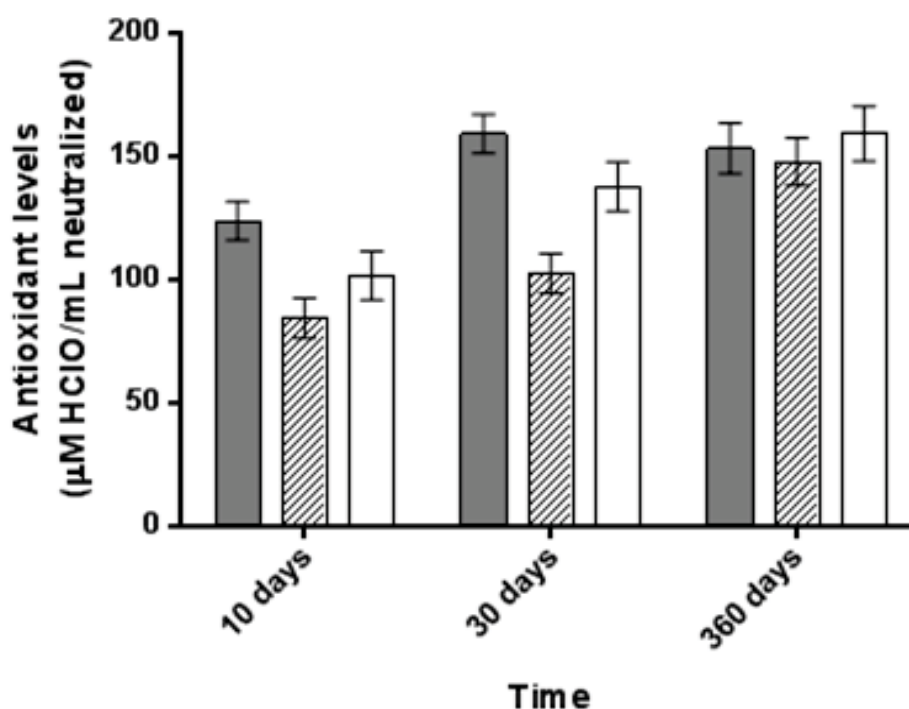


**Figure 4:** Flight performances expressed in maximum flight speed in the three experimental groups: enlarged broods (grey), reduced broods (white), and control broods (hatched) during different time periods (45 days vs. 360 days). Sample size at day 45: enlarged ( $n=30$ ), reduced ( $n=15$ ) and control broods ( $n=25$ ); sample size at day 360: enlarged ( $n=14$ ), reduced ( $n=13$ ) and control broods ( $n=20$ ). Bars represent  $\pm$  SE.



**Effects on oxidative stress, telomere length and survival**

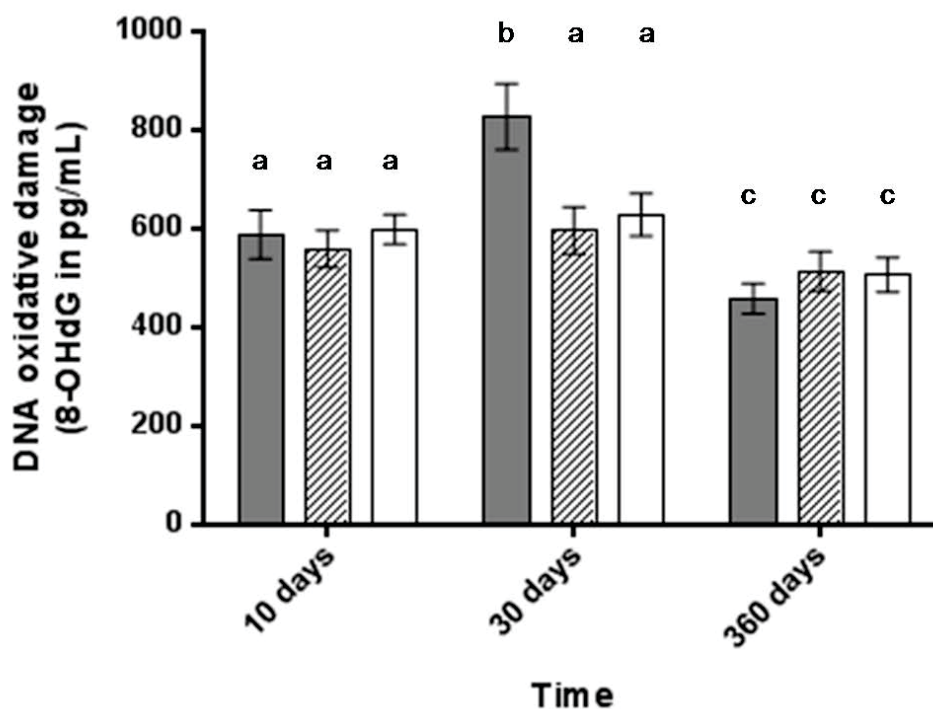
Brood size manipulation as well as the time period (10 days vs. 30 days vs. 360 days) had significant effects on antioxidant levels for both males and females (Fig 5, Table 1). All experimental groups exhibited an increase in antioxidant defences over the growth period. Independently of the time period, chicks raised into enlarged brood size displayed higher levels of antioxidant levels compared to the controls ( $p < 0.001$ ) but not significantly different when compared to the reduced group (Fig 5,  $p = 0.112$ ).



**Figure 5:** Antioxidant levels (measured as  $\mu\text{M HOCl/mL}$  neutralised) in the three experimental groups: enlarged broods (grey), reduced broods (white), and control broods (hatched) during different time periods (10 days vs. 30 days vs. 360 days). Sample size at day 10 and 30: enlarged ( $n=30$ ), reduced ( $n=15$ ) and control broods ( $n=25$ ); sample size at day 360: enlarged ( $n=14$ ), reduced ( $n=13$ ) and control broods ( $n=20$ ). Bars represent  $\pm$  SE.

The interaction between brood size manipulation and the time period significantly affected oxidative damage on DNA (Fig 6, Table 1). At 10 days, all experimental groups exhibited the same levels of oxidative damage. However, at the end of the growth period (30 days), birds

from the enlarged broods group displayed higher levels of oxidative damage compared to those from the control and reduced groups (Fig 6,  $p=0.09$  and  $p=0.011$  respectively). A year after the experiment, all groups displayed the same levels of oxidative damage on DNA.

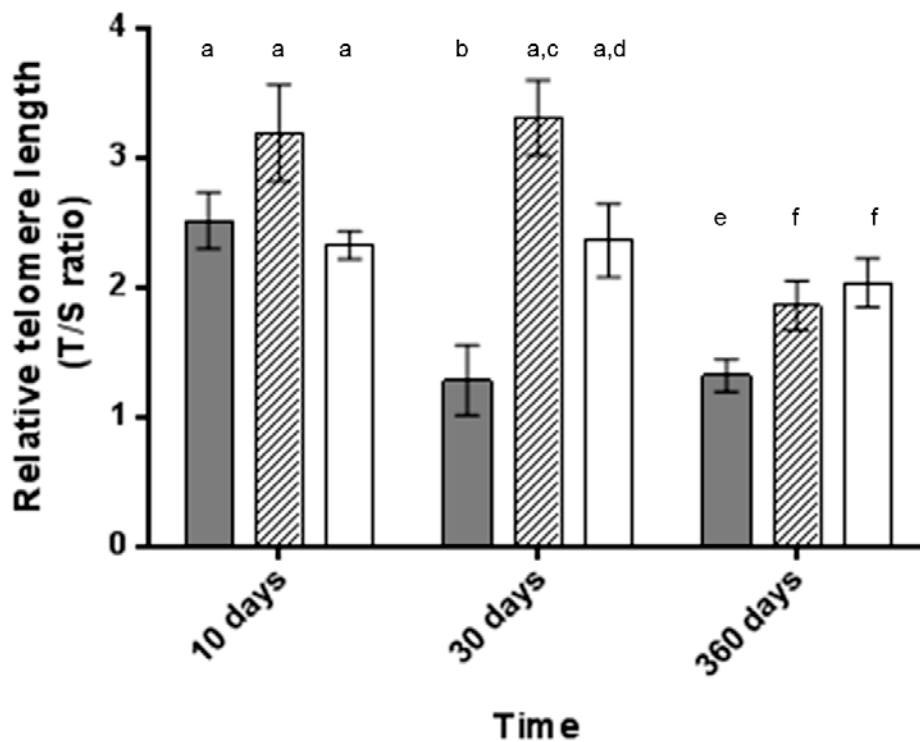


**Figure 6:** Oxidative damage on DNA (measured as 8-OHdG in pg/mL) in the three experimental groups: enlarged broods (grey), reduced broods (white), and control broods (hatched) during different time periods (10 days vs. 30 days vs. 360 days). Sample size at day 10 and 30: enlarged ( $n=30$ ), reduced ( $n=15$ ) and control broods ( $n=25$ ); sample size at day 360: enlarged ( $n=14$ ), reduced ( $n=13$ ) and control broods ( $n=20$ ). Bars represent  $\pm$  SE.

Brood size manipulation, the time period (i.e. 10 days vs. 30 days vs. a year after the experiment), as well as the interaction between brood size manipulation and the time period had significant effects on telomere lengths (Table 1). All birds presented a progressive decrease in their telomere length over one year (Fig 7). However, telomere loss was not observed in all experimental groups over the growth period (during the first 30 days), only chicks from the enlarged group exhibiting a significant decrease in telomere length during that period. Compared to chicks in the control and reduced groups, chicks raised in enlarged brood size were the ones that displayed the most important telomere loss at the end of the

growth period (30 days) and one year after, while there was no significant difference between the rates at which the control and the reduced groups lost their telomere repeats over the same time-period.

The damaging effects of brood enlargement on telomere length persisted a year after and the enlarged group displayed the shortest telomeres (LSD post hoc comparisons,  $P < 0.001$  for both groups respectively, Fig 7). Conversely, at day 360, birds from the control and reduced groups displayed no significant differences in their telomere lengths (LSD post hoc comparisons,  $P = 0.955$ , Fig 7). Offspring survival over the first year after manipulation did not differ between experimental groups (sex effect  $p = 0.910$ , brood size manipulation effect  $p = 0.985$ ). Telomere length, DNA oxidative damage and antioxidant levels at 30 days were not linked to survival either (respectively  $p = 0.462$ ,  $p = 0.275$  and  $p = 0.781$ ).



**Figure 7:** Relative telomere length (T/S ratio) in the three experimental groups: enlarged broods (grey), reduced broods (white), and control broods (hatched) during different time periods (10 days vs. 30 days vs. 360 days). Sample size at day 10 and 30: enlarged ( $n = 30$ ), reduced ( $n = 15$ ) and control broods ( $n = 25$ ); sample size at day 360: enlarged ( $n = 14$ ), reduced ( $n = 13$ ) and control broods ( $n = 20$ ). Bars represent  $\pm$  SE.

Variable	D.F	F	P
<b>(a) Body mass gain</b>			
Time (0-20 vs. 20-40 vs. 40-60 days)	2	29.4	<0.001
Sex	1	0.47	0.493
Brood manipulation	2	4.89	0.022
Time period x brood manipulation	4	3.15	0.016
Initial body mass	1	11.17	0.001
<b>(b) Body mass</b>			
Time (day 10, day 20, day 30, day 40, day 60, day 360)	5	133.99	<0.001
Sex	1	0.53	0.467
Brood manipulation	2	10.76	<0.001
Time x brood manipulation	10	0.86	0.574
<b>(c) Fledging date (in days)</b>			
Sex	1	1.32	0.255
Brood manipulation	2	18.27	<0.001
<b>(d) Flight performances</b>			
Time period (45 vs. 360 days)	1	0.77	0.383
Sex	1	2.23	0.142
Brood manipulation	2	2.35	0.116
Time period x brood manipulation	2	5.17	0.009
Body mass	1	14.26	<0.001
<b>(e) Antioxidant levels</b>			
Time period (10 vs. 30 vs. 360 days)	2	21.7	<0.001
Sex	1	3.28	0.072
Brood manipulation	2	11.58	<0.001
Time period x brood manipulation	4	2.14	0.079
<b>(f) DNA oxidative damage</b>			
Time period (10 vs. 30 vs. 360 days)	2	22.34	<0.001

Sex	1	0.34	0.56
Brood manipulation	2	1.82	0.179
Time period x brood manipulation	4	5.81	<0.001
Antioxidant levels	1	10.32	0.002

**(g) Telomere length**

Time period (10 vs. 30 vs. 360 days)	2	5.71	0.001
Sex	1	0.19	0.791
Brood manipulation	2	5.3	0.007
Time period x brood manipulation	4	2.48	0.008
DNA oxidative damage	1	7.93	0.003

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**Table 1:** Results of general linear mixed models on body mass gain, body mass, fledging date (in days), flight performances, antioxidant levels, DNA oxidative damage, and telomere length.

## Discussion

Our experimental manipulation of brood size at hatching in a captive population of zebra finches shows that chicks from reduced broods were growing at a faster rate and attained a heavier body mass, but without any apparent deleterious impact on plasmatic oxidative markers and telomere length, when compared to chicks from control broods. In contrast, although chicks from enlarged broods were growing at the same rates and exhibiting similar body masses than control chicks, they suffered from greater oxidative damage and telomere loss during growth than control broods, this latter effect being persistent at adulthood.

### ***Impact of brood size manipulation on nestlings' development and flight performances***

The brood size manipulation was successful in creating differences in growth trajectories. Chicks raised in reduced broods showed higher growth rates (until 20 days) and were heavier whatever the time compared to control and enlarged groups. Chicks reared in enlarged and control broods had similar body mass. These results are in accordance with previous literature relating the effects of brood size reduction on growth patterns (Dijkstra *et al.* 1990; De Kogel 1997; Pettifor, Perrins & McCleery 2001) but differ from some others where reduced and control chicks presented no differences in their body masses (Dijkstra *et al.* 1990). This actually raises two questions concerning the brood manipulation experimental design. The first one concerns the nature of the information that can be obtained from the control-reduced-enlarged two-by-two group comparisons, as in some studies the un-manipulated group is absent (e.g. (Alonso-Alvarez *et al.* 2007)). Secondly, it is not always clear whether chicks raised in reduced and enlarged clutches really differ in their body mass gain trajectories compared to control ones. For instance, since chicks reared in enlarged clutches often suffer from sub-optimal phenotype, it underlines that parameters other than growth rate participate to the deleterious impacts of growth variability. Therefore, costs hidden to the unique body mass measurements probably exist. Growth compensation that can follow an initial growth delay has been suggested to carry most of the costs usually associated to growth variability (De Kogel 1997; Metcalfe & Monaghan 2001; Alonso-Alvarez *et al.* 2006; Alonso-Alvarez *et al.* 2007). On the other hand, the degree of the initial growth stunt or its timing (the earlier in development being more

detrimental (Lindström 1999; Metcalfe & Monaghan 2001) may also be implicated. Given that in our study no detectable catch-up trajectories could have been characterised (enlarged brood chicks being smaller even when 1 year-old), our results rather suggest that the latter explanation should be mainly responsible of the observed patterns in the physiological consequences. This is further supported by the delay in the fledging date of chicks from the enlarged group, which stayed longer in the nest compared to those from the control and reduced groups. One hypothesis could be that compensatory growth response is, in this species, partially governed by parental ability to increase feeding rate, which was maybe over the capacity of our breeders. In addition, because chicks from the reduced clutch did not exhibit a particular phenotype, it also raises the possibility that under certain conditions, rapid growth *per se* does not obligatory carry substantial costs. Therefore, measuring additional physiological variables (*i.e.* oxidative stress and telomere loss) during growth may provide further information on how growth rate on one hand, and the developmental conditions faced by chicks on the other hand, could interact to ultimately produce a more complicated picture.

***Why could brood size enlargement have induced deleterious effects on ageing parameters?***

Brood enlargement is likely to have decreased food availability of chicks, parents having to feed more chicks than they originally intended to raise. For instance a neonatal energy stress is known to disturb the growth-body maintenance trade-off (Metcalfe & Monaghan 2001). It may also alter the fine-tuned processes that regulated the progressive development and maturation of the different organs specific to each species (Ricklefs 1979; Bize, Metcalfe & Roulin 2006). This is likely to lead to different body conditions at fledging and adulthood and to an altered functioning of key tissues, such as muscle, thereby partially explaining the reduced flight ability of enlarged brood chicks (Crisuolo *et al.* 2011). However, as enlarged birds were doing as well as control ones, it is rather likely that our differences in flight performances are coming from a better ability of flight escape in birds reared in reduced broods. In addition, long term flight performances were not affected by the experimental treatment probably because individuals were under mild-challenging conditions (Crisuolo *et al.* 2011).

The observed detrimental effects of growth on ageing might come from several other alternatives, like a lack in specific nutrients (Arnold *et al.* 2007), a restructuring of the growth hormone-insulin like growth hormone axis (Carter, Ramsey & Sonntag 2002), or an elevation in corticosterone levels. Indeed, chronic exposure to high levels of stress is known to increase oxidative damage (Costantini, Fanfani & Dell'Omo 2008; Zafir & Banu 2009). Chicks raised in enlarged brood were certainly facing food shortage and increased intra-brood competition. As a consequence, they may have responded by increasing their begging activity, a process that has previously been related to enhanced corticosterone levels (Kitaysky *et al.* 2003; Noguera *et al.* 2010). This may in return have triggered oxidative stress and telomere loss which have been recently shown to be increased following corticosterone exposure in early life (Hausmann *et al.* 2011), but the ways through which corticosterone alters the oxidative balance remain elusive (Hausmann & Marchetto 2010). When investigating effects on oxidative stress it is best to consider both aspects of the imbalance, antioxidant levels as well as oxidative damage which can be used as a proxy for ROS production, as measurements of only one of these parameters can give a misleading picture (Monaghan, Metcalfe & Torres 2009). Oxidative stress is due to the imbalance between ROS production (mainly by mitochondria) and the antioxidant networks (mitochondrial, cellular and systemic) (Halliwell & Gutteridge 1999). Interestingly in our study, chicks that grew up in enlarged broods exhibited higher antioxidant levels than control broods (but no different from reduced broods) and more DNA oxidative damage at the end of growth period than control and reduced broods. These results are in concordance with the findings of (Alonso-Alvarez *et al.* 2006; Alonso-Alvarez *et al.* 2007). Therefore, this elevated DNA oxidative damage displayed by chicks from enlarged broods at the end of growth (30 days) indicates that, despite an increased antioxidant barrier, these chicks were not able to compensate for an increased ROS production. This actually suggests that chicks from enlarged broods suffer mainly from mitochondrial malfunctioning, the main cell organelle responsible in ROS production (Balaban, Nemoto & Finkel 2005). This points out that growth perturbation could affect mitochondria potentially through mitochondrial membrane lipid composition and permeability or respiratory protein disfunctioning, both been associated to ROS production (Balaban, Nemoto & Finkel 2005; Hulbert *et al.* 2007).

Not only did brood size enlargement had an impact on oxidative stress, it also affected short and long term telomere dynamics. Compared to chicks in the control and reduced groups,



those raised in enlarged broods displayed a more severe telomere erosion during the growth period. Moreover, these birds were the ones with the shortest telomeres a year after the experiment. Our findings differ from Voillemot *et al.* (2012) study that shows that there was no effect of brood size manipulation on telomere length at the end of the growth period. This absence of effect in this previous study might be due to the fact that they sampled telomere length once during growth (at day 12) instead of using longitudinal measures to assess effects on the dynamic of telomere erosion. However, our results may also be interpreted as a support to Voillemot and colleagues conclusion that telomeres are not only controlled by intrinsic growth rates *per se* but may also be under the control of some extrinsic environmental factors that may get our understanding of the growth-ageing interaction more complicated. In our case, enlarged and control broods displayed the same pace of growth, but did not exhibit the same levels of oxidative stress or the same pattern of telomere erosion. This suggests that the environmental conditions in which an individual grows up might have predominant effects on ageing markers compared to growth rates *per se*.

***Why chicks reared in reduced brood size grew faster while avoiding any deleterious effects?***

Our results indicate that individuals raised in reduced broods did better in terms of growth and of short term flight performances, suggesting that they were raised in over optimal conditions compared to individuals that grew up in control and enlarged broods. Indeed, environmental stress (i.e. chicks competition, parent-offspring conflict) may be of prime importance given its impact on telomere dynamics in humans (Blackburn & Epel 2012). Yet, long term DNA oxidative damage being similar for all groups, the shorter telomeres of enlarged brood chicks at adulthood might be the consequence of a reduced capacity to repair damaged telomeres. Affecting telomerase function is likely to be an important issue. Chronic stress has been shown to increase telomere shortening (Epel *et al.* 2004; Kotrschal, Ilmonen & Penn 2007; Hatakeyama *et al.* 2008) and to lower telomerase activity (Epel *et al.* 2010). In addition, early exposure to corticosterone is associated to long term increase in stress reaction of the whole adreno-cortical system (Spencer, Evans &

Monaghan 2009). This may account for the shorter telomeres at adulthood of enlarged brood chicks (Hausmann *et al.* 2011).

Alternatively, in reduced broods, parents took care of fewer chicks than what was initially expected, thus investing more per chick and enhancing chick quality without immediate ageing costs. This idea is supported by the fact that reduced brood chicks presented an increase of their plasma antioxidant capacity at a similar level than the one recorded in chicks from the enlarged group. This means that rapid growth may have enhanced ROS production, but that good rearing conditions may have provided chicks from the reduced broods with adequate antioxidant resources (Beaulieu & Schaefer 2013). Future experiments should focus on the enzymatic and non enzymatic antioxidant buffering activities in a similar experimental design.

### ***Growth, ageing and fitness***

Our study extends our understanding of the proximate mechanisms involved in the trade-off between growth and longevity. It highlights that adverse environmental conditions during growth can come at a cost *via* increased oxidative stress and long term eroded telomeres. Previous studies conducted on laboratory rats show impairment of antioxidant functions and shorter telomeres after alteration of developmental conditions (Tarry-Adkins *et al.* 2008; Tarry-Adkins *et al.* 2009; Tarry-Adkins *et al.* 2013). However, growth acceleration following an initial growth delay often carries additional costs rather than just rapid growth (Metcalf & Monaghan 2001; Dmitriew 2011). In any case, the effects observed on telomere dynamics might be the consequence either the extrinsic growth conditions or of the intrinsic growth rates. Future studies should concentrate on distangling these two aspects. Despite their effects on oxidative stress, telomere dynamics and telomere length, rearing conditions did not modify survival rates a year after. However, following survival rate of adult zebra finches over a year is probably not long enough to highlight the impact of growth retardation on subsequent individual lifespan and fitness. It underlines that we still need to acquire more data on the accurate nature of the determinants of telomere length in early life (*i.e.* environmental stress) and on the way potentially important maintenance processes, such as telomerase activity, are affected on the long term.

Telomere length measured early in life or at adulthood has been found to predict subsequent survival and life expectancy (Cawthon *et al.* 2003; Hausmann *et al.* 2003; Bize *et al.* 2009; Heidinger *et al.* 2012). Merging cell and organismal levels of comprehension of ageing causes and consequences will certainly enable us to test whether telomere dynamics are a reliable proxy of individual fitness, as it is more and more often hinted (Monaghan & Hausmann 2006; Hausmann & Marchetto 2010; Monaghan 2010).

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## Study 5

### Increased brood size leads to persistent eroded telomeres

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## Introduction

Une des hypothèses centrales de la théorie des traits de vie est que la reproduction est une activité coûteuse qui peut entraîner des coûts à long terme, notamment sur la longévité des individus. Cependant, les mécanismes sous-jacents de ce compromis entre investissement reproducteur et longévité sont peu compris.

Le coût de la reproduction peut se diviser en deux parties. Tout d'abord, la reproduction en tant que telle, peut présenter des coûts dus aux changements des taux hormonaux et de leurs effets pléiotropiques sur l'immunité et le métabolisme. Dans un second temps, une fois que la reproduction a commencé, les parents sont supposés ajuster leur investissement reproducteur en fonction de leur condition (ou de leur qualité) ainsi que des conditions environnementales dans lesquelles ils se trouvent lors de la reproduction.

Récemment, il a été suggéré que les télomères pourraient faire partie des mécanismes sous-tendant les effets des coûts induits par l'effort reproducteur, sur la durée de vie des organismes. De plus, le stress oxydant étant un des paramètres régulant la dynamique d'érosion des télomères, il a également été identifié comme étant impliqué dans le coût de la reproduction. Bien que le lien entre le coût de la reproduction et la dynamique d'érosion des télomères ait été précédemment étudié, il reste néanmoins controversé. Pour déterminer si les télomères sont impliqués dans le compromis entre reproduction et longévité, nous avons testé si le niveau de l'effort reproducteur était susceptible d'affecter l'érosion des télomères à court et long terme. Pour répondre à cette question nous avons manipulé la taille des nichées (en créant trois groupes expérimentaux : nichées de taille augmentée, nichées contrôle, nichées de taille réduite) chez le diamant mandarin dans le but de faire varier l'investissement reproducteur, et nous en avons mesuré l'effet sur l'érosion des télomères et le stress oxydant.

## Principaux résultats

L'effort reproducteur a induit un effet significatif sur l'érosion des télomères à court et moyen terme. En effet, les parents dont la taille de la nichée a été augmentée (ajout de deux poussins par rapport à la taille de la nichée initiale) présentent une érosion télomérique plus importante à la fin de la reproduction, mais également un an après

l'épisode de reproduction. Cependant, cette accélération de l'érosion des télomères ne semble pas associée à une augmentation du stress oxydant, puisque les niveaux de dommages oxydatifs et d'antioxydants restent inchangés.

## Discussion

Plusieurs études montrent qu'une augmentation de l'effort parental peut induire des effets sur la fitness et la survie des individus. En accord avec cette hypothèse, notre étude montre qu'un évènement reproducteur coûteux est susceptible d'entraîner un raccourcissement des télomères chez les adultes à la fin de la reproduction, et que ce coût semble aussi être payé à moyen terme, puisque ces individus, un an après, présentent toujours des télomères courts comparés aux autres groupes. Dans le cas de notre étude, il semblerait cependant que le traitement expérimental n'ait pas d'effets sur le stress oxydant. Cette absence d'effet peut avoir plusieurs causes. Il est possible que cela soit dû à un problème dans le timing de la seconde prise de sang. Parallèlement, cette absence de lien conforte l'hypothèse selon laquelle pour l'instant le lien fonctionnel *in vivo* entre dynamique d'érosion des télomères et stress oxydant, serait incertain.

La régulation de la longueur des télomères passe aussi par des processus de réparation (activité télomérase). De ce fait, il se peut que dans les nichées augmentées, l'accélération de l'érosion des télomères soit due à une réduction de cette activité télomérase. Cependant, de plus amples études expérimentales seront nécessaires pour mettre en évidence ce lien. Les individus des groupes contrôle et réduit, ne présentent pas de perte télomérique. Ceci suggère que les organismes ajustent leur effort reproducteur en fonction de leur état physiologique afin de limiter les coûts potentiels de la reproduction.

De plus, dans la mesure où la longueur des télomères est associée à la survie des individus, les résultats de cette étude indiquent que la dynamique d'érosion des télomères semble être un bon repère pour expliquer le lien fonctionnel entre reproduction et longévité.



## Abstract

Costs of reproduction can be divided in mandatory costs coming from physiological, metabolic and anatomical changes required to sustain reproduction itself, and in investment-dependent costs that are likely to become apparent when reproductive efforts are exceeding what organisms were prepared to sustain. Interestingly, recent data showed that entering reproduction enhanced breeders' telomere loss, but no data explored so far the impact of reproductive investment. Telomeres protect the ends of eukaryote chromosomes. Shortened telomeres were associated with shorter lifespan, telomere erosion being then proposed to powerfully quantify life's insults. Here, we experimentally manipulated brood size in order to modify reproductive investment of adult zebra finches (*Taeniopygia guttata*) below or beyond their (optimal) starting investment and tested the consequences of our treatment on parents' telomere dynamics. We show that an increased brood size led to a reduction in telomere lengths in both parents compared to control and to parents raising a reduced brood. This greater telomere erosion was detected in parents immediately after the reproductive event and the telomere length difference persisted up to one year later. However, we did not detect any effects of brood size manipulation on annual survival of parents kept under laboratory conditions. In addition, telomere lengths at the end of reproduction were not associated with annual survival. Altogether, although our findings highlight that fast telomere erosion can come as a cost of brood size manipulation, they provide mixed correlative support to the emerging hypothesis that telomere erosion could account for the links between high reproductive investment and longevity.



## Introduction

A central tenet of life history theory is that reproduction can come as a cost of longevity. Accordingly, numerous empirical studies demonstrated that individuals prevented from reproduction live longer than those allowed to reproduce (SMITH 1958; Partridge & Farquhar 1981) and that an increase in parental reproductive effort can “precipitate death” (Daan, Deerenberg & Dijkstra 1996; Santos & Nakagawa 2012). The underlying mechanisms accounting for costs of reproduction on longevity remain however little understood and are the current topic of intense investigations (Harshman & Zera 2007; Speakman 2008; Monaghan, Metcalfe & Torres 2009).

Costs of reproduction can be divided in two complementary components. Firstly, reproduction itself is associated with numerous, mandatory, changes that stem chiefly from changes in sex hormone levels and of their pleiotropic effects on traits as diverse as immunity, metabolism and behaviours (Brown-Borg 2007). Experimental manipulation of sex hormone profiles, for instance using castration in males and progesterone implants in females, are supporting the existence of mandatory costs of engagement in reproduction on longevity (Tavecchia *et al.* 2005; Brown-Borg 2007; Min, Lee & Park 2012). Secondly, once reproduction has started, parents of species that display parental care to their offspring are expected to optimize their reproductive investment according to their own condition (or quality) and to environmental conditions encountered during reproduction (Stearns 1992; Santos & Nakagawa 2012). This optimization problem is commonly studied in birds by manipulating clutch size or brood size, and in so doing parental effort (Santos & Nakagawa 2012). Accumulating evidence demonstrates that parental costs of reproduction on longevity are “investment-dependent”, becoming apparent only when reproductive efforts are exceeding what parents were prepared to sustain (Santos & Nakagawa 2012).

Interestingly, although there is little doubt nowadays that reproduction can shorten lifespan through combinations of mandatory and investment-dependent costs (originating on whether one considers reproduction status, i.e. entering or not in reproduction, or levels of reproductive effort, respectively), one emerging hypothesis is that reproduction impacts adult survival and/or longevity through a shared, endpoint, aging mechanism: telomere erosion (Allsopp *et al.* 1992; Cawthon *et al.* 2003; Bize *et al.* 2009).

Telomeres are highly conserved non coding repetitive DNA sequences located at the end of linear chromosomes which hide the chromosome ends as being recognised as double stranded breaks (Blackburn 1991). Because of the inability of telomeres to be completely replicated by DNA polymerase, progressive telomere shortening occurs over cell divisions. Once reaching a lower critical size, telomere signalisation pathway induces cell division arrest and/or cell senescence (Harley, Futcher & Greider 1990; Blackburn 1991). Furthermore, the rate at which telomeres are lost is dependent on the balancing of pro- and anti-erosion factors. For instance, oxidative stress comes from the imbalance between the production of reactive oxygen species and the antioxidant capacity (Finkel & Holbrook 2000). Actually, increased reproductive effort has been previously associated with decreased protection against oxidative stress (Wiersma *et al.* 2004) or with altered cell resistance to an oxidative burst (Alonso-Alvarez *et al.* 2004). However, whether reproduction causes oxidative stress remains an open question and can only be answered using experimental approaches (*i.e.* brood size manipulation) (Stier *et al.* 2012; Metcalfe & Monaghan 2013). Oxidative stress accelerates telomere loss (Von Zglinicki 2002; Haussmann *et al.* 2011; Blackburn & Epel 2012) while telomerase activity (mainly active in germinal and stem cells) and shelterin protein complex stability favour telomere maintenance (Blackburn 2000; De Lange 2009). Therefore, telomeres are not just cell division counters but their rate of erosion is actually largely modulated by environmental stressors thereby explaining the age-matched individual variability observed in telomere length (Haussmann *et al.* 2003; Hall *et al.* 2004; Monaghan & Haussmann 2006). This telomere ability to “powerfully quantify life’s insults” (Blackburn & Epel 2012) makes them a potential proxy of future individual fitness, which starts now to be confirmed by the accumulation of an increasing number of studies, from humans to birds (Cawthon *et al.* 2003; Haussmann, Winkler & Vleck 2005; Pauliny *et al.* 2006; Bize *et al.* 2009; Salomons *et al.* 2009; Heidinger *et al.* 2012; Bauch, Becker & Verhulst 2013).

“Engaging in reproduction accelerated telomere shortening” was one the main results of Heidinger *et al.* (2012) in captive zebra finches (*Taeniopygia guttata*). However, this effect was not related to the number of reproductive events undertaken during this time period, and reproductive birds did not exhibit shorter telomeres than non-reproducers after two years. This is a rather surprising result in the light of the expected trade-off between

reproduction and adult lifespan (see (Kotrschal, Ilmonen & Penn 2007) for a suggested link between reproduction and telomere loss). One important point was that birds were only allowed to lay eggs and not to raise chicks during each reproductive attempt (Heidinger *et al.* 2012), therefore largely reducing reproductive costs due to brooding and chick feeding. Hence, one explanation may be that entering in the reproductive life induces sexual maturation costs, but that subsequent reproductive efforts are optimized in relation to telomere maintenance ability. If this hypothesis is right, finding a correlation between reproduction investment and telomere erosion in non-manipulated breeders, moreover under *ad libitum* feeding conditions will prove to be difficult. Adult reproductive investment and its impact on telomeres has been previously tested in two wild bird species. In the Adélie penguin (*Pygoscelis adeliae*), experimental increased reproductive effort (breeding workload being modulated through an increase of the adult foraging costs) was found to be associated with enhanced antioxidant defences and unchanged telomere length but also survival (Beaulieu *et al.* 2011). Alternatively, observations in the common tern (*Sterna hirundo*) showed that parents providing care to their brood beyond the 10<sup>th</sup> nestling day experienced greater telomere loss compared to parents that failed in their reproduction (Bauch, Becker & Verhulst 2013). Thus, evidence of costs of reproduction on telomere dynamics remains scarce and controversial.

To fully understand how telomere erosion is implicated in the trade-off between reproduction and longevity, we need to investigate how the level of reproductive effort during a single breeding event is affecting adult telomere loss and over which time window. To address this question, we experimentally manipulate zebra finches (*Taeniopygia guttata*) reproductive effort by increasing brood size and by determining the short- (end of reproduction) and mid-terms (one year) impact on adult telomere loss. If investment in reproduction is done at the expense of longevity, we expect that pro-ageing markers (oxidative damage and telomere loss) to be increased in adult zebra finches that raised enlarged broods. To assess the effects of reproductive effort on ageing markers, we measured telomere length by relative qPCR, as well as plasmatic oxidative stress with the d-ROMs and the OXY-ADSORBENT tests (Diacron International, Grosseto, Italy) and DNA damage (biosciences inc., Canada). Since telomere loss is further exacerbated by exposure to oxidative stress, at least *in vitro* (Von Zglinicki 2002), the oxidative balance is a good

candidate to mediate the relationship between telomere dynamics and the reproduction-ageing investment trade-offs. Therefore, we also tested whether oxidative stress was involved in the potential reproduction telomere loss relationship. If increased reproductive investment has persistent effects on telomere length (even more under *ad libitum* feeding conditions), this may suggest that telomere loss during an unexpectedly costly breeding attempt would potentially have fitness consequences. Thus we also checked brood size manipulation, oxidative stress and telomere length relationships with adult survival one year after the reproductive event.

## Experimental procedures

### *General procedures*

The study was conducted on captive zebra finches. We experimentally modified reproductive effort using a combined partial cross-fostering and brood size manipulation protocol. Twenty-five randomly formed pairs were placed in breeding cages (0.57 x 0.31 x 0.39 m) with food (a commercial mix of seeds for exotic birds enriched with vitamins and eggs) and water *ad libitum*. The cage placement within the room was random with regard to the treatment. Nest boxes and straw were provided for breeding. The cages were put in a room with a constant temperature of 24°C ( $\pm 1^\circ\text{C}$ ) and light conditions were 13L : 11 D. The brood size was manipulated in order to create three groups: 9 enlarged pairs, 9 reduced pairs, and 7 control pairs. Mean age in years of the parents was not significantly different for the three groups (enlarged:  $2.3 \pm 0.8$ , reduced:  $2.1 \pm 0.8$ , control:  $1.7 \pm 0.9$ ; total range for the three groups, 1 to 3 years; GLMM,  $F=2.68$ ,  $p=0.079$ ). Birds involved in the experiment had experienced at least one reproductive event prior the brood size manipulation. Pre-manipulated clutch size did not differ between groups ( $p=0.078$ ). Chicks from 1 to 3 days old were randomly cross-fostered to form 9 enlarged (2 chicks added, mean brood size  $4.8 \pm 0.75$ ), 9 reduced (2 chicks removed,  $1.8 \pm 0.64$ ) and 7 control pairs (number of chicks unchanged,  $2.8 \pm 0.64$ ). Brood size was constant in each of the treatments during the experiment. Small blood samples (50 $\mu\text{L}$ ) were collected from the brachial vein of the parents at the beginning of the reproductive period (when the breeding pairs were formed) and at the end of the reproductive period (when the chicks were 35 days old, which corresponds to

the moment when they were separated from the parents). After reproduction, birds were put back in different aviaries in the same room, males and females being separated.

In order to evaluate the long term effects of a manipulated reproductive effort on the breeding adults, we monitored the survival rate and collected a blood sample (50µL) on the birds that were still alive a year after the end of the breeding period (10 individuals in the enlarged group, 6 individuals in the control group, 11 individuals in the reduced group). The mean age of the birds which died was 2.8 years  $\pm$  0.5, because of undetermined natural causes.

All blood samples were centrifuged (2000g, 10 min at 4°C), and red blood cells and plasma once separated were immediately stored at -80°C until analyses.

### **Telomere measurements**

Telomere length measurements were done before, after reproduction, and a year after the experiment. Telomere length was measured on DNA extracted from red blood cells (stored at – 20°C until analysis), which are nucleated in birds, using DNeasy Blood and Tissue kit (Qiagen). Telomere length was assessed by the quantitative real-time amplification (qPCR) procedure (Cawthon 2002), previously used in zebra finches (Criscuolo *et al.* 2009). Relative telomere length is expressed as the ratio (T/S) of telomere repeat copy number (T) to a control single gene copy number (S). We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a single control gene. Forward and reverse primers for the GAPDH gene were 5'-AACCAGCCAAGTACGATGACAT-3' and 5'-CCATCAGCAGCAGCCTTCA-3' respectively. Telomere primers were: Tel1b (5'-CGGTTTGGTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3') and Tel2b (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'). qPCR for both telomere and GAPDH were performed using 5 ng of DNA with sets of primers Tel1b/Tel2b (or GAPDH-F/GAPDH-R), each used at a concentration of 200 nM/200 nM, in a final volume of 10 µl containing 5 µl of Power SYBR Green PCR Master Mix (Applied biosystems). Telomere and GAPDH real time amplification were performed on two different plates. qPCR conditions for telomeres were 10 min at 95°C followed by 30 cycles of 1 min at 56°C and 1 min at 95°C. PCR conditions for the GAPDH were 10 min at 95°C followed by 40 cycles of 1 min at 60°C and 1 min at 95°C. Each plate (telomere and GAPDH) included serial dilutions (10 ng, 5 ng, 2.5 ng, 1.25 ng) of

DNA of the same reference bird. This was used to generate a reference curve to control for the amplifying efficiency of the qPCR (efficiencies for GAPDH and telomere plates were between 95 % and 105 %). Samples were run in duplicate on each plate. Samples were randomly distributed and run on a total of 4 plates on which samples from all groups were mixed. To take into account the slight variation of efficiencies between telomere and GAPDH amplifications, we calculate relative telomere length using the method suggested by (Pfaffl 2001). The relative T/S ratios were calculated using the formula:  $((1 + E_{\text{telomere}})^{\Delta C_{\text{t telomere}} (\text{control} - \text{sample})} / (1 + E_{\text{GAPDH}})^{\Delta C_{\text{t GAPDH}} (\text{control} - \text{sample})})$ , in which “control” corresponds to the value obtained for the reference sample (Pfaffl 2001).

