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What modulates telomere dynamics?

Inheritance, developmental effects, and physiological challenges

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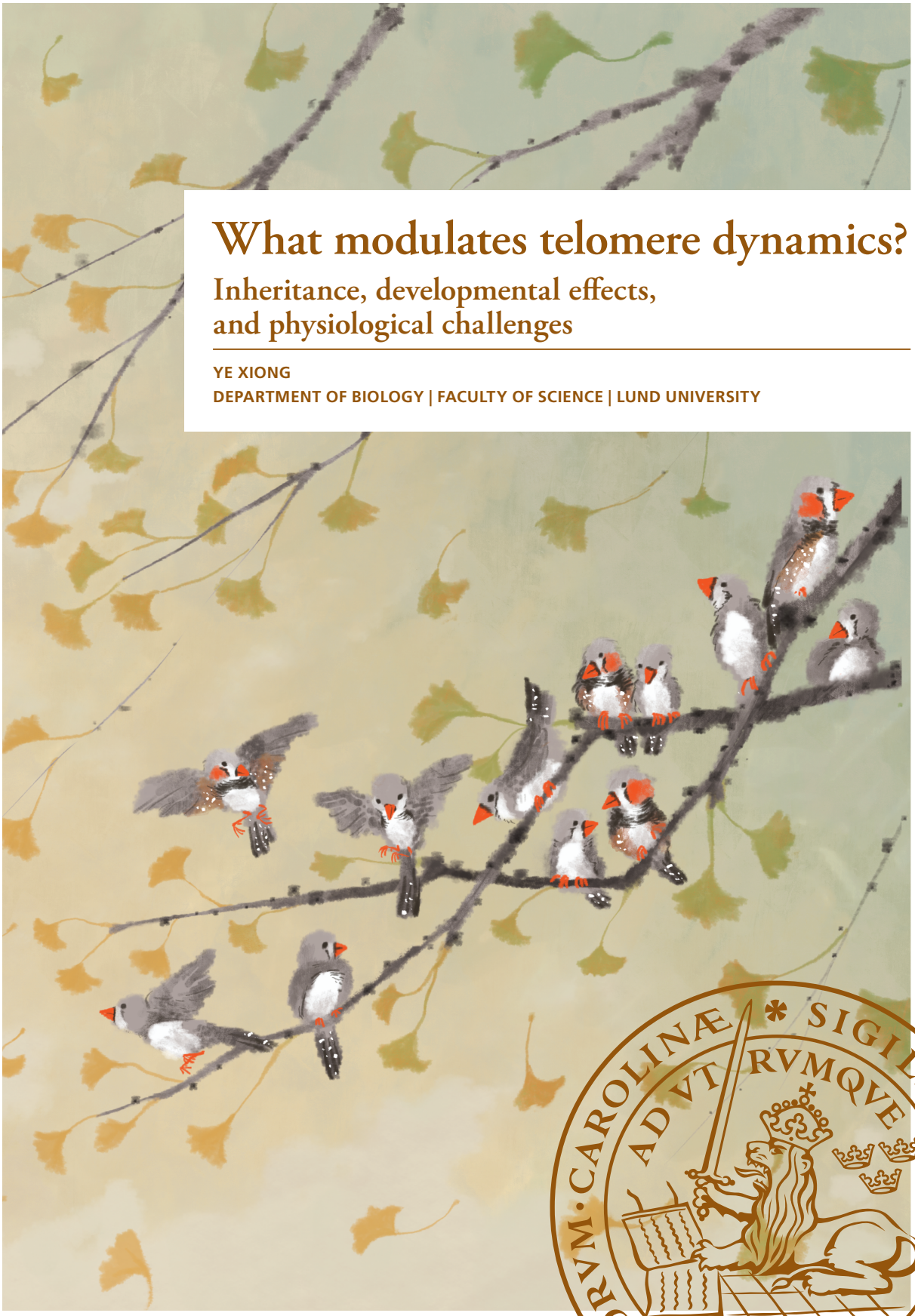
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Inheritance, developmental effects, and physiological challenges

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DEPARTMENT OF BIOLOGY | FACULTY OF SCIENCE | LUND UNIVERSITY

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Inheritance, developmental effects, and physiological challenges

Ye Xiong



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DOCTORAL DISSERTATION

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Title and subtitle: What modulates telomere dynamics? Inheritance, developmental effects, and physiological challenges

Abstract: Telomeres, repeated sequences located at the end of linear chromosomes, have been studied intensiviely over the last decades. Like a cap they protect the chromosome ends and prevent chromosome fusion and degradation. Telomere shortening is linked with age-related dysregulation of bodily functions. Telomere length and shortening also have relevance in ecology and evolution as indicators of individual quality and mediators of life-history trade-offs. Telomere length (TL) predicts life span in many animal species with considerable variation in TL and shortening rate between individuals, populations and species. How does this variation come about and how is it maintained? Paper I in this thesis discusses the most prominent hypotheses in ecology and evolution explaining variation in TL and telomere dynamics. It presents frameworks grouping the hypotheses based on research question or their underlying assumptions about effects of telomeres on performance. In Paper III we test if mild inflammation affects TL over time and leads to deterioration in physiological health. To measure health, we used the VetScan blood analyser, which is carefully evaluated for broader use in paper II. Immune activation had no measurable effects on TL, but changing environment appeared to induce substantial telomere elongation (up to 150 % increase) in the individuals with the shortest TLs. This supports the hypotheses that telomere restoration is costly and therefore primarily occurs under benign conditions. Possibly, telomere elongation was more frequent among individuals with the shortest TL, as they are closer to the lower critical threshold in TL. Paper IV and V investigate the genetic and non-genetic contribution to TL variation. In an breeding experiment we assortatively paired individuals with the shortest and longest TL at birth to test how parental effects contribute to TL during offspring development. The results show that offspring TL at birth, but not embryo TL, was linked to parental TL group. These results are not consistent with hypotheses assuming telomere trajectories to be predicted by TL at the very early stages of embryonic development. However, they support the idea that telomere shortening rate rather than TL itself is inherited. In paper V we further examined inheritance patterns of TL using an animal model and parent-offspring regressions and show that TL is moderately heritable, and that estimates vary considerably depending on the life stage/age of TL measurement. Altogether, this thesis presents a number of novel findings confirmimg and challenging current ‘telomere hypotheses’ discussed in paper I.

Key words: Telomere, telomere dynamics, telomere shortening, immune challenge, telomere inheritance, heritability, physiologial parameter, zebra finch (*Taenopygia guttata)*

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The only constant in life is change.

Heraclitus

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List of Papers

I. Tobler, M., Gómez-Blanco, D., Hegemann, A., Lapa, M., Neto, J. M., Tarka, M., **Xiong, Y**. and Hasselquist, D. (2022). Telomeres in ecology and evolution: A review and classification of hypotheses. Molecular Ecology 31: 5946–5965. John Wiley and Sons Inc.

II. **Xiong, Y.**, Tobler, M., Hegemann, A. and Hasselquist, D., Assessment of avian health status: validation and application of the 'VetScan blood analyser' for ecological and evolutionary studies. Submitted.

III. **Xiong, Y.**, Hegemann, A., Tobler, M. and Hasselquist, D., Telomere dynamics and physiological health in relation to repeated experimental immunisation and short-term changes in environmental conditions. Manuscript.

IV. **Xiong, Y.**, Melgar, J., Tobler, M. and Hasselquist, D., Pre- and post-natal development is critical to determination of early-life telomere length. Manuscript.

V. **Xiong, Y.**, Tarka, M., Tobler, M. and Hasselquist, D., Importance of life stages when estimating heritability of telomere length in a songbird. Manuscript.

Author's contribution to the papers

I. MTo and DH conceptualized the study; all authors contributed equally in data collection, interpretation and discussion of the results; MTo lead wrote the first draft; MTo and DH edited and revised the manuscript with support from AH, DGB, JMN, ML, MTa and YX; DH, AH and MTa received funding.

II. DH, MTo, and YX conceptualized and designed the study; YX, MTo and AH carried out the fieldwork; AH, MTo, and YX performed the laboratory work; YX performed the statistics analysis with supports from MTo and DH; MTo and YX wrote the first draft of the manuscript; all authors edited and revised the manuscript; DH and AH received funding.

III. DH, MTo, and YX conceptualized and designed the study; YX, MTo and AH, carried out the fieldwork, supported by DH; YX and AH performed the laboratory work; YX performed the statistical analyses, supported by MTo and DH; YX and MTo wrote the first draft of the manuscript; all authors edited and revised the manuscript; DH received funding.

IV. DH, MTo, and YX conceptualized and designed the study; YX carried out the fieldwork, supported by MTo; YX performed the laboratory work, supported by JM; YX performed the statistical analyses supported by MTo and JM; YX and MTo wrote the first draft of the manuscript; all authors edited and revised the manuscript; DH received funding.

V. DH, MTo, MTa and YX conceptualized and designed the study; YX carried out the fieldwork, supported by MTo; YX performed the laboratory work; MTa and YX performed the statistics analysis; YX and MTo wrote the first draft of the manuscript; all authors edited and revised the manuscript; DH received funding.

Abstract

Telomeres are the conservative sequence repeats located at the end of linear chromosomes that have been the focus of intensive research across many disciplines over the last four decades. They function as a cap to protect the chromosome ends from fusion to other chromosomes and to prevent chromosome degradation. Telomere shortening is thought to be involved in ageing, reflecting or even causing age-related dysregulation of bodily functions. Telomere length and telomere shortening have also gained considerable interest in the field of ecology and evolution as indicators of individual quality and mediators of life-history trade-offs. Telomere length (TL) predicts life span in many animal species but there is considerable variation in TL and shortening rate between individuals, populations and species. How does this variation come about and how is it maintained? Paper I in this thesis discusses the most prominent hypotheses in ecology and evolution that have been put forward to explain variation in TL and telomere dynamics (i.e., shortening and elongation of telomeres). It presents a framework that groups the different hypotheses based on research question or their underlying assumptions about the causal effects of telomeres on organism performance. Some of the key issues that are highlighted in this synthesis paper are that 1) the question of whether telomeres have a causal effect on ageing and life span is still debated, 2) the costs of telomere shortening and elongation remain elusive, and 3) the genetic and non-genetic contribution to variation in TL (and therefore the potential for selection to act on TL) is still not fully understood. The papers III-V in this thesis try to tackle some of these questions. Paper III aims to test whether mild inflammation (induced through repeated immune challenges), can have measurable negative effects on TL, both over the short- and the long-term and whether this results in concomitant deterioration in physiological health. To measure physiological health, we use the measurement method based on the VetScan blood analyser, which is introduced and carefully evaluated for broader use in paper II. Immune system activation had no measurable effects on TL, but the change in ambient and social environment as a consequence of the experiment design (from harsh, rather cold and large-group to benign, thermoneutral and same-sexed pairs conditions), appeared to induce substantial telomere elongation (up to 150 % increase) in the individuals with the shortest TLs. This supports the hypotheses that telomere restoration is costly and therefore primarily occurs under benign conditions. Moreover, telomere elongation occurred more frequently among individuals with the shortest TL, possibly because such individuals are closer to the lower critical threshold in TL. Paper IV and V

investigate the genetic and non-genetic contribution to the variation in TL. By creating parental groups that were based on the individuals with the shortest and longest TL at birth in the population (thus manipulating the expected genetic contribution from parents to offspring) it was possible to test how genetic and non-genetic parental effects contribute to TL during the prenatal and early post-natal life stages. The results show that offspring TL at birth, but not embryo TL, was predicted by parental early-life TL group. These results are not consistent with hypotheses assuming that telomere trajectories can be predicted based on TL at the very early stages of embryonic development. However, they support the idea that telomere shortening rate rather than TL itself is inherited. To study this further, paper V examines inheritance patterns of telomere length in detail using both animal model and parent-offspring regressions. The study shows that heritability estimates of TL are moderate magnitude, and, particularly, that these estimates vary considerably depending on at which life stage/age TL measurements were compared between parents and offspring.. Altogether, this thesis presents a number of novel findings that both confirm and challenge some of the current 'telomere hypotheses' discussed in paper I.

Popular science summary

Telomeres were first discovered in the 1940s by Hermann Mahler and Barbara McClintock. They noticed this tiny structure located at the ends of chromosomes, which were different from the broken end of DNA sequences that was often seen in DNA exposed to X-ray experiments. During the 1970s, Elizabeth Blackburn and Joseph G. Gall discovered the unique character and the function of telomeres and they were awarded the 2009 Nobel Prize in Physiology and Medicine for the discovery of how telomeres protected chromosomes and the function of the reverse transcriptase telomerase.

So, what are telomeres? Telomeres are repeated sequences of $(TTAGGG)_n$ at the end of linear chromosomes. During cell division, chromosomes untwist and replicate, however there is always some pieces of gene segment that cannot be fully copied (also known as the “end replication problem”). The function of telomeres is to protect the important coding regions of the DNA by extending the chromosome ends with this non-coding sequence that act as a protecting ‘cap'. As a cell continues to divide, chromosomes will reach a point when the telomere sequence reaches a critically short length that exposes coding DNA. Thus, the older a cell (and to some degree organism), the shorter the telomere length. This will lead to cell dysfunction and eventually cell death. Telomeres and the surrounding structures are also protecting DNA from oxidative stress. Oxidative stress is often generated during situations when organisms get sick or are living in challenging social and environmental life conditions. To sum up, telomeres are protective cellular structures that have evolved to reduce the immediate negative effects of DNA damage and that are known to shorten with age.

The next question is, if telomeres could be continuously repaired and thus remain long, would this result in considerably prolonged life spans? Sadly, the answer is most probably no. Another important function of telomeres is to prevent unlimited cell division, which is an important mechanism to prevent cancer cells to be formed. In other words, if telomeres do not shorten due to cell division this would make cells immortal and, if such a cell would contain a malign mutation it would be able to divide and spread relatively unhindered. Thus, we might not die of short telomeres but of cancer instead.

The biological meaning of life is to successfully pass genes to the next generation. There are multiple life history strategies, but the core idea is to produce relatively

more successfully reproducing offspring, and one important way of doing this is to live a healthy and long life. In this thesis, I used captive zebra finches as the study species and designed experiments to test hypotheses in the field of telomeres in ecology and evolution.

