

Telomere Dynamics in Magellanic penguins (*Spheniscus magellanicus*)

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**Abstract**

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Lifespans among taxa vary widely, however, most all species undergo a reduction in physiological function with increasing age, often termed *aging*. One theory suggest that aging occurs as a result of the accumulation of damage during life, and though maintenance and repair mechanisms have evolved, they are not sufficient to completely mitigate the accrual of damage, resulting in aging pathologies. This theory also suggests that the variation of lifespan among species could be a result of the variable ability of maintenance systems to mitigate damage. Particularly long-lived species should evolve processes that maintain physiological systems linked to survival. Magellanic penguins (*Spheniscus magellanicus*) are an outlier among bird species and live 26% longer than their mass-predicted maximum lifespan. Telomeres, tandem repeating, non-coding sequences that protect the coding regions of DNA during cell replication, are linked to survival. Telomeres shorten with age in most species. We show that Magellanic penguins maintain their telomeres over their lifespan, resisting the stressors of growth, reproduction and environment. This may be a key factor in their enhanced longevity. We found telomere lengths for adults from 4 years to more than 27 years of age were similar.

We found telomeres of adult Magellanic penguins remained similar in length over a 3-year period. Telomere length did not predict re-sighting, nor did telomeres shorten with more reproductive effort or reproductive success. Our results suggest that Magellanic penguins maintain their telomeres.

If telomere length is important to survival, adults should have strong mechanisms maintaining telomere length. Chick growth is energetically costly, and is characterized by high levels of cell proliferation, which is linked to shorter telomeres in other species. Mitochondria are the source of both energy production, and damaging reactive oxygen species that can shorten telomeres. We characterized the dynamics of telomere length and mitochondria number during the fastest growth and throughout the lifespan of Magellanic penguins. We tested how growth impacted telomeres by taking blood from wild known-age Magellanic penguin chicks as they grew and measured telomere length and mitochondria number. Telomeres shortened during early rapid growth but by fledging telomeres were similar to their length at hatching and stay at this length throughout life. Mitochondrial copy number increased significantly after hatch as the chick grew, probably to meet energetic demands, than returned to their number at hatching by 45 days of age. When the penguins reach 7 to 8 years of age the number of mitochondria decreased to the level at hatching, remaining at this abundance throughout adulthood. Our results indicate that while telomeres shorten during growth, characterized by increased mitochondria number, Magellanic penguins elongate telomeres and enter breeding age with telomeres similar to their hatch day.

Magellanic penguins also live in captivity, where the stressors they experience undoubtedly differ from those of wild penguins, but could have equally potent effects on

maintenance systems. In captivity, confinement and living in non-native habitats may be stressors, shortening telomeres. If so, telomeres in Magellanic penguins should shorten more in captivity than in the wild. We found that telomeres do not shorten with age in males or females, in captivity. Telomeres were similar in length in captive and wild Magellanic penguins. Magellanic penguins appear to maintain their telomeres in captivity, the same as in the wild, which is consistent with their longer than predicted lifespan.

Magellanic penguins' enhanced longevity may be explained, at least in part, by the maintenance of telomeres. By understanding the aging physiology of seabird species like Magellanic penguins we are likely to find insights into the evolutionary processes that drive the aging phenotypes we observe in nature.

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## CHAPTER ONE: BACKGROUND AND HYPOTHESES

### *Introduction*

Lifespans among taxa vary widely, but generally longevity can be divided into those species that are short-lived, and those that are long-lived, and reproductive strategies have evolved with the evolution of longevity. In all species, finite resources must be allocated toward three competing functions: survival (or maintenance), reproduction, and growth (Stearns 1976; Wikelski and Ricklefs 2001). Investment bias towards one or another function will lead to senescence in the others. Also, those species that invest in maintenance early in life, or grow slowly, often begin reproduction later in life (Stearns 1976). Long-lived or short-lived species should allocate resources differently among maintenance and reproductive systems. Two continua likely exist: (1) in the length of time until sexual maturity; and (2) investment in high quantity of offspring with limited parental investment, or in lower numbers of high quality offspring with considerable parental investment. Species can be generally categorized as either *r-selected* or *k-selected* (MacArthur and Wilson 1967). *r-selected* species are those that grow rapidly and produce many offspring (MacArthur and Wilson 1967; Ricklefs 1969b; Ricklefs 2000a). These species are generally short lived as they expend a high proportion of metabolic resources into rapid growth and adult reproductive systems, usually at the cost of maintenance systems (Wikelski and Ricklefs 2001; Ricklefs and Wikelski 2002). Short-lived species, like passerine birds, generally have low annual survival, develop rapidly and reach sexual maturity quickly (Ricklefs 2000a; Saether 1988; Promislow and Harvey 1990). With increased yearly mortality, the tradeoff between maintenance and reproductive systems favors rapid growth and reproduction, rather than maintenance

systems. *k-selected* species, however, tend to be large and develop slowly, with high probability of surviving to adulthood, and have generally lower fecundity with offspring needing more parental investment (Ricklefs 1969a; Ricklefs 1969b; Ricklefs 2000a; Ricklefs and Wikelski 2002; MacArthur and Wilson 1967). Though clutch sizes for long-lived species tend to be smaller (Ricklefs 2000a), long-lived *k-species* have the increased future reproductive potential with their increased survival (Ricklefs and Wikelski 2002; Wikelski and Ricklefs 2001).

While long-lived species will still incur the resource bias towards growth during development, they however, should evolve systems that favor maintenance (Ricklefs and Wikelski 2002; Wikelski and Ricklefs 2001; Austad 2001). Despite this, individual maintenance systems suffer degradation and senescence (Ricklefs and Wikelski 2002; Wikelski and Ricklefs 2001; Williams 1957). The analysis of aging deals with two kinds of questions: the *how* and *why* of aging. In other words, what are the proximate causes of aging physiology that we observe (*how*) and what are the ultimate evolutionary forces that drive them (*why*). The ultimate theories explain why the proximate mechanisms exist. I explore how life-history decisions can act as causal factors of aging and longevity in a long-lived seabird, providing insight into *why* these mechanisms evolved to provide the fitness advantage.

The evolution of aging is imbedded in life-history theory. Ultimately, aging and fitness are linked at reproduction. Aging is often described most generally as the loss of physiological function and the decrease in fertility with increasing age (Kirkwood and Austad 2000). Aging negatively impacts an individual's survival, and has implications for individual fitness and fecundity as well as population ecology and thus species

conservation. For instance, in populations without predators, individuals are not under pressure to reproduce rapidly. Thus, they evolved to age more slowly and reproduce later in life than individuals of the same species with predation pressures (Austad 1988, 1997). Similarly, the length of adult lifespan of *Drosophila* increased when only older individuals were given the opportunity to reproduce (Luckinbill and Clare 1985). Nevertheless, potential breeding opportunities increase along with increased lifespan (Austad 2001; Kirkwood and Austad 2000).

Darwin (1859) suggested that lifespan, like other species traits, should be affected by selective pressures. So, reproductive strategies should have influenced the evolution of longevity. There is a broad variation in the aging phenotype among taxa. Classical evolutionary models of aging predict that all species eventually age, and with the exception of a handful of biologically immortal species (Piraino et al. 1996; Martinez 1998; Tan et al. 2012), most eukaryotes undergo aging. There is, however, some debate of the theoretical framework that might govern the wide variation in aging phenomena among taxa. Three major evolutionary theories of aging exist: 1) the theory of programmed death, 2), the antagonistic pleiotropy theory of aging, and 3) the mutation accumulation theory of aging. These theories are not necessarily mutually exclusive, and it is likely that the reality of aging that we observe in nature is an aggregate of two or more of these theories (Kirkwood and Austad 2000; Kirkwood and Rose 1991).

### *Programmed Death*

The earliest of aging theories, first advanced by Weismann (1891), takes a species, rather than individual fitness, perspective. The programmed death theory states

that aging (and death) evolved to replace less fit individuals in a population with younger ones with more reproductive potential (Weismann 1891). There is, however, limited evidence of senescence directly linked to population mortality in the wild, and natural mortality is likely linked to extrinsic factors like predation, infection or environmental hazards (Kirkwood and Austad 2000). There are no known evolutionary mechanisms that could yield such a result, so though the theory was the foundation for later hypotheses, it can't be a driving evolutionary force. Weismann, in a secondary theory, suggested that aging probably evolved in organisms with separate reproductive germ and soma cells, because they must invest considerable resources into the germ cells, limiting the resources for maintenance of the soma (Weismann 1891).

### *Antagonistic Pleiotropy*

The power of natural selection declines with age once reproduction begins (Ackermann et al. 2007; Medawar 1952). Therefore, genes that result in a loss of fitness early in life, particularly before reproduction, are under strong negative natural selection (Medawar 1952; Ackermann et al. 2007). Conversely, genes that have negative effects later in life face little selective pressure because increasing proportions of individuals die before the genes exert their effects (Ackermann et al. 2007; Medawar 1952). For this reason, those genes that code for senescence, susceptibility to disease or perhaps physiological malfunction persist, leading to the aging phenomena we observe. This theory was further refined by Williams (1957), suggesting that single (or multiple; see Kirkwood and Austad 2000) genes could be both adaptive at early age and hazardous at older ages, or *pleiotropic* genes. Rose and Charlesworth (1980) demonstrated the



presence of these genes in *Drosophila melanogaster*, supporting the theory. Hunt et al. (2006) later showed that the selection for increased longevity leads to decreased reproductive effort, a classical example of *antagonistic pleiotropy*.

### *Accumulation Theory of Aging*

Weismann's secondary resource allocation theory was elaborated as the "Disposable Soma" theory by Kirkwood, where individuals must balance the allocation of resources between germ and soma (Kirkwood and Austad 2000; Kirkwood and Rose 1991). Aging occurs as a result of the accumulation of damage during life, and though maintenance and repair mechanisms have evolved, they are not sufficient to completely mitigate the accrual of damage, resulting in aging pathologies (Kirkwood and Austad 2000; Medawar 1952). This theory also suggests that the variation of lifespan for individuals within a species could be a result of the variable ability of maintenance systems to mitigate damage that an individual faces. Additionally, as an individual ages, the value of current reproductive opportunity increases relative to future opportunities (Kirkwood and Austad 2000; Ricklefs and Wikelski 2002; Wikelski and Ricklefs 2001). This occurs since with each increasing year the probability of mortality increases and the potential for future reproduction is diminished. This, however, may be at odds with evidence supporting Medawar's theory of *antagonistic pleiotropy*, since increased survival might delay reproduction and increase risk of exposure to mal-adaptive genes (Rose and Charlesworth 1980; Medawar 1952). There are a number theories that fall under the accumulation theory of aging, and provide proximate mechanisms for the aging we observe. The free-radical theory of aging proposes that reactive oxygen species

(ROS), produced from stress and metabolism lead to damage in both DNA and cellular material (Harman 1956; von Zglinicki 2002). Though all mechanistic relationships are not known in detail, reactive oxygen species can lead to DNA damage (Beaulieu et al. 2011; Kotrschal et al. 2007; Kirkwood and Austad 2000; von Zglinicki 2002). Oxidizing ROS are released as byproducts of mitochondrial metabolism (Miquel et al. 1980; Harman 1956). The mitochondrial theory of aging implicates the mitochondria as the chief target of damage and the primary producer of the damaging reactive oxygen species (Barja and Herrero 2000; Miquel et al. 1980; Harman 1956). The accumulation theory of aging is the basis for current research into the mechanistic relationship between stressors, metabolism and mitochondria, oxidative damage, and DNA maintenance, explored in this dissertation.

### *Telomeres*

Telomere length, a key factor in cell maintenance, shortens with age (Blackburn 1991; Prowse 1995; Haussmann et al. 2003; Aubert and Lansdorp 2008). Telomeres are the tandem repeating, non-coding sequences that protect ends of chromosomes during cell replication (Blackburn 1991; Greider and Blackburn 1985). During each cycle of cell replication, telomere sequences are lost because DNA polymerase cannot fully replicate the 3-prime end of the DNA strand (Watson 1972; Blackburn 1991; McClintock 1941). Telomere shortening may affect the number of generations a cell line has (Hayflick 1965), which could play a role in system senescence. For example, in elderly humans, telomeres in CD8 t-cells shorten earlier than in natural killer lymphocytes, which may be partly responsible for the preserved innate-immune response in old age

(Mariani et al. 2003). The telomere length and its rate of shortening may contribute to cell and organismal survival (Dong et al. 2005; Haussmann et al. 2007; Hornsby 2007; Blackburn 1991; Chan and Blackburn 2004; Greider and Blackburn 1985; Bize et al. 2009). However, the dynamics driving telomere shortening are significantly more complex than a simple age-based consequence of cell-replication (Haussmann et al. 2003; Speakman 2005; Monaghan and Haussmann 2006).

### *Telomeres and growth*

Growth is energetically costly, and is characterized by high levels of cell proliferation. All birds grow rapidly, many incurring a cost by allocating resources away from maintenance systems to growth (Ricklefs 1969b; Ricklefs 2000a). Furthermore, developmental conditions like brood size (Voillemot et al. 2012), growth rate (Geiger et al. 2012) and being smaller than brood-mates (Nettle et al. 2015) may affect both telomere length and survival (Pauliny et al. 2006; Boonekamp et al. 2014). While long-lived species will still incur the resource bias towards growth during the development period, they should also allocate resources to systems that enhance adult survival. We hypothesize that the continual investment cost to maintaining telomeres increases the potential of adult survival and future reproductive events. Growth requires high levels of available energy and is characterized by high levels of metabolism (Breuner 2003; Ricklefs 1969a; Ricklefs 1969b). A high metabolic state could release reactive oxygen species (ROS) and damage telomeric repeats (Monaghan and Haussmann 2006; von Zglinicki 2002). Both gluconeogenesis and the general inflammatory response of increased metabolism, correlate with shortened telomeres (Monaghan and Haussmann

2006). In King penguins (*Aptenodytes patagonicus*), chicks that had increased growth of body mass had more oxidative damage (Geiger et al. 2012). Small chicks that grew faster showed higher oxidative damage and accelerated telomere loss, showing the correlative link between growth and telomere degradation (Geiger et al. 2012). King-penguins chick telomere lengths did not recover to their length at hatching.

### *Telomeres and reproduction*

The relationship between increased reproductive effort and telomere length is well studied (Bauch et al. 2013; Beaulieu et al. 2011; Kotrschal et al. 2007; Sudyka et al. 2014). Reproduction can increase oxidative stress (Alonso-Alvarez et al. 2004), which can shorten telomeres (von Zglinicki 2002). Experimentally increased reproductive effort is correlated with increased telomere shortening, in both lab studies of zebra finch (*Taeniopygia guttata*), (Reichert et al. 2014; Heidinger et al. 2011) and wild blue tits (*Cyanistes caeruleus*) (Sudyka et al. 2014). Some long-lived seabirds show similar patterns; common terns (*Sterna hirundo*) that returned to the breeding colony earlier, and had larger brood sizes, had shorter telomeres (Bauch et al. 2013). However, individuals with the highest reproductive success showed the smallest loss of telomere length (Bauch et al. 2013), suggesting that long-lived seabirds of higher quality may better mitigate the cost of reproduction. While individual quality, body condition, or behaviors related to chick rearing could explain this result; it does suggest that adult seabirds may be able to mitigate the cost of reproduction on their telomeres. Beaulieu et al. (2011) showed that Adelie penguins with experimentally increased reproductive effort, by means of

handicapping, did not have significantly shorter telomeres, though they did feed on a higher anti-oxidant diet.

