The consequences of urbanisation and artificial light at night on wildlife health and physiology: Insights from avian systems.

Rachel Rose Reid

Great tit *(Parus major):* Photo by Rachel Reid

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School of Biodiversity, One Health, & Veterinary Medicine
College of Medical, Veterinary, and Life Sciences
University of Glasgow

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Contents

Abstract

Urbanisation is accelerating at an unprecedented rate globally, exposing urban wildlife to a suite of novel anthropogenic stressors that can significantly impact health and fitness. One of the most pervasive of these stressors is Artificial light at night (ALAN) which can impact species at multiple levels of the ecosystem through the disruption of natural light-dark cycles that are essential for the regulation of many biological and ecological processes. Despite growing awareness of these threats, our understanding of how urbanisation and specific urban stressors like ALAN affect wildlife health remains limited, as does our knowledge of the mechanisms driving these relationships. A deeper understanding is vital for developing effective mitigation strategies that balance human needs with the conservation of vulnerable species in the face of urban expansion. As urbanisation continues to intensify, assessing its effects on wildlife health and fitness becomes increasingly urgent. This requires the use of reliable health biomarkers to generate consistent and biologically meaningful evidence in ecological studies. This thesis integrates experimental, and meta-analytical approaches to provide a comprehensive assessment of how urbanisation influences physiology and health in avian study systems. It also addresses the methodological challenges of measuring health in ecological contexts, with a focus on high intraindividual variation in physiological biomarkers, which can obscure meaningful patterns.

To address these knowledge gaps, I first conducted a phylogenetically controlled global meta-analysis to evaluate the relationship between urbanisation and avian health, identifying the biological and methodological factors that influence this relationship. While some studies suggest urbanisation negatively impacts avian health, findings have been inconsistent, and this is likely due to variability in study locations, focal species, and biomarker types. By collating data across a variety of studies, I was able to identify key drivers of variation and reveal hidden patterns. Notably, urbanisation was found to negatively impact avian health only when it was measured as a continuous variable (urban score), rather than as a binary categorical variable (urban vs non-urban classification). This finding emphasises the importance of quantifying urbanisation in a way that captures its heterogeneity for the detection of subtle relationships. The results also highlight that the impacts of urbanisation on avian health are context dependent, influenced by factors such as life stage and biomarker type.

I then narrowed the focus to investigate the effects of ALAN exposure on multiple health biomarkers in birds, using both field and captive study designs. In the field, I exposed great tit *(Parus major)* nestlings to ALAN from hatching to fledging using artificial nest boxes, targeting the sensitive developmental period where exposure to stressors is likely to have stronger consequences. Additionally, in a captive experiment, I exposed adult zebra finches *(Taeniopygia guttata)* to ALAN over four months to assess its long-term physiological effects using a longitudinal design. To explore potential mitigation strategies, I also tested a partial light at night treatment in the captive experiment in which lights were turned off for half of the night, comparing its effects to those of the full night ALAN exposure.

In both experiments, I adopted a multi-biomarker approach to capture different dimensions of health, addressing its multivariate nature. In the field, ALAN exposure did not show any consistent negative effect on oxidative stress or corticosterone levels in nestlings, but it was shown to significantly reduce body condition. In the captive study, ALAN increased telomere loss and disrupted glucose circadian rhythms, although it had no detectable effects on the oxidative stress markers assessed. Importantly, these negative effects were absent in the partial light at night treatment group, suggesting that this mitigation strategy may provide a viable means of reducing the impact of ALAN on wildlife health. These findings highlight the complex and variable nature of the effects of ALAN on health and show the potential for mitigation through the use of altered light exposure regimes.

I then explored the methodological challenges involved in measuring health by focusing on oxidative stress biomarkers, which are commonly used to assess fitness and physiological condition, particularly in research focusing on ageing and ageing related diseases. Through a comprehensive meta-analysis, I demonstrated that oxidative stress markers exhibit generally low within-individual repeatability, regardless of taxa, sex, environment, biomarker type, or study design. Using a simulation study, I further showed that this low repeatability can obscure associations with other traits, including telomere length. While increasing samples sizes and the number of repeated measures per individual can help mitigate this issue, further work is needed to develop robust approaches that address these detection limitations. These findings highlight the importance of careful experimental design and cautious interpretation when using highly variable physiological biomarkers in ecological studies.

The findings of this thesis contribute to the growing body of literature looking into the effects of urbanisation and anthropogenic stressors on wildlife health and well-being. By examining these issues and considering species traits, life stages, environmental factors,

and employing a multi-biomarker approach, this work advances our understanding how urban environments shape wildlife health. Furthermore, insights into the use and limitations of oxidative stress markers will inform future studies and support the development of reliable assessment of health and physiology in wild populations. This research enhances our understanding of the complex interactions between urbanisation, anthropogenic stressors, and wildlife health, providing a foundation for developing evidence-based mitigation strategies to reduce the negative effects of urban stressors such as ALAN. This research also lays the groundwork for future research on key areas, including the mechanistic pathways underlying these relationships, the cumulative long-term effects of urbanisation, and the development of realistic, scalable interventions to reduce negative impacts of urbanisation on biodiversity.

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Declaration of authorship

I declare that this thesis is the result of my own work, except where explicit reference is made to the work of others. This thesis has not been presented in any previous application for a degree at this or any other institution.

Printed Name: Rachel Reid

Signature:

Co-authorship statement

Chapter two

Author contributions: Rachel Reid, Davide Dominoni and Jelle Boonekamp conceived and designed the study. Yacob Haddou calculated urban score for each set of coordinates. Rachel Reid ran the analysis with guidance from Pablo Capilla Lasheras. Pablo Capilla Lasheras contributed to writing out the R scripts. Rachel Reid wrote the paper with input from all authors. All authors gave approval for the publication of the final manuscript.

Chapter three

Author contributions: Rachel Reid, Davide Dominoni and Jelle Boonekamp conceived and designed the study. Rachel Reid, Christopher Mitchell, Neil Evans and Neal Dawson contributed to analysing the data in the lab. Rachel Reid conducted the final data analysis. Rachel Reid wrote the paper with input from all authors. All authors gave approval for the publication of the final manuscript.

Chapter four

Author contributions: Rachel Reid, Davide Dominoni, Robert Gillespie and Jelle Boonekamp conceived and designed the study. Rachel Reid, Claire Branston, Pablo Capilla Lasheras, Robert Gillespie and Davide Dominoni contributed to collecting data from the experiment. Rachel Reid, Christopher Mitchell, Neal Dawson, and Eleanor Duncan contributed to analysing the data in the lab. Rachel Reid conducted the final data analysis. Rachel Reid wrote the paper with input from all authors. All authors gave approval for the publication of the final manuscript.

Chapter five

Author contributions: Rachel Reid, Davide Dominoni and Jelle Boonekamp conceived and designed the study. Rachel Reid conducted the analysis. Rachel Reid and Jelle Boonekamp developed the simulation. Rachel Reid wrote the paper with input from all authors. All authors gave approval for the publication of the final manuscript.

List of abbreviations

ALAN – Artificial light at night

FLAN – Full light at night

PLAN – Partial light at night

PRISMA – Preferred reporting items for systematic reviews and meta-analyses

LMM – Linear mixed model

LRT – Likelihood ratio test

ANOVA – Analysis of variance

ICC – Intraclass correlation

CORT – Corticosterone

OXY – Antioxidant capacity of plasma

MDA – Malondialdehyde

ROS – Reactive oxygen species

SMI – Scaled mass index

LED – Light-emitting diode

HPLC – High performance liquid chromatography

qPCR- Quantitative polymerase chain reaction

ELISA – Enzyme-linked immunosorbent assay

CV – Coefficient of variation

Chapter 1
General introduction

1.1 Biomarkers of physiological health in wildlife

1.1.1 Defining and measuring health in ecological systems

As urbanisation and environmental changes increasingly affect wildlife, understanding the impacts of these stressors on wildlife health has become a significant area of ecological research. To develop effective mitigation strategies, it is crucial to gain a deeper understanding of how these environmental changes influence wildlife well-being. One of the main challenges in this area of research is defining and measuring health in wildlife, as health is a multifaceted and complex concept. The multivariate nature of health complicates the task of measuring and interpreting it (Stephen, 2014).

To measure health, researchers typically rely on biological markers, or biomarkers, which offer a small representation of the physiological state of an organism (Isaksson, 2020). These biomarkers are used to track various biological processes and help us understand how wildlife is responding to environmental stressors. Physiological processes and systems, including the redox system, inflammatory responses, metabolism, and endocrine function, contain numerous measurable components that serve as useful indicators of health (Isaksson, 2020). However, the interpretation of these markers can be challenging, and there are both benefits and limitations associated with each one. Selecting the appropriate biomarkers for specific research purposes and ensuring its reliability is an important yet difficult aspect of this research.

The ideal biomarker in ecological studies should go beyond the classical binary assessment of disease absence or presence, and instead support a more integrated, mechanistic understanding of organismal health (Aleuy *et al.,* 2023). Suitable biomarkers should ideally be sensitive to the specific environmental stressor/s of interest, serve as early indicators of physiological disturbance, offer predictive value for key fitness outcomes such as survival and reproduction, and measurements should be reliable across populations (Lotmartire, Marques and Goncalves, 2021; Shultz, Britnell and Harvey, 2021). However, it is rarely the case that a single marker fulfils all these criteria.

Importantly, biomarkers are not limited to physiological measurements. Behavioural biomarkers can provide valuable, often non-invasive, insights into health status and may reveal functional consequences of physiological changes (Mouneyrac and Amiard-Triquet, 2013; Shultz, Britnell and Harvey, 2021). Several studies have demonstrated the value of combining behavioural and physiological markers. For example, altered activity patterns in bycaught loggerhead turtles *(Caretta caretta)* have been linked to compromised health states (Arkwright *et al.,* 2020), while research on feral horses *(Equus caballus)* has shown that social instability correlates closely with changes in their physiological phenotype (Nunez *et al.,* 2014).

Thus, an optimal strategy for assessing health involves integrating markers across multiple levels, including fitness, behaviour, and physiology to generate a comprehensive picture of how organisms are responding to environmental challenges. Increasingly, researchers recognise that relying on single biomarkers may provide a misleading or incomplete understanding of health status. A multi-biomarker approach, spanning diverse biological systems, could offer more ecologically meaningful insights into the pathways by which external stressors may affect individual fitness (Shultz, Britnell and Harvey, 2021; Warne, Proudfoot and Crespi, 2015).

1.1.2 Benefits and limitations of physiological biomarkers of health.

Glucose and metabolic regulation

Glucose plays a vital role in energy metabolism, serving as the primary energy source for many organisms. Plasma glucose levels and insulin production are tightly regulated and are typically synchronised with feeding patterns to ensure proper metabolic function (Downs, Wellmann and Brown, 2010; Lobban, Downs and Brown, 2010; Lill, 2011). Disruption of this synchronisation can lead to metabolic abnormalities, including obesity, type 2 diabetes, and cardiovascular disease (Kumar Jha, Challet and Kalsbeek, 2015). High circulating glucose levels can lead to non-enzymatic reactions with proteins and sugars, which is a process known as glycation. This can result in the formation of Advanced Glycation End-Products (AGEs) which can accumulate in tissues, impair cellular functions, and contribute to oxidative stress and inflammation (Aragno and Mastrocola, 2017; Teodorowicz *et al.,* 2018; Vadekedath and Kandi, 2018). AGEs have been linked to various clinical conditions in humans and lab animals including diabetes mellitus, chronic kidney disease,

neurodegenerative diseases, skin diseases, ageing, and cancer (Aragno and Mastrocola, 2017; Brouwers *et al.,* 2011; Vadekedath and Kandi, 2018).

Glucose measurements provide valuable insights into an organism's metabolic state; however, they can present challenges in ecological studies. Glucose levels can vary significantly among individuals, even within the same species, making it difficult to detect consistent changes in response to environmental stressors (Sweazea, 2022). This variability must be carefully considered when using glucose as a biomarker for assessing physiological condition.

Telomere shortening

Telomeres are protective complexes found at the ends of eukaryotic chromosomes, protecting them from erosion and end to end fusion, therefore playing a critical role in maintaining cellular genomic integrity (Lin *et al.*, 2019). Telomere length will decline naturally with each cell division; however, oxidative stress and other cellular conditions can accelerate this process. Once telomeres shorten to a critical length, cells enter senescence and will eventually undergo apoptosis (Nussey *et al.*, 2014).

Due to their role in genomic stability and cellular replication, telomere length is widely used as a biomarker for ageing and fitness (Nussey *et al.*, 2014). Shorter telomeres, particularly in early life, have been linked to reduced survival probability in various species, which has led to telomere attrition becoming a valuable metric in ecological research (Eastwood *et al.*, 2019; Tricola *et al.*, 2018; Wilbourn *et al.*, 2018). However, the relationship between telomere length and lifespan is complex and has been shown in several species to only show strong correlations at early-life stages (Heidinger *et al.*, 2012; van Lieshout *et al.*, 2019). Telomere attrition has also been implicated in age-related diseases, particularly in humans (Herrmann *et al*., 2018; Xi *et al*., 2013). When senescent cells accumulate, they can contribute to tissue disfunction and increase the risk of cancer and other degenerative conditions (Fasching, 2018; Wentzensen *et al.*, 2011).

Despite its usefulness as a biomarker, there is ongoing debate regarding the most reliable method for measuring telomere length. Telomere restriction fragmentation (TRF) is a widely used method that introduces minimal variation and provides high accuracy. However, it is labour-intensive and expensive (Aubert *et al*., 2012; Fojtová *et al*., 2015; Yu *et al*., 2024). Quantitative PCR (qPCR) is a faster and more cost-effective alternative that

is gaining wider application; however, this method introduces higher measurement variability and error rates (Aubert, Hills and Lansdorp, 2012; Kärkkäinen *et al.*, 2022; Lai, Wright and Shay, 2018; Olsson, Wapstra and Friesen, 2018). These methodological inconsistencies must be accounted for when interpreting telomere length as a biomarker of physiological condition.

Glucocorticoid hormones

Glucocorticoid hormones such as corticosterone (CORT) are widely used biomarkers in the literature to infer physiological condition (Injaian *et al*., 2020; Jessop *et al*., 2015; Narayan *et al*., 2013; Rivers *et al*., 2012). When exposed to environmental stressors, vertebrate species including birds, mammals and amphibians release glucocorticoid hormones, which upregulate physiological and behavioural responses to maximise survival (Denver, 2009; Romero, 2004; Romero *et al*., 2008; Vitousek, Jenkins and Safran, 2014). As a result, CORT levels can provide important insights into an organism's health and its ability to cope with environmental stressors (Falso *et al.,* 2015; Romero, 2004; Romero *et al.,* 2008; Schoech, Rensel and Heiss, 2011; Vitousek, Jenkins and Safran, 2014). Prolonged exposure to elevated CORT levels can have detrimental effects on growth, behaviour, reproduction, metabolic rate, cognitive ability and the immune system (Braun *et al.,* 2013; Denver, 2009; Falso *et al.,* 2015; Schoech, Rensel and Heiss, 2011; Sterner and Kalynchuk, 2010; Quirici *et al.*, 2016; Vitousek, Jenkins and Safran, 2014; Vitousek *et al.,* 2018c).

In birds, CORT is most commonly measured from plasma samples to assess both baseline and stress-induced levels (Crino *et al*., 2020; Injaian *et al*., 2020; Ouyang *et al*., 2013; Rivers *et al*., 2012; Müller *et al*., 2010). However, circulating CORT levels can fluctuate rapidly in response to stress, therefore plasma measurements provide only a short-term snapshot of an individual's acute glucocorticoid activity (Cockrem, 2013; Kloet *et al*., 2008; Karaer *et al*., 2023). An alternative approach is to measure CORT in feather samples, where CORT is incorporated into the feather structure over the period of feather growth. This method offers a longer-term measure of CORT levels and provides a more integrated assessment of both baseline and stress-induced CORT responses (Palma *et al.*, 2020). However, interpreting CORT data still requires careful consideration of environment and biological factors to ensure accurate conclusions about the organism's physiological state.

1.1.3 Oxidative stress as an important physiological biomarker

Oxidative stress occurs when the balance between reactive oxygen species (ROS) and antioxidant defences is disrupted, leading to an excess of ROS in comparison to enzymatic and non-enzymatic antioxidants (Costantini *et al.*, 2010; Monaghan, Metcalfe and Torres, 2009). While ROS play important roles in cellular signalling and regulation, excessive ROS levels can cause significant damage to DNA, lipids and proteins due to their highly reactive nature (Costantini *et al.*, 2010; Monaghan, Metcalfe and Torres, 2009). Antioxidants can mitigate this damage by neutralising ROS, forming a complex and dynamic defence system (Monaghan, Metcalfe and Torres, 2009).

Oxidative stress has gained widespread attention as a biomarker due to its links to various diseases and conditions and its association to fitness-related traits, including survival and reproduction (Beaulieu and Costantini, 2014; Ito, Sono and Ito, 2019; Losdat *et al.*, 2013; Luo *et al.*, 2020). For example, oxidative stress is implicated in the formation of atherosclerotic plaques in diabetes, where lipid peroxidation is used as a biomarker for disease risk and progression (Ito, Sono and Ito, 2019). In ecological studies, oxidative stress has been associated with disease susceptibility to herpesvirus in magnificent frigatebird *(Fregata magnificens)* nestlings (Sebastiano *et al.*, 2017). It has also been linked to various fitness traits in great tits *(Parus major)* including fledgling success (Losdat *et al.*, 2013).

Oxidative stress also has a role in cellular senescence and has therefore become a central focus in ageing and ageing related disease research (Luo *et al.*, 2020; Monaghan, Metcalfe and Torres, 2009; Reichert and Stier, 2017; Speakman *et al.*, 2015). Telomeres, which are highly rich in guanine and have limited DNA repair capacity, are particularly susceptible to oxidative damage (Metcalfe and Olsson, 2021; Wang *et al*., 2010). As telomere damage accumulates this accelerates cellular senescence, contributing to ageing-related disease and carcinogenesis (Ahmed and Lingner, 2018; Barnes, Fouquerel and Opresko, 2019; Houben *et al.*, 2008; Luo *et al.*, 2020; Reichert and Stier, 2017). Despite the importance of oxidative stress in ageing research, there are studies that have failed to show significant links between telomere length and oxidative stress (Boonekamp *et al.*, 2017; Luo *et al.*, 2020; Reichert and Stier, 2017). This could be due to high within-individual variation in oxidative stress levels masking this relationship as oxidative stress factors are highly labile and variable over short and long periods (Costantini, 2019; Hõrak and Cohen, 2010). For

example, short-term variations in oxidative stress can result from immune challenges (Vider *et al.,* 2001; Schneeberger, Czirjak and Voigt, 2013), and fluctuations in food availability (Lemieux *et al.,* 2019), while long-term variation may arise due to seasonal cycles (Bhat *et al.,* 2008; Kosaruk *et al*., 2023), and broader fluctuations in weather patterns (Bilham *et al.,* 2018).

Unlike many biomarkers, oxidative stress provides a dual-faceted measure, and biomarkers fall broadly into two categories: those that measure oxidative damage to biomolecules including lipids, proteins, and DNA, and those that assess antioxidant defences which can either be enzymatic or non-enzymatic (Beaulieu and Costantini, 2014). This dual-faceted nature is important, as measuring both components offers a nuanced insight into an organism's physiological condition and its ability to cope with oxidative challenges. Several reviews have emphasised the value of simultaneously assessing oxidative damage and antioxidant defence to fully understand an organism's oxidative balance (Beaulieu and Costantini, 2014; Costantini, 2008; Metcalfe and Alonso-Álvarez, 2010. However, the oxidative system is highly complex, and markers of oxidative stress often exhibit weak correlations with each other (Dotan, Lichtenberg and Pinchuk, 2004; Hõrak and Cohen, 2010). This highlights the need for a multi-marker assessment conducted simultaneously to fully capture oxidative status (Costantini and Verhulst, 2009; Hõrak and Cohen, 2010; Romero-Haro and Alonso-Álvarez, 2014).

1.1.4 Repeatability and reliability of physiological markers with a focus on oxidative stress.

Repeatability is an essential tool for studying labile physiological traits, as high variability in biomarker measurements can lead to the misinterpretation of results (Rudeck *et al.*, 2020). Repeatability provides insight into the reliability of taking multiple measurements of the same trait in the same individual, as well as providing some understanding into the environmental variance and consistency of that trait (Nakagawa and Schielzeth, 2010). Within-individual repeatability can be used to examine the associations between successive oxidative stress measurements through measuring the proportion of total variation that is consistent across repeated measures of the same individual (Nakagawa and Schielzeth, 2010).

Several meta-analyses have investigated biomarker repeatability both within and among individuals (Kärkkäinen et al., 2022; Taff, Schoenle and Vitousek, 2018). This includes the

biomarker telomere length, which showed an overall moderate to high level of repeatability. Measurement error had a strong impact on telomere length repeatability with studies using TRF to measure telomere length exhibiting high repeatability estimates while repeatability for studies using qPCR methods to measure telomere length was lower and more variable (Kärkkäinen *et al.*, 2022). Another meta-analysis investigated within-individual repeatability of glucocorticoids in vertebrates, these measures exhibited moderate repeatability, though substantial variation was observed across species (Taff, Schoenle and Vitousek, 2018). There is no such comprehensive analysis of the within-individual repeatability of oxidative stress markers, despite oxidative stress being an important biomarker to infer fitness and survival.

Despite the growing use of oxidative stress as a biomarker of health and fitness, it's within-individual repeatability remains poorly understood and no comprehensive analysis investigating this exists. Given its highly variability, oxidative stress can present many challenges when trying to interpret its outcome (Costantini *et al.*, 2010). There are many factors that have been shown to contribute to this variability including environmental changes, geographical location, predation risk, seasonal shifts in diet, reproduction, life stage and life history (Cohen, Hau and Wikelski, 2008; Costantini *et al.*, 2010; Metcalfe and Alonso-Álvarez, 2010; Slos and Stoks, 2008), as well as natural variation that occurs throughout an individual's lifespan (Beaulieu and Costantini, 2014). An example of this is shown in the geographical variation in oxidative stress markers in land iguanas *(Conolophus subcristatus)* across the Galapagos (Costantini *et al*., 2009) and in mussels *(Mytilus edulis)* within the Baltic Sea (Prevodnik *et al*., 2007). It was also shown that diet type affects some circulating antioxidants in 95 bird species across and within species as well as across months and season had a substantial impact on this finding (Cohen, Klasing and Ricklefs, 2007).

Additionally, there are numerous assays used to measure oxidative stress and assay variability can mask any true biological variation (Hõrak and Cohen, 2010). Despite its importance, few studies report the within-individual repeatability of oxidative stress markers over time. This raises critical questions about the reliability of these markers in long-term studies, there is a real need to understand if oxidative stress is repeatable within individuals and to understand what factors both biological and methodological may be driving repeatability levels in oxidative stress markers.

1.2 Urbanisation and its ecological impacts

Urbanisation is accelerating at an unprecedented rate, with the global urban population projected to reach 6.3 billion by 2050. This growth, especially in low-income areas, is driven by various factors including natural population growth, migration, government policies, infrastructure development, and broader political and economic forces (Alirol *et al*., 2011; Huang *et al*., 2010; Zhang, 2016). This rapid expansion of urban areas poses significant challenges to biodiversity and ecosystems impacting the survival, fitness and health of wild animal populations (Hassell *et al*., 2017; Murray *et al*., 2019; Sepp *et al*., 2017). However, responses to urbanisation vary substantially both within and between species (Aronson *et al*., 2014). While some species struggle to adapt, others thrive, and urban areas can even support endemic species when properly managed, potentially maintaining or even increasing biodiversity (Aronson *et al.*, 2014). For example, Australian cities are hotspots for threatened species as they offer predictable food sources and a warmer microclimate (Ives *et al.*, 2016).

Understanding the relationship between urbanisation and animal health is critical for conservation and management. Identifying the contexts in which urbanisation impacts wildlife health can help conservation efforts become more targeted, allowing for the prioritisation of certain species and locations.

1.2.1 Anthropogenic stressors and wildlife health

Urban environments introduce various novel anthropogenic stressors that disrupt natural ecosystems and impact wildlife. These include noise pollution, altered food availability, air pollution, and artificial light at night (ALAN), all of which can negatively affect species physiology, morphology, and disease susceptibility (Brumm *et al*., 2021; Dorado-Correa *et al*., 2018; Injaian, Poon, *et al*., 2018; Injaian, *et al.,* 2018; Meillère *et al*., 2015b).

Noise pollution

Noise pollution primarily arises from transportation networks (e.g. highways, city roads and railways) and short-term human activities such as construction. It has been shown to interfere with communication, predator-prey interactions, and stress responses in wildlife (Abbaspour *et al.*, 2015; Isaksson, 2015; Shannon *et al.*, 2016). For example, in passerine species, traffic noise has been shown to disrupt cognitive function, delaying song

development as a result (Brumm *et al*., 2021). Traffic noise causes an avoidance response in Daubenton's bats *(Myotis daybentonii)* reducing foraging efficiency (Luo, Siemers and Koselj, 2015). Song sparrows *(Melospiza melodia)* in noisy areas increase vigilance behaviour at the expense of foraging, as they rely more on visual cues than auditory ones for predator detection (Sweet *et al.*, 2022).

The effects of noise pollution are not limited to terrestrial species. In marine environments, low-frequency noise causes severe acoustic trauma in cephalopods, impairing their sense of balance and position (André *et al.*, 2011). These disturbances can negatively impact species fitness and reproductive success. Noise pollution also poses health risks for humans, contributing to disturbed sleep, high blood pressure and psycho-physiological symptoms (Silva and Mendes, 2012).

Food availability

Food availability is a major constraint for wildlife in cities. Urban areas can create phenological mismatches between resource availability and breeding cycles, for example, great tits experience lower fledgling success in cities due to a prolonged but less concentrated caterpillar season, resulting in reduced peak prey availability during the critical nestling period (Capilla-Lasheras *et al.*, 2017; Seress *et al.*, 2020). Caterpillars provide essential nutrients including carotenoids, fatty acids and vitamin E which are crucial for nestling development (Biard, Surai and Møller, 2006; Surai, Speake and Sparks, 2001; Timmeleht *et al*., 2006). However, alternative food sources, including anthropogenic food, often lack these key nutrients (Derryberry and Coomes, 2020; Seress *et al.*, 2018).