I first looked into if telomeres shorten faster when an individual is fighting a disease. We know that when the immune system is activated, the body will increase its metabolism and produce a large amount of immune cells, such as white blood cells and antibodies to fight invading pathogens. Immune system activation also produces an excess of free radicals (harmful 'reactive' molecules that can damage DNA and cells). Both increased cell proliferation and the action of free radicals are known key processes that shorten telomere length. I immune-challenged zebra finches and measured their telomere length across a year. I predicted that telomeres would shorten in immune-challenged birds. Surprisingly, telomere length did not differ between experimental or control birds over one year, but to our surprise a huge telomere elongation occurred over only 30 days at the end of experiment, despite that some birds were supposed to be quite sick! This is a remarkable result because elongation of telomere length is a controversial issue among telomere researchers and some have even argued that it is only a methodological artefact. In the case of my study, moving the zebra finches from rather harsh outdoor to nicer indoor environment may have masked the effect of sickness, and instead allowed them to make very rapid and large investments in terms of telomere elongation (particularly in those with initially short telomere length). So maybe moving the birds away from the not always so pleasant March weather in Skåne and the "collective dormitory" of around 60 birds, and instead move them into a warmer (+ 22°C) environment where only two birds shared the same small "apartment" could have substantially eased social and physiological stress. A room with a heating system and no competition and easy access to plenty of good food may have made life so much easier for all the birds, apparently allowing them to invest much more into self-maintenance such as telomere elongation!

The second part of my work focuses on the inheritance pattern of telomeres. This knowledge is important because the level (high or low) and type (genetic or non-genetic) of inheritance determines how evolution can act on telomeres. I wanted to know how offspring inherit this trait from their parents. If parents have long telomeres, will their children have long telomeres too? I spent four years preparing and conducting matched pairing (based on if parents had short or a long telomere length early in life) breeding experiments to test these questions. The results of the experiment were: 1) offspring telomere length showed a moderate level of similarity to the Tl of their parents; 2) the difference did not appear at the embryonic development stage, but appeared later when chicks were 10-days old; 3) advanced 'animal model' analyses showed that the estimated heritability of telomere length was dependent on which life stages of both parents and offspring that were used in the models. These results tell us that genetic and environmental factors jointly shape

offspring telomere lengths. Thus, these studies show that it is important when in life telomere measurements are taken for analysis as this can influence the results and thus lead to different conclusions.

Altogether, my thesis presents a number of novel findings that both confirm and challenge some of the current ideas about the functional and evolutionary roles of telomeres.

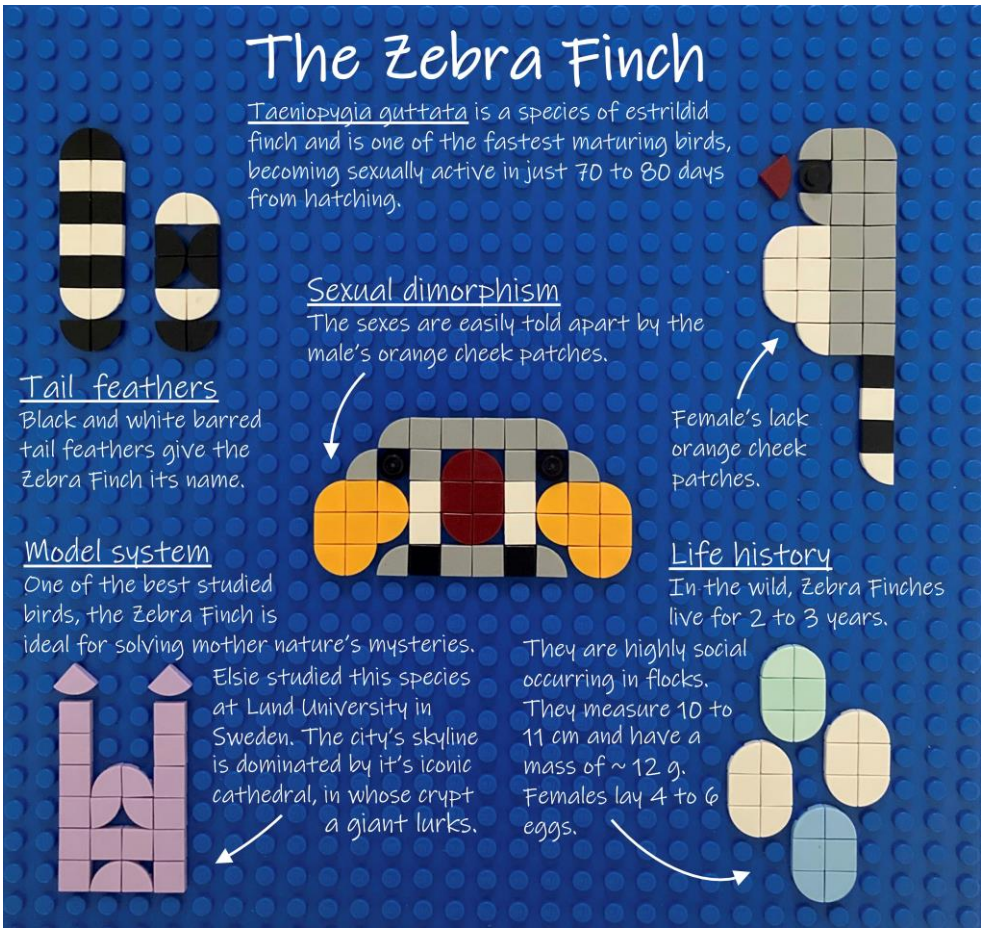


Figure 1. What we know about zebra finches (by Philip Downing)

Populärvetenskaplig sammanfattning

När Hermann Mahler och Barbara McClintock gjorde upptäckten av telomerer på 1940-talet, lade de märke till de små strukturerna på kromosomernas ändar som skiljer sig från DNA-kedjans vanliga ändstycken. På 1970-talet upptäckte Elizabeth Blackburn och Joseph G. Gall telomerernas unika karaktär och funktion. De fick sedan Nobelpriset i fysiologi och medicin år 2009, för att ha upptäckt hur telomerer skyddar kromosomer och den omvända transkriptasen telomeras.

Men vad är egentligen telomerer? Det är små upprepningar av sekvensen $(TTAGGG)_n$ som sitter i änden av våra kromosomer. När celler delar på sig löser kromosomerna upp sig och kopieras. Vissa gensegment kan inte kopieras fullständigt (detta kallas *ändreplikationsproblemet*). Telomerernas uppgift är då att förlänga kromosomernas ändstycken med denna icke kodande sekvens och därigenom skydda de viktiga kodande delarna av DNA:t.

Allteftersom cellen fortsätter dela på sig finns det till slut inte mer av de skyddande telomererna kvar och istället förbrukas de kodande delarna, vilket leder till celldysfunktion och slutligen celldöd. Förutom celldelning skyddar telomerer och omgivande strukturer även kromosomens stabilitet från oxidativ stress. Oxidativ stress kan uppstå vid sjukdom eller under dåliga livsförhållanden. Sammanfattningsvis är telomerer en skyddsmekanism som organismer utvecklat för att bekämpa DNA-skador. Forskning har visat att telomerer förkortas med åldern.

Men vad skulle hända om vi skulle kunna förlänga telomererna i all oändlighet, kunde vi då leva förevigt? Tyvärr är svaret nej. Telomerer som är alltför utsträckta är mer benägna att brytas sönder och skadas. Telomererna har även en annan viktig funktion, vilken är att hämma obegränsad celldelning. Detta är en mekanism som är avgörande för att förhindra bildandet av cancerceller.

Alla organismers främsta evolutionära syfte är att föra vidare våra gener till nästa generation. Genom att leva ett hälsosamt och långt liv förbättrar vi våra möjligheter att fortplanta och föra våra gener vidare. I min avhandling har jag använt zebrafinkar för att pröva befintliga hypoteser inom ekologi och evolution.

Inledningsvis undersökte jag hur sjukdom påverkar telomerförkortning hos zebrafinkar. När immunsystemet aktiveras ökar kroppens metabolism och producerar en stor mängd immunförsvarsceller, såsom vita blodkroppar och antikroppar, för att bekämpa inkräktande patogener. Detta leder under en kortare tid

till ökad celldelning och metabolism. Utifrån detta var mitt antagande att telomererna förkortas snabbare när de motarbetar en infektion. Tillsammans med mina kollegor tilldelade jag immunostimulanter till zebrafinkarna och mätte deras telomerlängder under ett år. Förvånande nog skilde sig inte telomerlängden mellan försöks- och kontrollfåglarna under året. Däremot observerade vi en förlängning under de sista 30 dagarna, även bland fåglarna som fått immunostimulanter. Resultatet är anmärkningsvärt eftersom förlängningen av telomerer fortfarande är ett omdiskuterat ämne. Orsaken varför telomererna inte förkortades snabbare hos de immunstimulerade fåglarna är oklart, men det verkar som att den behagliga och varma inomhusmiljön har överskuggat sjukdomseffekten. Fåglarnas tillvaro - med värmesystem och tillräckligt med föda - förbättrade tillvaron för alla fåglarna, som visat sig ta tillfället i akt och lägga energi på sitt välmående!

Jag studerade också hur en avkomma ärver arvsanlag för telomerer från sina föräldrar. Om föräldrarna har långa telomerer, kommer deras avkomma också ha det? Under fyra år genomförde jag avelsexperiment för att testa dessa idéer, där zebrafinkar parades baserat på deras telomerlängd. Som förväntat visade det sig att telomerlängden hos ungarna beror på föräldrarnas telomerlängd. Däremot visade sig inte skillnaden i telomerlängd under det embryonala stadiet, utan dök upp senare när ungarna var 10 dagar gamla. Slutligen visade vår djurmodell att telomerlängd beror till 30% på genetiska effekter och ca 15% på föräldraskötsel. Den återstående variationen verkar kunna tillskrivas miljöeffekter. Dessa resultat ger oss insikt i att genetiska och miljömässiga faktorer tillsammans formar telomerlängden hos avkomman.

Vilken berättelse skildrar jag då i min avhandling? Min forskning bidrar till vår förståelse av telomerernas dynamik i två viktiga tillstånd, nämligen vid födsel och under sjukdom, genom att tillföra ny kunskap om hur telomerer utvecklas under uppväxten och hur sjukdomar och förändringar i livsstil påverkar fåglars fysiologi.

大众科学

在 1940 年代, 赫尔曼・马勒和芭芭拉・麦克林托克两位科学家分别独立观察到了染色体端粒。相比于其他随意断裂的染色体，端粒这个处在染色体末端的结构显得尤为独特！

如果我们要了解染色体端粒的功能， 那首先我们就必须得先说到生物里最重要的遗传物质——脱氧核糖核酸，也就是我们生活中常常能听说的所谓的 DNA。于 1953 年剑桥大学的卡文迪许实验室里，弗朗西斯・克里克与詹姆・沃森共同发现了 DNA 的双螺旋结构。他们的这个发现令人类对生物遗传物质的认识产生了质的飞跃， 而且因此这两人也共同收获了 1962 年的诺贝尔生理及医学奖。 1970 年代，随着分子生物学家们对染色体的进一步研究，他们惊讶地发现在细胞分裂过程中，染色体 DNA 末端总有一段基因片段是无法被完整复制的。我们可以预想到， 随着细胞分裂次数的增加，这种机制会导致染色体不断地丢失末端的片段，直至遗传信息大量缺失而基因无法表达支持细胞正常生理功能的蛋白质，从而使细胞慢慢走向细胞衰亡。除了细胞分裂，细胞氧化代谢产生的氧化应激也会损伤染色体结构。为了应对以上这些情况，生物进化出了端粒。

端粒的位置在染色体的最末端，它的主要功能是保护染色体终端的完整性。然而，端粒的长度也是有限的，在一次又一次的细胞分裂过程中会逐渐缩短，直到再也无法起到保护的作用，于是细胞走向衰亡。我们不妨换个角度思考，如果染色体端粒无限长那我们是不是就可以长生不老了呢？答案是否定的。这主要是因为染色体端粒拥有另外一个重要的特性，能防止细胞无限制分裂。这也被认为是防御细胞癌变的重要机制之一。

搞清楚了染色体端粒是什么之后，现在来聊聊我的博士论文吧！我的论文主要分为两大部分：第一部分主要讨论了生病时（免疫系统激活）端粒长度可能发生的变化。免疫学的研究目前已经认识到，当机体处在免疫应答，免疫细胞如 B 细胞会在短期内大量增殖。 由于细胞分裂也需要消耗能量，因此机体的新陈代谢加速，细胞呼吸所产生的副产物氧自由基也相应增多，于是我们能预测到端粒的长度会因此浮动。在我的论文里，我以斑胸草雀作为实验对象，在为期一年的实验期间，它们多次接受脂多糖和血蓝蛋白皮下注射来

激活两种不同程度的的免疫反应，而我观测了它们血液红细胞中端粒在这一年中的动态变化。实验结果显示，实验组与对照组的端粒长度在这一年的时间里并无明显差异，然而在第二年实验结束时，端粒长度竟有了显著的增长！更出乎意料是，初始端粒长度较短的斑胸草雀个体在实验的最后一个月里端粒长度却是增加最多的。对于这个意想不到的结果，我们推测最可能的原因是生存环境的改变。三月份的斯科纳省户外气温在 0-10 摄氏度之间，在这个实验期间，鸟儿们从户外鸟舍被转移到了室内，打个比方，就相当于从容纳 60 只鸟的集体宿舍搬进了室内坏境恒温的小公寓（两只鸟）。 在这样温暖的环境和充足的食物条件下，鸟儿得以有更多的能量来支持机体的生理功能，通俗来说，就是吃好喝好身体倍儿棒！