### *Telomere length and longevity in Magellanic penguins*

Generally, those species with longer lifespans tend to have telomeres that shorten more slowly, though interestingly in some bird species, telomeres do not shorten and may even lengthen with age (Table 1) (Hausmann et al. 2003). Two theories - the *selection* and *elongation* hypotheses - have been advanced to explain this pattern, where similar data are consistent with either explanation (Hausmann and Mauck 2008). The *selection hypothesis* suggests that the individuals that survive through adulthood to older ages initially possessed longer telomere lengths than other individuals in the population. Thus, they may have long telomeres even though they actually shortened with age. The *elongation hypothesis*, however, suggests that individuals are able to maintain or extend their telomere lengths throughout life (Hausmann and Mauck 2008). Those studies showing a elongation of telomeres have been primarily cross-sectional (Hausmann et al. 2003), and data from longitudinal studies of the same populations support the *selection* hypothesis rather than true elongation (Hausmann and Mauck 2008).

Understanding of the factors that affect a species' longevity is limited but there are study systems that should provide insight into physiological aging and the tradeoffs between maintenance and reproductive systems. For the majority of species, body mass is positively correlated with longevity (Lindstedt and Calder 1976, Hausmann et al. 2003, Speakman 2005). While complex interactions govern the relationship between size and longevity, larger organisms live longer (Ricklefs 2000b; Speakman 2005; Hausmann et

al. 2003). Interestingly, bird species live significantly longer than mammalian species of similar body size, however knowledge of the complex criteria that affect the increased longevity of birds is limited (Hausmann et al. 2003, Speakman 2005). Among birds, there are a few species that exceed the lifespan predicted by their mass (Hausmann et al. 2003) (Table 1). Magellanic penguins are an outlier among species and live 26% longer than their predicted maximum lifespan, where predicted lifespan is based on body mass from the equation:  $lifespan = 17.6 (mass\ in\ kg)^{0.20}$  (Lindstedt and Calder 1976) (Table 1). African (*S. demersus*) and Little blue penguins (*E. minor*) also display this phenomenon, living 24% and 40% longer than mass-predicted, respectively (Table 1). Interestingly, this is not conserved in all penguins, or even within the *Spheniscus* genus as Humboldt penguins (*S. humboldti*) do not exceed their predicted lifespan (84%), nor in other penguin species (Table 1).

Wild Magellanic penguins have evolved in a variable environment, in which the quality of conditions for breeding varies spatially and temporally. Magellanic penguins arrive at the breeding colony in September, with males arriving about a week prior to the first females (Boersma et al. 2013). Males compete for nesting sites and mates. Females lay clutches of two eggs up to 3-4 weeks after the males have been on land fasting (Boersma et al. 1990), a considerable dietary stressor. After laying of both eggs, males typically will leave to forage and females will fast while incubating the eggs (Yorio et al. 1990; Boersma et al. 1990). Males and females exchange duties both incubating the eggs and feeding the chicks (Boersma et al. 2013). During breeding, both male and female penguins undergo stressful events, and excrete the stress hormones, corticosteroids (Walker et al. 2006; Walker et al. 2005). The longer the adults are fasting, the more

dramatic the release of corticosterone after capture, presumably in response to nutritional stress (Hood et al. 1998). Stressors, for males, include a combination of social and nutritional stress, including competition for nest sites, extended fasting and offspring defense (Boersma et al. 2013; Boersma et al. 1990). Similarly, females have long fasts before egg laying and during incubation, interact with conspecifics and defend offspring, and lay two nutritionally rich eggs (Boersma et al. 2013). Stressful events for birds such as reproduction (Kotrschal et al. 2007), trauma and injury (Fowler et al. 2013; Herborn et al. 2014), environmental stressors (Walker et al. 2006; Walker et al. 2005; Fowler et al. 2013) and high density living (Kotrschal et al. 2007) can degrade maintenance systems by diverting resources to production of stress hormones. In addition, stressful events (Epel et al. 2004) as well as chronic infection from pathogens (Asghar et al. 2015) can directly result in telomere shortening. Though the mechanism is not fully explained, stressors could lead the release of ROS that produce DNA strands breaks, which are difficult to repair when they occur in telomere DNA, leading to a loss of telomeres (von Zglinicki 2002).

Stressors can lead to the release of stress hormones (Walker et al. 2006; Walker et al. 2005). Magellanic penguins likely evolved systems that favored maintenance because of the highly variable reproductive success (Boersma 2008). Yearly, Magellanic penguins can lose nest contents due to chick starvation from decreased food availability (Boersma et al. 1990; Boersma and Stokes 1995), predation from terrestrial predators (Stokes and Boersma 1998) and an increasing frequency of severe storms (Boersma and Rebstock 2014). Adult penguins may also skip reproductive years if its breeding condition is sub-optimal (Boersma and Rebstock 2010). Therefore, mechanisms that enhance longevity

and therefore future reproductive opportunities, like the maintenance of telomeres or resistance to stress, are selected. Magellanic penguins generally have low baseline corticosteroid levels with little variation (Walker et al. 2015). Similarly, Adelie penguins (*Pygoscelis adeliae*) do not exhibit chronic stress hormone release in response to repeated handling (Vleck et al. 2000). Adelie penguins, though, do not display enhanced longevity in the wild (Table 1). Corticosterone release has been shown to increase in the losers of fights in copperheads (*Agkistrodon contortrix*) (Schuett and Grober 2000), fish (*Betta splendens*) (Verbeek et al. 2008) and mice (*P. leucopus*, *P. californicus*) (Oyegbile and Marler 2006). In contrast, corticosterone levels in Magellanic penguins are at baseline levels even after recent fights that they lose (Walker et al. 2015). Magellanic penguins appeared to have evolved maintenance mechanisms that allow individuals to suppress elements of the stress response and live longer.

#### *Magellanic penguins and captivity*

Magellanic penguins also live in captivity, where the stressors penguins experience undoubtedly differ from those of wild penguins, but could have equally potent affect on maintenance systems (Morgan and Tromborg 2007). While longevity in some penguin species increases in captivity (Table 2), there is considerable evidence for stressors that exist for captive individuals. Confinement (Morgan and Tromborg 2007; Hediger 1955, 1964), high-density living (Kotrschal et al. 2007), and non-native diets (Morgan and Tromborg 2007) can cause stressful behaviors in captive individuals of a number of species (Morgan and Tromborg 2007). Restricted movement due to confined space can cause abnormal behaviors in captive individuals (Hediger 1955, 1964), and is



correlated with increased aggressive behaviors in both mammals (Napolitano et al. 2004; Wiegand et al. 1994; Lammers and Schouten 1985; Cassinello and Pieters 2000) and birds (Buchwalder and Huber-Eicher 2004). Stressful behaviors like pacing and infant mortality in captivity were positively correlated with a species' home range size (Clubb and Mason 2003, 2007). In captive mice, high-density living is correlated with increased telomere shortening (Kotrschal et al. 2007). Captivity may provide a different set of life conditions than the wild, but the response may depend on species. However, in a phylogenetic study of 28 bird species, aging-related mortality was equivalent within zoo and wild population, suggesting that intrinsic causes of death kill independently of the environment (Ricklefs 2000b). In six penguin species, of variable size and habitat, however, longevity was increased in captivity for four species (Table 2). Interestingly, the two species of penguins where longevity in captivity is no longer than in the wild are Magellanic and Little blue penguins (*E. minor*; Table 2), both species that exceed their mass-predicted lifespan in the wild (Table 1). Two hypotheses consistent with this observation are, (1) other penguin species face higher predation pressure the wild than Magellanic or Little blue penguins, which would shorten their maximum observed lifespan, or (2) these species may already maximize their longevity, a component of which may be telomere maintenance. In Chapter 4, we characterize whether in captivity Magellanic penguins maintain their telomeres as they do in the wild.

#### *Telomere maintenance and telomerase*

Maintenance mechanisms that protect telomeres should be selected for under evolutionary theories of aging (Kirkwood and Austad 2000; Kirkwood and Rose 1991).

One hypothesis suggested in other long-lived penguin species (Beaulieu et al. 2011; Beaulieu et al. 2010) is an ability to protect telomeres by mitigating the effects of ROS, likely by bolstering the antioxidant ability of blood plasma. The anti-oxidants present within the blood plasma are, in part, a result of a diet that possesses a high anti-oxidant load (Beaulieu et al. 2011; Beaulieu et al. 2010; Corsolini et al. 2001). Magellanic penguins feed upon fish, squid and crustaceans (Boersma et al. 2013). Carotenoids, found primarily in crustaceans and squid, can be an important component of wild penguin diets (Wilson et al. 2005; Scolaro et al. 2013; Gandini et al. 1994). Carotenoids are considered minor dietary antioxidants in birds (Costantini and Møller 2008). While there is debate about the effect of diet carotenoids *in vivo* (Costantini and Møller 2008), they are considered to be indicators of an individual's greater antioxidant ability (Marri and Richner 2014) as carotenoids are bleached by oxidative stressors (Woodall et al. 1997). The anti-oxidant ability of blood plasma is largely a result of the intake of prey, high in anti-oxidants (Corsolini et al. 2001).

Telomerase, a ribonucleic reverse transcriptase, elongates telomeres, and may play a key role in governing lifespan (Hausmann et al. 2007; Shay et al. 1993; Vaziri et al. 1993; Dong et al. 2005; Forsyth et al. 2002; Counter et al. 1992). In humans, telomerase is down-regulated after development and it is only active in germ and stem cell lines and remains at considerably low levels in somatic cell lines of kidneys, lymphocytes and epithelial cells (Counter et al. 1992; Dong et al. 2005; Forsyth et al. 2002; Shay et al. 1993; Vaziri et al. 1993). Long-lived bird species, however, show telomerase activity in a number of tissues (Hausmann et al. 2007; Dong et al. 2005). Telomerase activity in bone marrow, gonads and intestine cell lines is increased in long-

lived adult species (*T. guttata*; *T. bicolor*) compared to short lived ones (*S. hirundo*; *O. leucorhoa*) (Hausmann et al. 2007). Increased telomerase activity in regions where cell lines develop, could allow mature somatic cells to have longer telomeres as the individual ages. Telomere lengths in human sperm cells display this phenomenon, with sperm cell telomeres from older individuals being longer as they are likely acted upon by enhanced telomerase activity in the gonads (Eisenberg et al. 2012). Additionally, telomerase activity is implicated in enhanced longevity observed in some taxa, including lobsters (Klapper et al. 1998), *hydra* and *planaria* (Tan et al. 2012), the latter two of which are thought to be biologically immortal.

In this dissertation, we demonstrate that Magellanic penguins maintain their telomeres over their lifespan, resisting the stressors of growth, reproduction and environment. Seabirds in general, and Magellanic penguins specifically, extend their lifespan beyond what is predicted by body mass. Enhanced longevity may be explained, at least in part, by the maintenance of telomeres. By understanding the aging physiology of seabird species like Magellanic penguins we are likely to find insights into the evolutionary processes that drive the aging phenotypes we observe in nature.

### *Hypotheses and predictions*

**Chapter Two:** Telomeres do not shorten in breeding Magellanic penguins (*Spheniscus magellanicus*)

**Hypothesis:** Magellanic penguins maintain telomeres over lifespan.

**Predictions:**

- (a) Telomere lengths are not correlated with an individual's age in the population (cross-sectional).

- (b) Telomere lengths do not shorten as an individual ages (longitudinal).
- (c) Shorter telomeres are correlated with increased reproductive effort, when controlling for age and sex.
- (d) Telomeres shorten more quickly with increased reproductive effort and output.
- (e) Longer telomeres are correlated with increased survival in adults.
- (f) Telomeres shorten more slowly in those individuals with increased survival.

**Chapter Three:** Telomeres shorten and then lengthen despite mitochondrial increase during growth in Magellanic penguins

**Hypothesis:** Magellanic penguin telomeres shorten and mitochondria number increases during growth.

**Predictions:**

- (a) Telomere lengths are shorter during growth.
- (b) Telomere length will return to hatch-day levels by the end of growth.
- (c) Mitochondria number is increased during growth to meet metabolic demands.
- (d) Mitochondria number is decreased in adults.
- (e) Shorter telomere lengths are correlated with more mitochondria on hatch day and in adults.
- (f) Longer hatch-day telomeres are correlated with increased fledging success.
- (g) Adults with longer telomeres had more fledglings return to the colony.
- (h) Adults that start reproducing at an earlier age have shorter telomeres.

**Chapter Four:** Magellanic penguin telomeres in captivity and in the wild are similar

**Hypothesis:** Magellanic penguins maintain telomeres in response to variable life conditions.

**Predictions:**

- (a) Male and female telomeres will be similar in zoos.
- (b) Captive penguin telomeres will shorten with age.
- (c) Telomere lengths of captive penguins are shorter than wild Magellanic penguins.