In some cases, urban food sources such as human waste and supplemental feeding (i.e birdfeeders) can be beneficial by providing a consistent year-round food supply. For example, white storks *(Ciconia ciconia)* in Cordoba exploit open rubbish dumps as a stable food resource, leading to a higher breeding success for individuals nesting closer to these sites (Tortosa and López, 2002). However, reliance on anthropogenic food can often come at a cost. Urban coyotes *(Canis latrans)* that consume more human-derived food sources assimilate less protein and are more likely to suffer from sarcoptic mange (Murray *et al*., 2015a). Similarly, non-breeding Cape gannets *(Morus capensis)* benefit from supplementing their diets with fishery waste, however breeding individuals will double their diving efforts to provide higher quality live fish for their chicks. When natural resources are unavailable, breeding efforts usually fail (Grémillet *et al.*, 2008).

Supplemental food sources, including the use of bird feeders, also poses disease transmission risks due to high-density feeding and food contamination (Lawson *et al.*, 2018; Meillère *et al.*, 2015a; Wilcoxen *et al.*, 2015). For example, in areas with a higher density of bird feeders, Mycoplasma gallisepicum transmission rates increase in house finches *(Haemorhous mexicanus)*. Similar patterns have also been observed for other avian diseases, including finch trichomoniasis, Paridae pox, and passerine salmonellosis (Aberle *et al.*, 2020; Lawson *et al.*, 2018; Sykes, Hutton and McGraw, 2021).

Air pollution and climate change

Cities are responsible for over 80% of global carbon dioxide emissions which are typically released from traffic and industrial activities, making them key drivers of climate change (Silva and Mendes, 2012). Urban air pollution consists of various greenhouse gases, including carbon dioxide, methane, nitrous oxide, and halocarbons (Baklanov, Molina and Gauss, 2016). This poses serious health risks for both wildlife and humans, increasing the incidence of heart disease and respiratory conditions (Mabahwi, Leh and Omar, 2014; Silva and Mendes, 2012).

Urban development also introduces the urban heat island (UHI) effect, where cities experience higher air temperatures than surrounding rural areas due to increased impervious surfaces (Abutaleb *et al.*, 2015). The UHI effect not only raises ambient temperatures, but it also worsens air pollution by trapping pollutants in the atmosphere (Baklanov, Molina and Gauss, 2016). The impact of air pollution varies across regions, for instance, South Asian megacities experience significantly higher pollution levels and morbidity risks than cities such as Los Angeles, New York and Tokyo (Gurjar *et al.*, 2010).

Light pollution

Light pollution, or artificial light at night (ALAN), is another pervasive urban stressor that interferes with natural circadian rhythms disrupting behaviours including reproduction, migration, feeding, and sleep (Isaksson, 2015). Despite growing research into the ecological effects of ALAN, its long-term physiological consequences remain poorly understood. Given its widespread and increasing prevalence in urban areas, understanding the relationship between ALAN and wildlife health is crucial for conservation and management. This novel stressor will be explored in more detail in section 1.3.

1.2.2 Life History Traits and Urban Adaptation

Urbanisation does not affect all species equally. Some, known as urban exploiters, thrive in urban environments by taking full advantage of human-modified landscapes and food sources. Examples include carrion crow *(Corvus corone),* rock pigeon *(Columba livia),* and small mammals such as brown rats *(Rattus norvegicus)* and rabbits *(Oryctolagus cuniculus)* (Isaksson, 2015). Other species, known as urban adapters, can survive in cities but do not necessarily thrive. These species often face environmental constraints that limit their success in urban habitats (Isaksson, 2015). Many songbird species, including blue tits *(Cyanistes caeruleus),* robins *(Erithacus rubecula),* and house finches *(Haemorhous mexicanus),* are considered urban adapters (Isaksson, 2015). Conversely, urban avoiders, which include many specialist species, struggle to survive in urban areas. These species often suffer population declines or will disappear altogether as urbanisation intensifies (Isaksson, 2015; Winarni *et al.*, 2022). Certain traits can determine an individual's ability to exploit urban environments. Urban exploiters tend to have broader diets and greater behavioural plasticity, allowing them to adjust to novel conditions (Palacio, 2020). In contrast, species with more specialised foraging needs may be unable to take full advantage of urban resources, limiting their ability to thrive in these environments (Palacio, 2020).

A large meta-analysis revealed that species with specific life-history traits, such as ground-nesting birds (skylarks, waders, waterfowl), are particularly vulnerable to urbanisation due to a lack of suitable habitats and increased predation risk (Lakatos *et al.*, 2022). Ground feeding birds also face challenges in cities due to the scarcity of suitable, undisturbed open grasslands (Lakatos *et al.*, 2022). Food and nesting availability are major constraints for specialist species, as urban environments typically lack the resources that these species require. In contrast, generalist species are better able to adapt to changing conditions and exploit available resources (Máthé and Batáry, 2015).

Additionally, certain life-stages may also be more vulnerable to urban stressors. For example, even a small increase in ambient temperature can trigger a stress reaction in nestling pied flycatchers *(Ficedua hypoleuca)* within the first 12 days of life (Skwarska *et al.*, 2022). It is known that anthropogenic change can cause biotic homogenization where biodiversity will decline due to the disappearance of some species while other species increase in abundance mainly due to species-specific traits playing a role (Willmott *et al.*,

2022). It is vital that we understand which conditions allow for which species to thrive to implement biodiversity management.

1.2.3 Variability in urban environments

One of the greatest challenges in studying the effects of urbanisation is the heterogeneity of urban environments. Local factors such as tree composition, air pollution, and temperature differ significantly between locations, making it difficult to draw general conclusions about the effects of urbanisation (Jensen *et al.*, 2023; Seto, Sánchez-Rodríguez and Fragkias, 2010). There are substantial differences both within and between cities in terms of climate, habitat structure, human population density, and socio-economic density (Luck, Smallbone and O'Brien, 2009; Richards *et al*., 2019). Any of these factors can influence how much of an effect urbanisation will have on wildlife (Krehl *et al.*, 2016; Ouyang *et al.*, 2018).

There is evidence that the degree of urbanisation can be a major factor in influencing wildlife health. For example, parasite prevalence in red-browed finches *(Neochmia temporalis)* was higher with an increasing degree of urbanisation (Delgado-V and French, 2015). Similarly, an increase in urban cover was also associated with influencing innate immune function in nestling black sparrowhawks *(Accipiter melanoleuscus)* (Nwaogu *et al.*, 2023). The level of urbanisation also explains significant variation in physiological biomarkers across several bird species (Chavez-Zichinelli *et al.*, 2013; Liker *et al.*, 2008).

Given this complexity, treating urban and non-urban environments as binary categories may be overly simplistic. Instead, considering gradients of urbanisation could provide deeper insights into how wildlife responds to urban stressors. Reviews and syntheses of the literature suggest that future research should move away from binary habitat classifications and instead focus on quantifying environmental factors within urban environments. This approach could improve comparability between studies and facilitate broader generalisations (Ouyang *et al.*, 2018; Murray *et al.*, 2019).

By identifying the factors that drive species responses to urbanisation, we can better inform urban management strategies aimed at maintaining or enhancing biodiversity and wildlife health.

1.2.4 Impacts of urbanisation on health and fitness

The cumulative effects of urban stressors can lead to negative consequences for wildlife health, although responses vary across species and habitats. These effects can be seen in several key areas of health, including physiological, and morphological changes, as well as disease susceptibility.

Physiological impacts

Urbanisation has been associated with various physiological changes in wildlife, including alterations in immune function, higher glucocorticoid levels and accelerated telomere shortening (Beaugeard *et al*., 2019; Capilla-Lasheras *et al*., 2017; Salmón *et al*., 2016; Zhang *et al*., 2011). For example, urbanisation has been linked to higher feather corticosterone levels in juvenile house sparrows *(Passer domesticus),* suggesting elevated chronic stress (Beaugeard *et al.*, 2019). Similarly, baseline corticosterone levels have been shown to be higher in urban tree sparrows *(Passer montanus)* although this effect varies across different urban habitat types (Zhang *et al.*, 2011). Gene expression analysis has shown that urban great tits have consistently higher expression of immune-related genes, likely due to increased pathogen exposure in urban environments (Capilla-Lasheras *et al.*, 2017). These findings suggest that the physiological impacts of urbanisation are species and habitat dependent.

Telomere shortening, a biomarker of ageing and cellular stress, appears to be accelerated in urban wildlife populations. Great tit nestlings exhibit faster telomere attrition after brief exposure to urban areas (Salmón *et al.*, 2016), while adult urban blackbirds *(Turdus merula)* have shorter telomeres than their forest counterparts, regardless of age (Ibáñez-Álamo *et al.*, 2018). There is also evidence that exposure to noise pollution can accelerate telomere shortening in juvenile zebra finches and developing house sparrows (Dorado-Correa *et al.*, 2018; Meillère *et al.*, 2015b). These impacts can reduce lifespan and long-term survival in urban populations (Boonekamp *et al*., 2014; Koren *et al*., 2011; Leishout *et al*., 2019; MacLeod *et al*., 2018).

However, not all studies report negative physiological effects of urbanisation. For example, urban blackbirds were found to have no significant differences in immune function or CORT levels compared to forest blackbirds (Ibáñez-Álamo *et al.*, 2020). These contrasting

results highlight the complexity of urbanisations effects and the need for species-specific investigations.

Morphological changes and body condition

Urban-dwelling birds often exhibit poorer body condition, often due to nutrient-deficient diets. Urban diets often lack essential nutrients such as carotenoids, fatty acids, and vitamins, which are critical for proper growth and development. Great tit nestlings in urban environments have been found to suffer from nutrient deficiencies, leading to a lower body condition compared to their rural counterparts (Bailly *et al.*, 2017). Studies have also reported a continuous negative correlation between increasing urbanisation and body condition in house sparrows (Jiménez-Peñuela *et al.*, 2019; Liker *et al.*, 2008). Similarly, urbanisation has been linked to changes in morphological traits, including a smaller body size and reduced tarsus length in nestling great tits (Caizergues *et al.*, 2021). However, there are contrasting results. One study found no significant effect of urbanisation on adult house sparrow body condition (Bókony *et al.*, 2012), indicating that the impacts may be age dependent as has been shown in previous studies (Caizegues *et al*., 2021; Meillère *et al.,* 2015a) or influenced by additional environmental factors such as local weather conditions (McCloy, Glasscock and Grace, 2023; McCloy and Grace, 2023) or fluctuations in food availability (Brown and Sherry, 2006).

Species-specific responses further complicate patterns. For example, in South Africa, the non-native house sparrow benefitted from urbanisation, exhibiting greater body mass and improved condition in highly urbanised areas. In contrast, the native Cape sparrow *(Passer melanurus)* showed the opposite trend, experiencing poor body condition in more urbanised areas (Naidoo, Chamberlain and Reynolds, 2024). These findings reinforce the idea that certain species possess traits that allow them to exploit urban landscapes, while others are negatively affected.

Disease and parasitism

Many studies suggest that urban wildlife face a higher risk of disease and parasitism. For example, urban populations of white ibis *(Eudocimus albus)* populations, exhibit less diverse gut microbiomes, which can compromise immune function and increase susceptibility to pathogens. Consequently, these urban birds show a higher Salmonella prevalence than their rural conspecifics (Murray *et al.*, 2020).

A global meta-analysis found that the impacts of urbanisation on parasitism can depend on the life cycle of the parasite (Werner and Nunn, 2020). Parasites that require multiple hosts tend to decline in urban habitats, reducing parasite burdens in urban wildlife (Werner and Nunn, 2020). In contrast parasites with simpler life cycles can thrive in urban environments, leading to higher infection rates (Werner and Nunn, 2020). The impact of urbanisation on haemosporidian parasite richness in the common chlorospingus *(Chlorospingus flavopectus)* varies depending on the parasite lineage, with some thriving in urban areas, while others rely on well-preserved forests (Hernández-Lara, Carbó-Ramírez and Santiago-Alarcon, 2020). Parasite burden can also differ between species. In red-browed finches *(Neochima temporalis)* the prevalence of parasites was higher in more urbanised areas, but urbanisation did not impact parasite burden in superb fairywrens *(Malurus cyaneus)* which could be due to many factors including behaviour, habitat and diet (Delgado-V and French, 2015).

Therefore, there are many studies that show urban environments may have profound effects on organismal processes linked to health including physiology, morphology and disease susceptibility. However, other studies have contrasting results, revealing that there are either no or limited effects of urbanisation on health. Indeed, in some cases urbanisation appears to have positive implications for health, such as through reducing parasite burden. There are many factors that may impact the relationship between urbanisation and wildlife health including life-history traits of species, the type of habitat, or the biomarker measured, and we need to understand the independent role of each in contributing to this complex, multivariate relationship.

As urbanisation continues to increase, there is a growing concern about the negative impacts of the novel stressors that are introduced in urban environments. One of these stressors is ALAN which can cause many physiological disruptions. The next section will focus specifically on ALAN as an urban stressor, its mechanisms, and its impact on wildlife health.

1.3 Artificial light at night as an urban stressor

1.3.1 What is artificial light at night?

Urbanisation introduces many novel stressors to wildlife, and artificial light at night (ALAN) has become an important and widespread factor. The rapid expansion of human

populations has led to the proliferation of artificial lighting into previously dark areas, fundamentally altering nocturnal environments (Dimovski and Robert, 2018). ALAN has become an integral part of modern life, providing safety, enabling the extension of work hours, and facilitating social interactions after dark (Gaston and Sánchez De Miguel, 2022). However, the transition to energy-efficient lighting to reduce emissions, particularly the use of white energy-efficient light emitting diodes (LEDs), has introduced new ecological challenges. White LEDs emit broad-spectrum light, with a strong peak in short, blue wavelengths, which have been linked to adverse environmental and health effects (Dimovski and Robert, 2018; Kyba *et al.*, 2017).

ALAN manifests in different forms, including sky glow, where upward-directed light is scattered by particles in the atmosphere, brightening the night sky and disrupting natural cycles (Gaston and Sánchez De Miguel, 2022). Light trespass occurs when artificial light spills into areas that would otherwise be dark, this can be caused by floodlights or streetlights. Over-illumination refers to the excessive use of artificial light beyond necessity (Chepesiuk, 2009; Gaston *et al.*, 2013). The sources of ALAN are diverse, ranging from streetlights and advertising displays to vehicle headlights and domestic lighting (Gaston *et al.*, 2013). Quantifying the full extent of ALAN remains a challenge, with global estimates often underrepresenting its spread and long-term environmental impacts (Gaston *et al.*, 2015; Gaston and Sánchez De Miguel, 2022). Over the past century, natural light patterns have been increasingly disrupted by ALAN, causing profound behavioural and physiological consequences for both wildlife and humans (Gaston *et al.*, 2013).

1.3.2 Mechanisms behind the impact of artificial light at night on wildlife

Daily light-dark cycles, driven by the earth's rotation, are crucial for regulating biological rhythms in most organisms (Russart and Nelson, 2018a; Yadav et al., 2022). Circadian rhythms, which operate on approximately 24-hour cycles, synchronise an organism's behaviour and physiology with environmental cues, including light (Russart and Nelson, 2018b; Yadav et al., 2022). This allows organisms to optimise activity patterns and resource use while minimising risks (Dominoni, Partecke and Partecke, 2015; Russart and Nelson, 2018b).

ALAN disrupts this synchronisation by artificially extending the perceived daylight. While some species can benefit from increased illumination at night, such as the common

redshank *(Tringa tetanus),* which can increase foraging efficiency under artificial light sources when overwintering in Northern Europe (Dwyer *et al.*, 2013), many other species experience negative consequences. Species with strong circadian regulation, such as birds, have shown to exhibit heightened nocturnal activity and will wake up earlier in response to ALAN exposure (Alaasam *et al*., 2018; Dominoni, Quetting and Partecke, 2013; Dominoni, Partecke and Partecke, 2015; De Jong *et al*., 2017; Yadav *et al*., 2022). For instance, breeding colonies of common swifts *(Apus apus)* in Israel exhibited higher levels of nighttime activity under intense artificial lighting (Amichai and Kronfeld-Schor, 2019).

ALAN also disrupts other critical behaviours like migration, as artificial lights disorient migrating birds, leading to excessive energy expenditure and increase the risks of predation and collision with manmade structures (Van Doren *et al.*, 2017). Species such as tammar wallabies *(Macropus eugenii)* use day length as an important cue for reproduction, as a result light pollution has been shown to mask seasonal changes in ambient light cues which delays birth and causes lower offspring survival due to a trophic mismatch (Robert *et al.*, 2015).

Many physiological processes, including hormone production and energy regulation, are under circadian control, and ALAN-induced circadian misalignment can disrupt these functions (Mason *et al.*, 2020). Many species, including humans, exhibit daily fluctuations in glucose levels that align with activity periods and feeding patterns (Downs, Wellmann and Brown, 2010; Lobban, Downs and Brown, 2010; Mason *et al.*, 2020). Disruptions in these cycles can impair energy homeostasis. Glucocorticoid hormones are also under circadian control and may become dysregulated with ALAN exposure (Guan *et al.*, 2022). There is also evidence that telomerase activity is under circadian control, in mice cultures, deficiency in the CLOCK gene lead to significant changes in telomere length and causes the loss of rhythmic telomerase activities (Chen *et al.*, 2014). Additionally, ALAN can suppress melatonin (De Jong et al., 2017; Russart and Nelson, 2018a), which is a hormone produced by the pineal gland at night. This can affect behaviours such as the time of singing in songbird species (Dominoni, Quetting and Partecke, 2013; Dominoni et al., 2013) and potentially increasing oxidative stress due to its antioxidant role (Jones *et al.*, 2015). These different mechanisms of disruption by ALAN may underpin many of the health and fitness consequences in wildlife exposed to ALAN.

1.3.3 Impacts of artificial light at night on physiology and health

ALAN has been linked to metabolic disturbances across species. In humans, circadian misalignment caused by shift work or jet lag has been associated with increased risks of metabolic disorders, such as type 2 diabetes (Mason *et al.*, 2020). Experimental studies show that ALAN-exposed mice exhibit disturbed feeding patterns, consuming more food during their inactive phase, leading to weight gain (Arble et al., 2009; Guan *et al*., 2022; Russart and Nelson, 2018b). Similarly, zebra finches *(Taeniopygia guttata)* exposed to ALAN displayed higher levels of fat deposition and metabolic abnormalities (Batra, Malik and Kumar, 2019). In contrast, developing birds exposed to ALAN showed lower body condition and body mass than their rural counterparts (Ferraro, Le and Francis, 2020; Grunst *et al.*, 2019).

Many hormones, including corticosterone, have been shown to be under circadian control and may therefore be vulnerable to disruption from ALAN exposure (Guan *et al.*, 2022). For example, great tit nestlings exposed to ALAN showed higher levels of feather corticosterone (Grunst *et al.*, 2019). Similarly, this has been shown in several bird studies where exposure to ALAN led to elevated CORT levels (Alaasam *et al.*, 2018; Dominoni *et al.*, 2021). Although, these effects have been shown to be dependent on the location of the experiment. In contrast, the opposite effect was found in adult cane toads *(Rhinella marina)* where CORT levels decreased as intensity increased (Secondi *et al.*, 2021). Exposure to ALAN was also shown to cause endocrine disruption in Eurasian perch *(Perca fluviatilis),* through the alteration of thyroid hormones (Kupprat *et al.*, 2020).

Oxidative stress and immune response have also been shown to be strongly linked with circadian rhythms. In rodents, reduced melatonin due to ALAN exposure has been shown to alter antioxidative defence and lead to oxidative damage (Verma *et al.*, 2022). In humans, night shift workers experience lower levels of antioxidant defence and higher levels of oxidative damage in comparison to daytime workers (Teixeira *et al.*, 2019). Similarly, great tit nestlings exposed to ALAN showed significant changes in inflammatory markers, which could limit their ability to mount an immune response. These birds also had 49% lower levels of melatonin, which may explain their reduced immune function (Raap *et al.,* 2016b; Ziegler *et al.*, 2021).

The relationship between ALAN and telomere shortening is well established in human studies, where women working consecutive night shifts experienced higher levels of

telomere shortening, which is linked to a higher risk of developing breast cancer (Erdem *et al.*, 2017). Another study was able to show a link between night shifts and sleep duration in humans where those who experienced less sleep had smaller telomeres than those that had over 10 hours of sleep (Liang *et al.*, 2011). However, research on birds has yielded limited evidence that ALAN impacts telomere shortening, with some studies finding no significant link (Grunst *et al.*, 2019; Grunst *et al.,* 2020; Ouyang *et al.*, 2017), despite evidence that urban environments accelerate telomere shortening (Salmón *et al.*, 2016).

Fitness consequences

Disruptions in metabolism, hormonal regulation, and immune responses can affect overall fitness and survival (Beale *et al*., 2024; Brown-Borg, 2007; Graham *et al*., 2010; Hanssen *et al*., 2003; Nelson and Chbeir, 2018; Ruff *et al*., 2013; Vitousek *et al*., 2018b). For example, ALAN-exposed pet Australian budgerigars *(Melopsittacus undulatus)* showed lower reproductive success, including fewer eggs laid and a decline in hatchability (Malek and Haim, 2019). ALAN could potentially have a stronger effect during the sensitive developmental period which may have knock on effects on their fitness and survival as adults (Monaghan, 2007; Blas *et al*., 2005). In many bird species, newly hatched young are entirely dependent on their parents for care and resources and even when parental quality is high, environmental stressors can still have a negative impact (Quirici *et al.*, 2016).

1.3.4 Potential mitigation strategies to target artificial light at night

As urbanisation continues, ALAN will only expand, necessitating the exploration of effective mitigation strategies to reduce its negative effects on wildlife. Potential strategies include adjusting the intensity, colour and duration of ALAN exposure.

Research suggests that the intensity of light can significantly influence the effects of ALAN. In human's, metabolic homeostasis was shown to be heightened with higher levels of light intensity (Abay and Amare, 2018). Reducing the intensity of light has also been shown to reduce any negative metabolic effects in lab mice (Fan *et al.*, 2022). It has also been shown in captive European starlings *(Sturnus vulgaris)* that birds exposed to a high frequency light exhibited higher basal corticosterone levels than the birds exposed to a low frequency light (Evans *et al.*, 2012). Birds have been shown to advance their activity when exposed to higher light intensities during the night and will stay active for longer at the end of the day. An increase in light intensity at night was also shown to further decrease the

hormone melatonin (De Jong et al., 2017). This shows that light intensity is an important factor to consider, however negative impacts of ALAN are still present even at low light intensities (Dominoni *et al*., 2013; Sanders *et al*., 2021).

Studies have also investigated the use of different ALAN colours on wildlife. It was shown that chickens exposed to green light experienced a reduction in body metabolism with an increase in both thyroid hormones and body mass (Gharahveysi *et al.*, 2020). These effects appeared to be dampened when the birds were exposed to yellow or red light (Gharahveysi *et al.*, 2020). This was also shown to be the case in another study where effects on physiology and behaviour appeared to be lessened when birds were exposed to green or red-light treatments compared to when they were exposed to white light treatments (Ouyang *et al.*, 2017). The insights offered from these studies reveal that alternative light spectra could potentially be utilised to mitigate physiological costs of ALAN exposure in wildlife.

Another mitigation approach which has remained understudied, is partial night lighting, where lights would be switched off for a few hours during the night to reduce exposure to ALAN. Studies looking into this strategy have yielded mixed results, for example partial night lighting reduced some of the negative effects of ALAN on behavioural daily rhythms in oysters (Botté *et al.*, 2023). However, other studies have shown that partial night lighting has no significant impact on the activity of several bat species when lights were turned off from midnight to 5am, these studies suggest that for the partial night lighting strategy to work lights would have to be turned off even earlier (Azam *et al.*, 2015; Day *et al.*, 2015). Most of the studies investigating partial night lighting have focused on behavioural outcomes and there has not been much focus on the benefits such a strategy could have on wildlife health. If it was shown that partial night lighting could have benefits for wildlife in urban areas, this strategy would also have huge economic benefits by reducing electricity usage, saving energy and reducing carbon emissions (Pagden, Ngahane and Amin, 2020). However, more has to be done to investigate the benefits of this strategy on different species, in different environmental contexts and on different outcomes including health.

1.4 Research focus and rationale

In this chapter, I provide a comprehensive review of the relationship between urbanisation and wildlife health. I begin by discussing the challenges of measuring health in ecological

systems and highlight the importance of understanding the repeatability and reliability of biomarkers that are used to assess individual health and physiology. I then outline the key stressors and environmental heterogeneity associated with urban habitats, followed by a discussion on how urbanisation impacts wildlife health and physiology. Finally, I focus on ALAN as a specific urban stressor, exploring its effects on behaviour and health.

In chapter 2, I explore the relationship between urbanisation and avian health on a global scale using a meta-analysis approach. While some evidence suggests that urbanisation negatively impacts avian health, findings remain inconclusive due to variability in species-specific traits, health biomarkers measured, and geographic locations. To address this, I conducted a meta-analysis encompassing all avian species, age classes, and health biomarkers to identify key variables driving this relationship. A crucial part of my approach was quantifying the level of urbanisation at each location used in the meta-analysis and using this as a continuous variable in meta-regression models. This approach aimed to uncover any subtle effects that may be hidden when urban areas are simply classified as urban versus non-urban. Many studies adopt this binary classification, which may mask nuanced negative impacts of urbanisation.

In chapter 3, I focus on ALAN as a specific anthropogenic stressor, investigating its effects on avian health during the sensitive developmental period. To achieve this, I exposed great tit nestlings to dim ALAN during their first two weeks of life using artificial nest boxes in a field setting. Most studies that focus on nestlings examine short-term exposure to ALAN, with the exception of Dominoni *et al.* (2021). Additionally, measuring health is complex as it is a multivariate trait. Therefore, to provide a comprehensive assessment, I examined four biomarkers of health which were scaled mass index (SMI), antioxidant capacity of plasma (OXY), malondialdehyde (MDA), and feather corticosterone (fCORT).

Building on this, chapter 4 extends the investigation to assess ALANs long-term effects on avian health. Using a longitudinal approach, I exposed adult zebra finches to dim ALAN for four months in a controlled captive setting. Most experimental studies, particularly in captivity, focus on short term exposure of ALAN, however there is a pressing need for longitudinal studies to assess long-term impacts. To address the complexity of measuring health, I analysed four biomarkers which were the circadian variation of glucose, telomere shortening, OXY and MDA. At least two samples were taken from each individual, at both the start and end of the experiment, enabling an assessment of physiological changes over

time. Additionally, given the growing need for mitigation strategies to reduce ALAN's negative impacts on wildlife, I tested a potential mitigation strategy which was partial night lighting. One group of birds was exposed to this reduced lighting condition, and I compared their health outcomes to the birds that were exposed to full-night lighting to assess whether this approach mitigated ALAN's effects.

