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Dietary nucleotides can prevent glucocorticoid-induced telomere attrition in a fast-growing wild vertebrate

Stefania Casagrande[1] | Jasmine L. Loveland[2] | Marlene Oefele[1] | Winnie Boner[3] | Sara Lupi[4] | Antoine Stier[5,6] | Michaela Hau[1,7]

[1]Max Planck Institute for Biological Intelligence, Evolutionary Physiology Group, Seewiesen, Germany

[2]Department of Cognitive and Behavioral Biology, University of Vienna, Vienna, Austria

[3]Institute of Biodiversity Animal Health and Comparative Medicine, University of Glasgow, Glasgow, UK

[4]Konrad Lorenz Institute of Ethology, Vienna, Austria

[5]Université de Strasbourg, CNRS, Institut Pluridisciplinaire Hubert Curien, UMR7178, Strasbourg, France

[6]Department of Biology, University of Turku, Turku, Finland

[7]Department of Biology, University of Konstanz, Constance, Germany

Correspondence
Stefania Casagrande, Max Planck Institute for Biological Intelligence, Evolutionary Physiology Group, 82319 Seewiesen, Germany.
Email: stefania.casagrande@bi.mpg.de

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Abstract

Telomeres are chromosome protectors that shorten during eukaryotic cell replication and in stressful conditions. Developing individuals are susceptible to telomere erosion when their growth is fast and resources are limited. This is critical because the rate of telomere attrition in early life is linked to health and life span of adults. The metabolic telomere attrition hypothesis (MeTA) suggests that telomere dynamics can respond to biochemical signals conveying information about the organism's energetic state. Among these signals are glucocorticoids, hormones that promote catabolic processes, potentially impairing costly telomere maintenance, and nucleotides, which activate anabolic pathways through the cellular enzyme target of rapamycin (TOR), thus preventing telomere attrition. During the energetically demanding growth phase, the regulation of telomeres in response to two contrasting signals – one promoting telomere maintenance and the other attrition – provides an ideal experimental setting to test the MeTA. We studied nestlings of a rapidly developing free-living passerine, the great tit (*Parus major*), that either received glucocorticoids (Cort-chicks), nucleotides (Nuc-chicks) or a combination of both (NucCort-chicks), comparing these with controls (Cnt-chicks). As expected, Cort-chicks showed telomere attrition, while NucCort- and Nuc-chicks did not. NucCort-chicks was the only group showing increased expression of a proxy for TOR activation (the gene TELO2), of mitochondrial enzymes linked to ATP production (cytochrome oxidase and ATP-synthase) and a higher efficiency in aerobically producing ATP. NucCort-chicks had also a higher expression of telomere maintenance genes (shelterin protein TERF2 and telomerase TERT) and of enzymatic antioxidant genes (glutathione peroxidase and superoxide dismutase). The findings show that nucleotide availability is crucial for preventing telomere erosion during fast growth in stressful environments.

KEYWORDS
cellular enzyme target of rapamycin (TOR), devoloping individuals, glucocorticoids, metabolic telomere attrition hypothesis (MeTA), mitochondrial bioenergetics, nucleotide availability, shelterin protein TERF2, telomere maintenance 2 (TELO2)

1 | INTRODUCTION

Growth is a delicate life-history stage, where new cells and tissues are produced at a very high rate. It involves an increase in body mass that requires a constant supply of external resources to support the energy demands of producing more cells (Delfarah et al., 2019; Glazier, 2015; Marchionni et al., 2020; Stamps, 2007). Long-term energy shortages during growth can have a lasting impact on cellular processes, potentially impairing the organism's functioning over time. One of those biomarkers is the shortening of telomeres (Marasco et al., 2022; Monaghan & Ozanne, 2018; Salmón et al., 2021; Sugimoto, 2014), complexes of DNA repeats and proteins that protect the coding part of the chromosome from incomplete DNA replication (Blackburn et al., 2015). Telomere attrition can be substantial during early growth because of the high rate of DNA replication (Monaghan & Ozanne, 2018; Salmón et al., 2021). Additionally, stressful conditions experienced during the early phases of life can exacerbate telomere attrition (Blackburn & Epel, 2012; Entringer et al., 2011; Epel, 2020). Importantly, early telomere attrition is related to health and life expectancy in animals and humans (Heidinger et al., 2012; Muñoz-Lorente et al., 2019).

The mechanisms underlying telomere erosion in individuals growing under adverse conditions are not fully understood, but a plausible candidate is the activation of the hypothalamus-pituitary–adrenal – 'stress' – axis, which culminates in the secretion of glucocorticoid hormones (Casagrande & Hau, 2019; Giraudeau et al., 2019). When secreted at high concentrations, glucocorticoids bind to the glucocorticoid (GR) receptor. The activation of GR triggers major metabolic changes, including a heightened reliance on internal resources to transform the necessary energy required for the organism to endure the challenge (Chrousos & Kino, 2005; Hau et al., 2016; Yudt & Cidlowski, 2002). Why this process should lead to telomere attrition is a matter of debate. One hypothesis is that glucocorticoids cause oxidative stress (Angelier et al., 2018; Costantini et al., 2011; Picard et al., 2018), where pro-oxidants are produced in excess or cannot be buffered sufficiently and, consequently, damage vital molecules like DNA and telomeres (Armstrong & Boonekamp, 2023; Reichert & Stier, 2017). However, evidence that physiological concentrations of glucocorticoids causes oxidative stress has not been generally established, as studies on free-ranging birds show (Casagrande & Hau, 2018; Vitousek, Taff, Ardia, et al., 2018). An alternative hypothesis suggests that glucocorticoids shorten telomeres because these hormones can change energy metabolism in a major way ('metabolic telomere attrition hypothesis' (MeTA), Casagrande & Hau, 2019). One cornerstone of the MeTA is that telomere length is costly to maintain, and when glucocorticoids signal the need to re-direct limited resources to processes that support immediate survival, telomeres can shorten as a result of this energetic trade-off.

The MeTA proposes that glucocorticoids act through specific metabolic pathways, which involve two key components: the mitochondria, where energy is transformed, and the enzyme target of rapamycin (TOR), a sensor of cellular energy supplies that controls growth and metabolism. Growth depends on the availability of resources like energy and specific nutrients (Martin & Hall, 2005; Valvezan & Manning, 2019; Wullschleger et al., 2006). TOR is able to sense the quantities of nutrients and adenosine triphosphate (ATP) available in the cell. If enough resources are present, TOR activates the anabolic pathways needed to grow (Avruch et al., 2009; Betz & Hall, 2013; Limson & Sweder, 2009; Zhang, Meng, et al., 2019) and to maintain long telomeres as observed in yeast, mice and humans (Ferrara-Romeo et al., 2020; Kupiec & Weisman, 2012; Schonbrun et al., 2009; Ungar et al., 2011; Zhou et al., 2003). TOR also receives and integrates different endocrine signals related to energy homeostasis, in order to synchronise energy-consuming processes with energy availability (Kupiec & Weisman, 2012; Martin & Hall, 2005; Schonbrun et al., 2009; Valvezan et al., 2017; Wang & Proud, 2009; Zhang, Liu, et al., 2019). Specifically, TOR is activated by a positive energetic state, that is, high levels of nitrogen-rich nutrients, nucleotides, high concentrations of ATP, and by anabolic endocrine signals, for example, insulin-like factors, growth hormones and sex steroids (Valvezan & Manning, 2019). When TOR is activated it inhibits catabolic and promotes anabolic pathways, through specific down-stream signals that lead to the biosynthesis of proteins, lipids and nucleotides (Schieke et al., 2006). Interestingly, an inability to deactivate TOR and thus to curb anabolic processes when nutrients are scarce leads to fatal outcomes, as observed in fasting mice carrying a genetic knockout for the main TOR inhibition pathway (Xu et al., 2014). Inhibition of TOR by nutrient deprivation or hormonal signals activates autophagy to recycle and replenish cellular supplies of vital amino acids and nucleotides (Sudarsanam & Johnson, 2010; Van Leene et al., 2019). Although TOR is a master regulator of cellular metabolism, its main role is the regulation of cell growth when contrasting or rapidly fluctuating signals are present (Ben-sahra et al., 2018; Bonawitz et al., 2007; Dibble & Manning, 2013; Schieke et al., 2006; Valvezan & Manning, 2019). From a more ecological perspective TOR can been seen as a regulator of trade-offs. Glucocorticoids are often viewed to regulate the trade-off between current and future survival (Crespi et al., 2013; Ouyang et al., 2016; Vitousek, Taff, Hallinger, et al., 2018), and the MeTA proposes that they do this by affecting the state of TOR. Exploring this idea may shed some light on the context-dependency of glucocorticoid mediated trade-offs (Breuner et al., 2008).