相比于论文的第一部分，第二部分侧重在端粒的遗传模式。据大量文献显示，端粒具有很高的遗传性，因此我们非常好奇如果父母的端粒长，这是否会遗传给后代使得他们也有长的端粒？对于这个问题，我们花了四年的时间来准备和实验，挑出端粒最长和最短的斑胸草雀个体进行定向繁育。实验结果如下：1）如我们所预测，鸟宝宝的端粒长度是会被鸟爸妈的端粒长度影响的；2）但是这个差异在胚胎发育的中期并没有显现，而出现在了鸟宝宝孵化 10 天后； 3）我们利用稍微复杂的动物模型试图区分可能决定鸟宝宝端粒长度的因素，结果显示 30%由基因决定，15%左右取决于鸟爸妈的个“鸟”因素，剩下的就是养育方式，比如喂食的频率，或者同窝鸟宝宝们的相互竞争也可能会导致个体间端粒长度的差异。这些实验结果告诉我们遗传因素和后天因素都会影响后代的端粒，尽管程度不同，但都很重要。

综上，我的论文到底讲述了一个什么故事呢？我以斑胸草雀作为研究对象，通过长期的纵向研究，分析了端粒在鸟生中的动态变化，探索了疾病和生活环境的改变是如何影响和改变鸟儿的生理状态；同时我也试图理解染色体端粒是如何遗传的。

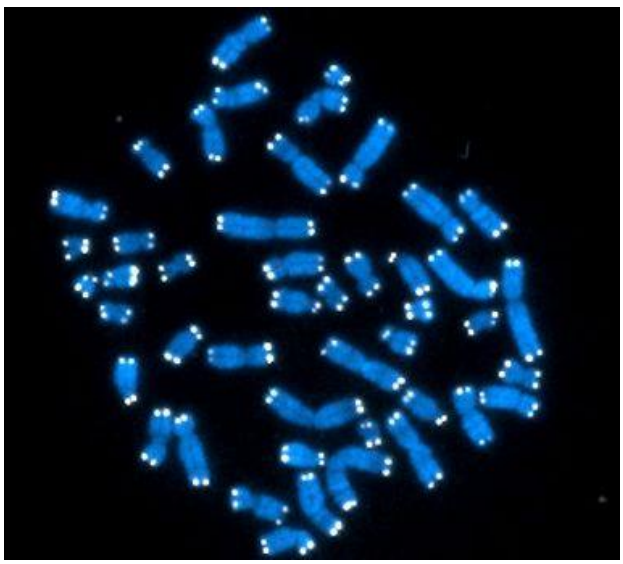
我花了四年的时间在这个领域溅起一个小小的水花，水波涟漪，一下就融进了大海里，希望这个小小的水花有贡献进科学的海洋里，至此

Background

Telomeres

What are telomeres?

Telomeres are distinctive sequences located at the end of linear chromosomes that help preserve genome stability, inhibit unnecessary recombination and prevent chromosome fusions (Blackburn 2000, Chan & Blackburn 2004). The shelterin complex (a protein structure that surrounds chromosome ends) and the enzyme telomerase, protect and maintain telomeres. The telomeric sequence is repeats of a short core sequence $(TTAGGG)_n$, that is highly conserved in all vertebrates which indicates that telomeres are an efficient system for protecting chromosome ends (Meyne et al. 1989).



Picture 1. A depictation of all 46 human chromosomes (blue), with telomeres (white) visualised with fluorescent markers. Copyright: Hesed M. Padilla-Nash and Thomas Ried, NCI (NIH in press).

Telomere attrition and restoration

What causes telomere loss?

In all somatic cells, the loss of telomeric sequences occurs progressively at each cell division due to the end replication problem (Levy et al. 1992). During cell division, chromosomes untwist and replicate but some pieces at the end of the chromosome will not be fully copied under the process of DNA replication, which is a result of inefficient initiation of Okazaki fragment synthesis (Chan & Blackburn 2004). Thus, at each cell division a chunk of telomeric repeats are lost, and because this inevitable loss of some distal bases are hitting telomeric repeats the core coding DNA region is protected from this grinding process (Verdun & Karlseder 2007). Consequently, telomeres shorten due to rapid cell division (e.g., during fast developmental growth) and with chronological age of the organism.

In addition, other commonly cited reasons for telomere shortening is oxidative stress, often mentioned in studies of ageing. The imbalance between reactive oxygen species (ROS) and antioxidants in the cell environment may damage DNA, and the guanine-rich telomeric sequence are particularly vulnerable to this oxidative damage. Oxidative stress can be elevated by, for example chronic inflammation, disease, adverse environment, physiological and psychological stress (Epel et al. 2004, López-Otín et al. 2013, Asghar et al. 2015a, Casagrande & Hau 2019, Shay & Wright 2019, Louzon et al. 2019).

Nevertheless, telomeres ensure that the coding-DNA regions remain intact during cell divisions and are less vulnerable to DNA damage and, therefore, telomeres help to secure regular cell function. However, the protective action of telomeres is limited by telomere length: when telomeres shorten beyond a critical point, there are too few telomere repeats left to protect the coding regions of the chromosome. Thus, telomeres rather offer a relaxation than a cure for cell death/aging/cell dysfunction.

Telomere shortening with age

Telomere shortening in somatic cells translates into a finite number of divisions and cells therefore have a limited capacity to proliferate, unless it is a cancerous cell (Kim et al. 1994). Telomere shortening has thus became a hot topic for research focusing on ageing and senescence, either as a causative agent of ageing or as a marker of biological age that might be linked to e.g., physiological or pathological state. A meta-analysis showed that telomere length in adulthood, on average declines with age, but the correlation is weak and varies across vertebrate classes (Remot et al. 2022). This suggests that the decline in telomere length with age can be complex in vertebrates. In another study, it was found that the absolute telomere length (measured by telomere restriction fragments, TRF, analysis) decreased with increasing age in the zebra finch, the tree swallow, the Adélie penguin and the common tern, but elongated with increasing age in the Leach's storm petrel (Vleck

et al. 2003). Longitudinal studies in human infants and feline cells found telomere shortening with age (Zeichner et al. 1999, Brümmendorf et al. 2002). However, a study on long–lived seabirds, the European shag and the wandering albatross found that telomere declined between the chick and the adult stage, but telomere length and age were no longer correlated after adulthood (Hall et al. 2004). Clearly, patterns of telomere length and age vary among species (Gomes et al. 2010). Some studies also suggested that the rate of telomere shortening may play an important role in the aging process, and act as predictor of the mean lifespan of a species (Whittemore et al. 2019). For example, a paper by Haussmann et al. (2003) showed that long-lived bird species have a relatively slower telomere shortening rate compared to short-lived bird species. In this paper, they also found that telomere rate of change (TROC) varies with maximum lifespan in birds, and they reported similar pattern for mammals, e.g., house mice, dog, sheep and southern pig-tailed macaque (Haussmann et al. 2003). TROC emphasize that telomeres are not always shortening over time, but they may also show net elongation between time points. Telomere elongation could be involved in determining maximum lifespan in long-lived species, and this may elucidate that perhaps the mean speed in which a species loses telomeric repeats is the main determinant for when telomere reaches its critical point. TROC could in turn be a consequence of many processes, for example differences in telomerase activity, different start-up telomere lengths, different rates of telomere shortening, and different cell division rates (Monaghan & Ozanne 2018).

Oxidative damage on telomere length

Regardless of the cell division rate, another main factor responsible for telomere shortening is oxidative stress (Reichert & Stier 2017). As mentioned above, reactive oxygen species (ROS) can be produced as metabolites by multiple cellular processes in response to different stimuli, e.g., during mitochondrial oxidative metabolism or during an infection (Houben et al. 2008, Reichert & Stier 2017) or as a consequence of the innate immune response in which phagocytes produce excess ROS to directly damage the pathogen (Schantz et al. 1999, Ilmonen et al. 2008). Oxidative stress refers to the imbalance that arises when the level of ROS exceeds the capability of the cell to be protected by internal antioxidant responses or external antioxidants (Schantz et al. 1999). Telomeres are highly sensitive to oxidative stress due to their rich content of guanines (von Zglinicki 2002) and lack of antioxidants may therefore lead to a failure in the prevention of oxidative damage at telomere regions. The complex formed by telomeres and surrounding proteins becomes less stable, eventually leading to an accumulation of DNA damage and telomere loss (Reichert & Stier 2017). Oxidative stress is often increased after periods of high metabolic demands (Dowling & Simmons 2009, van de Crommenacker et al. 2012). Harsh environmental conditions (e.g., low food availability, low temperatures) or challenging biological processes (e.g., reproduction, immune responses) are also

expected to increase metabolism, whilst they also increase cell division rate and therefore lead to a higher production of reactive oxygen species. Accumulating evidence suggests that a stressful environment affects telomere length and/or telomere shortening rate, especially in early life (Epel et al. 2004, Monaghan 2014, Chatelain et al. 2020).

Interestingly, mild stressors, such as mild disease or moderate environmental stress have sometimes been assumed to have negligible effect on survival and fitness. However, growing evidence suggests that moderate stressors may have a delayed "hidden cost" that manifests itself only after a threshold is reached (Asghar et al. 2015b), that is also known as the 'accumulating costs' hypothesis (Hasselquist & Tobler 2021).

Telomere restoration and mitigation of telomere shortening

Telomeres shorten over time due to the processes mentioned above, thus organisms have adaptive ways to mitigate or reverse telomere attrition. The most direct way could be decreasing the cell turnover rate, hence somatic cells would go through less cell division per lifetime. Cellular turnover rate varies among cell types within an organism, but also between species (Sender & Milo 2021). Studies showed that long-lived individuals have potentially lower cell turnover rate. For instance, the lifespan of individual erythrocytes apparently extends along with species life span, hence reducing the mean number of necessary cell divisions per lifetime (Röhme 1981).

Telomere restoration enhances genome stability and tissue renewal and it involves the activation of the enzyme telomerase, i.e., the essential reverse transcriptase that is active during telomere synthesis (Blackburn 1991). Telomerase contains an RNA template and a protein catalytic unit to add additional telomeric repeats to the end of the chromosome (Greider & Blackburn 1989, Blackburn 2005). Telomerase levels are typically higher in cells with a high prolific potential, such as embryonic and adult stem cells, as well as cells in the germ line. Moreover, it is also present in stem cells in the bone marrow that have high proliferation rates necessary to make many new red and white blood cells. Telomerase is expressed during embryogenesis in humans and presumably in all mammals, but telomerase activity is normally suppressed after embryonic development is completed, and it is thought that this suppression of telomerase in somatic tissues contributes to cancer prevention (Young 2018, Shay & Wright 2019). Suppression of telomerase activity is believed to prevent tumorigenesis, dividing cells will reach the Hayflick limit thereafter the cell will commit apoptosis if telomerase is absent. However, in cancerous cells where the telomerase gene expression has been activated, cell division will no longer be limited by the Hayflick limit and such cells then in principle become immortal resulting in malign cancer (Wu et al. 2017, Shay & Wright 2019).

Recent research found that telomerase activity varies largely among cell types, differently life stages and in different taxa (Gomes et al. 2010). A study on asexual and sexual animals showed different patterns in the maintenance of somatic telomere length. Whereas sexual animals achieve telomere elongation only through sexual reproduction, asexual animals were able to maintain somatic telomere length by upregulated telomerase activity during regeneration (Tan et al. 2012). Risques and Promislow (2018) summarized studies of telomerase activity in animals in relation to body size. In species that were smaller than roughly 2kg, telomerase was expressed throughout life, while telomerase activity was commonly suppressed after embryotic development in animals with higher body mass (Risques & Promislow 2018). Telomere elongation has previously often been viewed as a measurement error, typically in qPCR-based studies (Verhulst et al. 2015). However, recent studies on some mammals and a songbird reported telomere elongation that suggested a more complex pattern where elongation occurred under more benign environmental and conducive to self-maintenance (van Lieshout et al. 2019, Viblanc et al. 2022, Tissier et al. 2022, Brown et al. 2022), whereas in another songbird elongation occurred in individuals with short telomeres and without malaria infection (Gómez-Blanco 2023). A study by Haussmann and Mauck (2008) first brought up the idea of the *[excess resources] elongation hypothesis* which assumes that TL maintenance is relatively expensive; only high-quality individuals would be able to afford to invest resources in telomere elongation process. This seems to be the case in Seychelle warbler females that experienced lower stress (i.e., high food availability, assisted by helpers, without malaria) and had the chance to lengthen their telomere (Brown et al. 2022). We recently also proposed the *last resort elongation hypothesis* in paper I, suggesting that individuals would accept the disadvantages (e.g., energetic costs, risk of cancer) to invest and elongate telomeres when its telomere length is getting too close to the critical point (further discussed in paper III) (Tobler et al. 2022).

The balance between telomere shortening and telomere maintenance is essential for the health of organisms. The understanding of the patterns and pace of telomere attrition and restoration is likely to be very important for our understanding of life history evolution. When DNA damage accumulates and exceeds the capacity of DNA repair mechanisms, cellular senescence or apoptosis occurs, which may contribute to the ageing process. Thus, it has been suggested that telomeres and/or telomere dynamics is one of the best proxies for studying the accumulation of damage in somatic cells (Monaghan 2010).

The immune system and telomeres

Organisms are normally exposed to a wide range of pathogens (both in the wild and in captivity). In vertebrates, when pathogens are detected by immune cells, the innate and the adaptive immune system will be activated. As a first line of defense,

the innate immune system induces a series of reactions in a short time, including the activation of a complement system, the identification of antigens, activation of immune cells and the promotion of the clearance of antibody complexes. Large amounts of white blood cells will be produced and released into the blood stream to remove foreign substances. If the pathogen is not cleared from the body, the adaptive immune system will also be activated, which will produce antibodies against specific antigens. Antigens are highly specific to particular pathogens and this recognition guarantees a long-lasting protection in the case of re-infection (Ooi et al. 2010, Vanguri 2014). Maintaining an effective immune system is very important to protect organisms from diseases. It is a key component for organism function, however, the magnitude of investment in the immune system may differ depending on life history strategy. This is because the maintenance of an efficient immune system and the deployment of immune responses are costly (McKean et al. 2008), thus investment in immune function must be traded off against investment in other essential physiological functions, such as maintenance of TL, for example.