In chapter 5, I shifted focus to the methodological challenges of measuring health in ecological studies. Many physiological biomarkers in wildlife exhibit high within- and between- individual variability, leading to inconsistent results across studies. While meta-analyses have examined the within-individual repeatability of certain biomarkers (including glucocorticoids, metabolic rate and telomere length), no comprehensive review has focused on oxidative stress biomarkers. Since oxidative stress markers are widely used to infer fitness and survival prospects but are known to fluctuate over short and long periods of time. I conducted a meta-analysis to assess their within-individual repeatability across non-human species. I also explored biological and methodological factors influencing this variability, including taxonomic class, environment, sex, oxidative stress marker type, time between repeat measurements, and sample size. Given the relationship between oxidative stress and telomere shortening, oxidative stress markers are frequently used in ageing studies. However, if these markers have low repeatability, studies may require substantially larger sample sizes to achieve sufficient statistical power to detect an association between oxidative stress and telomere shortening. To address this, I conducted a simulation study to determine the minimum sample size required to detect an association between telomere length and oxidative stress at varying levels of repeatability for oxidative stress. Additionally, I assessed whether repeated sampling from the same individuals could reduce overall sample size requirements at low oxidative stress repeatability levels.

In chapter 6, I synthesis the findings from the data chapters, discussing their broader implications, discussing the key challenges encountered, and potential directions for future research as well as providing overall conclusions.

1.5 Thesis aims and objectives

1. To understand the complex relationship between urbanisation and avian health through a global meta-analysis across species, health biomarkers and age-classes. I also aimed to identify key factors influencing this relationship, including the degree

of urbanisation, the health biomarker measured, species-specific traits (e.g. behaviour, life stage), and latitude. [Chapter 2]

2. To assess the impact of ALAN on multiple health biomarkers including body condition, oxidative stress and feather CORT levels during the sensitive developmental period of a bird's life by exposing great tit nestlings to ALAN in the wild. [Chapter 3].
3. To examine the long-term impacts of ALAN on avian health by measuring circadian variation of glucose, oxidative stress and telomere shortening in adult zebra finches exposed to prolonged ALAN in captivity. Additionally, I aimed to evaluate whether partial night lighting mitigates the negative effects of ALAN on heath. [Chapter 4].
4. To investigate the within-individual repeatability of oxidative stress markers across non-human animals using a meta-analysis. I also aimed to identify factors influencing repeatability, including taxa, environment, sex, oxidative stress marker type, time between measures, and sample size. [Chapter 5].
5. To determine the minimum sample size required to achieve over 80% power for detecting a relationship between telomere length and oxidative stress at different levels of oxidative stress repeatability using a simulation approach. I also examined whether repeated sampling from the same individuals could reduce overall size requirements [Chapter 5].

Chapter 2

The impact of urbanisation on health depends on the health metric, life-stage and level of urbanisation: A global meta-analysis on avian species.

Rachel Reid[1*], Pablo Capilla-Lasheras[1], Yacob Haddou[1], Jelle Boonekamp[1], Davide M. Dominoni[1]

[1]School of Biodiversity, One Health and Veterinary Medicine, University of Glasgow, Graham Kerr Building, 82 Hillhead Street, G12 8QQ, Glasgow, UK

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2.1 Abstract

Stressors associated with urban habitats have been linked to poor wildlife health but whether a general negative relationship between urbanisation and animal health can be affirmed is unclear. We conducted a meta-analysis of avian literature to test if health biomarkers differed on average between urban and non-urban environments, and whether there are systematic differences across species, biomarkers, life stages, and species traits. Our dataset included 644 effect sizes derived from 112 articles published between 1989 and 2022, on 51 bird species. First, we showed that there is no clear impact of urbanisation on health when we categorised the sampling locations as urban or non-urban. However, we did find a small negative effect of urbanisation on health when this dichotomous variable was replaced by a quantitative variable representing the degree of urbanisation at each location. Secondly, we showed that the effect of urbanisation on avian health was dependent both on the type of health biomarker measured as well, with some effects also varying between life-stages. Our comprehensive analysis calls for future studies to disentangle specific urban-related drivers of health that might be obscured in categorical urban vs non-urban comparisons.

Key words: Urban Ecology, Avian populations, Health biomarkers

2.2 Introduction

Urbanisation is characterized by profound modifications of natural habitats, which includes the creation of impervious surfaces and buildings (Gong et al., 2012; Mathew, Khandelwal and Kaul, 2016), the introduction of high levels of chemicals and metals in the ground (Sharley *et al.*, 2016; Merga *et al.*, 2020), increased levels of air (Duh *et al.*, 2008; Gong *et al.*, 2012), noise and light pollution (Gallaway, Olsen and Mitchell, 2010; Abbaspour *et al.*, 2015), high amounts of refuse, including anthropogenic food sources (Murray et al., 2015b; Theimer et al., 2015), and increased ambient temperatures (the "heat island effect") (Mathew, Khandelwal and Kaul, 2016). In humans, many of these environmental factors have been associated with changes in physiological processes linked to health which has been discussed in great detail in a variety of reviews including Mabahwi, Leh and Omar, (2014) and Tong *et al.* (2022).

Several studies have suggested that these environmental factors associated to urbanisation may not only affect human but also affect wildlife health (Sepp *et al.*, 2019). Noise pollution has been shown to impair reproduction and territorial communication of wild species (Alirol *et al.*, 2011), and one of the suggested mechanisms for this is an increase in the concentration of glucocorticoid levels and inflammatory molecules (Brumm *et al.*, 2021). Similarly, artificial light at night (ALAN) has been shown to disrupt circadian rhythms, including that of several physiological processes, as well as leading to an increase activity and metabolic rate, which may have consequences for health (Isaksson, 2015; Dominoni, Borniger and Nelson, 2016). An increase in chemical and metal pollution has also been associated to downstream health and reproductive consequences (Chatelain *et al.*, 2021). The anthropogenic food that is readily available in urban environments, either directly or indirectly provided to wildlife by humans, often lacks many essential macro and micronutrients, which may result in poor diet and lead to knock-on effects on oxidative stress (Tan, Norhaizen and Liew, 2018; Bacou *et al*., 2021), gut microbiome (Sugden *et al.*, 2020), immunity (Watson *et al.*, 2017), and infection risk (Murray *et al.*, 2016).

Because of the profound effects that each urban environmental factor may have on organismal processes linked to health, there are many studies that show that wildlife populations living in urban habitats are in worse health than their non-urban conspecifics. This is particularly true for birds, one of the most studied animal taxa in urban ecology. Urban great tit (*Parus major*) nestlings have been shown to be smaller and in poorer

condition than non-urban chicks (Kaliński *et al.*, 2015; Salmón *et al.*, 2016; Biard *et al.*, 2017). Increasing levels of urbanisation have been shown to lead to higher feather corticosterone levels in juvenile house sparrows (*Passer domesticus*), which may constrain their development (Beaugeard *et al.*, 2019). Transcriptomics studies have shown that urban blue tits (*Cyanistes careuleus*) and great tits have higher levels of gene transcripts associated to inflammatory responses, compared to their non-urban cousins (Pollock *et al.*, 2017; Watson *et al.*, 2017). However, not all studies support the idea that urbanisation invariably and negatively affects health. In song sparrows (*Melospiza melodia)*, increased levels of urbanisation had no impact on their stress physiology or body condition (Grunst, Rotenberry and Grunst, 2014). Another study found that non-urban burrowing owls (*Athene cunicularia)* actually had higher levels of stress induced corticosterone when compared to urban birds and suggested this could be due to urban birds having adapted to deal with stress more effectively (Palma *et al.*, 2020). In two Australian passerines, Coccidian infection increased with increasing urbanisation in red-brown finches (*Neochima temporalis*), but not in superb fairy wrens (*Malurus cyaneus*) (Delgado-V and French, 2015). In black sparrowhawks (*Accipiter melanoleucus*), urbanisation was negatively associated with some biomarkers of immunity and oxidative stress, while the majority of such markers were not affected (Nwaogu *et al.*, 2023). Similarly, a large comparative analysis on the prevalence of *Salmonella* and *Campylobacter* in birds, two avian gastrointestinal bacteria, showed that while *Salmonella* prevalence was mostly affected by ecological factors including urbanisation where prevalence was higher in urban areas, *Campylobacter* prevalence was largely associated with life-history traits (Minias, 2020).

There are many factors that may impact the relationship between urbanisation and health, for example the early developmental stage for many bird species is a particularly sensitive time, and indeed a large proportion of the mortality in birds occur in early life (Grue and Shipley, 1984; Skwarska *et al.*, 2022; Diehl *et al.*, 2023). Therefore, negative impacts of urbanisation on health may be more apparent in younger birds than adults. A few studies have provided evidence of this, showing that nestling/juvenile birds tend to be exhibit poorer fitness and condition in urban areas while adults of the same species appear to be less affected (Bókony *et al.*, 2012; Meillère *et al.*, 2017; Catto *et al.*, 2021). However, studies have also shown no age-specific urban effects on avian health markers (Salmón *et al.*, 2016; Ibáñez-Álamo *et al.*, 2018). A comprehensive analysis of the literature is thus needed to assess the evidence for general age-dependent urban effects on health. Urbanisation may also impact different bird species in different ways, for instance because the way a species

responds to urban stressors may depend on specific traits, which may either help them to adapt or limit their ability to adapt to such stressors (Meillère *et al.*, 2015b), yet no study to date has looked into this at a global scale. Geographic location, and specifically latitude can also be a factor on how urbanisation impacts health. Recent rates of urbanisation vastly differ between different regions of the world (Seto, Sánchez-Rodríguez and Fragkias, 2010), with faster rates of urbanisation in tropical areas (Neiderud, 2015; Thaweepworadej and Evans, 2023). This means that animals living in different cities around the world have been exposed to different rates of urban-related environmental change in the last decades, and this may affect their ability to cope with such changes and as a result impact their health (Van Hemert, Pearce and Handel, 2014).

Taken all together, while previous research has suggested that urbanisation may impact upon the health of wild avian species, the evidence remains inconclusive and rather suggests that urban-related health change may be dependent on the studied species, the age of the individuals sampled, and location of the study. Moreover, results also strongly differ depending on which health biomarker was measured, and most studies focused only on one or few of such markers. Thus, there is a need to assess the effect of urbanisation on wildlife health by synthesizing and analysing the results of existing literature at the global scale, across all avian species, age classes and biomarkers of health. Here we build on the work of previous meta-analyses that have investigated the relationship between urbanisation and health. These previous works found contrasting results but overall suggests there is a relationship between urbanisation and health and that this relationship may depend on a variety of factors including the study species and health biomarker measured (Becker, Streicker and Altizer, 2015; Murray *et al.*, 2019; Iglesias-Carrasco *et al.*, 2020; Putman and Tippie, 2020; Werner and Nunn, 2020). Crucially, an important part of our approach was that we quantified the level of urbanisation at each location included in our meta-analysis, while sites were traditionally categorised as urban vs non-urban. Urban areas can be spatially heterogeneous and thus the level of urbanisation at each sampled location may strongly influence the difference between urban and non-urban populations (Capilla-Lasheras *et al.*, 2022). Using the level of urbanisation as a continuous variable in a meta-regression analysis may help us to uncover any hidden effects that we may not see when we group all urban areas in one category (Capilla-Lasheras *et al.*, 2022). Moreover, we also included a wider range of health biomarkers than what has been used previously. We believe that this study will be of importance to policymakers as identifying the contexts in which urbanisation impacts wildlife health is crucial to understand what areas need to be directly targeted for

conservation management. This includes a better understanding of what species are more sensitive to urbanisation and why, whether certain life stages are more at risk, as well as what health metrics are most likely to be impacted by urbanisation (and therefore could be prioritised as relevant biomarkers). Such understanding would be crucial to protect and enhance urban biodiversity in a world that is projected to become increasingly more urbanised.

We performed multiple meta-analyse to increase our understanding of the complex relationship between urbanisation and health in birds. We aimed to inform urban ecology theory as well as to guide future urban planning and management efforts. The questions we specifically want to answer are: -

1) Is there an overall impact of urbanisation on bird health? We would expect that there will be an overall negative effect of urbanisation on bird health as it is well established that urban areas have many novel stressors that may impact health (Sepp *et al.*, 2019; Ferraro, Le and Francis, 2020; Brumm *et al.*, 2021; Risi, Sumasgutner and Cunningham, 2021).

2) Is there a relationship between the degree of urbanisation and health? We expect to find a stronger negative relationship between urbanisation and health when the level of urbanisation increases, an increase in urbanisation will likely mean an increase in urban stressors that have the potential to impact health.

3) Are specific health traits more sensitive to urbanisation? We predict that certain health traits are likely to be more sensitive to urbanisation as several studies have found strong effects of urbanisation on health traits such as telomere shortening (Salmón *et al.*, 2016, 2017; Ibáñez-Álamo *et al.*, 2018), other studies have also found contrasting results on the same health trait, for example evidence for effects on corticosterone have been shown to be more variable (Grunst, Rotenberry and Grunst, 2014; Beaugeard *et al.*, 2019). This could suggest the relationship will be dependent on other variables.

4) Is the relationship between urbanisation and health dependent on species specific traits including maximum lifespan, trophic niche, migratory behaviour, primary behaviour, and life stage? We expect that there will be an effect of species-specific traits on this relationship. Studies have shown that having specific traits can either help a species adapt or will hinder their ability to adapt to urban environments (Catto *et al.*, 2021). For

example, it has been shown that nestlings will be more sensitive to environmental change than adults so we may find a stronger effect of urbanisation on health in this particular life stage (Grue and Shipley, 1984; Bókony *et al.*, 2012; Skwarska *et al.*, 2019). Species that have certain trophic niches may also be more sensitive to urbanisation for example we may find that insectivores are in poorer health due to reduced food availability (Máthé and Batáry, 2015)

5) Is the relationship between urbanisation and health dependent on latitude? We expect that there will be an effect of latitude on the relationship between urbanisation and health. Rates of urbanisation vastly differ around the world and species that inhabit regions where the rates of urbanisation are increasingly rapidly may be more sensitive to these changes (Van Hemert, Pearce and Handel, 2014; Smit, 2021).

2.3 Methods

2.3.1 Literature search

We first created a search string using key words that are relevant to the study, selected by reading relevant literature. The search string which we used for the meta-analysis is shown below.

(“City*” OR “Urban*”) AND (“Aves” OR “Avian” OR “Bird*” OR “Ornithol*” OR “Passerine*” OR “Passeriform*” OR “Songbird*”) AND (“Body condition” OR “Corticosterone*” OR “CORT*” OR “Disease*” OR “Infection*” OR “Immun*” OR “Oxidative stress” OR “Parasite*” OR “Stress*” OR "Telom*" OR "Telomere attrition" OR "Telomere shortening")

This search string was used to search for relevant peer-reviewed published papers on the web of science core collection. All the literature that was flagged using this search string was then extracted and we de-duplicated the initial search results in excel using the excel duplicate tool. We recorded number of results for each search iteration following Preferred Reporting Items for Systematic reviews and Meta-analysis guidelines (PRISMA) (Page *et al.*, 2021). The PRISMA diagram for the meta-analysis is shown in Fig. 2.1.

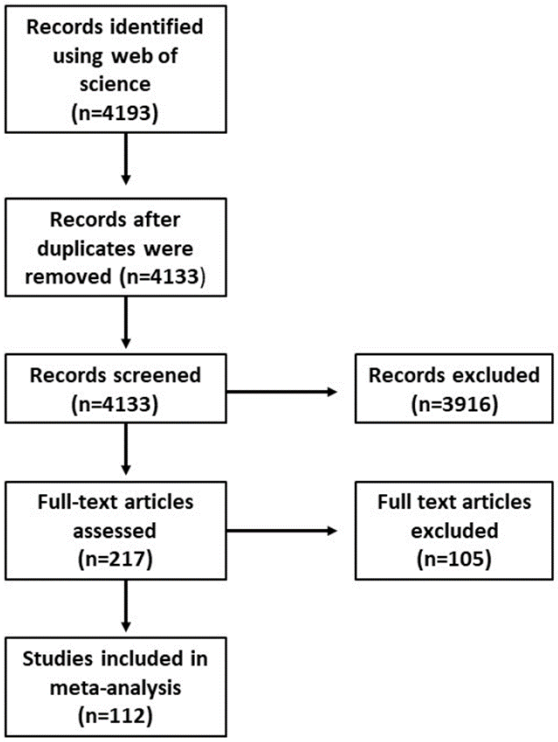

Figure 2.1*. PRISMA diagram showing the stages of the meta-analysis.*

2.3.2 Inclusion criteria

To be included in the meta-analysis studies must have been comparing paired urban and non-urban populations. In the case of a study where the sampling design was an urbanisation gradient, then we used data extracted from the populations located at the urban and non-urban extremes of the gradient which was the case for 45 studies. 4 studies used multiple urban and non-urban sites not really representing a gradient, and in these cases any descriptions of the sites presented was used to extract data from the most urban and non-urban populations. If this was not possible a random number generator was used to randomly select a non-urban and urban population to be included in the analysis. Studies were only included if the focal species they were measuring belonged to the avian class. The study must also have been measuring at least one health biomarker which we defined as any physiological or morphological measures or a measure of parasitism or disease that could have an overall effect on an organism's fitness and survival. We first screened all studies by reading the title and the abstract, marking all relevant studies based on whether or not they met these inclusion criteria (Fig. 2.1). After inspecting 4133 studies published between 1981 and 2022 (Fig. 2.1), our meta-analysis included 644 urban-non-urban paired comparisons from 112 studies published between 1989 and 2022 from 16 health biomarkers (described in

Tables S1.1 and S1.2) and included 51 bird species shown in Fig. S1.1 (Abolins-Abols, Hope and Ketterson, 2016; Amiot, Harmange and Ji, 2022; Andersson *et al.*, 2015; Atwell *et al.*, 2012; Auman, Meathrel and Richardson, 2008; Bailly *et al.*, 2016; Bailly *et al.,* 2017; Banbura *et al.*, 2013; Beaugeard *et al.*, 2019; Bichet *et al.*, 2014; Bókony *et al.*, 2012; Bonier *et al.*, 2007; Caizergues *et al.*, 2021; Calegaro-marques and Amato, 2010; Capilla-Lasheras *et al.*, 2017; Carbo-Ramirez and Zuria, 2017; Cavalli *et al.*, 2018; De Chapa *et al.*, 2020; Charmantier *et al.*, 2017; Chavez-Zichinelli *et al.*, 2013; Christie *et al.*, 2021; Corra and Sullivan, 2021; Cummings *et al.*, 2020; Davies *et al.*, 2015; Davies *et al.,* 2016; Davies and Sewall, 2016; Dominoni *et al.*, 2021a; Espin *et al.*, 2020; Evans, Newson and Gaston, 2009; Fokidis, Greiner and Deviche, 2008; Fokidis, Orchinik and Deviche, 2009; Fokidis and Deviche, 2011; Foltz *et al.*, 2015; Geue and Partecke, 2008; Glądalski *et al.*, 2016; Gregoire *et al.*, 2002; Grunst, Rotenberry and Grunst, 2014; Gryczyńska, 2018; Hamer, Lehrer and Magle, 2012; Heiss, Clark and Mcgowan, 2009; Hernández-Lara, González-García and Santiago-Alarcon, 2017; Hernández-Lara, Carbó-Ramírez and Santiago-Alarcon, 2020; Herrera-Dueñas *et al.*, 2014; Herrera-Dueñas *et al.*, 2017; Hõrak, Ots and Murumagi, 1998; Hõrak *et al.*, 2004; Ibáñez-Álamo *et al.*, 2018; Isaksson and Andersson, 2007; Isaksson *et al.*, 2009; Isaksson *et al.,* 2017; Jiménez-Peñuela *et al.*, 2019; Jiménez-Peñuela *et al.*, 2021; Jones, Rodewald and Shustack, 2010; Kaliński *et al.*, 2009; Kaliński *et al.*, 2014; Kaliński *et al.,* 2015; Kunca, Smejkalova and Cepicka, 2015; Liker *et al.*, 2008; Mackay *et al.*, 2017; Magallanes *et al.*, 2020; Markowski *et al.*, 2015; McClure, 1989; McGraw *et al.*, 2020; Meillère, *et al.*, 2015a; Meillère *et al.*, 2017; Minias, 2016; Minias, Jedlikowski and Włodarczyk, 2018; Morrissey *et al.*, 2014; Murray *et al.*, 2020; Phillips *et al.*, 2020; Pikus *et al.*, 2021; Plaza and Lambertucci, 2018; Randall, Blitvich and Blanchong, 2013; Redondo *et al.*, 2021; Ribeiro *et al.*, 2022; Rodewald and Shustack, 2008; Rouffaerl *et al.*, 2017; Russ *et al.*, 2015; Rzad *et al.*, 2014; Salmón *et al.*, 2016; Salmón *et al.*, 2018a; Salmón *et al.,* 2018b; Sándor *et al.*, 2022; Santiago-Alarcon *et al.*, 2020; Schoech and Bowman, 2003; Schoech *et al.*, 2007; Seewagen, Glennon and Smith, 2015; Senar *et al.*, 2017; Sitko and Zaleśny, 2014; Skwarska *et al.*, 2019; Strasser and Heath, 2011; Strubbe *et al.*, 2020; Sullivan, Corra and Hayes, 2021; Suri *et al.*, 2017; Sykes, Hutton and McGraw, 2021; Tinajero *et al.*, 2019; Vangestel *et al.*, 2010; Verhagen *et al.*, 2012; Weaver, Mcgraw and Mousel, 2014; Weaver, Gao and McGraw, 2018; Wemer *et al.*, 2021; Wright and Fokidis, 2016; Zhang *et al.*, 2011)

***Figure 2.2**. World map representing the geographic coverage of our study. The pink circles depict the locations where coordinates were known and could be extracted to calculate urban score.*

2.3.3 Data extraction

We read all relevant studies (n=217, Fig 2.1) in full. We then extracted any qualitative information about the study, including the focal species, health trait, authors, title, publication year, publishing journal, country the study took place in, how many years the study took place over. We also extracted information about the study location, and specifically about the geographic coordinates, if recorded. We then extracted the quantitative data needed to calculate effect sizes for each paired urban and non-urban location, which included the standard deviation, the mean, and the sample size of the health biomarker measured for each population. This was done in different ways depending on the study. For most studies we extracted data from tables or text. In some cases, we extracted data from figures, using the function metaDigitise () in the metaDigitise package (Pick, Nakagawa and Noble, 2018) in R version 4.3.2 (R Core Team, 2024). We also extracted data from the supplementary materials or directly from the raw data that was provided as part of the publication. When the study had missing data or did not report the information needed to calculate effect sizes, we contacted the corresponding author recorded on the study to request the missing data.

To calculate the standardised mean difference between paired urban and non-urban locations for the health traits measured in each study, we calculated Hedges' g (Brydges, 2019) as well as the sampling variance of the effect sizes, using the function escalc() in the Metafor package (Viechtbauer, 2010). To allow interpretation of the outcome of the meta-analysis, we assigned a direction of effect for each of the health traits included in the study. Specifically, if an increase in the value of a given health trait would have a predicted negative impact on health in urban environments, then this would be considered as a negative direction. The effect sizes for all health traits with a negative direction were flipped by multiplying them by -1. If a health trait was too complex to determine directionality, then this was excluded, this included biomarkers such as measures of gut microbiome, certain hormones (testosterone and oestradiol) as well as gene expression data. The directionality of each health trait is shown in Table S1.1.

2.3.4 Phylogeny

We extracted phylogenetic trees from The Open Tree of Life, using the interface provided by the R package rotl (Michonneau, Brown and Winter, 2016). We calculated tree branch length, built a phylogenetic correlation matrix, and included this in all phylogenetic multilevel meta-analytic models. The phylogenetic signal was assessed in the meta-analysis based on the proportion of variation explained by phylogeny. The phylogenetic tree used for analysis is shown in Fig. S1.1.

2.3.5 Additional variables

We also included additional variables to investigate the mechanisms mediating urban effects on avian health. We included species specific traits such as trophic niche (the major resource types utilised), primary behaviour (the dominant locomotory behaviour while foraging) and migratory behaviour, which we extracted from the AVONET database (Tobias *et al.*, 2022) (Table S3). The maximum lifespan of the species was also included as a moderator and was extracted from the AnAge database (Tacutu *et al.*, 2018). Other moderators included the life stage (nestling or juvenile/adult) and the latitude of the study area (Table S1.2).

2.3.5 Urban score

For the subset of the studies where exact coordinates were reported for both the non-urban and urban sites (Fig 2.2), or where the corresponding authors had provided this information upon request, we calculated the degree of urbanisation of each site, hereafter named "urban score" using previously developed methods by Capilla-Lasheras *et al.* (2022). This was done for 35 paired sites from 37 studies. We did so by using the Copernicus Climate Change Service ICDR Land Cover data (Defourny, Lamarche and Flasse, 2019) which provides consistent land cover per year with a global coverage and spatial resolution of circa 300m per pixel. Each pixel is classified as one of 22 land cover categories which are defined by United Nations Food and Agricultural Organisation Land Cover Classification System.

We first extracted the number of pixels belonging to each land cover category within a circular buffer around each urban and non-urban location. We performed this operation for each of 13 buffer radii from 150 m to 5000 m, in intervals of 250 m. We calculated the urban score as the proportion of each buffer area that was categorised as urban land cover type (Defourny, Lamarche and Flasse, 2019). We then verified that urban score was higher in the urban than in the non-urban location which was the case for each paired location.

2.3.6 Data analysis

All meta-analyses were conducted in R version 4.3.2 (R Core Team, 2024). We used the rma.mv () function from the metafor package (Viechtbauer, 2010) to run multilevel meta-analytic models with Hedges' g as the effect size (yi) and sampling variance (vi) as the variance input.

First, to evaluate the general effect of urbanisation on bird health, we fitted a phylogenetic multilevel (intercept only) meta-analysis. Second, to test whether the effect of urbanisation on health depended on the specific health trait considered, we ran another multilevel meta-analysis that included health trait as a moderator. Third, to investigate whether differences in health may be better captured by the level of urbanisation rather than a simple dichotomous urban/non-urban variable, we ran phylogenetic meta-regression models to explain differences in health between urban and non-urban populations (i.e., Hedges' g), where the difference in urban score between the two populations was included as a continuous moderator (hereafter "urban score"). We ran these models for every buffer radius

from 250-5000 m at 250 m intervals and then performed model selection to identify the model with the best fit which was determined to be the model with the lowest Akaike information criterion (AIC) value. This was found to be the model at 1000 m buffer radius. The results of this model were used for biological inference. The AIC values for models ran at each spatial scale can be found in Table S1.3. The 1000 m radius should be representative of the breeding home range of most species included in our study. We also ran this same model without urban score as a moderator to test whether the inclusion of urban score increased model fit. However, urban score was not used in the subsequent models as this information was only available for a small subset of the effect sizes used in the meta-analysis.