Nucleotides can also influence telomere dynamics independently of TOR (Figure 1). For instance, the activity of telomerase is influenced by nucleotide levels (e.g. Chen et al., 2018). Recent findings using CRISPR-Cas9 to genetically disrupt nucleotide metabolism pathways in cultured human cells have identified multiple telomere length control points (Mannherz & Agarwal, 2023). Specifically, reducing the salvage or de novo production of nucleotides resulted in shortened telomeres, whereas inhibiting nucleotide breakdown enzymes or supplementing with monophosphate nucleotides alone led to significant telomere elongation (Mannherz & Agarwal, 2023). These observations provide strong support for the critical role of nucleotides in telomere maintenance (Casagrande & Hau, 2019). Nucleotides are integral to almost all vital biological processes of the organism, not only as components of DNA and

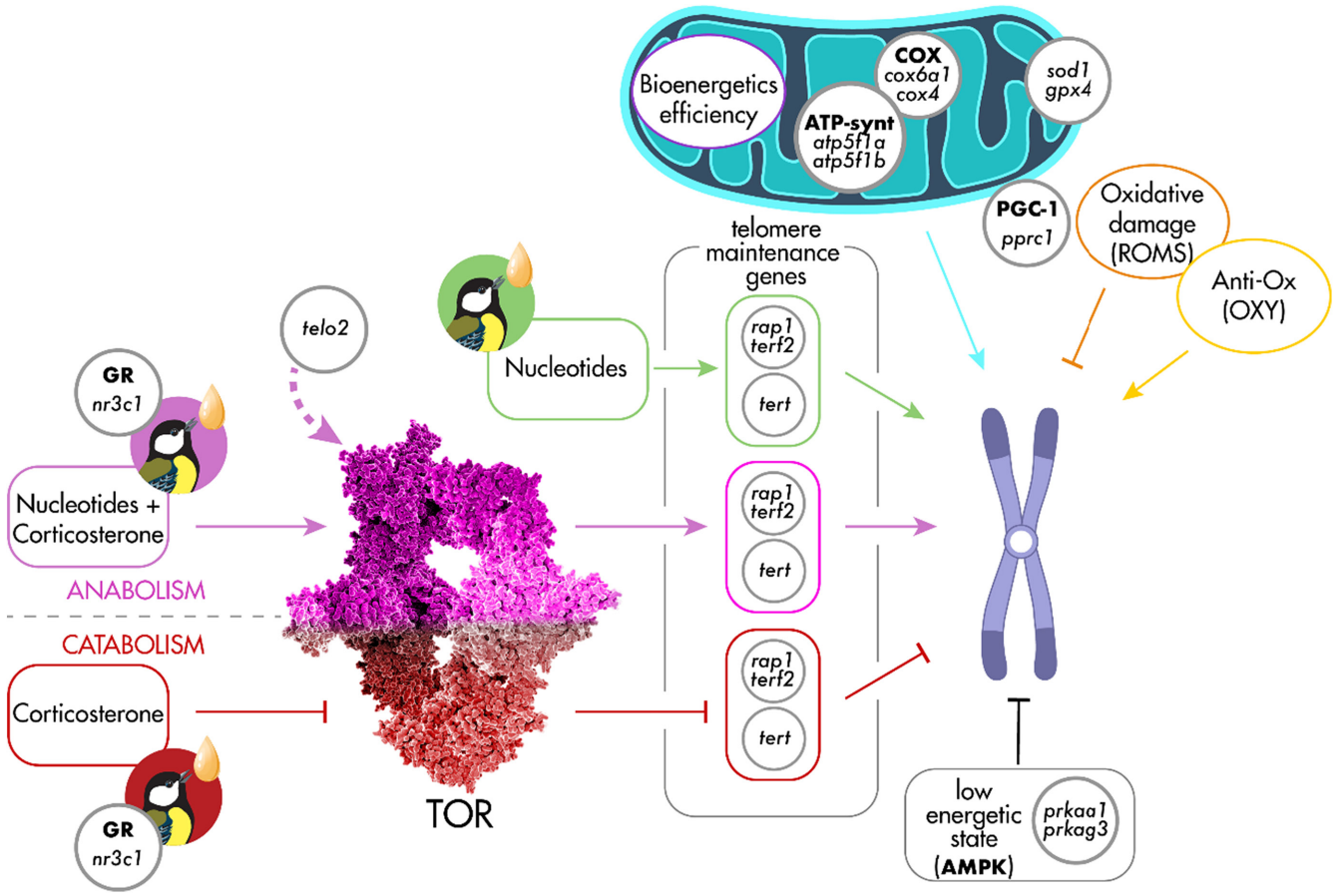

FIGURE 1 Basic conceptualisation of the study design. Pink pathway represents TOR activation expected for chicks receiving nucleotides and corticosterone while red pathways are for TOR non-activation/inhibition, as expected for Cort-nestlings. Green pathway represents effect of nucleotides on telomere length, independently of TOR. TOR activation in Nuc-birds was not expected because of the absence of an additional signal like elevated corticosterone (further details in Sections 1 and 4). Control chicks are not represented because we did not expect any of the pathways depicted here would be activated. Arrows represent activation while a blunt head arrow represents inhibition. Sharp and blunt-head arrows pointing at telomeres indicate maintenance-elongation or attrition of telomeres, respectively. White circles indicate gene expression (mRNA) for: mitochondrial enzymes of the electron system cytochrome oxidase (*cox6a1*, *cox4*); mitochondrial ATP-synthases (*atp5f1a*, *atp5f1b*), mitochondrial and intracellular antioxidants: superoxide dismutase (*sod1*) and glutathione peroxidase (*gpx4*); mitochondrial regulator PGC1 (*pprc1*); telomere maintenance proteins: shelterin proteins (*trf2*, *rap1*) and telomerase (*tert*); biomarker for low energetic state AMPK (*prkaa1*, *prkag3*). The effectiveness of corticosterone administration was assessed by measuring the level of circulating corticosterone (Cort) and of the gene expression of the glucocorticoid receptor GR (gene: *nr3c1*). See text for detailed explanations of expectations.

RNA but also as crucial molecules linked to energy metabolism, mitochondrial functions and redox processes (ATP, GTP, NADH, AMPK, etc.). Although organisms can synthesise nucleotides to fulfil their needs, the de novo production is energetically expensive, necessitating substantial amounts of ATP and glucose (Fan et al., 2019). Consequently, salvage pathways recycling nucleobases and nucleosides that are released during the breakdown of nucleic acids do exist (Austin et al., 2012). Conditions of increased demand, including rapid growth, activation of the immune system or decreased protein intake, may deplete nucleotide supplies (Ding et al., 2021), which for this reason are considered 'conditionally or semi-essential nutrients' (Sánchez-Pozo & Gil, 2002). In recent years, dietary nucleotides have been recognised as conferring specific benefits to both animal models (Hess & Greenberg, 2012) and humans (Carver et al., 1991). The largest part of nucleotide absorption occurs in the upper regions of the small intestine in the form of nucleosides (i.e. nucleotides without the phosphate group/s, which is/are removed by nucleotidases enzymes; Bissonnette, 1992; Salati et al., 1984; Sanderson et al., 1994), thanks to the action of specific carriers (i.e. concentrative nucleoside transporters – CNTs and equilibrative nucleoside transporters ENTs; Sinclair et al., 2001). The re-phosphorylation of nucleosides takes place primarily in the liver, although each cell has the capacity to convert them back into nucleotides by enzymes called nucleoside kinases (Theisinger et al., 2002). Once converted back into nucleotides, they are then circulated via the blood throughout the entire body, where they can be used in various physiological processes (Uauy et al., 1994).

Here we experimentally tested whether the catabolic actions of high glucocorticoid concentrations can be counteracted by the

effects of high nucleotide availability on telomere dynamics during growth using a wild avian model (*Parus major*, great tit) with known effects of glucocorticoids on telomere length during a phase of rapid growth (Casagrande et al., 2020). The great tit is a common passerine found throughout Europe and Asia that has become a popular model species for ecological studies in a variety of research areas (Hau et al., 2022; Laine et al., 2016; Ouyang et al., 2012; Regan & Sheldon, 2023; Verhagen et al., 2020), including telomere length (Atema et al., 2021; Casagrande et al., 2020; Stier et al., 2016, 2021), mitochondrial (Casagrande et al., 2020; Nord et al., 2021) and gene expression studies (Lindner et al., 2021). Great tits have been found to exhibit substantial variation in telomere length (Atema et al., 2019), which has been linked to various life history traits and environmental factors (Casagrande et al., 2020; Stier et al., 2016, 2021). Here we investigated the combined effects of administration of corticosterone and nucleotides on early-life telomere length. We provided additional nucleotides to free-living great tit nestlings because these can activate TOR (Valvezan & Manning, 2019) and are essential for telomere maintenance in different taxa (Chen et al., 2018; Hoxhaj et al., 2017; Sanford et al., 2021; Valvezan et al., 2017). Moreover, nucleotide shortage impairs cellular division and triggers the replicative stress response. During energy shortages, cells may promote nucleotide salvage pathways rather than, or in addition to, energetically costly new biosynthesis, by allocating recycled nucleotides to the encoding genome (Austin et al., 2012; Casagrande & Hau, 2019). In line with the MeTA, we expected that the effect of glucocorticoids on telomere length depends on the energy status of the cells, an information delivered by nucleotides. We therefore predicted that (1) chicks receiving both corticosterone (catabolic) and nucleotides (anabolic) are able to maintain telomere length as a result of the TOR-activating effect of nucleotides, which compensates for the catabolic effect of glucocorticoids (Figure 1, green arrows; Casagrande & Hau, 2019). We further predicted that (2) Nuc-chicks would exhibit longer telomeres than the control group, as nucleotides are expected to be a scarce resource in this phase of life (Casagrande & Hau, 2019) and because the positive effects of nucleotides availability on telomere length are directly linked to telomere maintenance processes (Mannherz & Agarwal, 2023). For these reasons, nucleotides in Nuc-chicks are not necessarily dependent on the TOR pathway (Figure 1). (3) We also expected that longer telomeres observed post-treatments (Figure 1, pink and green arrows) are associated with the enhanced expression of telomere maintenance genes (De Lange, 2005; Epel et al., 2004), with improved mitochondrial functions in producing ATP and with antioxidant defences that protect telomeres from oxidative insults (Armstrong & Boonekamp, 2023; Reichert & Stier, 2017). We also expected that post-treatment longer telomeres are negatively associated with the adenosine mono-phosphate activated kinase AMPK, expression of a lower energetic state of the cell, which is more abundant when the energetic state of the cell is low (Casagrande & Hau, 2019) and with a lower abundance of oxidative damage biomarkers (Armstrong & Boonekamp, 2023; Reichert & Stier, 2017).