Mean intra-plate coefficient of variation was 1.1% for the Ct values of the GAPDH assays and 3.0% for the Ct values of the telomere assays, and inter-plate coefficient of variation was calculated on four samples repeated on the different plates and was of 1.6% for the Ct values the GAPDH assays, 2.9% for the Ct values of the telomere assays. Mean coefficient of variation for the relative T/S ratios was 8.5%. Both a negative control (water) and melting curves were run for each plate to check for non-specific amplification and primer-dimer artefacts.

### ***Oxidative stress measurements***

Oxidative stress measurements were done before, after reproduction, and a year after the experiment. We investigated oxidative stress in plasma samples (stored at – 20°C until analysis), with the d-ROMs and the OXY-ADSORBENT tests (Diacron International, Grosseto, Italy) as previously described in birds (Costantini, Cardinale & Carere 2007; Costantini, Dell'Araccia & Lipp 2008; Beaulieu *et al.* 2010; Beaulieu *et al.* 2011) and mammals (Stier *et al.* 2012). The OXY-ADSORBENT test measures the total antioxidant capacity of the plasma using a colorimetric determination to quantify the ability of the antioxidant barrier to cope with the oxidant action of hypochlorous acid (HOCl). Measurements are expressed as mmol-1 HOCl neutralised. For the OXY-ADSORBENT assay mean intra-plate coefficient of variation was of 2.2% and mean inter-plate coefficient of variation was of 7%. The d-ROMs test measures the early exposure to oxidative stress by evaluating the concentration of hydroperoxide (ROOH) which is a reactive oxygen metabolite (ROM) resulting from the attack of ROS on macromolecules (lipids, protein, DNA). The concentration of hydroperoxyde

was then calculated by comparison with a standard solution whose oxidative activity on the chromogen is equivalent to the activity of H<sub>2</sub>O<sub>2</sub> (0.08 mg dl<sup>-1</sup>). Measurements were expressed as mg dl<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> equivalents. Mean intra-plate coefficient of variation was of 1.3% and mean inter-plate coefficient of variation was of 2%. Procedures for both tests were performed according to manufacturer instructions.

We also investigated DNA oxidative damage through measurements of 8-hydroxy-2-deoxy Guanosine (8-OHdG), using the 8-OHdG EIA kit (StressMarq Biosciences Inc., Victoria, BC Canada). 8-OHdG is the by-product of oxidative damage on DNA due to reactive oxygen species (ROS) and increased levels of this marker have been associated with the ageing process (Shen & Abate-Shen 2007). DNA was first extracted from blood cells with (DNeasy Blood and Tissue kit Qiagen) and then genomic DNA was totally digested following the instructions described in Quinlivan and Gregory III (2008). DNA damage measurements are expressed in pg/mL. Mean coefficient of variation was of 12%

### ***Statistical analysis***

All statistical analyses were performed using SPSS v. 18.0.

Generalized linear mixed models (GLMM) with sex and brood size manipulation as fixed factors and brood identity as a random factor were used to analyse body mass, telomere length, antioxidant levels, and oxidative damage levels before the reproductive period, and show no significant differences between treatments or sex (all P-values > 0.1).

### ***Short and long term effects of brood manipulation on body mass, telomere length and oxidative stress***

Body mass, telomere length, antioxidant levels, and oxidative damage levels (plasmatic and DNA) were measured at the three different periods (before, at the end, and a year after the breeding period). To compare the short and long term effects of brood size manipulation on these parameters, we used GLMMs with time period (beginning, end, and a year after the reproductive period) as a repeated variable, brood identity as a random factor, and sex, brood size manipulation, as well as the interaction between the brood size manipulation and the time period as factors in the model. For body mass, the interaction between sex and the time period was added. For telomere length analysis, oxidative damage levels (plasmatic and

DNA), antioxidant levels, body mass, and age were added as covariates. For plasmatic and DNA oxidative damage levels analysis, the interaction between sex and the time period was added, as well as antioxidant levels and body mass as covariates. For antioxidant levels analysis, body mass was added as a covariate.

Other interactions between explanatory variables were found to be non-significant (all  $p > 0.1$ ) and therefore were not included in the final models.

To identify differences between the breeding groups and the time periods we used LSD post-hoc tests. Results are expressed as mean  $\pm$  SE.

### *Effects on adult survival*

We used a binomial GLMM to analyse survival a year after the breeding period where the brood size manipulation, the sex, the age and telomere length at the end of the breeding period were entered as explanatory variables. Other interactions between explanatory variables were found to be non-significant (all  $p > 0.1$ ) and therefore were not included in the final model.

## **Results**

### *Effects of brood size manipulation on nestlings*

The effect of brood size manipulation had strong consequences on nestling development. Nestlings from enlarged broods stayed longer in the nest compared to those from control and reduced broods (mean age in days at fledging: enlarged broods:  $21.3 \pm 0.4$ , reduced broods:  $17.0 \pm 0.6$ , control broods:  $18.4 \pm 0.7$ ; LSD post hoc comparisons after a general linear model,  $p=0.001$  and  $p<0.001$  respectively). At the end of the growth period (35 days), nestlings from enlarged broods were lighter than those from control and reduced broods (mean body mass in grams: enlarged broods:  $12.1 \pm 0.3$ , reduced broods:  $13.6 \pm 0.5$ , control broods:  $13.8 \pm 0.6$ ; LSD post hoc comparisons after a general linear model,  $p=0.013$  and  $p=0.015$  respectively).



***Short and long term effects of brood size manipulation on adult body mass, oxidative stress and telomere length***

There was no effect of brood size manipulation, nor of the interaction between brood size manipulation and the time period on adult body mass (Table 1,  $p=0.439$  and  $p=0.864$ ).

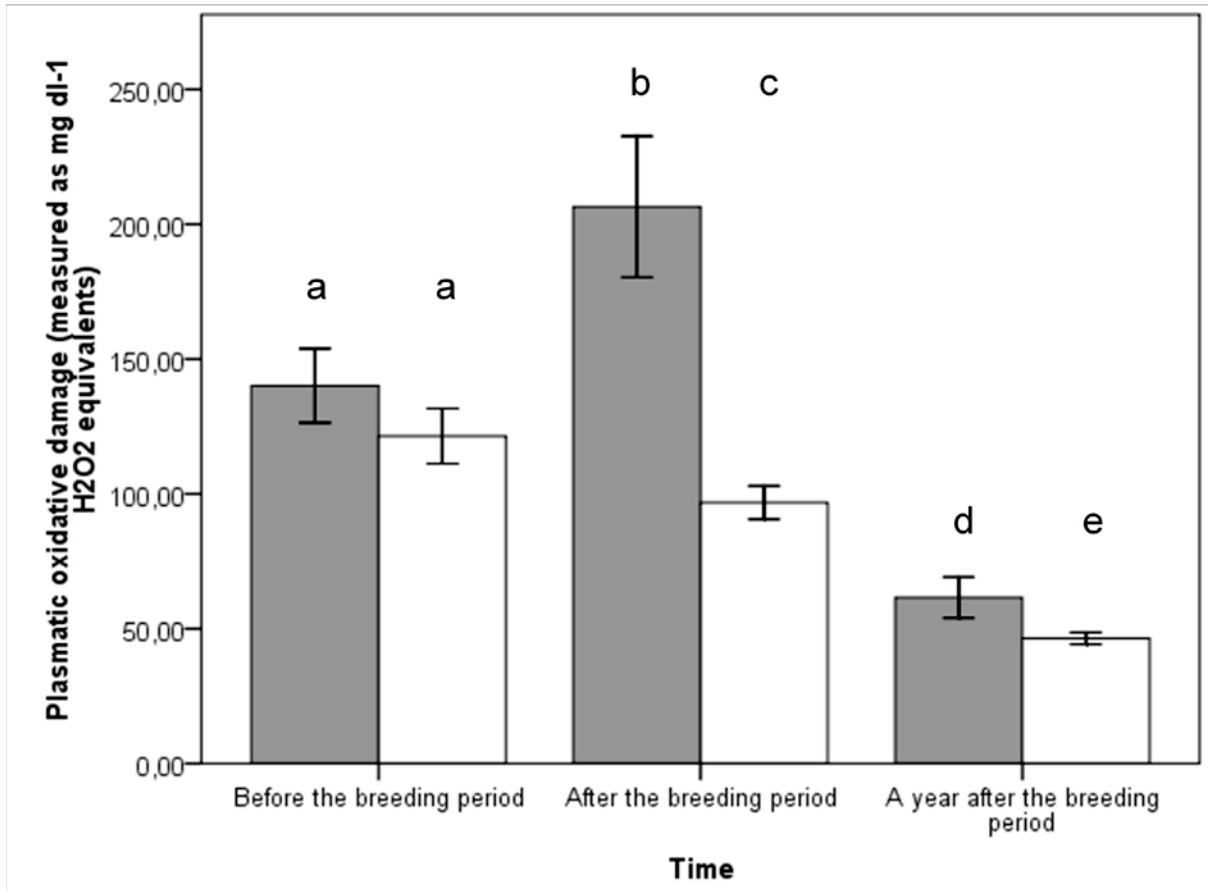
Variable		Estimate	SE	D.F	F	P
<b>Body mass</b>	<i>Random effect</i>	Estimate	SE			
	Brood identity	0,428	0,553			
	<i>Fixed effects</i>					
	Reproductive status			2	0,852	0,439
	Sex			1	8,333	<b>0,005</b>
	Time			2	3,851	<b>0,025</b>
	Reproductive status*Time			4	0,319	0,864
	Sex*Time			2	1,944	0,149
<b>Telomere length</b>	<i>Random effect</i>	Estimate	SE			
	Brood identity	0,070	0,030			
	<i>Fixed effects</i>					
	Reproductive status			2	2,020	0,157
	Sex			1	3,784	0,055
	Time			2	9,169	<b>&lt;0,001</b>
	Reproductive status*Time			4	4,756	<b>0,002</b>
	Plasmatic oxidative damage levels			1	0,261	0,611
	DNA oxidative damage levels			1	0,000	0,986
	Antioxidant levels			1	0,406	0,526
	Age			1	0,593	0,444
	Body mass			1	1,715	0,194
<b>Plasmatic oxidative damage levels</b>	<i>Random effect</i>	Estimate	SE			
	Brood identity	694,850	522,360			

	<i>Fixed effects</i>			
	Reproductive status	2	0,472	0,630
	Sex	1	15,181	<0,001
	Time	2	10,253	<0,001
	Reproductive status*Time	4	0,237	0,917
	Sex*Time	2	8,329	<0,001
	Antioxidant levels	1	0,998	0,320
	Body mass	1	1,240	0,268
<b>DNA</b>				
<b>oxidative damage levels</b>	<i>Random effect</i>	Estimate	SE	
	Brood identity	3305,216	3867,428	
	<i>Fixed effects</i>			
	Reproductive status	2	1,080	0,356
	Sex	1	0,442	0,508
	Time	2	1,168	0,316
	Reproductive status*Time	4	0,422	0,792
	Sex*Time	2	0,590	0,557
	Antioxidant levels	1	4,275	<b>0,041</b>
	Body mass	1	0,174	0,678
<b>Antioxidant levels</b>	<i>Random effect</i>	Estimate	SE	
	Brood identity	3305,216	3867,428	
	<i>Fixed effects</i>			
	Reproductive status	2	0,950	0,402
	Sex	1	0,904	0,344
	Time	2	12,933	<0,001
	Reproductive status*Time	4	0,964	0,431
	Body mass	1	0,290	0,591

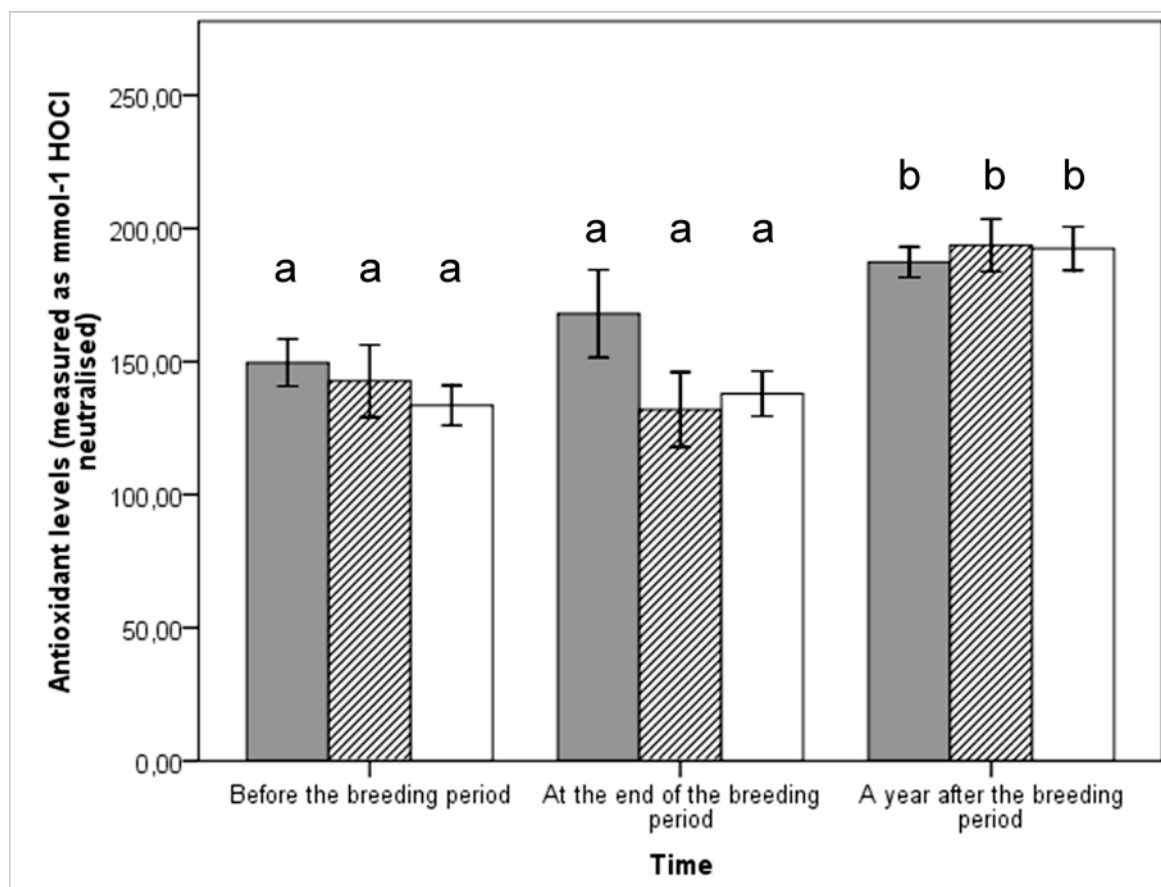
**Table 1:** Results of linear mixed model on body mass, telomere length, oxidative damage levels (plasmatic measured as mg dl-1 H2O2 equivalents and DNA measured as 8-OHdG pg/ml), and antioxidant levels (measured as mmol-1 HOCl neutralised).

However, body mass was affected by the time period (Table 1,  $p=0.025$ ), individuals being lighter during the breeding event compared to a year after the experiment (LSD post hoc comparisons, respectively  $p=0.026$  and  $p=0.008$ ).

All groups exhibited non-different mean plasmatic and DNA oxidative stress levels over the experiment (d-ROM:  $p=0.630$ ; 8-oxo-dG:  $p=0.356$ ) (Table 1). However, the time period (i.e. before vs. at the end vs. a year after the breeding event), sex, as well as the interaction between sex and the time period had significant effects on plasmatic oxidative stress (Table 1). Indeed, at the end of the breeding period and a year after reproduction, females exhibited higher plasmatic levels of oxidative stress than males (LSD post hoc comparisons, respectively  $p<0.001$  and  $p=0.027$ ) (Table 1) (Fig. 1). All groups exhibited similar plasmatic antioxidant levels over the entire experiment ( $p=0.402$ ) (Table 1). Nonetheless, antioxidant levels were higher in all groups when measured a year after the experiment compared to the reproduction period (Table 1, time effect  $p<0.001$ , LSD post hoc comparisons both  $p<0.001$ ) (Fig. 2).



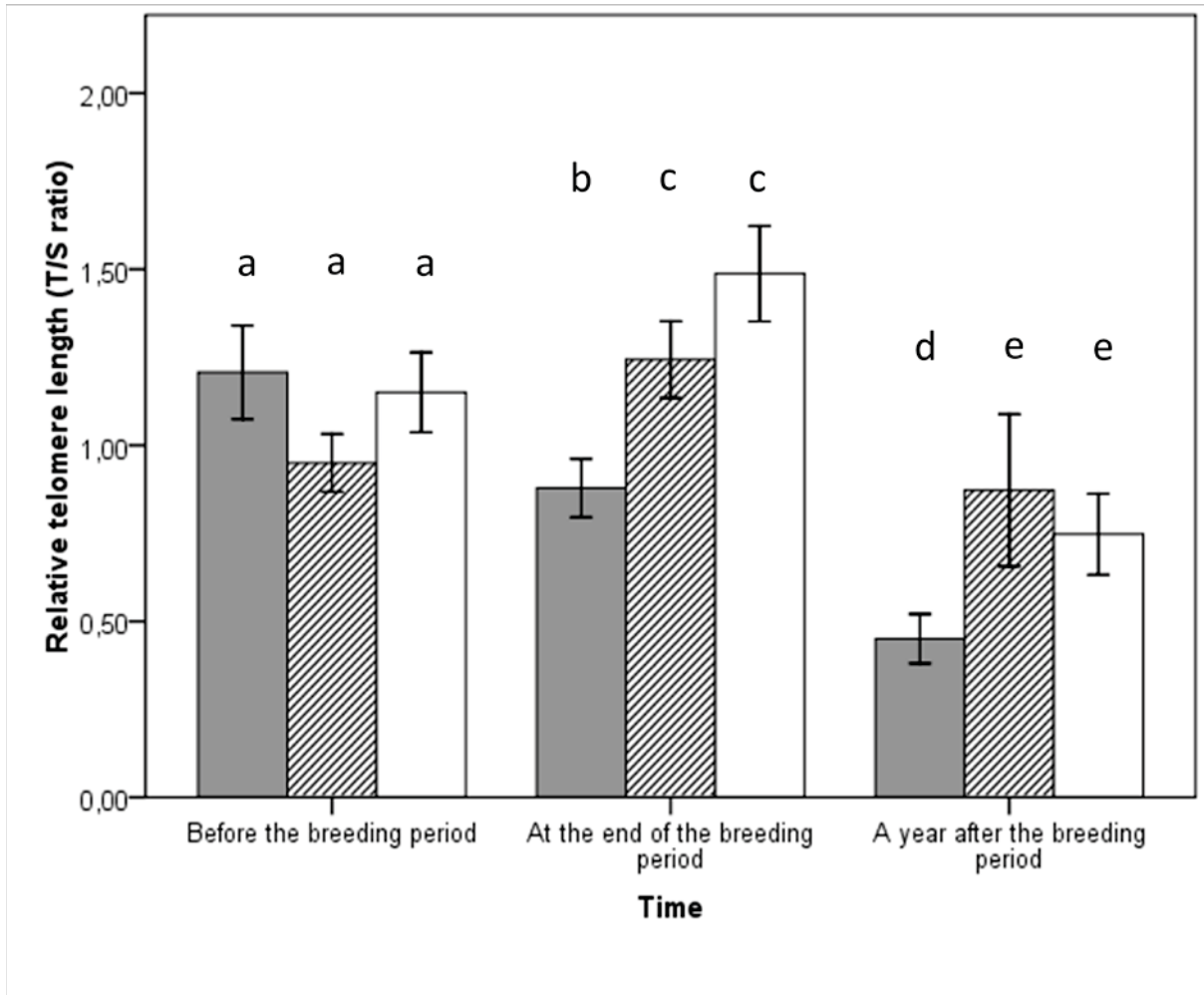
**Figure 1:** *Plasmatic oxidative damage at different time periods: before the breeding event (females n=25, males n=25), at the end of the breeding (females n=25, males n=25), a year after the breeding event (females n=10, males n=17). Bars represent  $\pm$  SE. Females in grey and males in white.*



**Figure 2:** Plasmatic antioxidant levels for both parents in the three different groups at different time periods: before the breeding event (enhanced group  $n=18$ , control group  $n=18$ , reduced group  $n=14$ ), at the end of the breeding event (enhanced group  $n=18$ , control group  $n=18$ , reduced group  $n=14$ ), a year after the breeding event (enhanced group  $n=10$ , control group  $n=6$ , reduced group  $n=11$ ). Bars represent  $\pm$  SE. Enhanced in grey, control in hatched, reduced in white.

The time period (i.e. before vs. at the end vs. a year after the breeding event), as well as the interaction between brood size manipulation and the time period, had significant effects on telomere length (Table 1). Parents forced to raise two additional chicks had reduced telomere lengths at the end of the reproductive period compared to parents raising a

control brood or a reduced brood (LSD post hoc comparisons,  $p=0.010$  and  $p<0.001$ , Fig. 3). There was no significant difference of telomere length between the control and the reduced group (LSD post hoc comparisons,  $p=0.194$ ). The same pattern was observed a year after the experiment. Indeed, the detrimental effect of brood enlargement on telomere length was amplified with time and the enlarged group displayed the shortest telomeres a year after reproduction compared to the control and reduced groups (LSD post hoc comparisons,  $p=0.040$  and  $p=0.022$  respectively, Fig. 3). Conversely, at the same time, parents from the control and reduced groups displayed non-different telomere lengths (LSD post hoc comparisons,  $p=0.666$ , Fig. 3). There was no relationship between telomere length and plasmatic antioxidant (OXY;  $p=0.526$ ) or oxidative damage levels (d-ROM;  $p=0.611$  and DNA oxidative damage (8-oxo-dG;  $p=0.986$ ) or between telomere length and body mass ( $p=0.194$ ) (Table 1). We also controlled for the potential effect of age on telomere length, but there was no significant link between telomere length and age ( $p=0.444$ ).



**Figure 3:** *Telomere lengths for both parents in the three different groups at different time periods: before the breeding event (enhanced group n=18, control group n=18, reduced group n=14), at the end of the breeding event (enhanced group n=18, control group n=18, reduced group n=14), a year after the breeding event (enhanced group n=10, control group n=6, reduced group n=11). Bars represent  $\pm$  SE. Enhanced in grey, control in hatched, reduced in white. Groups which are not significantly different from another are marked with the same letter.*

***Effects of brood size manipulation on survival***

Survival rate a year after did not significantly differ among experimental groups (Table 2,  $p=0.222$ ). Females had lower survival rates a year after reproduction than males (Table 2,  $p=0.048$ ). Telomere length after the breeding period was not predictive of survival a year after (Table 2,  $p=0.591$ ).

Variable	D.F	Khi2	P
<b>Survival</b>			
<i>Effects</i>			
Brood size manipulation	2	3,007	,222
Sex	1	3,922	<b>,048</b>
Age	2	4,305	,116
Telomere length (end of the breeding period)	1	,289	,591

**Table 2:** Results of the binomial GLMM on survival a year after the breeding event with the experimental group, sex, age and telomere length at the end of the breeding period were entered as explanatory variables.

**Discussion**

Daan, Deerenberg and Dijkstra (1996) were the first to uncover that increased parental effort can have persistent effects on adult future fitness, even when feeding conditions were not restricted, therefore suggesting non-energetic trade-offs. In direct line with this suggestion, our study shows that a single costly reproductive event shortens adult telomeres in a way that telomeres are not restored one year after despite the fact that, in between, birds were maintained as non-breeders under optimal environmental and feeding conditions.



***Did the experimental treatment increase adult investment in reproduction?***

Contrary to previous studies (Daan, Deerenberg & Dijkstra 1996; Alonso-Alvarez *et al.* 2004; Wiersma *et al.* 2004), increased reproductive effort did not induce a cost in terms of adult body mass loss in our study. However, reproduction triggered a short and mid-term rise in oxidative stress (D-ROM levels), as previously described (Alonso-Alvarez *et al.* 2004), but only in females and independently of the experimental treatment in our case. Despite the rise in antioxidant levels a year after the experiment, females still displayed higher plasmatic levels of oxidative damage. Still, we were unable to confirm this result while checking the effect of the experimental treatment on a DNA oxidative damage marker. Neither reproduction nor brood size manipulation had short or mid-term repercussions on DNA oxidative damage, underlying the importance to measure several oxidative markers before drawing definitive conclusion on oxidative costs (Selman *et al.* 2012).

The lack of impact of our brood size manipulation on oxidative damage may be attributed to the bleeding timing. In order to avoid disturbing parents' blood volume during their reproduction and to avoid potential chick abandonment, the second blood sample and body mass measurements were not taken during reproduction but when the chicks were independent (i.e. around 35 days). Interestingly, females still displayed higher plasmatic levels of oxidative damage than males when their chicks were 35 days old. Even though the level of plasmatic oxidative damage decreased a year after, it was still higher in females. In zebra finches, parental care is performed by both sexes; however, females take a greater part of the burden than males (Zann & Bamford 1996), which could explain the sex differences we observed. This difference in oxidative damage could be related to the lower survival rates that females exhibit a year after the reproductive event (see below).

Nestlings from the enlarged broods were lighter, and as they fledged later their period of dependency on the parents was longer than for the control and the reduced groups. These results are similar to what was found by Dijkstra *et al.* (1990) showing that brood size manipulation affects current reproduction. This also suggests that in the case of the enlarged broods, parents might not have been able to go beyond their physiological limits to care adequately for their increased number of chicks. Indeed, parents and offspring are expected to disagree over how long the period of parental investment should last and over the amount of parental investment that should be given (Trivers 1974; Stearns 1992). Therefore,

parents may have invested less in their young when the brood size was augmented to preserve their body maintenance, at least in terms of body mass. Nonetheless, it is interesting to note that brood size manipulation had some harmful effects on other components of adult body maintenance: telomere length.

### ***Reproductive investment and telomere length***

Our study suggests short- and mid-term deleterious effects of brood size manipulation on telomere length. Parents raising enlarged broods displayed shorter telomeres both at the end of the reproductive event (35 days) and one year after reproduction. This deleterious impact on telomere dynamics may be the result of an increased reproductive investment in enlarged broods. However, as the impact of our brood size manipulation on adult reproductive effort was not clear (lack of effects on adult body mass and oxidative damage), other explanations might be considered. A possibility is that telomere erosion is not directly related to increased parental energy expenditure (Daan, Deerenberg & Dijkstra 1996) and to an associated rise in oxidative damage but that an indirect link, such as a perceived stressful situation (through increased social stress within the pair and/or with the offspring) may took place (see Hausmann and Marchetto (2010); Blackburn and Epel (2012) for stress vs. telomere erosion relationship).

Surprisingly, we were unable to clearly establish a link between high levels of plasmatic oxidative damage or of DNA oxidative damage and a higher rate of telomere shortening. Firstly, even though females of all groups displayed immediate higher plasmatic oxidative damage, their telomere loss was not exacerbated and we did not find any correlation between telomere loss and oxidative damage at the end of the reproductive event or a year after the reproductive event. These findings support the idea that the link between oxidative stress and telomere erosion is unsettled at present *in vivo* (Chen *et al.* 2001). However, beside the fact that we may have failed to detect an oxidative unbalance in adults caring for enlarged broods (see above), the lack of apparent relationship between oxidative damage and telomere loss may also be explained by other factors. Telomere loss results not only from the deleterious effect of oxidant attacks on DNA, but is balanced by repairing systems such as telomerase activity (Greider & Blackburn 1985). In fact, our higher telomere loss in “enlarged breeders” may rather reflect the inability of birds, which were forced to raise

more chicks, to insure adequate telomere maintenance; mediated either by telomerase activity (Greider & Blackburn 1985), or the pattern of expression of protecting shelterin proteins (De Lange 2005). However, more experimental work is needed to understand accurately how telomere maintenance mechanisms might be involved into reproductive costs.

Another noteworthy point is that the effects of brood size manipulation found on telomeres at the end of the reproductive period persist a year after the experiment. Indeed, parents subjected to increased breeding constraints had the shortest telomeres even a year after the costly reproductive effort. These results enrich the recent finding of long-term telomere changes over the entire reproductive life of zebra finches, showing that an erosion of telomeres takes place independently of the number of reproductive events (Heidinger *et al.* 2012). This latter study actually suggested that entering the reproductive life is associated to a reduction of telomere length, as a consequence of the triggering of processes leading to sexual maturity. It will be interesting to test in future experiments the impact of the progressive rise in sexual steroids on telomere dynamics. However, in Heidinger *et al.* (2012) study, the breeding pairs were only allowed to produce and incubate the eggs, and not to raise any chick. Therefore, we think that both components of the reproductive costs (reproductive maturity and investment) are modulating adult telomere loss, and that chick brooding and feeding activities may have an important impact on adult cell senescence pathways (Bauch, Becker & Verhulst 2013).

After the breeding attempt, parents of the control and the reduced groups did not incur any cost in terms of telomere loss. In fact, telomere length increased slightly. This result indicates that in optimal conditions individuals probably may adjust their reproductive effort in order to avoid any potential costs that might be associated with increased reproductive effort, such as accelerated ageing rate, potentially through activation of telomere maintenance mechanisms such as telomerase. Haussmann *et al.* (2007) study might give support to this hypothesis by showing that telomerase activity is maintained throughout lifespan in different somatic tissues (including bone marrow) in birds. Therefore telomerase activity in the hematopoietic cells of the bone marrow (Broccoli, Young & De Lange 1995) might have maintained telomere length in the control and the reduced groups. In addition, when parents see their initial investment reduced (as it is the case in the reduced group),

there is no apparent positive impact on telomere loss, reinforcing the idea of a fine tuned parental effort related to individual physiological state.

Our data suggest that brood size manipulation can have short and long terms effects on telomere dynamics. This is likely to have different implications at the fitness level, particularly if those results apply in natural conditions (see Daan, Deerenberg and Dijkstra (1996) for correlative support in the wild). Several studies indicate that telomere length being a good predictor of survival and lifespan in wild species (Hausmann, Winkler & Vleck 2005; Bize *et al.* 2009; Salomons *et al.* 2009; Heidinger *et al.* 2012), short telomeres after a costly breeding event might compromise future survival. In this context, telomere dynamics could still be a good candidate to explain the mechanistic link between reproduction and survival, but we still lack a definitive demonstration of this ultimate consequence. In fact, as it was the case in the study of (Heidinger *et al.* 2012), we found that the level of breeding effort did not influence short term survival even for the parents that had the shortest telomeres. A possible explanation for that would be that the study was conducted in captivity and in *ad libitum* feeding conditions, thus creating favourable conditions which might have preserved individuals. Moreover, following survival rate of adult zebra finches over a year is probably not long enough to conclude. However, an interesting observation was that females exhibited higher oxidative damage and lower survival rates one year later, suggesting more rapid fitness consequences of an acute unexpected oxidative disorder. Such an observation between oxidative damage and annual survival rate was previously observed in free-living barn swallows (*Hirundo rustica*) (Saino *et al.* 2011) and male alpine swifts (*Apus melba*; (Bize *et al.* 2008), suggesting as well an important role of this process in immediate individual fitness.

In conclusion, our study highlights that brood size manipulation is related to adult long term eroded telomeres, but with no clear-cut impact on adult reproductive effort (i.e. adult body mass loss or oxidative damage) and over a year survival rate. In the future, particular attention should be given to experimental brood manipulation and their repercussions on social interaction and stress in order to understand how telomere maintenance mechanisms may be involved into parental reproductive investment and their potential costs.

## **Conflict of interest statement**

We declare no conflict of interest.

## **Authors' contribution**

Conception and design of the experiment was done by SR, SM, FC and PB. Data was acquired by SR, SM, FC, AS and SZ while analysis and interpretation of the data was done by SR, SM, FC, PB and AS.

## **Acknowledgments**

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## What link between telomere dynamics and individual maintenance?



Céline Bret





## Study 6

# Experimental increase in telomere length leads to faster feather regeneration

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Keywords: telomere, telomerase, feather renewal

### Highlights

TA-65 treated birds had longer telomeres.

TA-65 treated birds presented faster rates of tissue regeneration.

Long telomeres could be a good predictor of greater tissue regeneration.

Submitted – Experimental Gerontology.

## Introduction

La partie terminale des chromosomes, nommée les télomères, perd progressivement de l'ADN du fait de l'incapacité de l'ADN polymérase à répliquer le double brin d'ADN. Les télomères sont des structures d'ADN non codant, qui ont un rôle essentiel pour la protection et l'intégrité du génome. En effet, ils permettent notamment à la cellule de différencier les cassures double brin de l'extrémité des chromosomes. Ils ont également un rôle crucial, puisqu'ils permettent d'éviter la fusion entre différents chromosomes.

L'érosion des télomères est un paramètre dynamique qui résulte de l'équilibre entre des processus pro-érosion (tel que le stress oxydant) et des processus de restauration (tel que l'activité télomérase). De ce fait, la dynamique d'érosion des télomères est un paramètre clé susceptible d'indiquer la viabilité des cellules. En effet, l'utilisation de la dynamique d'érosion des télomères comme indicateur de la durée de vie, a été étendue au niveau de l'organisme chez un certain nombre d'espèces. Très rapidement, l'écologie évolutive a su tirer avantage de ces propriétés pour évaluer, non seulement, le taux de survie, mais aussi pour estimer les coûts des compromis évolutifs.

Cependant, une question reste sujette à débat : comment les télomères peuvent-ils être liés à la qualité des individus ?

A première vue, cette relation est susceptible d'être le reflet de mécanismes (stress oxydant et/ou activité télomérase) en amont, affectant la dynamique d'érosion des télomères. En effet, récemment, il a été proposé que l'activité télomérase puisse être un des facteurs limitant du vieillissement de l'organisme. La manipulation expérimentale de l'activité télomérase chez les souris, a montré que cette enzyme, restaurant la longueur des télomères, était associée à des effets positifs sur la santé, le vieillissement et la durée de vie chez les individus traités. En effet, chez ces individus, de longs télomères ont été associés à une meilleure viabilité des cellules, qui de ce fait, permettrait un meilleur fonctionnement de l'organisme.

Cependant, dans la mesure où, pour l'instant, ces travaux n'ont été réalisés que chez la souris, nous ne savons que très peu de choses sur les effets de la variation de l'activité télomérase chez des espèces non mammifères, et plus spécifiquement, sur ses effets potentiels sur les télomères et la qualité individuelle.

Pour répondre à cette question, nous avons manipulé l'activité télomérase avec un activateur de cette enzyme (le TA-65, trouvé dans les racines d'Astragale (*Astragalus membranaceus*)) chez le diamant mandarin. Nous avons évalué l'effet de notre traitement sur la longueur des télomères dans les globules rouges, et sur le taux de renouvellement des plumes de vol (évalué par la vitesse de repousse des plumes). Le maintien de la qualité des plumes est essentiel pour les performances individuelles des oiseaux, et peut donc servir d'indicateur pour la maintenance et la qualité des individus.

## Principaux résultats

Les résultats indiquent que la longueur des télomères est affectée par le traitement. En effet les individus traités au TA-65, présentent des télomères significativement plus longs au cours du traitement et à la fin du traitement. De plus, le traitement expérimental a également eu un effet significatif sur la vitesse de repousse des plumes. Les individus traités au TA-65 ont une vitesse de repousse des plumes plus rapide que les individus contrôle.

## Discussion

Les résultats de cette étude indiquent que les individus traités au TA-65 présentent un taux de renouvellement de leurs plumes plus rapide, et ont des télomères plus longs à la fin du traitement.

Ces données confirment donc ce qui a été précédemment décrit dans la littérature chez les souris, et étayent l'hypothèse stipulant que le TA-65 est susceptible de restaurer la longueur des télomères, et selon laquelle cette restauration s'accompagne d'une capacité de régénération cellulaire plus importante. En effet, l'augmentation de l'activité télomérase, du fait de son effet sur les télomères, a pu favoriser une accélération du taux de renouvellement cellulaire.

Ceci fut observé dans une étude menée chez la souris, qui montra qu'une augmentation de l'activité télomérase s'associait à un meilleur état de santé général et une prolongation de la durée de vie. De plus, cette augmentation de l'activité télomérase chez la souris, semble également provoquer une repousse plus rapide des poils.

En conclusion, notre étude étend les résultats mis en évidence chez les souris, aux oiseaux et démontre que la dynamique d'érosion des télomères est susceptible de refléter l'activité de

la télomérase. De ce fait, les télomères semblent un bon indicateur de la santé des organismes et de leur longévité potentielle.

## Abstract

Telomeres– the protective ends of linear chromosomes – reveal themselves as a good proxy in terms of longevity, but more recently also as a marker of healthy ageing in laboratory rodents. Telomere erosion is prevented by the activation of telomerase, an enzyme suspected to be also vital for tissue regeneration and which experimental activation improves health state in mice. One emerging hypothesis is that telomerase activity accounts for the frequently reported positive links between telomere lengths and individual quality in a wide range of organisms. Still, we lack an experimental approach testing the exact impact of inter-individual differences in telomere length on individual trait variability. In a first step study, we tested the impact of the TA-65, a plant-derived product stimulating the expression and the activity of telomerase, on telomere lengths and flight feather renewal capacity of captive zebra finches (*Taenopygia guttata*). Telomere length was longer in TA-65 treated finches while their feather grew faster than in controls. Our data support the idea that long telomeres could reflect high telomerase activity, and in so doing be a good predictor of greater telomerase-dependent tissue regeneration, which may ultimately explain variation in organism quality and longevity.

## Introduction

The very end of linear chromosomes is progressively losing DNA sequences because of the inability of DNA polymerase to replicate the whole DNA double-strand. In the same time, the DNA repairing mechanisms must be able to distinguish real chromosome-ends from DNA breaks. By doing so, wrong end-joining recombination or end-to-end fusion between different chromosomes will be avoided, preserving the cell genome integrity (De Lange 2009). In both of these end-replication and end-protection problems, telomeres are playing a crucial role (Blackburn 2000). Telomeres consist of non-coding DNA in association with proteins of the shelterin complex which forms a specific structure that caps the end of the chromosomes (Blackburn 1991; De Lange 2005), hiding them from general DNA-damage response pathways. Telomere length is dynamic and results from a balance between erosion (end replication-(Blackburn 1991), oxidative stress-(Von Zglinicki 2000)) and restoration (telomerase activity, (Greider & Blackburn 1985)) factors. So, telomere length remains a key feature that indicates the viability of a cell (Blackburn 2000). The utilisation of telomere dynamics as a proxy of lifespan has been widely and successfully extended to the organism level (Cawthon *et al.* 2003). In particular, evolutionary ecology studies took rapidly advantage of such a lifespan proxy, which provides them indication not only about survival rates (Bize *et al.* 2009; Heidinger *et al.* 2012) but also about the costs of life history trade-offs (Heidinger *et al.* 2012). For instance, in the common terns (*Sterna hirundo*), telomere erosion was enhanced by reproduction but highly successful individuals were also those losing less telomere length (Bauch, Becker & Verhulst 2013), suggesting that individual quality may be indicated by long telomeres.

Still, a question remains: how can telomere length be related to individual quality? At first glance, this relationship can result from up-stream mechanisms that both affect telomere length and the overall performances of the organism. As such, oxidative stress may be a major factor that can accelerate telomere erosion but also damage vital cellular functions (Von Zglinicki 2000). A premature loss in telomere length may also be induced by low quality growing conditions that have been previously associated with shortened telomeres at adulthood (Tarry-Adkins *et al.* 2008). The set-up of the organism as a “functioning nexus” during growth will be of tremendous importance for the future performances of the adult.