Fourth, to investigate whether species traits or latitude would modulate the effect of urbanisation on health, we ran another multilevel meta-analysis which included life stage, primary behaviour, trophic niche, migratory behaviour, and latitude (as an absolute value) as moderators. A separate model was then run with maximum lifespan as a moderator as this information was not available for all of the effect sizes. Last, since several biomarkers were measured either on nestlings or adult birds, we decided to subset the data by life stage (nestling vs adult) and conducted independent analyses on each subset using health trait as a categorical moderator.

A phylogenetic variance-covariance matrix was constructed using a trimmed Open Tree of Life phylogeny using the rotl (Michonneau, Brown and Winter, 2016) and ape packages (Paradis and Schliep, 2019), with branch lengths computed using Grafen's method (Grafen, 1989). We then included study ID and phylogeny as random effects in each model as well as a unique ID for each individual effect size to account for residual variation due to multiple observations from the same studies but only in the models where health trait was not included as a fixed effect (as only one observation per health trait and study was included in our models). We calculated the heterogeneity for each of the phylogenetic multilevel models using the i2_ml () function from the OrchaRd package (Nakagawa *et al.*, 2023). This allows us to calculate the percentage of total relative heterogeneity as well as the heterogeneity explained by each random effect included in the model.

We then checked the data for any signs of publication bias. This included looking for any signs of small study effects as well as time lag bias. Small study effects occur when smaller studies show different, in many cases larger treatment effects than larger studies. Time lag bias may occur when more statistically significant effects are published quicker than smaller

or non-statistically significant effects (Alirol *et al.*, 2011; Sepp *et al.*, 2019; Brumm *et al.*, 2021; Risi, Sumasgutner and Cunningham, 2021). To test for bias, we ran two extra multilevel meta-regressions with the same random structure as the previous models but using a moderator that was either i) the square root of inverse sampling variance to test for small study bias (Nakagawa *et al.*, 2022) or ii) year of study publication mean-centred for time lag bias (Koricheva and Kulinskaya, 2019; Nakagawa *et al.*, 2022). R^2 was then calculated using the R^2_ml() function from the OrchaRd package (Nakagawa *et al.*, 2023). This was done for the main dataset as well as for any subset data that were used for subsequent analysis.

2.4 Results

2.4.1 Does urbanisation impact avian health?

Overall, we did not detect any significant impact of urbanisation on avian health (mean estimate [95% Confidence Interval, CI] = 0.0276 [-0.1992, 0.544]; Table S1.4, Fig. 2.3A). Total heterogenity was high (I^2 = 89.63%) with 4.34% of it explained by phylogeny, while 18.28% of it was explained by differences between studies. The second model, which was run with health trait as a moderator showed that health, as determined by lower load of ectoparasites, was higher in urban environments (mean estimate [95% Confidence Interval, CI] = 1.728 [0.963, 2.493]; Table S1.4, Fig. 2.3B). This suggests birds had fewer ectoparasites in urban environments.

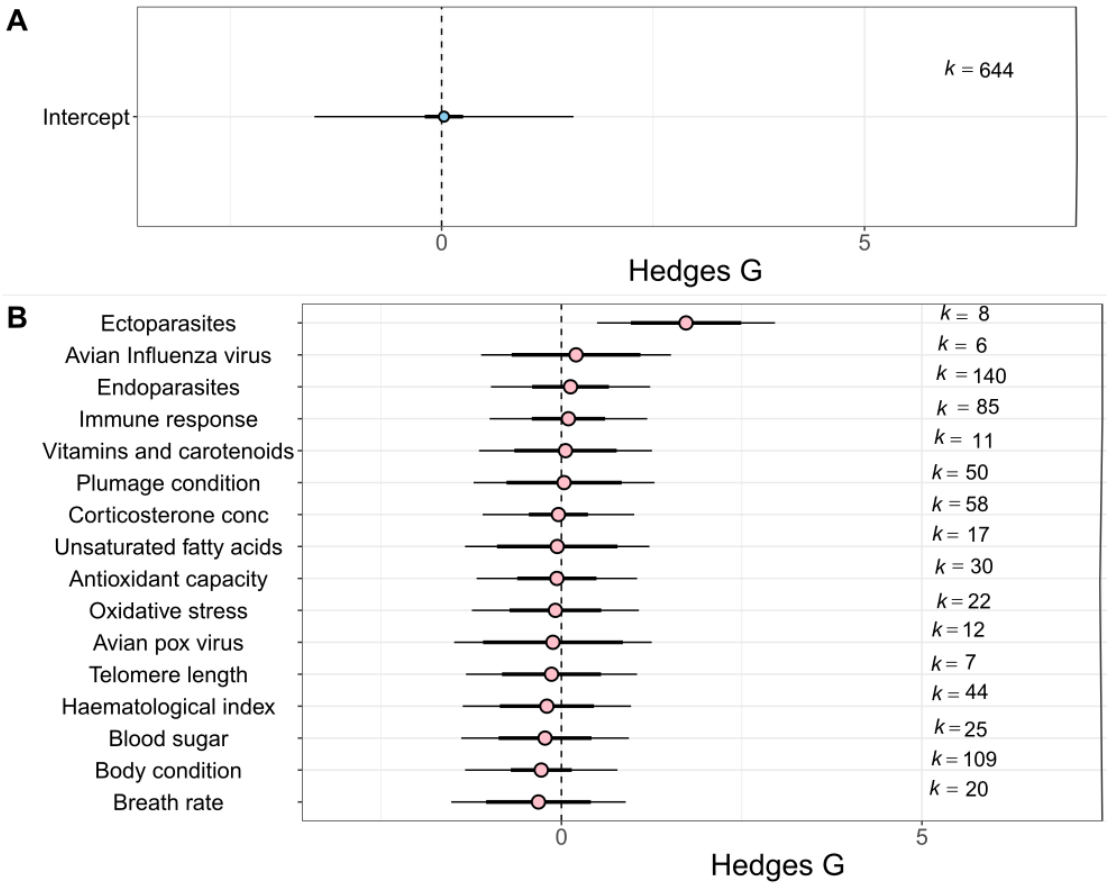

Figure 2.3. Urbanisation has no overall impact on avian health and most avian health traits are not affected by urbanisation. *A) Results from the intercept only meta-analytic model. The estimated health impact is reflected in the effect size (Hedges' g) which is displayed on the x axis. Model estimates for Hedges' g is shown along with its 95% confidence intervals (thick whisker), 95% prediction intervals are also shown (thin whisker). B) Second meta-analytic model where health trait was a moderator. The orchard plot shows different biomarkers of health and their estimated impact on the overall direction of health in urban environments. The health impact is reflected in the effect size (Hedges' g) which is displayed on the x axis. The health biomarkers that were measured are displayed on the y axis. Positive estimates assume a positive health consequence in urban bird populations. K represents the corresponding number of effect sizes.*

We found a significant negative relationship between urban score and overall health (mean estimate [95%CI][= -1.042 [-1.992, -0.092; Fig. 2.4). The regression line crosses 0 at an urban difference of 0.75, however the slope was negative which indicates that the urban effect is more negative the more urbanised a population is. The AIC score of the model

without urban score as a moderator was 2775 and the AIC score of the model when urban score was included as a moderator was 2707 therefore the model was a much better fit when urban score was included as a moderator. The effect of urban score and overall health at each spatial scale is shown in Fig. S1.2.

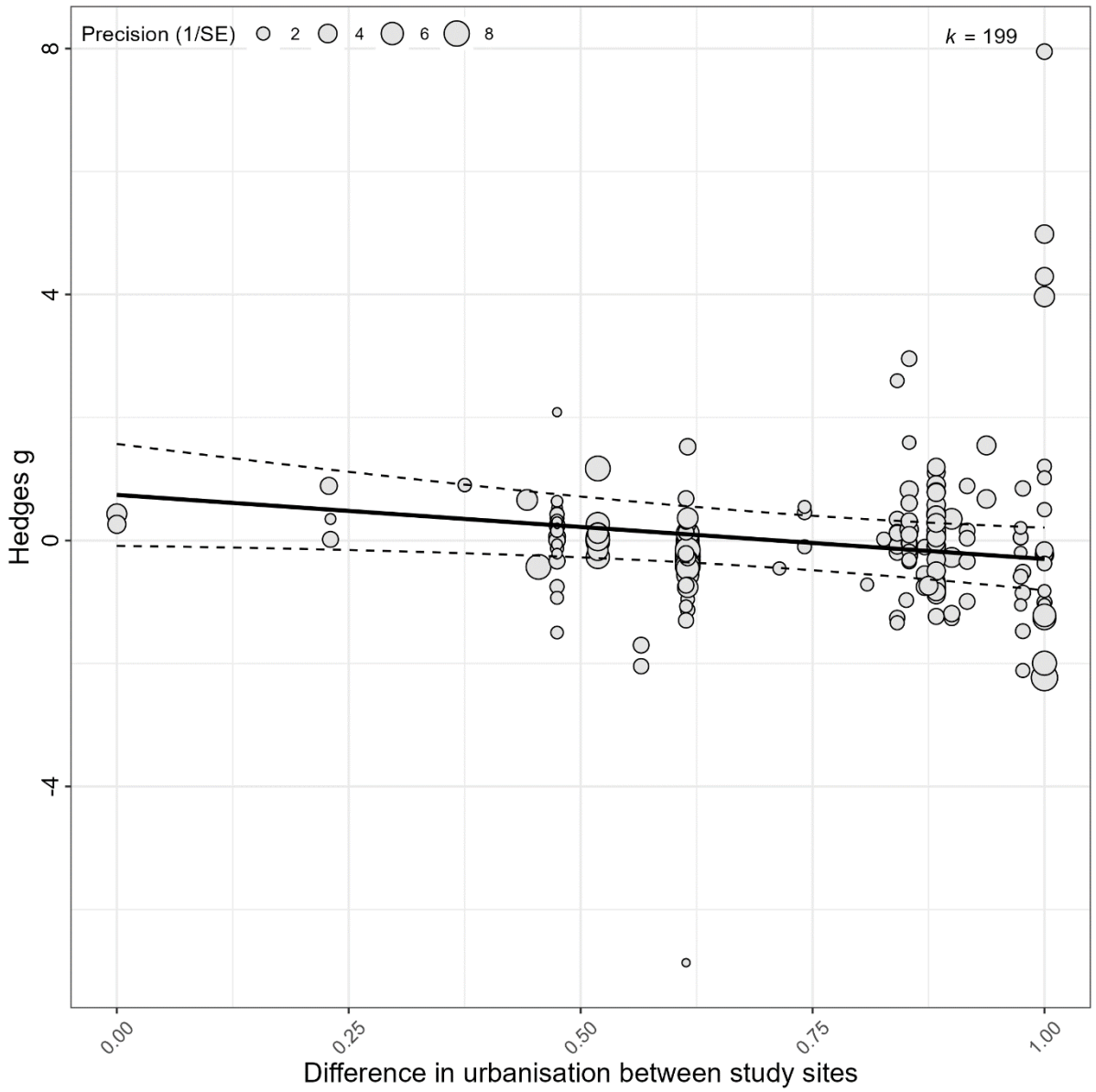

Figure 2.4. Larger differences in urbanisation lead to stronger effect on avian health. *The plot shows the effect of the difference in urban score between paired non-urban and urban populations on the health of bird species. The differences in urban score between two paired study sites is shown on the x axis, while Hedges' g is shown on the y axis. The individual effect sizes are scaled by their precision which is (1/SE), the more precise the effect size is the larger the circle. The solid line represents the model estimate, with the dashed lines representing the 95% confidence intervals. K shows the total number of effect sizes.*

2.4.2 Is the relationship between urbanisation and bird health impacted by species traits, latitude, or life stage?

The maximum lifespan of bird species did not have a significant impact on the relationship between urbanisation and health (mean estimate [95% CI] = 0.011 [-0.565, 0.543]; Table S1.4, Fig. S1.3). Similarly, trophic niche, migratory behaviour and primary behaviour did not affect the relationship between urbanisation and health (Table S1.4, Fig. 2.5). Latitude was also found to not affect the relationship between urbanisation and health (mean estimate [95% CI] = -0.005 [-0.02, 0.001]; Table S1.4, Fig. S1.4). The majority of studies included in the meta-analysis were located in the Northern Hemisphere (Fig. S1.4). Finally, life stage of birds does not appear to have a significant effect on their overall health in urban environments (adult data subset: mean estimate [95% CI] = -0.191 [-1.079, 1.154]; Table S1.4, Fig. 2.5A, nestling data subset: mean estimate [95% CI] = -0.132 [-1.418, 1.154]; Table S1.4, Fig. 2.5A).

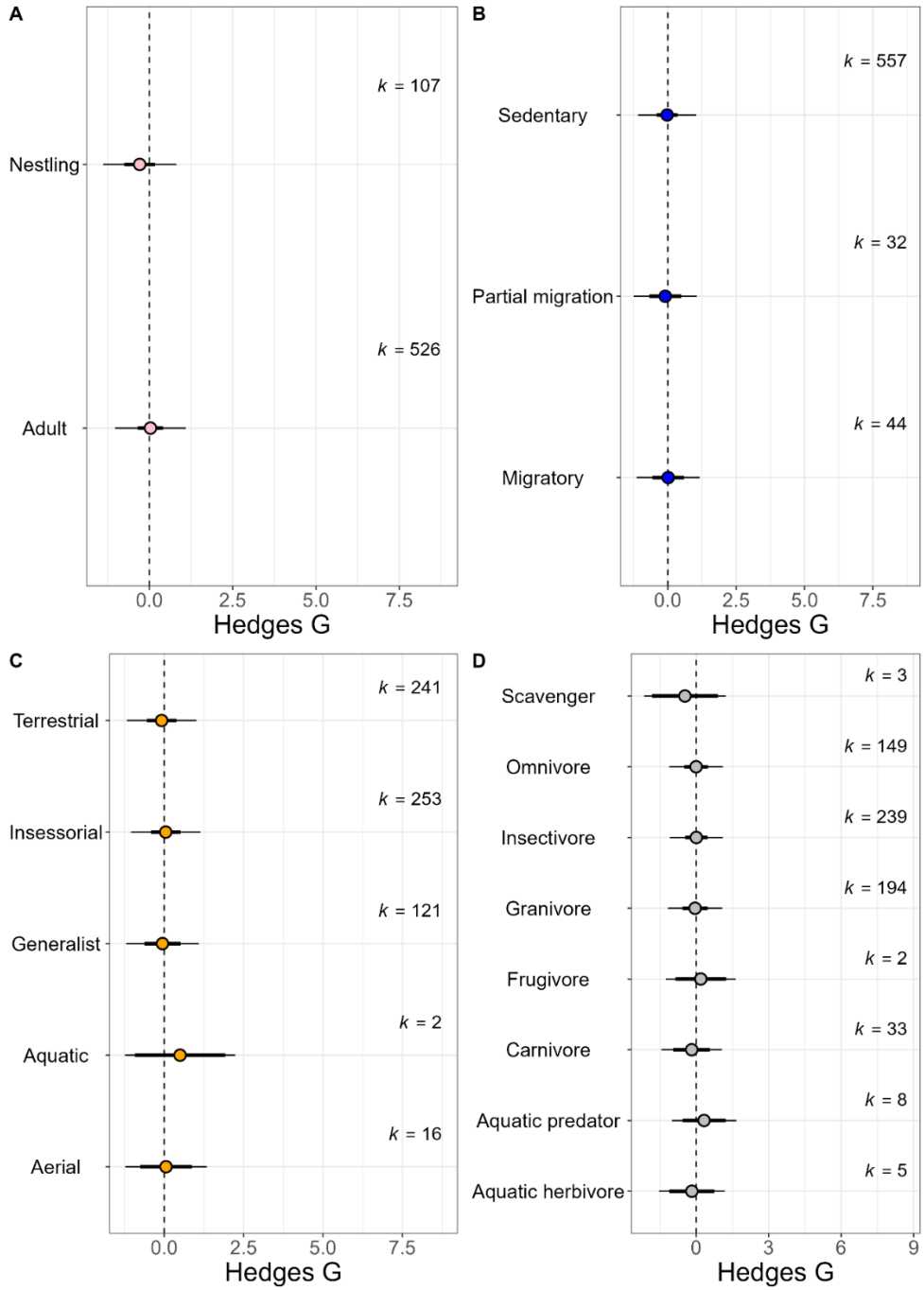

Figure 2.5: Species traits and life stage do not influence the relationship between urbanisation and avian health. *A) life stage, B) migratory behaviour, C) primary behaviour, D) trophic niche on the relationship between urbanisation and health. K represents the corresponding number of effect sizes.*

2.4.3 Decomposing the relationship between life stage, urbanisation, and health

While life stage did not appear to have a significant effect on the relationship between urbanisation and health, nestling health did go in a negative direction (Fig 2.5A). This, together with the fact that many health traits were not analysed in both adults and nestlings, prompted us to run two further models to decompose effects separately for adults and nestlings. We found that adult birds have less ectoparasite burden in urban environments compared to non-urban environments (mean estimate [95% CI] = 1.728 [0.972, 2.484]; Table S1.6 Fig 2.6A), while ectoparasite burden was never measured in nestling birds. Adult birds also tended to have higher blood sugar levels in urban environments, but this was not a significant relationship (mean estimate [95% CI] = -0.53 [-1.064, 0.005]; Table S1.6, Fig 2.6A). Unlike adults, whose body condition did not differ between habitats, nestlings were found to be in worse body condition in urban than non-urban environments (mean estimate [95% CI] = -0.499 [-0.901, -0.097]; Table S1.7, Fig 2.6B). To explore this effect further, we ran an additional meta-analysis with effect sizes of nestling body condition as response variable, and trophic niche and primary behaviour as moderators. There does not appear to be a significant effect of species trophic niche on nestling body condition in urban areas (Table S1.8, Fig. 2.7A). Birds with a terrestrial primary behaviour are in worse body condition in urban environments when compared to non-urban areas (model estimate [95% CI] = -0.9461 [-1.6854, -0.2067]; Table S1.8, Fig 2.7B). Birds with an insessorial primary behaviour also appear to be in worse body condition in urban areas but this relationship was not significant (mean estimate [95% CI] = -0.4487 [-0.9142, 0.0169]; Table S1.8, Fig 2.7B). Generalist species did not appear to have their health impacted by urbanisation (mean estimate [95% CI] = -0.515[-0.8043, 0.5012]; Table S1.8, Fig 2.7B).

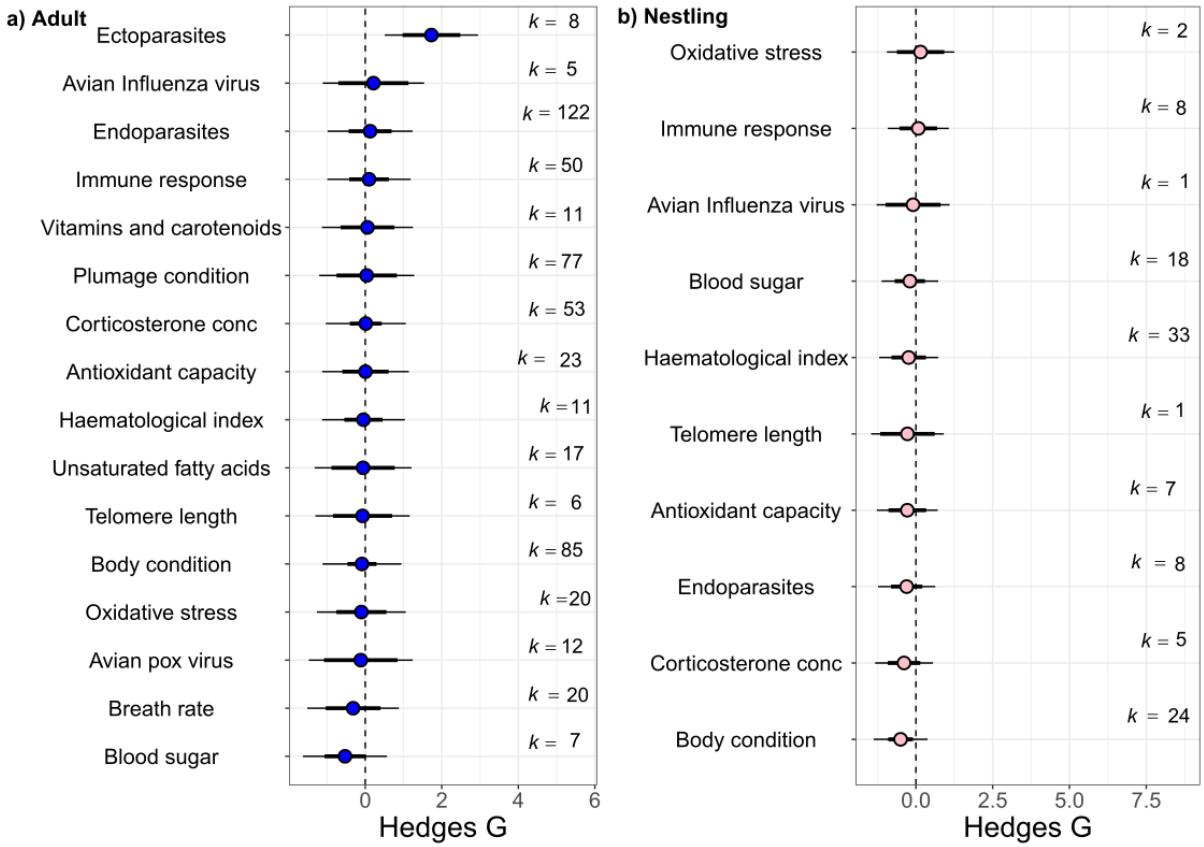

Figure 2.6. The relationship between urbanisation and specific health traits varies between life stage. *This orchard plot shows different biomarkers of health and their assumed impact on the overall direction of health in urban environments for A) adults and B) nestlings. K represents the corresponding number of effect sizes.*

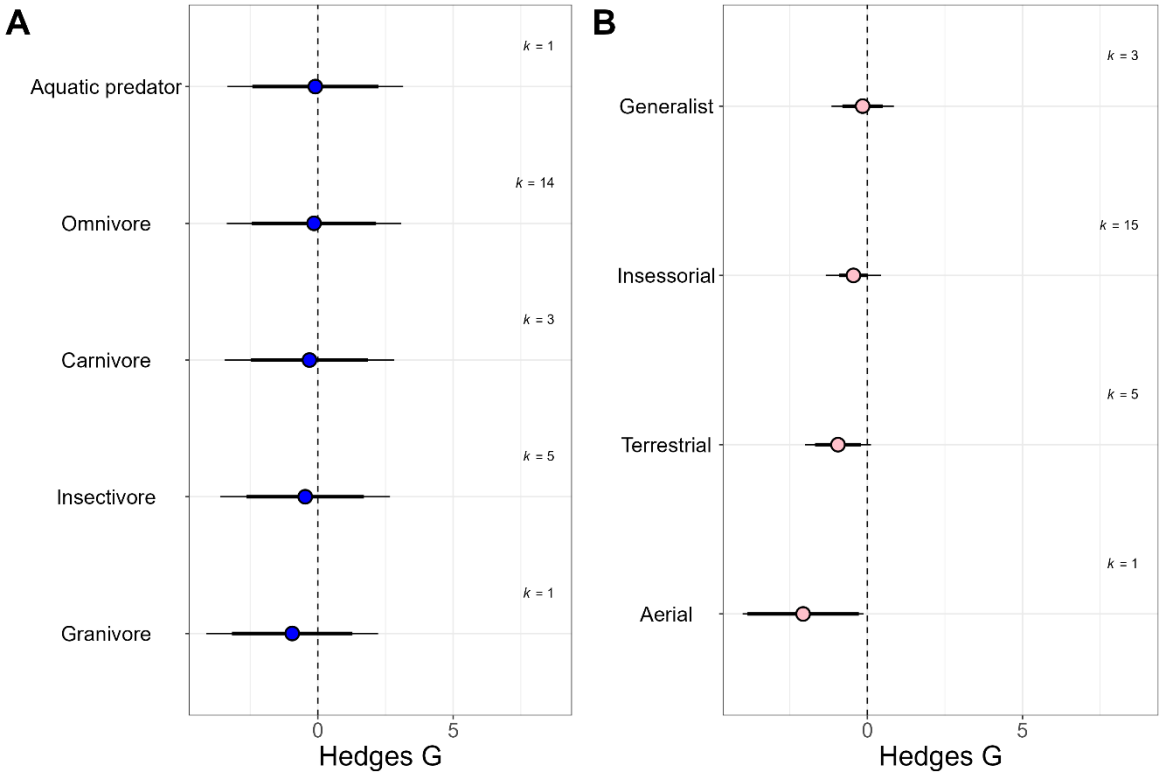

Figure 2.7: The impacts of urbanisation on nestling body size varies between functional traits. *This orchard plot shows the impact of different functional traits A) trophic niche, B) primary behaviour on the relationship between urbanisation and body condition in nestlings.*

2.4.4 Publication bias

We found no evidence of small study effects in the main dataset (mean estimate [95% CI] = -0.0482 [-0.565, 0.543]; Fig. S1.5) or time lag bias (mean estimate [95% CI] = 0.007 [-0.01, 0.024]). We also found no evidence of small study effects or timelag bias (mean estimate [95% CI] = 0.0002 [-0.018, 0.019]) in the data subset by "Adult". There was also no evidence of small study bias in the "nestling" subset dataset (mean estimate [95% CI] = -0.446 [-2,294, 1.402]). However, we did find evidence of time lag bias in the nestling subset dataset (mean estimate [95% CI] = 0.064 [0.003, 0.126]). There was no evidence of small study bias (mean estimate [95% CI] = -0.362 [-1.906, 1.183]) or time lag bias (mean estimate [95% CI] = 0.031 [-0.021, 0.083]) in the subset of data where urban score was calculated.