2 | METHODS

2.1 | Study design

The study was carried out in spring 2017 in a mixed forest located in southern Germany (47°99′ N, 11°39′ E). One-hundred and fifty nest boxes were checked weekly starting in late March to record the start of incubation, and from day 10 of incubation onwards every other day to record the date of hatching (day 0). After hatching, we randomly allocated nests to two major groups: experimental nests visited every day ($n = 23$) and control nests visited two times ($n = 10$). Experimental and control nests did not differ in mean (± SEM) clutch size, number of hatchlings or number of fledglings (data published in Casagrande et al., 2020). On day 5 after hatching, we identified the three-four heaviest nestlings of each brood by weighing them with a digital scale to the nearest 0.1 g. Nestlings from the four groups (Cort-, NucCort-, Nuc- and Cnt-chicks, see below for details) did not differ in body size before the treatment (body mass $F_{(3,47.34)} = 0.81$, $p = .49$; tarsus length $F_{(3,47.91)} = 2.21$, $p = .11$). Focal chicks were marked with one to two yellow or white dots on the skin or feathers of the head with permanent non-toxic markers to allow for quick individual identification. In each experimental nest, the three focal birds were each assigned a different treatment: from day 5 to day 14 Cort-nestlings received daily an oral dose of crystalline corticosterone dissolved in organic peanut oil; NucCort-nestlings received the same oral dose of corticosterone in addition to an oral dose of nucleotides (a mixture of AMP, GMP, CMP and UMP, Chemoforma AG., CH) dissolved in water; Nuc-nestlings received the same dose of nucleotides of NucCort chicks. To maintain the concentration of oral corticosterone at 0.85 $\mu g\, g^{-1}$ of body mass and of oral nucleotides at 70 $\mu g\, g^{-1}$ of body mass throughout the nestling period, we adjusted the volume of the oral dose to each nestling's body mass measured on days 5, 8 and 12 (range of volumes: 2.3–6.6 μl). This manipulation of corticosterone mimics natural repeated increases in this steroid, as usually observed during recurring stressful periods (Spencer & Verhulst, 2007). The three experimental nestlings in the same nest were therefore exposed to the same levels of disturbance, but differed in their exposure to exogenous corticosterone and nucleotides. Nestlings of control nests (control-nestlings, 3–4 per nest) were handled only two times during the nestling period (on days 5 and 15 and a brief visit on day 10 to refresh colour markings) and did not receive any treatments. We selected chicks from these nests as controls because handling them daily could trigger the secretion of corticosterone in nestlings (Herborn et al., 2014). This was something we needed to avoid to properly investigate the questions of our study. Therefore, we did not include a group to control for the potential effects of peanut oil. However, our previous study demonstrated that the vector did not play a role in telomere dynamics, mitochondrial bioenergetics or growth (Casagrande et al., 2020). Some nestlings disappeared from their nest for unknown reasons between our visits (control, $n = 3$, Nuc, $n = 5$, NucCort, $n = 4$ Cort, $n = 3$) while six nestlings in

control nests lost their colour marks and were not sampled on day 15. To calculate growth rate, body mass (to the nearest 0.1 g) and tarsus length (to the nearest 1 mm) were recorded on days 5 and 15 in experimental and control nests. All physiological markers considered in the study (see below) were measured for every chick in 80 µl of blood collected with a capillary tube on day 15 by puncturing the ulnar vein, within 3 min of opening the nest box. To minimise any variability due to daily fluctuations of the physiological parameters, nestlings were sampled between 08:00 h and 13:00 h. Blood was immediately stored on ice and within 4 h centrifuged at 2000 *g* for 10 min; plasma was stored at −80°C and analysed within 3 months. Red blood cells (RBCs) were washed immediately after sample centrifugation to measure mitochondrial activity as described below (see also Casagrande et al., 2020). An aliquot of RBCs from each individual was stored in newborn calf serum (NBCS) buffer at −80°C until analysis of telomere length. Another aliquot was stored at −80°C until RNA extraction in 2019.

2.2 | Parameters of interest

The effectiveness of corticosterone administration was assessed by measuring the level of circulating corticosterone (Cort) and of the gene expression of the glucocorticoid receptor GR (gene: *nr3c1*), both expected to be higher in birds receiving oral corticosterone (Casagrande et al., 2020).

To address the study questions, we measured the following genes and physiological traits in the blood:

1. Gene related to the energetic level of the cells: TELO2 (gene: *telo2*), an upstream activator of TOR (Brown & Gromeier, 2017; Fernández-Sáiz et al., 2013; Glatter et al., 2011; Pal et al., 2021) that physically binds to TOR (Glatter et al., 2011).
2. Genes related to telomere length regulation: (a) Telomerase reverse transcriptase (TERT, gene: *tert*), the enzyme that adds nucleotides to telomeres to buffer telomere shortening when cells are dividing (Blackburn, 2001); (b) RAP1 (gene: *rap1*), a subunit of the shelterin protein complex that is involved in changes of telomere length, both shortening and elongation (Zhang, Liu, et al., 2019); (c) TERF2 (gene: *terf2*) subunits of the shelterin protein complex that has a pivotal function in maintaining telomeres in their capped state and preventing their shortening (Ruis et al., 2021).
3. AMPK (subunit genes: *prkaa1* and *prkag3*, also known as *ampka1* and *ampkg3*. AMPK is activated when ATP levels are low (Rabinovitch et al., 2017) and it deactivates TOR (Martin & Hall, 2005; Seebacher & Little, 2017).
4. Peroxisome proliferator-activated receptor-gamma coactivator PGC-1 (gene: *pprc1*, a gene encoding a protein called 'PPARG related coactivator 1', functionally similar to 'PPARG Coactivator 1 Alpha' according to the GeneCards database (Stelzer et al., 2016)). PGC-1 is a master regulator of mitochondrial functioning (Lin et al., 2005; Zhu et al., 2019) that can activate TOR (Cunningham et al., 2007), with potential positive effects on telomere dynamics (Xiong et al., 2015).
5. Mitochondrial traits, specifically (a) the expression of mitochondrial enzyme subunits of the electron transport chain responsible for the final step of ATP synthesis (cytochrome oxidase – COX, genes: *cx6a1*, *cox4*) and subunits of the enzyme that synthesise of ATP (ATP-synthase, genes: *atp5f1a*, *atp5f1b*); (b) mitochondrial bioenergetics, specifically cell metabolic rate (CMR) and the component of aerobic metabolism allocated to ATP production (OXPHOS), the component associated with heat production (LEAK) and calculated indexes of mitochondrial inefficiency ($LEAK^{*}CMR^{-1}$), see below for definitions and descriptions of traits.
6. Oxidative stress status by measuring the expression of (a) glutathione peroxidase GPX (gene: *gpx4*) and superoxide dismutase SOD (gene: *sod1*) in RBCs including in the mitochondria as they encode proteins that convert ROS into hydrogen peroxide (SOD) and water (GPX); (b) extra-cellular biomarkers of oxidative damage (organic peroxides quantified as reactive oxygen metabolites – ROMs) and (c) oxidative defences like total non-enzymatic antioxidants (OXY).

2.3 | Telomere length measure

Absolute length of telomeres was measured in RBCs using a non-denaturing terminal restriction fragment (TRF) analysis following (Haussmann & Vleck, 2002). Full details of the protocol used are reported in Casagrande et al. (2020). Briefly, we measured telomere lengths (range: 2–40 kb) in 5–10 µl RBCs, after DNA extraction with Gentra Puregene Kit (Qiagen). RNA was removed with 2.5 µl RNase. Samples were restriction digested overnight prior to running them on an agarose gel with 0.5× TBE buffer. All samples were run using five gels and analysis was performed on singletons because of DNA quantity limitation and the low CVs associated with this protocol (Stier et al., 2020). Telomere oligos and 1 kb + ladder were radio-labelled with 32P. Each reaction was added to Sephadex spin columns and labelled products were stored at 4°C. We used a 0.8% agarose gel for pulsed field electrophoresis. To pre-hybridise the gel, we incubated it at 37°C for 60 min with 50 mL hybridisation solution. We then added 50 mL hybridisation/oligo solution to the gel and incubated it overnight, with the same conditions as described in the previous step. The following day, the gel was washed, dried and wrapped in cling film and placed in a phosphor screen cassette for 4 days, then visualised using a Typhoon Variable Mode Imager (Amersham Biosciences). Average telomere length was quantified by densitometry in the program ImageJ (version 2.0) within the limits of our molecular size markers (2–40 kb, Figure S2).