Telomere shortening as a response to immune activation

One potential cost of immune system activation may be accelerated telomere shortening (Hasselquist & Tobler 2021). There is a direct link between immune system activation and telomere shortening through cell proliferation and oxidative stress. Immune cells have a high proliferation rate during infections, and the activation of (especially) the innate immune system involves the production of excess ROS either through increased inflammatory mechanisms or through elevated metabolism (Ilmonen et al. 2008). Telomere length could be reducing at a faster rate as a response to immune activation when immune cells rapidly expand in numbers to fight infections or during inflammatory disease (Derting & Compton 2003, McKean et al. 2008, Eisenberg 2011). During periods of energy shortage, organism need to trade-off energy between immune function and telomere maintenance (telomere repair mechanisms). It has been suggested that under an energy emergency state, telomeres are shortened rapidly, possibly because immediate survival is prioritized over long-term somatic maintenance such as telomere protection and restoration (Casagrande & Hau 2019).

Telomere inheritance

Even though telomere length changes over life, a growing number of studies demonstrate that early-life telomere length (eTL) can predict telomere length at later stages of life (including total lifespan; (Heidinger et al. 2012, Eastwood et al. 2019, van Lieshout et al. 2019) as well as life history strategy (Herborn et al. 2014, Marasco et al. 2022). It is therefore of particular interest to understand how telomere length is inherited across generations, and how the parents may affect offspring physiology and behavior (survival) not only through genetic factors but also through

non-genetic effects (e.g., parental effects), and how environmental effects (e.g., epigenetic effects) are contributing. Taking into account both genetic and non-genetic effects will improve our understanding of the proximate mechanisms underlying the determination of telomere length (with potential implications for life history strategies).

Several studies have discussed the potential mechanisms for telomere inheritance. Early studies suggested X-linked inheritance of telomere length in humans (Nawrot et al. 2004), and such an association was confirmed in studies on a Dutch population and in a meta-analysis of humans in general (Broer et al. 2013, Nersisyan et al. 2019). However, studies on birds have provided different results. Studies in kakapos and great reed warblers have shown, that besides genetic effects, there was also rather strong maternal effects acting on telomere length (Horn et al. 2011, Asghar et al. 2015a) Maternal inheritance was also found in a study in king penguin, although they also noticed that the correlation become not significant as chicks grew older (Reichert et al. 2015). On the other hand, recent studies have reported stronger paternal inheritance of telomere length in humans and sand lizards (Nordfjäll et al. 2005, Njajou et al. 2007, Olsson et al. 2011). The suggested mechanisms behind such paternal/maternal telomere inheritance could be an independent or joint result of parent-specific imprinting, hormonal regulation and sex-chromosome linkage (Nordfjäll et al. 2005, Horn et al. 2011, Olsson et al. 2011).

The estimated telomere heritability reported in humans varies between 34% and 82% (Broer et al. 2013, Kim et al. 2020). Kim et.al (2020) reported a leukocyte telomere length heritability of 64%, estimated by measuring TL through three generations in families, including newborns, parents, and grandparents. However, it was problematic that telomere length was measured at a different time point in life for each individual. As compared with newborns, the telomere lengths of parents or grandparents are measured at very different life stage, and therefore environmental effects may have substantially affected their TL. This is a common drawback in studies of humans where environmental effects and non-genetic effects are not easily quantified. Studies on heritability in species other than humans provide a wide range of patterns, e.g., telomere length being inherited from father to daughter, or from mother to son (Dugdale & Richardson 2018). Atema et al. (2015) estimated a very high heritability (99%) in zebra finches. In contrast, a study on long-lived myotis bats only found a heritability of 1.1%–6.0% (Foley et al. 2020). In studies on animals where it is possible to separate genetic and non-genetic (e.g., parental) effects with the help of animal models, lower heritability estimates for the genetic components have been found 3.8 ± 6.9% in white-throated dippers, 18% in collared flycatcher, 35-48% in great reed warbler and 48% in tawny owls (Voillemot et al. 2012, Asghar et al. 2015a, Becker et al. 2015, Morosinotto et al. 2022). This variation in TL heritability estimates among studies is somewhat puzzling. It could be the result of biologically interesting variation in either the genetic background of the population or the amount of environmental variation experienced. This might be

able to explain the differences of mechanisms of inheritance on telomeres, either between wild versus captive populations, between humans and non-human animals, and between wild populations inhabiting environments with varying levels of temporal and spatial heterogeneity. However, study designs, methodological variation and biological or cultural differences across populations are likely to contribute also to this large variation. For example, the life stage in which telomere length is measured, or the use of different measurement method (TRF or qPCR-based), and statistical methods (parent-offspring regression or animal models) all could affect the results (Nussey et al. 2014, Eisenberg 2014).

Physiology and telomeres

Maintaining a good health status is a fundamental task for multicellular organisms. However, it is quite difficult to define 'being healthy', especially when it comes to animals. McGlone tried to describe it as a state of normal physiology and to live free from disease (McGlone 1993). Prolonging a good health status enables organisms to monopolize resources, pursue costly reproductive strategies, optimize reproduction and, ultimately, maximize Darwinian fitness. Though, physiological performance degrades with age (Boss & Seegmiller 1981, van Beek et al. 2016). Telomeres have been suggested to be an indicator of individual quality (i.e. longer telomeres signify better quality). If true, one might expect that individuals with longer TL would excel individuals with shorter TL in other measures of physiological health (especially given that TL has been shown to be associated with age-related degenerative diseases (Boss & Seegmiller 1981, van Beek et al. 2016).

Animal physiology involves intricate systems that do not work in isolation but form a coordinated physiological regulatory network (Cohen et al. 2012). One of the well-studied parameters that underly physiological performance is glucose (GLU). Glucose is the direct energy source for carbohydrate metabolism to generate energy for all kinds of fundamental cellular function. Blood glucose concentration is upregulated when exposed to energy demanding processes, such as immune activation (Braun & Sweazea 2008, Von Ah Morano et al. 2020). In addition, increased glucose levels in the blood may link to an increase in oxidative stress which could result in DNA (including telomere) and cell damage. However, this damage is mitigated through endogenous antioxidant mechanisms such as the production of uric acid (Cohen et al. 2007). Uric acid (UA) is the most abundant circulating antioxidant, and is derived from amino acid catabolism (Cohen et al. 2007). The oxidative tissue damage to skeletal and cardiac muscles induced as a response to immune activation is reflected in an elevated concentration of Creatine kinase (CK) and aspartate aminotransferase (AST) (Abdelmajeed 2009). Physiological mechanisms are tightly integrated, such as the immune response and the oxidative stress system are thought to be tightly linked with costs (Pamplona &

Costantini 2011), and many other physiological systems modulate each other and hence affect each other's outcomes (Costantini et al. 2011).

A wide range of assay are used to measure physiological parameters related to, e.g., metabolism and nutritional status; e.g., hormones (Davies et al. 2013, Huber et al. 2017, Fletcher et al. 2018), oxidative stress (Cohen et al. 2012, Costantini et al. 2016, Jensen et al. 2023), immune function (Hasselquist et al. 2001, Demas et al. 2011, Hegemann et al. 2017), and also telomere length (Chaney et al. 2003, Ilmonen et al. 2008, Hohensinner et al. 2011). Combining different measures of physiological status (e.g., TL and different blood analytes) can give a more comprehensive picture of the overall quality of an individual. Studies of physiological variation in response to biotic and abiotic stressors can help us to understand and predict how animals cope with stressful conditions (Enders et al. 2015, Lettoof et al. 2021), and improve our understanding of how different life-history strategies can evolve (Hasselquist et al. 2007, Evans & Gustafsson 2017, Hegemann et al. 2019, Andreasson et al. 2020, McWilliams et al. 2021).

Telomeres in an evolutionary ecological perspective

Telomere length and telomere dynamics is determined by both genetic and environmental effects. The variation in telomere length at adulthood within the population may result from one or a combination of environmental stressors, such as infection (Asghar et al. 2015b, Karell et al. 2017), reproduction (Sudyka et al. 2019, Pepke et al. 2022) and environmental conditions (Giraudeau et al. 2019, Chatelain et al. 2020). Recent studies have shown that the conditions individual experienced early in life can have profound effects on later life physiology and performance (Monaghan 2008). In vertebrates, the fertilized egg cell grows into an individual with complete functional organs during embryonic development, normally within a very limited time. During this period of development, from pre-natal to early post-natal, the offspring goes through a huge amount of cell proliferation and cellular differentiation. Hence, this period of high growth rate is usually also linked to a higher rate of telomere attrition (Haussmann & Marchetto 2010, Boonekamp et al. 2014, Monaghan & Ozanne 2018). As mentioned above, this is the suggested reason for higher telomerase activity during embryonic development, in order to compensate for the higher telomere attrition rate partially caused by the high cell division rate.

Earlier studies in humans showed that early-life stress, e.g., physiological or psychological stress, could affect long-term performance (e.g., metabolic disorders) into adulthood (Barker 1990, Marasco et al. 2022). The early-life stress in this context could mean a variety of physiological and behavioral responses, including, but not limited to, nutritional restrictions, limited parental resources, social

competition, predation pressures, pollutants, or hormones (Romero et al. 2015, Sapolsky 2015). Studies on song-birds have found shorter telomere length of nestlings reared in urban vs. rural environments (Salmón et al. 2016) or reared at higher ambient temperatures during the nestling period (Casagrande et al. 2020, Stier et al. 2020, Eastwood et al. 2022).

Parental traits, such as age, has been found to have an impact on offspring longevity (Carslake et al. 2019), which is also known as the Lansing effect (reviewed in Monaghan et al. 2020). Moreover, accumulating evidence suggest that offspring telomere length can be affected by parental age (Heidinger & Young 2020). One such mechanism is the quality decline of gametes with parental age, as some studies have found that gametes from older parents are more likely to contain DNA mutations and shorter telomeres (Monaghan & Metcalfe 2019). In addition to the genetic effect, there are non-genetic parental (environmental) effects that may change with age. For example, parental care may be reduced with age (e.g., lower feeding frequency or ability to defend the nest) and older parents may not be capable of occupying a high quality territory (Monaghan & Metcalfe 2019). However, there are also examples where older individuals seem to be of higher quality, such as older breeders being better at obtain resources for their offspring or older mothers producing offspring with longer telomeres (Asghar et al. 2015a, Dupont et al. 2018). To understand how offspring telomere length and telomer shortening may be affected by parental age and condition will help us to further reveal and understand patterns of telomere inheritance from an ecological and evolutionary perspective.



Figure 2. Watercolor masterpiece by Violeta Caballero López

Thesis aims

The aim of this thesis is to gain a better understanding of how telomere dynamics vary across life stages and how different factors such as inheritance, immune function and environmental conditions affect telomere length and shortening. The findings in this thesis add to our knowledge about the physiological mechanisms that shape-individual life history strategies and modulate fitness.

The thesis includes both theoretical and empirical work. Paper I describe a conceptual framework of the current main hypotheses in telomere ecology and evolution, highlighting controversies and gaps in knowledge. In paper II-V, a combination of experimental and laboratory methods are used to study inheritance of telomere length as well as physiology and telomere dynamics in captive zebra finches (*Taeniopygia guttata*) (Paper II-V) and nestling jackdaws (*Corvus monedula*) (paper II).

The main research questions are:

What are the current main research questions, controversies and conceptual gaps in telomere ecology and evolution?

In paper I, two conceptual frameworks are presented which group telomere-related hypotheses either based on their connection to research questions or in a hierarchical framework based on their assumptions of causality and proposed functional consequences of telomere length/shortening. The aim was to highlight the similarities and discrepancies between different 'telomere hypotheses' and, thus, to provide an overview of the controversies and theory 'gaps' in the field. The paper is also intended to bridge ideas that stem from different research disciplines and thus help researchers, both those familiar with and those new to the subject, to identify new avenues for research.

What tools can (field) ecologists use to obtain a comprehensive health measurement of birds?

Paper II is an evaluation of the usefulness of the VetScan blood analyser for the type of studies conducted in this thesis. The suitability of the blood analyser was tested in field or semi-field settings. The study describes potential constraints, e.g., in terms of blood volume (>80ul), how quickly analyses need to be conducted, as well as advantages in usage.

Do repeated episodes of immune system activation result in accumulated telomere shortening and what is the role of the ambient environment?

Paper III aims to elucidate how repeated immune challenges may induce physiological changes and affect telomere dynamics. TL was measured with the qPCR method and a portable VetScan VS2 analyser was used to measure physiologically important blood analytes. The major target was to experimentally test if accelerated telomere degradation may be a putative mechanism through which small immune costs can accumulate and be translated into senescence effects. However, it also revealed some unexpected and exciting results regarding the effects of the ambient environment on TL dynamics.

Are telomere length and telomere trajectories set for life already at conception?

Paper IV tests the idea that telomere length and telomere trajectories are determined already before birth (or even at conception), by studying trans-generational effects and parental age effects in relation to offspring sex, using an experimental set-up with assortative mating of parents based on their own early-life telomere length.

What are the genetic and non-genetic contributions to telomere length and are estimates of heritability consistent when they are based on the same or different life stages in parents and offspring?

In paper V, data on parental and offspring telomere length were used to estimate the heritability of telomere length when compared at different life stages in parents and their offspring. We used both animal model and parent-offspring regression analyses to disentangle genetic effects, non-genetic parental (maternal and paternal) effects and purely environmental effects.

Methods

Study species

The zebra finch (*Taenopygia guttata*) is native to Indonesia and Australia, it is the most common and widespread grass finch across the Australian continent. Zebra finches live in social flocks, their body size is ~10-12cm long and 9-1. In their natural habitat, zebra finches may live up to 4-5 years but individuals in captive populations can reach to 5-9 years. Typically, individuals reach sexual maturity at an age of three months and they breed all year round during the raining season. Zebra finches have a clutch size of 3-6 eggs and incubation normally takes 11-13 days. Both parents participate in feeding and taking care of the nestlings. Nestlings fledge around 18-22 days after hatching, and parents provision fledglings until they are independent (~circa 45 days). Zebra finches are easily kept and bred in captivity making them excellent study subjects for longitudinal studies.