2.5 Discussion

We compiled a global dataset of avian health biomarkers including physiological and morphological biomarkers as well as measures of disease and parasitism for paired urban and non-urban populations, to provide a holistic view on how urbanisation impacts avian health. A phylogenetically controlled meta-analysis revealed that urbanisation did not show a clear relationship with overall health. However, when the degree of urbanisation, rather than a dichotomous urban-non-urban category was used in the models, avian health did appear to decline with increasing urbanisation, suggesting this quantitative method is more sensitive. Although life stage was not a significant moderator in the overall model, subset analysis revealed that urbanisation may affect some health traits differently depending on life stage. For example, adult birds in urban environments showed lower ectoparasite burdens and slightly elevated blood glucose levels (although this was not significant). Urban nestlings also tended to be in worse body condition than adults. These patterns suggest that while life stage alone does not predict urban effects on health, it interacts with specific biomarkers shaping health outcomes.

It is well documented in the literature that organisms inhabiting urban environments can face negative implications to their health and wellbeing (Herrera-Dueñas *et al*., 2014; Watson *et al*., 2017; Nwaogu *et al*., 2023). Our results indicate that there is no significant effect of urbanisation on overall avian health. Moreover, our analysis showed a high total heterogeneity in Hedges' g. These findings indicate large variation among both studies and species in how urbanisation associates with changes in health biomarkers. While the lack of an overall difference in health between urban and non-urban birds might seem at first puzzling, our follow-up analysis that considered the urban score of each location, and specifically the difference in urban score between paired urban and non-urban locations, revealed a different picture. The negative slope indicates that as the difference in urban score increases health outcomes tend to worsen. The regression line crosses zero at an urban score difference of 0.75, suggesting that detectable negative effects of urbanisation on health begin to emerge when urbanisation levels are considerably higher than those of the paired non-urban site. In addition, or alternatively, the low levels of ectoparasite burden in urban areas could have balanced out the otherwise negative impact of urbanisation on health.

Regardless of the mechanistic explanation, this result does suggest that urban score may be a better predictor of avian health than a simple dichotomous classification of urban and non-

urban populations. Cities are a mosaic of heterogenous habitats that vary greatly from each other in their abiotic and biotic characteristics, including levels of pollution (light, noise, chemical and metal) (Duh *et al.*, 2008; Gallaway, Olsen and Mitchell, 2010; Gong *et al.*, 2012; Abbaspour *et al.*, 2015), human population density (Nowak *et al.*, 2005), amount and quality of green space (Nowak *et al.*, 2005; Gong *et al.*, 2012; Mathew, Khandelwal and Kaul, 2016), degree of anthropogenic food provisioning (Murray et al., 2015b; Theimer et al., 2015), and presence of invasive species (Potgieter *et al.*, 2019). Recent work suggests that although the degree of urbanisation can account for a large proportion of the variance found between habitats (Wang *et al.*, 2001; Melliger *et al.*, 2018; Sidemo-Holm *et al.*, 2022), it still may not be enough to describe more complex relationships. Instead, quantifying the impact of urban-specific environmental factors may be more revealing. This approach could be beneficial for future studies (see for instance Senzaki *et al.* (2020)), including in meta-analyses (Sanders *et al.*, 2021).

Our follow up analyses revealed some mechanisms by which avian health may be affected by urbanisation. We found that birds had a lower ectoparasite burden when they lived in urban areas in comparison to their non-urban conspecifics, which could have positive health outcomes. This result could be due to urban areas having a warmer microclimate which has been shown to reduce tick numbers (Brennan *et al.*, 2023). However, a warmer microclimate has also been shown to increase mosquito numbers, as these can feed and reproduce faster when it is warmer. Thus, this effect is likely dependent on the ectoparasite species being measured (Delgado- V and French, 2012). Adult birds in urban areas may also spend less time foraging due to availability of supplemental food sources. This could potentially mean they have more time for behaviours that would allow them to reduce their ectoparasite burden such as preening (Villa *et al.*, 2016). Previous work suggested that Ixodes ticks are studied more than any other arthropod parasite, likely due to them being easy to detect on a host through visual examination (Delgado- V and French, 2012), and that most studies on avian ectoparasites are clustered in Europe and North America on urban-adapted or introduced bird species (Delgado- V and French, 2012). The relationship shown in our current study is drawn mostly from studies looking into the burden of ticks on adult blackbirds in urban and non-urban populations in Europe (Gregoire *et al.*, 2002; Evans *et al.*, 2009; Gryczyńska, 2018). This limits our ability to make general assumptions about the relationship between urbanisation and ectoparasite burden on avian species and calls for future studies to look into other bird-parasite relationships in urban areas.

Our analysis showed that migratory behaviour, primary behaviour, trophic niche, or maximum lifespan did not affect the relationship between urbanisation and health in bird species. The ability of species to cope with and adapt to environmental changes such as urbanisation can be dependent on species traits such as their diet, dispersal ability and behavioural flexibility (Meillère *et al.*, 2015a). For instance, the meta-analysis by Lakatos *et al*. (2022) showed that the abundance of ground nesting and feeding birds was negatively impacted by urbanisation due to higher predation by domestic animals as well as a lack of suitable nesting sites (Lakatos *et al.*, 2022). This meta-analysis also showed that migratory birds were most likely to avoid cities (Lakatos *et al.*, 2022). Cavity nesters have been shown to have a better chance of survival in cities due to their ability to use artificial nesting sites such as nest boxes (Patankar *et al.*, 2021). The relationship between health and species traits might simply be absent in the context of urbanisation. Alternatively, this relationship may be dependent on the health biomarker measured. For example, insectivorous birds may be negatively impacted by urbanisation due to limited food sources, and this may have consequences for specific aspects of health such as body condition or carotenoid levels, but we may not see an impact on overall health. Future studies could look at this specific question.

It would also be expected that impacts of urbanisation on health may depend on the latitude of the study site, as there is latitudinal variation in rainfall, temperature and other climatic variables, and certain vector-borne diseases have been shown to be dependent on the climate (Nunn *et al.*, 2005). For instance, in the big brown bat *(Eptesicus fuscus)* the negative impact of fungal infections became more prevalent at higher latitudes, possibly because warming winter conditions in northern latitudes lead to insect declines reducing food availability (Simonis *et al.*, 2023). However, we did not find any relationship between latitude, urbanisation, and health in our study. The majority of studies included in our meta-analysis were conducted at similar mid-to-high latitudes, and thus we had little power to detect a latitudinal relationship between urbanisation and health, if this was present. There is a strong need for studies conducted at lower latitudes, particularly in tropical mega-cities where urbanisation rate has been high in recent decades (Seto, Sánchez-Rodríguez and Fragkias, 2010). The lack of studies looking at the impacts of urbanisation in the southern hemisphere is a massive gap in knowledge and is of particular importance as urbanisation in the global south is rapidly increasing. The differences in environment and the fact that many developing countries are located in the south may mean we would see a different relationship between urbanisation and health than what we are currently seeing in this meta-analysis (Smit, 2021).

We performed follow-up analyses on nestlings and adult birds separately because it is likely adult birds may be affected differently when living in urban environments than nestlings, as the developmental period is an extremely sensitive time, particularly when responding to environmental stressors (Grue and Shipley, 1984; Skwarska *et al.*, 2022; Diehl *et al.*, 2023). Although life-stage was not significant in the full model, our results show a strong tendency for urban adults to have higher levels of blood glucose than non-urban adults, although this difference was not significant. This could indicate fluctuations in the availability and quality of food found in urban areas as anthropogenic food that is widely available in urban environments is usually found to be higher in sugars than natural food sources and lead to an energetic imbalance when consumed (Kaliński *et al.*, 2016; Wist, Stolter and Dausmann, 2022). Higher glucose levels may also indicate that urban birds have higher metabolic demands than non-urban birds. Urban birds have been shown in a variety of studies to have higher levels of activity at night, which has been attributed to increased exposure to artificial light (Dominoni, Quetting and Partecke, 2013; Ouyang *et al.*, 2017; Yadav *et al.*, 2022). This increase in activity can lead to an increased demand for energy, in birds' this energy supply will mainly come from glucose (Braun and Sweazea, 2008). The relationship we found in our meta-analysis was only based on a small sample size therefore there is an increased need for more studies focusing on the metabolic demands that urban birds face, as this could constrain them when it comes to other energetically demanding activities including reproduction.

We also show that urban nestlings are in poorer body condition when compared to non-urban nestlings, but adult body condition did not differ between habitat types. One thing to note is that we did find evidence of time lag bias in the nestling data subset, this may indicate that studies within this subset with significant results may have been published earlier than those without significant results. The most likely cause of nestlings being in poorer body condition in urban environments is reduced food availability or quality. Nestlings that are raised in urban environments are more likely to be fed with poor quality food that may lack the nutrients required for development, as shown in a variety of bird species (Heiss, Clark and Mcgowan, 2009; Catto *et al.*, 2021; Sinkovics *et al.*, 2021). The availability of good quality food is important for both chick growth and condition which is shown in the meta-analysis by Grames *et al*. (2023). The fact that we see a negative relationship between urbanisation and health in nestlings but not adults could be seen as surprising as conditions during the development period have been found to have long lasting effects into adulthood (Metcalfe and Monaghan, 2001; Van De Pol *et al.*, 2006). However, this result may be a consequence

of the selective disappearance of birds that are in poorer condition before they reach adulthood in urban areas. This has been shown in great tits, where juvenile individuals with shorter telomeres disappeared from the urban population (Salmón *et al.*, 2017). Alternatively, the favourable winter conditions in urban areas, with availability of anthropogenic food and higher temperature compared to rural areas, may allow birds to recover from the stressors encountered during their developmental period, and thus increase winter survival and health (Nakagawa *et al.*, 2022).

We also found that the negative effects of urbanisation on body condition of nestlings were dependent on their primary behaviour, bird species with specialist behaviours including those classed as terrestrial were shown to be in poor body condition whereas generalist species were not impacted by urbanisation. Much of the literature indicates that an organism's ability to succeed in an urban environment will depend on them having specific traits that would allow them to take advantage of their surroundings. Species that are classified as "Generalists" can be more flexible to changing conditions including land modification as they are able to adapt to a wide range of both habitat and feeding conditions (Marques *et al.*, 2021). Species that specialise in certain behaviours do not have this luxury and are more likely to be negatively impacted by modifications to their habitat (Mennechez and Clergeau, 2006). For example, "Terrestrial" bird species spend most of their time on the ground where they will feed and nest however this behaviour makes them more vulnerable to urban stressors including predation by domestic cats and dogs as well as habitat modification or degradation caused by urban activities as shown in Lakatos *et al*. (2022). Nestlings that are in worse body condition and weigh less are less likely to successfully fledge and have a lower survival rate (Seress *et al.*, 2020). Thus, our results point to the early life exposure to urban stressors being a considerable challenge for urban bird populations. However, the demographic consequences of poor body condition and lower survival of urban nestlings are vastly overlooked, but it is essential that future studies quantify such effects to improve our understanding of whether or not urban bird populations are self-sustained (Balogh, Ryder and Marra, 2011; Stracey and Robinson, 2012).

2.6 Conclusions

Our meta-analysis contradicts the popular belief that urbanisation is generally bad for the health of wild birds. As health is a complex and multivariate trait, different components of health will be impacted in different ways by urbanisation. To reinforce this idea, we showed

that using a continuous rather than a dichotomous descriptor of urbanisation can improve the inference and does suggest an overall negative relationship between urbanisation and health. However, the effect size was rather small and only visible when the degree of urbanisation was very high. Moreover, heterogeneity was very high, highlighting that any effect may depend on the species studied, the life stage, the location and the measure of health used. We highlight the importance for future studies to measure the level of urbanisation as this could impact the strength and direction of the relationship with health.

We also highlight that the body condition of nestlings seemed to be negatively affected by urbanisation, which possibly points to lack of nutrients during growth as a key challenge in urban areas which may have negative fitness consequences. It is therefore clear that consideration must be taken to improve the body condition of birds during development. Planners could implement habitat improvement strategies aimed at increasing food resources for example insect abundance. The outcome in terms of insect availability and avian fitness could then be easily monitored. It is crucial that future studies will investigate what specific environmental factors may be more important for avian health, for example sensory pollutants including noise and light pollution, access to anthropogenic food, or lack of suitable habitat. This information could be pivotal for the management of urban environments to ensure the future conservation of the species that inhabit them and allow for a more targeted conservation management approach.

2.7 Availability of data and materials

All relevant data and R code used in this analysis are available on Zenodo: https://dx.doi.org/10.5281/zenodo.11208807

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2.10 Author contributions

Rachel Reid, Davide M Dominoni and Jelle Boonekamp conceived of and designed the study. Yacob Haddou calculated urban score for each set of coordinates. Rachel Reid ran the analysis with guidance from Pablo Capilla Lasheras, Pablo Capilla Lasheras contributed to writing out the R scripts. Rachel Reid drafted the manuscript. All authors read and approved the final version.

Chapter 3
Artificial light at night weakens body condition but does not negatively affect physiological markers of health in great tits.

Rachel R Reid[1]*, Neal Dawson[1], Neil P Evans[2], Christopher Mitchell[3], Jelle Boonekamp[1], Davide M Dominoni[1].

[1]School of Biodiversity, One Health and Veterinary Medicine, Graham Kerr Building, University of Glasgow, Glasgow, G12 8QQ, UK

[2] School of Biodiversity, One Health and Veterinary Medicine, Jarrett Building, University of Glasgow, Glasgow, G61 1GH, UK

[3] Environment and Sustainability Institute, University of Exeter, Penryn Campus, Penryn, Cornwall, TR10 9FE, UK

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3.1 Abstract

Urbanisation brings many novel challenges for wildlife through changes to the natural environment, one of the most unprecedented of these modifications is artificial light at night (ALAN). ALAN has been shown to have profound effects on the behaviour and physiology of many wildlife species which in turn have negative consequences for fitness and survival. Despite increasing knowledge of the mechanisms by which ALAN can affect health, studies that have investigated this relationship have found contrasting results. This study investigated the impact of ALAN on health biomarkers in 13-day old great tit (*Parus major*) nestlings including Malondialdehyde (a measure of oxidative damage), antioxidant capacity of plasma, feather corticosterone levels and scaled mass index. Immediately after hatching, broods were either exposed to 1.8 lux of ALAN until day 13 or left unexposed. Scaled mass index was lower in the birds exposed to the ALAN treatment when compared to the control group significantly, however there were no clear negative effects of ALAN

on malondialdehyde, antioxidant capacity, or corticosterone. This demonstrates that only certain aspects of health are impacted by early life ALAN, highlighting the importance of future studies measuring several biomarkers of health when investigating this relationship. Nestlings that fledge the nest in poor body condition have a decreased chance of surviving into adulthood. As urbanisation continues to expand, the negative effects of ALAN on wildlife are likely to become more pronounced. Therefore, it is crucial to gain a better understanding of this relationship.

Key words: Avian health, Avian physiology, Light pollution, Artificial light, Urbanisation, Avian morphology

3.2 Introduction

Urbanisation is increasing at an unprecedented rate due to the continued growth of the global human population. Urbanisation is characterized by extreme modifications of natural habitats which are typically accompanied by novel stressors for native animal and bird species including the introduction of high levels of chemicals and metals in the ground (Merga et al., 2020; Sharley *et al.*, 2016), increased levels of air pollution (Duh *et al.*, 2008; Gong *et al.*, 2012), and noise pollution (Abbaspour *et al*., 2015; Yang *et al*., 2020), high amounts of refuse which include anthropogenic food sources (Murray *et al*., 2015b; Theimer *et al*., 2015), and increased ambient temperatures (the "heat island effect") (Mathew, Khandelwal and Kaul, 2016). One of the most unprecedented of these modifications is light pollution (Bennie *et al.*, 2015; Gallaway, Olsen and Mitchell, 2010) and artificial light at night (ALAN). The global estimates into the spread of ALAN are limited and the impact of ALAN on the environment is not well understood (Gaston *et al.*, 2013). Recent estimates have shown that observable light emissions increased by 49% between 1992 and 2017 which is a faster rate of growth than the human population globally (Gaston and Sánchez De Miguel, 2022). ALAN is derived from various sources including street lighting, advertisements, security lighting, domestic lighting, and headlights from vehicles (Gaston *et al.*, 2013). ALAN has been shown to disrupt natural patterns of light through both direct illumination as well as skyglow, where ALAN that is emitted or reflected upwards is scattered by molecules or aerosols in the atmosphere which spreads ALAN even further and leads to the brightening of the night sky (Gaston *et al.*, 2013; Kouteib, Davies and Deviche, 2015).

There are many ways in which ALAN can cause negative consequences for wildlife. The disruption of natural light cycles by ALAN can have profound effects on the behaviour of many wildlife species impacting reproduction (Dominoni, Quetting and Partecke, 2013; De Jong *et al.*, 2017), foraging (Dwyer *et al.*, 2013; Santos *et al.*, 2010) and migration (Cabrera-Cruz, Smolinsky and Buler, 2018; Poot *et al.*, 2008). This is because most organisms synchronise their daily and seasonal activities to the light-dark cycles produced by the earth's rotation (Russart and Nelson, 2018b), to optimise the timing of behaviour and physiological responses with prevailing environmental conditions (Dominoni, Quetting and Partecke, 2013; Russart and Nelson, 2018b). For instance, many physiological processes exhibit circadian rhythms including plasma glucose (Mason *et al.*, 2020), testosterone (Xiao *et al.*, 2021) and glucocorticoid concentrations (Russart and Nelson, 2018a; Son *et al.*, 2008), insulin production (Challet, 2015), and immune responses (Labrecque and Cermakian, 2015; Logan and Sarkar, 2012). One mechanism through which ALAN can cause circadian disruption is by changes to activity patterns for example diurnal species will increase their activity levels during the night (Meléndez-Fernández, Liu and Nelson, 2023). This has the potential to disrupt the sleep-wake cycle which may have a knock-on effect on physiological processes (Meléndez-Fernández, Liu and Nelson, 2023). ALAN has been shown to suppress the nocturnal production of melatonin. Melatonin has an important role in driving biological rhythms and relays important information to the organism about day length (Jones *et al.*, 2015), but melatonin is also a strong antioxidant (Zhang and Zhang, 2014). Despite our knowledge of several mechanisms by which ALAN can have negative consequences for health, the plethora of studies that have investigated this relationship have found contrasting results. The lack of consensus in the field, highlights the need for additional high-quality studies to identify trends, mechanisms, or context-specific effects. This growing body of evidence could eventually support a comprehensive meta-analysis which to our knowledge has not yet been conducted.

There are many studies that have shown a negative relationship between ALAN and health particularly in human subjects. Studies that have focused on shift workers have documented that exposure to high intensity ALAN increased the risk of cancer, immune suppression, heart disease and metabolic dysregulation (Jones *et al.*, 2015; Russart and Nelson, 2018a). Negative impacts of ALAN exposure have also been shown in wildlife, for example ALAN decreased immune function in Siberian hamsters (*Phodopus sungorus)* (Bedrosian *et al.*, 2011) and great tit (*Parus major)* nestlings (Ziegler *et al.*, 2021). In the latter study, it was also documented that exposure of the nestlings to ALAN resulted in 49% lower levels of

melatonin during the night than the control birds and it is likely that this would have had effects on other physiological processes (Ziegler *et al.*, 2021)

There are also studies that have shown little or no effects of ALAN on health. For example, ALAN was found to have no effect on the level of oxidative stress markers in freshwater crustacean shredders (Czarnecka *et al.*, 2022). However, rats exposed to constant light at night exhibited higher oxidative stress levels and impaired antioxidant enzyme activity (Cruz *et al.*, 2003). Another study reported that ALAN had no effect on telomere shortening of great tit nestlings and had only a weak relationship with body condition (Grunst *et al.*, 2020) but this result is in contrast with other studies that show a strong influence on the body condition of nestlings (Ferraro, Le and Francis, 2020; Raap *et al.*, 2016a). Together these findings show that the relationship between ALAN and health may be complex and could be dependent on a variety of factors including species, life stage and the health biomarker measured.

Despite the increasing interest on the relationship between ALAN and wildlife health, there are limited field studies that have exposed birds to manipulated levels of ALAN to investigate the impact on health (Dominoni *et al.,* 2021; Grunst *et al*., 2019; Grunst *et al.,* 2020; Raap *et al*., 2016a; Raap *et al*., 2016b; Raap, Pinxten and Eens, 2016; Ziegler *et al.,* 2021). This type of experimental study is of particular interest in regard to exposure to ALAN during early developmental as this is a sensitive stage in a bird's life. Exposure to stressful conditions during early development have been shown to affect growth, metabolism, immunocompetence and sexual attractiveness later in life (Lindström, 1999). Most of the studies in the field have focused on adult birds (Dominoni, Quetting and Partecke, 2013; De Jong *et al.*, 2015; Ouyang *et al.*, 2017; Raap *et al.*, 2017b; Kernbach *et al.*, 2020). The few studies examining the developmental period typically exposed nestlings to ALAN for only 2-7 nights (Raap *et al.*, 2016a; Grunst *et al.*, 2020; Raap, Pinxten and Eens, 2018). In contrast, we exposed nestlings to ALAN for the first 13 days of their life, covering the bulk of their development. To our knowledge, the only other study with a similar exposure period to the study by Dominoni *et al.* (2021). This study was able to demonstrate that impacts of ALAN on feather corticosterone were population specific, with urban birds experiencing higher levels of feather corticosterone and forest birds experiencing lower levels of feather corticosterone when exposed to ALAN (Dominoni *et al.*, 2021).

The present study tested the hypothesis that exposure of great tit nestlings to dim ALAN throughout the night for the first two weeks of their life, until close to fledging, would have negative impacts on the nestlings' health. Health is a multivariate trait; therefore, it can be complex to measure and understand. We aim to address this complexity by measuring multiple markers of health to provide a more comprehensive assessment of how it is being impacted by ALAN. Most field studies investigating the physiological and health impacts of ALAN on birds have measured only one or two biomarkers (Dominoni, Quetting and Partecke, 2013; Dominoni *et al.*, 2021; Grunst *et al.*, 2020; Kernbach *et al.*, 2020; Raap, Pinxten and Eens, 2016; Raap *et al.*, 2017a) except for a few studies (Ouyang *et al.*, 2017; Raap *et al.*, 2016b). Here, we measured, four biomarkers of health including scaled mass index (SMI), antioxidant capacity of plasma (OXY), malondialdehyde (MDA), and feather corticosterone (fCORT) levels. SMI is a proxy of body condition, providing a measure of the fat content or nutrient reserves of an animal (Peig and Green, 2009; Schamber, Esler and Flint, 2009) and can be influenced by both exposure to stressful conditions and nutritional deprivation (Labocha and Hayes, 2012). Oxidative stress arises when pro-oxidants overwhelm antioxidants which leads to the highly reactive oxygen species which can damage the organism's own tissues. Oxidative stress has been linked to many important processes including senescence, expression of sexual ornamentation and sperm performance as well as survival and reproduction (Sepp *et al.*, 2012a; Speakman *et al.*, 2015). It has been highlighted in various reviews that to understand oxidative stress, it is beneficial to have at least one measure of oxidative damage as well as a measure of antioxidant defence (Costantini and Verhulst, 2009; Costantini, 2019; Speakman *et al.*, 2015). The measure of oxidative damage used in the current study was MDA, which is a product of lipid peroxidation. OXY was also used as a measure of antioxidant defence as it integrates the total non-enzymatic antioxidant defence that is found in the organism's plasma. Lastly, CORT is the main glucocorticoid and the effector of the hypothalamic-pituitary adrenal (HPA) axis in birds. CORT plays an important role in the regulation of physiological and behavioural responses to stressors (Bonier, 2012). Most studies that have focused on urban endocrine ecology have used measures of baseline and stress induced plasma CORT concentrations. Plasma CORT, however, only provides information about the short-term endocrine state of the individual. Feather CORT concentrations given a longer-term integrated measure of endocrine state, as CORT is deposited from the bloodstream into the feather structure during feather growth and therefore integrates both baseline and stress-induced CORT concentrations over time (Romero and Fairhurst, 2016). Feather CORT may therefore give a better measure of the HPA status of a nestling from birth until time of

sampling. The biomarkers measured in this study were selected as research has shown that they are regulated by circadian rhythms and are susceptible to circadian disruption (Borniger *et al.*, 2014; Budkowska *et al.*, 2022; Eikenaar *et al.*, 2020; Hardeland, Coto-Montes and Poeggeler, 2003; Hillebrecht *et al.*, 2024; Froy, 2010; Sani *et al.*, 2006). This increases the likelihood that these biomarkers may be particularly sensitive to ALAN exposure.

To address our overall hypothesis this study sought to address the following questions:

1) Does ALAN have an impact on the body condition of great tit nestlings? We predicted that there would be a negative effect of ALAN on body condition, as suggested in previous studies (Ferraro, Le and Francis, 2020; Raap *et al.*, 2016b)

2) Does ALAN impact fCORT levels of great tit nestlings? We predicted that ALAN would increase fCORT levels.
3) Does ALAN impact oxidative stress status of great tit nestlings through effects on MDA and OXY levels? We expected that ALAN will result in higher levels of oxidative stress, and this will be evidenced either by higher oxidative damage i.e., MDA, or lower antioxidant defence i.e., OXY, or a combination of both factors.

3.3 Methods

3.3.1 Study sites.

The research took place in 7 forest sites, with low anthropogenic disturbance, in the West of Scotland (Fig. 3.1), between April-June 2021. The exact location of study sites can be found in Table S1. At each site, existing nestboxes (Woodcrete Schwegler boxes, 17 x 26 x 18 cm, hole size = 32 mm) were used. Nestboxes were monitored weekly during the reproductive period and all reproductive activities recorded. Near the expected hatching date, the nestboxes were checked every other day to identify exact hatching dates.