2.4 | Corticosterone assay

Plasma corticosterone concentrations were determined using an enzyme immunoassay kit (Cat. No. K014-H1; Corticosterone ELISA Kit, Arbor Assays) following a double diethyl ether extraction of

a 15 μl plasma sample (for a detailed protocol see Casagrande et al., 2020). Samples were re-dissolved in assay buffer and allowed to reconstitute over-night. A buffer blank and two stripped chicken plasma controls (with corticosterone added at concentrations of 10 and 5 ng ml^{-1} respectively) were taken through the entire procedure. On the next day, 50 μl of each sample (in duplicate) were used. The inter-plate coefficient of variation (CV) was calculated as the average concentration of the four controls (for both high and low concentrations) of the two plates and was 2.40 ± 0.51%. The intra-plate CV was calculated as the average CV of the concentrations of all unknown samples run on six plates and was 3.72 ± 0.55%.

2.5 | Gene expression analysis

We quantified the expression of 14 genes of interest (*telo2*, *tert*, *terf2*, *rap1*, *atp5f1a*, *atp5f1b*, *cx6a1*, *cox4*, *gpx4*, *sod1*, *pprc1*, *prkaa1*, *prkag3* and *nr3c1*) relative to a single reference gene (*ube2d2*; Table S3A) following Casagrande et al., 2020. Briefly, we extracted RNA from RBC samples by mixing 2.5–5 μl RBCs with 230 μl of TRI Reagent BD (Sigma-Aldrich) and 5 μl of 2.5 M glacial acetic acid and then 60 μl of chloroform. We then centrifuged samples (12,000 *g*) for 15 min at 4°C, transferred the supernatant to a new tube and added an equal volume of 70% ethanol. This mixture was then applied to a RNeasy column (RNeasy Mini Kit, Qiagen), and followed the standard manufacturer's protocol with a final elution step in 30 μl of EB buffer. We measured RNA concentration and the A260/A280 ratio using a Nanodrop Spectrophotometer (ND-1000) and only samples with an A260/A280 ratio within the range 1.8–2.14 were used. For each sample, we used 400 ng of RNA as a template for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad) in a 20 μl reaction according to the manufacturer's instructions. We diluted cDNA 1:10 before use as template in final qRT-PCR assays.

We designed primers for all 14 genes with NCBI Primer-BLAST (Ye et al., 2012). We ensured that each amplicon spanned at least one exon–exon boundary. We ran standard curves to determine the efficiency of primer pairs. We ran standard curves with serially diluted cDNA from a single sample to calculate the amplification efficiency of each primer pair. The serial dilutions we tested were: three dilutions between 1:10 and 1:1000, and if there was no amplification in the 1:1000 dilution a second standard curve was run with four dilutions between 1:10 and 1:100. Efficiency was calculated with the Absolute Quantification tool and second Derivative Maximum method which uses the formula Efficiency = $10^{-1/\text{slope}}$ based on the quantification cycle [Cq, termed crossing point (Cp) in the software] and log concentration of template in each well. The theoretical efficiency of perfect amplification (i.e. exact doubling with each cycle) is 2. The efficiency of primer pairs ranged from 1.909 to 2.07; a detailed summary of standard curve results is listed in Table S3.

We performed qRT-PCR assays across 11 plates with a balanced combination of treatments in each plate. Plates were run in two separate batches: the first batch had four plates and the second batch had seven plates. The first batch contained target genes *nr3c1*, *prkaa1*, *prkag3* and *pprc1* and the second batch contained the remaining target genes listed in Table S3A. Data for *nr3c1* was previously reported for Cnt and Cort chicks in Casagrande et al., 2020. We ran assays on a LightCycler 480 II (Roche) machine using the SsoAdvanced Universal SYBR Green Super mix (Bio-Rad) in 384-well plates (Roche) and each reaction was run in duplicate. Each well consisted of a 10 μl reaction containing 1× SsoAdvanced Universal SYBR Green Super mix, 340 nmol of each primer and 3 ng of cDNA template (i.e. 1.5 μl of the 1:10 cDNA dilution; the estimate of template amount assumes a one-to-one correspondence between input RNA and synthesised cDNA). The cycling conditions were: pre-incubation step at 95°C for 30 s, 45 cycles at 95°C for 10 s, annealing and extension at 60°C for 30 s, with acquisition at the end of each cycle, followed by a melt curve (95°C for 5 s with five acquisitions per °C from 65 to 97°C with a 0.11°C ramp rate). We performed calculations from the raw amplification data using LightCycler 480 software (version 1.5.1.62) and used GraphPad Prism (version 7.05) for additional quality control analyses such as for testing for group differences on reference gene levels and calculating standard curve correlation coefficients. On every plate, we confirmed that each primer pair produced a single melt curve peak in the presence of cDNA template and showed no amplification when water was used as template. In case of primer dimer present in water controls, the melting temperature was clearly distinct from that of the target amplicon and primer dimer was not present in wells with cDNA template. We confirmed that the Cq values for *ube2d2*, pooled from all 11 plates, did not vary among the four treatment groups (Kruskal–Wallis $H(3) = 0.378$, $p = .945$; Cq mean ± SEM: 21.82 ± 0.25). Expression of target genes was calculated relative to the reference gene *ube2d2* in the software with the Advanced Relative Quantification analysis using the actual primer efficiencies from the standard curve instead of the preassigned value of 2.

2.6 | Mitochondrial metabolism analyses

The oxygen consumed by aerobic metabolism during mitochondrial respiration was measured in intact RBCs, which are metabolically active in birds (Engelhardt, 1932; Stier et al., 2013, 2017) following validated protocols (Casagrande et al., 2020; Stier et al., 2017). Briefly, 30 μl RBCs were transferred into 1 ml of cold buffer Mir05 (for details see Casagrande et al., 2020), washed by spinning at 500 *g* for 5 min and then resuspended in 1 ml of MiR05 buffer already equilibrated at 40°C in a Clark electrode high resolution respirometer (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). Mitochondrial respiration was quantified as the O_2 consumed in the following stages: (1) cellular metabolic rate (CMR) – basal respiration of the cells in their natural state; (2) oxidative phosphorylation (OXPHOS) – the process through which ATP is produced – after inhibiting ATP synthase by addition 1 μg ml^{-1} of oligomycin. (3) proton leak (LEAK) -remaining basal respiration that is not affected by oligomycin provided in step 2 and that is

uncoupled from ATP production because energy is dissipated in the form of heat. We also measured (4) the working capacity of the electron transport system (ETS) – by adding the mitochondrial uncoupler carbonyl cyanide m-chlorophenyl hydrazine (CCCP Mitochondria: titration of 1 µmol l^{-1} aliquots). The uncoupler causes the flow of electrons through the ETS to be independent from the transformation of ADP into ATP. All these traits were corrected for non-mitochondrial O_2 consumption by adding antimycin A (5 µmol l^{-1}), a potent suppressor of mitochondrial metabolism. From these measures we calculated mitochondrial inefficiency to produce ATP in relation to CMR (i.e. proportion of LEAK on CMR: LEAK/CMR). All measures were normalised by the volume of RBCs and expressed as pmol O_2*min^{-1}*ml^{-1} of RBCs.

2.7 | Oxidative stress

Levels of hydroperoxides produced by the oxidation of lipids, proteins and nucleic acids, that is, reactive oxygen metabolites (ROMs), were quantified with the d-ROM test (Diacron International, Grosseto, Italy; for details see Casagrande et al., 2020; Casagrande & Hau, 2019) using a microplate reader (Multiskan Go, Thermo Fisher Scientific, Vantaa, Finland). Measurements are expressed as mmol l^{-1} of H_2O_2 equivalents. All samples, the calibrator and controls for high and low concentrations were run in duplicate. The inter-plate CV was calculated as the average concentration of the four controls on each of the six plates and was 7.83 ± 3.2%. The intra-plate CV was calculated as the average CV of the concentrations of control samples run on six plates and was 3.18 ± 0.74%.

Plasma non-enzymatic antioxidants were quantified using the OXY-Adsorbent test (Diacron International; Casagrande & Hau, 2019; Casagrande et al., 2020) using reference standards and controls for high and low concentrations (all diluted 2:50 with distilled water) or blank (i.e. only water) using a microplate reader (Multiskan Go, Thermo Fisher Scientific). The antioxidant capacity is expressed in µmolHOClml^{-1}. All samples, standards and blank were run in duplicate. The inter-plate CV was calculated as the average concentration of the four controls on each of the six plates and was 15.86 ± 2.26%. The intra-plate CV was calculated as the average CV of the concentrations of control samples run on six plates and was 6.25 ± 1.34%.

2.8 | Statistical analysis

We obtained data from 69 chicks, but sample sizes differ across variables as some lab assays failed to produce reliable data (see below for further explanations; samples size reported in figures). To assess the effect of the treatments on the variables of interest we ran a model for each response variable with 'treatment' as a predictor (4 levels, Cnt, NucCort, Cort and Nuc) and 'nest' as a random factor. EIA plate number and TRF gel number were included as random factors in the models for corticosterone and telomere length, respectively. The 12 plates used to run RT-qPCR for gene expression analysis were not included in the models to avoid overfitting but their variance was limited as shown the plate contribution to the variance of each gene expression (Table S3B). The effect of the treatments was assessed by model estimates that took as reference group the unmanipulated group (Cnt) in order to assess differences of Cnt-chicks to NucCort- and Cort-chicks, respectively (see above for explanations on the use of this group as a valuable control for the questions addressed in the present study). We also provided full pairwise post-hoc comparisons (Tukey's Bayesian marginal means and 95% CI are shown in figures when meaningful; full comparisons are reported in Table S2).