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[Tables]

Species	Mean Mass (kg) <sup>h</sup>	Mass-Predicted Lifespan (yrs) <sup>a</sup>	Observed Lifespan (yrs) <sup>b</sup>	Percent of Mass-Pred. Lifespan <sup>m</sup>	Lifetime Telomere Rate of Change
Adelie Penguin <i>P. adeliae</i>	4.6	23.8	20 <sup>c</sup>	84	-23 ±48(SE) bp/yr <sup>*</sup>
African Penguin <i>S. demersus</i>	3.1	22.1	27.6 <sup>i</sup>	<b>124</b>	-
Magellanic Penguin <i>S. magellanicus</i>	4.6	23.8	≥30 <sup>h</sup>	≥ <b>126</b>	??
Emperor Penguin <i>A. patagonicus</i>	29.5	34.9	20 <sup>j</sup>	58	-
Humboldt Penguin <i>S. humboldti</i>	4.6	23.8	20 <sup>k</sup>	84	-
Little Blue Penguin <i>E. minor</i>	1.2	18.3	25.7 <sup>g</sup>	<b>140</b>	-
Zebra Finch <i>T. guttata</i>	0.012	7.3	5 <sup>c</sup>	69	-515±95(SE) bp/yr <sup>*</sup>
Tree Swallows <i>T. bicolor</i>	0.021	8.1	11 <sup>d</sup>	<b>135</b>	-391±65(SE) bp/yr <sup>*</sup>
Common Terns <i>S. hirundo</i>	0.12	11.5	25 <sup>f</sup>	<b>226</b>	-57±7 (SE) bp/yr <sup>*</sup>

**Table 1.1: Mass-predicted lifespan and Telomere Rates of Change in Birds.**

a: predicted lifespan based on body mass from the equation:  $lifespan = 17.6 (mass\ in\ kg)^{0.20}$  (Lindstedt and Calder 1976); b: based on published values for each species living in the wild; c: Zann (1996); d: Robertson et al. (1992); e: Williams (1995); f: Nisbet (2002); g: Dann et al. (2005); h: Boersma et al. (2013); i: Whittington et al. (2000); j: British Antarctic Survey – *estimated*; k: Woodland Park Zoo – *estimated*, l: Haussmann et al. (2003); m:  $(Observed\ LS/Mass-Predicted\ LS)*100$

Species	Wild Lifespan <sup>a</sup>	Captive Lifespan <sup>h</sup>
Adelie Penguin <i>P. adeliae</i>	20 <sup>b</sup>	39
Humboldt Penguin <i>S. humboldti</i>	20 <sup>g</sup>	39
Magellanic Penguin <i>S. magellanicus</i>	≥30 <sup>d</sup>	30
Emperor Penguin <i>A. patagonicus</i>	20 <sup>f</sup>	44
African Penguin <i>S. demersus</i>	27.6 <sup>e</sup>	39
Little Blue Penguin <i>E. minor</i>	25.7 <sup>c</sup>	25

**Table 1.2: Lifespan in the wild and captivity for penguin species.**

a: based on published values for each species living in the wild; b: Williams (1995); c: Dann et al 2005; d: Boersma et al 2013; e: Wittington et al 2000; f: British Antarctic Survey – *estimated*; g: Woodland Park Zoo – *estimated*, h: maximum lifespan observed in captivity, Weigl 2015 (*personal communication*)

## CHAPTER TWO: TELOMERES DO NOT SHORTEN IN BREEDING ADULT MAGELLANIC PENGUINS (*SPHENISCUS MAGELLANICUS*)

### [Abstract]

Long-lived species should evolve processes that maintain physiological systems linked to survival. Telomeres, tandem repeating, non-coding sequences that protect the coding regions of DNA during cell replication, are linked to survival. We investigated whether telomere length in Magellanic penguins (*Spheniscus magellanicus*) are maintained in adulthood as the penguins exceed their mass-adjusted predicted lifespan by approximately 26%. We found telomere lengths for all adults older than 4 years of age were similar and that telomeres of adults remained similar in length over a 3-year period. Telomere length was not well correlated with re-sighting of birds, nor did reproductive effort or output shorten telomeres. Our results suggest that Magellanic penguins maintain their telomeres, even if they reproduce more.

## [Introduction]

Physiological systems deteriorate as a result of aging in most species (Hausmann and Mauck 2008a, 2008b; Hausmann et al. 2007; Hausmann et al. 2003; Nakagawa et al. 2004; Austad 2001; Kirkwood and Austad 2000). Life-history theory predicts long-living species should invest more in maintenance systems like anti-oxidant defense or immune competence, as these are likely to enhance adult survival (Speakman 2005; Ricklefs and Wikelski 2002; Wikelski and Ricklefs 2001). Individual maintenance systems are expected to suffer the degradation of aging related decay and show significant senescence (Ricklefs and Wikelski 2002; Kirkwood and Austad 2000; Kirkwood and Rose 1991), however a long lifespan increases the number of potential reproductive events.

For the majority of species, longevity is positively correlated with body mass (Hausmann et al. 2003; Speakman 2005; Lindstedt and Calder 1976). Generally, bird species live significantly longer than their mammalian counterparts of similar body size, however knowledge of the complex criteria that affect the increased longevity of birds is limited (Holmes and Austad 1995b, 1995a; Holmes et al. 2001). Among birds, there are a few species that exceed the lifespan predicted by their mass (Table 1.1)(Hausmann et al. 2003). Magellanic penguins live 26% longer than their predicted maximum lifespan, where predicted lifespan is based on body mass from the equation:  $lifespan = 17.6 (mass \text{ in kg})^{0.20}$  (Lindstedt and Calder 1976). Investigating these long-lived species may provide clues to the physiological factors that govern longevity.

The shortening of chromosomal end-capping telomere sequences correlates negatively with adult survival (Hausmann et al. 2003; Hausmann and Mauck 2008b;

Salomons et al. 2009; Bize et al. 2009). Telomeres are the tandem repeating, non-coding sequences that protect the coding regions of DNA during cell replication (Blackburn 1991). During each cycle of cell replication, telomeres are shortened because DNA polymerase cannot fully replicate the 3' end of the DNA strand; this is one of the primary reasons that telomeres shorten with age (Blackburn 1991; Watson 1972; McClintock 1941). The initial telomere lengths and slow rate of shortening, as well as the regulation of telomerase, a ribonucleic reverse transcriptase that elongates telomeres, may correlate with increased adult survival (Blackburn 1991; Haussmann et al. 2007; Chan and Blackburn 2004; Greider and Blackburn 1985; Bize et al. 2009; Salomons et al. 2009).

The dynamics driving telomere shortening are significantly more complex than a simple age-based consequence of cell-replication (Haussmann et al. 2003; Speakman 2005; Monaghan and Haussmann 2006). Stressful events such as reproduction (Kotrschal et al. 2007), trauma and injury (Fowler et al. 2013; Herborn et al. 2014), environmental stressors (Walker et al. 2006; Walker et al. 2005; Fowler et al. 2013) and high density cohabitation (Kotrschal et al. 2007) are linked to the production of stress hormones and the shortening of telomeres (Kotrschal et al. 2007; Haussmann et al. 2012; Tissier et al. 2014; Herborn et al. 2014; Epel et al. 2004). Developmental conditions like brood size (Voillemot et al. 2012), growth rate (Geiger et al. 2012) and being smaller than brood-mates (Nettle et al. 2015) may affect both telomere length and survival (Pauliny et al. 2006; Boonekamp et al. 2014a). Studies also found that increased lifetime reproductive effort could reduce adult longevity as well as offspring quality (Kotrschal et al. 2007; Kirkwood and Rose 1991; Ricklefs and Wikelski 2002; Wikelski and Ricklefs 2001) and lifespan (Boonekamp et al. 2014b). Though the direct mechanistic relationship

is not fully understood, one explanation is oxidizing reactive oxygen species (ROS) damage DNA integrity, shortening telomeres (von Zglinicki 2002; Beaulieu et al. 2011; Kotrschal et al. 2007). Generally, reactive oxygen species produce DNA strands breaks, which are difficult to repair when they occur in telomeric DNA (von Zglinicki 2002). Telomerase is able to elongate telomeres (Greider and Blackburn 1985), however, its activity is selected against in most adults as it is implicated in tumorigenesis (Hausmann et al. 2007; Dong et al. 2005; Hornsby 2007; Stewart et al. 2002; Tollefsbol and Andrews 2001). There is some evidence, however, that telomerase may be more active in bone marrow, gonads and intestine cell lines in adult long-lived bird species (*T. guttata*; *T. bicolor*) compared to short lived ones (*S. hirundo*; *O. leucorhoa*) (Hausmann et al. 2007).

Magellanic penguins likely evolved systems that favored longevity because of the highly variable nature of their reproductive success. Yearly, Magellanic penguins can lose eggs and chick from starvation from decreased food availability (Boersma et al. 1990; Boersma and Stokes 1995), predation from terrestrial predators (Stokes and Boersma 1998) and an increasing frequency of severe storms (Boersma and Rebstock 2014). Adult penguins may also skip reproductive years if breeding conditions are sub-optimal (Boersma and Rebstock 2010). This would suggest that those mechanisms that impact longevity are under high selection pressure. Slowly shortening telomeres are observed in other bird species, at an individual level (Salomons et al. 2009), and a population trend (Hausmann et al. 2003), so we predicted we would observe similar patterns in Magellanic penguins. Our study tested if telomere length shortened with age in Magellanic penguins and if reproductive effort increased telomere shortening. We



hypothesized that (a) telomere lengths would shorten slowly with age, suggesting high levels of telomere maintenance, and (b) that telomeres would shorten with increased reproductive effort, when controlling for age and sex.

## **[Methods]**

### *Collection and Processing*

Beginning in 1982, we started banding Magellanic penguins at Punta Tombo, Argentina (44°02'S, 65°11'W) (Boersma et al. 1990). We banded chicks before they fledge, juveniles before they molt into adult plumage, and breeding adults (Boersma 2013). We collected blood samples from 73 known-age adult Magellanic penguins (*Spheniscus magellanicus*) from September to December 2007 at Punta Tombo, Argentina. We took blood from adults 4 to over 24 years of age of four age classes (Table 2.1). We also collected a subset of blood from females aged 15yrs (n=8) to determine variation in telomere length by sex. In 2010, three years later, we took blood from 32 (28 male, 4 female) individuals sampled in 2007 to measure the change in telomere length.

All birds in this study were known-age. We know the age of adults 19yo and younger because they were banded as chicks (year 0). Penguins in the oldest adult cohort, aged 24 years or more, were banded as adults in 1983. Birds were of known sex prior to sample collection (Boersma and Davies 1987). Sex is known from morphological traits, a method confirmed by genetic sexing to be accurate for >97% of adults (Bertellotti et al. 2002).

### *Sample Collection and qPCR*

We collected blood via the vein on the dorsal surface of the foot, distal to the tarsometatarsus, and released all penguins within 5 minutes of when they were first sighted. During venipuncture we used 22-25gauge hypodermic needles and collected blood via heparinized capillary tube (Thermo Fisher Scientific Inc.). We immediately placed the blood into anti-lyses buffer (10% EDTA/ 90% Newborn Bovine Serum), and placed the samples on ice. We froze the samples at -18°C within 1hr of collection. We stored the samples at -80°C at the University of Washington, until processing.

We isolated DNA using a Qiagen DNeasy Mini-kit for animal blood and tissue samples. DNA was extracted from a lightly centrifuged cell pack, consisting primarily of erythrocytes. DNA was quantified via nanodrop spectrophotometer (*mean 260/280 ratio*±*SE* = *1.85*±*0.02*). We measured telomere length by Quantitative Polymerase Chain Reaction (qPCR). This method has been used to measure telomere length in penguins (Geiger et al. 2012; Beaulieu et al. 2011).

For each sample, two PCRs were run: the first one to amplify the telomeric DNA and the second one to amplify a single-copy control gene (36B4, acidic ribosomal phosphoprotein PO), providing an internal control to normalize the starting amount of DNA. We included a four-point standard curve (2-fold serial dilutions from 10 to 1.25ng of DNA) in all PCRs to allow the transformation of Ct (cycle threshold) into nanograms of DNA. All samples were run in triplicates and the median was used for subsequent analyses.

We used a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) in a final volume of 20ul for all PCR reactions. Each reaction included: 1X PCR buffer

(Invitrogen, Carlsbad, CA), 0.2mM dNTPs, 0.4X SybrGreen (Molecular Probes, Eugene, OR), 2.5mM DTT, 1% DMSO and 5ng of DNA. The telomere PCR used 0.8ul of Platinum Taq (Invitrogen), 1.5mM MgCl, 300nM of each primer (tel1b: CGGTTTGTGGTTGGGTTGGGTTGGGTTGGGTTGGGTT; tel2b: GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT) and 30 cycles of amplification at 95°C for 15sec and at 56°C for 60sec. For the reference gene, we used 36B4 primers designed with Primer3 using the 36B4 gene sequence in the common chicken (*Gallus gallus*; *NW\_001471461.2*) and zebra finch (*Taeniopygia guttata*; *NW\_002197395.1*) from the nucleotide database, GenBank. The 36B4 control gene is single copy, confirmed by a melting (dissociation) curve at the end of every qPCR. This melting curve showed a single peak, confirming a single copy gene. The control-gene PCR used 0.5ul of Platinum Taq, 3.5mM MgCl, 300nM of forward primer (MAPE1: AGGGAGAAGAGGGACTGGAC) and 500nM of reverse primer (MAPE2-CAATCCCACACACACCTCAG) and 35 cycles of amplification at 95°C for 15sec and at 56°C for 20sec and 72°C for 20sec. Both PCR reactions had an initial denaturation step at 95°C for 15min.

### *Data Analysis*

The raw data from the Rotor Gene software were imported into Excel and transformed so as to align all amplification plots to a baseline height of 2% in the first 5 cycles of amplification. The fluorescence threshold for determination of the Ct was set at 20% of maximum products, which is at the beginning of the exponential phase of the plot. Cts were converted into nanograms of DNA using standard curves (mean telomere

efficiency  $\pm$  SE = 0.74 $\pm$ 0.01, mean 36b4 efficiency  $\pm$  SE = 0.88 $\pm$ 0.02; all  $R_{sq} > 0.99$ ).

The amount of telomeric DNA was divided by the amount of control-gene DNA, producing a relative measurement of the telomere length of the sample.

### *Reproducibility*

For each PCR, we visually evaluated the absorbance curves calculated by the Rotorgene. A key control of DNA quality was the intra-experiment replicates for each sample. Each sample was run in triplicate and we required that the three amplification curves from the Rotorgene aligned to help ensure qPCR products were consistent. Samples without aligned curves were rerun, if secondary run did not produce reproducible results, the sample was removed from analyses. Based on our prior experience we know that samples with low quality DNA consistently fail to produce reproducible amplification plots and, thus, qPCR data is not reliable. In each trial, two control samples were run to allow for normalization between trials and reproducibility trials to confirm correct measurements. The intra- and inter-trial variability (coefficient of variation) for the qPCR was 7% and 8%, respectively, which is typical for this assay (Martin-Ruiz et al. 2014).

### *Statistical Analysis*

To test if telomere length was predicted by age, we used a general linear model where telomere length was predicted by age in 2007 (n=73, Table 2.1). Because we found no difference in telomere length among males and females aged 15yrs, the data were pooled.

We defined telomere rate of change (TROC) to be  $= (2010 \text{ telomere length} - 2007 \text{ telomere length}) \div 3 \text{ years}$ . Since all birds were exactly 3 years older when resampled, we asked if the change in telomere length over those three years differed with the age of the individual. To test this, we used a linear regression where TROC is predicted by mean age of the individual, or 2007 age plus 1.5yrs.

We asked if telomere length changed for individuals over the three years 2007-2010, Since the change in age for all individuals was exactly three, and TROC did not vary by age, we compared each cohort of birds' mean telomere length in 2007 to the mean telomere length in 2010. Since all individuals are resampled, we used a paired t-test to compare means, an accepted technique when comparing telomere lengths of birds resampled longitudinally (Pauliny et al. 2006).

Next, we assessed the effect of reproductive effort on telomere length for all individuals sampled in 2007. For this analysis, for each bird, a year of reproductive effort was one where at least one egg was laid. To be more confident that we obtained the accurate measures of breeding attempts for an individual, we included in our analysis only penguins observed as adults before they were 7 years of age, as both sexes frequently are breeding by that age (Boersma et al. 2013). We regressed the individual's age against the total number of years of attempted breeding during that individual's lifetime and computed the residuals. These residuals were regressed against telomere length, with the added factor of sex. We also used a linear model to ask if previous reproductive output (number of chicks fledged) prior to 2007, predicted the TROC between 2007-2010.

Next, we included only samples for which we collected blood in both 2007 and 2010 ( $n=32$ ) in a general linear mixed effects model. In this model, reproductive success (chicks fledged/eggs laid) during the three years 2007-2010 was regressed against TROC, with mean-age as included as fixed effects. Subsequent models tested whether the number of reproductive attempts (years of breeding), number of eggs laid, or the number of chicks fledged, from 2007-2010 predict the TROC during the same period.