Then, if growth is disturbed, a bad set-up may lead to the concordance of short telomeres and low individual performances at adulthood (reviewed in (Metcalfe & Monaghan 2001) ), but without any direct functional link. Recent major advances in biomedicine are pointing toward a central role of “telomerase activity as a rate-limiting factor for organism ageing” (de Jesus *et al.* 2011). Experimental activation of telomerase activity in laboratory mice has demonstrated that this enzyme can rescue telomere length with concomitant positive impact on health (de Jesus *et al.* 2011), ageing and lifespan (Bernardes de Jesus *et al.* 2012). Of note, in mice long telomeres *per se* were also found to enhance cell viability and/or allow higher rate of cell renewal (Hao *et al.* 2005), thereby permitting a better functioning of the organism. Because most of our knowledge comes from laboratory mice, we still know very little about the generality of telomerase-increased activity on the phenotype of non-mammalian species, especially on its potential positive effects on telomere length and individual quality.

As a first attempt to fill this gap, we performed an experiment in a small bird species, the zebra finch, where captive adult males were treated with a stimulator of the telomerase activity (TA-65) (de Jesus *et al.* 2011). We then investigated the effects of our treatment both on telomere lengths in red blood cells (RBC) and on the renewal of flight feathers. In birds, RBC telomere length has been found to predict life expectancy (Heidinger *et al.* 2012), and the maintenance of flight feathers is paramount for bird individual performances, self-maintenance and survival (Lindhe Norberg 2002).

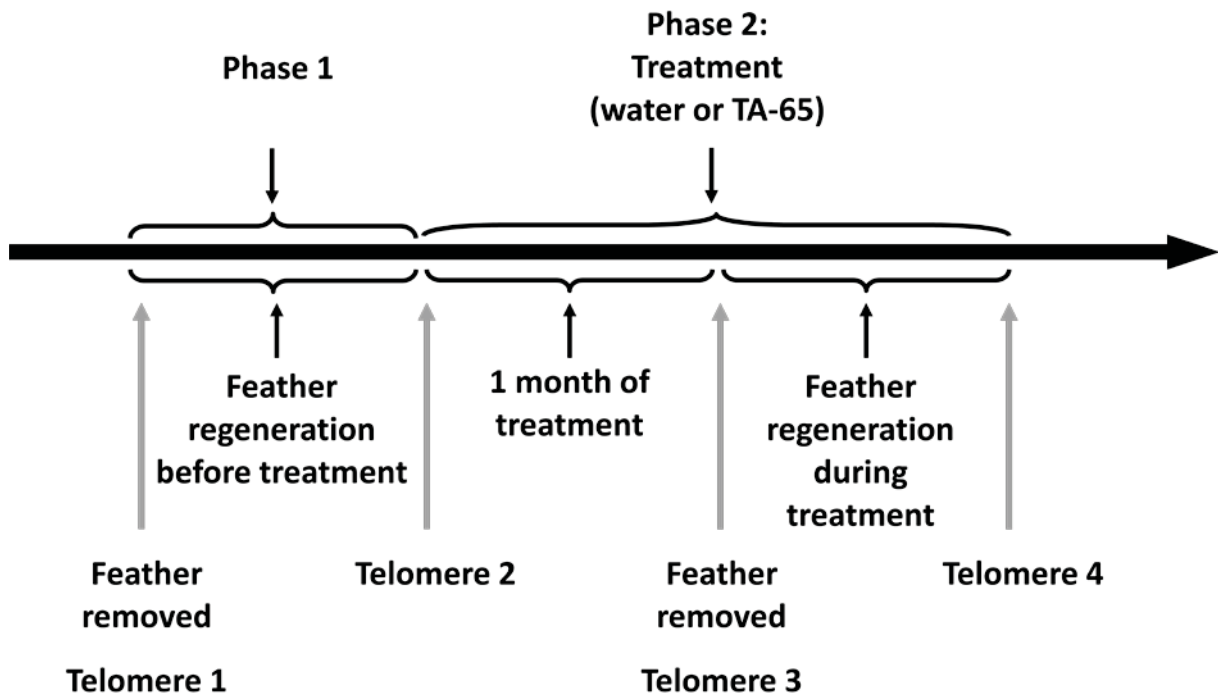


## Materials and Methods

### *Experimental design*

Twenty-eight adult male zebra finches were randomly assigned to two experimental groups: a control group treated with sterilised water (100µl) and a group treated with TA-65 (T.A. Sciences) (0.5 mg diluted in 100 µl of sterilised water per day). TA-65 is a small-molecule purified from the root of *Astragalus membranaceus* that stimulates telomerase activity (de Jesus *et al.* 2011).

Flight feather growth was used as a proxy for individual tissue regeneration capacity. This was achieved by plucking one feather and measuring its speed of re-growth with an electronic calliper every two days until it recovered its initial size. For each individual, we assessed feather growth capacity twice. During the **first phase** of our experiment, we removed the 7<sup>th</sup> primary feather on the right wing of each individual without treating them. It allowed us to obtain a baseline speed of feather regeneration. When the first bout of feather regeneration was over, individuals were then treated daily (using a pipette) either with sterilised water (n= 14 individuals) or with TA-65 (n = 14) for a month before undergoing a **second phase** of feather regeneration by plucking the same feather again. The treatment phase was terminated when the second bout of feather growth was over (Fig. 1. A).



**Figure 1a:** Experimental design: Times 1 and 2 are before the treatment (phase 1) while times 3 and 4 are those measured during the treatment (phase 2).

RBC telomere length was assessed by qPCR methodology as previously conducted in the same species (Crisciolo *et al.* 2009). Telomere lengths were measured before the plucking of the feather (**Time 1**), at the end of the first phase of feather regeneration (just before the start of treatment with water or TA-65) (**Time 2**), a month after the beginning of the treatment (just before the second phase of feather regeneration started) (**Time 3**), at the end of the second phase of feather growth (**Time 4**). Details on qPCR conditions are provided in the ESM.

#### **qPCR telomere length measurements**

To evaluate telomere length, blood samples were collected (50 $\mu$ L) before the first phase of the experiment, at the end of the first episode of feather regeneration (just before the treatment phase started), a month after the beginning of the treatment (just before the second bout of feather regeneration started), at the end of the second phase of feather growth. Telomere length was measured on DNA extracted from red blood cells (stored at –

20°C until analysis), which are nucleated in birds, using DNeasy Blood and Tissue kit (Qiagen). Telomere length was assessed by the quantitative real-time amplification (qPCR) procedure (Cawthon, 2002) adapted to birds and described by (Criscuolo et al., 2009). Relative telomere length is expressed as the ratio (T/S) of telomere repeat copy number (T) to a control single gene copy number (S). We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a single control gene. Forward and reverse primers for the GAPDH gene were 5'-AACCAGCCAAGTACGATGACAT-3' and 5'-CCATCAGCAGCAGCCTTCA-3' respectively. Telomere primers were: Tel1b (5'-CGGTTTGGTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3') and Tel2b (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'). qPCR for both telomere and GAPDH were performed using 5 ng of DNA with sets of primers Tel1b/Tel2b (or GAPDH-F/GAPDH-R), each used at a concentration of 200 nM/200 nM, in a final volume of 10 µl containing 5 µl of Power SYBR Green PCR Master Mix (Applied biosystems). Telomere and GAPDH real time amplification were performed on two different plates. qPCR conditions for telomeres were 10 min at 95°C followed by 30 cycles of 1 min at 56°C and 1 min at 95°C. PCR conditions for the GAPDH were 10 min at 95°C followed by 40 cycles of 1 min at 60°C and 1 min at 95°C. Each plate (telomere and GAPDH) included serial dilutions (10 ng, 5 ng, 2.5 ng, 1.25 ng) of DNA of the same reference bird. This was used to generate a reference curve to control for the amplifying efficiency of the qPCR (efficiencies for GAPDH and telomere plates were between 97 % and 100 %). Samples were run in duplicate on each plate. Samples were run on a total of 3 plates. To take into account the slight variation of efficiencies between telomere and GAPDH amplifications, we calculate relative telomere length using the method suggested by (Pfaffl, 2001). The mean values were used to calculate the relative T/S ratios using the formula:  $((1 + E_{\text{telomere}})^{\Delta C_t \text{ telomere (control - sample)}} / (1 + E_{\text{GAPDH}})^{\Delta C_t \text{ GAPDH (control - sample)}})$ . Mean intraplate coefficient of variation was 0.67% for the Ct values of the GAPDH assays and 1.6% for the Ct values of the telomere assays, and interplate coefficient of variation was calculated on four samples repeated on the different plates and was of 3.08% for the Ct values the GAPDH assays, 0.35% for the Ct values of the telomere assays and 15% for the relative T/S ratios.

### **Data analysis**

Effects of the treatment on telomere length and feather growth were analysed using general linear mixed models (SPSS 18.0), using Bird Identity as random factor, sampling Time as repeated factor, Treatment and the interaction between Treatment and Time as fixed factors. When the Treatment by Time interaction was significant, each Time point was subsequently analysed separately (using t-tests) to assess the Treatment effect.

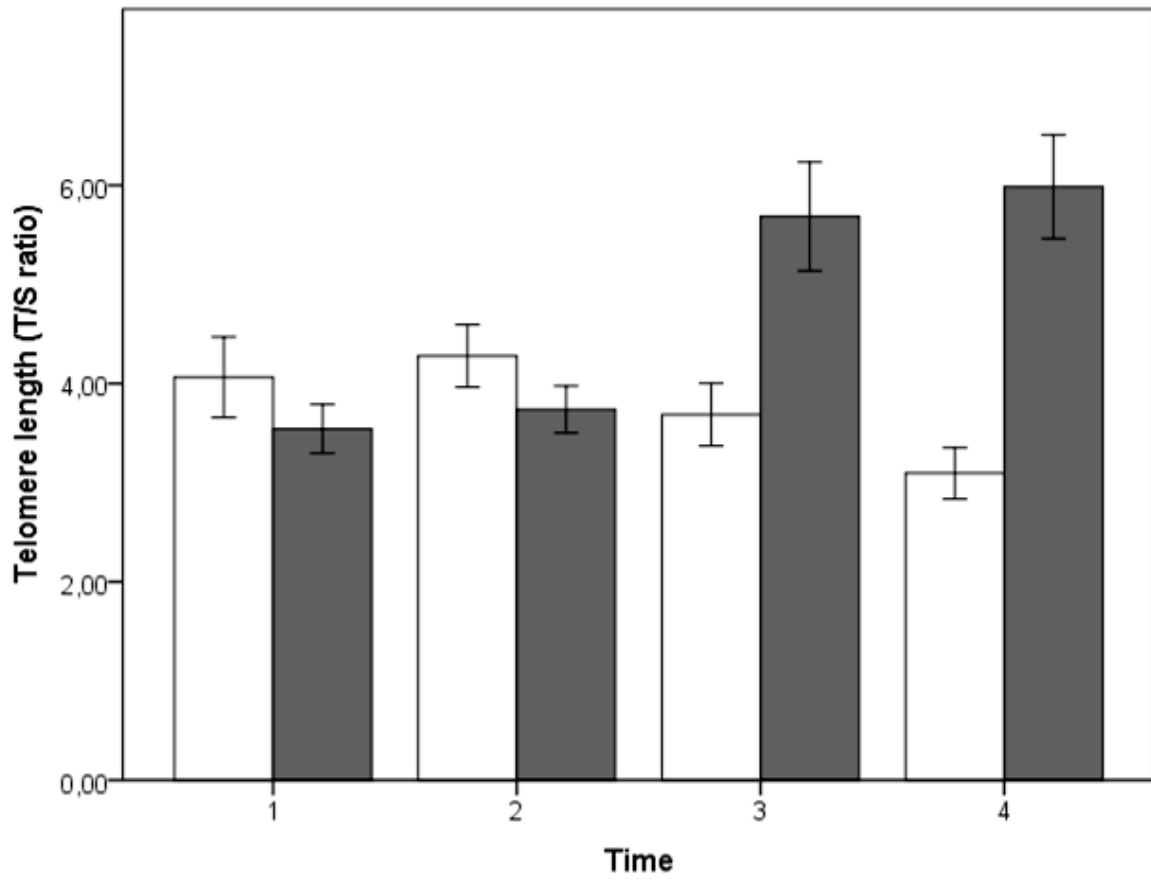
### **Results**

The two experimental groups did not show any significant difference in their initial or final body mass (Linear Mixed Model, treatment x Time interaction  $p = 0.318$ ). The mean value over the experiment was of  $16.87 \pm 2.73$  g (Control birds) and  $16.92 \pm 2.73$  g (TA-65 treated birds). Body mass was stable over the experiment (Initial value,  $16.60 \pm 2.69$  g; final value,  $16.56 \pm 2.69$  g). Linear mixed model showed no significant difference in initial wing length and age between the two experimental groups (respectively  $p=0.431$  and  $p=1$ ). There were no significant differences in age, wing length and body mass between the two experimental groups (see ESM).

1a Telomere length	ddl	F	<i>p</i>
<i>Bird Identity</i> 1.077 ± 0.362			
Treatment	1, 26	4.93	0.035
Time	3, 78	5.58	0.002
Treatment x Time	3, 78	24.31	<0.001
<hr/>			
1b Feather renewal rate	ddl	F	<i>p</i>
<i>Bird Identity</i> 0.001 ± 0.0004			
Treatment	1, 26.3	132.61	<0.001
Phase	1, 25.7	542.38	<0.001
Treatment x Phase	1, 25.7	360.13	<0.001

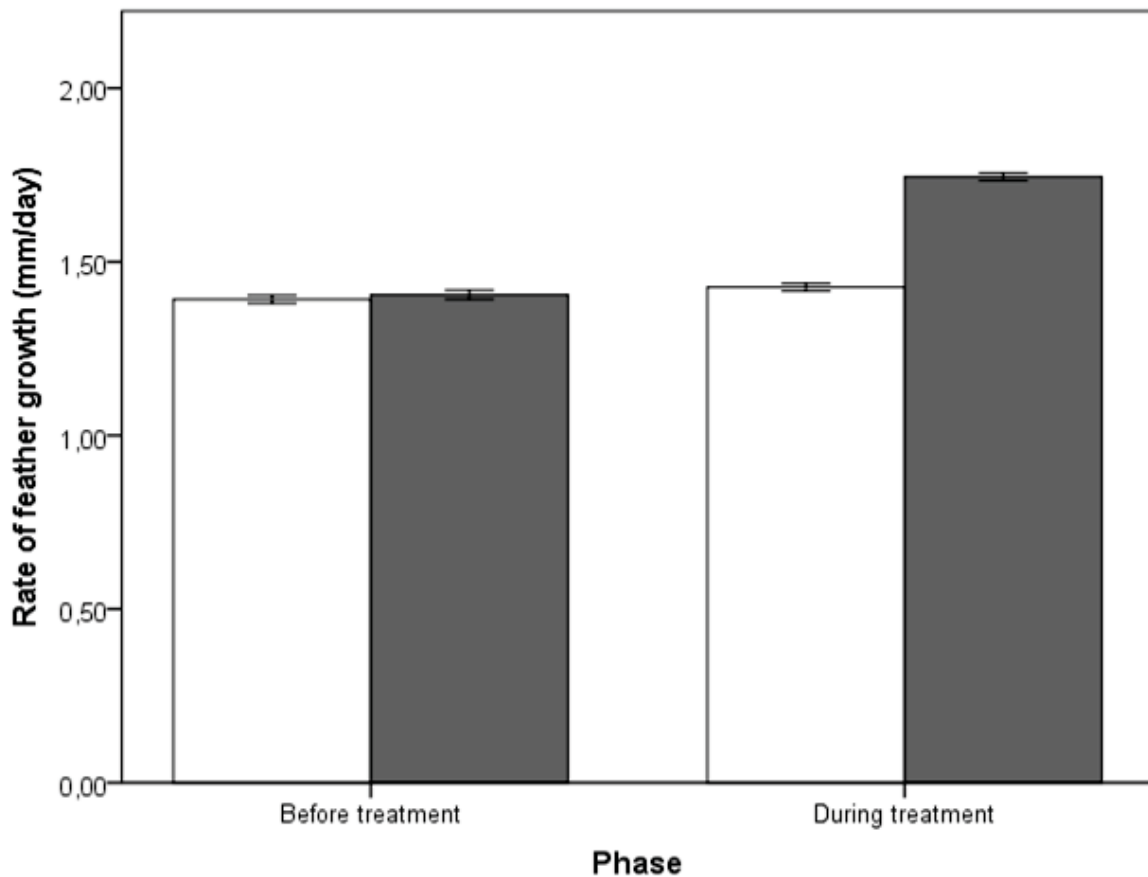
**Table 1:** Linear Mixed Model analysis explaining the impact of TA-65 treatment on (1a) telomere length and (1b) the rate of wing feather renewal of zebra finches.

The significant interaction Treatment x Time showed that telomere lengths were affected by the experimental treatment and by time (Table 1). Tests for each time showed that telomere lengths were not significantly different between control and TA-65 treated birds at Time 1 and 2 (*i.e.* before treatment, Time 1:  $p = 0.28$ ; Time 2:  $p = 0.18$ ; Fig. 1. B). However, TA-65 treated birds were characterised by longer telomeres than control during phase 2 (*i.e.* during treatment, Time 3:  $p = 0.004$ ; Time 4:  $p < 0.001$ ).



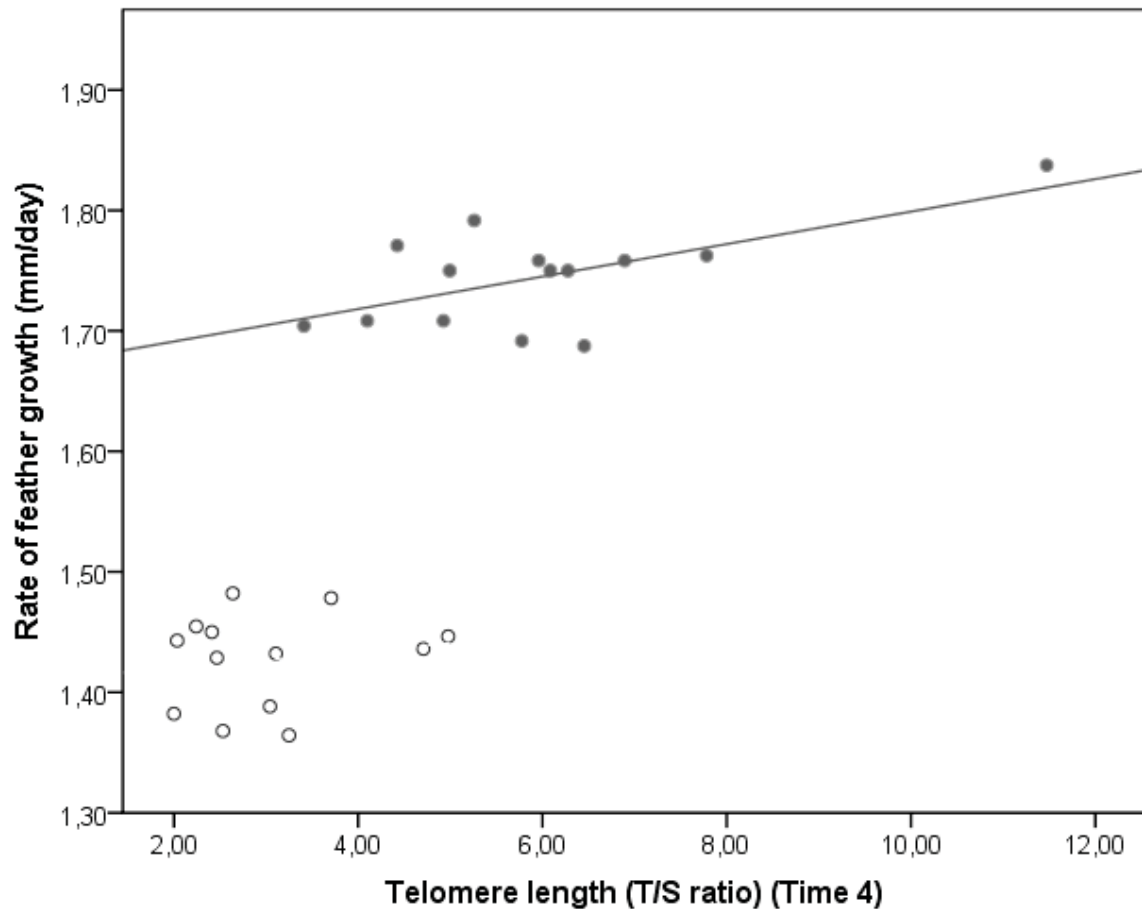
**Figure 1b:** Mean  $\pm$  SE telomere length in TA-65 (grey) or control birds (white) over the different times or phases of the experiment.

The experimental treatment also had a significant impact on wing feather renewal rate (Table 1, Fig. 1. C). The significant Treatment x Phase interaction indicate that TA-65 birds exhibited a faster feather growth than control ones after the treatment (Phase 2:  $p < 0.001$ ) while no differences were detected before the treatment (Phase 1:  $p = 0.45$ ).



**Figure 1c:** Mean  $\pm$  SE rate of feather re-growth in TA-65 (grey) or control birds (white) over the different times or phases of the experiment.

The rate of feather re-growth (Phase 2) was significantly correlated with telomere at the end of the treatment (Time 4) in TA-65 birds (Pearson's correlation,  $r = 0.63$ ,  $p = 0.015$ ) but not in control birds ( $r = 0.16$ ,  $p = 0.58$ ; Fig. 1. D).



**Figure 1d:** Correlation plots between the individual rates of feather growth with telomere length in treated and control birds.

## Discussion

We found that male zebra finches treated during one month with TA-65 presented longer RBC telomere lengths and higher rates of feather renewal than control birds treated only with water. In addition, there was a direct relationship between telomere length and feather re-growth rate in TA-65 birds, the longer the telomere, the faster the feather growth. Our data therefore supports the idea that TA-65 can rescue telomere length (de Jesus *et al.* 2011; Harley *et al.* 2011) with a global positive effect on the proliferating capacity of cells (i.e. epidermal in our case).



Enhanced telomerase activity and/or preservation of telomere length may favour a higher rate of cell renewal. This has been previously observed in mouse skin and subcutaneous adipose tissue, bone, lung or neuromuscular tissue (Bernardes de Jesus *et al.* 2012), thus being associated with an improvement of the global health status of adult mice (de Jesus *et al.* 2011). Indeed, telomerase gene therapy has recently emerged as a potential mean to counteract organism malfunction linked to ageing (Bernardes de Jesus *et al.* 2012). Premature loss of telomere length associated with mutation of telomerase protein component (TERT) is responsible of multiple pathologies (Aubert & Lansdorp 2008) that are likely to affect the future survival and reproduction of those individuals. Overall, the enhanced tissue fitness in TA-65 mice led to an extension of median lifespan by 24 to 40% (Bernardes de Jesus *et al.* 2012). Interestingly, TA-65 treatment in mice is also favouring hair re-growth (de Jesus *et al.* 2011). The authors proposed that the preservation of the skin layer and of the subcutaneous adipose tissue is important to delay the multiple skin lesion associated with ageing in this species.

Our study extends those findings from mice to birds. Our results provide encouraging support to the general idea that long telomeres might reflect high telomerase activity, and in so doing be good predictors of greater telomerase-dependent tissue regeneration and organism health and longevity. Still, we need to be prudent in our conclusions because, despite the fact that flight feather maintenance is paramount for bird survival, a higher rate of growth of feather is not always synonym of better plumage quality (Dawson *et al.* 2000). Studies are now required to determine whether stimulation of the telomerase activity has positive effects on bird fitness-related traits such as flying capacity, resilience to environmental stress, reproduction or lifespan as it has (at least for health and lifespan) in mice.

## **Acknowledgements**

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## **Box 1**

### **Telomerase activity, telomere length and self-maintenance: effects on the immune function**

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Unpublished data.

## Introduction

The results presented in study 6 support the hypothesis that the preservation of long telomeres by enhanced telomerase activity is associated with a physiological state that allows a faster renewal of feather. The significant and concomitant rises in both telomere length and feather renewal rate supports the idea that long telomeres could reflect high telomerase activity. In so doing, they could be a good predictor of greater telomerase-dependent tissue regeneration, which may ultimately explain variation in organism quality. Thus, good individual quality may be indicated by long telomeres. The next step is to determine if this enhanced telomerase activity is associated with global individual quality, and if other maintenance-related traits such as immunocompetence (*i.e.* the ability of an organism to prevent or control infection by pathogens and parasites) are enhanced.

The immune system plays a crucial role in defending animals against pathogens and parasites (Norris & Evans 2000). Moreover, if resources are limited, costly activities like immunity might be constrained / reduced to enable the allocation of available resources for other physiological processes (Sheldon & Verhulst 1996; Norris & Evans 2000). However, these trade-offs can vary with the physiological state of the individual, as it was shown in house sparrows (Martin II *et al.* 2006a). For instance in this latter study, authors showed that birds that had just completed postnuptial moult (*i.e.* then facing an energy stressful situation) had the weakest immune response. Indeed, it is generally assumed that providing immunological defences is costly in terms of necessitating trade-offs with other nutrient-demanding processes such as growth, reproduction, and thermoregulation (Lochmiller & Deerenberg 2000).

Immune defences are critical for individual maintenance and survival (Norris & Evans 2000). Indeed, reduction in immunocompetence is likely to lead to an increased risk of infection and therefore to a reduced ability for individuals to maintain themselves and to survive (Norris & Evans 2000). For instance house martins nestling which had poor cell-mediated immunocompetence were less likely to survive than more immunocompetent ones (Christe, Møller & de Lope 1998). Consequently, the ability to mount an immune response can be used as a proxy for individuals' ability to maintain themselves. As the immune response depends on cell proliferation capacity, increased telomerase activity might promote immune

cells proliferation and thus improve the immune response through enhanced cell replicative capacity.

In this context, in addition to the rate of renewal of wing feathers, we also evaluated how our TA-65 treatment (for details see study 6) affected immune activity, another parameter of importance for bird maintenance performances.

In our study, immune activity was evaluated using the phytohaemagglutinin (PHA) cutaneous test. PHA is a mitogen derived from the red kidney bean (*Phaseolus vulgaris*) that triggers cell proliferation. The PHA test is a currently used technique in evolutionary ecology to assess the cost of an immune response and individual immunocompetence (Blount *et al.* 2003; Kennedy & Nager 2006). It is likely that PHA triggers both innate and acquired immune responses (Kennedy & Nager 2006). PHA injection causes swelling and edema at the injection site, and incites local infiltration of immune cells including heterophils, macrophages, basophils and other lymphocytes (Kennedy & Nager 2006).

## Materials and methods

The PHA test was done at the end of the second experimental phase (time 4) (see Fig 1a in study 6). We also evaluated whether the TA-65 treatment actually affected telomerase activity, which was measured on samples collected at the end of the treatment period. To do so, seven birds of each group were euthanised, and their bone marrow and testes were removed. We chose to measure telomerase activity in the bone marrow and in the testes because there have been shown to be one of the few tissues in which telomerase is active in the zebra finch (Hausmann *et al.* 2007).

### **Measuring telomerase activity**

Telomerase activity was determined using Quantitative Telomerase Detection Kit (QTD Kit, US Biomax Inc.) which is designed to detect telomerase activity with real-time PCR. In the assay, tissues (with enzymatically active telomerase) are digested and the telomerase activity in the cell extract is determined through its ability to synthesise telomeric repeats onto an oligonucleotide substrate *in vitro* upon the addition of the appropriate buffer conditions and dNTPs. Telomerase from the cell extract adds telomeric repeats onto a substrate oligonucleotide and the resultant extended products are subsequently amplified

by the polymerase chain reaction (PCR). The PCR products are then visualised using highly sensitive DNA fluorochromes SYBR Green.

For each individual, one testis was homogenised on ice in 100 µl lysis buffer, with a pellet pestle (Sigma # Z359971). 1 µl of anti-proteases was added (Sigma # P8340). The samples were incubated on ice during 30 minutes for lysis, and then centrifuged at 12000 g for 30 minutes at 4°C. The supernatant was immediately collected and frozen at -80°C until analysis. One aliquot was used for protein determination, using Quick Start Bradford Microassay protocol (Bio-Rad # 500-0201). For the normalisation of the results, the quantitative PCR has been processed with 350 ng of protein. For each sample, a control of inactivation (85°C for 10 minutes) of the telomerase was done to check the absence of amplification without telomerase. An oligonucleotide with a sequence identical to telomere primers (TSR) is used to generate a standard curve. This standard curve allows the calculation of the amount of template with telomeric repeat created by telomerase in a given extract. The calculated CT resulting of the quantitative PCR conducted on biological samples were compared to those of the standard curve of TSR to determine the telomerase activity present in each sample. Telomerase activity is expressed in amol/ml of TSR.

### ***PHA measurement***

Just before the end of the treatment, we subjected each bird to a PHA skin test, which assays an individual's T-lymphocyte responsiveness to a foreign plant protein (Smits, Bortolotti & Tella 1999). To conduct the PHA test, we measured the right wing web of each bird three times with a digital micrometer (to the nearest 0.05 mm) to obtain an average pre-swelling measurement and then injected this area with 100µg of PHA-P (Sigma Chemical Co., St. Louis, MO) in 20 µl of phosphate-buffered saline (PBS). Birds were immediately placed back in their cages and the swollen area was measured 24h later. Presented results correspond to the difference between mean post-injection swellings and mean pre-injection swellings.

### ***Statistical analysis***

Due to small sample size and non-normal distribution, effects on telomerase activity were tested using non parametric Mann-Whitney tests. For the testis samples there were

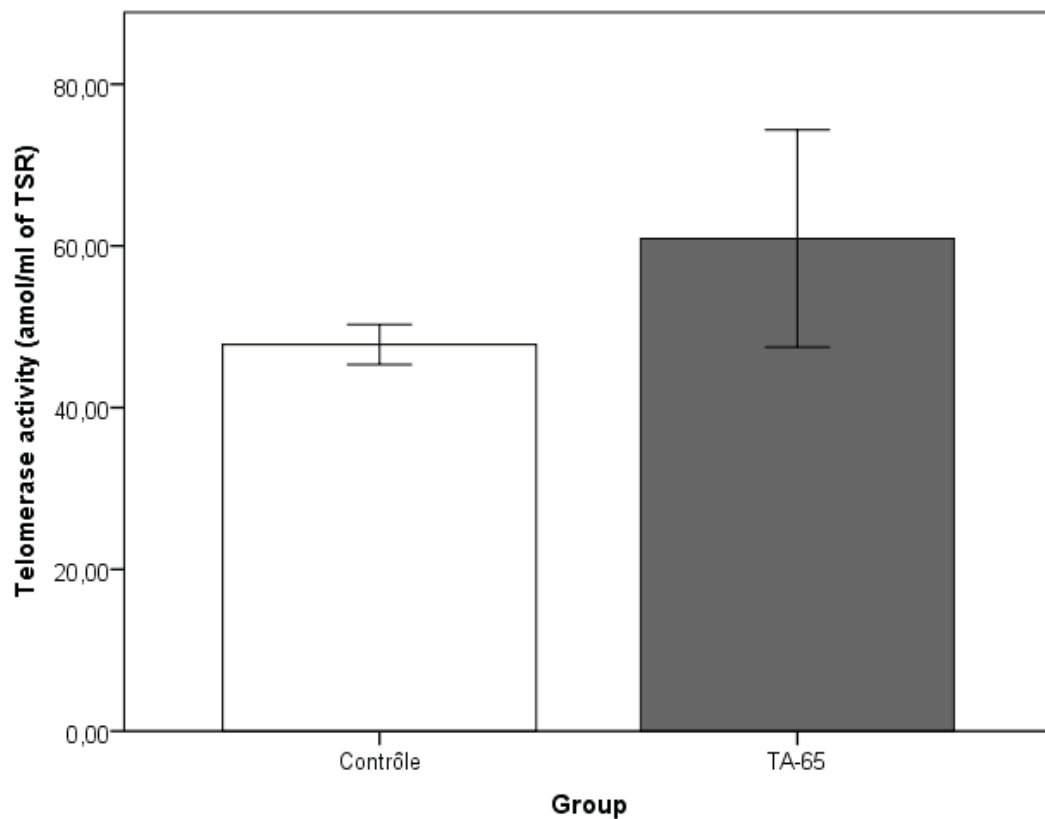
seven individuals per group, and for the bone marrow samples there were only three individuals per group as the rest of the samples were damaged due to bad conservation.

Effects of the treatment on the PHA response were analysed using general linear mixed models (SPSS 18.0), using Bird Identity as random factor and Treatment as fixed factor. Means are quoted  $\pm$  S.E. All statistical analyses were performed using SPSS v. 18.0.

## Results

### *Effects on telomerase activity*

The experimental treatment had no significant effects on testis telomerase activity ( $p=0.749$ , Fig 1) and on bone marrow telomerase activity ( $p=0.1$ ).

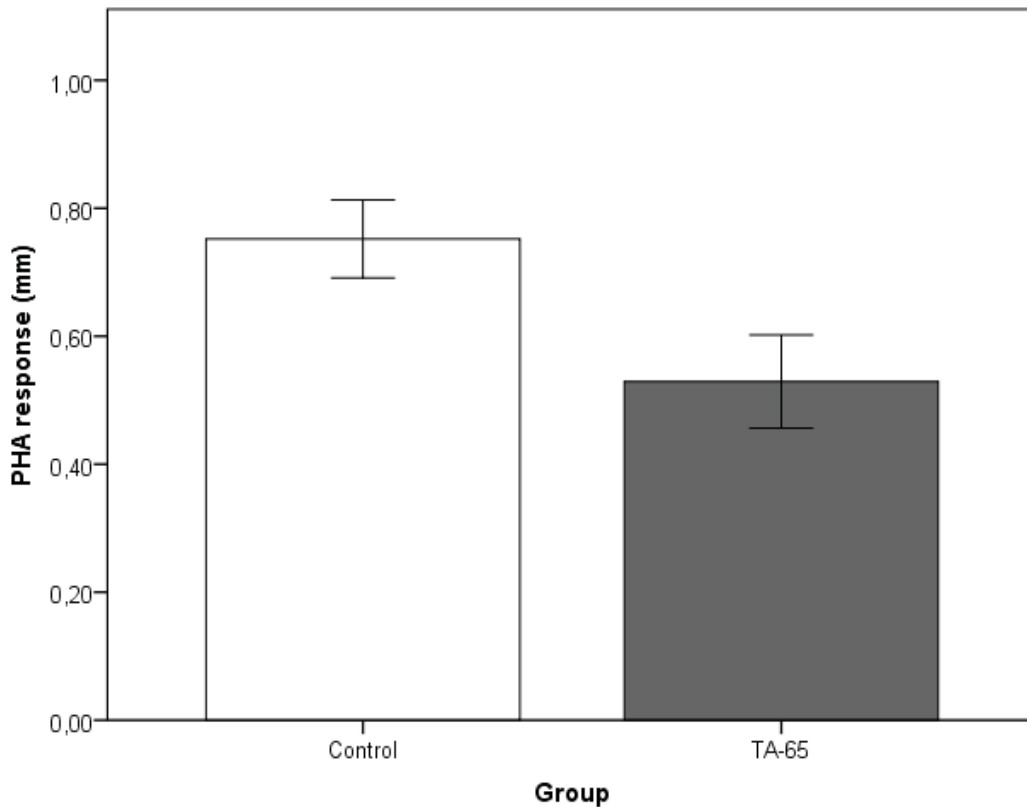


**Figure 1:** *Telomerase activity at the end of the treatment in the testes in the control group (n=7) (in white) and the TA-65 group (n=7) (in grey).*



***Effects on the PHA response***

Interestingly – and in contrast to telomerase activity – the experimental treatment significantly affected the PHA response ( $F=5.5$ ,  $p=0.027$ , Fig 2). Birds treated with TA-65 displayed reduced web wing thickness after PHA injection compared to control birds.



**Figure 2:** Response to PHA injection at the end of the treatment (time 4) in the control group ( $n=14$ ) (in white) and the TA-65 group ( $n=14$ ) (in grey).

**Discussion**

The aim of this study was to test whether long telomeres could reflect high telomerase activity, and in so doing be a good predictor of greater telomerase-dependent tissue regeneration, which may ultimately explain variation in organism quality and longevity. To do so we tested the impact of TA-65 (a plant-derived product stimulating the expression and the activity of telomerase) on telomere lengths, flight feather renewal capacity and PHA immune responses. Overall, our results show that the individuals treated

with TA-65 presented longer telomeres at the end of the treatment, more rapid rates of feather renewal capacity and weaker PHA responses.

Surprisingly, treatment with TA-65 did not affect telomerase activity in testes nor in the bone marrow, as the level of telomerase activity was not significantly different from the one measured in the control group. Interestingly, in de Jesus *et al.* (2011) study, they only found increased telomerase activity following TA-65 treatment in the liver, whereas in other tissues (kidney, lung and brain) telomerase activity remain non significantly different than the levels observed in the control group. This suggests that the treatment might have differential effects depending on the type of tissue considered. Therefore, the tissues we sampled (testes and bone marrow) might not have been the main target affected by our treatment. Another possible explanation would be that, as the level of telomerase activity is already elevated in the testis (Hausmann *et al.* 2007), additional increase of this activity by an extrinsic chemical treatment would be less likely to be detected. Nonetheless, despite the absence of an evident effect on telomerase activity, telomere lengths in the TA-65 group were increased (see study 6), indicating that our treatment was somehow having the expected impact on telomeres.

As for telomere length and the rate of feather regeneration (see study 6 for details), the experimental treatment had an effect on the PHA response. In our study, birds treated with TA-65 exhibited a lower PHA response than birds from the control group. In the light of the findings presented in Fauce *et al.* (2008), our results might seem surprising. Indeed, in the latter study, they showed that CD8+ T lymphocytes exposed to a small telomerase activator (TAT2) displayed retarded telomere shortening, increased proliferative potential and increased antiviral activity, thus suggesting an enhancement of the immune function.

Our contrasting results might be partially explained by the fact that we did not test the same part of the immune system. Alternatively, our findings may be the result of trade-offs between different components of the immune system and/or between costly physiological activities.

***Trade-off between components of the immune system***

Usually, in the case of PHA injection, the basic assumption stipulates that more swelling represents a better cell-mediated immune response or a stronger immunocompetence. Therefore, we would expect that if the TA-65 treatment ameliorates individual maintenance abilities (as suggested by the faster rate of feather regeneration and the longer telomeres) it would also increase the ability to mount an immune response. However, one has to be cautious with PHA response data interpretation (Kennedy & Nager 2006; Martin li *et al.* 2006b) as it is likely that the PHA swelling triggers both innate and acquired immune responses. Consequently larger swellings might not indicate greater cell-mediated immunocompetence. As the PHA response triggers both innate and acquired immunity, it is possible that the two systems, although highly integrated, are affected differentially by the PHA and at a different pace, and that a trade-off exists between the two. For instance, it has been shown that a strong innate immunity might come to the detriment of acquired immunity (Gross *et al.* 1980). Indeed, a large swelling response to PHA injection might reveal a poor acquired immunity (Kennedy & Nager 2006) or might indicate allergy or unrestrained local inflammatory activity (Martin li *et al.* 2006b); hence suggesting that more swelling is not always better.

***Trade-off between two costly physiological activities***

On the other hand, our results might also reflect a trade-off between feather regeneration and immune capacity. Indeed, several studies previously showed that PHA induced swelling are traded off with other physiological functions (Nelson & Demas 1996; Martin II *et al.* 2006a), with PHA responses being weaker when other costly activities are concomitant. For instance, in house sparrows, immune responses are weaker in individuals being in reproductive state or just after moult (Greenman, Martin li & Hau 2005; Martin li 2005; Martin II *et al.* 2006a). Martin *et al.* (2005) found slower feather regeneration in birds after a PHA injection. Later on, they found a suppression of immune function after the completion of moult (Martin II *et al.* 2006a), suggesting delayed trade-offs between moult and immune function. Therefore, in our case, PHA swelling might have been weaker in birds treated with TA-65 because they experienced a faster rate of feather regeneration.