Zebra finches have been used as a captive model organism in a wide range of biological studies, ranging from behavior such as song learning (Slater et al. 1988, Clayton et al. 2009) to physiology and genomics (Westneat 1997, Wada et al. 2006, Warren et al. 2010, Balthazart et al. 2017) and aging/telomere studies (Haussmann & Vleck 2002, Atema et al. 2015, Salmón et al. 2021). The population used for my work originate from a large population maintained at the Max Plank Institute, Seewiesen, Germany.

My study population is kept in outdoor aviaries at Stensoffa Ecological Station, Lund, Skåne Sweden (N 55°69′, E 13°44′). Zebra finches were taken indoors during the immune challenge experiments in papers II and III, and selective breeding experiments in papers IV and V. During these experiments, they were exposed to natural timing of day/night regime, a mixed seed diet (Finkfrö, Franks Zoofor AB), drinking water and sepia shell were provided *ad libitum* at all times.



Picture 2. Zebra finches in outdoor aviary in Stensoffa Ecological Station (Photo by Elsie Ye Xiong)



Picture 3. Breeding experiment at indoor aviary and a pair of zebra finches in a breeding cage at Stensoffa Ecological Station (Photo by Elsie Ye Xiong)

Paper II involved western jackdaws (*Corvus monedula*) from a wild nest box colony at Revingehed (N 55°43′, E 13°26′) in southern Sweden (Aastrup & Hegemann 2021). Jackdaws are mostly resident (i.e., non-migratory), their clutch size 3-5 eggs, and our study only involved nestlings (26 to 29 day-old). Their larger body size that allowed for repeated blood sampling in a relatively short time period.

Immune challenge experiment

The immune response is particularly of interest to us given that the maintenance of an efficient immune system is costly (see introduction). We applied two types of immune stimulants to zebra finches in chapter III. Lipopolysaccharides (LPS) is an

endotoxin that is found on the cell wall of gram-negative bacteria (Johnson et al. 1993) and Keyhole limpet hemocyanin (KLH), is a large metalloprotein that is found in the hemolymph of the giant keyhole limpet- Both two compounds are commonly used to trigger innate and adaptive immune responses respectively (Hasselquist & Nilsson 2012, Merrill & Grindstaff 2015)

We were interested in how physiological parameters and telomere length are altered under different scenarios of immune responses. We repeatedly stimulated the immune system of zebra finch adults within a month per year (two times in total, 16th March to 16th April 2019 and 17th March to 16th April 2020; Figure 3.). LPS injection was administered via injection every 10 days from when the experimental month started (16th March 2019 and 17th March 2020) for three times (day0/day10/day20 and day365/day375/day385). KLH injection was administered twice at day0/day20 and day365/day385 within the experimental month. All birds were blood sampled at each timepoint before immune challenge. The control birds were only handled, and blood sampled, but not given an immune stimulant.

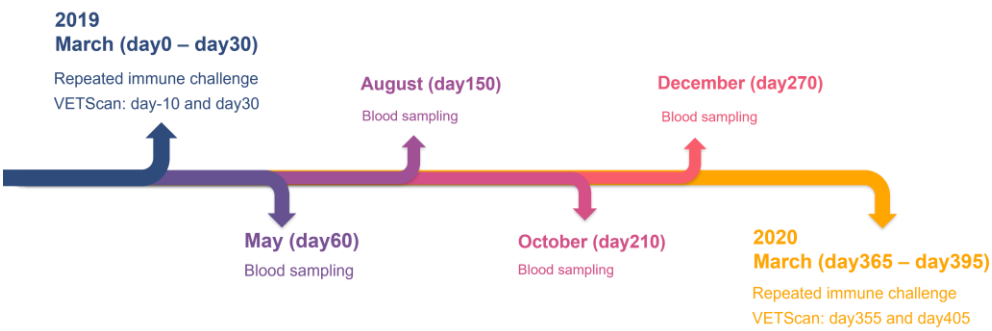


Figure 3. Experiment setup of the longitudinal immune challenge experiment.
The long-term experiment was preformed over the course of a year from 2019 March to 2020 April. Zebra finches were moved in from the outdoor to the indoor aviary on March 2019 and March 2020 for a month per year. During the months they were housed indoors, we used a VetScan VS2 blood analyser to measure physiological blood analytes on day-10 (10 days before immune challenge experiment start), day355 and day405. We also gave LPS (Lipopolysaccharides) and KLH (Keyhole limpet hemocyanin) to treatment groups (details please see above in text), and only handled the control group. Blood samples were collected at multiple time points through the year.

VetScan – physiological health

In papers II and III, we measured physiological parameters with VetScan VS2 machine. The VetScan VS2 machine is a small stationary blood analyzer that can be used to measure up to 12 different physiological parameters from one single

sample (80 µL) of avian or reptilian blood (blood parameters: Asparate Aminotransferase (AST), Creatine Kinase (CK), Uric Acid (UA), blood Glucose (GLU), Phosphorous (PHOS), Calcium (Ca^{++}), Total Protein (TP), Albumin (ALB), Globulin (GLOB), Potassium (K+), and Sodium (Na+)). These physiological measurements helped us to evaluate individuals' health status by examining blood parameters on electrolyte status, liver integrity and renal function. Therefore, it provided an overview of the animal's health while focusing on specific organ functions. We followed individuals (2-3 times) to collect this longitudinal dataset therefore allowing us to assess whether there are any signs of physiological senescence (paper II) or responses to immune challenge (paper III). In paper II, we examined if VetScan could capture individual consistency on physiological traits in the long-term but also account for how handling stress in the short-term can affect blood analytes. In paper III, we included physiological measurements were accompanied to understand the effect of a moderate stressor (immune challenge) on telomere dynamics.

Breeding experiment

Telomere inheritance has been well studied in humans and many other organisms, including zebra finches (Horn et al. 2011, Eisenberg 2014, Atema et al. 2015, Noguera et al. 2018). Although, how telomere heritability is potentially affected by genetic or non-genetic factors at depending on developmental stages, i.e. from conception to postnatal development stage is still unclear (Perez & Lehner 2019). To investigate the telomere inheritance pattern at different life stages, we conducted assortative breeding experiments where we selected zebra finch adults with known early-life telomere lengths? (eTL, measured by day10 blood sample) and age cohort. Experimental individuals were checked for relatedness before pairing to avoid inbreeding (we did not breed siblings or half siblings). We performed the assortative breeding experiment twice between July-December 2020 and between July-November 2021. Breeding zebra finch adults were selected based on their eTL group (long or short) and age (cohort/age group: young 6-9 month or old 27-32 month). The pairing method was different between year 2020 and year 2021. In year 2020 (paper V), we made four distinct breeding groups by producing pairs within the same eTL group and age groups (long × long and short × short for old and young cohorts respectively). In the year 2021, we also paired birds within the same eTL group but not between age groups (long eTL: young female × old male or young male × old female, same for short eTL groups).

The breeding processes were monitored from nest building until nestlings were independent from their parents. In addition, we collected the first clutch of eggs laid by the breeding pairs in 2020 and only the first egg laid in 2021, the collected eggs were moved and incubated in an incubator for 6 days with controlled temperature and humidity. Pairs were allowed to continue with breeding and produce a new

clutch from which they raised offspring. More specifically, in paper IV, we compared telomere length of 6-days-old embryos and 10-day old nestlings ((produced by the same parents) in relation to parental eTL group and parental age group based on the breeding round in year 2020. In paper V, we used animal models and parent-offspring regressions to estimate heritability between parents and offspring at different life stages in both parents (at nestling and at adult (when breeding) stages) and their offspring (at embryo and at nestling stages).

Telomere measurement

DNA extraction

Studies have shown that tissue types, sampling collection methods, and storage of DNA extractions, can lead to conflicting telomere measurements (Cunningham et al. 2013, Nussey et al. 2014). We used DNA extraction kits from Machery-Nagel to extract DNA from blood and embryo samples to avoid unnecessary inconsistency by sample type or method (Reichert et al. 2013, Asghar et al. 2016, Demanelis et al. 2020). However, the NucleoSpin™ Blood QuickPure kit was used to extract DNA from whole blood for paper III, and blood from parent samples in papers IV&V. Additionally, to extract DNA from embryo and nestling blood samples in papers IV&V, we used a NucleoSpin™ Tissue kit. Extractions were conducted according to the manufacturers' protocols.

Quantitative real-time PCR

Relative telomere length (RTL or TL) was measured by quantitative real-time PCR (qPCR) (Cawthon 2002), and is a relative measure of the telomere repeated copy number (t) in relation to control copy gene (s). Hence, the relative telomere length is measured as a ratio (t/s). We optimized our qPCR protocol based on Criscuolo's method (Criscuolo et al. 2009) which has previously been validated for studies involving birds. We designed new primers for a single copy gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GenBank ID: NM_001198610), telomere primers remained the same as in the paper. We updated the qPCR protocol by decreasing the amount of DNA required for each reaction to 5ng per reaction and reduced reaction cycles for both telomere and GAPDH. We also changed to Skirted Hard-Shell® 96-Well PCR Plates (Bio-Rad, model.id: HSP9601) to improved qPCRs performance in the CFX96™ Realtime System C1000 Touch® Thermal Cycler (Bio-Rad).

In papers III, IV and V, we measured the relative telomere length by using our established qPCR method (details see paper III). DNA samples that on each qPCR plate were ran at least in duplicates for paper III, and as triplicates for papers IV and

V. Further, we also ran every sample for at least two distinct rounds (same DNA sample but different dilutions) to confirm the results.



Figure 4. Illustration of zebra finch cuties playing with DNA tree.

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Result and Discussion

The combination of abiotic and biotic stressors shapes an organism's health status. Telomeres have been considered one of the best proxies for evaluating the effects of environmental stressors on health status at both the individual and population level, and in laboratory and wild study systems (e.g., Asghar et al. 2015b, Bateson 2016, Entringer et al. 2018, Angelier et al. 2019). Since telomeres are heritable, this allows researchers to investigate how transgenerational patterns shape fitness-related life history traits (Heidinger et al. 2012, Voillemot et al. 2012, Brown et al. 2022). Specifically, this thesis investigates the effects of environmental conditions (i.e., immune challenge and transfer to a benign ambient environment) on telomere dynamics and estimates heritability of TL based on different life stages in a set of experiments and using different methods.

Here, I present an overview of some of the most important results found in the five papers of my thesis, and also include a brief discussion of these results.

The current main research questions, controversies and conceptual gaps in telomere ecology and evolution (paper I)

For the past two decades, there has been a surge in the number of hypotheses involving telomeres in the field of ecology and evolution. These have mainly been related to associations between telomere length/shortening and life-history traits or fitness-facilitating processes, sometimes with similar or just slightly different angles on a certain research question. To get a comprehensive understanding of the existing hypotheses, how to test them and to provide a quick overview over the results from previously published studies, it is helpful to synthesize the hypotheses in a systematic way. In paper I, we review name-given 'telomere hypotheses' in the field of ecology and evolution and provide two conceptual frameworks that help understand their differences (or similarities). The aim of paper I is to frame and provide a clear overview that can help researchers from different fields to critically test the different hypotheses and to identify conceptual gaps.

The hypotheses were grouped in two ways; (i) based on their main research question, and (ii) in a hierarchical framework based on the assumptions of causality (i.e., if telomere length/shortening has a causal effect on organism performance) and if functional consequences of telomere length/shortening have been proposed. We

found that many of the existing hypotheses from different research areas have generated parallel, or sometimes even overlapping, ideas. However, several of the hypotheses are also competing, thus generating opposing predictions. When we grouped the hypotheses based on the research questions they address (Figure 5), we found that some hypotheses have been formulated in a broad way so that they end up in more than one cluster. We then grouped the hypotheses in a hierarchical way, based on assumptions of causality and the proposed functional consequences of telomere attrition on performance (Figure 6). This hierarchical framework helps to get a new angle on the similarities and differences among the many hypotheses. Hence, we believe that this paper would help other researchers to conduct more rigorous hypothesis testing and identify conceptual gaps.

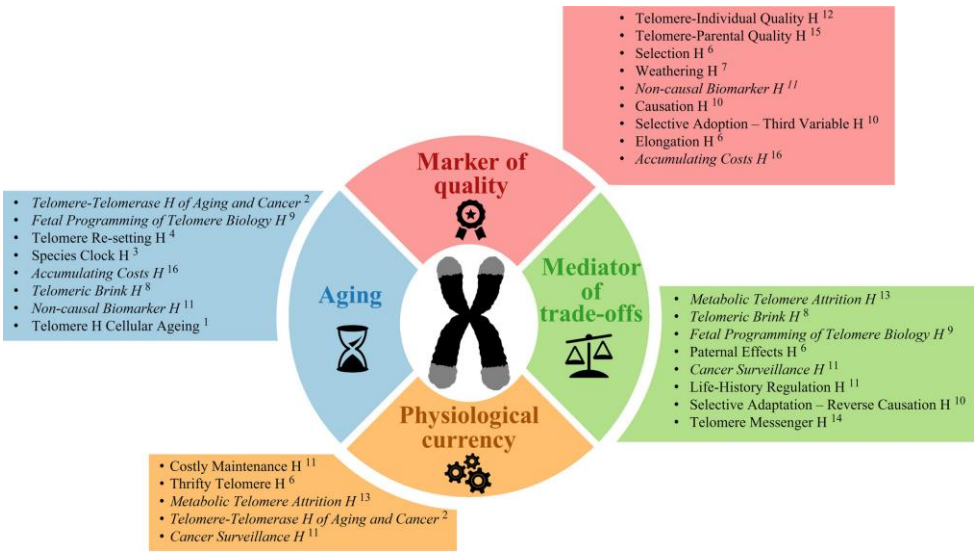


Figure 5. Grouping of the hypotheses based on research questions (Grouping I).
Hypotheses in italics are included in more than one research question because they have been described so broadly that they cover more than one of these contexts.