Since Magellanic penguins can skip reproductive years (Boersma and Rebstock 2010), we tested for the effect of telomeres on survival by determining whether adult penguins sighted in 2007 were re-sighted during the combined three-year period: 2010-2013. If individuals were not sighted at any point during this three-year period, we assumed they were dead. We used a binomial general linear mixed model with both age and sex as fixed effects to test whether telomere length predicts re-sighting of adults.

Finally, we tested for the effect of TROC on survival by using a binomial general linear mixed model to test whether TROC from 2007-2010 predicts re-sighting of adults during the combined three-year period: 2010-2013.

## **[Results]**

Telomeres of adult male and female Magellanic penguins 15 years of age were similar in length, so we pooled them in subsequent analyses ( $t=0.44$ ,  $p=0.66$ ,  $n=25$ ). Telomere lengths for the 73 penguins sampled in 2007 were similar among age groups ( $Rsq<0.001$ ,  $p=0.9156$ ,  $n=73$ , Figure 2.1). Telomere rate of change (telomere length change/year) did not correlate with mean age of individual, suggesting that telomere attrition is not higher at increasing age ( $Rsq=0.06$ ,  $p=0.57$ ,  $n=33$ ). The telomere lengths

of the 32 adults sampled exactly 3 years later were similar in length (All  $p > 0.05$ ; Figure 2.2). Reproductive attempts were not correlated with telomere length for penguins aged 15yo and 19yo, even when we controlled for sex (Figure 2.3,  $Rsq = 0.027$ ,  $p = 0.663$ ,  $n = 37$ ). Additionally, the number of chicks fledged (reproductive output) prior to 2007 did not predict the TROC from 2007-2010 ( $Rsq = 9.393e-05$ ,  $p = 0.96$ ,  $n = 20$ ). Likewise, the number of eggs laid ( $p = 0.48$ ), chicks fledged ( $p = 0.77$ ), number of reproductive attempts ( $p = 0.86$ ), or reproductive success ( $p = 0.46$ ) during 2007-2010 did not predict TROC during the same period. Additionally, telomere length in 2007 did not correlate with re-sighting within the three years, 2010-2013 ( $z = 0.95$ ,  $p = 0.34$ ,  $n = 73$ ). Furthermore, the TROC during 2007-2010 was not well correlated with the re-sighting of individuals during the following three years, 2010-2013 ( $z = 1.15$ ,  $p = 0.24$ ,  $n = 33$ ).

## **[Discussion]**

Telomeres of Magellanic penguins were of similar length for adults aged 4 to 24yrs of age, and did not shorten over a 3-yr period, suggesting a high level of maintenance.

During breeding, both male and female penguins undergo stressors. Stressors for males include competition for nest sites, extended fasting and offspring defense (Yorio et al. 1990; Boersma 2013; Boersma et al. 2013). Similarly, females have long fasting periods before egg laying and during incubation, interact with conspecifics, defend offspring, and lay two eggs that require nutritional investment (Boersma et al. 2013; Boersma et al. 2004). We found, however, no relationship between an individual's reproductive effort and telomere length, nor the TROC. Magellanic penguins may have

an increased ability to neutralize ROS through antioxidant defense, relative to other bird species. The anti-oxidants present within the blood plasma are, in part, a result of the intake of prey that possess high anti-oxidant load (Cohen et al. 2009). Adelle penguins (*Pygoscelis adeliae*) can preferentially feed within prey assemblages that consist primarily of krill (*E. superba* and *E. crystallorophias*), a high anti-oxidant prey, in response to oxidative stress (Beaulieu et al. 2010). Magellanic penguins at Punta Tombo feed upon fish, squid and crustaceans (Boersma et al. 2013). Squid (*Loligo spp.*, *Illex argentinus*) is an important component of their diet, and can account for 1–19% of prey observed in stomach contents (Gandini et al. 1994; Wilson et al. 2005). Squid ink significantly increases the antioxidant ability in chickens (*Gallus gallus*) (Liu et al. 2011). Chickens that ate a diet of just 2% squid ink over a 42-day study showed significantly increased anti-oxidant ability (Liu et al. 2011). The diet of Magellanic penguins may help mitigate the effects of ROS increases and minimize shortening of telomeres by bolstering their antioxidant ability.

Telomerase elongates telomeres, and may play a key role in governing lifespan (Hausmann et al. 2007; Shay et al. 1993; Vaziri et al. 1993; Dong et al. 2005; Forsyth et al. 2002; Counter et al. 1992). In humans, telomerase is only active in germ and stem cell lines and remains at considerably low levels in somatic cell lines of kidneys, lymphocytes and epithelial cells (Counter et al. 1992; Dong et al. 2005; Forsyth et al. 2002; Shay et al. 1993; Vaziri et al. 1993). Long-lived bird species may, however, not immediately down-regulate the expression of telomerase (Hausmann et al. 2007; Dong et al. 2005). Some long-lived seabirds have increased telomerase activity in bone marrow and spleen, where some somatic cell lines develop (Hausmann et al. 2007). This could allow mature



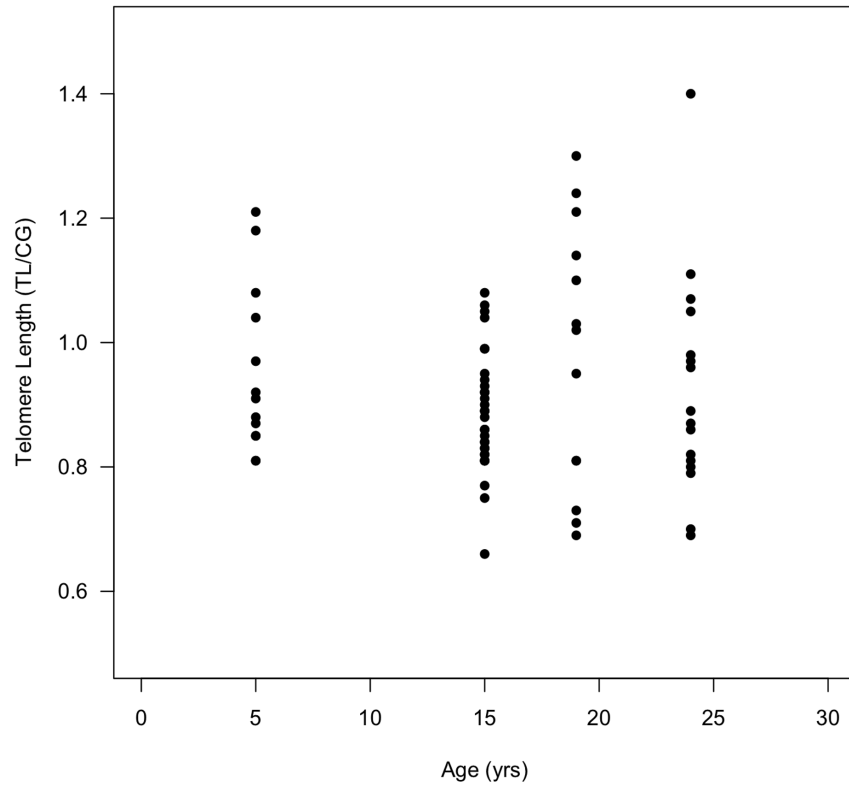
somatic cells to have longer telomeres as the individual ages. Telomere lengths in human sperm cells display this phenomenon, with sperm cell telomeres from older individuals being longer as they are likely acted upon by enhanced telomerase activity in the gonads (Eisenberg et al. 2012). In other long-lived avian species, telomerase is more active than in short-lived species (Hausmann et al. 2007), and this could be a component of the maintenance mechanism our results suggest.

While we show that lifetime reproductive effort was not correlated with telomere length for penguins aged 15yo and 19yo. The relationship between increased reproductive effort and shortened telomere length is well studied (Pauliny et al. 2006; Kotrschal et al. 2007; Bauch et al. 2013; Sudyka et al. 2014; Voillemot et al. 2012). Reproduction can increase oxidative stress (Alonso-Alvarez et al. 2004), which can shorten telomeres (von Zglinicki 2002). Increased reproductive effort could lead to increase levels of ROS. Experimentally increased reproductive effort is correlated with increased telomere shortening, in both lab studies of zebra finch (*Taeniopygia guttata*), (Reichert et al. 2014; Heidinger et al. 2011) and wild blue tits (*Cyanistes caeruleus*) (Sudyka et al. 2014). Some long-lived seabirds show similar patterns; common terns (*Sterna hirundo*) that returned to the breeding colony earlier, and had larger brood sizes had shorter telomeres (Bauch et al. 2013). However, individuals with the highest reproductive success showed the lowest loss of telomere length (Bauch et al. 2013), suggesting that long-lived seabirds of higher quality may better mitigate the cost of reproduction. While individual quality, body condition, or behaviors related to chick rearing could explain this result; it does suggest that adult seabirds may be able to mitigate the cost of reproduction on their telomeres. Our results, showing no relationship

between reproductive effort and telomere length, are similar to results for other penguin species. Beaulieu et al. (2011) showed that Adelie penguins with experimentally increased reproductive effort, by means of handicapping, did not have significantly shorter telomeres.

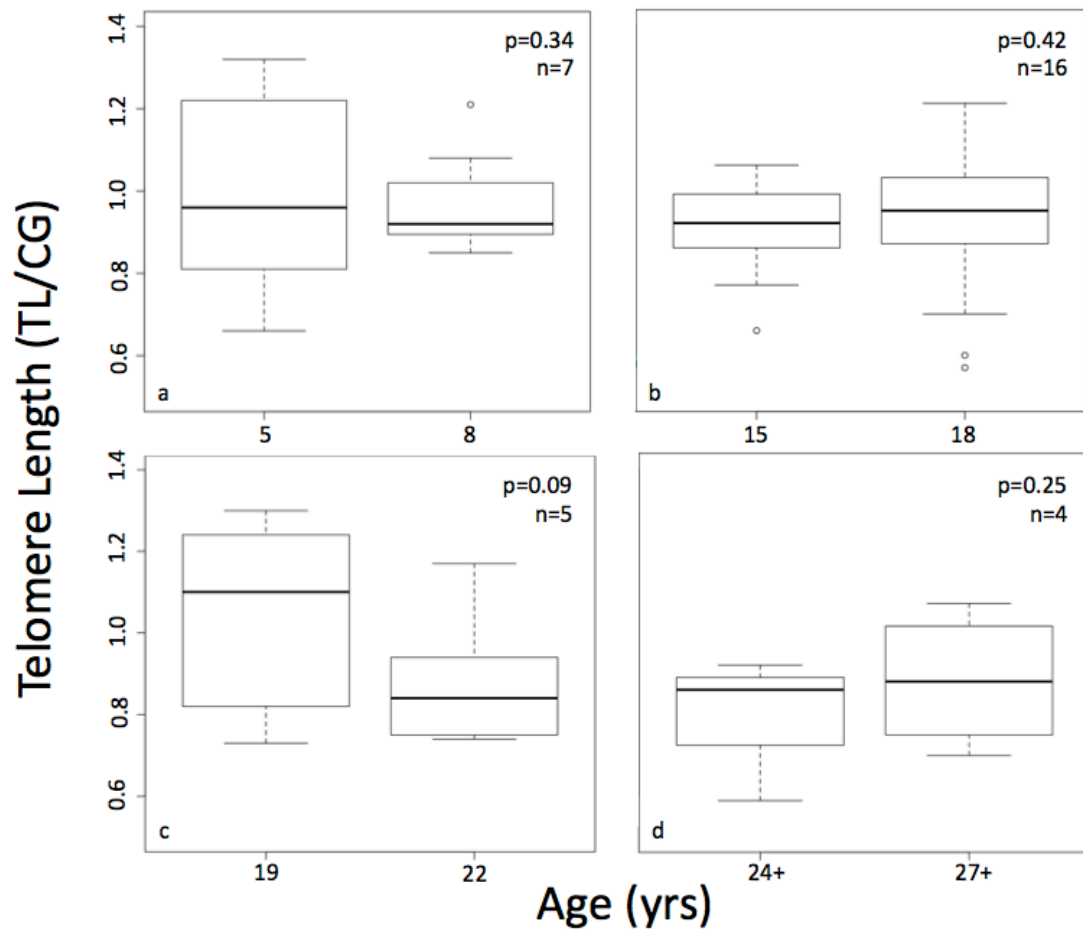
Interestingly, we observed that longer telomeres did not correlate with increased re-sighting, a measure of survival. This contrasts with studies, where telomere shortening correlated with a decreased survival (Bize et al. 2009), likelihood of return (Salomons et al. 2009) or lifelong reproductive success (Pauliny et al. 2006), even with adult maintenance of telomeres (Salomons et al. 2009). Our results are consistent with two hypotheses: (1) within a population of increased longevity, those physiological systems (telomere maintenance) that correlate with a survival advantage are maximized by evolutionary pressures or, (2) those individuals with significantly shorter telomeres, or those that shorten with stressors, may have already been culled from the population. We explored telomere change only in breeding age adults. Periods of high energetic investment in chick and juvenile growth may show telomere attrition, an area explored in Chapter 3.

[Figures]



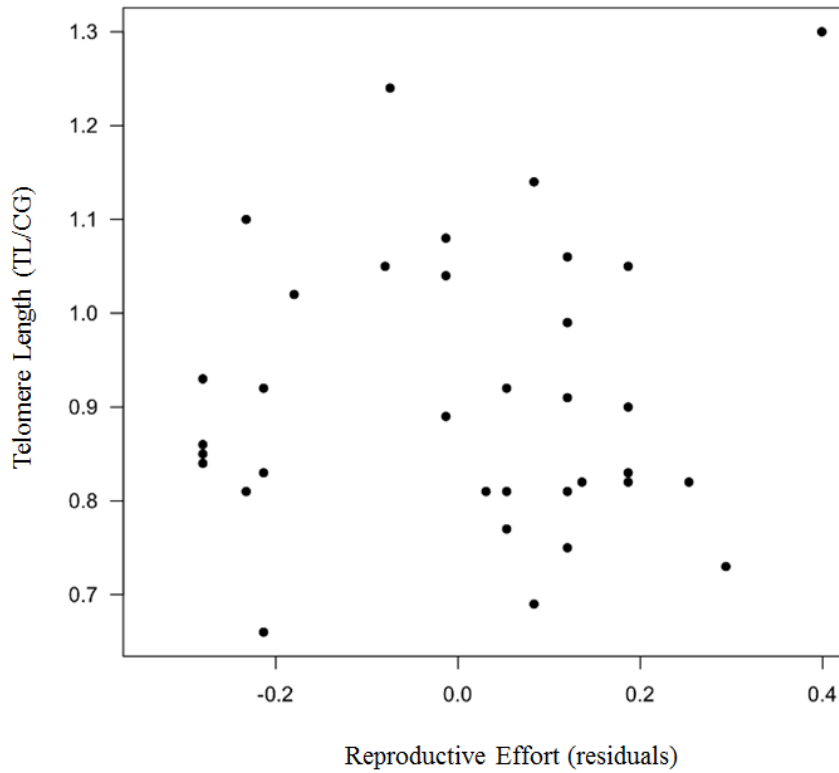
**Figure 2.1: Telomere length is similar in adult Magellanic penguins aged 4 to older than 24 years of age.**

Telomere lengths for the penguins collected in a cross-sectional sample in 2007 showed no relationship with age, suggesting high telomere maintenance through 24+ years ( $Rsq < 0.001$ ,  $p = 0.9156$ ,  $n = 73$ ).