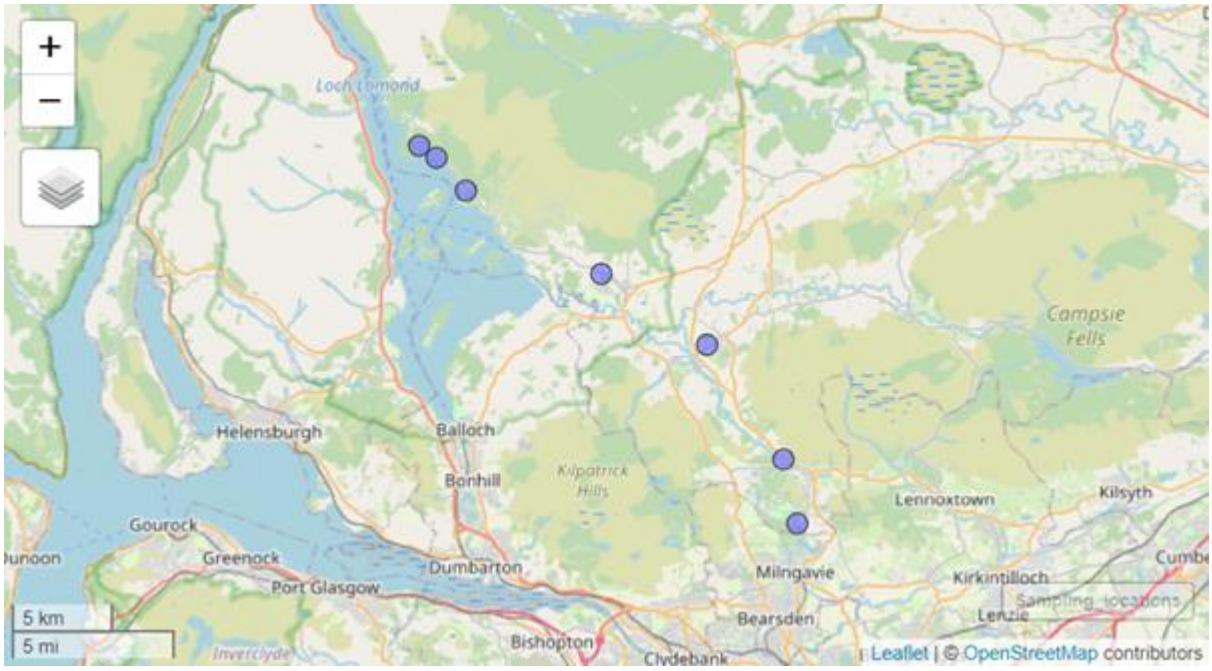

Figure 3.1: *The above map shows each study site used for the field experiment. The map was created in R (R Core Team, 2024) using the package Leaflet and the function Mapview ()* (Appelhans *et al.*, 2015)

3.3.2 Manipulation of artificial light at night

Upon hatching, whole broods were either exposed to ALAN (experimental group) or left unexposed (control group). Nest boxes were allocated to either an experimental or control group alternately based on their brood size and hatching date. For each box (control or experimental), the entire brood, including the original nest cup, was transferred into a new

nest box equipped with a single broad spectrum, cool-white LED bulb attached to the nestbox's ceiling. This bulb was powered by a 12 V, 3.2Ah battery that sat outside of the nest box on the forest floor. 200 A photometer (Li-COR Environmental, UK ltd, Cambridge, UK) was used to calibrate all lights to a standardised intensity of 1.8 lux (±1 lux) that has been shown to simulate the light in an urban environment (Dominoni, Quetting and Partecke, 2013). A sensor (Bioelectronics department, University of Glasgow) was attached to the outside of the battery box and turned the light on or off dependent on the ambient light intensity, to ensure that the experimental light manipulation only occurred at nighttime. The control nestboxes were also fitted with an LED light on the inside of the nest box roof, but these lights remained turned off. 14 experimental nestboxes and 15 control nestboxes were used. The exact allocation of control and experimental boxes to study site can be found in Table S2.1.

3.3.3 Sample collection.

To measure physiological biomarkers, blood samples (used to measure MDA and OXY) and feather samples (used to measure CORT) were collected 13 days after the day the first chick in a brood hatched. For every nest, four nestlings were removed from the brood for sampling. All nestlings were first placed in a bird bag, and four were then selected. This was consistently applied across both control and ALAN nest boxes to reduce the risk of bias. To obtain blood samples, the brachial vein was punctured with a sterile needle, and blood was collected into a 75 µl capillary tube. Our primary aim was to ensure that no more than 1% of each chick's body weight was taken, while still attempting to fill the tube whenever possible. Blood was immediately dispensed into a prelabelled 1.5 mL Eppendorf tube. The samples were kept on ice when out in the field. Within four hours of collection, the samples were spun for 10 min at 10,000 rpm to separate the plasma from the red blood cells. The plasma was then pipetted into a new 0.5 mL Eppendorf tube. Both the red blood cells and plasma were then stored in a -80^{o}C freezer until further analysis.

Two tail feathers no more than 20 mm in length were clipped from each of the 4 nestlings and transferred into a 1.5 mL Eppendorf tube prelabelled with a unique ID. The feathers were stored in a dry place at room temperature until further analyses.

Every nestling in each nest box was also ringed by a trained ringer. The tarsus length, body mass and wing length of each nestling was also recorded.

In total, we sampled 107 nestlings. For oxidative stress markers (OXY and MDA), we collected samples from 54 nestlings in control nest boxes and 53 nestlings in ALAN nest boxes. For fCORT analysis, we sampled 31 nestlings from control boxes and 30 from ALAN boxes. Differences in sample size across analyses were due to limited sample availability for certain metrics.

3.3.4 Scaled mass index

Body condition was calculated for each individual using scaled mass index which standardises body mass to a specific fixed linear measurement of the organism, which was tarsus length, as per equation (1) taken from Peig and Green (2009):

$$SMI = M^i \left(\frac{L_0}{L_i}\right) b^{SMA} \quad (1)$$

Where M^i is body mass and L_i is tarsus length. The scaling exponent b^{SMA} is estimated using a standardised major axis (SMA) regression of log transformed M^i on log transformed L_i. This was calculated using the sma () function from the smatr package in R (Warton *et al.*, 2012).

L_0 represents the mean tarsus length of the study population, and SMI is the predicted body mass for an individual when their tarsus length is standardised to L_0 (Peig and Green, 2009).

3.3.5 Lab work

DNA extractions

DNA was extracted from the red blood cells using a Puregene kit (QIAGEN, Hilden, Germany), following the manufacturer protocol. DNA concentration and quality were quantified using a Nanodrop-8000 spectrophotometer (Thermoscientific, Massachusetts, USA). DNA integrity was checked in a subset of 29 samples, using an Agilent 2200 TapeStation (Agilent, Santa Clara, California, USA). Genomic DNA samples were stored at -80ºC.

Molecular sexing

Molecular sexing of the nestlings was carried out using RT-PCR with primers P2 and P8, as described previously following Griffiths *et al.* (1998).

Measuring malondialdehyde levels in plasma.

Plasma concentrations of MDA were determined using high-performance liquid chromatography (HPLC) as described previously (Nussey *et al.*, 2009). In summary, an aliquot of plasma was transferred to a reaction tube pre-loaded with 20 µl of butylated hydroxytoluene (BHT). 40 µl of thiobarbituric acid (TBA) and 160 µl of phosphoric acid were added and the reaction tubes vortexed for 5 s. The sample were then placed in a dry heat bath (100ºC) for 1 h, before being cooled on ice. Once the reaction tubes had cooled, 160 µl of butanol was added and the tubes vortexed for 10 s, The samples were then centrifuged at 4°C at 12,000 x g for 3 min. An aliquot (60 µl) of the upper phase which contained MDA-TBA adducts was drawn off and placed in a HPLC vial.

20 µl of the prepared samples were injected into a HPLC system (Agilent 1200 series, Santa Clara, California, USA) fitted with a Hewlett-Packard Hypersil 5 µl ODS 100 x 4.6 mm column and a 5 µl ODC guard column (Thermo Fisher Scientific Inc., Massachusetts, USA) kept at 37ºC. The mobile phase was MeOH/buffer (40:60 v/v) at a flow rate of 1 mL min^{-1}. The buffer was 50 mM potassium monobasic phosphate, adjusted to pH 6.8 using 5 M KOH. Fluorescence detection was then performed using an Agilent 1260 at 515 nm excitation and 553 emission (Agilent, Santa Clara, California, USA). The MDA concentrations were calibrated using an external standard of 1,1,3,3- tetrathoxypropane (TEP) serially diluted with 40% ethanol. The intra-plate coefficient of variation (CV) for this assay was calculated using 12 standards run in duplicate for the calibration curve, yielding an intra-plate CV of 4.16%. Due to limited sample availability, repeatability between plates could not be directly assessed. However, this assay has been demonstrated to be repeatable in previous analyses conducted in the same laboratory (Bodey *et al.*, 2020; Nussey *et al.*, 2009)

Measuring antioxidant capacity of plasma.

OXY was measured in triplicate using an OXY-Adsorbent test (Diacron International, Grosseto, Italy) following the manufacturer's instructions with modifications. 2 µl of plasma was first diluted 1:100 and then 5 µl of the diluted sample was added to the plate along with 190 µl hypochlorous acid (HOCI; an endogenously produced oxidant). Once the oxidant solution was added the plate was incubated at 37ºC for 10 min. After incubation 5 µl of chromogen was added to each of the wells. The plate was read using a SpectraMax Plus spectrophotometer (Molecular Devices, San Jose, California, USA) at a temperature of 37ºC and a wavelength of 505 nm. The results of the OXY-Adsorbent test were expressed as µmol of HCI0/mL of sample. Repeatability was calculated as $R^2 = 0.67$ (n = 15). Repeatability was estimated by conducting the complete protocol on 15 samples in duplicate. A linear mixed-effects model (LMM) was fitted with sample ID as a random intercept to model within-individual variability. The repeatability metric was calculated as the ratio of the random intercept variance to the total variance. Inter-plate and intra-plate CVs were 10.62% and 7.03% respectively, which are within the range described by Costantini (2011).

Measuring feather corticosterone levels

Feather samples were washed (10 min) in an orbital shaker once with 1 mL of 20% methanol and twice with UltraPure water, before being left to air dry. This washing will remove any contamination and dirt as well as CORT from other sources such as faeces or preening oils from the surface of the feathers. Once dried, the feathers were cut into less than 5 mm lengths and weighed. Any samples that weighed <1 mg were excluded. All remaining samples weighed between 1-26 mg. To extract CORT from the feathers, 2 mL of high-performance liquid chromatography (HPLC)-grade methanol was added to each feather, and it was incubated at 52ºC in an orbital shaker at 175 rpm for 19 h. After incubation, 1 mL of methanol was removed into a 12 x 17 mm borosilicate glass tube and dried in a sample concentrator (Thermo Savant SC210A Speedvac concentrator). The samples were reconstituted in 150 µl of assay buffer in a multi-vortexer (SMI, Newmarket, UK) for 10 min. The CORT concentration for each sample was determined using a commercial ELISA kit (Cayman chemical Corticosterone ELISA Kit, Item No. 501320, Ann Arbor, Michigan, USA) following the manufacturer's instructions.

All samples were run in duplicate, and samples from the same nest boxes were split across different plates to avoid nest effects. Eight standards were run in duplicate on each plate to

generate a standard curve and assess plate sensitivity. Optical density was measured at a wavelength between 405 nm and 420 nm using a LT-4500 absorbance plate reader (Labtech International, Heathfield, East Sussex, UK). Assay Zap computer software (Biosoft, Acropolis Computers Ltd, Cambridge, UK) was then used to calculate the feather CORT concentration. The results were corrected against the weight of the feathers. This was following previous studies including Dominoni *et al.* (2021). Intra-plate CV was assessed using the eight standards, each run-in duplicate per plate, yielding an average CV of 7.09%. Due to limited sample size, we were unable to formally assess inter-plate repeatability, however this assay has previously been validated in the same laboratory (Dominoni *et al.*, 2021).

3.3.6 Statistical analysis

All analysis were performed in R (version 4.0.4) (R core team, 2024).

Effects of artificial light on scaled mass index, antioxidant capacity of plasma, malondialdehyde and corticosterone.

The effects of ALAN on the various health biomarkers measured were examined, using linear mixed models (LMMs) run with the R package lme4 (Bates *et al.*, 2015). The models were run separately for SMI, MDA, OXY and fCORT. Sex, treatment, brood size, and date of sampling were included in the models as predictors. Date of sampling which refers to the date on which biological samples were collected was converted into Julian date so that it could be added to the models as a continuous variable. We also included Julian date as a quadratic term. Treatment x sex was included as an interaction effect because males and females differ in size, with males potentially outcompeting females for food (Michler *et al.*, 2010; Teather, 1992), which may result in sex-specific treatment responses.

Treatment x date of sampling was also included as an interaction because phenological changes throughout the breeding season can impact reproductive success due to phenological mismatch with insect prey (Grüebler and Naef-Daenzer, 2010; Grimm *et al.*, 2015). Birds may therefore respond differently to the treatment depending on the time of breeding.

We included nest box ID nested into study site as a random effect in all models, to take into account the non-independency of samples collected from the same brood, and from broods at the same site. In all models excluding the one where SMI was used as the response

variable, SMI was included as a continuous predictor and treatment x SMI were included as an interaction effect. This interaction was included because birds in poorer body condition may be more sensitive to physiological changes induced by the treatment. Backward selection was always completed for every model using likelihood ratio testing (LRTs) with the drop1 () function from the lme4 package. To do this we began with a full model that included all of the relevant main effects and interactions. We sequentially removed non-significant interaction terms, starting with the interaction that had the highest P value. All main effects were retained in the final model, even if non-significant. This approach is common in statistical modelling and the removal of non-essential interactions which may absorb variance helps to prevent overfitting and reduce model complexity. This method also allows for more accurate model estimates for all variables of interest (Johnson and Omland, 2004; Lewis, Butler and Gilbert, 2011).

We performed diagnostic checks on al LMMs to ensure model assumptions were met. Normality of residuals was assessed using Q-Q plots, which showed no substantial deviations, indicating that residuals were approximately normally distributed. Homoscedasticity was evaluated through visual inspection of residuals plotted against fitted values, which did not reveal any major patterns that suggested heteroscedasticity. To assess multicollinearity among predictor values, we calculated variance inflation factors (VIF) using the car package (Fox and Weisberg, 2019). All VIF values were below 3, indicating no problematic collinearity among the fixed effects.

To explore significant interaction effects, post hoc pairwise comparisons were conducted using the emmeans package (Lenth *et al*., 2024). Tukey's Honest Significant Difference method was applied to adjust for multiple comparisons, and adjusted p-values along with confidence intervals were extracted using the contrast function.

The effect of antioxidant capacity of plasma on malondialdehyde.

To examine whether there was an effect of OXY on MDA levels, an LMM model was run for MDA where OXY was included as a continuous predictor. A three-way interaction between treatment, sex, and OXY was also tested in this model.

The effects of physiological biomarkers on scaled mass index.

To examine the effects the physiological biomarkers measured in the study had on SMI, an LMM model was run and included OXY and MDA as continuous predictors. We ran a separate model where feather CORT was included as a continuous predictor as this was only known for 61 out of 107 samples. Interaction effects of OXY, MDA and Feather CORT with treatment were also included in these models as we were interested if treatment causes physiological changes that as a result could affect body condition. We also performed a Pearson's correlation using the cor package (Guo, Song and Zhu, 2024) to assess relationships between the four biomarkers.

3.4 Results

3.4.1 Does artificial light at night impact body condition?

Great tit nestlings exposed to ALAN, regardless of sex, had a significantly lower SMI (χ^2 = 11.73, P = 0.001, Table 3.1), indicating a lower body condition, than the control birds (Fig. 3.2A).

Table 3.1: *The output of the linear mixed-effects model with values from likelihood ratio testing where scaled mass index was used as a response variable. The output shows the model estimate, standard error, 95% confidence intervals, degrees of freedom and P values. The fixed effects can be seen in bold, and any factor levels are shown in italics. Significant P values are indicated by a *. Random effect variance components are provided to indicate distribution of variance across grouping levels.*

Effect	Estimate	SE[A]	95% CI[A]	χ^2	df	p-value
Fixed effects						
Intercept	17.68	0.32	17, 18			
Treatment				11.73	1	**0.001***
ALAN	—	—	—			
Control	1.26	0.28	0.71, 1.8			
Sex				0.07	1	0.797
F	—	—	—			
M	0.04	0.22	-0.40, 0.48			
Brood size	0.00	0.18	-0.35, 0.36	0.01	1	0.928
Date of sampling	0.31	0.18	-0.05, 0.67	2.53	1	0.111
Random effects						
Nestbox: study site	0.178	0.422				
Study site	0.337	0.581				
Residual	1.166	1.08				
[A] SE = Standard Error, CI = Confidence Interval						

3.4.2 The effects of artificial light at night on corticosterone levels.

There was no significant impact of ALAN on fCORT concentrations (χ^2 = 0.68, P = 0.411, Table 3.2, Fig. 3.2B) in either sex.

Table 3.2: *The output of the linear mixed-effects model with values from likelihood ratio testing, where feather corticosterone levels was used as a response variable. The output shows the model estimate, standard error, 95% confidence intervals, degrees of freedom and P values. The fixed effects can be seen in bold, and any factor levels are shown in italics. Significant P values are indicated by a *. Variance components from the random effects structure are included to show the distribution of variance across grouping levels.*

Fixed effect	Estimate	SE[A]	95% CI[A]	χ^2	df	p-value
Intercept	-0.92	9.42	-19, 18			
Treatment				0.68	1	0.411
Control	—	—	—			
ALAN	-1.04	1.42	-3.8, 1.7			
SMI	0.32	0.49	-0.63, 1.3	0.44	1	0.507
Sex				0.49	1	0.485
F	—	—	—			
M	-1.11	1.20	-3.5, 1.2			
Brood size	-1.43	0.70	-2.8, -0.07	3.45	1	0.063
Date of sampling	0.37	0.67	-0.95, 1.7	0.38	1	0.540
Random effects						
Nestbox: Study site	0	0				
Study site	1.209	1.1				
Residual	21.243	4.609				
[A] SE = Standard Error, CI = Confidence Interval						

3.5.3 The effects of artificial light at night on antioxidant capacity of plasma.

The interaction between treatment and sex was not significant (χ^2 = 3.086, P = 0.079) Treatment was shown to have a significant impact on OXY levels (χ^2 = 5.66, P = 0.017,

Table 3.3), birds exposed to ALAN experienced higher levels of OXY than the control birds (Fig. 3.2C).

The analysis showed a trend towards an interaction between SMI and treatment on OXY, however this was not significant ($\chi^2 = 3.331$, $P = 0.068$). For the birds exposed to ALAN as SMI increased OXY decreased and the opposite trend can be seen in the control group (Fig. S2.1).

Table 3.3: *The output of the linear mixed-effects model with values from the likelihood ratio testing, where antioxidant capacity of plasma was used as a response variable. The output shows the model estimate, standard error, 95% confidence intervals, degrees of freedom and P values. The fixed effects can be seen in bold, and any factor levels are shown in italics. Significant P values are indicated by a *. Variance components from the random effects structure are included to show the distribution of variance across grouping levels.*

Effects	Estimate	SE[A]	95% CI[A]	χ^2	df	p-value
Fixed effects						
Intercept	222.33	93.19	40, 405			
Treatment				5.66	1	**0.017***
Control	—	—	—			
ALAN	28.03	11.91	4.7, 51			
SMI	-0.47	4.93	-10, 9.2	0.01	1	0.917
sex				2.82	1	0.093
F	—	—	—			
M	-17.79	10.78	-39, 3.3			
Brood size scaled	4.13	6.39	-8.4, 17	0.45	1	0.504
Date of sampling	-9.11	6.89	-23, 4.4	1.83	1	0.176
Random effects						
Nestbox: Study site	0	0				
Study site	5.871	2.423				
OXY plate ID	1005.709	31.713				
Residual	2851.833	53.403				
[A] SE = Standard Error, CI = Confidence Interval						

3.4.4 The effects of artificial light at night on malondialdehyde.

When investigating the effects of ALAN on MDA levels the results show that the interaction between sex and treatment was significant ($\chi^2 = 4.27$, P = 0.039, Table 3.4). Whereas both males and females exposed to ALAN had similar levels of MDA, within the control group, the males had the lowest levels of MDA when compared to all other groups (Fig. 3.2D). The post hoc analysis showed that control females and control males had the biggest difference from each other in their MDA levels (mean estimate [95% Confidence Interval, CI] = 1.629 [-0.088, 3.35]; Table S2.2). However, this was not statistically significant (Table S2.2).

There was no significant effect of OXY on MDA levels ($\chi^2 = 0.673$, P = 0.412, Fig. S2.2) and no interaction effect between OXY and treatment on MDA levels ($\chi^2 = 1.599$, P = 0.206).

***Table 3.4**: The output of the linear mixed-effects model with values from likelihood ratio testing, where malondialdehyde was used as a response variable. The output shows the model estimate, standard error, 95% confidence intervals, degrees of freedom and P values. The fixed effects can be seen in bold, and any factor levels are shown in italics. Significant P values are indicated by a *. Variance components from the random effects structure are included to show the distribution of variance across grouping levels.*

Effects	Estimate	SE[A]	95% CI[A]	χ^2	df	p-value
Fixed effects						
Intercept	8.43	3.89	0.80, 16			
Treatment						
Control	—	—	—			
ALAN	-1.10	0.73	-2.5, 0.33			
SMI	-0.05	0.21	-0.45, 0.35	0.07	1	0.789
sex						
F	—	—	—			
M	-1.63	0.63	-2.9, -0.40			
Brood size	0.18	0.33	-0.47, 0.83	0.36	1	0.548
Date of sampling	-0.48	0.35	-1.2, 0.20	2.18	1	0.140
Treatment * sex				4.27	1	**0.039***
*ALAN * M*	1.82	0.87	0.11, 3.5			
Random effects						
Nestbox: Study site	1.079	1.039				
Study site	0	0				
Residual	4.195	2.048				
[A] SE = Standard Error, CI = Confidence Interval						

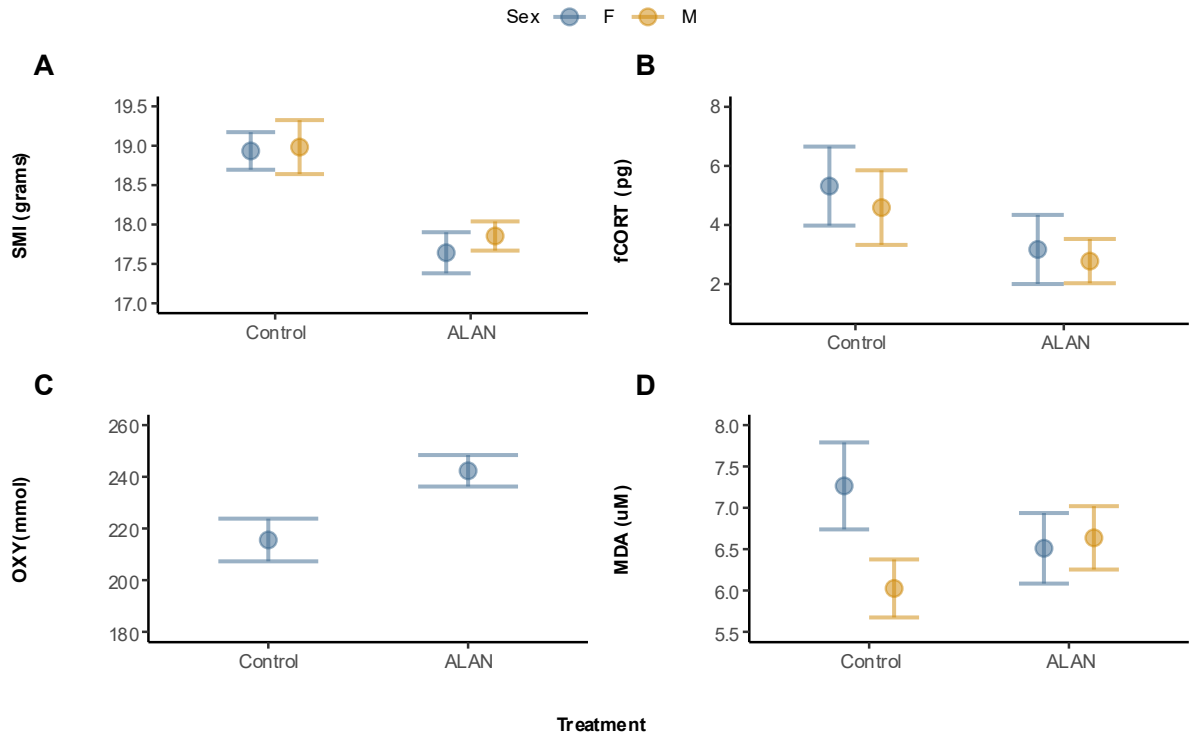

Figure 3.2: Impacts of treatment group (Control, ALAN) on health biomarkers in male and female great tit nestlings. *Each panel shows means ± S.E.M.* ***(A)*** *Scaled mass index (SMI) with sample sizes: ALAN female (n = 24), ALAN male (n = 28), Control female (n = 33), Control male (n = 22).* ***(B)*** *Feather corticosterone (fCORT) with sample sizes: Female ALAN (n = 13), Female Control (n = 20), Male ALAN (n = 18), Male Control (n = 15).* ***(C)*** *Antioxidant capacity of plasma (OXY), adjusted for experimental plate effects using residuals from the linear mixed model (LMM); sample sizes: Female ALAN (n = 27), Female Control (n = 33), Male ALAN (n = 27), Male Control (n = 25). D) Malondialdehyde (MDA) with sample sizes: Female ALAN (n = 27), Female Control (n = 33), Male ALAN (n = 27), Male Control (n = 25). Females are shown in blue, and males are shown in yellow.*

3.4.5 The impacts of the physiological biomarkers on scaled mass index.

The interaction of fCORT, OXY and MDA with treatment had no statistically significant effect on SMI and so were dropped from the model.

The results of the final model indicated that there was no significant relationship between the biomarkers measured (MDA: $\chi^2 = 0.16$, P = 0.694, OXY: $\chi^2 = 0.96$, P = 0.693, fCORT: $\chi^2 = 0.47$, P = 0.753) and SMI. Pearson's correlation analysis revealed very weak correlations between all four biomarkers as shown in Fig. 3.3.