The paper I review relates directly to several of the other papers included in my thesis. For instance, I investigated hypotheses in the cluster "physiological currency" in grouping I as well as in the context of "cost maintenance models" in grouping II. It is well known that telomeres shorten with age and that cell function would collapse if telomere length reached a critical point. At the basis of many of the hypotheses is the question 'why is there such large between-individual variation in telomere length within populations?' If having long telomeres is beneficial for the organism, why is there no directional selection for longer telomeres? The current view is that long telomeres also entail costs. The *costly maintenance hypothesis*, the *thrifty telomere hypothesis* and the *metabolic telomere attrition hypothesis* assume

that telomere maintenance is energetically costly and that organisms therefore must trade-off investment in maintenance with investment in other energetically demanding functions or behaviours. Hence, organisms have to tolerate some telomere shortening because they have to trade-off limited resources with other fitness-improving functions. The studies described in paper IV and V test hypotheses in the cluster that is termed "static and dynamic signal models" that suggest telomeres are pre-determined by parental effects and the early-life environment (pre-natal and early postnatal). Hypotheses highly relevant for my thesis in this cluster are the *fetal programming of telomere biology hypothesis* that assumes a maternal effect modulate the initial setting of telomere length and telomerase expression with long-term influence on the offspring's life-history or behavioural strategies, and the *telomere messenger hypothesis* that proposes that environmental cues are transferred to offspring via TL.

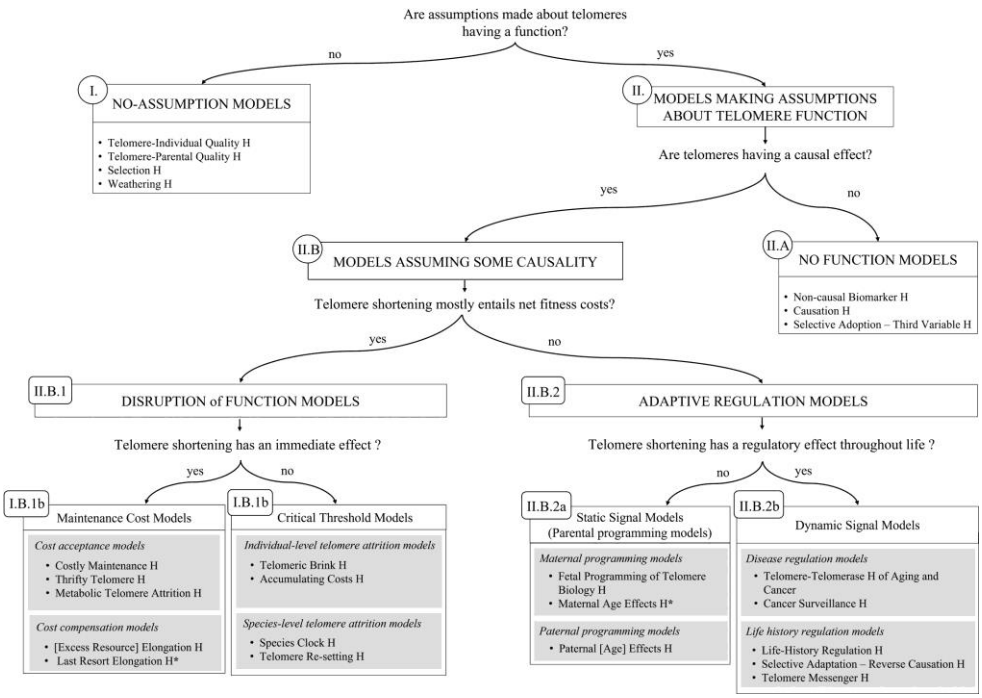


Figure 6. Hierarchical classification of the telomere hypotheses (Grouping II).
Specific assumptions for each hypothesis and the rationale of clustering them in a certain group are discussed in more detail in paper I. With causality or causal effect, we mean that telomere length or shortening *per se* can affect performance and function of the organism. * denotes new hypotheses that were added by us in paper I.

A tool for (field) ecologists to obtain a comprehensive health measurement of birds (paper II)

The assessment of VetScan VS2 in a study with semi-field and field settings

Physiological mechanisms work as a complex network, meaning that many physiological systems cooperate and they modulate each other's outcomes within different pathways (Costantini et al. 2011). Our aim was to use analyses of physiological health in conjunction with telomere dynamics in immune-challenged individuals. However, the measurement of physiological parameters can be difficult, for example because the sample volume is small and only few parameters can be assayed, or because there are financial and logistical limitations when working with wild animals. The VetScan blood analyser may be a valuable tool to overcome some of these limitations. We therefore conducted a methodological study to validate the usefulness of this instrument for ecological and evolutionary studies, such as the ones described in paper II and III of this thesis. We tested the repeatability of the measurements and the sensitivity to stress under different conditions and across different timepoints in jackdaw nestlings and zebra finch adults (paper II).

Other physiological studies have shown that storage condition and time can affect the measurement values for different blood analytes (Männistö et al. 2007). In paper II, we checked consistency of blood parameter values in relation both to short- and long-term storage time. We found no effect of short-term storage (2 hours) on the concentrations of seven out of 10 blood parameters, and four blood analytes remained stable even after long-term storage (12 hours). Our results generally agree with studies done on other blood analysers (Christopher & O'Neill 2000, Turchiano et al. 2013, Hoppes et al. 2015, Lee et al. 2016).

Animals can show rapid reactions to stress (Davies et al. 2013, Deviche et al. 2016, Li et al. 2019). To investigate how sensitive VetScan analyte measures were to effects of capture and handling stress, we sampled blood from jackdaw nestlings at multiple times (<3min, 16 min and 30 min after handling began). Eight out of 10 parameters were significantly affected by handling stress already after 16 min (Figure 7). These results show that the Vetscan VS2 blood analyser can capture rapid changes in blood chemistry, and thus can be used to study stress effects on multiple analytes simultaneously. However, our study also underlines that blood samples should be collected as quickly as possible after capture (e.g., within 3 min) if wanting to avoid confounding effects of handling stress.

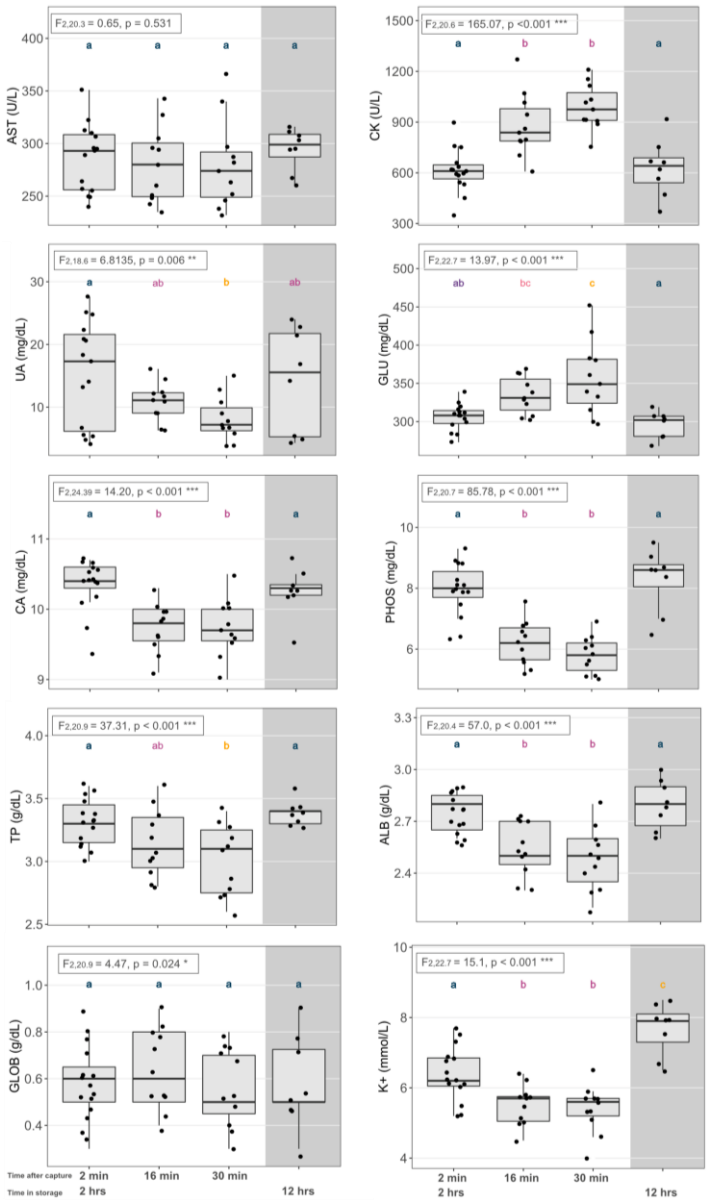


Figure 7. VetScan measurement results from handling stress and the 12-h storage experiment conducted on jackdaw *Corvus monedula* nestlings.
The x-axis labels denote the time from capture to when the blood sample was taken (2min (baseline), 16min and 30min (stress samples)) and the maximum storage time for jackdaw samples (12h, shaded area). See methods for detailed handling and storage protocols. The y-axis denotes the range of the values for the different blood analytes. The box in the upper left corner of each panel reports the statistics of the repeated measures analyses for the 2-min, 16-min and 30-min sample. Different letters above the error bars reflect significant differences ($p<0.05$) and are based on Tukey post-hoc tests for the repeated measures. Statistical differences between the measurement values analyzed after 2h and after 12h, respectively, were based on Wilcoxon signed rank tests.

Effects of repeated episodes of immune system activation on accumulated telomere shortening and the role of the ambient environment (paper III)

In paper III, I and my colleagues investigated how telomere dynamics and other physiological health measures were affected by repeated immune challenges and environmental conditions. Controls and immune challenge groups showed significant differences in two blood analytes, GLU and CK. Changes in CK are difficult to interpret since most of the treatment effect is due to changes in the control individuals between day 60 and day 365. However, in the case of glucose, the two immune challenged groups showed higher levels than controls, suggesting that blood glucose levels were upregulated to facilitate energy demanding inflammatory immune reactions.

We found no significant difference between treatment groups in terms of telomere length (TL) change over the entire experimental period. However, we found an unexpected pattern of a mean increase in telomere length (i.e., telomere elongation) independent of treatment when birds were moved from harsh outdoor to benign indoor conditions over the second immunization period (day365 to day395). Moreover, the magnitude of telomere elongation (and shortening) depended critically on the initial TL value prior to the immune challenge treatments (Figure 8). Individuals with initially short telomeres showed the largest elongation (a mean increase of almost 70 % and a maximum of > 150%) over just one month, while individuals with longer telomeres tended to retain or even shorten their telomere length (Figure 9). To the best of our knowledge, such a large and fast telomere elongation effect has not been observed before in any vertebrate. These results suggest that ambient environmental factors contributed unexpectedly much to telomere dynamics in this case. Indeed, other studies have shown that zebra finches increased aggressive behaviors when living at high social density, leading to a stronger activation of reactive oxygen metabolites, and this, in turn, means that less energy can be used for body maintenance (Poot et al. 2012, Quque et al. 2022). The level of inter-individual competition (about 60-ish same-sexed in the outdoor aviary vs only 2 birds in an experimental cage) was likely strongly reduced during the immune challenge period. Additionally, the benign and mild indoor environment with constant room temperature likely improved living conditions (and reduced their costs). These changes in living environment singular or combined probably masked the cost of immune stimulation.

The results from paper III showing a rapid elongation in telomere length are notable also because elongation in telomere length is still debated in the field (previously often viewed as a methodological artefact), and is at odds with the hypotheses listed under “Aging” in the grouping based on research questions in paper I. Moreover, it is worth noting that the *individual quality hypothesis*, the *excess resources elongation hypothesis* and the *costly maintenance hypothesis* all assume that

telomere maintenance is costly and only high-quality individuals can afford to have long telomeres (i.e., are able to invest heavily in TL maintenance). The results in paper III appear to partly support this. Zebra finches elongated their telomeres after they were moved indoors which suggests that the telomere maintenance costs were alleviated when the birds were moved inside. However, it was mostly the individuals with the shortest telomere length (at the experiment start) that elongated TL over 30 days during the second immunisation period. These results support the *critical threshold models* (see paper I) and the *last resort telomere elongation hypothesis* where birds are expected to invest in TL maintenance (including elongation) when coming close to a lower critical threshold in TL. The results also to some extent support the *excess resources elongation hypothesis* as it was when moved into the benign indoor conditions that substantial telomere elongation was induced, although note that this pattern was particularly evident in individuals with short TL which according to this hypothesis would be considered low-quality individuals. Still, maybe the indoor environment was so benign that even low-quality short TL individuals could afford a very high investment in telomere maintenance that it even resulted in telomere elongation. Furthermore, drawing conclusions about the quality of an individual based on a few adult TL measurements may potentially be misleading (see Figure 8). However, it is not clear whether such a rapid change in ambient environment like the one that was created in the experiment would occur in nature.

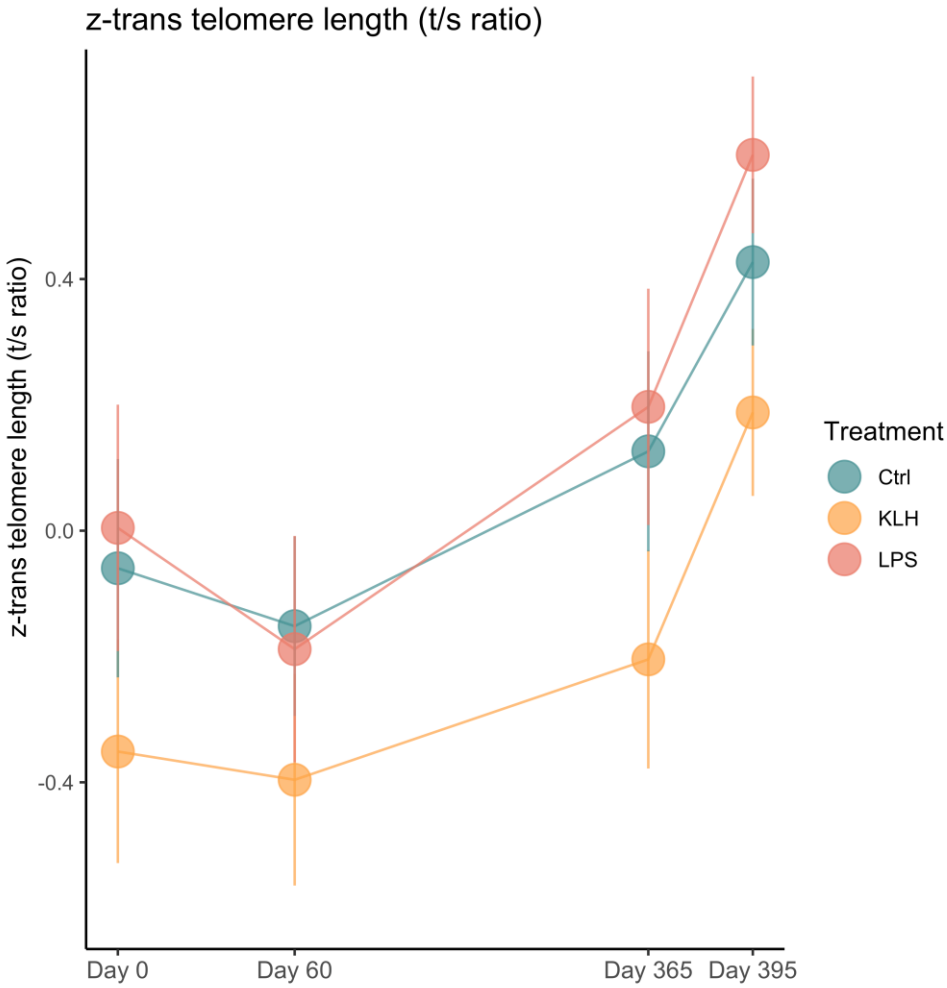


Figure 8. Telomere dynamics throughout experiment from day0 to day395.
Relative telomer length (z-trans log-t/s) on y-axis and four timepoints represent at x-axis. Despite of KLH group had a relative lower t/s from the start (by chance), all immunization treatment groups have the same pattern of an telomere elongation, especially from day365 to day395.