**Figure 2.2: Longitudinal sampling of adults over a 3-year period shows no telomere change.**

Longitudinal resampling, individuals sampled after 3-years (2007-2010). Regardless of age, telomere length of Magellanic penguins did not shorten over a three-year period (paired t-test; a:  $t=-0.40$ ,  $p=0.34$ ,  $n=7$ ; b:  $t=-0.19$ ,  $p=0.42$ ,  $n=16$ ; c:  $t=1.53$ ,  $p=0.09$ ,  $n=5$ ; d:  $t=0.73$ ,  $p=0.25$ ,  $n=4$ ).



**Figure 2.3: Telomere lengths of adult Magellanic penguins are not predicted by reproductive effort.**

Linear model of residual values for the number of reproductive attempts by age of individuals regressed against telomere length, controlling for sex of individual ( $Rsq=0.027$ ,  $p=0.663$ ,  $n=37$ ).

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[Tables]

<b>Age (2007)</b>	<b><i>n</i></b>	<b>Age (2010)</b>	<b><i>n</i></b>
4-5yrs (Male)	15	7-8yrs	7
15yrs (Male)	17	18yrs	12
15yrs (Female)	8	18yrs	4
19yrs (Male)	15	22yrs	5
≥24yrs (Male)	18	≥27yrs	4
<b>TOTAL</b>	<b>73</b>	-	<b>32</b>

**Table 2.1: Age groups and sample size.**

## CHAPTER THREE: TELOMERES SHORTEN AND THEN LENGTHEN DESPITE MITOCHONDRIAL INCREASE DURING GROWTH IN MAGELLANIC PENGUINS

### [Abstract]

Increased adult survival enhances fitness. In some long-lived species, longer telomeres are correlated with increased survival. If telomere length is important to survival, adults should enter breeding with long telomeres to maximize their lifespan. Chick growth is energetically costly, and is characterized by high levels of cell proliferation, which is linked to shorter telomeres. Mitochondria are the source of both energy production, and damaging reactive oxygen species that can shorten telomeres. We characterized the dynamics of telomere length and mitochondria number for Magallanic penguins from hatch to old age with a special focus during growth. We tested how growth impacted telomeres by taking blood from wild known-age Magallanic penguin chicks (*Spheniscus magellanicus*) every 15 days from hatching to 60 days of age. We also sampled a set of 1-year-old juvenile penguins, and adults aged 4 to 27+ years. We used qPCR to measure telomeres and NADH-dehydrogenase, which reflects mitochondria number, an indicator of metabolism. Telomeres were shorter on day-15 than on hatch day but returned to length at hatching when the chicks were 45 and 60 days old. Length of telomeres of newly hatched chicks, chicks aged 45 and 60 days, juveniles, and adults aged 4-5yrs were similar. Mitochondrial copy number increased significantly from hatch to age 15 days and remained similar until 30 days of age. Mitochondria number then dropped significantly to hatch-day levels in by 45 days of age. The number of mitochondria decreased in adult penguins by age 7-8 years and remained similar throughout adulthood.



Our results indicate that while telomeres shorten during growth, characterized by increased mitochondria number, Magellanic penguins elongate telomeres and enter breeding with telomeres similar to their hatch day.

## **[Introduction]**

Long-lived species have the potential for future breeding opportunities, however, physiological systems deteriorate with age in most species (Wikelski and Ricklefs 2001, Ricklefs and Wikelski 2002, Haussmann et al. 2003, Haussmann et al. 2007). Thus, life-history theory predicts long-living species should invest more in maintenance systems that increase adult survival (Wikelski and Ricklefs 2001, Ricklefs and Wikelski 2002).

Telomere length, a key factor in cell maintenance, shortens with age (Blackburn 1991, Prowse 1995, Haussmann et al. 2003, Aubert and Lansdorp 2008). Telomeres are the tandem repeating, non-coding sequences that protect ends of chromosomes during cell replication (Greider and Blackburn 1985, Blackburn 1991). During each cycle of cell replication, telomere sequences are lost because DNA polymerase cannot fully replicate the 3' end of the DNA strand (McClintock 1941, Watson 1972, Blackburn 1991). Telomeres, consequently, shorten with age in most species (Blackburn 1991). The telomere length and its rate of shortening may contribute to survival (Greider and Blackburn 1985, Blackburn 1991, Chan and Blackburn 2004, Dong et al. 2005, Haussmann et al. 2007, Hornsby 2007, Bize et al. 2009). Stressful events are linked to the production of stress hormones (Walker et al. 2005, Walker et al. 2006, Fowler et al. 2013) and the shortening of telomeres (Epel et al. 2004, Kotrschal et al. 2007). Though the mechanistic relationship is not known in detail, one explanation is oxidative stress, which leads to DNA damage and shorter telomeres (Kirkwood and Austad 2000, von Zglinicki 2002, Kotrschal et al. 2007, Beaulieu et al. 2011). Specifically, reactive oxygen species (ROS), produce DNA strands breaks, which are difficult to repair when they occur in telomeric DNA, leading to abrupt losses of fragments of telomeres (von

Zglinicki 2002). Oxidizing ROS are released as byproducts of mitochondrial metabolism (Harman 1956, Miquel et al. 1980). The mitochondrial theory of aging implicates the mitochondria as the chief target of damage and the primary producer of the damaging reactive oxygen species (Harman 1956, Miquel et al. 1980, Barja and Herrero 2000). Thus, telomere shortening might be the consequence of increased oxidative damage produced by mitochondria (von Zglinicki 2002, Passos et al. 2007).

Growth is energetically costly, and is characterized by high levels of cell proliferation. All birds grow rapidly, many incurring the cost to this allocation of resources away from maintenance systems in favor of growth (Ricklefs 1969b, Ricklefs 2000). However, even with energetically demanding and stressful growth periods, species should evolve processes that maintain physiological systems linked to survival. Short-lived species, like passerines, can have low survival rates, develop rapidly and reach sexual maturity quickly (Saether 1988, Promislow and Harvey 1990, Ricklefs 2000). With this increased mortality, the tradeoff between maintenance and reproductive systems swings highly in the favor of rapid growth and reproductive, rather than maintenance systems, like telomeres. Those avian species with high yearly mortality and shorter lifespans have telomeres that shorten more rapidly, demonstrating this bias (Hausmann et al. 2003). With a short-lived life-history strategy, the allocation cost of resources towards telomere maintenance does not provide a beneficial increase in fitness. In long-lived species, however, there is a fitness advantage to increased survival. While long-lived species will still incur the resource bias towards growth during the development period, they should also allocate resources that enhance adult survival, like

telomeres. We hypothesize that the continual investment cost to maintaining telomeres increases the potential of adult survival and future reproductive events.

To study the changes of both mitochondria and telomeres within a wild long-lived species, individuals must be tracked as they age. Since 1983, we have banded and studied Magellanic penguins (*Spheniscus magellanicus*) at Punta Tombo, Argentina (Boersma et al. 1990, Yorio et al. 1990, Boersma and Rebstock 2014). Magellanic penguins in the wild are known to live more than 30 years (Boersma et al. 2013), exceeding their maximum, mass-predicted lifespan by 26% (where  $lifespan = 17.6 (mass\ in\ kg)^{0.20}$ ) (Lindstedt and Calder 1976). Telomere maintenance could influence this longevity.

If growth is a considerable stress, and evolution favors adult penguins having the highest chance of survival, Magellanic penguins should finish the growth period with the longest possible telomeres. We quantified telomere and mitochondrial copy number from hatch to more than 27 years of age in Magellanic penguins to characterize the potential changes in telomere length and mitochondria when high cellular proliferation and energy demands were high. We predicted: (a) during growth, the lengths of telomeres would decrease, and (b) mitochondria expression would increase during growth to meet metabolic demands.

## **[Methods]**

### *Sampling of Birds*

Beginning in 1982, we banded Magellanic penguins at Punta Tombo, Argentina (44°02'S, 65°11'W) (Boersma et al. 1990). We banded chicks before they fledge, juveniles before they molt into adult plumage, and breeding adults (Boersma 2013). We

collected blood samples from 15 known-age adult Magellanic penguins, aged 4-5years (*Spheniscus magellanicus*) from September to December 2007 at Punta Tombo, Argentina. We also collected blood samples from wild Magellanic penguin chicks from hatching to fledging (or death) beginning in November of 2010 and ending in January of 2011. We took blood from adult penguins in 2010 of four age classes (n=39; Table 3.1), as part of this sample, we resampled 7 individuals from 2007, now aged 7-8years (Table 3.1).

We know the lay date of individual eggs, and because we checked the nests daily before hatching, and we know the exact hatch day and sex of individuals (Boersma and Davies 1987, Yorio et al. 1990, Boersma and Rebstock 2014). We measured the length and width of eggs on lay date. Chicks were bled on their hatching day (day 0) and then captured and bled every 15 days until they were 60 days of age, before they fledged. High chick mortality reduced sample sizes during the nestling period: hatching day (“day 0”, n=20 chicks), 15 days old (n=12), 30 days old (n=11), 45 days old (n=11), and 60 days old (n=11, Table 3.1). We also collected blood from 20 juveniles (1yo). We measured morphological traits (bill, flipper, foot and weight) of all individuals (Boersma et al. 1990).

### *Sample Collection and Processing*

We collected blood via the vein on the dorsal surface of the foot, distal to the tarsometatarsus, and released all penguins within 5 minutes of when they were first sighted. During venipuncture we used 22-25gauge hypodermic needles and collected blood via heparinized capillary tube (Thermo Fisher Scientific Inc.). We immediately

placed the blood into anti-lyses buffer (10% EDTA/ 90% Newborn Bovine Serum), and placed the samples on ice. We froze the samples at -18°C within 1hr of collection. We stored the samples at -80°C at the University of Washington, until processing.

We isolated DNA using a Qiagen DNeasy Mini-kit for animal blood and tissue samples. DNA was extracted from a lightly centrifuged cell pack, consisting primarily of erythrocytes. DNA quantity was confirmed via nanodrop spectrophotometer (*mean 260/280 ratio*±*SE* = 1.85±0.02). We measured telomere length by Quantitative Polymerase Chain Reaction (qPCR). This method has been used to measure telomere length in penguins (Beaulieu et al. 2011, Geiger et al. 2012).

For each sample, two PCRs were run: the first one to amplify the telomeric DNA and the second one to amplify a single-copy control gene (36B4, acidic ribosomal phosphoprotein PO), providing an internal control to normalize the starting amount of DNA. We included a four-point standard curve (2-fold serial dilutions from 10 to 1.25ng of DNA) in all PCRs to allow the transformation of Ct (cycle threshold) into nanograms of DNA. All samples were run in triplicates and the median was used for subsequent analyses.

We used a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) in a final volume of 20ul for all PCR reactions. Each reaction included: 1X PCR buffer (Invitrogen, Carlsbad, CA), 0.2mM dNTPs, 0.4X SybrGreen (Molecular Probes, Eugene, OR), 2.5mM DTT, 1% DMSO and 5ng of DNA. The telomere PCR used 0.8ul of Platinum Taq (Invitrogen), 1.5mM MgCl, 300nM of each primer (tel1b: CGGTTTGTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT; tel2b: GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT) and 30 cycles of

amplification at 95°C for 15sec and at 56°C for 60sec. For the reference gene, we used 36B4 primers designed with Primer3 using the 36B4 gene sequence in the common chicken (*Gallus gallus*; *NW\_001471461.2*) and the zebra finch (*Taeniopygia guttata*; *NW\_002197395.1*) from the nucleotide database, GenBank. The control-gene PCR used 0.5ul of Platinum Taq, 3.5mM MgCl, 300nM of forward primer (MAPE1: CAGCAAGTGGGAAGGTGTAATCC) and 500nM of reverse primer (MAPE2-CAATCCCACACACACCTCAG) and 35 cycles of amplification at 95°C for 15sec and at 56°C for 20sec and 72°C for 20sec. Both PCR reactions had an initial denaturation step at 95°C for 15min.

#### *Mitochondrial Copy Number*

For the measurement of mitochondria, we used qPCR to target NADH-dehydrogenase pseudogene. NADH-dehydrogenase is a key protein component of the electron-transport-chain, within the cell wall of mitochondria (Weiss et al. 1991). Measuring the amount of nucleic DNA of this gene can inform us of the copy number of mitochondria within the erythrocytes. We used primers designed with Primer3 using the Genbank sequence for Magellanic penguins (*GQ354792.1*). The PCR used 0.5ul of Platinum Taq, 3.5mM MgCl, 300nM of forward primer (NADH1: TCCCACCACTAACAGGCTTT) and 500nM of reverse primer (NADH2-GCTGTTGGTTTGTGGATGTG) and 35 cycles of amplification at 95°C for 15sec and at 56°C for 20sec and 72°C for 20sec. The PCR also had an initial denaturation step at 95°C for 15min.

### *Data Analysis*

The raw data from the Rotor Gene software were imported into Excel and transformed so as to align all amplification plots to a baseline height of 2% in the first 5 cycles of amplification. The fluorescence threshold for determination of the Ct was set at 20% of maximum products, which is at the beginning of the exponential phase of the plot. Cts were converted into nanograms of DNA using standard curves (mean telomere efficiency  $\pm$  SE =  $0.74 \pm 0.01$ , mean 36b4 efficiency  $\pm$  SE =  $0.88 \pm 0.02$ ; all  $R_{sq} > 0.99$ ).

The amount of telomeric DNA was divided by the amount of control-gene DNA, producing a relative measurement of the telomere length of the sample. Similarly, the amount of NADH-dehydrogenase DNA was divided by the amount of control-gene DNA to give a relative mitochondrial copy number. In each trial, two control samples were run to allow for normalization between trials and reproducibility trials to confirm correct measurements. The intra- and inter-trial variability (coefficient of variation) for the qPCR was 7% and 8%, respectively, which is typical for this assay (Martin-Ruiz et al. 2014).

### *Chick Growth and Statistics*

In 60 days, Magellanic penguins develop from newly hatched chicks (~76g) to fledging weight (~1800g). Since we used repeated samples of the same individuals all analyses of chicks used a mixed-effects model approach with age as a fixed effect and chick-ID as a random effect to test if telomere length or mitochondrial copy number were predicted by age. A TukeyHSD test was performed to test for difference between age groups in chicks and adults.



Since no adults were resampled, we used a linear model, controlling for age, to determine if mitochondria copy number predicted telomere length in adults. We used a binomial general linear mixed model with both age and sex as fixed effects to test whether telomere length predicts survival of fledglings, or breeding rate of adults.

We used a linear model to determine if the rate of growth of body parts predicted telomere length. A t-test was used to compare telomere lengths for those chicks that fledged and those that died during growth. Statistics were completed with R Statistical software (R Foundation for Statistical Computing: Development Core Team (2008)).