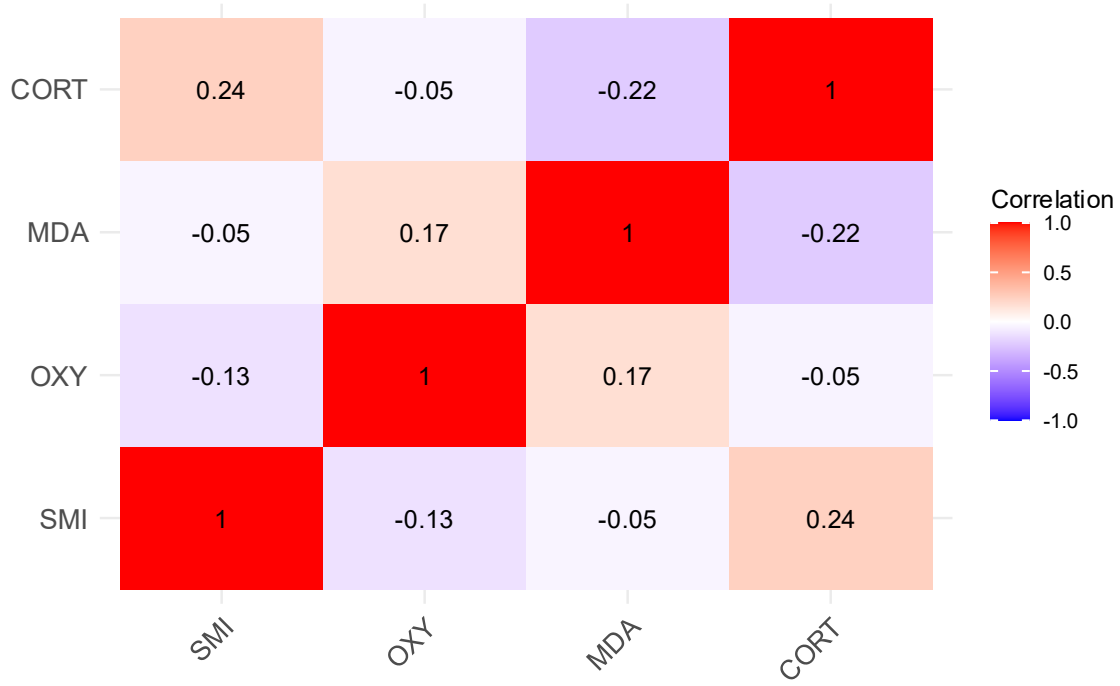

Figure 3.3: This heatmap presents the Pearson's correlation values between biomarkers. *The intensity of the colour represents the strength of the correlation, with red indicating a strong positive correlation and blue showing a strong negative correlation. Pearson's correlation coefficients are overlayed on top of the heatmap.*

3.5 Discussion

It is well documented that organisms that are exposed to ALAN face both behavioural and physiological changes that can be detrimental to health. The results of this study show that exposure to ALAN leads to lower body condition in great tit nestlings which may have negative consequences for fitness and survival later in life. However, there was no conclusive evidence that the changes in body condition were associated with effects of ALAN on oxidative stress or feather corticosterone levels.

3.5.1 The impacts of artificial light at night on body condition.

The findings of this study indicated that the nestlings in the ALAN treatment group had a significantly lower SMI than control birds. This result agrees with findings in previous studies where it has been shown that ALAN can have a negative impact on body condition and development in young birds (Ferraro, Le and Francis, 2020; Raap *et al.*, 2016a) and other species including cane toads (*Rhinella marina)* (Secondi *et al.*, 2021). There may be various mechanisms behind this relationship. Nestlings have been shown to beg for food more frequently throughout the night when exposed to ALAN (Raap, Pinxten and Eens, 2016), however it's unlikely that this heightened begging behaviour led to an increased feeding rate by the parents as the light treatment was only used inside of the nest box and the dark nighttime conditions outside would not provide optimal conditions for the parents to forage. This increase in begging behaviour can be costly as it may divert important resources from physiological processes including growth and metabolism. One study was able to show that as begging behaviour intensified in captive house sparrow (*Passer domesticus)* nestlings, metabolic expenditure increased, and body condition declined (Soler *et al.*, 2014).

The decrease in SMI could also be an indirect effect of ALAN through changes in the provisioning behaviour of the parents. It has been shown that when nestboxes were exposed to ALAN it disrupted the provisioning behaviour of female tree swallows *(Tachycineta bicolor)* and resulted in nestlings being fed less frequently than the control birds (Injaian *et al.*, 2021). Furthermore, female great tits exposed to ALAN within their nest box, have delayed sleep onset, and their sleep duration was reduced by half (Raap, Pinxten and Eens, 2016). Disruption to the sleep pattern of parents while they are rearing chicks could impact

parental care and as a result may negatively impact the condition of the nestlings. The impacts of a decrease in body condition prior to fledging are potentially lifelong and it has been shown that coal tits *(Periparus ater)* and great tits that leave the nest in poor condition, have a lower chance of survival after fledging (Naef-Daenzer, Widmer and Nuber, 2001).

3.5.2 The impact of artificial light at night on feather corticosterone levels.

There was no statistically significant effect of ALAN exposure on fCORT levels in great tit nestlings. The lack of an effect of ALAN was unexpected and contrasts with several studies that show exposure to ALAN can lead to increased levels of CORT including in developing birds (Alaasam *et al.*, 2018; Grunst *et al.*, 2020; Ouyang *et al.*, 2015). However, only one of these studies measured CORT levels in the feathers (Grunst *et al.*, 2020) while the remaining studies measured CORT in the plasma (Alaasam *et al.*, 2018; Ouyang *et al.*, 2015). CORT levels measured in plasma represent a short-term snapshot of CORT in time and may not be directly comparable with the chronic measure of CORT used in the current study.

The reason why we may be seeing no significant effect of ALAN on fCORT levels could be an adaptive response of neonates where they will stop chronically high levels of CORT during early development to avoid negative consequences later in life (Romero, 2004). This has been shown in neonate mammals where they will have a dampened response to stressors in the first few days of life to protect from the harmful effects of continuous glucocorticoid elevation (Romero, 2004). The environment where the nestlings are reared may also impact how ALAN affects CORT levels, for example an earlier study (Dominoni *et al.*, 2021) showed that ALAN has different effects on CORT concentrations in urban and non-urban blue tit nestlings. In this study urban birds had higher fCORT levels in response to ALAN whereas non-urban birds had lower levels (Dominoni *et al.*, 2021). It could be the case that birds in the urban field site are exposed to other external stressors as well as the ALAN manipulation such as poor diet and the combined effect of these stressors may explain the increase in feather CORT. This corresponds with the mild effect seen in the current study as the current ALAN experiment took place in non-urban environments. Grunst *et al*. (2020) found that developing great tits exposed to ALAN had elevated feather CORT levels, which contradicts our results. However, in the Grunst *et al.* (2020) study, nestlings were not exposed to ALAN until they were 8 days. It is possible that the birds in this previous experiment were able to mount a more robust stress response when exposed to ALAN, whereas the nestlings in our study may have become habituated, having been exposed from

a much earlier developmental stage. The interpretation of CORT levels must also be carefully considered. Researchers cannot say for certain if elevated levels of CORT reflect chronic stress as CORT has many other functions including energy mobilisation (Romero, 2004). Glucocorticoid response to chronic stress may also depend on many factors including species, sex, age, season as well as the type of external stressor they are exposed to (Cyr and Romero, 2007).

3.5.3 The impacts of artificial light at night on oxidative stress

In this study we found a significant effect of treatment group on OXY levels, with birds exposed to ALAN exhibiting higher OXY levels than the control groups. This suggests that ALAN exposure may cause the birds to upregulate their antioxidant defences, possibly in response to higher oxidative damage caused by ALAN. This aligns with findings from previous studies, which have shown that several bird species increase antioxidant parameters in response to environmental stressors (Cohen, Hau and Wikelski, 2008; Casagrande and Hau, 2018). However, we cannot conclude from this single biomarker alone if this increase has a negative impact on the organism. While antioxidant defences can be energetically costly, they are also vital for mitigating oxidative stress and can serve a variety of other biological functions (Costantini *et al.*, 2010). Diet can also play an important role in antioxidant levels, as parents may provide nestlings with antioxidant-rich food, potentially influencing the observed levels (Møller, Erritzoe and Karadas, 2010).

When assessing the relationship between ALAN and MDA levels, MDA levels in ALAN exposed males and females appeared to be lower than the control females and only slightly higher than the control males. This coupled with the higher oxidative defence found in the ALAN exposed birds could indicate that ALAN caused higher levels of oxidative damage which was successfully combatted by a robust antioxidant response. It is not possible to conclude that this is the case as we only collected one sample from each individual and the balance between pro-oxidants and antioxidants is a dynamic state which is constantly changing. The females in the control group had higher levels of OXY and MDA than the control males which may suggest that females have naturally higher levels of oxidative stress than males. If this is the case, then it highlights the importance of considering sex when investigating these types of relationships.

There is evidence of sex differences in oxidative stress responses in the literature. Our study agrees with a meta-analysis that looked at oxidative stress levels across vertebrates and found

that levels were usually higher in females than males across a range of tissues (Costantini, 2018). The findings in the literature into whether ALAN impacts oxidative stress levels are mixed. Several studies looking into a variety of species suggest that ALAN does not impact on oxidative stress (Czarnecka *et al.*, 2022; Dimovski and Robert, 2018; Raap *et al.*, 2016a). One study on great tit nestlings found no effect of ALAN on a wide range of oxidative stress biomarkers including antioxidant enzymes and measures of oxidative damage. This study also reported that there were no effects of sex influencing the way in which ALAN impacted oxidative stress (Raap *et al.*, 2016a). Another study showed that there was no effect of ALAN on lipid peroxidation or antioxidant capacity in female tammar wallabies (*Macropus eugenii)* (Dimovski and Robert, 2018). These results suggest that impacts on oxidative stress may be dependent on a range of factors including sex specific differences and species.

It is important to highlight that one should ideally measure several biomarkers of both oxidative damage and antioxidant defence in multiple tissues (Costantini, 2019). We also need to be careful when interpreting static measures of oxidative stress as it is a dynamic process which is constantly changing limiting our ability to capture the process at a single point in time (Azzi, 2022). It has also been discussed in several reviews that an increase of oxidative stress with age can be a major source of damage to cellular function and structure (Costantini, 2011; Yu and Chung, 2006). Organisms have therefore evolved many sophisticated mechanisms to prevent the accumulation of oxidative damage (Costantini, 2018; Miura and Endo, 2010). Therefore, it could be the case that we see limited evidence of oxidative stress in the current study as maintaining oxidative processes are critical to survival and longevity. If this is the case, then it could highlight the need for future studies to include fitness or first year survival into their experiment.

3.5.4 The relationship between physiological biomarkers and body condition.

No relationships were found between any of the physiological biomarkers measured and SMI. High levels of Glucocorticoids have been suggested to be associated with low relative fitness as discussed in a review by Bonier *et al*. (2012). Some studies have found that birds in better body condition tend to have lower levels of blood CORT (Strange *et al.*, 2016; Vágási *et al.*, 2020). However, other studies focusing on fCORT levels have found no correlation between fCORT and body condition (Beaugeard *et al.*, 2019; Gormally *et al.*, 2020). It may therefore be the case that only certain indices of CORT are correlated to body condition for example CORT levels measured in plasma represent a snapshot measure of

CORT while CORT levels measured in feather samples represent long-term chronic levels of CORT.

High levels of oxidative stress have also been associated with negative impacts on overall fitness and survival in previous studies. One study found that Alpine swift *(Tachymartptis melba)* females laid clutches that were smaller and less likely to hatch than females that had a higher resistance to oxidative stress (Bize *et al.*, 2008). There are limited studies that have investigated the relationship between oxidative stress and body condition, and the studies that have investigated this relationship reported contrasting results. It was shown in zebra finch nestlings that when they experienced high levels of oxidative stress in early life this could result in phenotypic changes including a shorter tarsus length than the control birds (Romero-Haro and Alonso-Álvarez, 2020). Larcombe *et al*. (2015) looked at captive adult parrots and was able to show an association with higher levels of oxidative damage and increased body mass. They did, however, report that not all oxidative stress biomarkers were associated with body mass (Larcombe *et al.*, 2015). It may, therefore, be the case that the relationship between condition and oxidative stress is a complex one that depends on various factors including life stage and environment (Boonekamp *et al*., 2014).

3.5.5 Limitations and directions for future research.

There are some limitations to the current study that must be considered and offer scope for future research. This study did not delve deep into the relationship between ALAN and body condition, it would be beneficial for future studies to investigate the mechanisms behind this relationship, for instance whether it was a consequence of a direct effect due to the increased energy expenditure of the begging nestlings or an indirect effect due to changes in the provisioning behaviour of the parents. This type of behaviour could be measurable using cameras set up within the nest box (Grzędzicka, 2017; McCowan and Griffith, 2014; Mutzel *et al.*, 2019; Raap, Pinxten and Eens, 2016; Soler *et al.*, 1999).

Secondly, our study was limited to a single field season. While incorporating multiple years of data would have strengthened our results by accounting for external factors such as weather variability between years, multi-year studies are not always feasible. Many previous field studies have also relied on single-season data (Dominoni *et al.*, 2021; Grunst *et al.*, 2019; Grunst *et al.,* 2020; Raap *et al.*, 2016b; Raap *et al.*, 2017a), contributing to a growing body of research that can help identify broader trends and mechanisms. These individual

studies may support future comprehensive meta-analysis on this topic. However, in order to assess annual variation, multiple field seasons would be necessary in future studies.

Lastly, our study was limited by a relatively small sample size. As some interactions showed weak or marginal significance, these results should be interpreted with caution. Further studies with larger sample sizes are therefore needed to confirm these patterns which would strengthen the findings in the current study.

3.6 Conclusions

As urbanisation continues to increase globally, wildlife is being exposed to increasing amounts of urban stressors including ALAN. Therefore, it is becoming more important to understand the impacts of these urban stressors on the health and fitness of the wildlife that are exposed to them. The results of this study show that the body condition of great tit nestlings is negatively impacted by exposure to ALAN which could have negative consequences for their future fitness and survival. However, the study provided no evidence of ALAN having effects on the fCORT levels of the nestlings. We did observe a sex-dependent effect of ALAN on oxidative stress, although it remains unclear whether this change reflects a negative impact. These findings highlight the complex, variable nature of ALAN on physiological effects and reinforces the need for future studies to include more biomarkers of health to better understand what aspects of health and physiology are impacted by ALAN. It is also important for future research to focus on the mechanisms behind ALAN impacting body condition. If we can better understand this relationship, then it would help future conservation efforts in urban areas to be better targeted. There is also a need for future studies to further investigate the implications of ALAN for oxidative balance taking into consideration differences between sexes.

3.7 Ethics statement

All work involving feather clipping was conducted under a British Trust for Ornithology licence (12138) issued to Davide M Dominoni. Permission for bird ringing was granted by the British Trust for Ornithology with licences to Davide M Dominoni (permit number: 6822). All blood samples were collected under the project licence CPP20193834 issued to Davide M Dominoni.

3.8 Availability of data and materials

All relevant data and R code used in this analysis are available on Zenodo: https://doi.org/10.5281/zenodo.14922693

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3.11 Author contributions

Rachel Reid, Davide M Dominoni and Jelle Boonekamp designed the study. Rachel Reid, Christopher Mitchell, Neal J Dawson and Neil P Evans analysed the data in the lab. Rachel Reid conducted the data analysis. Rachel Reid wrote the initial draft of the manuscript with all contributing authors editing and revising the manuscript.

Chapter 4
Partial night lighting mitigates the effects of artificial light at night on glucose and telomere loss in a songbird.

Rachel R Reid[1*], Neal Dawson[1], Eleanor Duncan[1], Robert Gillespie[1], Christopher Mitchell[2], Claire J Branston[1,3], Pablo Capilla-Lasheras[1,4,5], Jelle Boonekamp[1], Davide M Dominoni[1].

[1]School of Biodiversity, One Health and Veterinary Medicine, Graham Kerr Building, University of Glasgow, Glasgow, G12 8QQ, UK

[2]Environment and Sustainability Institute, University of Exeter, Penryn Campus, Penryn, Cornwall, TR10 9FE, UK

[3]School of Health and Life Sciences, University of the West of Scotland, Hamilton International Technology Park, Stephenson Pl, Glasgow, G72 0LH, UK

[4]Swiss Ornithological Institute (Vogelwarte), Seerose 1, Sempach, 6204, Switzerland

[5]Doñana Biological Station, Spanish National Research Council (EBD-CSIC), Sevilla, 41092, Spain

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4.1 Abstract

Background: Artificial light at night (ALAN) continues to increase at an unprecedented rate globally every year. ALAN can disrupt circadian rhythms and cause behavioural and physiological changes which may have knock on effects for health, yet we still understand very little about these effects. It is becoming increasingly important to investigate potential mitigation strategies; through understanding what aspects of ALAN negatively impact wildlife health.

Methods: Here we present the results of an experiment where we investigated the impact of ALAN on various health biomarkers in captive adult zebra finches *(Taeniopygia guttata)* over the course of four months. The health biomarkers measured included glucose concentration, relative telomere length, malondialdehyde and antioxidant capacity of plasma. The birds were separated into treatment groups and exposed to full light at night, partial light at night or dark nights (control).

Results: We show that exposure to full light at night impacted the circadian pattern of glucose levels and accelerated the shortening of telomeres, but these effects were not present when birds were exposed to partial light at night.

Conclusion: These results highlight that a partial night lighting strategy could be enough to reduce the negative effects on ALAN on wildlife health and therefore has the potential to be included in future mitigation strategies aimed to reduce negative impacts of ALAN in urban habitats.

Key words: Avian health, Avian physiology, Artificial light, Light pollution, Urban ecology

4.2 Introduction

As our world has become increasingly urbanised due to the continued growth of the human population, the levels of various novel stressors associated with urban environments have increased. One of these stressors is artificial light at night (ALAN). It has been estimated that over 80% of the human population globally now lives with light pollution (Guan *et al.*, 2022). ALAN can be attributed to various sources including street lighting, architectural lighting, security lighting, domestic lighting and vehicle lighting (Gaston *et al.*, 2013). ALAN can also have impacts via skyglow where ALAN that is emitted or reflected upwards is scattered by molecules in the atmosphere (Gaston *et al.*, 2013). Recent estimates indicate that observable light emissions are increasing faster than that of the human population globally (Gaston and Sánchez De Miguel., 2022). While ALAN is an important part of everyday life for most people and outdoor lighting allows humans to extend work time, socialize and improve safety (Gaston and Sánchez De Miguel., 2022), the loss of darkness can also come with negative consequences, particularly for wildlife and ecosystems.

There are various direct and indirect ways in which exposure to ALAN can lead to negative consequences for wildlife. The earth's rotation produces natural daily dark-light cycles that

influence an organism's behaviour (Russart and Nelson, 2018b). Most organisms have circadian clocks that allows them to align behavioural and physiological activities to the external environment, thus maximising fitness (Yadav *et al.*, 2022). However, ALAN can disrupt this natural alignment by desynchronizing the timing of essential behaviours such as activity, sleep, foraging, orientation and migration (Aulsebrook *et al.*, 2018; Hoffmann, Palme and Eccard, 2018; Russart and Nelson, 2018b; Van Doren *et al.*, 2017). For example, many diurnal species have been shown to be active throughout the night when exposed to ALAN, expending more energy than needed and diverting resources away from other important physiological functions (Dimovski and Robert, 2018; Dominoni *et al*., 2013; De Jong *et al*., 2017; Yadav *et al*., 2022). Various physiological processes are also under circadian control and as a result are vulnerable to ALAN exposure. These physiological processes include, but are not limited to, the secretion of corticosterone (Helm, Greives and Zeman, 2024) and sex steroids (Helm, Greives and Zeman, 2024), and the production of glucose and insulin (Kumar Jha, Challet and Kalsbeek, 2015; Lobban, Downs and Brown, 2010). If ALAN causes temporal misalignment of these processes, then it could lead to serious health consequences.

The misalignment of internal body rhythms with the external environment is well-known to negatively impact human health, including causing a higher risk of metabolic syndromes, obesity, breast and prostate cancer (Erdem *et al.*, 2017; Russart and Nelson, 2018a), however the health impacts of ALAN on wildlife have only recently been appreciated. In both nocturnal and diurnal rodents, ALAN reduces survival rates and reproductive success (Vardi-Naim *et al.*, 2022). ALAN has been shown to reduce reproductive success and impair both larval development and pupal diapause in moth species discussed in detail in Boyes *et al*. (2020). There is also evidence of a negative relationship between ALAN and health in bird species. In male great tits *(Parus major)*, circadian disruption altered tissue function related to timing, memory, metabolism and immunity (Dominoni *et al.*, 2022). Another study showed exposure to white light led to higher levels of nighttime activity and lower oxalic acid levels in great tits, indicating sleep deprivation which correlated with a higher likelihood of avian malaria infection (Ouyang *et al.*, 2017).

Although many studies show negative impacts of ALAN on health, others report minimal or no effects. One study showed that ALAN had little effect on the fitness and reproductive success of two songbird species (De Jong *et al.*, 2015). Similarly, in great tits, no difference was found in telomere length between birds exposed to white light and the control group,

despite a higher prevalence of malaria infection (Ouyang *et al.*, 2017). It has also been shown that there was no direct evidence for ALAN exposure to increase oxidative stress levels in tammar wallabies (*Notamacropus eugenii)* (Dimovski and Robert., 2018). These contrasting findings suggest that the impact of ALAN on health is complex and may vary with factors such as biomarkers measured, and environment, as observed for urbanisation health effects on wildlife (Reid *et al.*, 2024). Most studies on ALAN focus on one or two biomarkers of health within a single study, including those by Dominoni *et al.* (2018), Evans *et al.* (2012), Malek and Haim, (2019), and Raap *et al.* (2017a), which limits our understanding of its full physiological impact. As health is multivariate, increasing the number of biomarkers could provide a holistic view of ALAN's impacts by revealing trade-offs between interconnected health systems. Moreover, most experimental studies, particularly in captivity, involve short-term ALAN exposure (days to weeks). However, in one long-term study, Eurasian blackbirds *(Turdus merula)* exposed to ALAN for two years experienced a complete reproductive shutdown after the first year (Dominoni, Quetting and Partecke, 2013). This highlights the potential for dramatic long-term effects of ALAN and the need for longitudinal studies (Alaasam *et al.*, 2024; Beaugeard, Brischoux and Angelier, 2024; Dimovski and Robert, 2018; Itay and Haim, 2024; Jha and Kumar, 2017; Malek, Haim and Izhaki, 2020).

Given the growing evidence of ALAN's impact on wildlife health and biodiversity (Sanders *et al.*, 2021), developing mitigation strategies is becoming increasingly important. Adjusting ALAN properties, such as intensity and spectrum, is a promising avenue. Studies indicate that reducing light intensity (Dominoni *et al.*, 2018; Evans *et al.*, 2012; Raap, *et al*., 2017b) and shifting the light spectrum to longer wavelengths (Aulsebrook *et al.*, 2020; De Jong *et al.*, 2017) can significantly lessen ALAN's behavioural and physiological impacts. Another strategy involves reducing ALAN duration, such as turning off streetlights after midnight when fewer people need it. This not only could benefit wildlife but could also lower economic costs (Gallaway, Olsen and Mitchell, 2010; Gaston *et al.*, 2012; Gaston *et al.,* 2015; Pagden, Ngahane and Amin, 2020). However, studies looking into partial night lighting have found contrasting results. One study found stronger negative effects on aphid colony growth under partial light compared to exposure to full light at night (Heinen *et al.*, 2023). Studies on bats reported no benefits of partial light on bat activity and suggested to be beneficial the lights would need to be switched off before midnight (Azam *et al.*, 2015; Day *et al.*, 2015). However, in contrast, partial lighting reduced disruptions to oyster behavioural rhythms (Botté *et al.*, 2023). Therefore, there is mixed evidence to date for the benefits of partial night lighting as a mitigation strategy. This highlights the need for further

experiments to determine whether partial ALAN exposure has weaker health impacts than full ALAN exposure.

In this study, we exposed adult zebra finches (*Taeniopygia guttata)* to ALAN for four months in captivity to examine effects on health over time. Birds were divided into three groups: A natural photoperiod without ALAN (hereafter DARK), ALAN throughout the night (FLAN), and ALAN during the first half of the night (PLAN). The PLAN group was designed to expose the birds to light during the first half of the night to test the effects of intermittent darkness on health. The timing used in the treatment groups was aligned with the winter photoperiod at the time of the experiment. To capture the multivariate nature of health, we measured several biomarkers including glucose concentration, relative telomere length (RTL), antioxidant capacity of plasma (OXY) and malondialdehyde (MDA).

Glucose levels, tightly regulated across species, are influenced by habitat quality, reproductive state, and body condition. Glucose is also under strong circadian control making it vulnerable to ALAN induced disruptions (Kaliński *et al.*, 2014; Lobban, Downs and Brown, 2010). Disruption to glucose levels can lead to metabolic disturbance and even cell death (Kawahito, Kitahata and Oshita, 2009). Telomere attrition is linked to survival, mortality risk, fitness, and reproductive success in several species (Beirne *et al.*, 2014; Wilbourn *et al.*, 2018; Wood and Young, 2019) and environmental stressors are known to accelerate telomere loss (Monaghan and Haussmann., 2006). Lastly, oxidative stress is linked to many important processes including senescence, ornamentation, sperm performance, survival, and reproduction (Sepp *et al.*, 2012a; Speakman *et al.*, 2015). Several reviews have highlighted that to understand oxidative stress it is recommended to measure both a biomarker of oxidative damage as well as a biomarker of antioxidant defence (Costantini and Verhulst, 2009; Costantini, 2019; Speakman *et al.*, 2015). We measured malondialdehyde (MDA), which is a product of lipid peroxidation. Antioxidant capacity of plasma (OXY) was used to measure antioxidant defence as it integrates the total non-enzymatic antioxidant defence that is found in the organism's plasma.

Using these biomarkers, we aimed to address the following questions:

1) Does ALAN impact the circadian rhythm of glucose? We predict that ALAN will disrupt glucose circadian variation, causing abnormal peaks, particularly in the FLAN group, and to a lesser extent in the PLAN group. We also predict that there will be no disruption to glucose circadian variation in the control group.

2) Does ALAN impact glucose concentrations over time? We predict that ALAN will increase glucose concentrations over time by disrupting circadian rhythms, causing mistimed activity and feeding patterns (Kumar Jha, Challet and Kalsbeek, 2015; Rumanova *et al.*, 2022). We predict this effect will be stronger in the FLAN group. We also predict that there will be no impact on glucose levels over time in the control group.

3) Does ALAN impact telomere length over time? We predict that ALAN will accelerate telomere shortening over time, with a stronger effect in the FLAN group. We predict that there will be no effect on telomere shortening in the control group.
4) Does ALAN exposure impact oxidative stress levels over time? We predict that ALAN will increase oxidative stress levels over time, evidenced by higher MDA levels and lower OXY levels, with a stronger effect in the FLAN group. We predict that there will be no impact on oxidative stress in the control group.

4.3 Methods

4.3.1 Experimental protocol

On the 7th of September 2022, 44 adult (>three months old) zebra finches (21 females and 23 males were brought to a facility based at the University of Glasgow having been bred together in captivity from the same breeding stock. The birds were given a two-week period of acclimatisation where they were exposed to a photoperiod of 9 hours daylight and 15 hours of darkness (lights on from 7 am to 4 pm) which was close to the local photoperiod. Between the 21st of September 2022 and the 23rd of January 2023, the birds were split into and kept in three treatment groups which varied in their lighting regimes, these are described in Table 4.1. The males and females were housed separately, and individuals were randomly selected for each treatment group.