Some variables were highly correlated with each other and were therefore first subjected to a principal component analysis to avoid redundancy. Specifically, we found a strong correlation between mass growth (expressed as the difference in body mass between day 15 and day 5) and tarsus growth (expressed as the difference in tarsus length between day 15 and day 5; $r = .61$, $n = 69$, $p < .0001$). The principle component analysis indicated that PC1 explained 80.4% of the variation in growth of both traits and was therefore used in subsequent analyses. The model for growth included initial tarsus length to account for any starting imbalances (initial body mass was not included because of collinearity; see correlation analysis above). For the gene expression analysis, we replaced highly correlated genes with the first principal component factor calculated from a principal component analysis for the following traits: two genes encoding subunits of ATP synthase F1 (*atp5f1a*, *atp5f1b*, see Table S3A for more specifications) and two genes encoding subunits 4 and 6A1, respectively, of cytochrome c oxidase *cox4* and *cox6a1*, (Table S3A; all $r \geq .52$ see Table S4 for full correlation matrix; PC1 is referred to as 'cytochrome oxidase and ATP-synthase' and represents 79.20% of the combined traits); *sod1* and *gpx4* (Table S3A, $r = .48$, $p < .0001$; PC1 is referred to as 'GPX and SOD' and represents 73.94% of the combined traits).

In order to understand the physiological mechanisms that allowed NucCort birds to maintain relative long telomeres despite the high levels of circulating corticosterone (see Section 3) we ran a linear mixed model to assess which of the traits that responded to the treatment (i.e. TERT, TERF2, PC1 cytochrome oxidase and ATP-synthase, PC1 GPX and SOD and PC1GPX_SOD and PC1 growth) predicted telomere length (response variable of the model) with nest as random factor (Table 2). We also included 'LEAK' among the predictors because it was enhanced in Cort- but not in NucCort-chicks, leading us to think that the higher CMR observed in Cort- versus NucCort-chicks had different meanings (i.e. driven by LEAK in Cort- and by an enhancement of mitochondrial complexes in NucCort-chicks, see results). We did not include 'treatment' to avoid a post-treatment bias as these factors were also the ones that the responded to the treatment (McElreath, 2020). We did not include TELO2, because it is considered up-stream of TOR activation and, indeed, was highly correlated with most of the

covariates included in the explanatory model for telomere length (TERT, 0.38 [0.16,0.60]; TERF2, 0.66 [0.47,0.85]; PC1 cytochrome oxidase and ATP-synthase, 0.62 [0.43,0.81]; PC1 GPX and SOD, 0.52 [0.30,0.73]; PC1 growth, −0.20 [−0.4,−0.01]); not correlated with leak (0.12 [−0.17,0.35]). To run these explanatory models, the missing values caused by random events (i.e. assay failure or insufficient sample volume) were imputed as the mean value of the respective treatment group (see McElreath, 2020). Results with and without computed values did not change substantially (see Table S1 for statistical comparison between data set with and without missing data computation). In constructing our models, we adhered to best practices to mitigate potential overfitting by maintaining a minimal number of predictors. Specifically, we followed the widely recommended rule of thumb of ensuring at least 10 observations per level of each fixed or random factor. This yielded a necessary minimum sample size of 50, given our four-level treatment factor and single-level random factor. In all our analyses, we exceeded this minimum, with no analysis involving fewer than 54 samples. While this rule is typically advocated for frequentist analyses, we applied it despite our use of Bayesian estimates, which generate a posterior distribution and thus provide more robust results when working with small sample sizes (McElreath, 2020). To further ensure the robustness of our findings, we also conducted additional analyses using computed missing values in a larger/full dataset, with these analyses confirming the results obtained using the original dataset. These results, along with our adherence to sample size guidelines, should assuage any concerns about the robustness of our findings. To test our predictions, that were formulated a priori in the grant proposal referred in the appropriate statement, we ran several models without correcting for multiple testing, which is not needed when using Bayesian multilevel modelling (for details, see Berry & Hochberg, 1999; Gelman, 2012).

When used as covariates, variables were z-score normalised. We checked whether variables met the assumptions of homogeneity of variance and normal distribution by visually analysing the graphical distributions of fitted values versus their residuals. Then, all factors were log transformed except for telomere length, non-enzymatic antioxidants OXY and bioenergetics traits (among the latter only ETS was log transformed). All statistical models were fitted using a Bayesian framework implemented in the statistical software R (version 4.2.2, R Core Team, 2022) using the R-package 'rstanarm'. For all models, we used parameter-flat priors (Korner-Nievergelt et al., 2015). The number of iterations chosen to ensure that the minimum Markov chain Monte Carlo entailed an effective sample size was of 4000 iterations and four chains. All models showed absolute autocorrelation values lower than 0.1, satisfied convergence criteria based on the Heidelberger and Welch convergence diagnostics, had an effective sample size ('neff') close to expected iterations, while none had an 'rhat' value above 1.0. We drew inferences from the posterior distribution and 95% credible interval (CI), considering fixed effects to be meaningful if the range 2.5–97.5% CI did not include zero.

3 | RESULTS

3.1 | Effects of treatment on corticosterone, GR receptor, body size and telomere length

The main goal of the study was to assess whether additional nutrients could counteract the effects of daily increases in corticosterone concentrations as is typical when stressful conditions occur. Both groups receiving corticosterone, that is, Cort- and NucCort-chicks, had higher corticosterone concentrations and a higher expression of GR receptor mRNA compared to Cnt- (Table 1; Figure S1a,b) and to Nuc-chicks (post-hoc difference in corticosterone marginal means: Cort-Nuc: 0.42 [0.10,0.75]; NucCort-Nuc: 0.28 [−0.01,0.63]). Post-hoc differences in GR marginal means: Cort-Nuc: 0.61 [0.21,1.06]; NucCort-Nuc: 0.38 [0.01,0.78]. This result indicates that the treatment was effective in mimicking stressful early life conditions.

Telomere length was shorter only in Cort-chicks (Table 1; Figure 2a), whereas NucCort-birds were able to maintain telomeres at similar length as Nuc- and Cnt-chicks (Post-hoc in Figure 2a). NucCort-chicks and Cort-chicks were smaller at fledging in comparison to controls (95% CI slightly overlapping 0) but were not different from Nuc-chicks, which did not differ from controls (Table 1; Figure 2b).

3.2 | Effects of the treatments on TOR state and down-stream variables

NucCort-birds had higher gene expression levels of the proxy for TOR activation (TELO2), and of shelterin protein TERF2 (Table 1, Figure 3b) in comparison to all other groups (Table 1, Figure 3a,b). NucCort-chicks had also higher expression of TERT, but only in relation to Cnt-chicks (Table 1, Figure 3c). NucCort-chicks had also a higher expression of genes encoding cytochrome c oxidase and ATP synthase (Table 1, Figure 4a) and antioxidant enzymes GPX and SOD compared to all other groups (Table 1, Figure 4b). None of the treatment groups significantly differed from controls in oxidative damage (ROMs; Table 1, Figure S1d) or non-enzymatic antioxidants (OXY; Table 1, Figure S1c). The treated chicks also did not differ from control chicks for RAP1, AMPK and PGC1 (Table 1, Figure S1e–g).

3.3 | Effects of treatments on mitochondrial bioenergetics

Cort- and NucCort-chicks had higher CMR (Table 1, Figure 5a) and a marginally higher ETS (Table 1) while OXPHOS was not different from controls (Table 1, Figure S1h,i). Cort-chicks had higher levels of CMR also in relation to Nuc-chicks, while NucCort-chick CMR did not differ from that of Nuc-chicks (Figure 5a). Only Cort-chicks had a higher LEAK (Table 1, Figure 5b) and consequently a higher

TABLE 1 Statistical outputs of models used to assess the effect of the treatments on the variables of interest (reference group was the control group). Estimates of fixed (β) and random (σ^2) parameters are shown as posterior modes with 95% credible intervals (CI). In bold statistically meaningful results. We considered results to be marginally meaningful when slightly overlapping 0 (≤0.03 – bold italics).