## **[Results]**

Telomeres were significantly shorter when chicks were 15 days of age than on their hatch day, and by 45 days of age, telomeres were similar to length at hatching, where they remained unchanged through fledging ( $p=0.002$ ,  $n=65$ , TukeyHSD, Figure 3.1). We investigated a trend between day 60 and adults 27 yrs or older, however found no evidence of shortening ( $Rsq=0.03$ ,  $p=0.11$ ,  $n=76$ ). Additionally, there was no relationship between the growth of morphological traits (bill length, bill depth, flipper length, foot length and weight) and the shortening of telomere length between day 0 and day 15 ( $BL: Rsq=0.04$ ,  $p=0.585$ ;  $BD: Rsq=0.07$ ,  $p=0.486$ ;  $FL: Rsq=0.02$ ,  $p=0.719$ ;  $FT: Rsq=0.0004$ ,  $p=0.956$ ;  $WT: Rsq=0.102$ ,  $p=0.388$ ;  $n=9$ ). Additionally, those individuals that fledged and those that died during growth did not have different telomere lengths ( $t=0.792$ ,  $p=0.43$ ,  $n=20$ ). Telomeres do not shorten from 1yo juveniles through adults aged 27 years and older ( $Rsq=0.009$ ,  $p=0.44$ ,  $n=64$ , Figure 3.1).

Mitochondrial copy number increased from hatch day until day 15 ( $p=0.003$ ) and then was similar through day 30 ( $p=0.99$ ) then decreased to hatch day levels by day 45 (ANOVA:  $p=0.99$ ; TukeyHSD:  $F=6.489$ ,  $p=0.001$ ,  $n=45$ , Figure 3.2). Mitochondria numbers in penguins continued to decrease from day 45 to adults aged 7-8yrs ( $p=0.036$ ), and remained at hatch-day levels through adults aged 27yrs ( $F=1.04$ ,  $p=0.384$ ,  $n=44$ ; TukeyHSD, 3.2). Chicks that hatched from larger eggs (by volume) had fewer mitochondria at age ( $Rsq=0.22$ ,  $p=0.02$ ,  $n=20$ ). On hatch day, there was a near significant negative correlation between telomere lengths and mitochondria number ( $Rsq=0.17$ ,  $p=0.06$ ,  $n=20$ ). In all chicks from hatch to day 45, there was no relationship between mitochondrial number and telomere length ( $Rsq=0.015$ ,  $p=0.41$ ,  $n=44$ ). In adults, there is a near correlation between increased mitochondria number and shorter telomere lengths ( $Rsq=0.145$ ,  $p=0.07$ ,  $n=36$ ).

In adults, the probability that an individual's fledglings would be recaptured as adults was independent of the adult's telomere length ( $z=0.301$ ,  $p=0.76$ ,  $n=40$ ). Also, adult telomere length did not predict the number of offspring that returned ( $Rsq=0.002$ ,  $p=0.77$ ,  $n=40$ ). Telomere lengths did not predict the number of breeding attempts for adult penguins, defined as the number of reproductive years, for either males or females ( $Rsq=0.004$ ,  $p=0.69$ ,  $n=40$ ). Telomere lengths for adults did not correlate with their age at first breeding ( $Rsq = 0.0003$ ,  $p= 0.89$ ,  $n=51$ ).

## **[Discussion]**

Telomeres shorten from hatching to day 15, but telomeres lengthened by day 45. This supports our hypothesis that, as a long-lived species, Magellanic penguins elongate

telomeres and enter breeding with telomeres similar to hatch length, presumably to optimize adult survival. Telomeres of newly hatched chicks, 1-year-old juveniles, and adults aged 4-5yrs were similar in length.

Growth requires high levels of available energy and is characterized by high levels of metabolism (Ricklefs 1969a, Ricklefs 1969b, Breuner 2003). A high metabolic state could release reactive oxygen species (ROS) and damage telomeric repeats (von Zglinicki 2002, Monaghan and Hausmann 2006). Both gluconeogenesis and the general inflammatory response of increased metabolism correlate with shortened telomeres (Monaghan and Hausmann 2006). The correlation we found during the early growth period and shortening of telomeres is likely a result of oxidative damage to telomeres. In King penguins (*Aptenodytes patagonicus*), chicks that had increased growth of body mass had more oxidative damage (Geiger et al. 2012). Small chicks that grew faster showed higher oxidative damage and accelerated telomere loss, showing the correlative link between growth and telomere degradation (Geiger et al. 2012). King-penguins chicks however, did not recover their telomere lengths to their length at hatching. In contrast, Magellanic penguin chicks showed telomere elongation. In times of rapid growth, evolution should favor factors protecting the telomeres in species with long lifespans. Wild, adult King penguins live only 73% of their mass-predicted lifespan, where lifespan =  $17.6 (mass \text{ in } kg)^{0.20}$  (Lindstedt and Calder 1976, Hasley et al. 2008). Magellanic penguins lifespan is 126% of what is predicted by their mass. Since Magellanic penguins exceed their predicted maximum lifespan, it suggests they may possess enhanced telomere maintenance mechanisms compared to other penguin species like King penguins.

An increase in telomere length from day-15 to day-60 suggests elongation of telomeres, likely through increased activity of telomerase. Telomerase, a ribonucleic reverse transcriptase, elongate telomeres (Blackburn 1991, Haussmann et al. 2007). In vertebrates, species show increased telomerase activity during development (mammals included) (Youngren et al. 1998, Chan and Blackburn 2004, Monaghan and Haussmann 2006, Geiger et al. 2012), a maintenance strategy that ensures adults start with the full complement of telomeres after the energetically demanding growth period. After birth in mammals, the telomeres in somatic cells begin shortening (Bekaert et al. 2004), and, in humans, telomerase is only found up-regulated in germ and stem cell lines and remains at low levels in somatic cell lines of kidneys, lymphocytes and epithelial cells (Tollefsbol and Andrews 2001, Stewart et al. 2002, Dong et al. 2005, Hornsby 2007). There is some evidence that telomerase may remain active in some adult long-lived seabirds (*S. hirundo*; *O. leucorhoa*) (Haussmann et al. 2007), which could explain this period of elongation as it is the only known reverse-transcriptase to elongate telomeres (Prowse 1995, Tollefsbol and Andrews 2001, Forsyth et al. 2002, Stewart et al. 2002, Dong et al. 2005, Hornsby 2007, Flores and Blasco 2010, Jaskelioff et al. 2011, Bernardes de Jesus et al. 2012). We did not measure telomerase directly, but we observed an increase in telomere length from lowest levels on day-15 back to hatch day length by day 45.

Elongating telomeres during growth helps mitigate the damage done by increased ROS, produced in high volume during energetically taxing events. The processes of cellular metabolism within mitochondria can directly lead to the production of ROS (Harman 1956, Miquel et al. 1980). Our results show elevated levels of mitochondria activity throughout growth (Figure 3.2), when energetic demands are high (Ricklefs

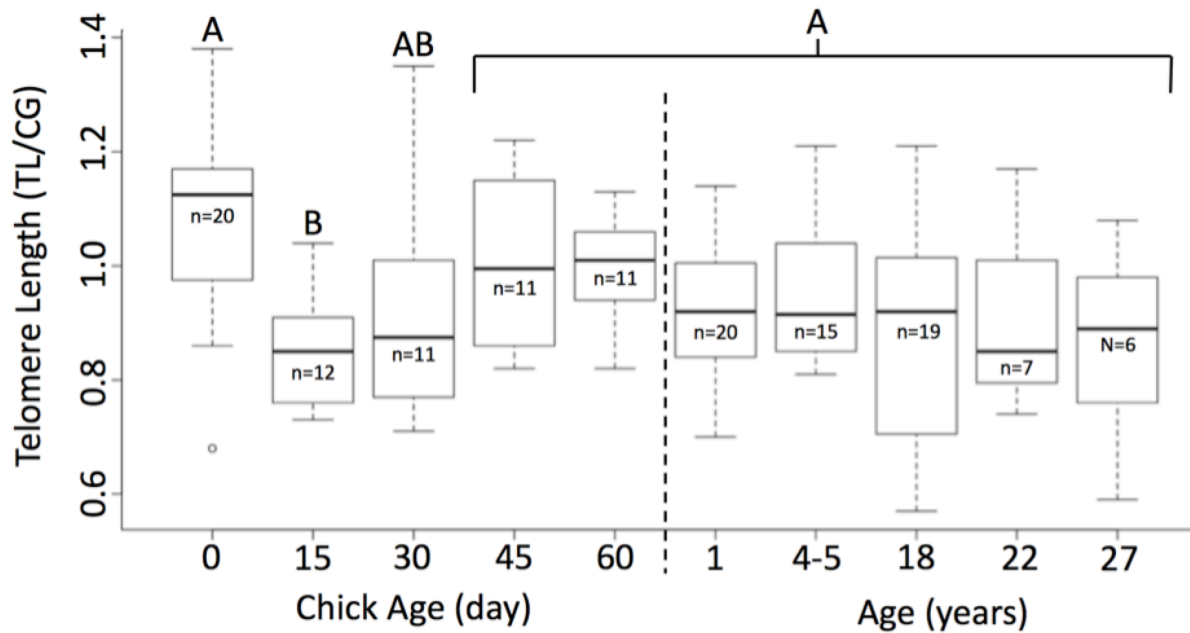
1969a, Ricklefs 1969b), suggesting an increase in overall ROS proliferation.

Mitochondrial DNA accrues damage faster than nucleic DNA; superoxide radicals produced in the mitochondria can lead to nucleic mutagenesis and telomere degradation (Harman 1956, Miquel et al. 1980, Yakes and Van Houten 1997). The resultant damaged mitochondria DNA form a positive feedback loop wherein damaged components lead to increased production of oxidative agents (Harman 1956, Miquel et al. 1980, Becker et al. 2003). We do, however, show a decrease in mitochondria number by age 7-8 years, when individuals are fully-grown. This is advantageous as it decreases the level of ROS proliferation and DNA damage during adulthood. For example, mammalian species that age more slowly show lower levels of oxidative damage indicators (Barja and Herrero 2000).

In adult Magellanic penguins, we observed that longer telomeres did not correlate with increased breeding rate, or the probability of an adult's offspring returning to the colony, both measures of fitness. This suggests that within a population of enhanced longevity either evolutionary pressures maximize systems that correlate with fitness, or other environmental factors are more important in affecting survival and fitness than telomere length.

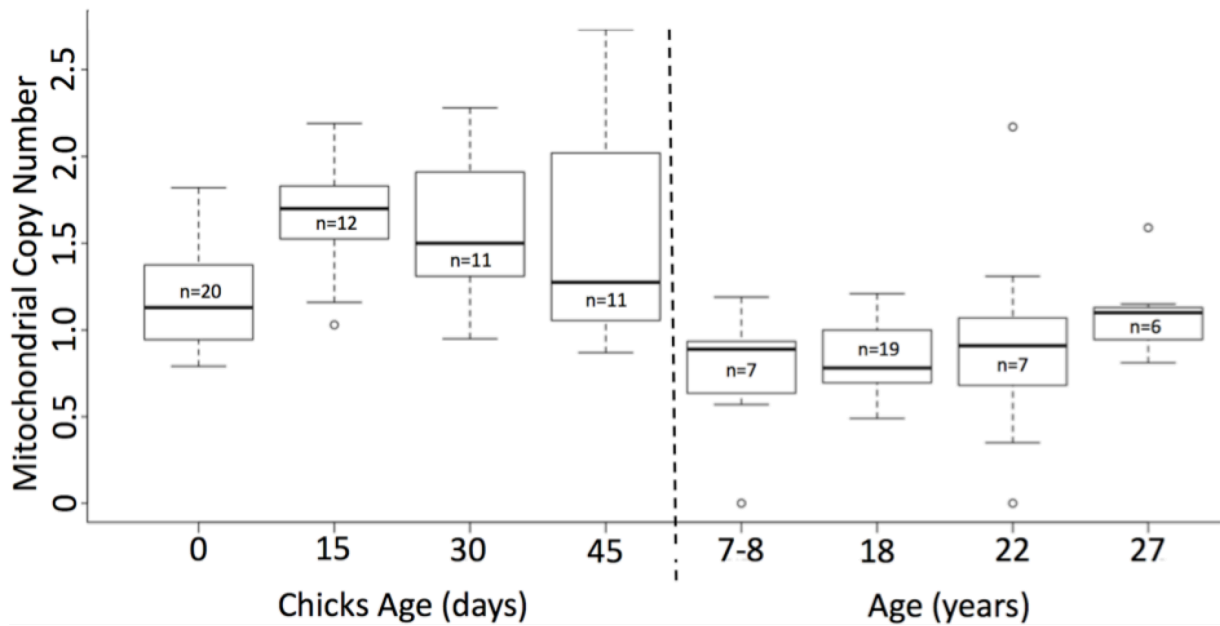
Our assay does not explore the mechanism for telomere maintenance, but shows that Magellanic penguins appear to have evolved a physiological system that maintains telomeres during rapid growth, even with high mitochondria number. This mechanism allows for Magellanic penguins to enter adulthood with telomere comparable to lengths at hatching.

[Figures]



**Figure 3.1: Telomere length returns to hatch-day levels after shortening in early growth.**

Groups that do not share the same heading letter are significantly different. Telomeres of newly hatched chicks, 1-year-old juveniles, and adults aged 4-5yrs were similar in length ( $p=0.14$ ,  $n=55$ ). Telomeres were shorter when chicks were 15 days of age than on their hatch day, but at 45 days of age, telomeres were similar to length at hatching ( $p=0.002$ ,  $n=65$ , Tukey HSD). Telomeres do not shorten from 1yo juveniles through adults aged 27 years and older ( $Rsq=0.009$ ,  $p=0.44$ ,  $n=64$ ). We investigated a trend between day 60 and adults older than 27yrs, however found no evidence of shortening ( $Rsq=0.03$ ,  $p=0.11$ ,  $n=76$ ). Sample sizes presented under means, bars are standard error.



**Figure 3.2: Mitochondria number increases during growth but drops from growth to adults.**

Mitochondrial copy number increased from hatch day until day 15 ( $p=0.003$ ) and then was similar through day 30 ( $p=0.99$ ), but then decreased to hatch day levels by day 45 ( $p=0.99$ ; TukeyHSD:  $F=6.489$ ,  $p=0.001$ ,  $n=45$ ). Mitochondria numbers in penguins continued to decrease from day 45 to adults aged 7-8yrs ( $p=0.036$ ), and remained at hatch-day levels through adults aged 27yrs ( $F=1.04$ ,  $p=0.384$ ,  $n=44$ ).

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[Tables]

Chicks		Adults	
Age	<i>n</i>	Age	<i>n</i>
Day 0	20	1yr	20
Day 15	12	4-5yrs*	15
Day 30	11	7-8yrs*	7
Day 45	11	18yrs	19
Day 60	11	22yrs	7
		≥27yrs	6

**Table 3.1: Age groups and sample size.**

Chicks were sampled in 2010, adults aged 4-5years were sampled in 2007. Adults aged 1-yr, as well as 7-8years and older were sampled in 2010. Adults aged 4-5yrs were resampled after 3 years, at age 7-8yrs, these are marked with an asterisk in the table.