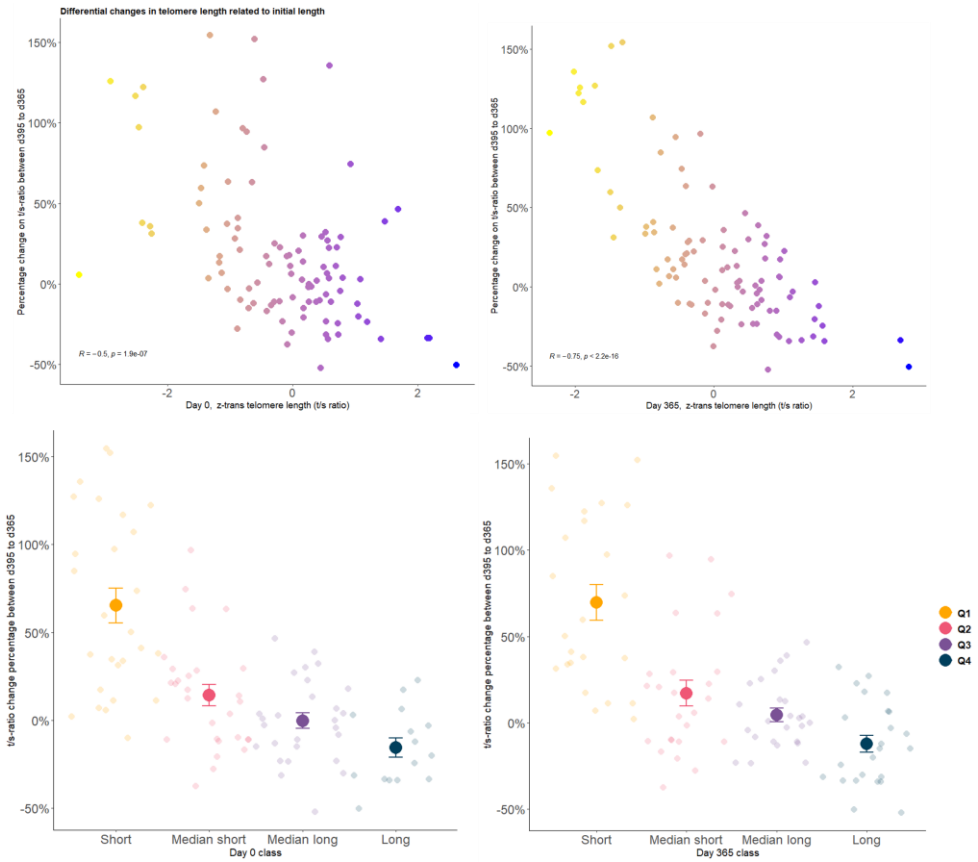


Figure 9. The percentage change in TL (z-tans log t/s-ratio) at population level.
Percentual TL change (t/s ratio) between day365 and day395 plotted against TL at day0 (top left) and against TL at day365 (top right). The same variables plotted against each other, but separately per TL group (day0: bottom left; day365: bottom right).

Inheritance of telomere length (paper IV and V)

Increasing evidence shows that early-life environmental conditions affect telomere length and that these effects can carry over into adulthood (Noguera et al. 2016, Bijnens et al. 2017, Stier et al. 2020, Martens et al. 2021). Moreover, early-life telomere length (eTL) has been shown to predict lifespan (Heidinger et al. 2012), survival into adulthood (Fairlie et al. 2016, van Lieshout et al. 2019), and lifetime reproduction success (Nowicki et al. 1998, Monaghan & Ozanne 2018, Eastwood et al. 2019). In paper IV and V, attention was moved from how adult telomere length responds to challenges (immune responses and ambient conditions) to how environment and genes determine the telomere length at different life stages.

Telomere trajectories but not telomere length may be set already at conception (paper IV)

Telomere length and telomere shortening are key physiological traits that during early development may contribute to the programming of individual life trajectories (Entringer et al. 2018). Our selective breeding experiment allowed investigation of telomere inheritance patterns at the embryo and nestling life stages. For the study in paper IV, two main scenarios of telomere inheritance were considered: 1) TL is strictly genetically determined already at conception and in general there is a parallel pattern between individuals in terms of TL change over time (Noguera et al. 2016); 2) (early-life) TL is predominately affected by factors occurring after conception during the late pre- and early post-natal periods. The later scenario matches with the fetal programing of telomere biology hypothesis (Entringer et al. 2018) (see paper I). We tested the two scenarios by creating two parental groups with distinct early-life telomere length (eTL, long eTL and short eTL). Based on the first scenario, our prediction was that the offspring's TL during pre- and postnatal stages would match up with their parent's eTL (Figure 10A). We also predicted two alternative outcomes based on the second scenario (Figure 10B & 10C, for detailed information see paper IV).

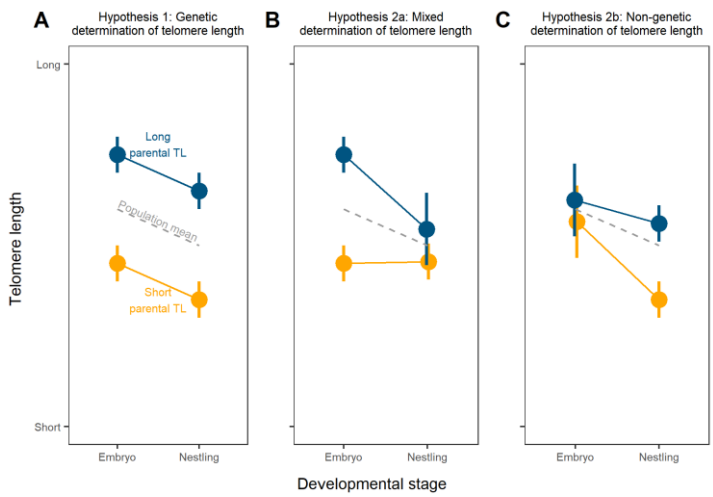


Figure 10. Schematic figure of the three alternative outcomes that can be predicted based on the experimental set-up

Parental pairs were mated assortatively based on their early life telomere length (eTL; i.e., TL measured on blood taken from parents when 10-days old) creating pairs originating from the same eTL group (i.e., either short or long eTL). Predictions of mean TL patterns in offspring from the two disticnt parental eTL groups when measured at embryo or nestling life stages; (A) Offspring TL at both the embryo and nestling stages reflect their parental eTL group (long or short). This will create a pattern of parallel TL shortening rate in the two paternal eTL groups over the early developmental period, (B) embryo TL differs between the short and the long parental eTL group but the difference fade away towards the nestling stage. The reason for this could be that genetic effects apparent at the embryo stage are later masked by non-

genetic (parental) effects acting at the late pre- and early post-natal stages, (C) mean embryos TL is similar for the two parental eTL groups (due to weak genetic effects) but later at the nestling stage mean TL differs and matches the parental eTL groups (due to a heritable component of the TL rate of change).

The results in paper IV show that embryo TL was on average 28% longer than nestling TL which is expected as embryos have undergone fewer cell divisions compared to nestlings (Schaetzlein et al. 2004, Tan et al. 2012) and telomerase activity is usually higher during the embryo stage (Wright et al. 1996, Shay & Wright 2019). Furthermore, our results show that nestling TL, but not embryo TL, matched with their parental eTL group (Figure 11). This result fits with the third prediction we made (Figure 10c). It could be explained by either genetic or non-genetic programming factors kicking in at the late pre- and early post-natal stages. Such factors could be e.g., genetically based co-variation between parents and offspring in the physiological factors determining telomere maintenance and/or restoration, or maternal effects acting on egg constituents and incubation effort. Nevertheless, our findings imply that parents and offspring TL have been influenced in the same way during early development resulting in similar eTL at the nestling stage. These findings suggest that telomere length at conception maybe not be a good predictor of telomere length at later life stages. Instead, this study highlights that the late pre- and early post-natal periods may be decisive in shaping early-life TL and studying these effects deserves further attention.

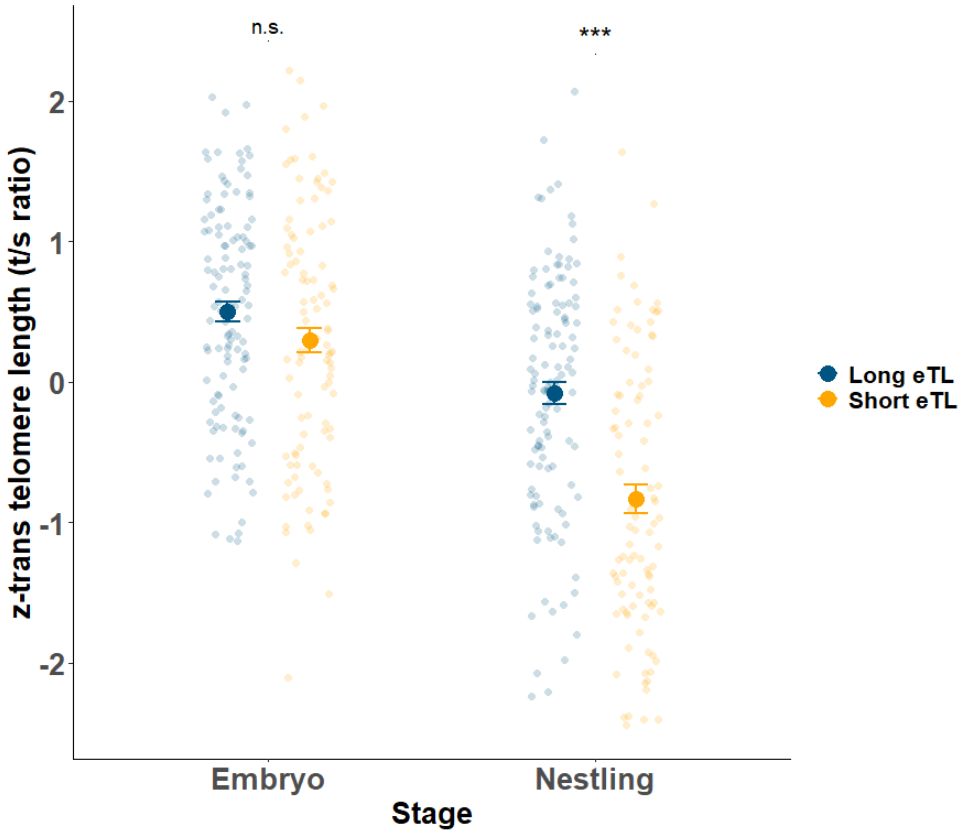


Figure 11. Telomere length (TL; z-transformed t/s ratio) at embryo and nestling stage, between long or short parent eTL groups.
The embryo TL is 28% longer than nestling TL in general. The result also showed no difference in TL between long or short parent eTL groups at embryos but the nestlings TL matched to their parentl eTL parental group at 10-days-old.

We also found sex-specific effects of our assortative pairing on nestling TL. Sons from parents of the short parental eTL group had significantly shorter telomeres than sons from parents of the long parental eTL group. Meanwhile, this difference is less pronounced in daughters that have intermediate TLs (Figure 12). These findings support previous studies that have found patterns of sex-specific parental imprinting in telomere maintenance genes (Barrett & Richardson 2011). Another explanation for these results could be that telomere restoration is regulated differently in males and females due to the action of sex hormones (Barrett and Richardson 2011). In this scenario, telomere maintenance or restoration mechanisms may have been upregulated in male offspring from the long parental eTL group but downregulated in the short parental eTL group. In comparison, female offspring of both parental eTL groups had an intermediate TL.

We found no effect of parental age on either embryo or nestling TL. Although there was a trend that male embryos produced by old parents had longer telomeres, but this trend disappeared when reaching the nestling stage. Therefore, our results do not seem to support the *telomere messenger hypothesis*.