Variable (R2)	Fixed effects	β [95% CI]	Random effects	σ^2 [95% CI]
Corticosterone (0.42)	Intercept	0.56 [0.26,0.86]	Nest	0.19 [0.01,0.37]
	NucCort	**0.40 [0.02,0.75]**	Assay	0.08 [0.004,0.53]
	Cort	**0.54 [0.17,0.84]**	Residual	0.42 [0.34,0.53]
	Nuc	0.11 [−0.23,0.47]		
GR (0.36)	Intercept	−1.02 [−1.35,−0.71]	Nest	0.22 [0.01,0.52]
	NucCort	**0.51 [0.05,0.95]**	Residual	0.54 [0.42,0.68]
	Cort	**0.74 [0.29,1.22]**		
	Nuc	0.12 [−0.31,0.59]		
Growth (0.86)	Intercept	8.23 [6.10,10.26]	Nest	0.73 [0.47,1.04]
	NucCort	**−0.85 [−1.61,−0.10]**	Residual	0.61 [0.49,0.78]
	Cort	***−0.46 [−1.16,0.03]***		
	Nuc	−0.38 [−1.14,0.38]		
	Tarsusinitial.s	**−0.97 [−1.21,−0.73]**		
Telomeres (0.67)	Intercept	14.29 [13.41,15.17]	Nest	0.58 [0.08,1.03]
	NucCort	−0.66 [−1.66,0.38]	Trf gel	0.34 [0.01.1.14]
	Cort	**−1.61 [−2.59,−0.60]**	Residual	0.89 [0.69,1.19]
	Nuc	−0.72 [−1.78,0.35]		
TELO2 (0.21)	Intercept	−1.75 [−2.01,−1.48]	Nest	0.11 [0.005,0.34]
	NucCort	**0.65 [0.22,1.08]**	Residual	0.55 [0.45,0.67]
	Cort	0.09 [−0.34,0.51]		
	Nuc	0.18 [−0.23,0.58]		
TERF2 (0.59)	Intercept	−0.65 [−1.1,−0.19]	Nest	0.40 [0.05,0.72]
	NucCort	**1.02 [0.31,1.61]**	Residual	0.64 [0.49,0.83]
	Cort	0.01 [−0.59,0.60]		
	Nuc	0.21 [−0.37,0.79]		
RAP1 (0.17)	Intercept	−1.61 [−2.10,−1.12]	Nest	0.18 [0.009,0.51]
	NucCort	0.48 [−0.27,0.85]	Residual	0.77 [0.63,0.95]
	Cort	0.41 [−0.25,1.04]		
	Nuc	0.46 [−0.19,1.04]		
TERT (0.15)	Intercept	−0.24 [−0.57,0.11]	Nest	0.14 [0.007,0.43]
	NucCort	**0.61 [0.09,1.14]**	Residual	0.65 [0.54,0.81]
	Cort	0.32 [−0.18,0.82]		
	Nuc	0.18 [−0.33,0.67]		
GPX_SOD (0.25)	Intercept	−0.37 [−1.01,0.31]	Nest	0.40 [0.02,0.97]
	NucCort	**1.10 [0.12,2.08]**	Residual	1.18 [0.93,1.47]
	Cort	−0.01 [−1.04,0.98]		
	Nuc	0.13 [−0.75,1.03]		
PC1 ETC (0.23)	Intercept	−0.43 [−1.19,0.35]	Nest	0.35 [0.018,0.93]
	NucCort	**1.09 [0.01,2.15]**	Residual	1.49 [1.25,1.87]
	Cort	0.13 [−1.10,1.32]		
	Nuc	−0.21 [−1.30,0.9]		
AMPK-*prkag3* (0.55)	Intercept	−0.89 [−1.21,−0.58]	Nest	0.27 [0.03,0.48]
	NucCort	0.00 [−0.42,0.42]	Residual	0.45 [0.36,0.58]
	Cort	−0.18 [−0.59,0.24]		
	Nuc	−0.12 [−0.54,0.32]		

(Continues)

TABLE 1 (Continued)

Variable (R2)	Fixed effects	β [95% CI]	Random effects	σ^2 [95% CI]
AMPK-*prkaa1*–(0.11)	Intercept	−0.14 [−0.53,0.21]	Nest	0.16 [0.007,0.46]
	NucCort	−0.08 [−0.64,0.51]	Residual	0.78 [0.65,0.95]
	Cort	0.28 [−0.19,0.88]		
	Nuc	0.36 [−0.18,0.91]		
PGC1 (0.09)	Intercept	−1.63 [−1.87,−1.40]	Nest	0.10 [0.005,0.29]
	NucCort	0.23 [−0.12,0.34]	Residual	0.46 [0.38,0.57]
	Cort	0.11 [−0.24,0.46]		
	Nuc	0.02 [−0.33,0.35]		
CMR (0.41)	Intercept	2.59 [1.78,3.4]	Nest	0.65 [0.07,1.23]
	NucCort	***1.05 [−0.01,2.15]***	Residual	1.31 [1.04,1.64]
	Cort	**1.22 [0.17,2.31]**		
	Nuc	0.38 [−0.72,1.42]		
OXPHOS (0.12)	Intercept	1.82 [1.37,2.27]	Nest	0.19 [0.010,0.55]
	NucCort	0.46 [−0.08,1.01]	Residual	0.81 [0.66,0.98]
	Cort	0.24 [−0.35,0.85]		
	Nuc	0.00 [−0.18,1.08]		
LEAK (0.54)	Intercept	0.80 [0.27,1.32]	Nest	0.47 [0.06,0.79]
	NucCort	0.57 [−0.13,1.30]	Residual	0.79 [0.63,1.01]
	Cort	**0.97 [0.28,1.68]**		
	Nuc	0.36 [−0.34,1.06]		
ETS (0.59)	Intercept	1.5 [1.18,1.88]	Nest	0.35 [0.09,0.56]
	NucCort	***0.45 [−0.02,0.90]***	Residual	0.47 [0.37,0.62]
	Cort	***0.41 [−0.03,0.84]***		
	Nuc	0.13 [−0.32,0.58]		
LEAK/CMR (0.38)	Intercept	0.30 [0.22,0.38]	Nest	0.06 [0.005,0.11]
	NucCort	0.04 [−0.16,0.15]	Residual	0.13 [0.10,0.16]
	Cort	**0.14 [0.03,0.25]**		
	Nuc	0.09 [−0.02,0.19]		
ROMs (0.18)	Intercept	−0.08 [−0.34,0.18]	Nest	0.14 [0.006,0.35]
	NucCort	0.03 [−0.36,0.40]	Residual	0.50 [0.41,0.62]
	Cort	−0.13 [−0.49,0.23]		
	Nuc	0.06 [−0.33,0.43]		
OXY (0.68)	Intercept	208.08 [160.58,251.8]	Nest	49.56 [26.49,75.13]
	NucCort	43.48 [−16.17,101.12]	Residual	56.77 [45.57,73.31]
	Cort	23.77 [−33.84,80.80]		
	Nuc	41.75 [−13.81,102.62]		

mitochondrial inefficiency (calculated as the ratio LEAK/CMR, Table 1, Figure 5c) in relation to controls and NucCort-chicks (see Figure 5b,c for further pair comparisons).

3.4 | Explanatory model for telomere length

The only parameters that significantly explained telomere length were the gene expression of the respiratory system producing ATP (cytochrome oxidase and ATP-synthase), which was positively associated with telomere length (Table 2), and LEAK, which was negatively related to telomere length (Table 2). The effects were confirmed when all non-significant predictors were dropped from the model (Table 2).

4 | DISCUSSION

We experimentally tested a core idea of the MeTA: that telomere length dynamics are linked to the state of the energy metabolism

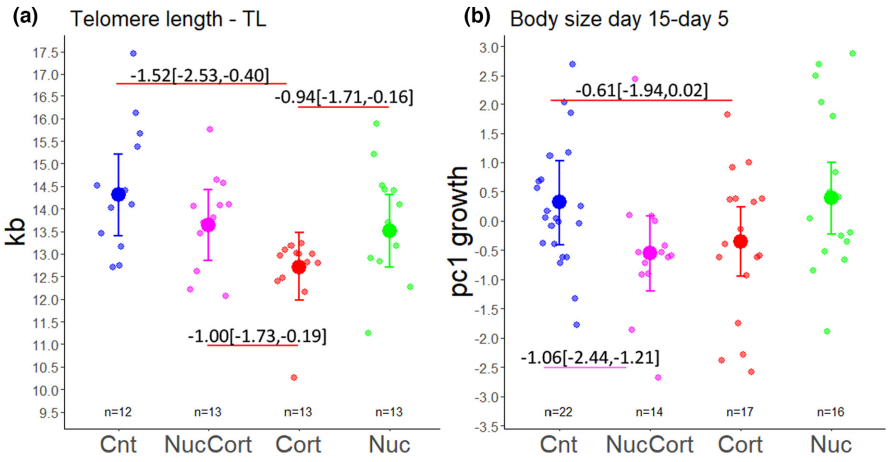

FIGURE 2 Group differences in telomere length (a) and growth (b). Growth is expressed as the first factor of a PCA including differences between day 5 and day 15 in tarsus length and body mass. Small circles represent individual raw values, while larger circles represent predicted mean values with 95% credible intervals (bars) as calculated by the statistical models. Pairwise Tukey contrasts are reported when between-group differences were significant (missing contrasts indicate lack of significant differences; see SEM for full comparisons). Contrasts were placed above coloured lines connecting groups of interest; colour refers to the group to which the mean difference was referred.