## **CHAPTER FOUR: MAGELLANIC PENGUIN TELOMERES IN CAPTIVITY AND IN THE WILD ARE SIMILAR**

### **[Abstract]**

Lifespan, like other species traits, should be affected by natural selection. Life-history decisions can have physiological consequences that affect an individual's fitness. Aging impacts an individual's maintenance systems and over time these systems suffer degradation and senescence. Telomeres are the tandem repeating, non-coding sequences that protect the ends of chromosomes. Maintenance of telomeres is linked to increased longevity, though dietary stress, trauma and injury, and high-density living can degrade maintenance systems and, in some cases, affect DNA integrity and shorten telomeres. In wild Magellanic penguins, however, telomere lengths do not shorten and are maintained from early breeding adulthood through old age. Stressors in captivity are unlikely to be the same as in the wild. In captivity, confinement and non-native habitat conditions may be stressors, shortening telomeres. If so, telomeres in Magellanic penguins would shorten more in captivity than in the wild. We show, however that telomeres do not shorten with age in males or females, in captivity. Telomeres were similar in length in captive and wild Magellanic penguins when sex and age were included. Our results suggest Magellanic penguins maintain their telomeres in captivity, the same as in the wild, which is consistent with their longer than predicted lifespan.

## [Introduction]

Darwin (1859) suggested that lifespan, like other species traits, should be affected by selection. More breeding is possible with increased lifespan, increasing an individual's fitness (Kirkwood and Rose 1991, Kirkwood and Austad 2000, Austad 2001, Wikelski and Ricklefs 2001, Ricklefs and Wikelski 2002). Maintenance systems, however, suffer degradation and senescence (Williams 1957, Wikelski and Ricklefs 2001, Ricklefs and Wikelski 2002). Nonetheless, all species, but especially long-lived ones, should evolve systems that favor maintenance and resist damage, at least to an extent (Austad 2001, Wikelski and Ricklefs 2001, Ricklefs and Wikelski 2002). While complex interactions govern the relationship between body size and longevity, larger organisms typically live longer (Hausmann et al. 2003, Speakman 2005). Moreover, birds live significantly longer than mammals of similar body size (Hausmann et al. 2003, Speakman 2005). Magellanic penguins (*Spheniscus magellanicus*) live 26% longer than their mass-predicted maximum lifespan for birds (Lindstedt and Calder 1976, Cerchiara et al. 2015), an outlier among penguins (Table 4.1). We are exploring the relationship between life history decisions and physiological mechanisms of Magellanic penguins that may contribute to their enhanced longevity.

One potential mechanism promoting longevity is the maintenance of telomeres, which are the tandem repeating, non-coding sequences that protect the ends of chromosomes during cell replication (Blackburn 1991). During each cycle of cell replication, telomeres are shortened because DNA polymerase cannot fully replicate the 3' end of the DNA strand (Watson 1972, Blackburn 1991). Telomere shortening may affect the number of potential generations a cell line has (Hayflick 1965), which could



play a role in system senescence. Maintenance of telomeres is linked to increased longevity (Hausmann et al. 2003, Monaghan and Hausmann 2006, Hausmann et al. 2007, Hausmann and Mauck 2008). However, stressful events such as reproduction (Kotrschal et al. 2007), trauma and injury (Fowler et al. 2013, Herborn et al. 2014), environmental stressors (Walker et al. 2005, Walker et al. 2006, Fowler et al. 2013) and high density living (Kotrschal et al. 2007) can degrade maintenance systems by diverting resources to production of stress hormones. In addition, it has been reported that stressful events can result in telomere shortening (Epel et al. 2004). Though the mechanistic relationship is only partially understood, one explanation may be the production of oxidizing reactive oxygen species (ROS) under stressful situations (Kirkwood and Rose 1991, Epel et al. 2004). Specifically, ROS can cause DNA strand breaks, which are difficult to repair when they occur in telomeric DNA (von Zglinicki 2002). This can lead to an abrupt losses or fragments of telomeres (von Zglinicki 2002).

Wild Magellanic penguins have evolved in an environment where stressors vary over time. Stressors, for males, include a combination of social and nutritional stress, including competition for nest sites, extended fasting periods and offspring defense (Boersma et al. 1990, Boersma et al. 2013). Similarly, females have long fasts before egg laying and during incubation, interact with conspecifics and defend offspring, and lay two nutritionally rich eggs (Boersma et al. 2013). In a variable environment, in which the quality of conditions for breeding varies spatially and temporally, mechanisms that enhance longevity, like the maintenance of telomeres or resistance to stress, should be highly selected for. In the wild, Magellanic penguins telomeres do not shorten with age

(Cerchiara et al. 2015), so telomere maintenance in wild penguins appears effective in offsetting the effects of environmental stressors.

Captivity is generally regarded as stressful (Morgan and Tromborg 2007). Habitat confinement (Hediger 1955, 1964, Morgan and Tromborg 2007), high-density living (Kotrschal et al. 2007), and non-native diets (Morgan and Tromborg 2007) can cause stress-linked behaviors in captive individuals of a number of species. Restricted movement due to confined space can also cause abnormal behaviors in captive individuals (Hediger 1955, 1964), and is correlated with increased aggressive behaviors in both mammals (Lammers and Schouten 1985, Wiegand et al. 1994, Cassinello and Pieters 2000, Napolitano et al. 2004) and birds (Buchwalder and Huber-Eicher 2004). Additionally, stress-linked pacing behavior, and increased infant mortality in captivity were both positively correlated with a species' home range size, in carnivores (Clubb and Mason 2003, 2007). In captive mice, high-density living was correlated with increased telomere shortening (Kotrschal et al. 2007). However, in a phylogenetic study of 28 bird species, aging-related mortality was equivalent within zoo and wild population, suggesting that intrinsic causes of death kill independent of the environment (Ricklefs 2000). In six penguin species, of variable size and habitat, however, longevity was increased in captivity for four species (Table 1.2). Interestingly, the two species of penguins that do not experience increased longevity in captivity are Magellanic and Little blue penguins (*E. minor*), both species that exceed their mass-predicted lifespan in the wild (Table 4.1). Two hypotheses could support this observation, (1) other penguin species face higher predation pressure the wild than Magellanic or Little blue penguins, which would shorten the maximum observed lifespan, or (2) these species may already

maximize their longevity, a component of which may be telomere maintenance.

Regardless, captivity provides very different conditions than the wild. For this reason, we expected that the different stressors in captivity would negatively affect telomere length in Magellanic penguins. Here we characterize whether in captivity, Magellanic penguins maintain their telomeres as they do in the wild. We predicted that, captive penguin telomeres (1) would shorten with age, and (2) would be shorter than those of wild Magellanic penguins.

## **[Methods]**

### *Study Site and Individuals*

Whole blood from known-age adult Magellanic penguins (n=15; aged 1-22yrs) was collected by veterinary staff at SeaWorld, San Diego, CA (USA), where age and sex were known. All samples were of breeding age adults aged 1 to 22yrs (7 males and 8 females). In addition, from September to December 2007, we collected blood samples from 75 known-age adult Magellanic penguins at Punta Tombo, Argentina. Magellanic penguins at Punta Tombo, Argentina (44°02'S, 65°11'W) have been intensively studied since 1982 (Boersma et al. 1990, Boersma 2008). Yearly, we band chicks before they fledge, juveniles before they molt into adult plumage and breeding adults. The penguin samples were aged 4-5yrs, 15yrs, 19yrs and older than 24yrs (Table 4.2). We know the age of adults 19 yrs and younger because they were banded as chicks (year 0). Penguins in the oldest adult cohort, at least 24 years of age, were banded as adults in 1983. Sex is known from morphological traits (92% accuracy) (Boersma et al. 2013), this method has been confirmed by genetic sexing to be accurate for >97% of adults (Bertellotti et al. 2002).

### *Sample Collection*

We collected blood via the vein on the dorsal surface of the foot, distal to the tarsometatarsus, and released all penguins within 5 minutes of when they were first captured. During venipuncture we used 22-25gauge hypodermic needles and collected blood via heparinized capillary tube (Thermo Fisher Scientific Inc.). We immediately placed the blood into anti-lyses buffer (10% EDTA/ 90% Newborn Bovine Serum), and placed the samples on ice. We froze the samples at -18°C within 1hr of collection, and stored the samples at -80°C at the University of Washington, until processing.

We isolated DNA using a Qiagen DNeasy Mini-kit for animal blood and tissue samples. DNA was extracted from a lightly centrifuged cell pack, consisting primarily of erythrocytes, since avian erythrocytes are nucleated. DNA was quantified via nanodrop spectrophotometer (*mean 260/280 ratio*±*SE* = *1.85*±*0.02*). We measured telomere length by Quantitative Polymerase Chain Reaction (qPCR). This method has been used to measure telomere length in penguins (Beaulieu et al. 2011, Geiger et al. 2012).

For each sample, two PCRs were run: the first one to amplify the telomeric DNA and the second one to amplify a single-copy control gene (36B4, acidic ribosomal phosphoprotein PO), providing an internal control to normalize the starting amount of DNA. We included a four-point standard curve (2-fold serial dilutions from 10 to 1.25ng of DNA) in all PCRs to allow the transformation of Ct (cycle threshold) into nanograms of DNA. All samples were run in triplicates and the median was used for subsequent analyses.

We used a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) in a final volume of 20ul for all PCR reactions. Each reaction included: 1X PCR buffer

(Invitrogen, Carlsbad, CA), 0.2 mM dNTPs, 0.4X SybrGreen (Molecular Probes, Eugene, OR), 2.5mM DTT, 1% DMSO and 5ng of DNA. The telomere PCR used 0.8ul of Platinum Taq (Invitrogen), 1.5mM MgCl, 300 nM of each primer (tel1b: CGGTTTGTGGTTGGGTTGGGTTGGGTTGGGTTGGGTT; tel2b: GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT) and 30 cycles of amplification at 95°C for 15sec and at 56°C for 60sec. For the reference gene, we used 36B4 primers designed with Primer3 using the 36B4 gene sequence in the common chicken (*Gallus gallus*; NW\_001471461.2) and zebra finch (*Taeniopygia guttata*; NW\_002197395.1) from the nucleotide database, GenBank. The 36B4 control gene is a single copy, confirmed by a melting (dissociation) curve that showed a single peak. The control-gene PCR used 0.5ul of Platinum Taq, 3.5 mM MgCl, 300 nM of forward primer (MAPE1: AGGGAGAAGAGGGACTGGAC) and 500 nM of reverse primer (MAPE2-CAATCCCACACACACCTCAG) and 35 cycles of amplification at 95°C for 15sec and at 56°C for 20sec and 72°C for 20sec. Both PCR reactions had an initial denaturation step at 95°C for 15min.

### *Data Analysis*

Raw data from the Rotor Gene software were imported into Excel and transformed so as to align all amplification plots to a baseline height of 2% in the first 5 cycles of amplification. The fluorescence threshold for determination of the Ct was set at 20% of maximum products, which is at the beginning of the exponential phase of the plot. Cts were converted into nanograms of DNA using standard curves (mean telomere efficiency  $\pm$  SE = 0.74 $\pm$ 0.01, mean 36b4 efficiency  $\pm$  SE = 0.88 $\pm$ 0.02; all Rsq > 0.99) .

The amount of telomeric DNA was divided by the amount of control-gene DNA, producing a relative measurement of the telomere length of the sample. In each trial, two control samples were run to allow for normalization between trials and reproducibility trials to confirm correct measurements. The intra- and inter-trial variability (coefficient of variation) for the qPCR was 7% and 8%, respectively, which is typical for this assay (Martin-Ruiz et al. 2014).

### *Statistical Analysis*

To determine if telomere length was correlated with sex in captive penguins, we used a linear regression with sex as the explanatory factor, age was included as a covariate in the model.

To test if telomere length was predicted by age, we used a linear regression. Males and females were included together in this regression, as their telomere lengths were similar in a previous analysis (Cerchiara et al. 2015).

To compare wild and captive telomere lengths, we used a generalized linear model where age and sex are included in the model as random effects. All statistics were compiled in R (R Foundation for Statistical Computing: Development Core Team (2008)).

### **[Results]**

Neither sex (Figure 4.1) nor age (Figure 4.2) predicted telomere length in male and female captive Magellanic penguins ( $Rsq=0.05$   $p=0.703$ ,  $n=15$ ). The telomere length of captive Magellanic penguins was not correlated with their age ( $Rsq=0.01$ ,  $p=0.703$ ,  $n=15$ ; Figure 4.2). Visual examination of this plot suggested that one individual may

overly influence our analyses. To test this data point as an outlier, we performed a Bonferroni outlier test and found the point was not a significant outlier ( $p=0.22$ ). The telomeres of captive penguins were similar in length to those of wild Magellanic penguins, when age and sex are included in the model as random effects ( $Rsq= 0.04$ ,  $p=0.39$ ,  $n=86$ ; Figure 4.3).

## **[Discussion]**

Telomeres were not shorter in captive than wild penguins, demonstrating Magellanic penguins, wherever they live, maintain their telomere lengths. While the stresses that captive penguins experience undoubtedly differ from those of wild penguins, captive conditions could have equally potent stressors (Morgan and Tromborg 2007). Nevertheless, we find that captive Magellanic penguins do not appear to suffer more rapid telomere shortening in captivity than do wild penguins (Cerchiara et al. 2015).

Various taxa respond to captivity differently, some with enhanced longevity and others with increased physiological stress (Mason 2010). While corticosterone response to these stressors might affect telomeres, Magellanic penguins generally have low baseline corticosterone levels with little variation (Walker et al. 2015). Also, Magellanic penguins that are losers of fights do not increase corticosterone release from baseline (Walker et al. 2015); unlike losers of fights in copperheads (*Agkistrodon contortrix*) (Schuett and Grober 2000), fish (*Betta splendens*) (Verbeek et al. 2008) and mice (*P. leucopus*, *P. californicus*) (Oyegbile and Marler 2006). Furthermore, fasting Magellanic penguins did not show any elevated baseline corticosterone until they fasted for several

weeks (Hood et al. 1998). This low corticosterone response could diminish the negative effect of stress response on telomeres and is consistent with the maintenance we observe.

Diet may also play a role in telomere maintenance, likely by bolstering the antioxidant ability of blood plasma, a hypothesis proposed for other penguin species (Beaulieu et al. 2010, Beaulieu et al. 2011). The anti-oxidants present within the blood plasma are, in part, a result of a diet high in anti-oxidants (Corsolini et al. 2001, Beaulieu et al. 2010). Magellanic penguins feed upon fish, squid and crustaceans (Boersma et al. 2013). Squid (*Loligo spp.*, *Illex argentinus*) can account for up to 19% of their stomach contents (Gandini et al. 1994, Wilson et al. 2005). Squid ink, even in small amounts can increase the antioxidant ability in avian plasma (Liu et al. 2011). Carotenoids, found primarily in crustaceans and squid, are considered minor dietary antioxidants in birds (Costantini and Møller 2008). While the effect of diet carotenoids *in vivo* is debated (Costantini and Møller 2008), they are considered to be indicators of an individual's greater antioxidant ability (Marri and Richner 2014) as carotenoids are bleached by oxidative stressors (Woodall et al. 1997). The diet of wild Magellanic penguins may help mitigate the effects of ROS, protecting telomeres.