Given these contrasting results, it seems that more work is needed on immune activity to disentangle the positive from the negative effects of high telomerase activity on the highly proliferating cell immune system. In addition, this would bring additional information on how telomeres may act on individual fitness.



## Chapter VI

# General discussion, limits and perspectives



## I – General discussion and overall synthesis

### 1.1 Summary of the main results

Over the past few years, the role of telomere dynamics in evolutionary biology and evolutionary ecology has drawn a lot of attention, thus hinting the great importance of this mechanism in evolutionary processes. In this context, the overall object of this thesis was to identify the different parameters determining telomere dynamics and to understand how telomere dynamics could be considered as a putative mechanism underlying evolutionary trade-offs.

In an attempt to answer these questions, the studies that were conducted during this thesis aimed to assess the following points:

- What is the inheritance pattern of telomere length? What is the relative influence of the genetic component on telomere length compared to the impact of environmental effects in two species? (Studies 2 and 3)
- How is telomere length affected by extrinsic factors, i.e. growth conditions and reproductive effort? What indications do these effects give us regarding the potential role of telomere dynamics in the growth-lifespan and in the reproduction-lifespan trade-offs? (Studies 4 and 5)
- Finally, is telomere length associated to individual maintenance, and therefore is it indicating anything about individual quality? (Study 6)

A summary of the main results of this thesis is presented in table VI.1.



Species	Experimental manipulation	Genetic component		Effects of extrinsic factors on telomere dynamics	Role in evolutionary trade-offs	Link to individual quality and fitness ?
		<i>Inheritance pattern</i>	<i>Heritability</i>			
King penguins (study 2)	No	Maternal inheritance	Low h <sup>2</sup>	Yes (time over growth)	Not measured	Not measured
Dippers (study 3)	No	Maternal inheritance	Low h <sup>2</sup>	Yes (year and nest effects)	Not measured	Positive link with inbreeding
Zebra finches	Yes					
Chicks (study 4)	Brood size manipulation	Not measured	Not measured	Yes (growth conditions effects)	Yes (growth-lifespan trade-off)	Not measured
Adults (study 5)	Brood size manipulation	Not measured	Not measured	Yes (reproductive effort effects)	Yes (reproduction-lifespan trade-off)	Not measured
Adults (study 6)	Telomerase inhibitor treatment	Not measured	Not measured	Not measured	Yes (link to self-maintenance)	Yes (link to individual quality)

**Table VI.1:** Summary of the main results of this work.

In the following part, I will present the main findings of my work and discuss how they provided answers to the initial questions.

1.2 What are the characteristics of proximate mechanisms of life history trade-offs?

The term trade-off implies that the traits considered are negatively correlated. A fundamental aspect in the notion of trade-off is that, in addition to the fact that traits may compete for a limiting pool of energy resources, the negative association between two traits has to be caused by a negative functional interaction (Zera & Harshman 2001). It is not just the sign (positive or negative) of the link between two traits that determines the presence of a trade-off; this relationship has to present a functional basis. There are multiple pathways through which two traits can be negatively related, and part of our work is to uncover the underlying mechanisms shaping this negative association in order to understand the functional interaction between these traits. A powerful approach to investigate life history trade-offs is to manipulate environmental conditions (nutrients, stress, reproductive rates, immune challenges) to generate phenotypic trade-offs. Coupling this type of approaches to the study of innovative functional aspects is essential to understand the mechanistic causes of life history trade-offs (Zera & Harshman 2001).

Consequently, for a mechanism to be considered as a functional link between traits and to underlie evolutionary trade-offs, it has to be sensitive to environmental variations. Secondly, it has to reflect the impact of one trait on another. Therefore, if a mechanism is indeed implicated in evolutionary processes, it has to be linked to fitness related traits and/or directly to individual fitness. Thirdly, as trade-offs are not static (they can change over lifetime), the mechanism envisaged has to be dynamic over time to allow us to quantify the changing aspect of evolutionary trade-offs. The two main mechanisms that have been mainly studied and which fulfil these requirements and which have drawn attention as possible functional links for evolutionary trade-offs are hormone regulation (Ketterson & Nolan Jr 1992; Finch & Rose 1995) and immune function (Sheldon & Verhulst 1996; Norris & Evans 2000).

Finally, as natural selection can lead to an evolution of traits through generations mainly if it acts on a heritable character, our first step is to determine its pattern of transmission and heritability.

### 1.3 Importance of the inheritance and heritability patterns

#### *Heterogametic pattern of telomere length inheritance hypothesis*

The genetic component has been shown to play an important part in the determination of telomere length in humans (Slagboom, Droog & Boomsma 1994; Graakjaer *et al.* 2004; Nordfjäll *et al.* 2005; Unryn, Cook & Riabowol 2005; Njajou *et al.* 2007), and in a couple of other vertebrate species (the sand lizard – (Olsson *et al.* 2011a) and the kakapo – (Horn *et al.* 2011)). Given the scarcity of the data investigating the patterns of telomere length inheritance and heritability in wild animals, and more particularly in birds, we tested if similar patterns were found in two wild populations of birds: the king penguin and the dipper. In addition, we also tested whether the pattern of telomere inheritance was maintained over time and how it might be affected by environmental conditions.

In both studies, offspring and parental telomere lengths were found to be associated, maternal telomere length having a stronger influence than paternal telomere length in these

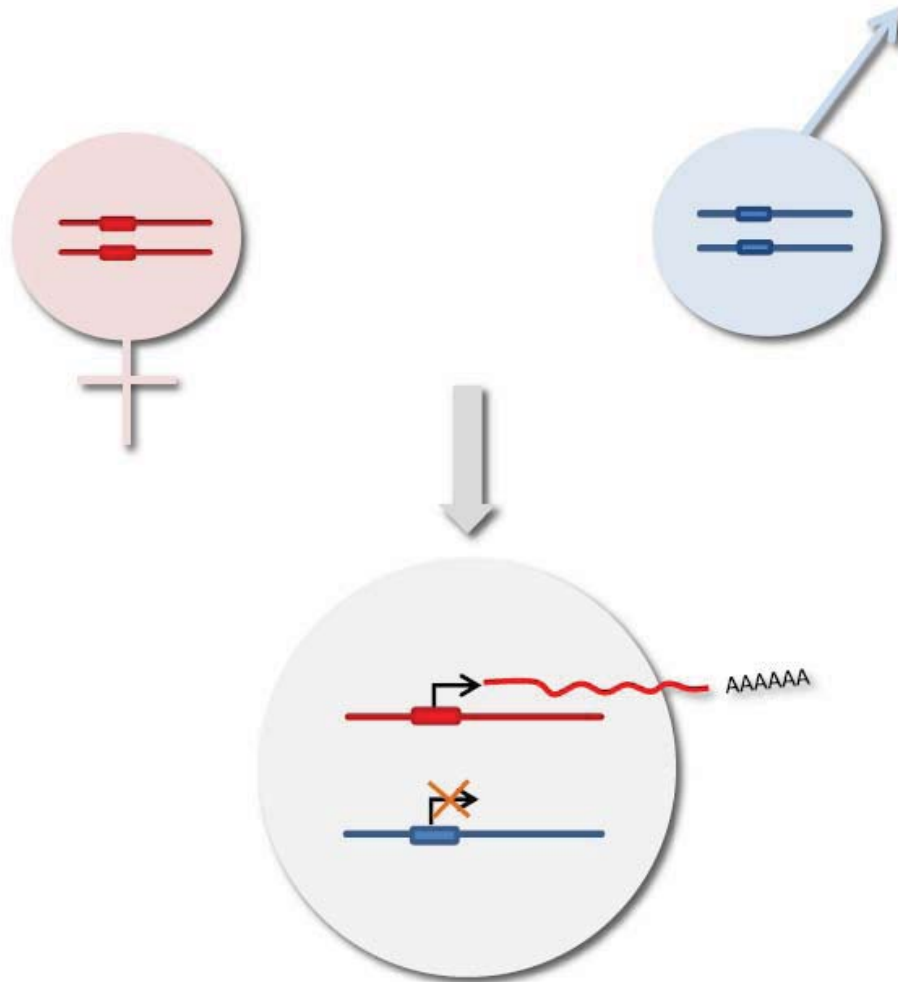
species. In the dipper, this bond is particularly strong as it was significant even though parents' telomere lengths were measured at the chick stage and not at reproduction.

Overall, our results are in accordance with previous data on the kakapo, which also displayed maternal inheritance of telomere length. This mirrors the pattern of telomere length inheritance found in most studies in humans (Nordfjäll *et al.* 2005; Njajou *et al.* 2007) and indicates the potential importance of the sex determination system in offspring telomere length determination. It has been previously suggested that the parental heterogametic sex might play a role in the pattern of telomere length inheritance (Horn *et al.* 2011).

#### *Mechanistic basis of telomere length inheritance by the heterogametic sex*

If telomere length inheritance patterns were indeed going through heterogamy, a possible mechanism that could explain it might be the implication of gene imprinting.

Parental imprinting is a process that leads to the differential expression of alleles depending on their parental origin (da Rocha & Ferguson-Smith 2004) (Fig VI.1). Genomic imprinting is explained by the parental conflict hypothesis (Moore & Haig 1991) stating that the maternal genome tends to restrain resource allocations to preserve the mother and future offspring, whereas the paternal genome tends to allocate resources to produce stronger offspring during the ongoing reproduction. Imprinting is associated with several molecular signatures which differentiate paternal and maternal inherited chromosomes to influence transcription (Bartolomei & Ferguson-Smith 2011). The processes regulating genomic imprinting involve molecular marks implicating DNA methylation and histone modifications (Pfeifer 2000). These epigenetic mechanisms influence transcription patterns and allow distinguishing paternally and maternally inherited chromosomes (Bartolomei & Ferguson-Smith 2011). Whether similar mechanisms of genomic imprinting are present in birds is still an open question (Frésard *et al.* 2013). However, studies in birds have shown that DNA methylation is also involved in the regulation of gene expression (Li *et al.* 2011), thus indicating that genomic imprinting is not to be excluded in birds (Frésard *et al.* 2013).



**Figure VI.1:** Principle of genomic imprinting (from (Frésard *et al.* 2013)). Each pair of chromosome consists of a maternal chromosome (in red) and a paternal chromosome (in blue). In this example, the offspring only expresses its maternal allele (red), since the paternally inherited allele is inactive.

A recent study has identified a paternal imprinting on sperm chromatin that is essential for the inheritance of telomere length in *Drosophila melanogaster* (Gao *et al.* 2011). Some studies have also shown that nucleotide polymorphisms in a large number of loci that code for proteins regulating DNA and histone methylation are associated with telomere length variability in humans (Gatbonton *et al.* 2006; Blasco 2007). Therefore, imprinting mechanisms might regulate expression of genes modulating telomere length (genes coding for telomerase and shelterin proteins), thus explaining the different patterns of telomere

length inheritance observed. The regulation of telomere length by epigenetic factors (Blasco 2007) also supports the idea that imprinting mechanisms might be involved in telomere length inheritance. Indeed, histone modifications and DNA methylation seem to act as negative regulator of telomere length in humans / mice (García-Cao *et al.* 2004; Gonzalo *et al.* 2006). Epigenetic factors not only impact telomeric and subtelomeric DNA, but they are also able to regulate telomerase activity (Cong, Wright & Shay 2002). The *hTERT* gene promoter has been reported, as a rare exception, to be up-regulated by methylation (Shin *et al.* 2003; Nordfjäll *et al.* 2005). Establishing the inheritance patterns of these mechanisms, especially in different classes of animals, might help better understand telomere length heredity.

#### *Alternatives to the heterogametic pattern of telomere length inheritance hypothesis*

Even though most studies conducted in humans suggest a paternal inheritance of telomere length (i.e. the mammalian heterogametic sex), some of them also display maternal inheritance (Nawrot *et al.* 2004; Broer *et al.* 2013). These contrasting results indicate that the implication of the heterogametic sex is less straightforward than initially hypothesised. In mammals, the most plausible mechanism accounting for the concordance of telomere length noted both between mothers and offspring and between fathers and offspring is an X-linked mechanism of inheritance (Nawrot *et al.* 2004). In sand lizards, which present a ZW sex determination and in which females (ZW) are the heterogametic sex (males being ZZ), telomere lengths showed differences in sex-specific heritability (Olsson *et al.* 2011a). Here, paternal and maternal telomere length transmission suggests that the genetic determinants of telomere length are Z-linked. Mechanistically, a possible explanation for X and Z linked regulation of telomere length inheritance patterns might involve differential chromosome silencing depending on paternal or maternal inheritance. X and Z linkage also suggests the potential presence of telomerase-regulating genes on these chromosomes which might be determinants of telomere length. This hypothesis is supported by the presence of telomere maintenance genes on sex chromosomes: the *DKC1* gene, which is important for telomerase activity, on the X chromosome (Mitchell, Wood & Collins 1999); and the *POT1* gene on the Z chromosome in the chicken (Wei & Price 2004).

The heterogametic pattern of telomere length inheritance observed in most studies might also be the result of confounding factors such as parental effects. Alternatively, it might be attributed to the fact that the link with the non-heterogametic sex is too weak to be detected. Investigation of telomere length inheritance patterns in more species (in complement to mammals and birds) with different sex determination systems, such as haplodiploidy (in insects) and environmental sex determination (i.e. temperature dependent in turtles), would help solve whether telomere length is determined by the heterogametic sex.

#### *Telomere length heritability in wild populations*

In accordance with Voillemot *et al.* (2012), both the king penguin and the dipper studies showed low values for telomere length heritability (respectively  $h^2=0.2$  and  $h^2=0.038$ ), which may be attributed to several possibilities.

As telomere length is suspected to be related to individual fitness (Bauch, Becker & Verhulst 2013), a possible explanation would be that natural selection has tended to deplete genetic variation of the locus regulating telomere length in early life. Alternatively, low values of heritability indicate that environmental effects explain large parts of phenotypic variation. Environmental factors may have a significance importance as determinants of early life telomere length (Jennings *et al.* 1999; Hall *et al.* 2004; Tarry-Adkins *et al.* 2008; Foote *et al.* 2011b; Geiger *et al.* 2012 - appendix 2), thereby depleting genetic influence on telomeres. In both of our studies, early life environmental factors seem to significantly influence telomere length. Indeed, in the king penguin study, we found that the link between offspring and maternal telomere length early in life disappeared over the growth period, hence suggesting that environmental factors have a strong influence on telomere loss in king penguin chicks. In addition, the dipper study shows significant effects of the year of sampling and of the nest on telomere length, which also indicates the crucial importance of environmental factors as determinants of early life telomere length.

Over all, these results show that even though genetic factors are a key determinant of early life telomere length, the exact mechanisms involved in the telomere length inheritance pattern still need a clear identification. Moreover, both studies (2 and 3) underline the prime

influence of early life environmental conditions as determinants of telomere length. Indeed, telomere length seems to be determined by the collective effects of genetic, environmental, early life experience and lifestyle factors. The question that comes next is how much do lifestyle factors contribute to telomere length differences?

#### 1.4 Importance of the extrinsic determinants of telomere length variability

The second step to assess how telomere dynamics might be involved in evolutionary trade-offs is to determine the nature of the extrinsic factors that are driving the variability observed in age-matched individuals.

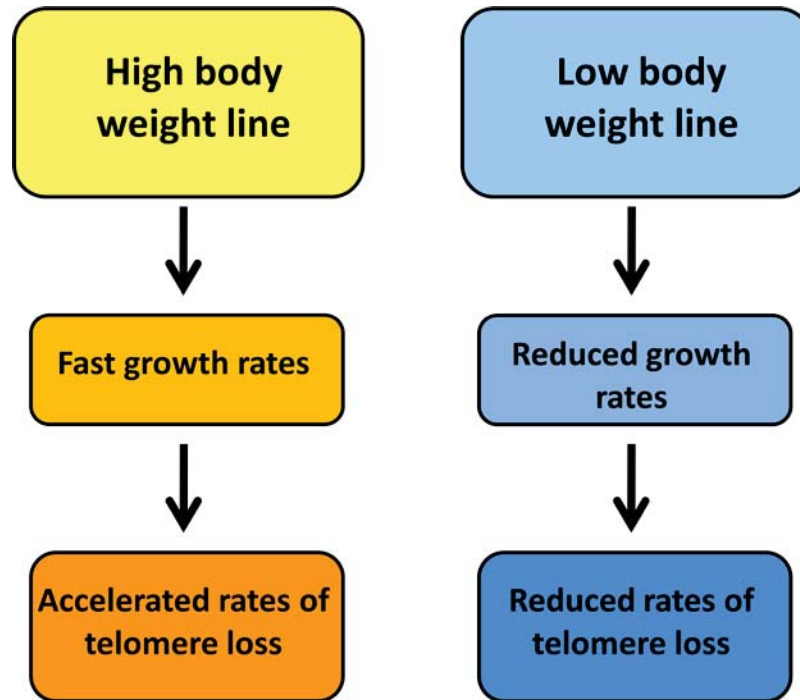
##### *Early life conditions and growth*

As most of telomere loss occurs during development and growth (Zeichner *et al.* 1999; Hall *et al.* 2004; Salomons *et al.* 2009; Foote *et al.* 2011b), conditions experienced during this time of life are expected to be of crucial importance, as bad or temporally disrupted conditions could further exacerbate this telomere loss (Tarry-Adkins *et al.* 2008; Foote *et al.* 2011b; Geiger *et al.* 2012; Voillemot *et al.* 2012). In accordance with these previous studies, the dipper data, together with our experimental manipulation of growth conditions in the zebra finch (study 4), indicate the apparent predominant influence of environmental early life conditions on telomere length, thus highlighting the importance of macro and micro environments in telomere length individual variability.

The importance of early life conditions on telomere dynamics were experimentally tested using modified growth conditions in zebra finches using clutch size manipulation. In this study, nestlings that displayed the fastest growth rates (reduced clutch-size group) were not the ones suffering the most from telomere erosion. Hence, environmental conditions in which an individual grows up, such as environmental stress (crowding, offspring-parent conflict), might have predominant effects on ageing markers compared to growth rates *per se*.

A possible way to disentangle the effects of growth rate vs. growth conditions might be to use genetic selection lines for different growth traits. Investigating telomere dynamics in chickens that have undergone long-term selection for high (HW) and low (LW) body weight

(Barbato, Siegel & Cherry 1983) might help determine the exact effects of growth rates on telomeres. If growth rates directly affect telomere length, we might expect accelerated telomere loss in fast growing individuals (Fig VI.2).



**Figure VI.2:** Use of selection lines to disentangle growth rates versus growth environmental conditions effects on telomere dynamics. If growth rates have a predominant impact, we may expect accelerated telomere loss in fast growth lines and reduced telomere loss in slow growth lines.

### *Reproductive effort*

Events over the adult lifetime might also participate to the telomere length variability among individuals. An evident costly parameter that can greatly impact telomere dynamics at that life stage is reproduction (Harshman & Zera 2007; Speakman 2008). However, evidence of costs of reproduction on telomere dynamics remains scarce and controversial (Kotrschal, Ilmonen & Penn 2007; Beaulieu *et al.* 2011; Heidinger *et al.* 2012; Bauch, Becker & Verhulst 2013). Except for Beaulieu's study (appendix 1), the impact of the cost of

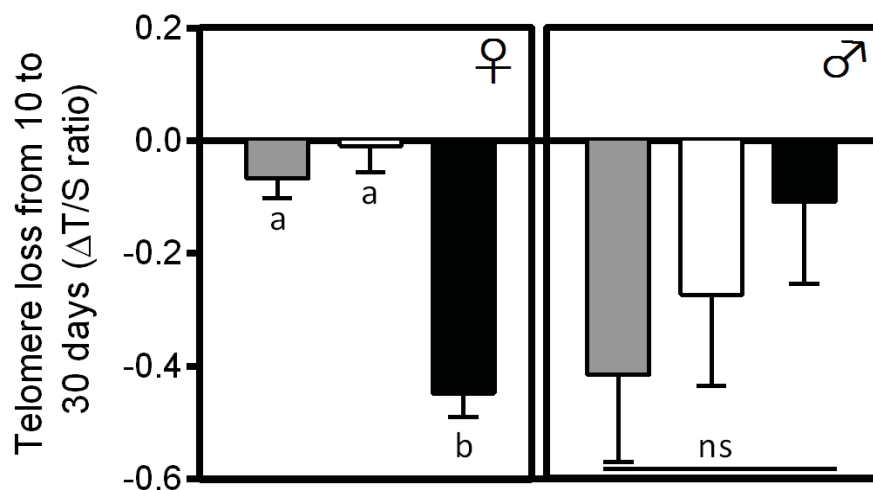


reproduction on telomere dynamics was only assessed by comparing reproductive individuals *versus* non-reproductive ones (Kotrschal, Ilmonen & Penn 2007; Heidinger *et al.* 2012; Bauch, Becker & Verhulst 2013). However, as suggested by Stier *et al.* (2012) and Metcalfe and Monaghan (2013), it is only when reproductive effort is manipulated that such a putative ageing cost can be determined. Reproductive effort can be manipulated with clutch or brood manipulations ((Alonso-Alvarez *et al.* 2004; Wiersma *et al.* 2004), study 5), with manipulation of the energetic costs of incubation by modifying ambient temperature (Nord, Sandell & Nilsson 2010), or with manipulation of foraging costs (Beaulieu *et al.* 2011). In the long-lived Adélie penguin, increased reproductive effort was not associated with changes in telomere length, as it is probably protected by increased maintenance mechanisms (i.e. high levels of antioxidants) (Beaulieu *et al.* 2011). Therefore, the pace-of-life is probably a confounding factor in this evaluation, and we expect more deleterious impact on telomere length in short-lived species or during a terminal reproductive attempt (Velando, Drummond & Torres 2006). To assess what happens in short lived species, we experimentally manipulated reproductive effort in zebra finches (study 5). We show that an increased workload led to a reduction in telomere lengths in both parents compared to control parents and to parents raising a reduced brood. This suggests that in short-lived species accelerated telomere loss might come as a cost of reproduction whereas long-lived species tend to protect and maintain their telomeres to insure individual maintenance.

#### *What about stress and hormone regulation?*

To better understand how developmental and growth conditions, and environmental factors in general affect telomere dynamics, we need to investigate the effects of other extrinsic factors such as diets, pollutants, immune challenges or, more importantly, stress (Blackburn & Epel 2012). Environmental as well as physiological stressors have been shown to accelerate telomere loss in mammals and birds (Epel *et al.* 2004; Kotrschal, Ilmonen & Penn 2007; Haussmann & Marchetto 2010; Haussmann *et al.* 2012). Haussmann *et al.* (2012) particularly emphasises that the period over which the effects of stressors are tested is important. It is usually found that the earlier an individual's life is disturbed, the stronger are the effects (Desai & Hales 1997), which highlights the importance of an optimal embryonic

growth. Indeed, embryonic growth is the period of highest cell division rate (Zeichner *et al.* 1999; Forsyth, Wright & Shay 2002), making the alteration of conditions during embryogenesis of particular importance in determining early life telomere dynamics. Studies investigating early life exposure to corticosteroids might also help to understand how maternal effects may shape offspring telomere dynamics. It is likely that maternal effects can modulate early life telomere dynamics by transferring steroids to the yolk is one way for mothers to translate environmental information into potential adaptive signal for their offspring (Hayward & Wingfield 2004). Given the effects of corticosteroids on oxidative stress (Liu & Mori 1999; Lin, Decuyper & Buyse 2004; Costantini, Fanfani & Dell'Omo 2008) and telomerase activity (Choi, Fauce & Effros 2008; Hausmann & Marchetto 2010), one might expect that maternal derived hormones may have adverse consequences on early life telomere dynamics and possibly accelerate telomere loss (Fig VI.3. Tissier *et al.* in prep, appendix 3).



**Figure VI.3:** Effect of the maternal hormonal treatment on telomere loss of chicks from  $E_2$ -treated females (grey), control females (white) and CORT-treated females (black) zebra finch.

Given that early life telomere length is a strong predictor of individual lifespan (Heidinger *et al.* 2012) establishing how early life conditions influence telomere dynamics during growth is of tremendous importance. If the mothers, through maternal effects, are also influencing

their offspring life expectancy, it reinforces the role of this non-genetic mode of information transmission over generations.

This accumulating evidence (Epel *et al.* 2004; Kotrschal, Ilmonen & Penn 2007; Haussmann & Marchetto 2010; Haussmann *et al.* 2012) highlights the great impact of hormones on telomere length regulation. Even though telomere length sex differences are not straightforward across taxa (Barrett & Richardson 2011), sex hormones (oestrogens and testosterone) are still expected to affect telomere dynamics through different mechanisms, like ROS production or telomerase activity. Oestrogen is a potent antioxidant (Sugioka, Shimosegawa & Nakano 1987), reduces ROS production (Viña *et al.* 2005) and stimulates telomerase activity (Calado *et al.* 2009). Testosterone has been shown to increase oxidative stress (Alonso-Alvarez *et al.* 2007a). This influence of sex hormones on telomere maintenance might partly explain the sexual dimorphism in telomere found in several species, the general trend being that males tend to display shorter telomeres than females (Barrett & Richardson 2011). Studies in humans (Barrett & Richardson 2011), mice (Coviello-McLaughlin & Prowse 1997), and rats (Cherif *et al.* 2003) show that males have smaller telomeres than females as adults. This pattern of sexual dimorphism was also found in ants (Jemielity *et al.* 2007) and some species of reptiles, such as the sand lizard (Olsson *et al.* 2011b) and the water python (Ujvari & Madsen 2009). However, in birds, sex difference in telomere length is less evident (Barrett & Richardson 2011).

Altogether, the results of these studies (2, 3, 4 and 5) confirm and underline the crucial importance of environmental factors as a determinant of telomere dynamics, especially during the growth period, they also support and highlight that telomere dynamics may reflect individual life events as previously suggested (Monaghan & Haussmann 2006; Lin, Epel & Blackburn 2012). As such, they appear to be a good candidate to mediate the link between lifestyle factors and individual senescence at adulthood. But can we really conclude on their role as a mechanism underlying, at least, the reproductive and growth evolutionary trade-offs?

### 1.5 Telomeres and evolutionary trade-offs

*Do our findings indicate a role for telomere dynamics in life history trade-offs?*

As mentioned in paragraph 1.2, for a mechanism to be considered as a potential functional link between life history traits, it has to fulfil certain characteristics. First of all, it has to be sensitive to environmental variations. The findings presented in the previous paragraph indicate that telomere dynamics fulfil this first requirement. Indeed, the effects displayed in study 4 (impact of growth conditions) and study 5 (impact of the level of reproductive investment) in the zebra finch on the rate of telomere loss suggest that telomere dynamics is sensitive to extrinsic variation and thus may very well be considered as one of the mechanisms linking life history trade-offs.

Secondly, for a mechanism to be considered as a functional link to underlie evolutionary trade-off, it has to reflect the impact of one trait on the other. As telomere length and telomere dynamics have been shown to be good indicators of survival and individual lifespan (Cawthon *et al.* 2003; Bize *et al.* 2009; Salomons *et al.* 2009; Heidinger *et al.* 2012), the alteration of growth conditions and its consequences on the rate of telomere loss observed in study 4 supports that telomere dynamics may reflect the impact of growth on individual longevity and provide a mechanistic link between early growth conditions and senescence, as previously suggested (Metcalf & Monaghan 2003; Monaghan & Hausmann 2006). Similarly, the influence of the level of reproductive effort on telomeres shortening rates strengthens the emerging hypothesis that telomere erosion could account for the links between high reproductive investment and longevity. However, our results only suggest an implication of telomere dynamics in these trade-offs as we found no treatment or telomere length link with mid-term survival (i.e. a year after the experiment). This issue will be further discussed in the paragraph II of this chapter.

Over all, the findings from studies 4 and 5 indicate that telomere dynamics seem to fulfil the second requirement (i.e. reflecting the impact of one trait on another). Moreover, given that individual telomere length decreases with age (Hall *et al.* 2004) and is a potential marker for individual lifespan (Heidinger *et al.* 2012), it suggests that telomere dynamics might reflect trade-offs changes over time thus fulfilling the third requirement. Altogether, this supports the idea that telomere dynamics is a good candidate to functionally link life history traits and

hence underlie evolutionary trade-offs. However, the question that remains is whether telomere dynamics are directly involved in evolutionary trade-offs or do they just reflect upstream mechanisms such as oxidative stress.

*What role for oxidative stress in evolutionary trade-offs?*

As telomeres dynamics, oxidative stress is of prime interest as it is a good candidate mechanism that might mediate life history trade-offs (Monaghan, Metcalfe & Torres 2009; Metcalfe & Alonso-Alvarez 2010). Indeed, as all activities demand energy production, they are likely to generate ROS, thus managing oxidative stress may be a major determinant of life histories. Trade-offs involving oxidative stress might occur at different time scales. The trade-off may be immediate by dedicating resources to neutralise ROS. Conversely, there may be longer term trade-offs, with ROS accelerating senescence by accumulation of unrepaired cellular damage (Monaghan, Metcalfe & Torres 2009). Over the past few years, different components of oxidative stress have been involved with several life history trade-offs (Monaghan, Metcalfe & Torres 2009). For instance, several studies have found that oxidative stress could be linked to the cost of reproduction (Alonso-Alvarez *et al.* 2004; Wiersma *et al.* 2004; Stier *et al.* 2012), with increased reproductive effort being associated with reduced antioxidant capacity. At the individual level, increased oxidative damage also appeared to be a potential cost of accelerated somatic growth in zebra finches (Alonso-Alvarez *et al.* 2006; Alonso-Alvarez *et al.* 2007b).

Therefore, given the potential role of oxidative stress in evolutionary trade-offs and its deleterious impact on telomere dynamics (Von Zglinicki 2000), it was interesting to check the relationship with telomere loss in studies 4 and 5. Surprisingly, the level of reproductive investment did not affect oxidative stress and no *in vivo* link was found between the level of oxidative stress and telomere dynamics. On the contrary, growth conditions significantly altered oxidative stress. While some methodological bias (timing of our blood sampling or the fact that individuals were fed *ad libitum*) may be responsible for this partial lack of an expected relationship between telomere dynamics and oxidative stress (Von Zglinicki 2000), our results highlight the necessity to further question the accuracy of the link between oxidative stress and telomere dynamics *in vivo* (see paragraph II). These findings also show

the necessity to consider molecular determinants of telomere dynamics when investigating its role in evolutionary trade-offs (see paragraph 1.7).

*What next?*

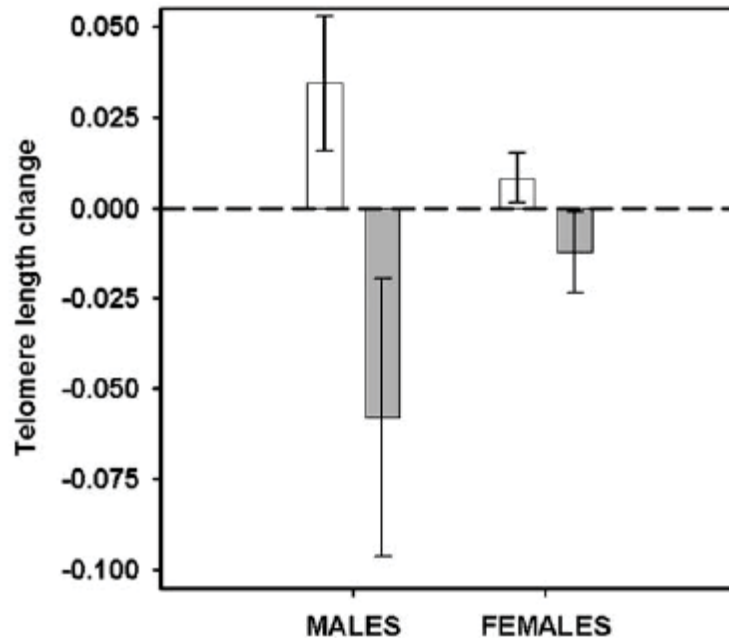
- Short-lived species versus long-lived species

To further understand to what extent telomere dynamics are implicated in evolutionary trade-offs, we need to assess how telomere dynamics might be linked to a wide range of life histories and over which time window. Moreover, as suggested by the comparison between study 5 and Beaulieu's study, short-lived and long-lived species responses to ecological and biological constraints in terms of telomere dynamics are not similar. Indeed, short-lived species might be more susceptible to pay the price of accelerated telomere loss when subjected to a constraint, whereas long-lived species would be more inclined to maintain their telomere length. A powerful way to answer these questions is to apply experimental manipulation involving longitudinal analysis.

- Telomere dynamics and self-maintenance: what about immunity?

Although the link between telomeres and growth/cost of reproduction have been given certain attention (Jennings *et al.* 1999; Kotrschal, Ilmonen & Penn 2007; Tarry-Adkins *et al.* 2008; Beaulieu *et al.* 2011; Geiger *et al.* 2012; Bauch, Becker & Verhulst 2013; Tarry-Adkins *et al.* 2013), there is very little indications on the potential implication in individual maintenance in non-human models. As suggested in the previous section, a first step would be to test the potential effects of different factors on telomere dynamics such as embryonic growth conditions, diet, pollutants or immune challenges. Testing the link between telomere dynamics and immune function in particular would be of great interest as it would give indications on whether telomere dynamics could be tied to individual maintenance. Up until now most studies investigating the relationship between immune activity and telomeres have been conducted in humans (Weng 2008; Weng 2012). For instance, short telomeres of leukocytes have been described in association with a number of immune-related diseases (Andrews *et al.* 2009). Studies of chronic viral infection also show that exhausted viral reactive T cells have very short telomeres (van de Berg *et al.* 2010). In non-humans models,

repeated experimental infection of mice with *Salmonella* triggered accelerated telomere loss (Ilmonen, Kotrschal & Penn 2008) (see Fig VI.4).



**Figure VI.4:** Consequences of infection on telomere dynamics in white blood cells from sham-infected controls (white bars) versus infected (grey bars) mice (Ilmonen, Kotrschal & Penn 2008).

To better understand how telomere dynamics could be linked to immune function, this relationship should be tested in a wider range of non-human species, which could help determine if telomere dynamics might be indicative of immunocompetence and thus reflect individual maintenance capacity. For instance future studies should examine if telomere length or telomere dynamics is associated with parasites load or the ability to mount an immune response after a specific immune challenge (with PHA or Newcastle disease virus treatment), which would give some indications on the potential link between telomeres and individual maintenance.

Indeed, clarifying the link between telomere dynamics and individual maintenance could not only help understand how telomeres are implicated in evolutionary trade-offs, but also how they might be indicative of individual state/quality and therefore of individual fitness.

### 1.6 Telomeres dynamics and individual maintenance

*What can correlative approaches tell us about fitness and individual quality?*

One of the main goals of evolutionary biology is to understand how different life history traits affect individual fitness and longevity, fitness being the resultant of individual survival and reproduction. The ability of an individual to maximise its fitness will not only depend on external circumstances, but also on the organisms' biological and physiological state (McNamara & Houston 1996; Wilson & Nussey 2010). An individual's body maintenance capacity is crucial for its fitness, and this capacity is influenced by the individual biological state (i.e. its quality: its ability to access food resources and to circumvent trade-offs). Accordingly, a way to uncover the potential link between telomere dynamics, individual quality and fitness would be to determine if telomeres are representative of individual maintenance capacities.

Over the past few years, short telomeres and increased telomere loss have been associated with high disease risks and poor individual biological state (Hausmann, Winkler & Vleck 2005; Monaghan & Hausmann 2006; Foote *et al.* 2011a; Lin, Epel & Blackburn 2012). Since diseases may reduce organisms' capacity to face environmental challenges, this suggests that telomere dynamic is linked to individual quality. Such a relationship is even more directly supported by studies showing that telomeres are associated with survival (Cawthon *et al.* 2003; Hausmann, Winkler & Vleck 2005; Bize *et al.* 2009) and reproductive success (Pauliny *et al.* 2006; Bauch, Becker & Verhulst 2013).

To have some indications of the nature of this link, we made the assumption that determining the effects of inbreeding depression on telomere length could help to further understand the relationship between telomere length and fitness (study 3). As inbreeding has negative effects on survival or fitness (Keller & Waller 2002), and since telomere length is suspected to be positively related to both, we might expect a negative relationship



between inbreeding depression and telomere length. Contrary to what we expected, we found that telomere length was positively linked to inbreeding coefficient, the most inbred dippers also presenting longer telomeres. A previous study conducted in laboratory mice displayed a positive effect of inbreeding on telomere length, but without giving any mechanistic explanation (Manning *et al.* 2002). Telomere length might be indirectly and positively affected by inbreeding coefficient because of offspring developmental history. Indeed, inbred organisms display slower embryo growth (Kulkarni & Kurian 1990), which might entail lower cell division rates, thus causing less telomere loss due to cell division. Given the lack of data on the link between inbreeding and telomeres, further studies are needed to understand inbreeding effects on telomere dynamics. For instance, investigating this relationship in laboratory inbred populations might help us uncover the ultimate causes and the proximate mechanisms implicated in this link.

Ultimately, even though correlative approaches allow us to uncover phenotypic links, they give little indications on the causal nature of the relationship and do not allow us to uncover functional links.

#### *Experimental manipulation of individual maintenance*

More and more studies give indications on a link between telomere dynamics and fitness, and thereby suggest a link to individual quality, most of the time these relationships are correlative (Pauliny *et al.* 2006; Bauch, Becker & Verhulst 2013). Consequently, to better understand these links, experimental approaches are needed to test the exact impact of inter-individual differences in telomere lengths on individual maintenance variability. Assessing the link between telomeres and maintenance is crucial to determine if telomere dynamics might somehow indicate individual quality and thus give indications on whether telomeres could be tied to individual fitness. Our experimental activation of telomerase activity (study 6 and box 1) gave some insights on the question.

Although we fail to show a link between telomere length and immunocompetence (box 1), study 6 still shows that telomere length is associated with another aspect of individual maintenance - cell regeneration. Longer telomeres were associated with a better ability of

feather re-growth. This suggests that long telomeres may be good indicators of better biological state and greater individual maintenance. These findings support the connection between telomere length and individual quality. As individual quality is closely related to individual fitness, these results also suggest a connection between telomere length and fitness.

#### *What to do next?*

Our understanding of the link between telomeres, individual quality and fitness is still at its infancy, and several questions arise from this potential relationship. Do telomeres simply reflect individual quality, in other words do they simply correlate with markers of quality and fitness, or do they have a direct effect on these parameters? To answer these questions, future experimental work should focus on determining if there is a direct causal link between telomere dynamics and different measures of individual quality and fitness, such as sexual secondary traits and lifetime reproductive success.

Another question that arises is whether this link is direct or is it mediated by other mechanisms? Indeed, even though the idea that telomere dynamics might provide an indication of individual quality and fitness is exciting, do not telomere dynamics just reflect up-stream mechanisms? As a consequence, to determine whether the link between telomere dynamics and fitness and individual quality is direct or whether it is mediated by up-stream mechanisms, more attention should be given to the molecular determinants of telomere length (oxidative stress, telomerase activity, expression of shelterin proteins).

#### 1.7 Importance of telomere length molecular determinants in evolutionary processes

To reach this objective, we need to include determinants of telomere length, both at the evolutionary trade-off level but also at the cell level. As telomere length is regulated by several molecular determinants (oxidative stress, telomerase activity, expression of shelterin proteins), can we use this single marker as an indicator for fitness and individual quality? Except for a couple of studies (Beaulieu *et al.* 2011; Geiger *et al.* 2012), in general the work in evolutionary ecology investigating the role of telomere dynamics only focuses on

telomeres and does not take into account the molecular determinants of telomere length. But given the importance of the molecular determinants in telomere regulation, taking into account some of these parameters would be of great interest.