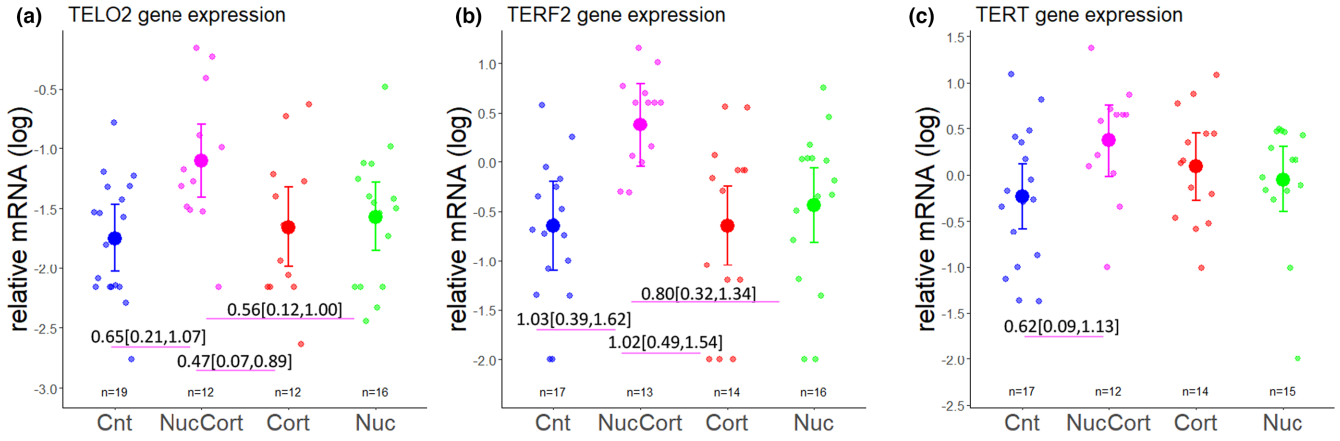

FIGURE 3 Gene expression of the TOR proxy TELO2, and telomere maintenance genes TERF2 and TERT. Small circles represent individual raw values, while larger circles represent predicted mean values with 95% credible intervals (bars) calculated by the statistical models. Contrasts were placed above coloured lines connecting groups of interest; colour refers to the group to which the mean difference was referred.

of an individual (Casagrande & Hau, 2019). Within this framework, we assessed whether the magnified telomere shortage that is often observed when offspring grow up under stressful circumstances (which requires responses that are energetically costly), was counteracted by the availability of sufficient nucleotides. By providing daily oral corticosterone doses to free-living great tit nestlings during their rapid growth phase, we mimicked a protracted exposure to stressful conditions, which did affect their telomere lengths (Casagrande et al., 2020). Specifically, chicks treated with corticosterone alone had the shortest telomeres while siblings that received a combination of corticosterone and nucleotides were able to maintain their telomere lengths similar to that of the control group. We showed that NucCort-chicks were the only group that had a higher gene expression of TELO2, a proxy for the activation of the enzyme TOR.

Cellular metabolism is inherently a self-regulated process that can proceed independently of TOR. However, when the cell receives contrasting signals, such as increased corticosterone and nucleotide levels, TOR triggers a cellular line of communication within the cell to ensure that energy produced in the mitochondria matches energetic needs (Valvezan & Manning, 2019). The need for such a coordination is particularly pressing during the intense cell proliferation that occurs during energetically demanding rapid growth (Wullschleger et al., 2006). Indeed, when only one signal is present, like in Nuc-birds that received additional nucleotides but had low levels of corticosterone, TELO2 was not increased (and by extension TOR was not activated). Chicks treated with corticosterone (NucCort- and Cort-chicks) could not maintain growth at the same rate as individuals that were not treated with corticosterone (in Cort-chicks the 95% CI slightly overlapped 0),

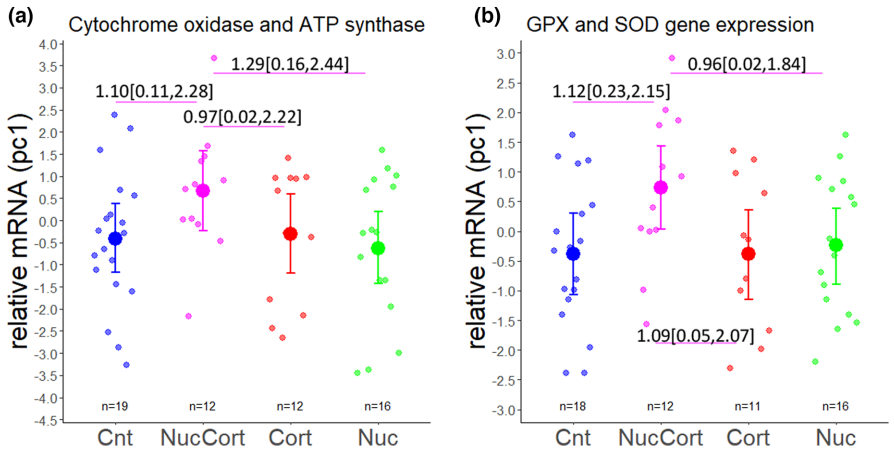

FIGURE 4 Gene expression of (a) four subunits of the two mitochondrial enzymatic complexes IV and V (ATP-synthase F1 subunits (*atp5f1b*, atp5f1a), cytochrome c oxidase subunits 4 and 6A1 (*cox4*, *cx6a1*)) expressed by PC1 of a principal component analysis, (b) gene expression of two intracellular enzymatic antioxidants (*gpx4*, *sod1*) represent by a PC1 of a principal component analysis (see Section 2 for more details). Pale circles represent individual raw values, while saturated larger circles represent predicted mean values with 95% credible intervals (bars) calculated by the statistical models. Contrasts were placed above coloured lines connecting groups of interest; colour refers to the group to which the mean difference was referred.

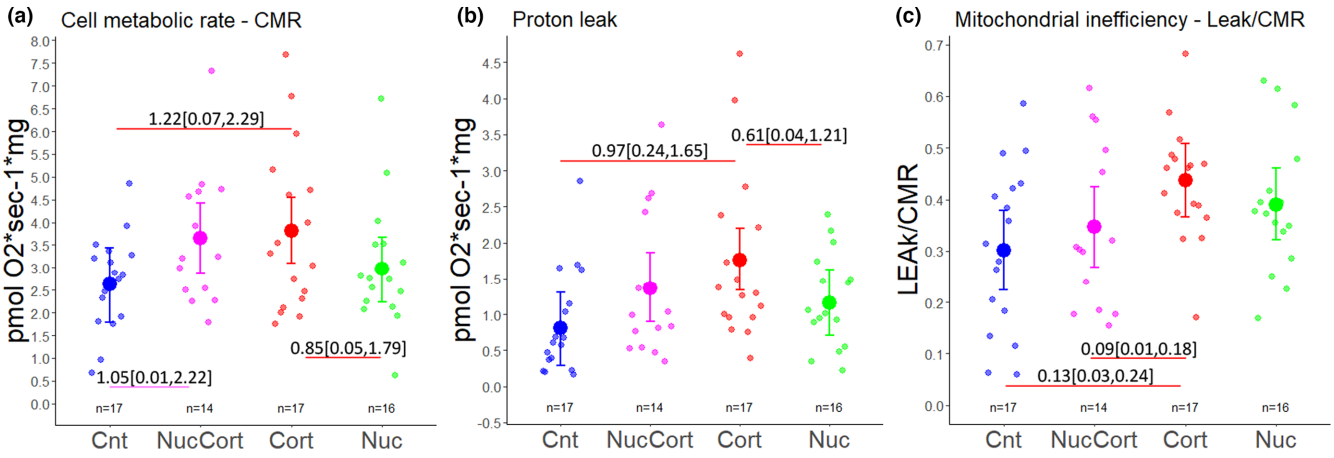

FIGURE 5 Effect of the treatment on mitochondrial bioenergetics CMR (a), LEAK (b) and mitochondrial inefficiency (c). Pale circles represent individual raw values, while saturated larger circles represent predicted mean values with 95% credible intervals (bars) calculated by the statistical models. Contrasts were placed above coloured lines connecting groups of interest; colour refers to the group to which the mean difference was referred.

indicating that the catabolic effects of corticosterone on growth were acting in both groups. In Cort-chicks, where the effect on growth was marginal, it could be speculated that Cort mediated the expected trade-offs between immediate survival (growth) at the expense of future, long-term benefits represented by telomere lengths (because it is rare that telomere attrition can affect individual survival at this early stage of life in the absence of specific pathologies, Monaghan & Ozanne, 2018). By contrast, NucCort-chicks prioritised long-term benefits (telomeres) over immediate benefits (growth), which is puzzling and needs some considerations. First, the enhanced performance of mitochondria observed in NucCort chicks (see explanations about the role of mitochondria below) was not used to boost growth but instead to maintain telomeres, highlighting the importance of limiting telomere loss during this stage of life. Indeed, several studies provide evidence that early telomere attrition constrains future survival (Heidinger et al., 2012; Wood & Young, 2019; but see van de Crommenacker et al., 2022), likely being the reason why telomere maintenance was prioritised over growth. Second, our findings show that growth and telomere downstream pathways are independent processes in this species. TOR is a complex kinase, comprised of several components that can also act independently of each other. For example, Rapamycin is effective in inhibiting the TOR-Complex-1, but not TOR-Complex-2, and the two units have different roles in controlling growth (Cybulski & Hall, 2009). It would be important in the future to investigate the specific pathways that were activated to maintain telomeres in NucCort-birds. This approach would allow us to biochemically 'visualise' the mechanisms underlying the trade-off between growth and telomere length that is often observed in vertebrates (Geiger

TABLE 2 Explanatory model for telomere length analysis.

Full model					Minimal model				
Variable (R2)	Fixed effects	β [95% CI]	Random effects	σ^2 [95% CI]	Variable (R2)	Fixed effects	β [95% CI]	Random effects	σ^2 [95% CI]
Telomere length (0.69)	Intercept	−0.02 [−0.27,0.25]	Nest	0.56 [0.26,0.87]	Telomere length (0.70)	Intercept	−0.02 [−0.27,0.25]	Nest	0.59 [0.34,0.89]
	ATP_COX	**0.46 [0.09,0.84]**	Trf gel	0.26 [0.02,0.92]		**ATP_COX**	**0.32 [0.12,0.52]**	Trf gel	0.23 [0.01,0.83]
	LEAK	**−0.36 [−0.58,−0.13]**	residual	0.71 [0.57,0.90]		**LEAK**	**−0.32 [−0.54,−0.09]**	residual	0.69 [0.56,0.87]
	Growth	0.16 [−0.07,0.39]							
	TERT	0.00 [−0.24,0.24]							
	GPX_SOD	−0.05 [−0.42,0.30]							
	TERF2	−0.06 [−0.32,0.21]							

et al., 2012; Monaghan & Ozanne, 2018; Spießberger et al., 2022; van de Crommenacker et al., 2022).