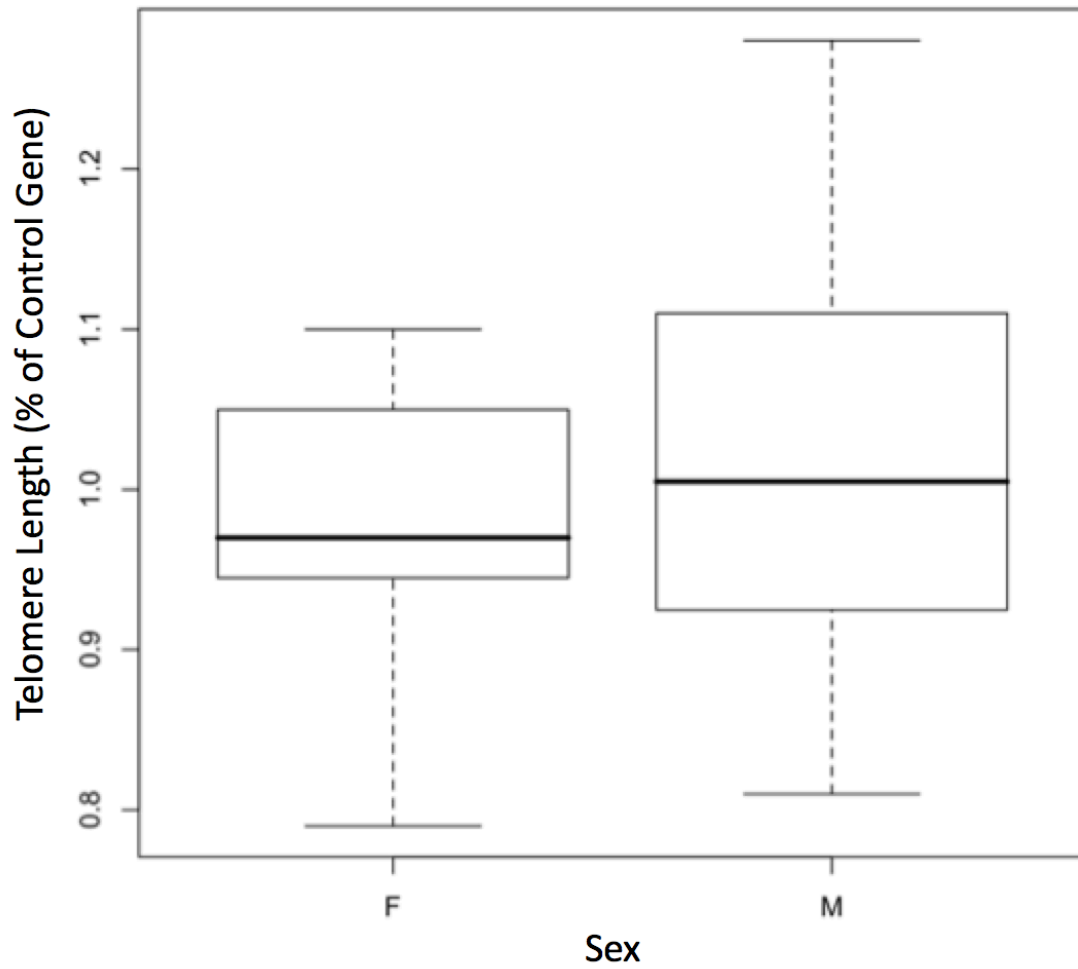
Additionally, telomerase elongates telomeres (Counter et al. 1992, Shay et al. 1993, Vaziri et al. 1993, Forsyth et al. 2002, Dong et al. 2005). In humans, telomerase is active only in germ and stem cell lines and remains at considerably lower levels in somatic cells (Counter et al. 1992, Shay et al. 1993, Vaziri et al. 1993, Forsyth et al. 2002, Dong et al. 2005). It has been shown that telomerase may remain at higher activity in bone marrow, gonads and intestine cell lines in adult long-lived bird species (*T. guttata*; *T. bicolor*) compared to short lived ones (*S. hirundo*; *O. leucorhoa*) (Hausmann



et al. 2007), this could be a component of the maintenance mechanism our results suggest.

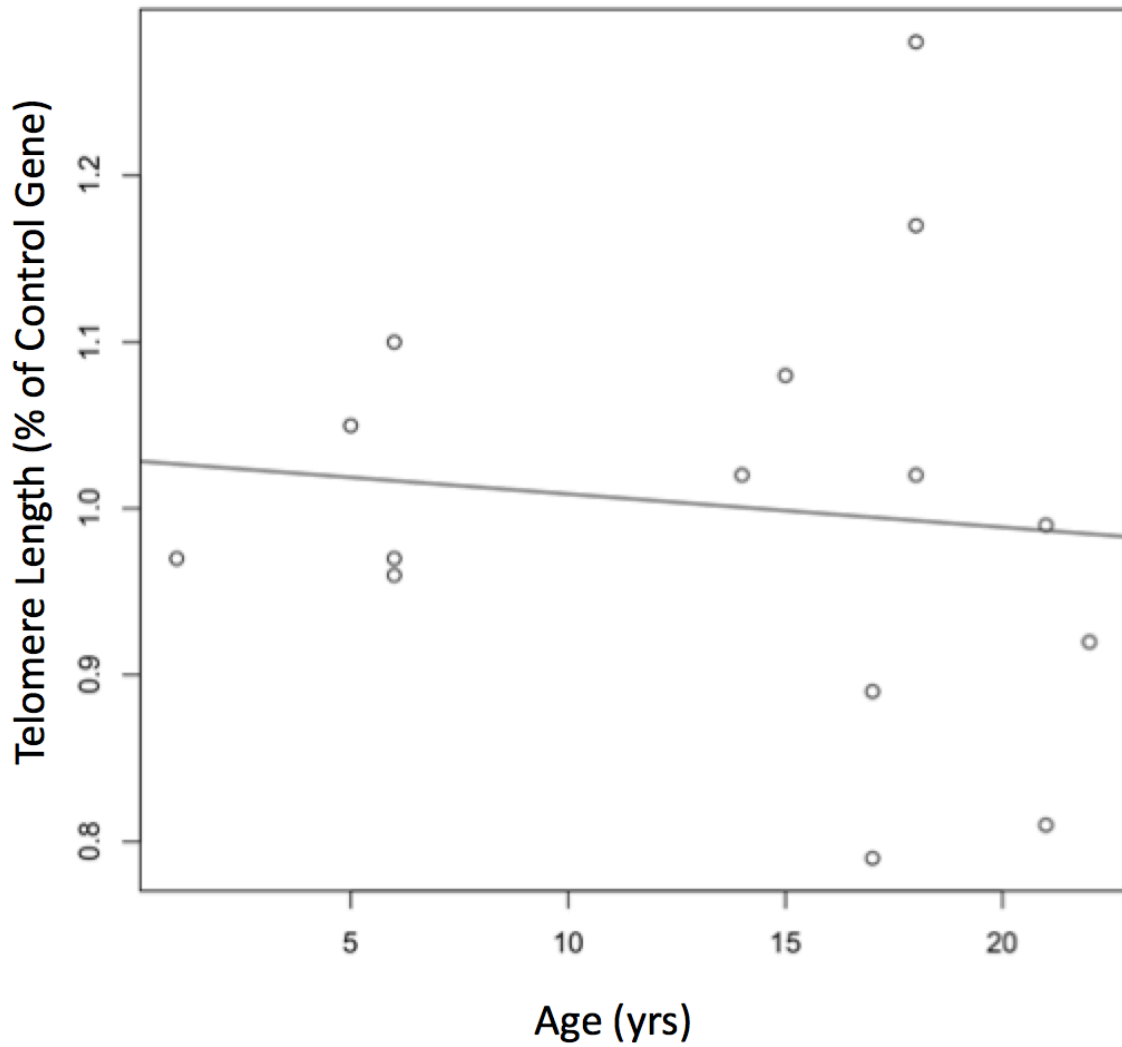
Our results are consistent with the premise that Magellanic penguins, as a species, have a robust system of telomere maintenance. Magellanic penguins exceed their mass-predicted lifespan in the wild and do not experience increased longevity in captivity. They may already maximize their longevity, a component of which may be telomere maintenance. Magellanic penguins likely evolved this maintenance due to the yearly variation in the quality of breeding conditions in their environment. Magellanic penguins can lose nest contents due to chick starvation (Boersma et al. 1990, Boersma and Stokes 1995), predation (Stokes and Boersma 1998) and severe weather (Boersma and Rebstock 2014). Adult penguins may also skip reproductive years if breeding conditions are sub-optimal (Boersma and Rebstock 2010), as future reproductive attempts hold a considerable value. For this reason, having a telomere maintenance system that may enhance adult survival provides the future reproductive opportunities that are a vital component of the fitness of long-lived species. Without this system, having to frequently skip reproductive years would be a detriment to life-long fecundity. Magellanic penguins under many conditions appear to protect their telomeres, both in captivity and the wild. Magellanic penguin enhanced longevity may be explained, at least in part, by the maintenance of telomeres demonstrated by these findings. By understanding the aging physiology of seabird species like Magellanic penguins we are likely to find insights into the evolutionary processes that drive the aging phenotypes we observe in nature.

[Figures]



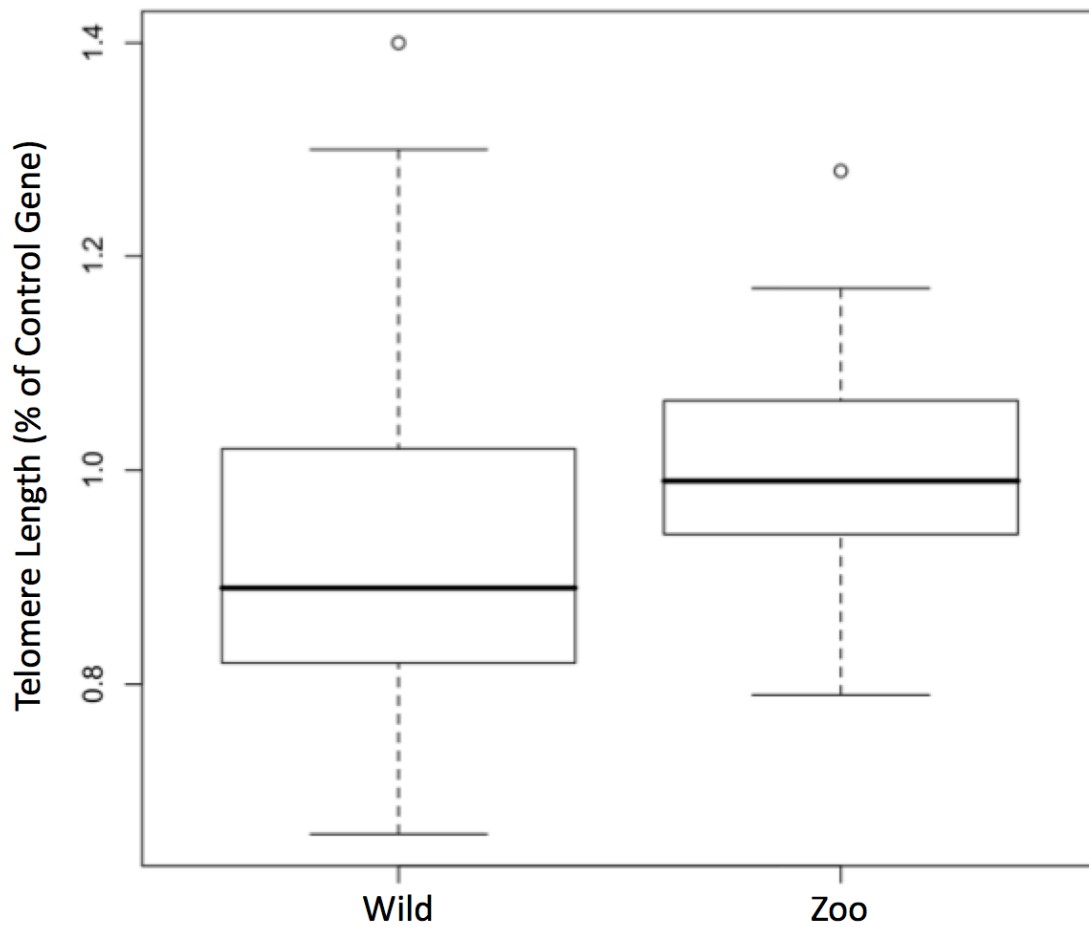
**Figure 4.1: Telomere lengths of male and female penguins in zoos are similar.**

Regression analysis where telomere length is the response of sex as the explanatory variable, age was included as a random effect in the model ( $Rsq=0.05$   $p=0.703$ ,  $n=15$ ).



**Figure 4.2: Telomeres in penguins living in zoos did not shorten with age.**

Both males and females were included in the model, because their telomere lengths were similar ( $Rsq=0.01$ ,  $p=0.703$ ,  $n=15$ ).



**Figure 4.3: Zoo and wild Magellanic penguin telomeres are similar in length.**

Colony location, age and sex do not predict penguin telomere lengths ( $Rsq= 0.04$ ,  $p=0.39$ ,  $n=86$ ). Age and sex are included in the model as random effects.

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<https://www.zoo.org/>

[Tables]

Species	Mass (kg)	Mass-predicted Lifespan	Observed Lifespan	Percent Mass-Adj. Lifespan
Chinstrap Penguin <i>P. Antarctica</i>	4.2	23.5	20 <sup>a</sup>	85%
Adelie Penguin <i>P. adeliae</i>	4.6	23.8	20 <sup>b</sup>	84%
Humboldt Penguin <i>S. humboldti</i>	4.4	23.7	20 <sup>e</sup>	84%
Magellanic Penguin <i>S. magellanicus</i>	4.6	23.8	30 <sup>a</sup>	<b>126%</b>
King Penguin <i>A. patagonicus</i>	12.8	29.3	20 <sup>a</sup>	68%
Emperor Penguin <i>A. forsteri</i>	30.7	34.9	20 <sup>d</sup>	57%
Little Blue Penguin <i>E. minor</i>	1.2	18.25	25.7 <sup>c</sup>	<b>140%</b>

**Table 4.1 – Mass-predicted lifespan for penguin species.**

Mass-predicted from the equation:  $lifespan = 17.6 (mass\ in\ kg)^{0.20}$  (Lindstedt and Calder 1976). a: Borboroglu and Boersma 2013, b: Williams (1995); c: Dann et al 2005; d: British Antarctic Survey – *estimated*; e: Woodland Park Zoo – *estimated*.

<b>Age (2007)</b>	<b><i>n</i></b>
4-5yrs (Male)	15
15yrs (Male)	17
15yrs (Female)	8
19yrs (Male)	15
≥24yrs (Male)	18

**Table 4.2: Age groups and sample size.**