#### *Telomerase activity*

The role of telomerase activity is not to be neglected when considering life history trade-offs; lifespan in particular. Indeed, the pattern of telomerase activity observed across taxa is partly determined by environmental stressors, suggesting that telomerase activity is likely to be one of the many factors that are to be responsible for species variation in telomere length and specific lifespan (Monaghan 2010). The pattern of telomerase activity varies greatly between species. For instance, studies in mice have shown that telomerase activity is maintained throughout adult lifespan in a number of somatic tissues (Prowse & Greider 1995; Forsyth, Wright & Shay 2002). In the species of fishes so far studied, telomerase activity seems to be high in somatic tissues at all ages (Hartmann *et al.* 2009). In birds, telomerase activity also varies between age classes and tissue types. Telomerase activity is higher in hatchlings compared to older adults and it is highest in proliferative tissues such as the bone marrow, gonads and intestines (Taylor & Delany 2000; Hausmann *et al.* 2004; Swanberg *et al.* 2010). Exceptions are the Leach's storm-petrel and common tern, which maintained telomerase activity in their somatic tissues at adulthood (Hausmann *et al.* 2007). Moreover, it was found that the long-lived species were not more likely to down-regulate telomerase compared to the short-lived species (Hausmann *et al.* 2007). Therefore, more *in vivo* studies on this topic are needed to understand the link between the substantial variation in telomerase activity, telomere dynamics and the consequences on longevity.

#### *What role for the shelterin proteins?*

Another essential component implicated in telomere dynamics regulation is the shelterin protein complex (De Lange 2005). Indeed, the shelterin complex is crucial for the stabilisation of the telomere structure. It also enables cells to distinguish their natural

chromosome ends from DNA breaks and regulates telomerase-based maintenance (Palm & de Lange 2008). Data from molecular studies show the importance of this protein complex for telomeres protection has been particularly studied in mammals (De Lange 2005; Palm & de Lange 2008) and only a few studies highlight similar results in the chicken (reviewed in Swanberg *et al.* 2010). Investigating how the shelterin complex is implicated into telomere dynamics regulation in the context of evolutionary questions could give some insights to better understand the balance of telomere erosion and repair and its link to life-histories.

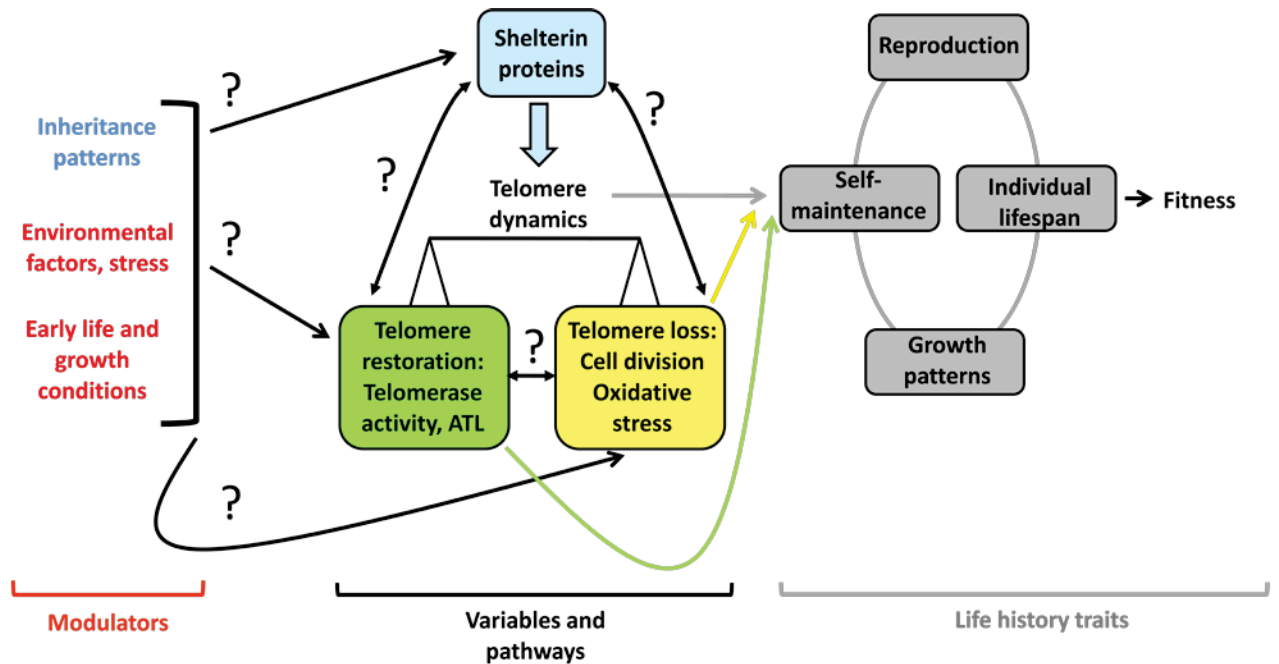
#### *Links between molecular determinants*

Data suggest that different links might exist between the several determinants regulating telomere length. Indeed, oxidative stress has been shown to affect directly telomerase activity and localisation: increased levels of enzymatic antioxidant enhancing telomerase activity (Borrás *et al.* 2004) and increased intracellular ROS leading to loss of TERT activity (Haendeler *et al.* 2004); telomerase is also transferred from the nucleus to the mitochondria under oxidative stress conditions, suggesting a protective role (Ahmed *et al.* 2008). Alternatively, telomerase activity has also been shown to be regulated by telomere binding proteins (de Lange 2002). The shelterin complex may inhibit telomerase activity. Finally, DNA oxidative damage is also prone to affect shelterin - telomere loop formation, as the presence of 8-oxoG reduces the binding affinity of TRF1 and TRF2 to telomeres (Opresko *et al.* 2005).

Altogether, these findings suggest that there are several levels of telomere length regulation and that the link between telomeres, shelterin proteins, oxidative stress, telomerase activity is more complex than initially stated. As such, this link should not be restricted to the simplistic and classical view; oxidative stress accelerating telomere loss and telomerase activity restoring telomere length. It should be kept in mind that these molecular determinants are likely to interact with one another (Fig VI.5).

Consequently, not only are these molecular determinants of great importance for telomere length regulation, they also might be a potential determinant of life histories, either in a direct manner or through their effect on telomere dynamics. Therefore, to understand how

these mechanisms are intimately linked and how they are involved in evolutionary trade-offs, it is essential when studying the role of telomeres dynamics in the evolution of life histories to consider these mechanisms at the same time and not to dissociate them from telomeres.



**Figure VI.5:** Importance of telomere length molecular determinants in evolutionary processes. Black arrows indicate intrinsic and extrinsic factors potentially influencing telomere length molecular determinants, and links between the different molecular determinants. The yellow and green arrows indicate the possible involvement of telomerase activity (green) and oxidative stress (yellow) in evolutionary trade-offs.

## II – Limits to our studies

### 2.1 Limit of the relative qPCR measure on red blood cells

All the studies of this work, as for most studies in evolutionary ecology in birds, measured telomere lengths in red blood cells. The choice of tissue type was based on different reasons. Red blood cells have been previously identified as a good candidate for telomere analysis (Hausmann & Vleck 2002) and red blood cells are an easily accessible tissue which allows repeated measures on the same individuals thus permitting longitudinal studies. However, telomere lengths vary among tissue types in several species (Forsyth, Wright & Shay 2002), therefore raising the issue of the pertinence of this measure. Partially solving this problem, study 1 shows that telomere length measures in avian red blood cells seem to be correlated to telomere lengths in several somatic tissues in adults, and thus showing that this measure can be representative of organismal telomere dynamics. Further studies should concentrate on verifying this link in early life stages and in a wider range of species. For instance, it might be interesting to study telomere dynamics in species which present negligible senescence such as turtles (Miller 2001). Do such species present lower levels of oxidative stress or higher telomerase activity that allow them to display slower rates of senescence?

Moreover, this relationship should also be tested in senescing individuals, even more so, as in the “oldest old” humans telomere length loses its predictive power for survival (Martin-Ruiz *et al.* 2005), thus suggesting that the link between telomere dynamics and biological might disappear in senescent individuals.

There is also the question of the technique used to measure telomere length. Indeed, although the qPCR presents several advantages, it measures relative telomere lengths preventing any inter-species comparisons. To overcome this issue, O'Callaghan *et al.* (2008) adapted the qPCR method described by Cawthon (2002) to measure absolute telomere length, using a standard curve established by dilution of known quantities of a synthesised oligonucleotide. A similar adaptation of this technique to non-human species would allow to measure absolute telomere length and compare results between species.

In addition, although mean telomere length quantification has been widely used in cancer, ageing and evolutionary ecology studies, recent findings suggest that the ability to measure

the abundance of short telomeres might be more informative (Hao *et al.* 2005; Vera *et al.* 2012; Vera & Blasco 2012). Indeed, the rate of increase in the percentage of short telomeres, rather than the rate of telomere shortening per month, was a significant predictor of lifespan (Vera *et al.* 2012).

Finally, the qPCR method has been validated for zebra finches by comparison with the TRF method (Criscuolo *et al.* 2009), but not for king penguins and dippers. Ideally, the qPCR protocols for these two species should be validated by comparison with TRF.

## 2.2 Limit of the oxidative stress measure

As discussed in paragraph 1.5, in studies 4 and 5, along with telomere dynamics we also assessed plasmatic antioxidant levels as well as plasmatic and DNA oxidative damage. However except for the link between DNA oxidative damage and telomere length found in the chicks (study 4), we found no *in vivo* relationship between oxidative stress and telomere dynamics. Previous studies in the penguins also investigated both telomere dynamics and oxidative stress and found contrasting results (Beaulieu *et al.* 2011; Geiger *et al.* 2012). The study conducted in adult Adélie penguins showed a link between telomere loss and plasmatic oxidative damage however this link disappeared when controlling for initial telomere length (Beaulieu *et al.* 2011). In king penguin chicks, telomere loss during growth was correlated with plasmatic oxidative damage (Geiger *et al.* 2012).

These contrasting findings raise several issues. First of all, it seems that for the moment the link between oxidative stress and telomere erosion is unsettled *in vivo* as previously suggested (Chen *et al.* 2001). For instance, studies directly manipulating oxidative stress (with a pro-oxidant treatment such as paraquat (Bus & Gibson 1984)) could help clarify the nature of the *in vivo* link between oxidative stress and telomere dynamics.

In study 4, telomere length was correlated with the levels of DNA oxidative damage. However, the same relationship was not found with plasmatic oxidative damage, which might question the pertinence d-ROM measurements. This suggests that the answer of oxidative stress to an external constraint might differ in function of the biological compartment (Selman *et al.* 2012). In addition, even though the d-ROM test has been largely used in several studies and proven to give pertinent results (Costantini & Dell'Omo 2006a; Costantini & Dell'Omo 2006b; Costantini, Fanfani & Dell'Omo 2008; Costantini, Monaghan &

Metcalfe 2011), the validity of this test has nonetheless been questioned (Harma, Harma & Erel 2006). Accordingly, it might be more informative to complete d-ROM measurement with other oxidative damage proxies such as DNA oxidative damage, when studying the link between telomere dynamics and oxidative stress.

Finally, the results displayed in study 5 highlight the importance of the moment of the oxidative stress measure. Therefore, it is essential to consider the dynamics/kinetic of response when studying the effect of a constraint on oxidative stress and its relation to telomere dynamics (Stier *et al.* 2012; Metcalfe & Monaghan 2013). Indeed, the impact of the constraint on oxidative stress may occur at a different pace than the one on telomere dynamics.

### 2.3 Lack of data on individual lifespan and LRS

As mentioned in paragraph 1.5, one of the main drawbacks of studies 4 and 5 is that we do not have any information regarding individual lifespan and we found no treatment or telomere length link with mid-term survival. This lack of data on individual lifespan prevents us linking for certain our experimental treatment to telomere dynamics and individual lifespan, and to appropriately demonstrate a clear-cut implication of telomere dynamics in these trade-offs.

In addition, even if study 6 hints that long telomeres might be representative of better individual quality, therefore suggesting a link between telomeres and fitness, we believe the only way to unambiguously validate this link is to tie telomere dynamics to lifetime reproductive success (LRS), which is also lacking in the present work.

A way to overcome this shortcoming would be to have access to long term longitudinal datasets following individual reproductive success and individual survival associated with blood sampling in order to measure telomere length. For instance, telomere length analysis coupled with life history traits analysis on the long term dipper dataset might give some further indications. A preliminary investigation of the link between telomere length, LRS and lifespan in this population revealed no relationship between these parameters.



## 2.4 Having three different models - is it an issue?

In this work, we studied diverse aspects of telomere dynamics in three different species: the king penguin, the dipper and the zebra finch. These species live in contrasting environments and present very different life-histories. The king penguin is long-lived whereas the dipper and the zebra finch are short-lived birds. At first glance, it might have been better to conduct all studies on the same species as this disparity of models might present certain disadvantages. For instance, it makes it more difficult to compare studies with one another (i.e. direct comparisons of telomere length between species).

A positive aspect of expanding the range of species studied is that more variable life-histories are covered, a necessary point that will enable the scientific community to understand how the cellular mechanisms have evolved to allow adaptation in different environments.

## **III – Future studies**

### 3.1 How to tackle the question of telomere length inheritance patterns?

To understand the importance of the genetic determinants of telomere length and the inheritance pattern of telomere length in birds, we need to examine this issue in a controlled environment. Indeed, up until now, this question was only studied in semi-controlled environment (Horn *et al.* 2011) or in wild populations (studies 2 and 3).

Most telomere loss occurs during embryogenesis (Zeichner *et al.* 1999; Forsyth, Wright & Shay 2002) and telomere length has been shown to be reset during embryogenesis in mammals (Schaezlein *et al.* 2004), suggesting that the early life period, especially embryogenesis, is of crucial importance in determining telomere dynamics. Therefore, it would be of great interest to assess the patterns of telomere lengths inheritance at this early stage of development. To tackle this question, several experimental designs are worth considering.

First of all, to avoid the potential confounding effects of parental quality and of environmental conditions on early life telomere length, cross-fostering designs might be conceivable. Indeed, as suggested by Voillemot *et al.* (2012), cross-fostering designs are better to have a clear idea of the patterns of telomere length inheritance. With this

experimental design we would avoid the biases of correlative approaches and disentangle the effects of the genetic component from the effects of the nest environment (i.e. parental quality). Cross-fostering chicks or eggs between broods would allow us to analyse whether offspring telomere length is better explained by biological parents telomere lengths or by adoptive parents telomere lengths.

Secondly, another way to overcome the confounding effects previously mentioned would be to test the hypothesis of the pattern of telomere length inheritance using artificially incubated fertilised eggs or using species for which parental care is limited/absent (i.e. in precocial species, such as chickens).

Finally, telomerase activity and the shelterin complex are paramount for telomere length regulation and determination (see paragraph 1.7). Therefore, assessing the relative implication of these parameters in telomere length inheritance must be one of the next steps that needs to be explored. In fact, such a study is at the moment being undertaken as part of my thesis work. Unfortunately, the patterns of inheritance of shelterin proteins and telomerase RNA expression are not yet resumed.

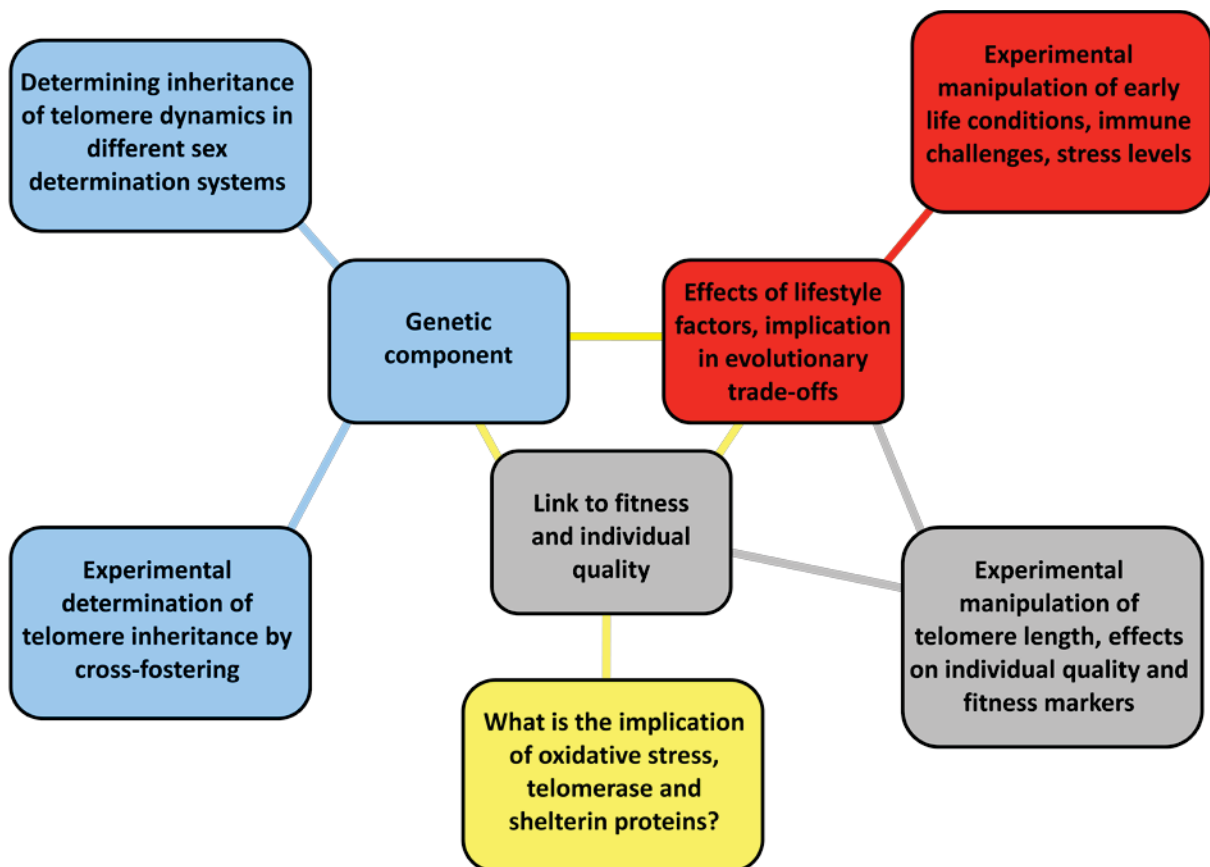
### 3.2 How to link telomere dynamics to fitness and individual quality?

Most studies suggesting a link between telomeres and fitness or individual quality are correlative. One way to compensate for this problem would be to experimentally and directly manipulate telomere length from an early stage and to tie telomere length to different parameters of individual performances.

Different approaches might be used to manipulate telomere length. For instance, artificial selection is a powerful tool to investigate and understand evolutionary processes (Conner 2003). To perform artificial selection, the trait of interest is measured in a population and individuals presenting the most extreme values are bred to produce the next generation. Therefore, in our case, individuals with extreme telomere lengths (long or short) would be selected to create two selection lines: “short telomeres” and “long telomeres”. However, even though artificial selection is very powerful, this type of experiments is not always practical, particularly in birds with relatively long generation times, as it can require a lot of time to create the different lines (Conner 2003).

Telomere length manipulation can also be achieved by treating the animals with telomerase activators (i.e. TA-65, as displayed in study 6; or TAT2, (Fauce *et al.* 2008)) and with telomerase inhibitor (GRN163L, see Herbert *et al.* 2005). By following a similar design as the one proposed in study 6, individuals would be treated during the growth period to create a “long telomeres” group and a “short telomeres” group and followed from birth to death. To link telomere dynamics to individual fitness, different parameters of individual performances would be measured: growth rates, flight performances, secondary sexual signals, immunity, reproductive success and lifespan.

#### IV – General conclusion



**Figure VI.6:** Future directions for understanding the role of telomere dynamics in evolutionary trade-offs.

The work presented here aimed to identify the determinants of telomere dynamics and to understand how telomere dynamics could underlie life history trade-offs. Identifying the mechanisms involved in evolutionary processes is considered crucial for our comprehension of life histories evolution. In this context, there is clearly a great potential in the study of telomeres. Indeed, measures of telomere dynamics may provide an indication on individual lifestyle and individual history (*via* the effects of environmental factors) and therefore offer a way to measure individual quality.

Indeed, several clues indicate that telomere dynamics might be a good candidate to functionally mediate evolutionary trade-offs. First of all, we know that telomere length is determined by the effects of genetics, but it is also affected by environmental and lifestyle factors. Therefore, telomeres being a heritable character can be a potential target of natural selection which could lead to an evolutionary response. Furthermore, as telomere length is sensitive to environmental variations it is prone to reflect the impact of one trait on the other. In addition, the dynamic aspect of telomere length regulation suggests that this mechanism may allow us to quantify the change of evolutionary trade-offs over time. Finally, evidence is accumulating that telomere dynamics could be linked to individual fitness and individual quality. Altogether, these features highlight that telomere dynamics is a potential mediator for evolutionary processes.

Nonetheless, our understanding of the role of telomere dynamics in evolutionary processes is still at its beginning and several questions remain to be answered (Fig VI.6). More work is needed to assess which extrinsic factors are important, how these effects change over the different stages of life and how it varies across taxa. Indeed, experimental approaches involving longitudinal studies with individuals under different environmental conditions (stress, immune challenge, pollutants etc) would help uncover causal links. This type of approaches is also needed to determine if telomeres reflect individual quality.

In addition, studies in a greater diversity of animals would be beneficial in order to broaden the range of life histories covered. Comparing species with variable life histories could provide information on novel mechanisms that have evolved to enable organisms to avoid the costs associated with high cell division rates and/or high levels of oxidative stress. It would also allow us to examine how the balance between telomere restoration and telomere loss is set across life stages in different taxa. Indeed, as mentioned previously, mechanisms involved in telomere length regulation, such as oxidative stress and telomerase

activity and shelterin proteins expression, need to be considered more in telomere dynamics studies.

To conclude, linking telomeres biology and more generally molecular mechanisms to evolutionary questions is exiting and promising, as it may further our understanding of life history strategies. However, one has to keep in mind that ageing is an integrative process involving several mechanisms and that considering only one of them independently of the others might be too simplistic. Indeed, the key to our understanding of the ageing process might be to consider a more global (and thus more complex) model which would integrate the different mechanisms suspected to be involved in organisms' ageing (telomeres, telomerase, shelterin proteins, oxidative stress and its link to mitochondrial activity or well-known ageing pathways like IGF1). Determining how all these pathways interact may enable us to have a more integrated view of ageing rate evolution.





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## Résumé de la thèse

Une des questions fondamentales de la biologie évolutive porte sur l'identification et la compréhension des mécanismes sous-tendant les processus évolutifs et l'évolution des compromis entre les traits d'histoire de vie. En effet, les ressources, ainsi que le temps nécessaires à leur acquisition, sont limités. De ce fait, la théorie des traits d'histoire de vie prévoit des compromis d'allocation de ces ressources entre les différents traits d'histoire de vie.

Dans ce contexte, les mécanismes impliqués dans le vieillissement sont susceptibles d'avoir une importance considérable, pour comprendre comment des compromis se sont mis en place. Parmi ces mécanismes, les télomères suscitent un intérêt tout particulier.

Les télomères sont des séquences d'ADN non codant formées d'un motif répété un grand nombre de fois, et localisées à l'extrémité des chromosomes des cellules eucaryotes. La perte des séquences télomériques, au cours du temps, limite le nombre de divisions qu'une cellule peut entreprendre, et régule, à l'échelle de l'organisme, la dynamique de perte et de renouvellement cellulaires. Les télomères participent ainsi à la régulation de la durée de vie des cellules, et vraisemblablement des individus. La plus grande partie de la recherche consacrée aux télomères a été réalisée dans un contexte cellulaire et médical, principalement axé sur l'homme ou la souris. A l'échelle de l'organisme entier, la taille moyenne des télomères diminue avec l'âge. Cependant, cette longueur télomérique varie également entre les individus d'une même espèce, ce qui signifie que des individus de même âge peuvent présenter des télomères plus ou moins longs. Cette variation entre individus de même âge, suggère que l'érosion de la longueur des télomères reflète plus que les effets de l'âge, mais que celle-ci est susceptible de donner des indications concernant le mode de vie et l'état physiologique des organismes.

Le but de mon travail de thèse a été de comprendre l'incidence de cette variabilité :

Quelles en sont les causes (nature des activités et des mécanismes favorisant la perte télomérique) et les conséquences (quel lien avec les performances des individus et leur longévité) ?

Ainsi, la vitesse à laquelle les télomères sont perdus, ainsi que leur taille résiduelle, seraient des indicateurs de la qualité des individus et de leurs chances de survie future. Tous ces travaux récents ont ouvert un nouvel axe de recherche, qui vise à intégrer les connaissances acquises sur les télomères en biologie cellulaire, dans un contexte écologique et de biologie évolutive. Cela a conduit au développement récent d'une approche intégrative du rôle de la dynamique des télomères, comme mécanisme sous-jacent des compromis évolutifs. Cependant, de nombreuses questions restent en suspens.

#### Questions posées et modèles biologiques :

La taille des télomères est-elle une caractéristique héritable, et si oui, quel est l'impact de cette héritabilité sur la croissance et la survie du jeune oiseau ? Quelle est la résultante d'une perte faible ou importante des télomères sur la fitness des individus ?

En quoi le taux de perte des télomères peut-il être affecté par des facteurs environnementaux ? Quel est le rôle des mécanismes participant à l'intégrité des télomères, tels que l'activité de la télomérase ou de l'expression des protéines associées aux télomères ?

Afin de répondre à ces questions, nous travaillons sur trois modèles d'oiseaux :

- sur le manchot royal (*Aptenodytes patagonicus*)
- ainsi que sur le cincle plongeur (*Cinclus cinclus*) en collaboration avec l'Université de Zürich, pour l'étude en condition naturelle de l'héritabilité de la taille des télomères ;
- sur le diamant mandarin (*Taeniopygia guttata*) en captivité (soumis à des protocoles expérimentaux de manipulation de la taille des télomères) pour mettre en évidence les effets de différents facteurs extrinsèques.

#### Principaux résultats :

Dans un premier temps, nous avons cherché à déterminer l'héritabilité de la longueur des télomères en travaillant sur deux populations naturelles : une population de manchots royaux et une population de cincles plongeurs. En effet, bien que plusieurs études

aient mis en évidence l'héritabilité de la longueur des télomères chez l'homme, seule une étude s'est intéressée à l'oiseau. Celle-ci montre que, contrairement à ce que l'on trouve chez l'homme, la longueur des télomères chez l'oiseau semble être héritée par la mère. Grâce à notre étude, nous confirmons ce résultat. En effet, nous trouvons une corrélation positive entre la longueur des télomères des poussins et celle de la mère. Ces résultats indiquent que chez les oiseaux, de même que chez l'homme, la longueur des télomères semble être transmise par le sexe hétérogamétique.

Dans un deuxième temps, nous avons cherché à mettre en évidence quels étaient les facteurs influençant la dynamique d'érosion des télomères, et plus particulièrement, s'il y avait un lien entre les conditions de croissance et la perte télomérique pendant la croissance d'une part, et d'autre part, s'il y avait un lien entre investissement reproducteur et perte télomérique chez l'adulte. Afin de répondre à ces questions, une expérience de manipulation de la taille de la nichée a été mise en place. La manipulation de la taille de la nichée a permis d'altérer les conditions de croissance des poussins, et de modifier l'effort de reproduction produit par les parents, soit en le diminuant (réduction de la taille de la nichée), soit en l'augmentant (augmentation de la taille de la nichée).

Dans le cadre de cette expérience, la manipulation de la taille de la nichée a induit une modification du taux de croissance des poussins. Les poussins, issus d'une nichée de taille réduite, présentent une croissance optimale et un faible taux de raccourcissement des télomères, alors que les poussins, issus d'une nichée de taille augmentée, ont une croissance plus lente et plus longue, ainsi qu'un taux plus important d'érosion des télomères.

En ce qui concerne les parents, nous avons déterminé les effets de la variation de l'effort reproducteur sur la masse, sur le stress oxydant et sur la dynamique d'érosion des télomères. Les résultats de cette expérience indiquent qu'il n'y a pas d'effet de l'effort reproducteur sur la masse des parents. En effet, les individus, dont la taille de nichée a été augmentée, ne présentent pas de perte de masse plus importante que ceux dont la taille de nichée a été diminuée. En revanche, on observe un effet de l'effort de reproduction sur la dynamique d'érosion des télomères, les parents dont la taille de la nichée a été augmentée ayant une érosion des télomères plus importante que les autres. Cette première étude nous a permis de mettre en évidence qu'une augmentation du coût de la reproduction entraîne

une accélération de la perte télomérique, ceci étant une première explication mécanistique du coût de la reproduction en termes de réduction potentielle de la longévité chez l'adulte. L'ensemble de ces résultats nous a permis de mettre en évidence certains facteurs susceptibles d'influencer la dynamique d'érosion des télomères. En effet, nous montrons ici que le coût de la reproduction, ainsi que la modification des trajectoires de croissance, entraînent une variation du taux de perte télomérique.

Enfin, dans le but de poursuivre ces recherches sur le lien entre télomères et maintenance de l'organisme (en utilisant la régénération cellulaire comme indicateur de la maintenance), nous avons mis en place un protocole qui a permis de manipuler expérimentalement la taille des télomères. Cette expérience a permis de mettre en évidence le fait que les individus ayant de longs télomères, sont aussi ceux dont la régénération cellulaire est la plus rapide, indiquant ainsi un lien direct entre la longueur des télomères et la maintenance individuelle, - ce qui suggère que les télomères sont susceptibles de donner des indications sur la qualité des individus.

En conclusion, les résultats de ce travail de thèse démontrent que la dynamique des télomères est un mécanisme sous-jacent des compromis évolutifs, et de ce fait, présente un intérêt considérable pour la compréhension des processus évolutifs.





## Appendices





## **Appendix 1 –**

Oxidative status and telomere length in a long-lived bird  
facing a costly reproductive event



# Oxidative status and telomere length in a long-lived bird facing a costly reproductive event

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## Summary

1. Life-history theory predicts that high reproductive investment alters self-maintenance. Several mechanisms underlying the cost of reproduction have been previously suggested, but how parental effort may impact cell and organism maintenance remains largely unknown. The effects of oxidative stress – the imbalance between oxidative damage and defences – on telomere dynamics may underlie this relationship. Indeed, oxidative stress is associated with costly activities like breeding, and impacts telomere length that is known to predict survival in birds. According to life-history theory, long-lived species are expected to minimize the adverse effects of current reproduction on their body maintenance and should therefore enhance their antioxidant capacity and preserve their telomeres when breeding workload increases.

2. In this study, we tested this hypothesis by determining experimentally how the oxidative status and telomere length were modified when long-lived Adélie penguins (*Pygoscelis adeliae*) faced a costly reproductive event. The breeding workload was increased through a handicapping procedure that increased the cost of foraging and therefore chick-provisioning.

3. In agreement with our hypothesis, Adélie penguins substantially increased their antioxidant defences during a costly breeding effort, while oxidative damage and telomere length remained unchanged.

4. As expected in long-lived species, Adélie penguins subjected to increased breeding constraints appear to prioritize self-maintenance as shown by their increased antioxidant capacity. Moreover, the absence of effects of our experimental procedure on telomere length suggests no apparent impact of breeding workload on the senescence of this long-lived bird. However, to better understand the role of the couple 'oxidative status/telomeres' in the regulation of life-history strategies, further studies should examine: (i) the nature and the cost of additional antioxidant protection; (ii) the changes in the oxidative status of animals throughout their annual cycle and the consequences on telomere dynamics; and (iii) the repartition of antioxidant resources between young and parents.

**Key-words:** Adélie penguin, breeding effort, long-lived species, oxidative stress, telomeres

## Introduction

Individuals have to deal with limited resources to support competing life functions (Stearns 1992). Because of this energetic conflict, living organisms may be constrained to reallocate resources under specific circumstances to a given function at the expense of another one. These trade-offs are a fundamental mechanism driving the evolution of diversified life histories observed in the wild (Roff 2002). The

trade-off between reproduction and self-maintenance represents the best illustration of this allocation conflict, with an increased breeding effort being associated with a subsequent lower fecundity or survival (Gustafsson & Pärt 1990; Reed *et al.* 2008). However, even though many studies focus on the conflict between reproduction and survival (e.g. Sanz, Kranenborg & Tinbergen 2000; Golet *et al.* 2004; Navarro & González-Solís 2007), we still only have a rough idea about the nature of the underlying physiological mechanisms. To buffer the deleterious effects of increasing reproductive demands on self-maintenance, adults can modulate five physiological components: secretion of hormones with pleiotropic effects, intermediate metabolism

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and fuel allocation, immune function, mating costs and defence against stress (reviewed in Harshman & Zera 2007). Ultimately, some of these physiological processes are likely to act directly and negatively on subsequent fecundity and longevity by accelerating ageing rates (Partridge & Gems 2006).

In this context, one proximate factor that has recently been explored is oxidative stress (Monaghan, Metcalfe & Torres 2009), that is, the imbalance between the production of reactive oxygen species (ROS) and the antioxidant capacity of the organism (Finkel & Holbrook 2000). ROS production mainly results from mitochondrial metabolic processes that consume oxygen and may damage proteins, lipids and DNA and generate reactive oxygen metabolites (ROM). To overcome this potential damage, living organisms are able to raise an antioxidant barrier, consisting of exogenous diet-derived (e.g. vitamin E, carotenoids) and endogenous (e.g. uric acid, superoxide dismutase, glutathione peroxidase) molecules converting ROS into less reactive molecules (Vleck, Haussmann & Vleck 2007; Cohen & McGraw 2009). As a result, antioxidant levels are expected to track ROS production. In the short-lived zebra finch *Taeniopygia guttata*, it has been shown that reproduction decreases antioxidant defences, illustrating that oxidative stress may mediate the cost of reproduction (Alonso-Alvarez *et al.* 2004; Wiersma *et al.* 2004). In addition, besides its implication in reproduction, resistance to oxidative stress has also been related to higher survival rates in breeding male Alpine swifts *Apus melba* (Bize *et al.* 2008). Oxidative stress therefore appears to be an important mediator between reproductive effort and the probability to survive.

The link between oxidative stress and self-maintenance may be explained by the deleterious effects of free radicals on key biological molecules (i.e. oxidative damage). These effects are considered as the principal proximate mechanism explaining cell senescence (Beckman & Ames 1998). Oxidative stress triggers cell death *via* membrane damage (cell necrosis) or by activating pro-apoptotic cellular pathways (Finkel 2003). This activation may be triggered by the disruption of DNA chromosome end structures, telomeres (Von Zglinicki 2002). Telomeres are well conserved non-coding sequences of a repeated motif  $(T_2AG_3)_n$  that cap the ends of chromosomes and stabilize the genome (Blackburn 2000; Monaghan & Haussmann 2006). Telomeres shorten because of the end-replication problem of the 5' → 3' template strand (Blackburn 1991), and this erosion can be accelerated by different stress factors (Epel *et al.* 2004) such as oxidative stress (Von Zglinicki 2002; Richter & Von Zglinicki 2007). Once telomere length reaches a critical lower threshold in absence of appropriate maintenance responses (Blackburn 2000), replicative senescence and apoptosis occur. As a result, telomere length can be used at the organism level, as an index of the biological (rather than chronological) age, 'indicative of the current position of the individual in its journey through life' (Monaghan & Haussmann 2006). Telomere dynamics (the rate at which telomere sequences are lost with time) have recently been described as a better predictor of adult survival than age

in wild populations of Alpine swifts and jackdaws *Corvus monedula* (Bize *et al.* 2009; Salomons *et al.* 2009). Therefore, if individuals with short telomeres are less likely to survive, the acceleration of telomere shortening rate, after a costly breeding event, may be a good candidate to explain how current reproduction alters adult self-maintenance and survival. However, there are no studies to date that experimentally tested whether increased breeding effort may actually modify telomere dynamics.

Even though evidence is accumulating to implicate oxidative stress in the trade-off between reproduction and self-maintenance, most of the work has been done on passerines which are short-lived birds that compromise oxidative defences when faced with increased reproduction workload (Alonso-Alvarez *et al.* 2004; Wiersma *et al.* 2004). According to evolutionary theory, long-lived birds should conversely avoid long-term impact on adult survival and therefore sacrifice current reproduction and prioritize self-maintenance. Accordingly, long-lived birds should exhibit higher oxidative defences in response to increased breeding effort and ROS production, and no acceleration in telomere loss should be detected.

To test this hypothesis, the breeding effort of adult Adélie penguins (*Pygoscelis adeliae*; Fig. 1) was modified by increasing the cost of foraging, since that is the most costly breeding activity in this species (Chappell *et al.* 1993). We increased breeding constraints by reducing the hydrodynamic properties and therefore the foraging efficiency of these streamlined animals. As expected, handicapped penguins expended a higher effort foraging at sea than their partners and control birds, as demonstrated by 70% longer foraging trips and



**Fig. 1.** Adélie penguin on its nest (copyright permitted by Michaël Beaulieu).

60% higher body mass loss (Beaulieu *et al.* 2009). In addition, handicapped parents sacrificed their current reproduction since their reproductive success and the body mass of their chicks were significantly decreased by our experimental procedure (Beaulieu *et al.* 2009). The impact of parental workload level was followed in a longitudinal study implying repeated blood and fitness measurements on the same individuals. During the subsequent breeding season, the lower resight rate of handicapped males and the decreased fecundity of handicapped females (Beaulieu *et al.* 2009) indicated that our experimental procedure had increased the cost of reproduction. Provided that handicapped penguins exhibited a higher breeding effort, we expected their production of ROS to be increased. These birds may then show either: (i) higher rates of telomere shortening or (ii) enhanced antioxidant capacities to buffer any deleterious impact on telomeres. According to life history of long-lived animals (Stearns 1992), we predict that long-lived Adélie penguins should set-up efficient antioxidant networks that will favour adult maintenance.

## Materials and methods

### FIELDWORK

The study took place in Dumont d'Urville (66°40'S; 140°01'E), Adélie Land, Antarctica during austral summers 2006–2007 and 2007–2008.

In 2006–2007, at the end of the courtship period (from 11 November to 15 November), 46 pairs of Adélie penguins were identified with a subcutaneous transponder and a symbol painted on their chest feathers with Nyanzol-D. Among this group, 30 penguins from 30 different pairs were equipped with rectangular cuboids (25 × 35 × 60 mm, 60 g), attached to the middle-back feathers to increase the cost of foraging. Diving behaviour of instrumented penguins is negatively impacted when the cross sectional area (CSA) of the equipment represents > 1% of the animal's CSA (Culik & Wilson 1991). Here, penguins were handicapped with instruments representing ~4% of their CSA; this size is within limits such that birds were not overly disadvantaged (see Beaulieu *et al.* 2009). The remaining 16 pairs were used as controls. Birds were captured a second time during the breeding season while rearing chicks (40–45 days after laying, from 23 December to 4 January), after a nest relief and just before leaving the colony to forage at sea. Sex was determined *a posteriori* by using a combination of parameters including cloacal inspection before egg laying, copulatory position and incubation routine (Taylor 1962; Kerry, Clarke & Else 1993). We were thus able to determine that 17 males and 13 females had been handicapped. Each time the penguins were captured, they were weighed with an electronic balance (Ohaus, ± 2 g) so that we were able to quantify body mass loss between the courtship and chick-rearing periods (data already published in Beaulieu *et al.* 2010a). The rectangular cuboids were left on handicapped penguins during the entire breeding season, and were naturally shed at the next moult (February–March). No bird lost the handicap during the breeding season.