TELO2 gene expression, and the related possible activation of TOR, in birds treated with both corticosterone and nucleotides was accompanied by an enhanced functionality of the mitochondria that we observed at several levels. NucCort-birds enhanced gene expression of the ETS, in particular for genes encoding ATP synthase F1 subunits alpha and beta and cytochrome C oxidase subunits 4 and 6A1, which were positively associated with telomere length. This finding suggests that mitochondria are key elements for telomere dynamics as suggested by the MeTA (Casagrande & Hau, 2019). Oxidative phosphorylation is the process through which the mitochondria produce the chemical energy in the form of ATP to fuel anabolic processes in the cell. Indeed, in NucCort-chicks, mitochondrial metabolic rate was enhanced without a concomitant increase in LEAK; indeed, nestlings in this group were more efficient than Cort-chicks in coupling aerobic metabolism with ATP. Since TOR activation depends on sufficient ATP concentrations, this could have further ensured the activated state of TOR. It is also important to consider that mitochondria are not only crucial for ATP production but also for biosynthetic processes. Specifically, they are the core elements in which the biosynthesis of nucleotides takes place. The synthesis of nucleotides in the mitochondria relies on the Krebs cycle (Harrison & Lane, 2018). The rate at which the Krebs cycle runs, in turn, is directly associated with the respiratory function of mitochondria (Lane & Fan, 2015), which could explain why the expression of genes encoding for respiratory enzymes was the variable that best explained telomere length. In other words, we suggest that a higher mitochondrial efficiency in producing ATP was not only associated with the energy availability of cells but also related to nucleotide synthesis in the mitochondria. Nucleotides are a limiting resource for both telomere maintenance and body growth (Delfarah et al., 2019; Robitaille et al., 2013; Valvezan et al., 2017). They are needed for DNA replication during cell division, as well as the for the RNA required for protein synthesis. The supply of nucleotides is usually guaranteed by salvage pathways that recycle them, but in certain phases like growth, this is not sufficient and their de novo synthesis becomes crucial (reviewed in Casagrande & Hau, 2019).

Contrary to expectations, we did not find longer telomeres in Nuc-birds, indicating that nucleotides are not a limited resource for these nestlings. Another explanation is that to elongate telomeres independently of TOR, another set of nucleotides should have been used, that is, those that include thymidine (TMP), which was missing from the mix we provided (see Section 2 for further details). A recent study investigating the role of nucleotide metabolism for telomere dynamics in cultured human cells found that thymidine nucleotides are essential for inducing telomere elongation (Mannherz & Agarwal, 2023). In the absence of thymidine, telomeres cannot be elongated even when other nucleotides are fully provided (Mannherz & Agarwal, 2023). This study was published after we conducted our experiment. When we designed our study, we did not actively exclude thymidine from our mix as we had no reason to do so, but unfortunately, it was not commercially

available as a food additive. Given the recent findings on the importance of thymidine nucleotide for inducing telomere elongation, we advise future studies to pay attention to thymidine and other key nucleotides when investigating telomere dynamics in similar contexts.

In NucCort-birds, the increase in mitochondrial metabolic rate was paralleled by an upregulation of key antioxidant enzymes that may have prevented oxidative insults caused by a greater production of reactive oxygen species (ROS) due to the increase in mitochondrial metabolic rate (e.g. increase in CMR and ETS as observed in NucCort-chicks). However, higher mitochondrial metabolic rate is not necessarily associated with higher production of ROS (Koch et al., 2021; Salin et al., 2015; Speakman et al., 2004). Indeed, we also did not find evidence that Cort- and NucCort-birds had higher oxidative damage despite having a high CMR. It is therefore currently unclear why Cort-chicks did not incur oxidative damage as measured by our assays since they did not upregulate enzymatic antioxidants. One possibility is that the inefficiency of mitochondria in linking respiration to oxidative phosphorylation, due to the increase in proton leak, limits the production of reactive oxygen species ('uncouple to survive' hypothesis; Brand, 2000; Brand et al., 2016; Speakman et al., 2004). Indeed, there is evidence suggesting that corticosterone can be negatively correlated with mitochondrial reactive oxygen species emission (Voituron et al., 2017). It is also relevant to note that antioxidant enzymes were not down-regulated in Cort-chicks, thus we did not find any evidence that corticosterone impaired antioxidant enzymes, as hypothesised to be one reason for why corticosterone can exert pro-oxidant functions (see Angelier et al., 2018; Costantini et al., 2011).

We can exclude that Cort treatment acted via AMPK, because gene expression for two subunits (*prkaa1*, *prkg3*) of this kinase, which is upregulated when ATP is low, did not differ across groups. We can therefore conclude that even though the mitochondria of Cort-chicks were less efficient in producing ATP (because their LEAK also increased), the higher cell metabolic rate induced by corticosterone was likely sufficient in offsetting the inefficiency in producing enough ATP (Picard et al., 2014, 2017, 2018).

Gene expression for mRNAs encoding the enzyme that elongates telomeres (TERT) and a protein from the shelterin protein complex (TERF2) were higher in NucCort-birds compared to all other groups. The capping state of the telomeres is the critical element that determines cell senescence and thus impaired tissue renewal. Only when telomeres are uncapped because they have become too short, or because shelterin proteins are not adequately produced, do they exert their signalling function to promote cellular senescence (Chang et al., 2016; El Maï et al., 2020; Pańczyszyn et al., 2020; Ruis & Boulton, 2021). We know very little about the factors that determine the capping state of telomeres, regardless of their length (Timashev & De Lange, 2020), but it is worthwhile to consider that DNA integrity is checked at multiple phases of the cells, not only during cell replication (Chao et al., 2017). This would explain why the expression of shelterin proteins is important for non-replicating RBCs, the tissue used in our study to measure genes encoding shelterin proteins and other target parameters. Recently, the most comprehensive study on blood-tissue correlations of telomere length in samples from humans of different ages and sex showed that blood telomere length is a proxy for telomere length in 18 tissues out of 23 (associations were not significant for ovary, breast, thyroid, oesophagus and coronary tissue; Demanelis et al., 2020).

5 | CONCLUSIONS

We simulated a stressful environment during development by providing daily doses of corticosterone to chicks of a fast-growing bird, showing that the shortening effects of this hormone on telomeres are attenuated when chicks also receive additional nucleotides. This finding suggests that the energetic state of the organism is a crucial factor in the context-dependent actions of glucocorticoids (Jaatinen et al., 2014; Schoenle et al., 2021). We therefore encourage future studies on the effects of glucocorticoids to also evaluate the energetic or nutritional state of individuals. This would be in line with theoretical models formulated to explain the physiological and behavioural outcomes of stress mediators like glucocorticoids – the allostasis model (McEwen & Wingfield, 2003) and the reactive scope model (Romero et al., 2009) – for which the effects of glucocorticoids are not invariant but differ in relation to internal resources and the energy obtainable from the environment. Investigating the complex interactions among different physiological systems as proposed by the MeTA – cellular energy availability, mitochondria functioning and metabolic hormones like glucocorticoids – helps us to illuminate some of the pathways connected to telomere maintenance. This is relevant considering that premature cellular aging, caused by early-life telomere shortening in individuals raised in stressful conditions, can also be observed in human newborns (Ridout et al., 2018; Send et al., 2017). It is slowly starting to emerge that telomeres are not passive accumulators of damage; rather, they are targeted by several regulatory systems and tightly linked with mitochondrial function (Casagrande & Hau, 2019; Lin & Epel, 2022; Metcalfe & Olsson, 2021). By further investigating the mechanisms that regulate telomere dynamics and their interactions with other cellular systems, we may gain a deeper understanding of their biology.

AUTHOR CONTRIBUTIONS

Conceptualisation: S.C., M.H.; Lab analysis: S.C., A.S., M.O., J.L. Formal analysis: S.C.; Data curation: S.C.; Writing - original draft: S.C.; Revision: S.C., M.H. and all co-authors; Project administration: S.C., M.H.; Funding acquisition: S.C., M.H.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing or financial interests.

DATA AVAILABILITY STATEMENT

The data will be publicly accessible upon manuscript acceptance on Dryad: https://datadryad.org/stash/share/EUHOPb2khVr077ujZD3fdjTZtUI7dSQnIPuxa6D4B0A.

ETHICS STATEMENT

All experimental procedures were conducted according to the legal requirements of Germany and were approved by the governmental authorities of Oberbayern, Germany.

All experimental procedures followed the strict ethical and animal welfare guidelines of animal experimentation laws of the European Union (Directive 2010/63/EU), the German Animal Welfare Act and were conducted under the approval of Regierungspräsidium von Oberbayern.

ORCID

Stefania Casagrande https://orcid.org/0000-0002-4264-8062
Antoine Stier https://orcid.org/0000-0002-5445-5524

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