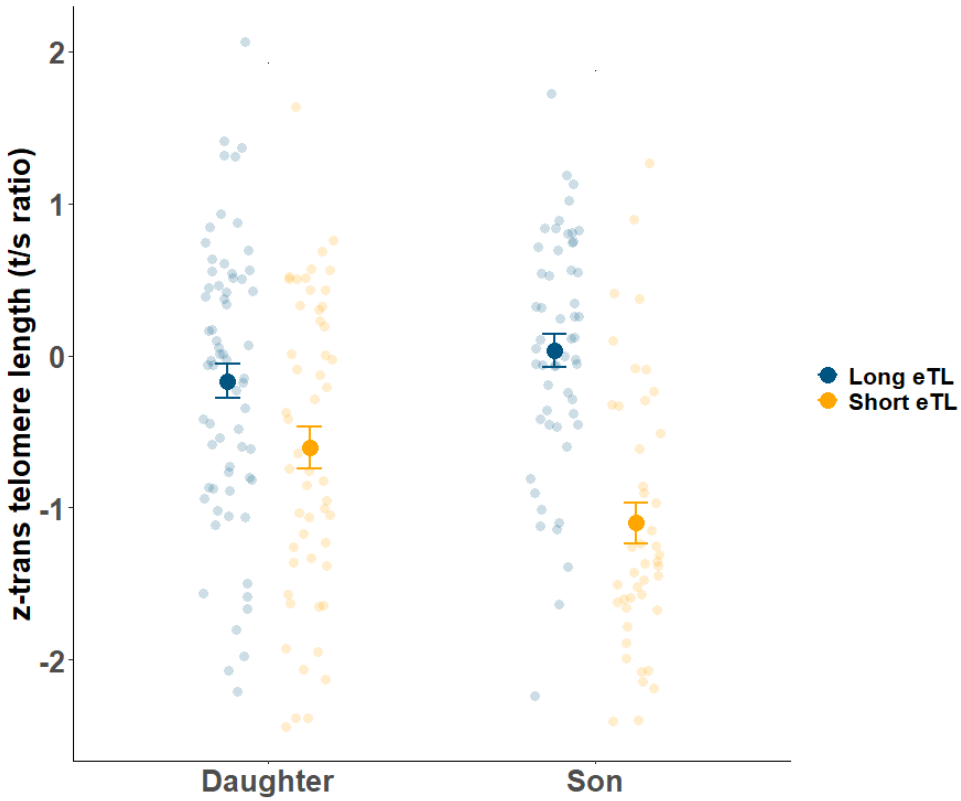


Figure 12. Nestling telomere length (TL; z-transformed t/s ratio) for sons and daughters separately for the short and long parental eTL group.
There was no significant difference in nestling TL between daughters originating from the two Parental eTL groups. For sons there was a strong significant difference; sons originating from parents with long eTL had the longest TL and sons from parents with short eTL had the shortest TL.

Genetic and non-genetic contributions to telomere length and variation in estimates of heritability of telomere length between life stages (paper V)

Telomere length in an individual at any given point is determined by three processes: the initial telomere length at conception and the amount of shortening and the amount of restoration it has experienced over time. It may therefore not be

surprising that heritability estimates of TL vary considerably between studies, as many studies, particularly in wild study systems and in humans, use telomere length measurements of parents and offspring that were taken at different ages and life stages and/or under different environmental conditions. Paper V uses captive zebra finches which allow repeated sampling at precise age and given life stages and housing under relatively stable environmental conditions to further investigate telomere inheritance with two different methods: 'animal models' and 'parent-offspring regression'. The results from paper V show that early-life telomere length for both parents and offspring is heritable to a moderate degree (h^2 = 27 – 31 %, Figure 13). The highest heritability estimate was obtained when parent and offspring TL measurements were taken at the same life stage (parent and offspring eTL). No significant and numerically relatively low maternal or paternal effects were found in all comparisons. The results at least partially support the hypothesis that environmental effects are accumulating with age and that therefore heritability estimates should be highest when based on measurements taken early in life and lower when based on measurements taken at later life stages.

Since heritability estimates varied depending on at which life stage the TL measurements were taken, the results do not support the idea that TL is set for life at conception or birth. The results also appear not to support the *telomere messenger hypothesis* (TMH). Under the TMH one would expect that environmental factors gradually change TL in parents over life, and it is this environmentally-altered TL that is inherited by the offspring (therefore higher heritability estimates would have been expected when parent TL was measured at breeding (or conception) and offspring TL measurements taken very early in life).

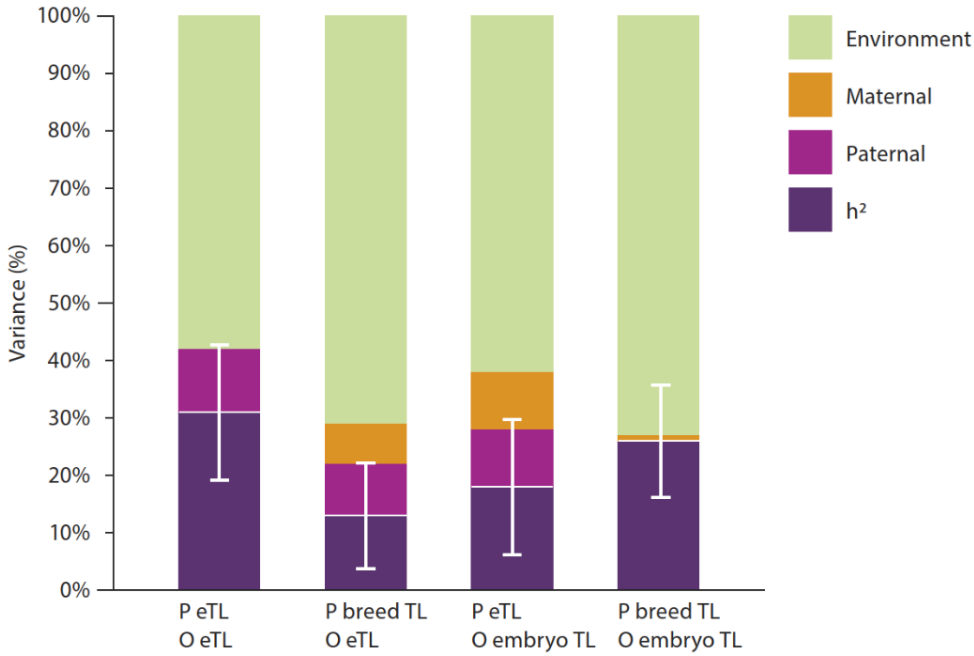


Figure 13. Variance partitioning of the phenotypic variance in telomere length (TL) in a captive population of zebra finches.
Bars show results from 'animal model' analyses based on TL was measured at different life stages in parents and offspring; P eTL – O eTL: both parents and offspring measured at nestling stage, P breedTL – O eTL: parents measured at breeding and offspring at nestling stage, P eTL – O embryoTL: parents measured at nestling stage and offspring at embryo stage, P breedTL – embryoTL: parents measured at adult stage (when breeding) and offspring at embryo stage. All models included genetic (narrow sense heritability), maternal, paternal and environmental (residual) effects. Heritability was significant in the P eTL – O eTL (0.31 ± 0.12 SE, p-value 0.013) and P breedTL – embryoTL (0.26 ± 0.10 SE, p-value: 0.023). Parental effects were not significant in any of these four models. Whiskers in figure show the ± 1 SE for the genetic effect. For more details, see paper V.

Conclusion and future perspective

General conclusion

Research on telomeres in an ecological and evolutionary framework is currently a hot research topic, as can be seen from the rapidly expanding number of published studies (paper I). However, we still have a limited understanding of how telomere length is inherited, which environmental factors that can influence telomere shortening or telomere elongation (still a debated issue), and to what degree an individual's fitness is directly affected by (either constrained or elevated) or merely correlated to telomere dynamics.

In this thesis, I used a captive songbird species, the zebra finch, to conduct immune challenge and assortative breeding experiments and applied state-of-the-art laboratory techniques for blood analysis. The work united hypotheses and methods of evolutionary ecology, immunology and molecular biology. The research conducted in the framework of my thesis has shed new light on the evolutionary background and ecological relevance of telomere length and telomere dynamics. Reviewing a plethora of hypotheses regarding the role of telomeres in ecology and evolution, allowed for a thematic hierarchical clustering of the hypotheses that can help researchers to explore and test ideas proposed in different research fields. Furthermore, me and my colleagues validated that a portable and cost-effective blood analyser can provide measurements of a spectrum of physiological parameters collected from birds under field and semi-field conditions, which may help to broaden the perspectives of the role of telomeres in bird ecology and ecophysiology. In the immune challenge experiment, there was physiological effects of immune treatment but no effects on telomere dynamics. However, there was an exceptionally strong and rapid telomere elongation effect over the second 30-day long immune challenge period in all birds, likely a consequence of them being transferred into a benign indoor environment. In addition, our TL-based assortative breeding experiment showed that the telomere length of nestlings, but not of embryos, is predicted by the nestling stage TL of their parents. These results suggest that it is the telomere rate of change, rather than the telomere length itself, that is the key heritable trait for determining early-life TL. Finally, we revealed that up to 30% of the telomere length variation in the offspring is determined by the telomere length of their genetic parents when measured at the same (i.e., nestling) life stage, but that the parents' telomere length at breeding was the main determinant (26 %) of embryo telomere length. Overall, these results highlight that the life stage when TL

measurements are taken fand later used for heritability analyses is important and may explain some of the huge variation in heritability estimates that have been reported in the literature.

Future perspectives

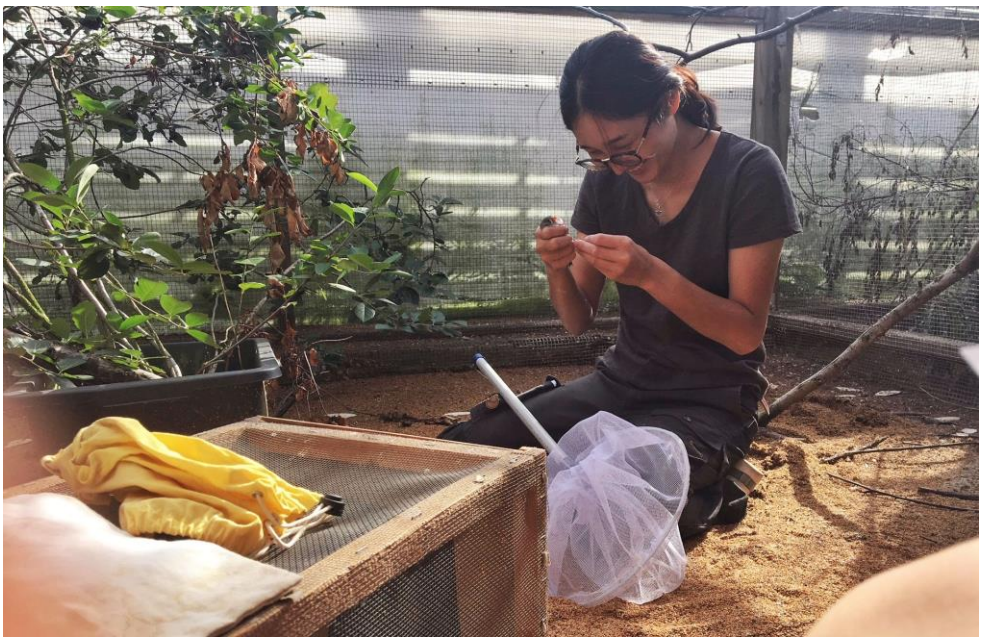
This thesis raises several exciting new questions to be studied in the future. For example, the remarkable telomere elongation pattern found in paper III may change our view of how we look at telomere dynamics, not the least seeing telomere shortening as an always ongoing and almost unavoidable process in living organisms. Although captive animal models are different from wild populations and results therefore need to be interpreted with this in mind, the results in paper III strongly suggest that changes in adult telomere length may be much more dynamic in terms of both magnitude and timeframe, than hitherto thought. These results were true for blood cells and it would therefore also be important to study if these effects also occur in other somatic cells (see below). Moreover, previous studies have often investigated changes in TL over longer periods (e.g., between years), but my results now call for studies that also focus on short-term dynamics (especially in wild study systems).

Whether or not an individual may be able to adjust investment in telomere maintenance over the short term may be more important than previously thought. If so, the role of telomerase, the main enzyme that regulates telomere restoration, should be also investigated more closely. Previous studies on vertebrates mostly suggest that telomerase is suppressed after early development (due to the risk of promoting cancer) and that there is little telomerase activity in adulthood. However, if there is a lower critical TL that affects systemic performance (and assuming TL measured in the blood reflects TL in the hematopoietic stem cells) and thus organism performance, it would be expected that telomerase is upregulated (at least for short periods) also in adults under certain circumstances e.g., when closing in on the lower critical threshold in TL. It might be possible to conduct experiments where offspring that have short or long telomeres (e.g., created in the same way as described in paper IV) are exposed simultaneously to multiple stressors (e.g., both controlled change in ambient environment and immune activation) and test whether they invest differently in telomere maintenance. Another interesting avenue for future research would be to test whether changes in TL in other tissue than blood may be equally rapid, or whether investment in telomere maintenance is different depending on tissue. Most studies measure TL from DNA of circulating blood cells. Thus, measuring TL also in tissue cells could potentially add to the bigger picture in our understanding about how an organism adapts to environmental stressors during costly life-history events e.g., migration or reproduction.

Telomere length is a heritable somatic trait. The heritability estimate based on the parents TL at breeding and embryo TL found in paper V is puzzling and needs

further investigation. For example, one might specifically test father-son or mother-daughter regressions to examine parental contributions more closely. Moreover, conducting animal model analyses only on siblings would give further insights. Furthermore, it would be very interesting to determine the TL of the offspring from paper IV and V when they were adults and conduct heritability analyses also based on these TL measurements.

Altogether, my thesis clearly shows that even though telomeres have been the target of research for several decades, there are still many questions that remain to be answered.



Pitcure 4. Elsie caught a female zebra finch at the outdoor aviary, Stensoffa.
On 11th August 2019, it was a warm and beautiful summer day (Photo by Farisia Polwijk)

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List of papers

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II. Xiong, Y., Tobler, M., Hegemann, A. and Hasselquist, D., Assessment of avian health status: validation and application of the 'VetScan blood analyser' for ecological and evolutionary studies. Submitted

III. Xiong, Y., Hegemann, A., Tobler, M. and Hasselquist, D., Telomere dynamics and physiological health in relation to repeated experimental immunisation and short-term changes in environmental conditions. Manuscript

IV. Xiong, Y., Melgar, J., Tobler, M. and Hasselquist, D., Pre- and post-natal development is critical to determination of early-life telomere length. Manuscript

V. Xiong, Y., Tarka, M., Tobler, M. and Hasselquist, D., Importance of life stages when estimating heritability of telomere length in a songbird. Manuscript



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