In 2007–2008, at the end of the courtship period (from 12 November to 16 November), all the nests of the colony were checked with a hand held antenna to find experimental birds identified with a transponder the previous year. Surrounding colonies were also checked but no identified bird was found in these colonies. Even if some female penguins moved from a nest to another, all the identified penguins

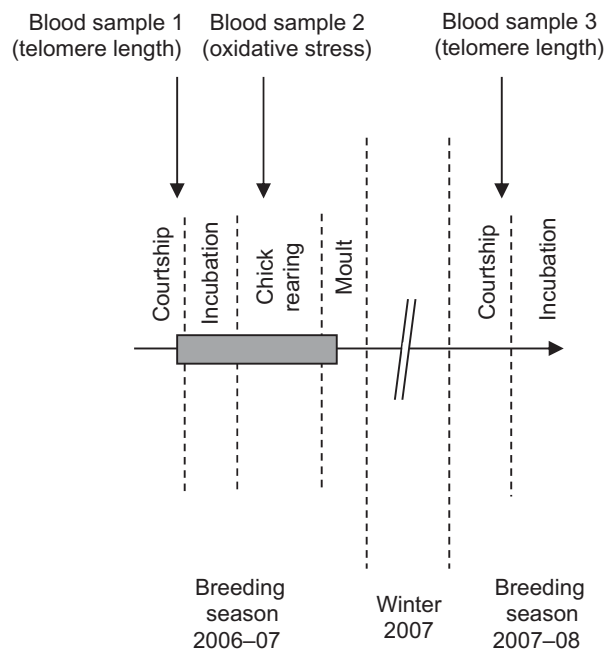
were found in the same colony. They were visually identified as described above and the sex determination carried out in 2006–2007 was re-confirmed in 2007–2008.

The return of penguins to the colony was also examined in 2008–2009 and 2009–2010. We considered penguins dead when unsighted during the three consecutive years.

Blood was collected from the wing vein with a heparinized syringe just after each capture, i.e. before treatment (courtship period in 2006–2007), during treatment (chick rearing 2006–2007) and after treatment (courtship 2007–2008; Fig. 2). After centrifugation, plasma and red blood cells were separated in Eppendorf tubes and stored at –20 °C until laboratory analyses. Blood was collected during the treatment for birds that were still breeding 40–45 days after egg-laying in 2006–2007 and for which we had adequate blood volume to measure parameters of oxidative status ( $n = 60$  birds) in addition to other physiological parameters (see Beaulieu *et al.* 2010a). Telomere length was measured in birds for which we had enough red blood cells for this measurement and telomere loss was measured only in birds that returned to the colony in 2007–2008 ( $n = 72$  birds in 2006–2007 and  $n = 51$  birds in 2007–2008).

### LABORATORY ANALYSES

Analyses of oxidative stress and telomere length were carried out at the IPHC-DEPE, France. To measure oxidative stress in plasma samples, we used the d-ROMs and the OXY-ADSORBENT tests (Diacron International, Grosseto, Italy). The d-ROM test measures the concentration of hydroperoxide, a ROM resulting from the attack of ROS on organic substrates, while the OXY-ADSORBENT test measures the total antioxidant capacity of plasma (for details on the procedure, see Beaulieu *et al.* 2010b). Moreover, plasma antioxidant capacity may be correlated with plasma uric acid levels, an end-product of protein metabolism in birds (Cohen & McGraw 2009). Its blood level directly reflects dietary input of purines and muscle



**Fig. 2.** Consecutive breeding cycles of Adélie penguins in 2006–2007 and 2007–2008 and study protocol. The grey area represents the period when penguins were instrumented.

catabolism. As our handicapping procedure clearly affected the feeding behaviour of penguins, increased body mass loss and hence probably muscle catabolism (Beaulieu *et al.* 2010a), it is likely that the antioxidant capacity of handicapped penguins was modified because of an increase in uric acid levels. Consequently, we also measured the concentrations of uric acid in the plasma of penguins, using enzymatic colorimetric tests (Sigma diagnostic, St. Louis, MO, USA).

Telomere measurements were conducted following the procedure described by Criscuolo *et al.* (2009) and used by Bize *et al.* (2009). DNA was extracted from 5 µL of red blood cells using a commercial kit (DNeasy® Blood and Tissue kit, Qiagen, Germany). Quantitative real-time amplification (qPCR) of the telomere sequence, initially set-up on humans (Cawthon 2002), has been adapted to birds (Criscuolo *et al.* 2009). Briefly, this method is based on the determination of a number of amplification cycles necessary to detect a lower threshold of fluorescent signal, the cycle number being proportional to the telomere length (T), or to the number of copies of a control gene (S). A ratio (T/S) of telomere repeated copy number (T) to single control gene copy number (S) is then calculated for each sample that will reflect relative inter and intra-individual differences in telomere length. For a detailed description of the analysis of qPCR results, refer to Criscuolo *et al.* (2009). As a single control gene, we used *P. adeliae* zinc finger protein (ZENK, Chubb 2004), with primer sequences defined using Primer 3 software (Adel1: 5'-CAACTGCCGTTTAAAGTTTTTC-3'; Adel2: 5'-AATATGGCCCTGCAAA TTCC-3'). Primer sequences for telomere amplification were Tel1b (5'-CGGTTTGGTTGGGTT-TGGGTTTGGGTTTGGGTTTGGGTT-3') and Tel2b (5'-GGC-TTGCCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'). Telomere and control gene PCR conditions were 10 min at 95 °C followed by 30 cycles of 30 s at 56 °C, 30 s at 72 °C and 60 s at 95 °C. qPCR amplicon sizes were determined after electrophoresis on a 1.5% agarose gel run in standard TBE (Tris/Borate/EDTA) buffer (90 V for 10 min and 130 V thereafter for 30 min) and using ethidium bromide staining. Amplification of the zinc finger protein gene led to an expected product size of 250 kb, predicted by primer alignment of the ZENK gene sequence. Telomere amplification produced a smear of several amplicons with a darker band between 50 and 100 bp, the lowest predicted size given by the primer sequences (78 bp, Cawthon 2002). Amplification of this shortest amplicon was proportional to the total sample telomere length. Telomere and ZENK real-time amplifications were carried out in duplicate on each plate and twice on two different plates and the mean values of the four measurements of telomere and control gene were used to calculate the final T/S ratio for each sample. For each qPCR run, we confirmed that the amplification efficiency was between 95% and 105% (telomere, mean 101.8, range 95–105; ZENK, mean 97.8, range 92–105), using the dilution curve calculation method (Larionov, Krause & Miller 2005), and inter-plate standardization was achieved with a reference bird sample in each qPCR (Criscuolo *et al.* 2009). Mean coefficient of variation was 2.92% for the telomere essays and 2.21% for ZENK essays. Telomere shortening rate was obtained on a 1-year scale, by subtracting the telomere length after treatment to the telomere length before treatment (Fig. 2). One limitation of this method is that birds have interstitial telomeric sequences in the centromeric region which are targeted by qPCR primers and may confound the measure of telomere length. However, even though it has not been demonstrated in Adélie penguins, we previously validated the qPCR method against absolute measurements of telomere length in two different bird species: the zebra finch and the Alpine swift (Criscuolo *et al.* 2009). Moreover, the telomeric sequences in the centromeric region are not affected by ageing and can thus be neglected when examining the change in telomere length within an individual.

## STATISTICAL ANALYSES

Male and female Adélie penguins allocate energy differently during reproduction: males' metabolic expenditure is higher than that of females over the breeding season but the reproductive effort associated with feeding chicks is lower in males than in females (Chappell *et al.* 1993). Moreover, the inter-annual survival is higher in males than in females after a breeding attempt (Ainley & DeMaster 1980). For these reasons, we could expect oxidative status and telomere dynamics to be sex-biased in Adélie penguins. Consequently, the factor 'sex' was included in every statistical model. To analyse whether oxidative status, uric acid levels and telomere length were modified by the breeding effort and were related to return rates the subsequent year, we used general linear mixed models (GLMM) with the sex of the birds, the treatment [control, handicapped or partner of handicapped individuals (further referred to as partners)], the return to the colony the subsequent year [coded as 0 (no return) and 1 (return)] and their interaction as fixed factors. We differentiated the partners of handicapped penguins from control penguins since their time-budget was also affected by the experiment. Indeed, because of the longer foraging trips of handicapped penguins, partners had to fast longer on the nest than control penguins (Beaulieu *et al.* 2009). In addition, we previously showed that partners modified their foraging behaviour compared to control penguins and exploited the same niche as handicapped penguins (Beaulieu *et al.* 2010a). Moreover, since we previously found that the oxidative status was positively correlated between males and females belonging to the same control pairs of Adélie penguins (Beaulieu *et al.* 2010b), the two parents cannot be considered as independent and we consequently added the identity of the pair as a random factor in the models. For initial telomere length, we run a GLMM including all the individuals ( $n = 72$ ) and another GLMM with the individuals for which the oxidative status was also measured ( $n = 60$ ). We also checked whether the initial telomere length was related with the rate of telomere shortening and the oxidative status with Spearman correlations. When the initial telomere length was correlated with the change in telomere length or the oxidative status, it was added as a covariate in the GLMM. The residuals of all the models were normal (test of Shapiro-Wilk). All analyses were conducted using SPSS 16.02 (SPSS Inc., Chicago, IL, USA). Results are expressed as means  $\pm$  SE and significance level was set at  $\alpha = 0.05$ .

## Results

### INITIAL TELOMERE LENGTH

Initial telomere length did not significantly differ between control, handicapped and partner birds (T/S ratio:  $1.04 \pm 0.10$ ,  $0.93 \pm 0.09$  and  $0.65 \pm 0.06$ , respectively; Table 1), all groups starting then the experiment with comparable mean telomere length. Initial telomere length was not related to the return rate to the colony the subsequent year [T/S ratio:  $0.91 \pm 0.11$  (non-return) and  $0.87 \pm 0.06$  (return); Table 1]. Finally, initial telomere length was similar in males and females ( $0.94 \pm 0.08$  and  $0.81 \pm 0.06$ , respectively; Table 1). The same statistical results were obtained when considering the initial telomere length of the individuals for which the oxidative status was also measured [GLMM:  $F_{2,49} = 1.54$ ,  $P = 0.22$  (treatment);  $F_{1,49} = 0.78$ ,  $P = 0.38$  (return rate);  $F_{1,49} = 0.41$ ,  $P = 0.53$  (sex)].

There was no correlation between initial telomere length and antioxidant capacity of penguins (Spearman correlation:

**Table 1.** Results of GLMM examining the effect of the sex, the treatment and the return rate of Adélie penguins on telomere length, oxidative status, uric acid levels and telomere dynamics

	Initial telomere length ( <i>n</i> = 72)		Antioxidant capacity* ( <i>n</i> = 60)		Uric acid ( <i>n</i> = 60)		Oxidative damage† ( <i>n</i> = 60)		Change in telomere length† ( <i>n</i> = 51)	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Sex	0.20	0.66	1.26	0.26	1.34	0.27	0.77	0.47	0.13	0.72
Treatment	1.00	0.37	3.32	0.04	0.43	0.65	0.52	0.60	0.28	0.76
Return	1.16	0.29	0.11	0.74	0.48	0.49	3.43	0.07		
Sex × Treatment	1.15	0.32	0.04	0.96	0.17	0.84	1.11	0.34	1.64	0.21
Sex × Return	0.02	0.89	2.43	0.12	0.01	0.91	1.06	0.31		
Treatment × Return	1.39	0.26	0.72	0.49	0.03	0.86	0.34	0.56		
Sex × Treatment × Return	3.17	0.08	0.23	0.63	2.08	0.16	0.07	0.79		

\*The change in body mass between the courtship and the chick-rearing period was added as a covariate in the model.

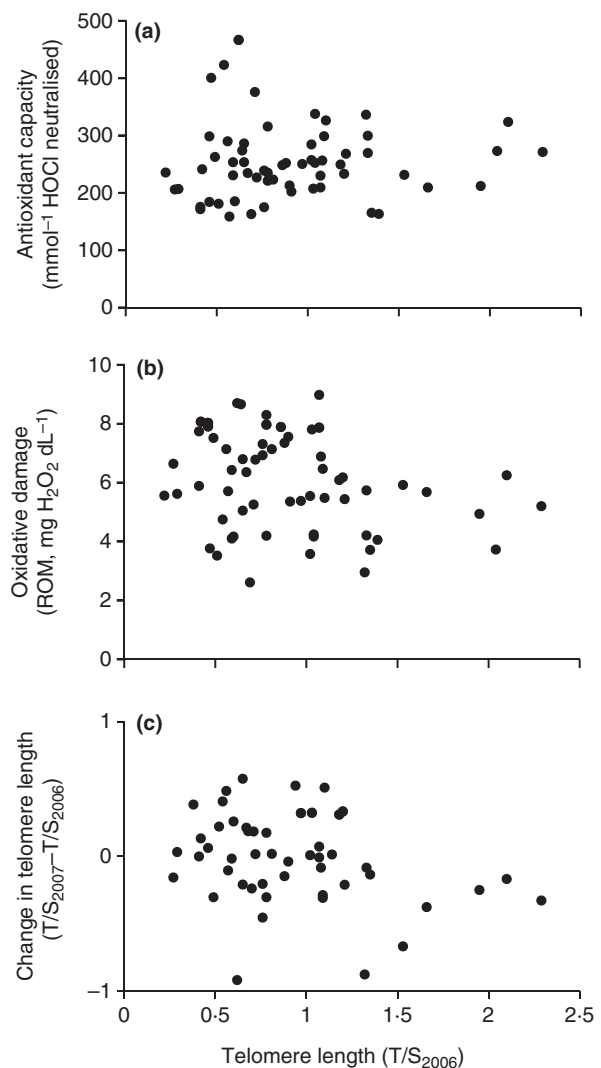
†The initial telomere length was added as a covariate in the model.

$r_s = 0.15$ ,  $P = 0.25$ ; Fig. 3a) or between initial telomere length and uric acid levels (Spearman correlation:  $r_s = -0.06$ ,  $P = 0.63$ ). However, the initial telomere length was weakly but significantly correlated with ROM levels (Spearman correlation:  $r_s = -0.26$ ,  $P = 0.049$ ; Fig. 3b), birds with the shortest telomeres also being those with the highest levels of oxidative damage. Finally, because the change in telomere length and the initial telomere length were not independent (change in telomere length = telomere length<sub>2007</sub> – telomere length<sub>2006</sub>), these two parameters were also correlated (Spearman correlation:  $r_s = -0.60$ ,  $P < 0.001$ ; Fig. 3c). This is due to the so-called regression effect in repeated measurements: individuals with values above the mean at the first measurement will, on average, have lower values at the second measurement whereas individuals with values below the mean will have higher values (Kelly & Price 2005). We then corrected this regression effect by the method described by Kelly & Price (2005). However, the negative correlation between the initial telomere length and the corrected change in telomere length persisted (Spearman correlation:  $r_s = -0.31$ ,  $P = 0.03$ ). This suggests that the change in telomere length was more important for individuals with long initial telomeres.

Consequently, the initial telomere length was added as a covariate in the GLMM examining ROM levels and the change in telomere length (corrected for the regression effect) in the different groups of penguins.

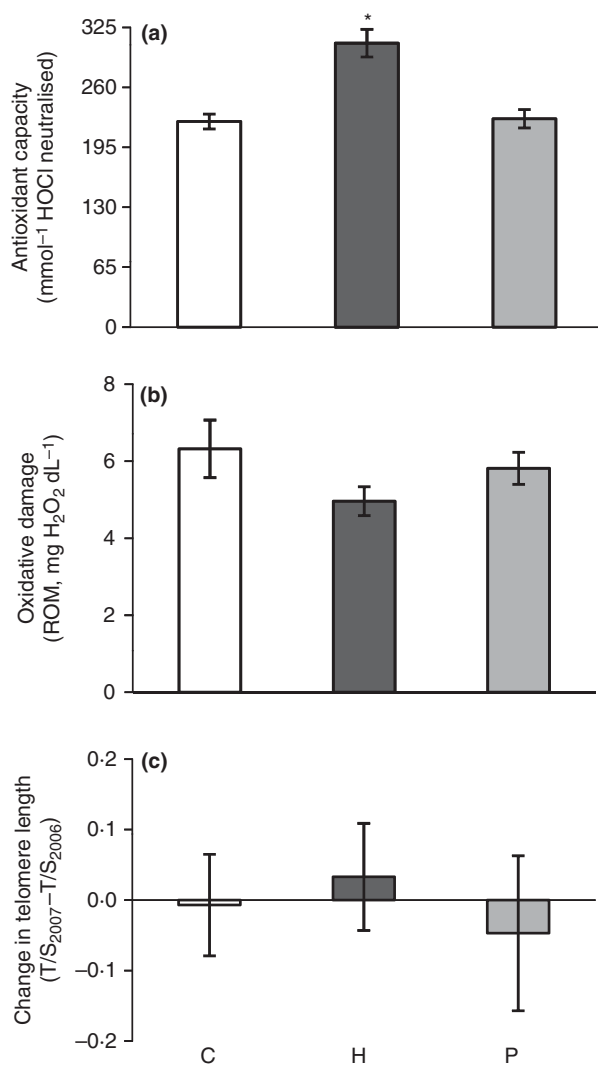
#### OXIDATIVE STATUS

Handicapped penguins displayed 35% higher antioxidant levels ( $308 \pm 15 \text{ mmol}^{-1}$  HOCl neutralized) than control and partner birds [ $223 \pm 8$  and  $226 \pm 10 \text{ mmol}^{-1}$  HOCl neutralized, respectively; GLMM:  $F_{2,49} = 3.45$ ,  $P = 0.04$ , *post hoc* tests:  $P = 0.04$  (handicapped vs. controls),  $P = 0.04$  (handicapped vs. partners); Fig. 4a]. However, plasmatic antioxidant levels did not differ between penguins that returned and those that did not return to the colony the subsequent year ( $250 \pm 9$  and  $260 \pm 22 \text{ mmol}^{-1}$  HOCl



**Fig. 3.** Relationship between the initial telomere length and the antioxidant capacity (a), the oxidative damage (b) and the change in telomere length between two consecutive breeding seasons (c) in Adélie penguins. The values for the change in telomere length were corrected for the regression effect.





**Fig. 4.** Antioxidant capacity (a) and oxidative damage (b) during the chick-rearing period and change in telomere length between two consecutive breeding seasons (c) in control (C), handicapped Adélie penguins (H) and the partners of handicapped individuals (P). The values for the change in telomere length were corrected for the regression effect and the data for the oxidative damage and the change in telomere length correspond to the estimated means with the initial telomere length as a covariate. Presented are means  $\pm$  SE. \*Indicates a significant difference between control and handicapped penguins.

neutralized, respectively; GLMM:  $F_{1,49} = 0.01$ ,  $P = 0.92$ ) or between males and females ( $258 \pm 12$  and  $248 \pm 11$  mmol<sup>-1</sup> HOCl neutralized, respectively; GLMM:  $F_{1,49} = 0.97$ ,  $P = 0.35$ ). As our handicapping procedure affected the feeding behaviour of penguins thereby increasing body mass loss and hence probably muscle catabolism (Beaulieu *et al.* 2010a), it is likely that the antioxidant capacity of handicapped penguins was increased because of increased body mass loss and/or uric acid levels. Indeed, we found a positive relationship between the antioxidant capacity of penguins and body mass lost between the courtship and chick-rearing periods (Spearman correlation:  $r_s = 0.39$ ,  $P = 0.003$ ). However, even after controlling for body mass loss, the treatment

still had a significant effect on the antioxidant capacity of penguins (Table 1) with handicapped penguins exhibiting higher antioxidant capacity than control birds (*post hoc* test:  $P < 0.05$ ) or their partners (*post hoc* test:  $P = 0.04$ ). The increase in the antioxidant capacity of handicapped penguins was not due to an increase in uric acid levels since they were similar in controls, handicapped penguins and their partners ( $0.34 \pm 0.03$ ,  $0.33 \pm 0.03$  and  $0.32 \pm 0.03$  mmol L<sup>-1</sup>, respectively; Table 1). Moreover, uric acid levels were not correlated to the antioxidant capacity of penguins (Spearman correlation:  $r_s = -0.08$ ,  $P = 0.53$ ).

Plasmatic ROM levels measured in the breeding season 2006–2007 were similar between control birds, handicapped birds and their partners ( $6.32 \pm 0.75$ ,  $4.96 \pm 0.38$  and  $5.82 \pm 0.42$  mg H<sub>2</sub>O<sub>2</sub> dL<sup>-1</sup>, respectively; Table 1; Fig. 4b). These results show that, for an equal level of oxidative damage, the handicapped birds had a higher antioxidant plasmatic capacity than their partners or control birds. There was no significant difference in ROM levels between birds that returned to the colony and those that did not ( $6.24 \pm 0.21$  and  $5.16 \pm 0.58$  mg H<sub>2</sub>O<sub>2</sub> dL<sup>-1</sup>, respectively; Table 1) or between males and females ( $5.94 \pm 0.31$  and  $6.03 \pm 0.28$  mg H<sub>2</sub>O<sub>2</sub> dL<sup>-1</sup>, respectively; Table 1).

#### CHANGE IN TELOMERE LENGTH

The change in telomere length was evaluated between the breeding years 2006–2007 and 2007–2008 (Fig. 4c). There was no difference between control, handicapped and partner birds ( $-0.007 \pm 0.072$ ,  $0.033 \pm 0.076$ ,  $-0.047 \pm 0.110$  T/S<sub>2007</sub> - T/S<sub>2006</sub>, respectively; Table 1; Fig. 4c) or between males and females ( $0.029 \pm 0.073$ ,  $-0.033 \pm 0.071$  T/S<sub>2007</sub> - T/S<sub>2006</sub>, respectively; Table 1). The change in telomere length between the 2 years was not significant for all the birds (one-sample *t*-test:  $t_{50} = 0.46$ ,  $P = 0.65$ ) and for each treatment separately (controls:  $t_{21} = -0.86$ ,  $P = 0.40$ ; handicapped:  $t_{18} = 0.22$ ,  $P = 0.83$ , partners:  $t_9 = 0.49$ ,  $P = 0.64$ ). The change in telomere length was not correlated with the antioxidant capacity of penguins (Spearman correlation:  $r_s = -0.02$ ,  $P = 0.89$ ) but was positively related to ROM levels (Spearman correlation:  $r_s = 0.33$ ,  $P = 0.03$ ). However, this significant relationship between ROM levels and change in telomere length was suppressed after controlling for the initial telomere length (partial correlation:  $r = 0.19$ ,  $P = 0.22$ ).

#### Discussion

The present study is, to our knowledge, the first that experimentally tested the role of the oxidative stress/telomere couple as a proximate mechanism explaining how long-lived species could counteract the negative impact of increased demands of reproduction on self-maintenance. As expected in long-lived species, handicapped penguins subjected to increased breeding constraints appear to have solved the conflict between reproduction and self-maintenance by giving priority to their own maintenance. Indeed, our longitudinal

study showed that these birds increased their antioxidant capacity, while oxidative damage and telomere length remained unchanged. In contrast, in the short-lived zebra finch, antioxidant defences decline with increased reproductive effort (Alonso-Alvarez *et al.* 2004; Wiersma *et al.* 2004). This discrepancy in the oxidative status relative to breeding effort between the zebra finch and the Adélie penguin is in agreement with life-history theory predicting that short-lived organisms are expected to give lower priority to self-maintenance than long-lived animals when breeding constraints increase. Finally, we did not find any differences in terms of oxidative status or telomere dynamics between males and females, the differences in the breeding investment between the two sexes (Chappell *et al.* 1993) presumably being too small to affect these parameters.

In a previous study (Beaulieu *et al.* 2010b), we showed that Adélie penguins could modulate their oxidative status by feeding in different niches, with penguins feeding more predominantly in pelagic areas also exhibiting lower oxidative stress. Our hypothesis was that penguins could select prey with different antioxidant concentrations while foraging in different areas; krill containing higher antioxidant levels than fish being found in more pelagic areas. Therefore, one hypothesis explaining the increased antioxidant capacity of handicapped penguins could be that they exploited a more pelagic niche to enhance their antioxidant capacity. However, we found that handicapped penguins exploited a more coastal niche presumably because they did not have the same foraging capacity as control penguins (Beaulieu *et al.* 2010a). This suggests that handicapped penguins did not modify their oxidative status by selecting the antioxidant rich prey. In addition, while the handicapped penguins and their partners exploited the same niche (Beaulieu *et al.* 2010a), they exhibited different oxidative statuses. We also found that the increased antioxidant capacity of handicapped penguins was not due to increased uric acid levels that could have resulted from a change in feeding behaviour or increased muscle catabolism. The regulation of the oxidative status of handicapped penguins had therefore different bases than that observed in our previous study conducted in control individuals (Beaulieu *et al.* 2010a). The most probable explanation, which would be worth examining, is that handicapped penguins increased the expression of endogenous antioxidants (e.g. superoxide dismutase, catalase, glutathione peroxidase). Increased expression of these molecules presumably had a cost that could have resulted in deleterious effects such as lower body condition and survival probability. Indeed we found a positive relationship between the body mass that Adélie penguins lost and the increase in their antioxidant capacity. However, we did not find any relationship between the antioxidant capacity of penguins and their survival 1 year later. This absence of relationship between antioxidant capacity and survival probability may be due to the combined and opposite effects on survival of the protection conferred by increased antioxidant capacity and the cost of this protection. It is indeed probable that there is a balance between antioxidant protection and the cost associated with this protection.

For instance, in the long-lived Alpine swift, it has been shown that males with the highest oxidative resistance were also those having the highest survival rates (Bize *et al.* 2008). In that case, the positive effects of a higher antioxidant capacity may have been higher than the negative effects related to the cost of this protection. In contrast, in our study, we did not find any relationship (positive or negative) between the survival probability and the antioxidant defences of Adélie penguins. This suggests that the positive effects of the antioxidant capacity of penguins were presumably counterbalanced by the cost of this protection. However, for the moment all these hypotheses on the cost of antioxidant protection are speculative and more work is needed in the future to assess their validity.

Biological age (estimated through initial telomere length) was related to the oxidative damage of penguins. Indeed, we found a weak but significant negative relationship between oxidative damage and telomere length (Fig. 3b), suggesting that older Adélie penguins experience higher levels of oxidative damage than younger individuals [here the adjectives old and young are relative to the biological age of birds but they may also be extended to chronological age since telomere length (i.e. biological age) and chronological age are negatively correlated in Adélie penguins; Haussmann *et al.* 2003]. In a recent study conducted on greater flamingos *Phoenicopterus ruber roseus*, Deveney *et al.* (2010) also found a weak relationship between the chronological age and another marker of the oxidative status of birds (the resistance of red blood cells to an oxidative attack). Since in Adélie penguins and in greater flamingos, the relation between age (biological and chronological ages, respectively) and oxidative status is weak, this suggests that age is not the only factor regulating the oxidative status in birds but other factors (environment, diet, breeding effort, etc) are also involved. That may explain why we did not find any relationship between telomere length and antioxidant capacity, the influence of the biological age being probably attenuated by the strong effects of different breeding efforts on the antioxidant capacity of penguins.

We also found that penguins with longer telomeres lost them more rapidly than individuals with shorter telomeres. Again, this relationship, even though it is significant, is characterized by an important variability (Fig. 3c) probably due to other factors that also affect telomere dynamics. Nevertheless, this relationship suggests that in general, reproduction has a stronger cost in terms of telomere dynamics in young individuals than in older penguins (again the adjectives young and old are relative to the biological age of birds). It may thus be possible that younger birds have to provide a higher breeding effort due to their inexperience and therefore lose more telomeres from 1 year to another. However, in contrast to this hypothesis, we did not find any differences in telomere dynamics between males and females who allocate energy differently in reproduction (Chappell *et al.* 1993). Moreover, in our experimental study, the additional breeding workload of handicapped penguins did not have any impact on telomere dynamics. Three non-exclusive reasons may explain this result. (i) Deleterious impacts on telomere length due to the

handicap procedure may appear after a time longer than 1 year. Indeed, telomeres shorten slowly in long-lived birds like the Adélie penguin (Haussmann *et al.* 2003) and in our study, no significant telomere loss was observed between two consecutive years. In addition, the relationship between telomere size and survival rates appears significant only after 3–4 years in Alpine swift (Bize *et al.* 2009), a bird with a comparable longevity. Another hypothesis explaining why telomeres were not altered in handicapped penguins may be that (ii) DNA repair mechanisms such as telomerase were activated, leading to the maintenance of telomere size. However, this seems unlikely since telomerase is inactivated in adulthood in the somatic cells of most species studied so far (Monaghan & Haussmann 2006; Vleck, Haussmann & Vleck 2007; but see Hatakeyama *et al.* 2008). Finally (iii) handicapped penguins may have protected their DNA from extra-damage due to ROS attack by increasing their antioxidant barrier. Without ruling out the two first hypotheses and even though the telomere dynamics over 1 year was not related to the antioxidant capacity of penguins during the breeding season, our data provide some support to the third hypothesis. In this context, it would be interesting to compare our results obtained in a long-lived species with the results from the same experimental design but carried out in a short-lived species that sacrifice antioxidant capacity when breeding constraints increase (Alonso-Alvarez *et al.* 2004; Wiersma *et al.* 2004) and in which telomeres shorten faster than in long-lived animals (Haussmann *et al.* 2003).

The absence of a relationship between telomere dynamics over a 1-year time span and oxidative status during the breeding season may be due to constraints other than breeding constraints that penguins had to undergo during the whole year. For instance, penguins were still handicapped after the breeding season when they had to forage and store reserves to prepare for moult (Fig. 2), another costly process in penguins (Croxall 1982). In addition, the constraints that Adélie penguins undergo in winter (distant foraging areas, harsh weather, low food availability, etc) may also affect their oxidative status and telomere dynamics. In this context, a longitudinal approach examining the oxidative status of organisms throughout the different stages of their annual cycle will be valuable. It would enable us to examine how the sum of these oxidative statuses is related with telomere dynamics over 1 year (however, this approach does not seem applicable to Adélie penguins for obvious methodological aspects).

Telomere dynamics predicts life expectancy in the wild (Bize *et al.* 2009; Salomons *et al.* 2009). However, we did not find any relationship between initial telomere length and the return rate of penguins the subsequent year. Since telomere dynamics necessarily implies the presence of breeding birds in two consecutive years, our results only apply to birds who returned to the colony the second year. Therefore, our procedure may have resulted in the selection of the most competitive birds and may hide an immediate relationship between telomere loss and survival (what was the telomere dynamics of the missing birds remains unknown). Without these data,

our experimental procedure suggests that there will be no long-term consequences for those handicapped Adélie penguins who survived and returned to the colony to breed 1 year after the experiment, all experimental groups presenting comparable telomere dynamics.

To better understand the role of the oxidative status in mediating the conflict between maintenance and reproduction, one important challenge in the future will be to determine how this physiological parameter is balanced between parents and offspring. In our study, it is possible that handicapped parents that managed to increase their own antioxidant capacity did this by sacrificing the antioxidant status of their offspring. Further studies investigating how antioxidant resources are shared between the parents and the young in different breeding conditions could be a first interesting step to explore.

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## **Appendix 2 –**

Catching-up but telomere loss: half-opening the black box of growth and ageing trade-off in wild king penguin chicks



# Catching-up but telomere loss: half-opening the black box of growth and ageing trade-off in wild king penguin chicks

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## Abstract

One of the reasons for animals not to grow as fast as they potentially could is that fast growth has been shown to be associated with reduced lifespan. However, we are still lacking a clear description of the reality of growth-dependent modulation of ageing mechanisms in wild animals. Using the particular growth trajectory of small king penguin chicks naturally exhibiting higher-than-normal growth rate to compensate for the winter break, we tested whether oxidative stress and telomere shortening are related to growth trajectories. Plasma antioxidant defences, oxidative damage levels and telomere length were measured at the beginning and at the end of the post-winter growth period in three groups of chicks (small chicks, which either passed away or survived the growth period, and large chicks). Small chicks that died early during the growth period had the highest level of oxidative damage and the shortest telomere lengths prior to death. Here, we show that small chicks that grew faster did it at the detriment of body maintenance mechanisms as shown by (i) higher oxidative damage and (ii) accelerated telomere loss. Our study provides the first evidence for a mechanistic link between growth and ageing rates under natural conditions.

*Keywords:* ageing, bird, growth compensation, oxidative stress, telomere

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## Introduction

The incredible size variability among organisms has attracted much attention of many studies in the context of life history theory (Arendt 1997). Of particular interest to ecological studies is the display of intra-specific size variability and its fitness consequences (Altmann & Alberts 2005; Gagliano & McCormick 2007). The way that size impacts upon fitness is not simply a question of 'eating when big or getting eaten when small' but also depends on how optimal size is reached for a given life stage. Intuitively, one would expect large size to be achieved quickly through fast growth, and thus organisms would always grow as fast as possible. However, it is now well-known that growth rates are slower

than the maximum potential, one of the reasons (other than insufficient food availability or physiological constraints, such as changes in developmental maturity of tissues, Ricklefs 1969) being that high growth rates carry fitness costs: in addition to the higher risk of predation associated to increased foraging activities, physiological costs of rapid growth have been largely documented (see Munch & Conover 2003). Therefore, whether it became evident that a trade-off with growth must exist, understanding the mechanisms through which rapid growth may induce a suboptimal adult phenotype (for a given genotype), remains a central focus in evolutionary ecology and medicine (Lindstrom 1999; Metcalfe & Monaghan 2001; Gluckman & Hanson 2004); for example, given the strong relationship between size and fitness (Richner 1989), growth compensation is expected once feeding conditions turn adequate again for individuals that have had to face a bad

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nutritional period. Growth compensation can be reached either through delaying age at maturity and extending the growth period (De Block *et al.* 2008) or by displaying accelerated growth rates over the normal period of time growth usually occurs (Metcalf & Monaghan 2001). Despite its short-term benefit (i.e. reaching an adequate size at fledgling), catch-up growth carries several physiologically associated costs (Crisuolo *et al.* 2008; Auer *et al.* 2010), among which one important long-term drawback is an associated reduction of longevity (Ozanne & Hales 2004). However, this suggestion is mainly based on experiments conducted under controlled environmental conditions, and, despite the fact that compensatory growth is known to occur in wild conditions (Bjorndal *et al.* 2003; Johnsson & Bohlin 2005; Bize *et al.* 2006), only scarce evidence of reduced longevity as a cost of accelerated growth has been described in free-living species (Johnsson & Bohlin 2010).

The proximal mechanisms underlying the growth-ageing relationship remain to be defined (Metcalf & Monaghan 2003). Among the potential candidates, oxidative stress has been suggested to play an important role (Alonzo-Alvarez *et al.* 2007; De Block & Stoks 2008). Oxidative stress is one of the main factors implicated in ageing theories (Beckman & Ames 1998) and may trigger quicker ageing notably via accelerated telomere erosion (von Zglinicki 2002; Tarry-Adkins *et al.* 2009). Indeed, telomeres form the termini of chromosomes and their reduction with age is one of the main mechanisms explaining cell senescence (Monaghan & Hausmann 2006). Until now, however, there has been no data examining whether oxidative stress and telomere erosion may be observed in young individuals that had to catch-up from a bad start in wild conditions.

The king penguin (*Aptenodytes patagonicus*) is the only seabird species in which the chick has to face the sub-antarctic winter period alone, while its parents are foraging at sea. During this time, roughly half of the chicks are never fed by their parents and then suffer from a high mortality (Weimerskirch *et al.* 1992). Therefore, complete growth spreads out over 11 months (one of the longest growing period in birds), mainly because parental feeding and consequently growth is interrupted during winter. Interestingly, if chicks differ in body mass and size at the beginning of the last phase of growth (when the winter fast ends), they, nevertheless, all seem to reach similar values at fledging (3 months later, Verrier 2003), suggesting that different growth rates may be exhibited by chicks during the post-winter growth phase. It is likely that these chicks must reach a threshold in either size or body condition (body lipid and protein content) before moulting and

departure at sea. These natural variability and evolutionary constraints acting on king penguin growth trajectories are unique opportunities to test the hypothesis that compensatory growth actually occurs in that species and induces an oxidative stress, which ultimately results in higher telomere shortening rates over the growth period.

## Methods

Our study was conducted in the colony of 'La Grande Manchotière' (approximately 24 000 breeding pairs), Possession island, Crozet archipelago (Terres Australes Antarctiques Françaises) located 46°25'S; 51°52'E. The study began at the end of the austral winter and ended the next summer when chicks started moulting (end of the main second growth period).

### The growth period in king penguin

Briefly, the growth period of king penguin chicks can be divided into three phases (Fig. 1). Following hatching, a first rapid growth phase (roughly 2.5 months) occurs, which is interrupted by the arrival of the winter period (i.e. a winter break phase of approximately 4.5 months). Indeed, following a sharp drop in marine resources in the sub-Antarctic area in early May, parents are required to rejoin the marginal sea ice (some 1800 km south off their colonies) to forage at sea (Bost *et al.* 2004). Winter is then the most sensitive period for king penguin chicks that are left fasting in the colony

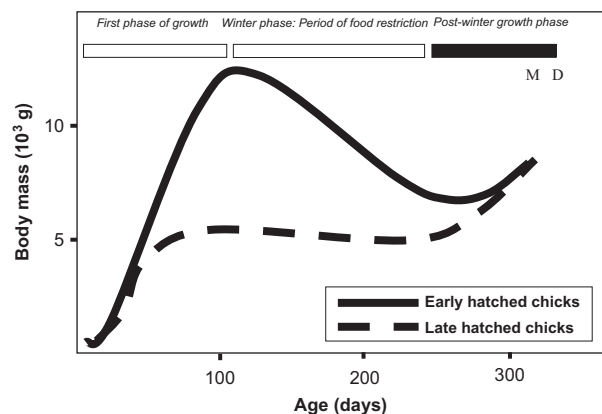


Fig. 1 Representation of body mass dynamics of king penguin chicks followed over their three successive phases of growth. Both early hatched chicks (January–early February) and late-hatched chicks (late February to early March) are represented. The whole period of development lasts around 11 months. In the present study, we focused on the last part of development (black bar) preceding the moult (M) and departure at sea (D). Adapted from Stonehouse 1960 and Verrier 2003.

over prolonged periods of time (Cherel *et al.* 1987; Descamps *et al.* 2002). A second growth period occurs in the subsequent austral summer, leading to complete maturation of the chicks (roughly 3 months, Stonehouse 1960; Descamps *et al.* 2002).

### Bird sampling

To obtain a broad range of post-winter growth trajectories, we visually chose large chicks ( $n = 14$ ; BM between 6.1 and 10.35 kg) in September, while small chicks ( $n = 44$ ; BM between 2.6 and 5.4 kg) were banded until October 2008 to compensate for the high mortality rate of this group. Indeed, while no large chicks actually died from September until fledging, 30 chicks of the small group were victims of predation and starvation. We have no data concerning the hatching date of these birds, but small chicks were expected to grow faster, as offspring have a comparable size when leaving the colony for their first trip to sea, independently of their hatching date (Verrier 2003). Chicks were identified with a plastic band on the flipper. We ended the study with 28 birds at fledging, of which 14 were initially small chicks. After the beginning of November, no death was recorded. Whereas only values from the surviving small chicks were taken into account for analysis of changes in oxidative stress and telomere length in relation to growth rate, we, nonetheless, checked whether the small birds that died early in the study presented differences in initial values of these variables (only on 16 small chicks that died for logistic problems).

### Growth measurements

Chicks were captured using a hooked pole (2 m long) and were immediately immobilized by hand. Upon capture, chicks were hooded to reduce handling stress. They were carried to a facility close to the colony where a blood sample was taken and where body size and mass were measured. No manipulation exceeded 20 min in total, and the chicks were released at the place of capture. From the beginning of September, the chicks were weighed ( $\pm 4$  g) every 15 days using a platform balance (Kern IT60K2LIP) and morphometric measurements were taken: the fully extended flipper length was taken from the ventral side of the humeral head to the tip of the flipper. Initial and final body conditions of chicks were calculated using the residuals of the linear regression between body mass and flipper length. Plastic flipper bands were removed after the beginning of the moult. All chicks departed to sea on average 15 days (range 7–30 days) after the last manipulation. To evaluate the post-winter growth rate in body mass and flipper length,

we used the slope of the linear regression fitting for body mass or flipper length in relation to time for each individual. A linear relationship was the best-fitting model for our growth data, given that we were only working on a part of the growth period (mass growth, mean  $r$  value =  $0.896 \pm 0.023$ ; flipper growth, mean  $r$  value =  $0.942 \pm 0.011$ ,  $n = 28$ ).

### Oxidative stress and telomere measurements

Blood samples from the flipper vein were taken both at the end of the winter (August–September), i.e. during the first morphometric measurement, and when the plastic flipper band was removed (during moult, last capture, November–January). Samples were centrifuged, plasma and red blood cells separated and frozen at  $-80$  °C until analysis was carried out at the laboratory (Department of Ecology, Physiology and Ethology, Strasbourg, France). Plasma concentrations of reactive oxygen metabolites (d-ROMs) and of the antioxidant capacity (OXY-ADSORBENT) were measured using Diacron tests (Diacron International, Italy) as previously described in penguins (Beaulieu *et al.* 2010; Costantini *et al.* 2006 for a detailed description of the methodology). Mean coefficient of intra-individual variation was of  $6.91 \pm 1.95\%$  and  $3.94 \pm 1.33\%$ , while mean inter-plate variation was of  $9.73 \pm 1.31\%$  and of  $10.29 \pm 2.06\%$ , for OXY-ADSORBENT and d-ROMs tests, respectively.

Telomere length was determined using DNA extracted from red blood cells following the quantitative real-time amplification (qPCR) protocol adapted for birds and described by Criscuolo *et al.* 2009;. Primer sequences for telomere amplification were similar to previous studies (Bize *et al.* 2009; Criscuolo *et al.* 2009), and as a single control gene, we used the *Aptenodytes patagonicus* zinc finger protein (NCBI accession number AF490194), with primer sequences defined by Primer 3 software as: (Royal1: 5'-TACATGTGCCATGGTTTTGC-3'; Royal2: 5'-AAGTGCTGCTCCCAAAGAAG-3'). Primer concentrations in the final mix were 200 nM for telomere length (T) determination and 300 nM for the control gene (S). Final telomere values are expressed as the ratio between telomere and the single control gene number of amplification cycles (T/S ratio, Criscuolo *et al.* 2009). Telomere and control gene PCR conditions were 10 min at 95 °C followed by 30 cycles of 30 s at 56 °C, 30 s at 72 °C and 60 s at 95 °C. Standard electrophoresis on a 1.5% agarose gel run in standard TBE buffer (90 V for 10 min and 130 V thereafter for 30 min, ethidium bromide staining) was used to determine qPCR amplicon sizes of the control gene (170 bp) and of the telomere amplification product (darker band

between 50 and 100 bp). All samples were measured on one plate for telomere amplification and another plate for the control gene amplification. Amplification efficiencies of the qPCR run were 104% and 100% (telomere and ZENK, respectively). Mean intra-individual variation was  $1.56 \pm 0.20\%$  for the telomere assay and  $1.47 \pm 0.17\%$  for ZENK assay. Because of either lack of volume of plasma or of poor DNA quality, only 16 birds were used in the analysis of oxidative status (d-ROMs and OXY-ADSORBENT) and telomere dynamics, respectively. Sex determination was performed on DNA extracted from red blood cells following a method adapted from Griffiths *et al.* 1998.

#### Data analyses

Differences in initial values of growth, oxidative stress and telomere length (recorded at the beginning of the post-winter growth) among small chicks that resumed growth, small chicks that died early in the study and big chicks were tested using a linear model with group as a fixed factor. Post hoc comparisons were carried out using Tukey's tests. Linear mixed models (SPSS V. 18) were used to examine the dynamics of changes in body mass, wing length, d-ROM and OXY-ADSORBENT, and telomere length experienced by the chicks over the study period. To analyse our data set, random effect of individual identity (both intercept and slope) was accounted for to control for nonindependence of repeated measurements on the same individual. Group and sex were used as fixed factors, and the duration of the post-winter growth period was included as a covariate to control for the fact that large birds took a shorter time to resume their post-winter growth (on average 50 days) than did small chicks (100 days). Normality was tested afterwards, using the residuals of the model

(Kolmogorov–Smirnov test, all  $P > 0.05$ ). A regression analysis was finally used to explicitly test the links between oxidative damage levels, growth rate, body condition and telomere loss. All tests were two-tailed, and  $P < 0.05$  was considered significant. Values are given as means  $\pm$  SE.

## Results

### *Initial characteristics of small chicks that died early in the study*

Table 1 describes the initial state of the chicks followed during the study (small chicks that died and survived until fledging and big chicks). Chicks differed not only in body mass, as a result of our experimental design, but also in body size: small chicks (of both subgroups) were lighter and smaller than big ones. Interestingly, those small chicks that did not survive the growth period exhibited lower plasma antioxidant capacities, higher plasma levels of oxidative damages and lower initial telomere lengths than other surviving small chicks and big chicks. However, telomere length and oxidative damage were comparable among the two surviving groups of big and small chicks at the beginning of the experiment. Males and females presented similar initial body masses ( $6.1 \pm 0.5$  vs.  $5.9 \pm 0.3$  kg,  $F_{1,27} = 0.11$ ,  $P = 0.746$ ), structural sizes ( $284.8 \pm 4.9$  vs.  $283.5 \pm 2.7$  mm,  $F_{1,27} = 0.11$ ,  $P = 0.826$ ), antioxidant capacities ( $262.6 \pm 13.8$  vs.  $295.9 \pm 18.6$   $\mu\text{mol HClO neutralized/mL}$ ,  $F_{1,15} = 1.71$ ,  $P = 0.208$ ) and oxidative damage levels ( $62.2 \pm 26.5$  vs.  $73.6 \pm 19.6$  mg  $\text{H}_2\text{O}_2/\text{dL}$ ,  $F_{1,15} = 0.10$ ,  $P = 0.756$ ). Body condition was significantly different between small chicks that naturally died and big chicks, but did not differ between sexes ( $0.05 \pm 0.52$  vs.  $-0.18 \pm 0.29$ ,  $F_{1,27} = 0.01$ ,  $P = 0.908$ ).

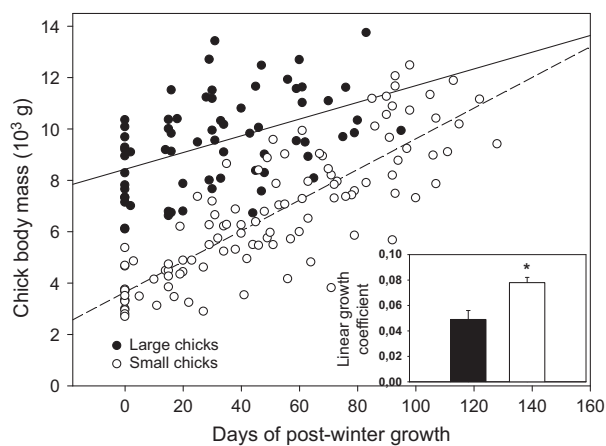
**Table 1** Mean ( $\pm$ SD) body mass, flipper length, body condition, oxidative balance indices and telomere length of king penguin chicks at the beginning of the post-winter growth. Values are indicated for the three experimental groups of birds, large chicks ( $n = 14$ ), small chicks that died before ( $n = 16$ ) or survived until fledging ( $n = 14$ ). Duration of this post-winter growth period is also indicated for both groups of birds

	Large chicks	Small chicks that died	Small chicks that survived	<i>F</i>	<i>P</i>
Variable					
Body mass ( $10^3$ g)	$8.1 \pm 1.1^a$	$3.2 \pm 0.4^b$	$3.9 \pm 0.8^b$	133.4	<0.001
Wing length (mm)	$307.9 \pm 5.9^a$	$259.8 \pm 4.0^b$	$260.4 \pm 4.0^b$	48.3	<0.001
Body condition (residuals mass/size)	$0.3 \pm 0.4^a$	$-0.6 \pm 0.3^b$	$-0.3 \pm 0.4^b$	4.7	0.015
Antioxidant capacity ( $\mu\text{mol HClO neutralized/mL}$ )	$309.5 \pm 14.8^a$	$167.1 \pm 8.8^b$	$257.1 \pm 13.4^c$	52.0	<0.001
Oxidative damage (mg $\text{H}_2\text{O}_2/\text{dL}$ )	$36.5 \pm 15.8^a$	$132.6 \pm 16.2^b$	$81.1 \pm 13.5^a$	4.0	0.029
Telomere length (T/S ratio)	$1.31 \pm 0.2^a$	$0.54 \pm 0.1^b$	$1.48 \pm 0.2^a$	23.6	<0.001
Recorded post-winter growth period (days)	$57.3 \pm 23.4^a$	$10.5 \pm 4.0^b$	$100.1 \pm 15.5^c$	129.2	<0.001

Differences among groups were tested using a linear model. Normality was verified in all cases using Kolmogorov–Smirnov tests (all  $P > 0.16$ ). Columns with different letters indicate a significant difference among chick groups (using Tukey's test).

### Post-winter growth rates

The growth period was longer in small than in large birds (Table 1), pointing out that those small birds took longer to resume their growth and to reach the adequate body condition or size for their departure at sea. There was an overall significant effect of chick group (i.e. initial body size) on body mass growth, small chicks accumulating body mass at a higher rate than large chicks (slope values, small chicks:  $0.078 \pm 0.004$ , mean coefficient of variation 6.7%; large chicks:  $0.049 \pm 0.007$  g/day, mean coefficient of variation 24.2%, Fig. 2, Table 2a). The same effect was found in flipper length growth (small chicks:  $0.504 \pm 0.036$  mm/day, mean coefficient of variation 8.0%; large chicks:  $0.212 \pm 0.040$  mm/day, mean coefficient of variation 29.9%, Table 2b), thereby indicating growth rate differences reflected both changes in body reserves and body size. The higher growth rate exhibited by small chicks resulted in a body mass comparable with large chick when all birds left the colony ( $10.51 \pm 0.32$  (small) vs.  $11.38 \pm 0.33$  kg (large chicks),  $F_{1,27} = 3.607$ ,  $P = 0.164$ ), although small chicks still had smaller flippers ( $303.4 \pm 1.8$  vs.  $320.9 \pm 1.8$  mm,  $F_{1,27} = 47.7$ ,  $P < 0.001$ ). Therefore, small chicks were initially smaller in mass and size, but grew more rapidly after the winter and did compensate totally in body mass and partially in size for their initial bad start. There was no effect of sex on final mass (males,  $11.74 \pm 0.47$  vs.



**Fig. 2** Body mass growth trajectories of two groups of small (open circles) and large (filled circles) king penguin chicks measured during the post-winter growth period (between September and December). Linear regression values: small chicks,  $r^2 = 0.69$ ,  $F_{1,108} = 241.0$ ,  $P < 0.001$ ; large chicks,  $r^2 = 0.21$ ,  $F_{1,67} = 17.9$ ,  $P < 0.001$ . Inset: mean linear growth coefficients differ significantly between the two groups, small chicks growing at a higher rate than large chicks during the post-winter growth period ( $P < 0.001$ , see Table 2 for statistics). Bars represent mean  $\pm$  SE.

**Table 2** Results of separate linear mixed models analysing the differences between our two experimental groups (large and small king penguin chicks at the end of the winter) on changes over time of: (a) body mass, (b) flipper length, (c) plasma antioxidant capacity, (d) plasma levels of oxidative damages and (e) telomere loss

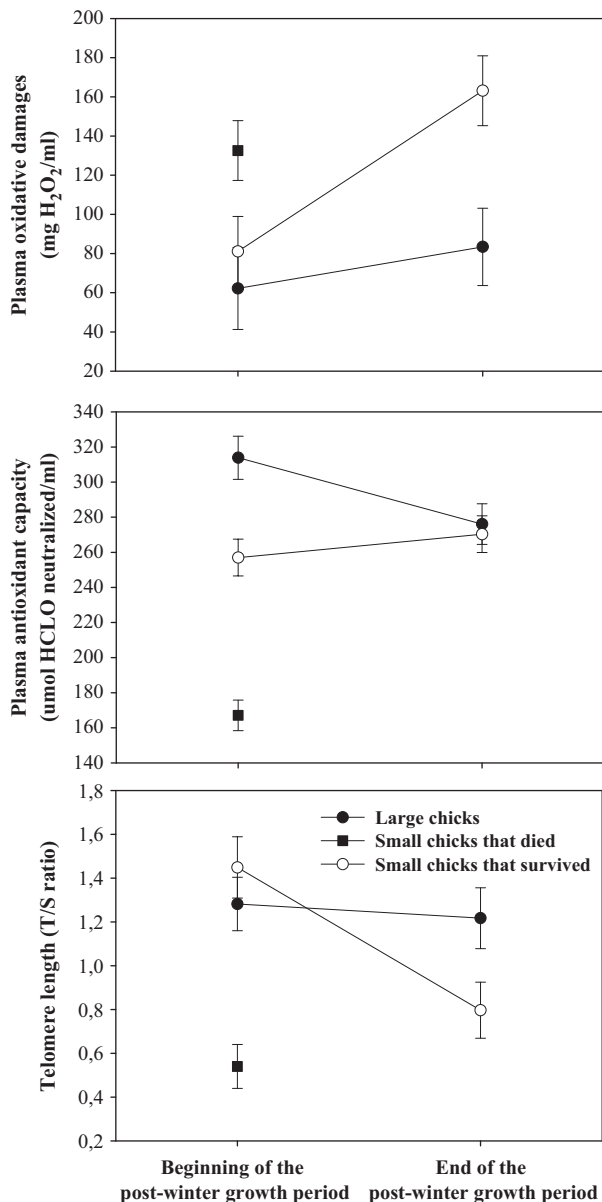
Variable and explanatory factors or covariates	D	F	P
<i>a. Body mass growth</i>			
Group	1, 32.1	62.92	<b>&lt;0.001</b>
Post-winter growth period (days)	1, 150.4	303.98	<b>&lt;0.001</b>
Group $\times$ Post-winter growth period (days)	1, 150.4	7.83	<b>0.006</b>
Sex	2, 22.3	0.42	0.66
<i>b. Flipper length growth</i>			
Group	1, 28.8	116.39	<b>&lt;0.001</b>
Post-winter growth period (days)	1, 146.6	353.73	<b>&lt;0.001</b>
Group $\times$ Post-winter growth period (days)	1, 146.6	52.40	<b>&lt;0.001</b>
Sex	2, 24.1	0.15	0.86
<i>c. Modification of the antioxidant capacity</i>			
Group	1, 31.7	8.97	<b>0.005</b>
Post-winter growth period (days)	1, 20.0	2.70	0.116
Group $\times$ Post-winter growth period (days)	1, 20.1	5.56	<b>0.029</b>
Sex	2, 18.5	0.33	0.57
<i>d. Modification of the oxidative damage level</i>			
Group	1, 30	0.78	0.385
Post-winter growth period (days)	1, 30	6.09	<b>0.021</b>
Group $\times$ Post-winter growth period (days)	1, 30	4.88	<b>0.037</b>
Sex	2, 30	0.07	0.80
Body mass growth	1, 30	4.21	<b>0.044</b>
<i>e. Total telomere loss</i>			
Group	1, 35.9	1.13	0.30
Post-winter growth period (days)	1, 18.8	4.12	0.057
Group $\times$ Post-winter growth period (days)	1, 18.8	5.41	<b>0.031</b>
Sex	2, 23.2	0.72	0.41

In each case, chick gender was used as a fixed factor and post-winter growth duration (time) as a covariate to correct for the difference in the total length of the growth period observed between the two groups of chicks. Significant terms are reported in bold, and nonsignificant terms were dropped sequentially from the model.

females,  $10.68 \pm 0.26$  kg,  $F_{1,27} = 3.66$ ,  $P = 0.067$ ) or body size (males,  $313.8 \pm 2.7$  vs. females,  $311.6 \pm 1.5$  mm,  $F_{1,27} = 0.45$ ,  $P = 0.509$ ).

### Post-winter oxidative stress

The dynamic in change of plasma antioxidant capacity was also different among the two groups (small,  $+1.5 \pm 18.7$  vs. large,  $-36.5 \pm 21.6$   $\mu\text{mol HClO}$  neutralized/mL, Fig. 3, Table 2c), small chicks starting with lower antioxidant levels but ending with comparable



**Fig. 3** Mean ( $\pm$ SE) plasma oxidative damage levels (upper panel), plasma antioxidant capacity (middle panel) and telomere length (lower panel) in king penguin chicks are indicated both at the beginning and the end of the post-winter growth period. Chicks were separated into three groups at the end of the winter in relation to their size [big chicks ( $n = 14$ ) and two groups of small chicks that either passed away naturally at the early beginning of the longitudinal following ( $n = 16$ ) or that resumed growth successfully ( $n = 14$ )]. Letters indicate significant differences, see tables for detailed statistics.

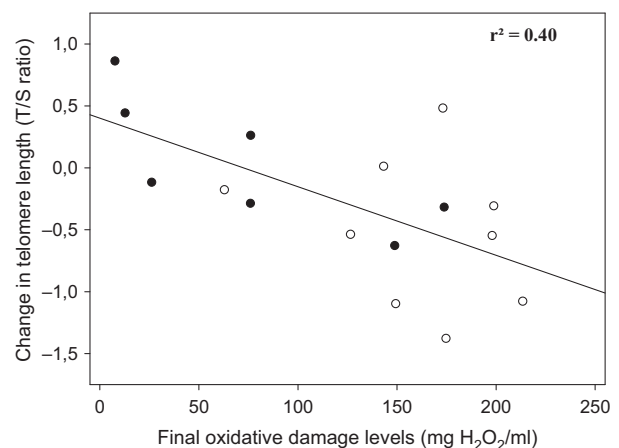
final antioxidant capacities (small,  $270.3 \pm 10.5$  vs. large,  $276.1 \pm 11.6$   $\mu\text{mol HClO neutralized/mL}$ , Fig. 3, Table 2c). On average, small chicks presented a lower mean antioxidant capacity of the plasma over the study than large chicks (Table 2c). Small chicks also exhibited a greater increase in their oxidative damage plasma

levels over growth than large birds ( $+74.7 \pm 20.6$  vs.  $+27.2 \pm 23.9$   $\text{mg H}_2\text{O}_2/\text{mL}$ , Fig. 3, Table 2d). Chicks that were initially small exhibited overall higher levels of oxidative damage than large chicks at the end of growth ( $163.1 \pm 17.8$  vs.  $83.4 \pm 19.7$   $\text{mg H}_2\text{O}_2/\text{mL}$ , Fig. 3, Table 2d). There was a positive regression between the final oxidative damage levels and the rates of body mass growth (residual oxidative stress,  $r^2 = 0.41$ ,  $F_{1,15} = 7.57$ ,  $P = 0.019$ ). Again, there was no sex effect on these parameters ( $P > 0.05$ ).

#### Post-winter telomere shortening

At the end of the winter, both small and large chicks started their growth period with a similar telomere length (Table 1), with no sex differences (males,  $1.4 \pm 0.2$  vs. females,  $1.4 \pm 0.1$  T/S,  $F_{1,15} = 0.03$ ,  $P = 0.875$ ). On average, telomere length was shortened during the post-winter growth (from  $1.4 \pm 0.1$  to  $1.1 \pm 0.1$  T/S, Table 2e). Males and females presented a comparable decrease in their telomere length, and there was no impact of growth period duration on telomere loss (Table 2e). However, the total telomere loss was higher in small than in large chicks ( $-0.6 \pm 0.2$  vs.  $-0.01 \pm 0.2$  T/S ratio, Table 2e, Fig. 3), the duration of the growth period being nonsignificant (Table 2e). Small chicks ended with a mean telomere length of  $0.8 \pm 0.1$  T/S ratio and large birds with  $1.2 \pm 0.1$  T/S ratio, telomere length being comparable between sexes at that stage (males,  $1.3 \pm 0.2$  vs. females,  $1.2 \pm 0.1$  T/S,  $F_{1,22.9} = 0.65$ ,  $P = 0.43$ ).

Interestingly, there was a regression between the absolute values of total telomere loss and of plasmatic levels of final oxidative damage, i.e. the higher the damages, the



**Fig. 4** Significant linear relationship between telomere loss and oxidative damage levels at the end of the growth period of king penguin chicks, which were either small (open circles) or large (filled circles) at the beginning of the post-winter growth period. See text for details.

larger the telomere loss throughout the post-winter growth period (regression,  $y = -0.006x + 0.402$ ,  $r^2 = 0.56$ ,  $F_{1,15} = 9.13$ ,  $P = 0.009$ , Fig. 4, the impact of antioxidant capacity being non significant,  $F_{1,15} = 1.21$ ,  $P = 0.29$ ). Additionally, those birds that resumed their growth with a better body condition were also those having lost more telomere sequences, independently of their sex ( $F_{1,15} = 4.84$ ,  $y = -0.238x + 0.007$ ,  $r^2 = 0.27$ ,  $P = 0.047$ , sex effect,  $F_{1,15} = 4.18$ ,  $P = 0.062$ ).

## Discussion

To sum up, chicks from the small group were growing faster and displayed higher final oxidative damages than chicks from the large group. In addition to their implications in ageing theories (Nemoto & Finkel 2004; Monaghan & Haussmann 2006), oxidative stress and telomere loss have been recently negatively linked to both reproductive success and survival rates in several free-ranging bird species (Bize *et al.* 2008, 2009; Salomons *et al.* 2009). Therefore, our results highlight that in natural conditions, oxidative stress and telomere loss are likely to link growth to ageing. The present work gives, to our knowledge, one of the first proximal explanations about how growth can modulate ageing rate in a wild species.

### *Impact of fast growth on oxidative stress*

Fast growth has been previously reported to have adverse effects on longevity (Ozanne & Hales 2004; but see Johnsson & Bohlin 2005). Chronic production of harmful molecules by aerobic metabolism is inevitable in the form of reactive oxygen species (ROS), and one simple (but not always obvious) assertion is that fast growth implies higher metabolic/oxygen consumption rates and exposition to ROS, leading, if organism defences are inadequately low, to a premature accumulation of important cell damages (Beckman & Ames 1998; Mangel & Munch 2005). Even if the core of this hypothesis can be discussed (Crisuolo *et al.* 2008), two previous studies have pointed out that decreased antioxidant defences may result from fast growth and suggested, despite presenting no data on oxidative damages, that oxidative stress may be part of the link between growth and lifespan (Alonzo-Alvarez *et al.* 2007; De Block & Stoks 2008).

### *Impact of fast growth on telomere loss*

Long-term impairment of antioxidant functions has been found 12 months after a growth acceleration in rats (Tarry-Adkins *et al.* 2008), and chronic oxidative stress is known to be at the origin of many age-related

diseases (Valko *et al.* 2007). In parallel, correlation between oxidative stress and telomere shortening is well-known from *in vitro* studies, because of the extreme frailty of telomeric DNA to ROS (von Zglinicki 2002). Association between growth rate and oxidative stress or between growth rate and telomere loss has previously been described *in vivo* (see Monaghan & Haussmann 2006; Monaghan *et al.* 2009 for review), but this is the first characterization of a potential relationship between growth rate, oxidative stress and telomere loss in a wild species. The impact of fast growth in king penguin chicks on telomere loss is in accordance with the previous work conducted on laboratory rats (Tarry-Adkins *et al.* 2009). Still, other factors may be responsible for the higher telomere loss encountered by small chicks. It is well-known, for example, that telomere dynamics are related to physiological and social stress (Epel 2004; Kotrschal *et al.* 2007; Haussmann & Marchetto 2010) that are regulated primarily by endocrine networks such as the hypothalamic–pituitary–adrenal axis. Given the particular role of corticosterone in fasting physiology (i.e. increases when body reserves are low) and its impact on growth rate (delayed growth), small chicks may have experienced higher chronic corticosterone levels. High corticosterone induces a decrease in antioxidant capacities (Stier *et al.* 2009), which may be ultimately responsible for a sharper decrease in telomere length. One direct impact of stress on telomere length is mediated via the reduction of specific enzyme activities such as the telomerase (Haussmann *et al.* 2007; but see Tarry-Adkins *et al.* 2009), which is also known to be decreased by cortisol in humans (Choi *et al.* 2008). As telomerase activity is maintained throughout life in long-lived birds (Haussmann *et al.* 2007), irreversible ability of triggering telomerase because of bad early life programming could also lead to accelerated telomere loss after the growth period ceased, amplifying the early-growth modulation of ageing.

### *Fast growth in king penguin chicks*

King penguins are set apart from other bird's life histories by the exceptionally long chick growth pattern, which is interrupted by a winter break (Stonehouse 1960; Fig. 1). Parental feeding events are irregular throughout the winter, leading to a large variety of body mass dynamics, which ranges from mass stability to important loss and sometimes even death by starvation (Stonehouse 1960; Cherel *et al.* 1987; Weimerskirch *et al.* 1992; Verrier 2003). Our data confirmed that body mass and body size can be extremely different among chicks at the end of the winter phase. But, importantly, we also show that small chicks are able to grow faster

than large chicks during the 2 months prior to fledging, thereby catching-up for their initial delay. The fact that individuals with the lowest body mass exhibited the fastest growth rate when good conditions are restored is consistent with the previous studies in the field (Johnsson & Bohlin 2005) and the laboratory (Jobling & Johansen 1994).

Compensatory growth has previously been described in wild animals (Suttie *et al.* 1983; Bjorndal *et al.* 2003; Johnsson & Bohlin 2010), and our study raised some intriguing questions: why and how might an altricial chick actually exhibit compensatory growth? The former question relates to developmental constraints such as a body condition threshold or the time schedule necessary to resume growth (Carrier & Auriemma 1992), which penguin chicks may be subject to. This is likely to be the case not only before the winter period starts (to reach a sufficient size and mass to successfully survive the winter food shortage), but also after the post-winter growth when chicks have to accumulate enough energy (i.e. proteins) stores to resume complete feather development, a prerequisite for departure at sea (Corbel *et al.* 2009).

The second question concerns the nature of the mechanisms enabling small chicks to show a higher growth rate than large ones. If compensatory growth is likely to involve hyperphagia (Niecieza & Metcalfe 1997), we did not determine how frequently small chicks were fed by parents. This is likely to be a key point, as parental feeding of king penguin chicks during the third phase of growth can be assumed either by both parents or only by one of them (for 65% of the chicks during the last month before moult, Corbel 2008). In addition, alloparental feeding has been previously observed in this species, and it may allow small chicks to be fed at a higher rate once the big chicks have started to quit the colony. However, alloparental feeding is more frequent during the winter period (Lecomte *et al.* 2006), and parents have little to gain by feeding unrelated chicks. Hyperphagia is not the unique parameter enabling full growth catch-up, and adaptations of other components of the energetic balance (e.g. energy expenditure) obviously appear as important modulators of growth rate.

In the king penguin, an entire year is needed to successfully fledge a chick with the consequence that adults can only attempt to breed on time every second year (Olsson 1996). Therefore, breeding late in the season is likely to produce small chicks (both at the entrance and at the end of the winter). In our study, we did not determine the hatching date of our chicks, and we cannot rule out the possibility that large and small chicks are rather early and late chicks. In that case, their growth trajectories may differ, because these chicks are not sampled at the same point of the growth trajectory,

and instead of growth compensation, we are measuring absolute growth rates, more or less high depending on growth stages. However, a previous study outlined that early and late chicks strongly differed in their prewinter growth rates, suggesting that chicks must reach the same degree of organism maturation when the winter fast begin (VanHeezik *et al.* 1993). We indeed observed in a recent field work that the first-fledging chick of a 2009 cohort was a late-hatching chick. In this particular species, with a unique growth pattern, the winter growth break may also synchronize all chicks before entering their last maturation phase before fledging.

#### *Oxidative stress and telomere loss as a constraint on growth*

In our bird model, it is likely that the growth fast induced a higher and unbalanced ROS production, with the consequence that it is hardly possible for penguin chicks to sustain both rapid development and adequate investment in body maintenance. Few data are available about how ROS production may be modified by growth rates. Rapid growth of transgenic mice showed enhanced lipid peroxidation or ROS production (Rollo *et al.* 1996). On the other hand, poor early nutritional conditions are known to impair long-term functioning of antioxidant network (Blount *et al.* 2003). We cannot exclude that our observations result from a bad start (during the first phase of growth) of the post-winter small chicks (e.g. dead small chicks suffered higher initial oxidative stress) rather than catch-up per se. Therefore, without more detailed data on the ontogeny of mitochondrial and antioxidant mechanisms during chick development, we are yet in the expectative concerning the exact nature of the growth/oxidative stress relationship. We also pointed out that, independently of the growth trajectory, ending with a good body condition was also associated with a higher loss of telomere sequences. This further suggests that fat accumulation (which can reach more than 20% in pre-moulting chicks, Cherel *et al.* 1993) is balanced against telomere maintenance mechanisms in penguins. Pathological fat accumulation through obesity has been previously found to be associated with higher levels of oxidative stress (Keaney *et al.* 2003; Furukawa *et al.* 2004) or shorter telomere length in humans (Valdes *et al.* 2005; Zannolli *et al.* 2008). Knowing that juvenile survival rates are positively related to body condition at departure in king penguin chicks (Saraux *et al.* 2011), the question of the potential long-term detrimental long-term effects (physiological/fitness) of ample fat accumulation evolved by penguins merit further consideration.

Small chicks that died early in the experiment were those presenting the shortest telomere length, highest

oxidative damage and least antioxidant capacity. This supports the view that telomere length may be a good predictor of future survival rate (Hausmann *et al.* 2005; Bize *et al.* 2009). Even if it deserves future experimental confirmation and long-term longitudinal studies to assess the ultimate consequences of increased telomere loss during early life in penguins (e.g. shortened lifespan), our study supports the view that telomere loss represents one proximal explanation of the growth-ageing trade-off (Metcalf & Monaghan 2003). This contributes to explain why, in general, growth rates are constrained by selection to a submaximal level.

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S.G. and M.L.V. resumed a PhD on fasting physiology in 2010 and on foraging performances of king penguins in 2011. S.R. and A.S. are currently working on a PhD program on telomere and oxidative stress as fitness indicators in birds, respectively. Y.L.M. is an ecophysiologicalist, specialist of penguin biology and F.C. is an evolutionary biologist working on ageing mechanisms and mitochondrial biology.

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### **Data accessibility**

Data deposited in the Dryad repository: doi: 10.5061/dryad.jh174st3.



## Appendix 3 –

Maternal effects, mediated by maternally derived hormones,  
underlie ageing costs of growth in the zebra finch  
(*Taeniopygia guttata*)



**Maternal effects, mediated by maternally derived hormones, underlie ageing costs of growth in the zebra finch (*Taeniopygia guttata*)**

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# Abstract

## Background

Maternal effects provide a mechanism to adapt offspring phenotype and optimize the mother's fitness to current environmental conditions. Transferring steroids to the yolk is one way mothers can translate environmental information into potential adaptive signal for their offspring. However, maternally-derived hormones might also have adverse effects for offspring, *e.g.* recent data in birds suggested that ageing related-processes (*i.e.* oxidative stress and telomere shortening) were increased after egg-injection of corticosterone (CORT). Still, we have no information on the biological relevance of maternally-derived hormones as direct or indirect modulators of ageing phenotype in offspring.

## Results

To fill this gap, we chronically treated pre-laying zebra finch females (*Taeniopygia guttata*) with 17- $\beta$ -estradiol (E<sub>2</sub>) or CORT, and followed offspring growth and cellular ageing rates. CORT injections decreased growth in male chicks and increased rate of telomere shortening in female offspring. E<sub>2</sub> increased body mass gain in male offspring, while reducing oxidative stress in both sexes but without affecting telomere loss.

## Conclusions

Since short telomeres were previously found to be a proxy of individual lifespan in zebra finches, maternally-derived hormones may, through pleiotropic effects, be important determinants of offspring life-expectancy by modulating ageing rate during embryo and post-natal growth.

## Key-words

Maternal effects, hormones, CORT, ageing, oxidative stress, telomere

## Introduction

Embryonic and post-natal growth are key phases of the life-cycle affecting final body size in organisms which can be positively correlated with subsequent fitness, *e.g.* through effects on size-dependent fecundity, or reproductive success [1,2]. Since growth is energetically costly (requiring abundant resources or/and parental care), it is clear that “external” constraints can limit growth [3]. Parental care, predation, social environment, litter size, within-brood hierarchy or parasitism can interact and modulate offspring growth [4,5,6]. In addition, “internal” constraints can limit growth due to the cost of growth itself [2]. Many studies have been focusing on physiological costs of fast growth, revealing among others an accelerated ageing process, for instance through a decreased resistance to oxidative stress [7, 8, 9] looking for a proximal explanation of the reduced lifespan of fast-growing individuals [10]. Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defenses, causing physiological damage such as lipids, proteins or DNA deteriorations, among which telomere attrition [11,12]. Telomeres are the ends of eukaryotic chromosomes formed by repeated sequences of non-coding DNA – (TTAGGG)<sub>n</sub> in vertebrates – ensuring the protection of the encoding part of the chromosome. Their role in cell senescence and apoptosis is now well known, short and dysfunctional telomeres triggering an emergency cell signaling cascade [13]. Telomeres were more recently used as an organism longevity proxy, first in humans [14] and in wild animals, where survival rate and lifespan were associated with the longest telomeres and/or the shortest rate of telomere loss [15,16,17,18]. By demonstrating that telomere length and telomere loss in early life can predict the lifespan of individuals in zebra finches, and that those having long telomeres at the end of the growth period had consistently longer telomeres over life, Heidinger *et al.* [18] have highlighted the importance of telomere dynamics during pre- and post-natal growth in terms of future fitness.

Mothers may affect the way their offspring grow up by modulating some physiological costs of growth, notably through hormonally-mediated maternal effects, an idea that received recent experimental support [19]. Maternal transfer of steroid hormones to egg yolk can have significant effects on offspring phenotype, *e.g.* through the down-regulation of offspring growth following CORT treatment [20, 21, 22], a hormone also known to cause a



decrease in immune response [23], standard metabolic rate [24] or survival rate [6]. Haussmann *et al.* [19] actually shown that an embryonic exposure to corticosterone by egg injection increases oxidative stress and reduces telomere length in domestic chicken chicks (*Gallus domesticus*), when these chicks were separated from their parents. Maternally derived hormones are also known to have a positive impact on ageing mechanisms, *e.g.* E<sub>2</sub> increases telomerase activity in humans, directly activates a promoter of telomerase [25] but also down-regulates oxidative stress by protecting neurons during development [26], reducing ROS production and being a potent antioxidant [27, 28] (but see also [29]).

These studies therefore suggest that hormones can have a significant impact on oxidative stress and telomere loss during growth and potentially influence individual telomere length at fledging, a fitness-related trait in captive zebra finches [18]. Still, the actual link between hormonally-mediated maternal effects, oxidative stress and telomere dynamics remains largely unknown. Here, we attempt to fill this gap by examining the impact of these two important maternally-derived yolk hormones – CORT and E<sub>2</sub> – on the body mass gain, oxidative stress and telomere dynamics during early growth in zebra finches. We injected mothers with hormones during egg formation – based on the evidence that an increase of E<sub>2</sub> or CORT plasma levels is followed by an increase into the yolk of eggs produced by these females [20, 23, 30, 31] – and looked at the impact of hormone treatment on offspring early development. Because of the antagonistic role of these hormones either on growth or oxidative stress (see above) we predicted that (1) exposure to CORT influences growth and ageing processes by decreasing body mass gain and increasing oxidative stress and telomere loss during the juvenile growth period, whereas (2) exposure to E<sub>2</sub> influences growth and ageing processes by increasing body mass gain and decreasing oxidative stress and telomere loss during this period.

## Material and Methods

### *(a) Animal care and breeding protocol*

Zebra finches were maintained in controlled environmental conditions (temperature 19–23°C; humidity 35–55%; constant light schedule, 14L: 10 D, lights on at 07.00). Breeding pairs were provided with a mixed seed diet (*Panicum* and white millet, 1:3, 11.7% protein, 0.6% lipid and 84.3% carbohydrate by dry mass), water, grit and cuttlefish bone (calcium) *ad*

*libitum*. They were complemented with a multivitamin supplement in the drinking water once per week and 6 g/pair per day of egg food (eggs, bread crumbs and cornmeal: 20.3% protein, 6.6% lipid) between pairing and fledging. Breeding pairs were placed in separate breeding cages (51cm x 39cm x 43cm) equipped with nest boxes (14cm x 14.5cm x 20cm) and nesting material. Nest-boxes were checked daily between 09:00 and 12:00 and all new eggs were weighed (to 0.001 g) and numbered. Chicks were raised by their parents until 30 days and then moved to same-sex cages (46 x 46 x 46 cm). Experiments and animal rearing were conducted under a Simon Fraser University Animal Care Committee permit (901B-94), in accordance with guidelines from the Canadian Committee on Animal Care (CCAC).

*(b) Female injections and chick growth*

A total of 45 females were weighed (0.1 g) and blood sampled (~ 80µL from the brachial vein) at the time of pairing, at clutch completion (CC, defined as 2 days after the last egg was laid [32]) and at chick independence (30 days after hatching). Females were randomly assigned to one of the following treatments: CORT, E<sub>2</sub> or Control (15 females *per* treatment). CORT and E<sub>2</sub> groups received hormones diluted in canola oil (CORT: 19.2ng/30µL of canola oil, E<sub>2</sub>: 16µg/30µL of canola oil) while the Control group received only canola oil. Steroids were first dissolved in isopropanol and then re-suspended in canola oil. Females received intramuscular injections every day between 10:00 and 11:00 from day 3 after pairing until CC. Doses were chosen to raise hormones levels in females' plasma while remaining within physiological levels [30, 33].

Of the 45 females paired, 30 (9 CORT, 8 Control, 13 E<sub>2</sub>) laid eggs within 2 weeks (66%) while the other 15 were removed from the experiment. Nest boxes were checked twice a day from day 8 after CC until all the eggs hatched. The hatching sampling size was of 15 CORT, 16 E<sub>2</sub> and 9 Control chicks (see **Electronic Supplementary Material, ESM**). Chicks were marked with a non-toxic p and en for individual recognition, weighed to the nearest 0.001g at hatching and were weighed every two days until 30 days of age. They were blood sampled (about 30µL, from the brachial vein) at 10, 20 and 30 days of age for oxidative stress and telomeres measurements. Blood samples were kept on ice and centrifuged at 6000 rpm for 10 minutes within an hour. Chick growth rate was estimated by calculating the mean body mass gain per day during the initial phase of rapid growth (0 to 10 days), and then during the subsequent growth period before separation with the parents (10 to 30 days) [34].

*(c) Telomere length and oxidative stress assessments*

DNA was extracted from 4µL of red blood cells – nucleated in birds – using the DNeasy Blood and Tissue kit (Qiagen, Germany). QIAGEN protocol for animal blood (Spin-Column protocol) was followed, replacing the 10min of incubation at 56°C by 3 hours at the same temperature in a water bath. Telomere measurements were conducted by quantitative real-time amplification (qPCR) of the telomere sequence (**ESM**), following the procedure described by Criscuolo *et al.* [35], an adapted procedure for zebra finches of a protocol initially setup on human samples [36], widely used since 2009 (*e.g.* [16, 17]).

Oxidative stress was analyzed in plasma samples by measuring DNA damage, ROMs (reactive oxygen metabolites) and total antioxidant capacity of the plasma [37,38], using the DNA Damage EIA kit (Enzo Life Sciences, USA), the d-ROMs test (Diacron International, Grosseto, Italy) and the OXY-ADSORBENT test (Diacron International, Grosseto, Italy), following manufacturer protocols (**ESM**).

*(d) Statistical analyses*

In all our models, hormonal treatment, chick sex and sex\*treatment interaction were used as fixed factors, while brood size and hatching rank of chicks were included as covariates. We first checked whether (i) egg mass, (ii) hatching body mass and (iii) chick body masses at 10 and 30 days differed among groups, using Linear mixed models (LMM). The following covariates were included, *i.e.* respectively (i) mother's mass, (ii) egg mass and (iii) previous chick body mass (*i.e.* at hatching when testing for differences at day 10, and at day 10 when testing for differences at day 30).

Then, an independent LMM was used to test if the changes in body mass during the 30 days of growth differed among hormonal treatments, with the previous mass (*i.e.* the body mass at hatching for the 0 to 10 days period and body mass at 10 days for the 10 to 30 days period) included as a covariate. We controlled for the non-independence of individuals of the same nest and the non-independence of repeated measurements on the same individual by including individual identity nested into the nest identity as a random effect, time period (0 to 10 days and 0 to 30 days) being the repeated fixed factor in this model. Finally, we conducted LMM *per* period to test whether the treatment affects ROMs and OXY levels (at 10 days first and at 30 days) and whether it affects telomere loss from 10 to 30 days. We included as covariates in those models (i) previous ROMs and OXY levels in the models

controlling for oxidative stress levels and (ii) body mass gain, ROMs and OXY at 10 days in the model testing the impact of treatment on telomere loss.

We also used a path analysis, to test the significance of multiple relationships among potentially inter-related variables, and to assess the sign and strength of those relationships [39]. Path analysis aims mainly to establish the links that should be further explored in future experiments [40]. Here, we estimated and quantified hypothesized causal relationships between (i) body mass gain from 0 to 10 days and (ii) from 10 to 30 days, (iii) ROMs and (iv) OXY at 10 days and (v) telomere loss from 10 to 30 days. Path analyses were conducted independently in each experimental group, to assess how these relationships may have been affected by E2 and CORT treatments (*i.e.* this is not used to test how treatment affect each variable). We present here the path analysis yielded by a significant threshold of  $p < 0.05$  (we check also for results using  $p < 0.1$  which may be more adapted to small sample sizes [40] and found similar results, data not shown). Path coefficients  $> 0.4$  (absolute values) are typically considered as strong, while those  $> 0.2$  are moderate.

Final LMM selection was based on the best AICc value (*i.e.* the small-sample-corrected version of the AIC). Normality was tested by a Kolmogorov-Smirnof test and the comparisons 2 by 2 were realized with a post-hoc LSD (least significant difference) test. All the tests were conducted on SPSS (V.20) and  $p < 0.05$  were considered significant.

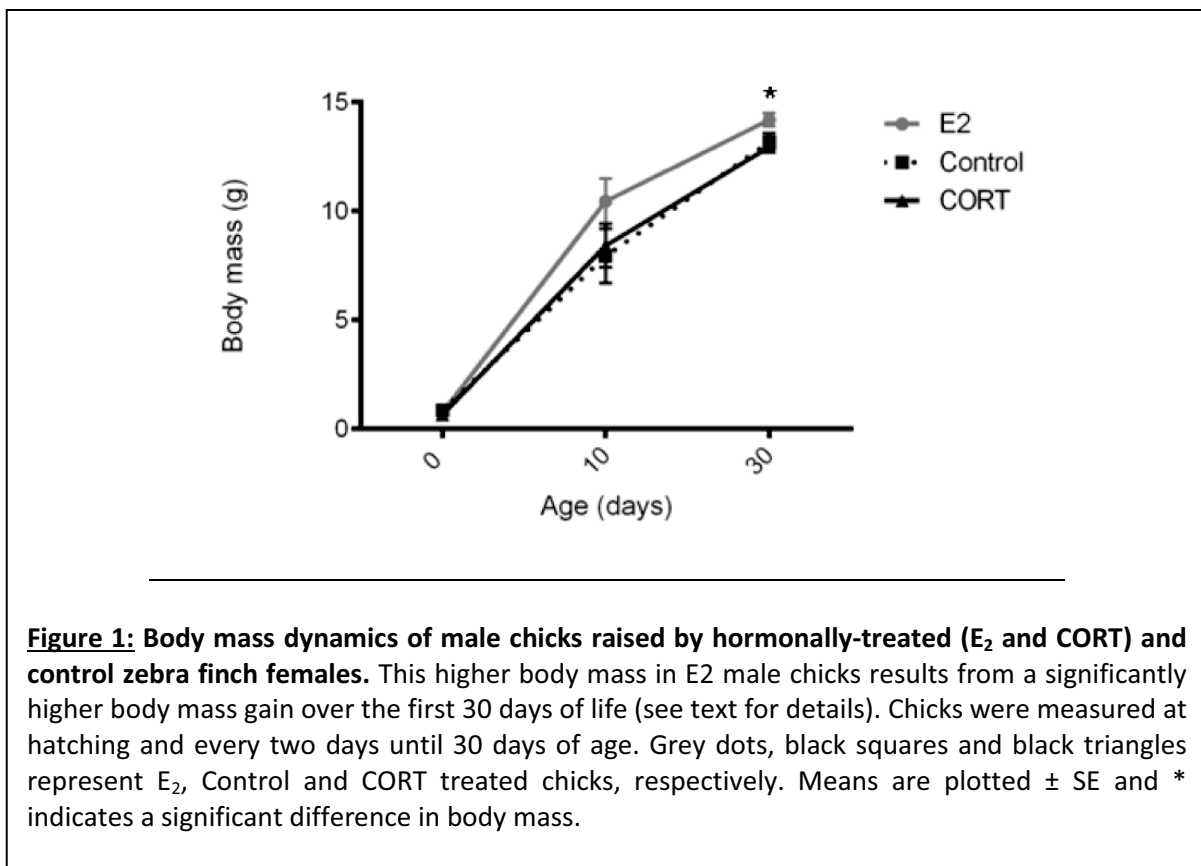
## Results

### *(a) Treatment effects on embryonic and post-natal growth*

Egg mass differed significantly among treatments ( $F_{2,154}=6.873$ ,  $p=0.001$ ), with a significant impact of mother mass ( $F_{1,154}=37.736$ ,  $p<0.001$ ): eggs produced by E<sub>2</sub> females were significantly heavier than eggs produced by Control and CORT treated females ( $1.101\pm 0.055$ ,  $1.047\pm 0.056$ ,  $1.044\pm 0.056$ ,  $p=0.003$  and  $p=0.002$ , respectively). Similarly, hatching body mass of chicks was significantly affected by egg mass ( $F_{1,30}=4.275$ ,  $p=0.047$ ) and mothers' hormonal treatment ( $F_{2,30}=5.918$ ,  $p=0.007$ ), but in this case CORT chicks were lighter than Control and E<sub>2</sub> chicks ( $0.635 \pm 0.062$  g vs.  $0.783 \pm 0.066$  g and  $0.767 \pm 0.065$  g,  $p=0.002$  and  $p=0.017$  respectively).

Body mass gain of chicks was significantly affected by chicks' previous mass ( $F_{2,26.725}=438.628$ ,  $p<0.001$ ) and by the sex\*treatment interaction ( $F_{2,29.671}=4.276$ ,  $p=0.023$ ),

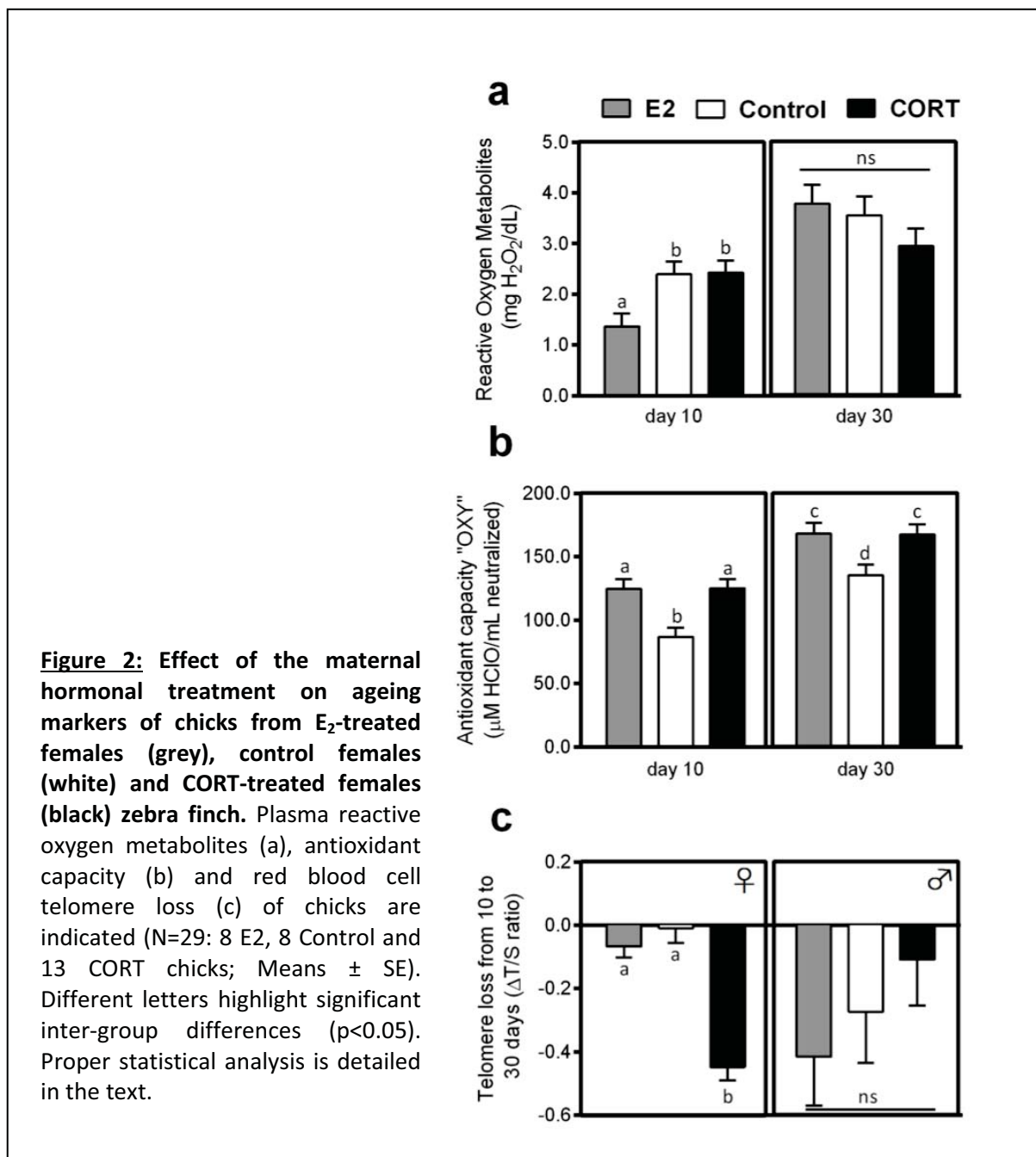
indicating that hormonal manipulation of mothers differently affects growth of male and female chicks. We conducted a separated analysis of treatment effect by chick gender which highlighted that treatment affected growth rate in males but not in females ( $F_{2,10.560}=4.276$ ,  $p=0.008$  and  $F_{2,11.510}=0.063$ ,  $p=0.939$ ). In fact, E2 males gained on average significantly more mass than CORT males (Figure 1,  $p=0.002$ ) but not than Control males (Figure 1,  $p=0.063$ ). This gender-biased effect is confirmed when looking at body mass of chicks at 30 days of age ( $F_{2,22}=4.156$ ,  $p=0.029$ ), E<sub>2</sub> males being heavier than Control and CORT males (Figure 1,  $p=0.030$  and  $p=0.009$  respectively).



*(b) Treatment effects on chick oxidative stress and telomere dynamics.*

Hormonal treatment of pre-laying females and chicks' body mass gain from 0 to 10 days had a significant impact on ROMs levels of 10 days-old chicks ( $F_{2,21}=4.524$ ,  $p=0.023$  and  $F_{1,21}=14.628$ ,  $p=0.001$  respectively). E<sub>2</sub> chicks from both sexes exhibited lower ROMs levels than Control and CORT chicks (Figure 2a,  $p=0.013$  and  $p=0.008$  respectively). Chick ROMs levels also differed between sexes at 10 days ( $F_{1,21}=6.109$ ,  $p=0.022$ ), male chicks being characterized by lower ROMs levels than females at this age ( $1.732 \pm 0.465$  mg H<sub>2</sub>O<sub>2</sub>/dL and  $2.398 \pm 0.461$  mg H<sub>2</sub>O<sub>2</sub>/dL respectively). However, there was no significant effect of

treatment or sex on ROMs at 30 days (Figure 2a,  $F_{2,21.300}=1.586$ ,  $p=0.228$ ) but a significant effect of chicks' body mass gain from 10 to 30 days ( $F_{1,21.358}=5.556$ ,  $p=0.028$ ) and of brood size ( $F_{2,22.365}=5.171$ ,  $p=0.033$ ). Treating mothers with CORT and  $E_2$  had also a significant impact on the plasma antioxidant capacity of their chicks at 10 days ( $F_{2,22}=8.575$ ,  $p=0.002$ ).  $E_2$  and CORT chicks had a higher antioxidant capacity than Control chicks (Figure 2b,  $p=0.006$  and  $p=0.001$  respectively). The same difference was observed at 30 days ( $F_{2,22.949}=7.617$ ,  $p=0.003$ ) with post-hoc analyses indicating that Control chicks had reduced antioxidant capacity compared to  $E_2$  and CORT chicks (Figure 2b,  $p=0.003$  and  $p=0.002$  respectively). We found no treatment effect on the plasma levels of DNA damage measured in 20 days-old chicks ( $F_{2,3.132}=1.372$ ,  $p=0.373$ ).



The hormonal treatment had also a significant impact on telomere loss of chicks from 10 to 30 days (Figure 2c), with a sex\*treatment interaction effect ( $F_{2,20}=3.227$ ,  $p=0.048$ ). While no significant treatment effect was found in males ( $F_{2,5.26}=0.871$ ,  $p=0.471$ ), there was a significant treatment effect in female chicks ( $F_{2,9}=6.652$ ,  $p=0.017$ ). Post-hoc tests indicated that CORT chick females had greater telomere loss compared to Control and  $E_2$  ones (Figure 2c.,  $p=0.009$  and  $p=0.025$  respectively). Telomere loss of chicks was also affected by their body mass gain from 10 to 30 days ( $F_{1,20}=5.575$ ,  $p=0.028$ ).

(c) *Path analysis, multiple relationships between:*

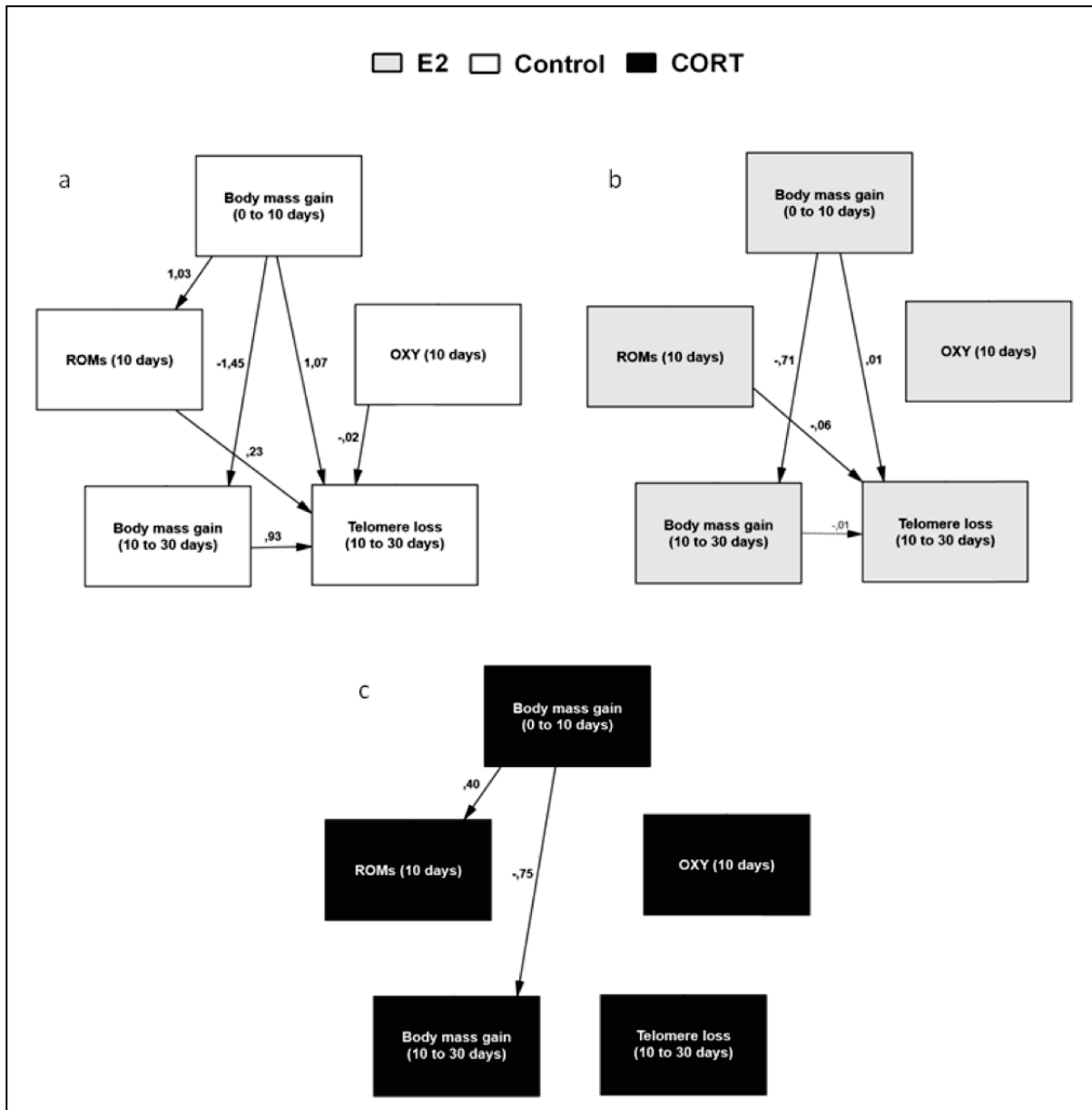
*Body mass gain and ageing variables*

The results of the multiple regression analysis (done *per* treatment) show that in the Control and CORT groups, body mass gain from 0 to 10 days was significantly and positively correlated with ROMs levels (Figures 3a and 3c,  $p<0.001$  and  $p=0.025$ ) while this was not observed in the  $E_2$  group (Figure 3b,  $p>0.05$ ). Body mass gain from 0 to 10 days also contributed directly but negatively to the rate of body mass gain recorded between 10 to 30 days in Control and CORT groups (Figures 3a and 3c,  $p<0.001$ ) but positively in the  $E_2$  group (Figure 3b,  $p=0.005$ ). In other words,  $E_2$ -treated chicks with the fastest growth between 0 and 10 days also had the fastest growth between 10 and 30 days but without any associated increase in their d-ROMs plasma levels.

Finally, body mass gain from 0 to 10 days contributed directly to telomere loss between 10 and 30 days in two situations: (i) positively in the Control group (Figure 3a,  $p=0.035$ ), the faster growing chicks losing more telomere length and (ii) negatively in the  $E_2$  group (Figure 3b,  $p=0.011$ ). This suggests counter-intuitively that the chicks raised by  $E_2$  females that grew faster from 0 to 10 days had reduced telomere loss subsequently. No significant interactions were observed in the CORT group between these two variables. In the Control group only, there was also a significant and positive impact of the body mass gain recorded from 10 to 30 days and the associated telomere loss during this period (Figure 3a.,  $p<0.001$ ).

*Oxidative stress and telomere loss*

ROMs levels at 10 days significantly contributed to the rate of telomere loss between 10 and 30 days in the Control (positively, Figure 3a,  $p=0.032$ ) and  $E_2$ -treated chicks (negatively, Figure 3b,  $p=0.05$ ). No significant relationships between these variables were found in the CORT group (Figure 3c,  $p>0.05$ ). In the control group, there was also a significant and negative contribution of the antioxidant levels at 10 days on the subsequent telomere loss (Figure 3a,  $p<0.001$ ).



**Figure 3:** Path analysis diagrams for intra-group relationships between growth and ageing variables measured on chicks, in response to the hormonal treatment of the reproductive mothers. Control (a),  $E_2$  (b) and CORT group (c). Arrow represent causal link between variables, and the  $\beta$  coefficients (which can be positive or negative) are indicated along their respective paths. Significant paths are indicated with numbers in bold. See text for statistical details. **ROMs:** reactive oxygen metabolites; **OXY:** plasma antioxidant capacity.



## Discussion

A recent study suggested that pre-natal exposure to CORT seems to modulate telomere-driven ageing process in birds [19]. However, this study did not assess maternal effects *per se*, since the treatment was done independently of the parents with eggs being injected directly and subsequently artificially incubated. Our study design actually examined the effect of an experimental manipulation of the maternal hormonal state during laying on chick growth rate. We subsequently studied the direct or indirect consequences of this hormonal treatment on chick ageing processes – *i.e.* oxidative stress and telomere dynamics – during early growth. Using two steroid hormones that are suspected to have pro- (CORT) or anti-ageing effects ( $E_2$ ), we showed that maternal exposure to CORT lead to a reduced hatching body mass in both sexes, and to an increased telomere loss in female chicks. Conversely, maternal exposure to  $E_2$  increased body mass gain and body mass at the end of growth in male chicks, without inducing any negative impact on ageing parameters. Uncoupling of the links between those inter-related variables in chicks reared by hormonally treated females suggests that the whole balance between pro- and anti-telomere erosion processes was likely affected by our treatment. Possible underlying mechanisms are further discussed below.

### *Indirect effects through the modulation of maternal behavior*

CORT-treated male chicks had reduced growth and a reduced body mass at 30 days compared to  $E_2$  male chicks, with Control male chicks showing intermediate values. The complexity of interpretation of the impact of CORT on growth (depending on which is the reference group) is reflected in the literature, some authors finding that CORT did not impact growth or fledging body mass [19, 23] while some others found that CORT reduces chick body mass growth [20, 21, 41]. Exposure to CORT and  $E_2$  in pre-laying females could have affected offspring growth in different ways. First, the treatment could have indirectly affected chick growth by modulating maternal investment into her progeny. Indeed, the increased egg mass of  $E_2$ -injected females in our study suggests a greater energy transfer of

these females to the embryo, as previously noted by Williams [42]. This high investment in reproduction should then lead to a better fitness for the mother, through “better quality” offspring (not necessarily of heavier hatching chicks but maybe *via* better growing chicks), which is consistent with the finding of increased body mass growth and increased 30 days-old body masses of E<sub>2</sub> compared to CORT offspring males.

*Indirect effects through the modulation of chick growth rate*

Alternatively, our results could be due to a direct modulation of chick growth by maternal estradiol. Unfortunately, to our knowledge, studies connecting E<sub>2</sub> and growth are still lacking, particularly in birds (but see [43, 44]). Interestingly, the impact of CORT on embryonic development is clearer. Indeed, treatment slowed embryonic development, chicks of CORT-injected females being lighter than others at hatching. This finding – consistent with previous studies [41, 45] – and associated with the increased egg mass of E<sub>2</sub>-treated females suggests that our hormonal treatment was physiologically relevant and transferred in some way into the egg. The treatment has also affected ageing parameters of mothers, with CORT negatively affecting oxidative stress and telomere maintenance (**ESM**). Indeed, CORT-treated females had higher levels of ROMs and shorter telomeres at fledging, suggesting an ageing cost of CORT injections [19].

*Indirect effect through the modulation of growth associated costs*

Alternatively, increased yolk E<sub>2</sub> could have had an effect on growth through the down regulation of oxidative stress (lower levels of oxidative damage and higher levels of plasma antioxidant capacity). Indeed, as explained above, oxidative stress has been suggested as having constraining effects or at least being inter-related with growth rate [12; 46]. By down regulating oxidative stress, E<sub>2</sub> could have indirectly affected growth capacity or optimality. Interestingly, E<sub>2</sub> males grew faster but did not suffer increased oxidative damage which is consistent with estrogen’s capacity to reduce ROS production [27] (reviewed in [28], but see also [47]) or its ability to be a potent antioxidant [28]. However, contrary to our predictions – and unlike a previous study [19] – we found no impact of CORT on oxidative damage (*i.e.* ROMs levels) either at 10 or 30 days. This finding can be due to higher levels of plasma antioxidant capacities (*i.e.* OXY) in CORT chicks from our experiment. Moreover, we worked on altricial birds, less likely to be affected by oxidative stress than self-feeding chicks [19]

because parents could buffer CORT deleterious effects – notably through increased feeding rates. Altricial birds are also more likely to be affected by a modulation of free CORT and corticosterone binding globulin (CBG) capacity by environmental conditions [48]. Nevertheless, in a recent study [49], cortisol levels were not directly but indirectly – *i.e. via* fungal infections – related to oxidative damage. This latter study highlighted the complexity of the relationships existing between the endocrine system and metabolism, thereby pointing out that caution should be applied in interpreting the relationships between a “single hormone and indicators of oxidative balance or other fitness proxies”. As such, even if CORT is not directly related to oxidative damage in our study, levels of CORT might be related to another hormone level, *e.g.* testosterone or prolactin, which could also affect oxidative damage.

Surprisingly, even though we found no effects of CORT-treatment on chick’s oxidative damage, and no correlation between ROMs levels and telomere loss (figure 3c), female CORT-treated chick had greater telomere loss between 10 and 30 days than female chicks of the other groups. This telomere loss is not related to the body mass gain, ROMs levels or OXY levels (figure 3c) contrary to what was observed in the control group (figure 3a). Under enhanced CORT-levels, telomere erosion due to cell division might have been costly because of the inhibition of telomere maintenance process by CORT, *i.e.* the inhibitory effect of CORT on telomerase [50]. On the other hand, there was no impact of the treatment or of the increased growth on telomere attrition in E<sub>2</sub> chicks. As indicated in figure 3b, the link between oxidative damage and telomere loss and the link between body mass gain from 0 to 10 days and subsequent telomere loss are the reverse of the links observed in Control chicks (figure 3a.). This suggests that the relationships between growth, oxidative stress and telomere loss have been changed by E<sub>2</sub> treatment, hypothetically through a positive impact of E<sub>2</sub> on telomerase activity. Indeed, besides its antioxidant capacities, estrogen is also known to be an activator of telomerase [25] and to reduce telomere shortening in humans [27].

## Conclusion

In a conclusion, E<sub>2</sub> treatment seems to have a sex-biased positive impact on offspring post-natal growth – and no impact on embryonic development, unlike CORT – associated

with short-term physiological benefits (*i.e.* decreased oxidative damage and increased plasma antioxidant capacities), but no costs on telomere attrition perhaps due to a positive impact of E<sub>2</sub> on telomerase activity. On the other hand, maternally derived CORT seems to have an impact on offspring embryonic development (both in males and females) and a sex-biased long term cost on ageing processes, affecting only female's telomeres in zebra finches, again potentially because of a decreased activity of telomerase in females. These sex-biased effects could be explained by a different investment of mothers into males and females, triggered by E<sub>2</sub> or CORT injections or by different direct effects of these hormones according to the sex of the chicks. An experiment based on egg injections would allow us to discriminate maternal-derived and direct effects of hormones on chicks' phenotypes and to understand if maternal hormones act directly on ageing parameters. Hausmann *et al.* [19] showed that CORT injection in eggs influence chick ageing in chicken. Here we show that maternal-hormonal status can also modulate growth and ageing in chicks. The next step would be to test whether natural variability in maternal E<sub>2</sub> and CORT levels is of importance in determining chick phenotype at fledging.

### **Acknowledgement**

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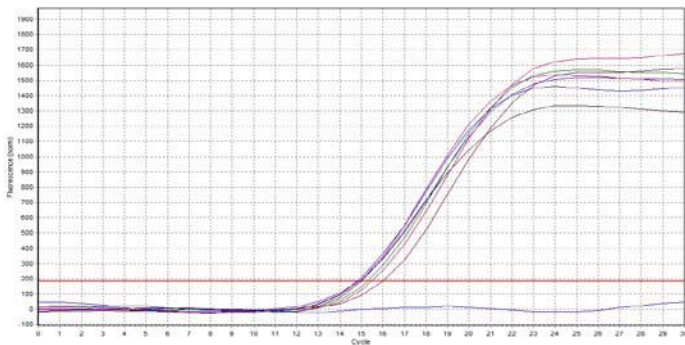
## ELECTRONIC SUPPLEMENTARY MATERIAL

### Material and Methods

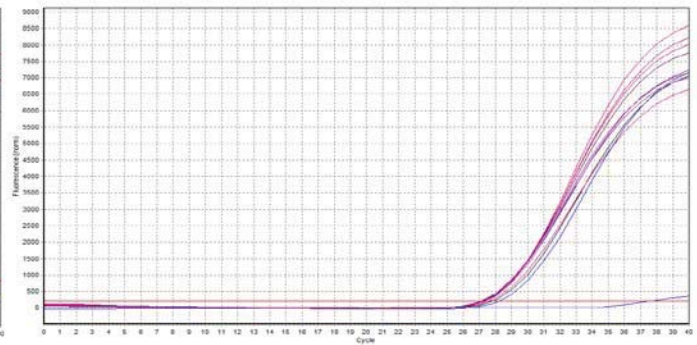
Hatching occurred in 17 of the 30 nests and the 17 “successful” nests were composed of 4 CORT, 9 E<sub>2</sub>, and 4 Control nests. Total hatching success – *i.e.* number of egg which hatched / total number of eggs – in CORT, E<sub>2</sub> and Control groups was of 45%, 29% and 25%, respectively, resulting in a final sample size of 15 CORT, 16 E<sub>2</sub> and 9 Control chicks at hatching. Chicks’ survival rate during growth was of 86.67% in the CORT group, 50% in the E<sub>2</sub> group and 100% in the control group, resulting in a sample of 13 CORT chicks, 8 E<sub>2</sub> chicks and 9 Control chicks at fledging. The sex of chicks dead before sexual maturation was molecularly determined using a method adapted from Griffiths *et al.* (1998). Briefly, this method is composed of three steps (i) DNA extraction, (ii) polymerase chain reaction (PCR) and (iii) electrophoresis on an agarose gel to determine the sexual chromosomes of the individuals (ZZ in males and ZW in females).

Telomere length measurement is based on the determination of a number of amplification cycles necessary to detect a lower threshold of fluorescent signal. Cycle number is proportional to the sample telomere length (T), or to the number of copies of a non-variable copy number gene (or control gene S, Smith *et al.* 2011). A ratio (T / S) is then calculated for each sample that will reflect relative inter and intra-individual (when repeated samples are taken from the same individual) differences in telomere length. As a control gene, we used the GAPDH (coding for the glyceraldehyde 3-phosphate dehydrogenase), amplified by oligonucleotide primers GAPDH-F (5'-AACCAGCCA-AGTACGATGACAT-3') and GAPDH-R (5'-CCA-TCAGCAGCAGCCTTCA-3'). For telomeres amplification, primers used were of the following sequences: Tel1b (5'-CGTTTGGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3') and Tel2b (5'-GGCTTGCCTTACCCTTACCCTTACCCTTAC-CCTTACCCT-3'). Primers – at a concentration of 200nM/200nM – were mixed with 5µL of SYBR Green (a nucleic acid stain), water and 2.5 ng of DNA by well, in a 10µL final solution. Cycling conditions included an initial hold at 95°C for 2 mins for the enzyme activation followed for telomere amplification by 30 cycles of: 1 min denaturation step at 95°C, 30 secs hybridation step at 56°C, and 30 secs elongation step at 72°C including the fluorescence signal readings. A 20 min final melt step was included on each run with the temperature ramping from 56°C to 95°C. The non-

VCN gene qPCR conditions were characterized by a 2 mins activation step at 95°C followed by 40 cycles of: 1 min 30 secs denaturation step at 95°C, and one step of both hybridation and elongation at 60°C for 1 min, with fluorescence readings recorded at this step. Again, a final melt step ended each run with the temperature ramping from 60°C to 95°C within 20 min. Cycling was performed on a Mastercycler real-time PCR instrument (Eppendorf, Germany). All the samples are run in duplicate and in each run, we included 6 replicates of DNA samples taken from an even number of individuals from each treatment group, as well as a no-template negative control. Mean amplification efficiencies of the qPCR runs were comprised between 0.96 and 0.99 for telomere and between 0.98 and 1.05 for the non-VCN gene (see figures below for example of amplification curves of samples and of the negative control).



**Figure ESM 1:** telomeres amplification



**Figure ESM 2:** gapdh amplification

Intra-plate coefficients of variation (based on CT values) were really low: 0.9% for telomere assay and 0,5% for the non-VCN gene assay. Inter-plate coefficients of variation based on repeated samples were of 1,9% for the telomere assay and 0.8% for the non-VCN gene assay (Ct values again). The melting curves showed a single peak of amplification for samples and no peak for negative control (water).

The DNA damage kit (used on chick's plasma sample at 20 days of age) allows a measurement of the plasmatic concentration of 8-hydroxy-2'-deoxiguanosine (8-OHdG), a form of free radical-induced oxidative lesion on DNA, which is a common marker of oxidative stress. All samples were measured in the same run, and intra-plate variation was 4.1%. The d-ROMs test measures the concentration of hydroperoxides, reactive oxygen metabolites produced following an attack of ROS on organic substrates (Costantini *et al.* 2006), while the

OXY-ADSORBENT test measures total antioxidant capacity of plasma (see Beaulieu *et al.* (2010) for details on the procedures). ROMs and antioxidant concentrations into plasma were assessed by colorimetry (at 510 nm) and expressed in mg of H<sub>2</sub>O<sub>2</sub>/dL and μmol of HClO/mL respectively. Intra-plate variation based on duplicates was below 10% (6.3% and 7.3% for ROMs and OXY respectively) as well as inter-plate variation based on a sample repeated over plates (CV = 5.5% and CV = 6.9% respectively).

## Results

### *Oxidative stress and telomere length of the mothers*

ROMs levels and relative telomere loss of mothers were affected by our experimental treatment ( $F_{2,6}=6.872$ ,  $p=0.028$  and  $F_{2,7}=4.723$ ,  $p=0.050$ ). Post-hoc analyses indicated that mothers injected with CORT had more ROMs than Control mothers ( $11.927 \pm 1.543$  mg H<sub>2</sub>O<sub>2</sub>/dL and  $6.067 \pm 1.579$  mg H<sub>2</sub>O<sub>2</sub>/dL respectively,  $p=0.010$ ) when their chicks were 30 days old, relatively to their levels at pairing. CORT-treated mothers had also higher rate of telomere loss than Control ones ( $-0.631 \pm 0.138$  vs.  $-0.032 \pm 0.139$ ,  $p=0.018$ ). We found no significant differences in those parameters between E<sub>2</sub>-treated females and the other ones. OXY levels and DNA damage of mothers were not affected by our hormonal treatments ( $F_{2,6}=0.657$ ,  $p=0.552$ ,  $F_{2,4}=0.575$ ,  $p=0.603$ ).

## References

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## Abstract

Little is known about the processes mediating and underlying life histories diversity, even though it is considered crucial to understand how life histories have evolved. Individual life history trajectories are shaped by the trade-offs that are made among traits that compete for limited a pool of resources. Thereby, ageing processes are likely to be of importance since they are changing across life stages, moreover at different rates, among individuals.

In this context, there is clearly a great potential in the study of the repetitive DNA sequences that cap eukaryotic chromosomes, the telomeres. Telomeres are structures made of double stranded non coding DNA that are involved in the cells' replicative capacity and are thought to play an important role in linking life conditions and senescence. Indeed, when the telomeres reach a certain length, cells stop dividing and enter a state of senescence. This suggests that telomeres could be part of the basic mechanisms that determine the rate of ageing. Although telomeres generally shorten with age in vertebrates, in most species studied there is considerable variation among same aged individuals. This age-matched individual variability suggests that telomere length might reflect more than just the effects of age, and that telomeres could act as markers of life style and of past-historical levels of stress. In other words, telomeres could inform on individual's current physiological quality.

The aim of this thesis is to determine whether telomeres could act as a mechanism underlying life history trade-offs, using an integrative approach. In this context, I organised my work into the following steps: (i) establishing the pattern of heritability of telomere length (genetic influence), a characteristic necessary to allow evolution of fitness-related parameters (ii) characterising the nature of determinants of telomere length (environmental influences) that may explain age-matched telomere length individual variability and (iii) testing the nature of the relationships between telomere length and individual maintenance, and ultimately with individual quality.

The present work shows that telomere dynamics is determined by the effects of genetic, but is probably predominantly affected by environmental and lifestyle factors. As such, both environmental conditions experienced during the growth period, as well as conditions experienced during adulthood (i.e. the level of reproductive effort) have a strong impact on individuals' telomere length. Finally, experimental manipulation of telomerase activity showed that telomere length could be linked to individual maintenance and thus might be indicative of individual quality. Altogether, these results highlight that telomere dynamics might provide a functional link between life history traits and therefore support the previously proposed idea that telomeres are of great interest in evolutionary biology.

**Key words:** telomere dynamics, oxidative stress, telomerase activity, telomere length inheritance, growth conditions, reproductive investment, self-maintenance, birds.





## Determinants of telomere length and implications in life history trade-offs

### Facteurs déterminant la longueur des télomères et implications dans les compromis évolutifs

#### Résumé

Une question fondamentale de la biologie évolutive porte sur la compréhension des mécanismes sous-tendant les processus évolutifs et l'évolution des compromis entre les traits d'histoire de vie. Parmi ces mécanismes, les télomères suscitent un intérêt particulier. Les télomères sont localisés à l'extrémité des chromosomes eucaryotes et participent à la sénescence cellulaire et au vieillissement des individus. La longueur des télomères est susceptible de donner des indications sur le mode de vie et l'état physiologique des organismes. Le but de cette thèse a été de comprendre quels sont les facteurs déterminant la longueur des télomères et leur implication dans les compromis évolutifs, ceci en établissant : si la taille des télomères est-elle héritable? Le taux de perte des télomères est-il affecté par des facteurs environnementaux? Quel lien entre les télomères, la maintenance individuelle et la qualité des individus? Il résulte de ce travail que la longueur des télomères est partiellement déterminée par les facteurs génétiques, elle semble aussi influencée par les facteurs environnementaux. En effet, le coût de la reproduction, ainsi que la modification des trajectoires de croissance, ont des effets néfastes sur la longueur des télomères. L'effet de la manipulation expérimentale de l'activité télomérase indique un lien entre les télomères et la maintenance individuelle, suggérant que les télomères sont susceptibles de donner des indications sur la qualité des individus. Ce travail de thèse montre que la dynamique des télomères est un mécanisme sous-jacent des compromis évolutifs, et présente un intérêt considérable pour la compréhension des processus évolutifs.

**Mots-clés:** télomères, stress oxydatif, télomérase, hérédité longueur télomérique, croissance, effort reproducteur, maintenance, oiseaux.

#### Résumé en anglais

Evolutionary pathways through which life histories may have evolved are numerous. Consequently identifying the underlying mechanisms of those processes is crucial for our overall comprehension of the origin of life diversity. Thus, there is clearly a great potential in the study of repetitive DNA sequences that cap eukaryotic chromosomes, the telomeres. Telomeres are structures involved in cell senescence and determine the rate of ageing. They are thought to reflect more than just the effects of age and to play an important role in linking life conditions and senescence. Indeed, telomeres could act as markers of life style and of past-historical levels of stress and inform on individuals' current physiological quality. This thesis aims to determine whether telomeres could act as a mechanism underlying life history trade-offs by establishing the pattern of heritability of telomere length; characterising telomere length's determinants; testing the nature of the relationship between telomere length and individual maintenance, and ultimately with individual quality. The present work shows that telomere dynamics is determined by genetic factors, but is probably predominantly affected by lifestyle factors. As such, environmental conditions experienced during the growth period, as well as during adulthood (i.e. level of reproductive effort) have a strong impact on individuals' telomere length. Experimental manipulation of telomerase activity showed that telomere length could be linked to individual maintenance and thus might be indicative of individual quality. Altogether, these results highlight that telomere dynamics might provide a functional link between life history traits.

**Key words:** telomere dynamics, oxidative stress, telomerase activity, telomere length inheritance, growth conditions, reproductive investment, self-maintenance, birds.