Each treatment took place in separate rooms to ensure the treatments did not interfere with each other. Each treatment room had a layout of four cages (2 m x 1 m x 0.5 m) each of which had 2-5 birds. Each cage was fitted with two broad spectrum white LED lights that covered a wavelength of 400-700 nm and were standardised to produce an intensity of 1.5 lux at perch level. Lights were controlled via a mechanical timer. The daytime illumination was produced by lights installed on the ceilings and produced an intensity of 400 lux at perch level. Birds were provided with dry finch seed mix and water ad libitum.

Table 4.1: *Code names, description and time of light exposure for each of the treatment groups that will be referred to throughout the main text. The associated sample sizes, in number of individuals, are also shown.*

Code	Treatment	Time lights were on	Sample size
Full darkness at night (DARK)	Control group – lights switched off during the night	Lights on from 7 am to 4 pm and then 15 hours of darkness	12 (6 males and 6 females)
Partial light at night (PLAN)	Group exposed to partial dim artificial light at night during the dark photoperiod	7 am to 4 pm and 9 pm-2 am therefore experienced 10 hours of darkness	15 (8 males and 7 females)
Full light at night (FLAN)	Group exposed to dim artificial light at night throughout the dark photoperiod	9 am to 4 pm and 7 pm-9 am therefore experienced 3 hours of darkness	16 (9 males and 7 females)

4.3.2 Sample collection.

We blood sampled each bird at the beginning (September) and end (January) of the experiment. Blood samples were taken from the brachial vein with a sterile needle into a 75 µl capillary tube, taking no more than 1% of each individual's body weight. The blood was immediately dispensed into a prelabelled 1.5 mL Eppendorf tube; the samples were kept on ice until processed. Within four hours of collection, we spun the samples using a centrifuge at 2000 rpm at 4^{o}c for 10 min to separate the plasma from the red blood cells (RBC's) and then transferred the samples into a -80^{o}C freezer. During each sampling attempt every bird was also weighed using an electronic balance.

To ensure that we could investigate the effect of treatment on the circadian rhythm of glucose, at the end of the experiment each bird was blood sampled twice at different times of day which included 1 am, 6 am, 1 pm and 8 pm. These times were chosen to ensure we could obtain measurements over a 24-h period. Half of the birds were sampled at each timepoint to ensure each bird was only sampled twice and had a break (12-14 h) in between sampling efforts. This staggered sampling approach meant that there were birds from every treatment group within each sampling time.

4.3.3 Lab work

Glucose measurements

During the start and end of experiment sampling attempts, a drop of blood from each individual bird was used to measure Glucose levels using the Exactive EQ real-time blood glucose test (MicroTech Medical, Zhejiang, China). As handling stress can elevate glucose levels (Malisch *et al.,* 2018; Le Maho *et al.,* 1992), handling time was kept to a minimum, and bleeding time was recorded for each individual. A linear mixed-effects model (LMM) fitted in R (R Core Team, 2024) showed that bleeding time had no significant effect on glucose levels ($\chi^2 = 0.104$, df = 1, P = 0.747).

Measuring relative telomere length

DNA was extracted from RBC's using the Puregene kit (QIAGEN, Hilden, Germany) following the manufacturers protocol. DNA concentration and quality were quantified using a Nanodrop-8000 spectrophotometer (Thermoscientific, Waltham, Massachusetts, USA), and sample were diluted to 1.25 ng/µl and stored at -80ºC.

Relative Telomere Length (RTL) was measured using real-time quantitative polymerase chain reaction (qPCR) which has been validated by various studies (Cawthon, 2002; Criscuolo *et al.*, 2009). RTL was measured as the T/S ratio (telomere repeat copy number (T) to control gene copy number (S) relative to a reference sample). The control gene, RAG-1 (Groth and Barrowclough, 1999; Liu *et al.*, 2018), was chosen for being highly conserved across species, including zebra finches (Kumar *et al.*, 2015). Telomere primers were Tel1b 5′ CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′ and Tel2b 5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′. RAG-1 primers were 5′-GCAGATGAACTGGAGGCTATAA-3′ and 5′-CAGCTGAGAAACGTGTTGATTC-3′.

Primer optimisation tests were performed with concentrations of 50 nM, 100 nM, 300 nM, 500 nM. qPCR assays were conducted with 7.5 ng of DNA per reaction with a primer concentration of 50 nM (RAG-1) and 500 nM (Telomere) in a final reaction volume of 20 µl containing 10 µl (1x) of absolute blue qPCR SYBR green low ROX mix. Thermal profiles were as follows: Telomere assay - 15 min at 95ºC, 27 cycles of 15 s at 95ºC, 30 s at 58ºC, and 30 s at 72ºC; RAG-1 assay - 15 min at 95ºC, 40 cycles of 15 s at 95ºC and 30 s at 60ºC. Melting curve analysis was performed after the reaction. Reactions were performed in triplicate, with plates balanced for cage number and treatment groups. A standard curve (6 points, 40 ng to 1.25 ng) was run on each plate using a serial dilution of pooled DNA samples. On each plate 6 µl of water was used for the non-template controls. Two inter-plate control samples were also run on each plate by mixing two separately selected batches of samples, using 7.5 ng of DNA per reaction. These were used to calculate inter-plate variation.

The qPCR data was analysed using the QBASE software (Hellemans *et al.*, 2008) which calculates RTL (T/S ratio) as calibrated normalized relative quantities, controlling for amplification efficiency and inter-run variation by including three inter-run calibrators from the standard curve. The mean telomere assay efficiency was 99.2 ± 2.3% and 104.1 ± 1.9% for the RAG-1 assays. Mean intra- and inter-plate of C_t values were 2.14 and 2.97% for telomere reactions and 1.02 and 1.10% for the RAG-1 reactions.

Measuring malondialdehyde levels in plasma.

Plasma concentrations of MDA were determined using high-performance liquid chromatography (HPLC) following Nussey *et al.* (2009). In summary, we transferred an aliquot of plasma to a reaction tube pre-loaded with 20 µl of butylated hydroxytoluene (BHT). We then added 40 µl of thiobarbituric acid (TBA) and 160 µl of phosphoric acid. Next, we vortexed reaction tubes for 5 s and placed them in a dry heat bath at 100ºC for 1 h, before being cooled on ice. Once the reaction tubes had cooled, we added 160 µl of butanol and then vortexed the samples for 10 s, we then centrifuged the samples at 4ºC at 12,000 x g for 3 min. An aliquot (60 µl) of the upper phase which contained MDA-TBA adducts was drawn off and placed in a HPLC vial.

We then injected 20 µl of the samples into a HPLC system (Agilent 1200 series) fitted with a Hewlett-Packard Hypersil 5 µl ODS 100 x 4.6 mm column and a 5 µl ODC guard column (Thermo Fisher Scientific Inc., Massachusetts, USA) kept at 37ºC. The mobile phase was

MeOH/buffer (40:60 v/v) at a flow rate of 1ml min^{-1}. The buffer was 50 mM potassium monobasic phosphate which we adjusted to pH 6.8 using 5 M KOH. We then performed Fluorescence detection using an Agilent 1260 at 515 nm excitation and 553 emission. We calibrated the MDA concentrations using an external standard of 1,1,3,3- tetrathoxypropane (TEP) serially diluted with 40% ethanol. The intra-plate coefficient of variation (CV) for this assay was calculated using 12 standards run in duplicate for the calibration curve, yielding an intra-plate CV of 4.16%. Due to limited sample availability, repeatability between plates could not be directly assessed. However, this assay has been demonstrated to be repeatable in previous analyses conducted in the same laboratory (Bodey et al., 2020; Nussey *et al.*, 2009).

Measuring antioxidant capacity of plasma.

Antioxidant capacity of plasma (OXY) was measured using an OXY-Adsorbent test (Diacron International, Grosseto, Italy) following the manufacturer's instructions with modifications. All samples were run in triplicate on the plate. 2 µl of plasma was first diluted 1:100 and then 5 µl of the diluted sample was added to the plate along with 190 µl hypochlorous acid (HCIO; an endogenously produced oxidant). Once the oxidant solution was added, the plate was incubated at 37°C for 10 min. After incubation we added 5 µl of chromogen (alkyl-substituted aromatic amine) to each of the wells. The plate was read using SpectraMax plus spectrophotometer (Molecular devices, San Jose, California, USA) at a temperature of 37ºC and a wavelength of 505 nm. The results of the OXY-Adsorbent test were expressed as µmol of HCI0/mL of sample. Repeatability was calculated as R^2 = 0.57 (n = 20). Repeatability was estimated as the proportion of total variance explained by the random intercept. Using the same 20 samples, the intra-plate CV was 9.35%, while the inter-plate CV was 10.97%.

4.3.4 Statistical analysis

All analyses were performed in R (version 4.0.4) (R core team, 2024) using the lme4 package (Bates *et al.*, 2015). Model selection was conducted via backward selection using likelihood-ratio tests (LRTs) with the "drop1" function. Non-significant interactive terms were removed from the initial full models however all noninteractive fixed effect predictors were retained in the full model even if not significant. To explore significant interaction effects, post hoc pairwise comparisons were conducted using the emmeans package (Lenth *et al*., 2024). Tukey's Honest Significant Difference method was applied to adjust for multiple

comparisons, and adjusted p-values along with confidence intervals were extracted using the contrast function.

We performed diagnostic checks on all LMMs to ensure model assumptions were met. We assessed the normality of residuals using Q-Q plots which indicated that residuals were approximately normally distributed. Homoscedasticity was evaluated through visual inspection of residuals plotted against fitted values. To assess multicollinearity among predictor values, we calculated variance inflation factors (VIF) using the car package (Fox and Weisberg, 2019). All VIF values were below 3, indicating no problematic collinearity among the fixed effects.

To investigate the impact of treatment on glucose circadian variation over time, we ran a linear mixed model (LMM) with time of day, treatment, sex, body mass and a three-way interaction with time of day, sex and treatment as explanatory variables. This interaction tested whether treatment influenced glucose variation over time and if this effect was sex dependent.

We then analysed glucose levels over time with an LMM, using glucose level as the response variable. Experiment timepoint (Start, End), body mass, sex, treatment, and a three-way interaction with experiment timepoint, sex and treatment were included as explanatory variables. Data was subset to include samples from midday (1pm) and early evening (8pm) matching sampling times at the start of the experiment, to account for the circadian pattern in glucose levels.

To investigate effects of treatment group on changes in RTL, MDA and OXY between the start and end of the experiment, we ran LMMs for each metric, with sex, treatment, experiment timepoint, body mass and a three-way interaction between sex, treatment and timepoint as explanatory variables. Plate ID was included as a random effect where appropriate. In all models, we also tested any associated two-way interactions as well as the three-way interactions and compared model fit using LRTs.

4.4 Results

4.4.1 The impact of artificial light at night on the circadian rhythm of glucose

Our analysis revealed a significant three-way interaction between sex, treatment and time of day on glucose levels ($\chi^{2} = 31.90$, df = 17, P = 0.015 Fig.4.1, Table S3.1). At 1 pm, the

FLAN group had higher glucose levels than the PLAN and DARK groups in both males and females. At 1 am females in the FLAN group had higher glucose levels than both the PLAN and DARK groups, PLAN also had higher glucose levels than the DARK group. This also was the case for males, though less pronounced. Post hoc analysis revealed that males in the FLAN group had significantly higher glucose levels than the DARK group at 1 pm (mean estimate [95% Confidence Interval, CI] = -6.535 [-12.85, -0.224], P = 0.048, Fig. 4.1, Table S3.2). For females' glucose levels in the FLAN group were significantly higher than in the DARK group at 1 am (mean estimate [95% Confidence Interval, CI] = -5.364 [-11.08, 0.347], P = 0.054, Fig. 4.1, Table S3.1). No significant pairwise differences were found between DARK and PLAN or FLAN and PLAN (Table S3.2). In the DARK (females) and PLAN (both sexes) groups glucose levels peaked at 8 pm, however in the FLAN group glucose peaked for males and females at 1 am (Fig.4.1).

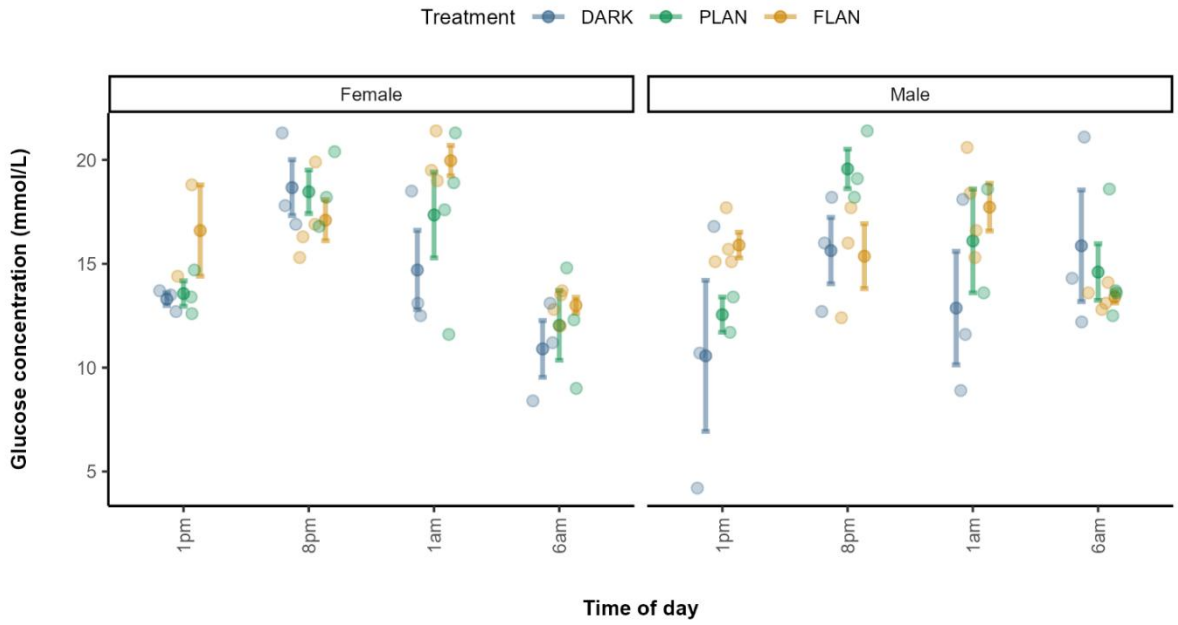

Fig. 4.1: Exposure to FLAN treatment impacts the circadian pattern of glucose. *The relationship between treatment group and glucose levels in zebra finches at various times of day. The DARK group are shown in blue, the PLAN group are shown in green, and the FLAN group are shown in yellow. The means are shown on the plot along with the standard errors. The raw data points can also be seen. Time of day is shown on the x axis and glucose concentration is shown on the y axis. The first panel of the plot represents the females, and the second panel represents the males. Sample sizes ranged from n = 2-4.*

4.4.2 The impacts of artificial light at night on glucose concentrations over time

A trend for a three-way interaction between treatment, sex, and experiment timepoint was observed when comparing glucose levels at the start and end of the experiment ($\chi^2 = 12.97$, df = 7, P = 0.073), however there was no effect of any of these factors individually (Table S3.3). To explore this further, we modelled PLAN and FLAN separately with the DARK group. No significant effect of treatment on glucose levels was found between the DARK and PLAN groups ($\chi^2 = 0.42$, df = 1, P = 0.519). However, a significant three-way interaction between sex, experiment timepoint, and treatment was found when comparing the DARK and FLAN groups ($\chi^2 = 10.66$, df = 4, P = 0.031).

Fig. 4.2 shows that, in females, glucose levels increased over time in all treatment groups, with the FLAN group having the highest levels by the end, however this was not significant. In males, the FLAN group had higher glucose levels at the start of the experiment than both PLAN and DARK. The males in the DARK group showed a decrease in glucose over time, with FLAN males experiencing a less pronounced decline. Post hoc analysis comparing the FLAN and DARK treatment groups showed a significant difference in glucose levels between the start and end of the experiment in DARK males (mean estimate [95% Confidence Interval, CI] = 3.26 [0.403, 6.12], P = 0.027). However, the effect size for this contrast was small (Cohen's d = 1.38) suggesting limited practical significance. Additionally, there was a significant difference between males and females in the DARK group at the end of the experiment (mean estimate [95% Confidence Interval, CI] = 3.78 [0.010, 7.54], P = 0.049), with a small effect size (Cohen's d = 1.62).

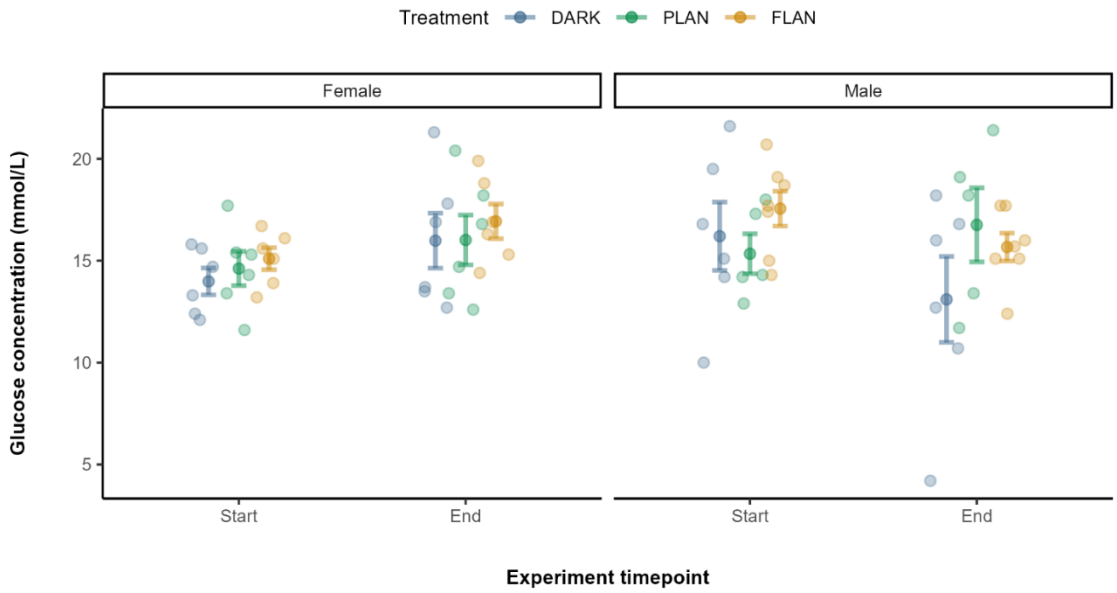

Fig. 4.2: No clear relationship between treatment group and glucose concentration over time. *The relationship between treatment groups (DARK, PLAN, FLAN) and glucose levels in zebra finches from the start to the end of the experiment. The DARK group are shown in blue; the PLAN group are shown in green, and the FLAN group are shown in yellow. The panels represent the different sexes. The means are shown along with the standard errors; the raw data points are also shown. Samples sizes range from n = 5-7.*

4.4.3 The impacts of artificial light at night on telomere length over time

We first included all three treatment groups in the same model to look at the effect on telomere length over time. There was a trend between treatment and experiment timepoint on telomere length however this was not significant ($\chi^2 = 4.81$, df = 2, P = 0.090). To explore this further, we modelled PLAN and FLAN separately with the DARK group. When comparing the DARK and PLAN groups there was no significant effect of treatment on telomere length ($\chi^2 = 0.407$, df = 1, P = 0.524, Fig.4.3). However, when comparing the DARK and FLAN treatment groups there was a significant interaction between treatment and experiment timepoint ($\chi^2 = 5.80$, df = 1, P = 0.016, Fig.4.3). Telomere length declined from the start to the end of the experiment in the FLAN group, whereas this was not observed in the DARK or PLAN groups as shown in Fig. 4.3.

We also tested if there was an effect of average MDA levels per individuals on telomere loss, but we found no significant effect ($\chi^2 = 0.12$, df = 1, P = 0.732, Fig.S3.1). All treatment groups were shown to overlap in their telomere shortening over time (Fig. S3.1).

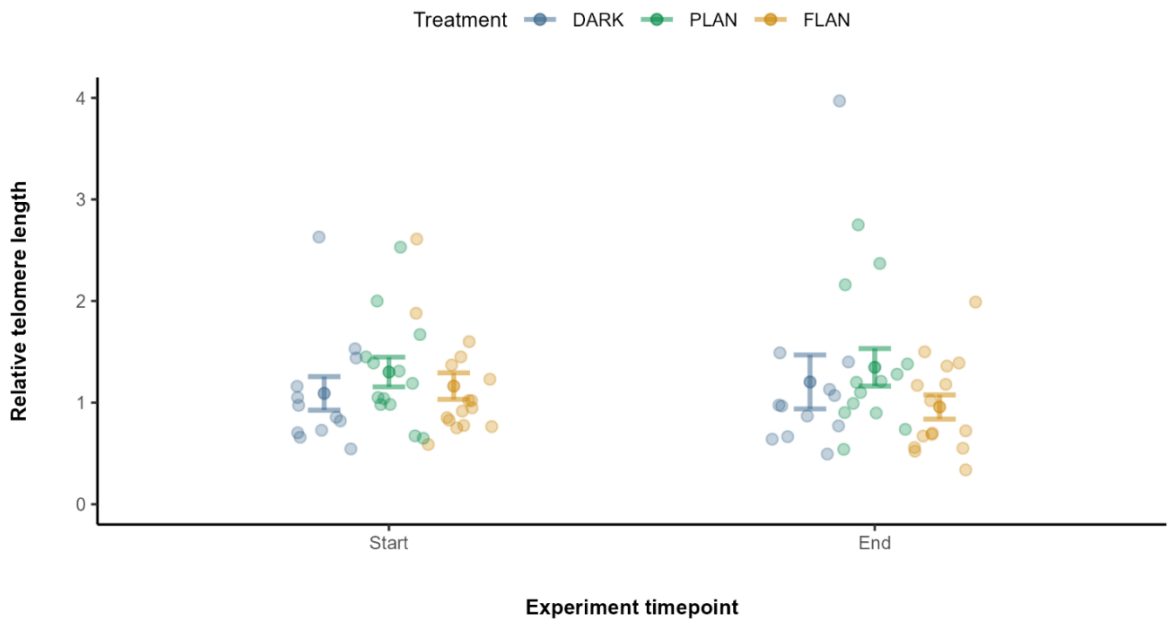

Figure 4.3: Telomere length decreases when birds are exposed to FLAN. *The relationship between treatment groups and telomere length in zebra finches at the start and end of the experiment. The DARK group are shown in blue; the PLAN group are shown in green, and the FLAN group are shown in yellow. The model residuals (without any plate effects) corrected by mean telomere length along with the standard errors, the raw data points are also shown. Experiment timepoint is shown on the x axis and relative telomere length is shown on the y axis. Sample sizes are DARK (13), PLAN (15), FLAN (16).*

4.4.4 The impact of artificial light at night on malondialdehyde levels over time

There was no significant effect of any of the interactions tested on MDA levels. Treatment did not have a significant impact on MDA levels ($\chi^2 = 0.23$, df = 2, P = 0.891, Table S3.4). There was a significant effect of sex on MDA levels ($\chi^2 = 5.49$, df = 1, P = 0.019, Table S3.4). Fig. 4.4 shows that, when comparing the treatment groups for females, MDA levels increased from the start to the end of the experiment only in the PLAN group while levels

decreased in the FLAN and DARK groups. However, when we consider the males their MDA levels only increased in the FLAN group.

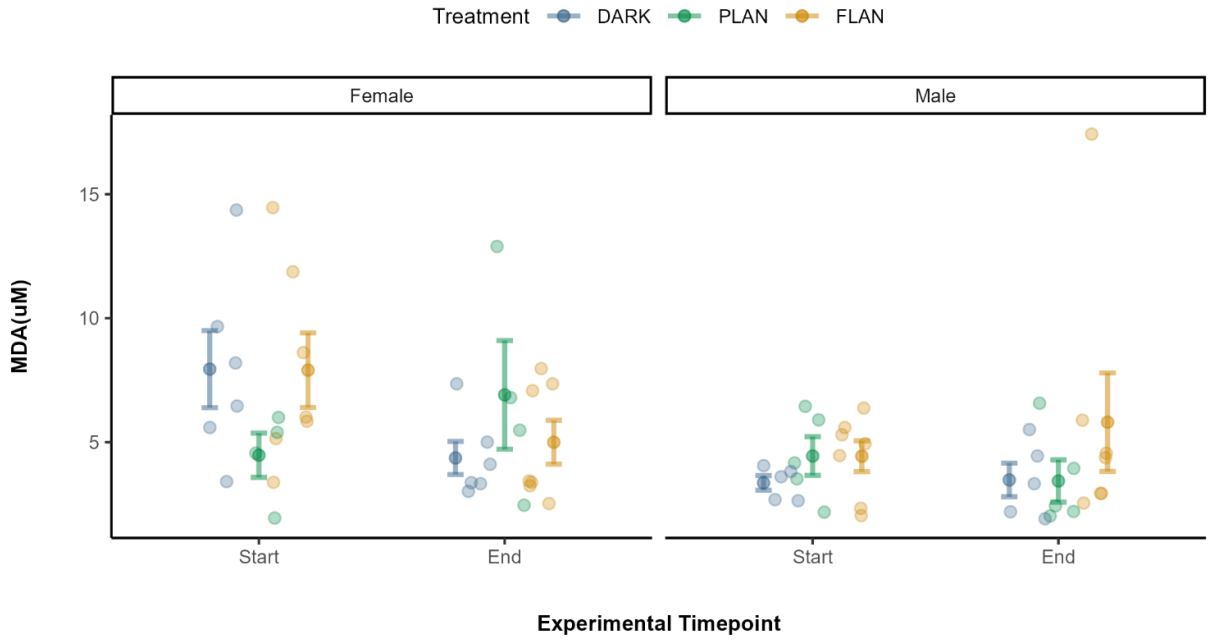

Figure 4.4: No clear effect of ALAN on MDA. *The relationship between treatment groups and MDA in zebra finches at the start and end of the experiment. The DARK group are shown in blue, the PLAN group are shown in green, and the FLAN group are shown in yellow. The means are shown along with the standard errors; the raw data points are also shown. Experiment timepoint is shown on the x axis and MDA is shown on the y axis. The separate panels represent the different sexes. Sample sizes range from n = 4-7.*

4.4.5 The impact of artificial light at night on antioxidant capacity of plasma over time

There was no significant effect of any of the interactions tested on OXY levels. There was no significant effect of treatment ($\chi^2 = 1.08$, df = 2, P = 0.582, Table S3.5) on OXY levels. In both the DARK and PLAN groups OXY levels did not appear to change by much from the start to the end of the experiment (Fig. 4.5). The birds exposed to FLAN appeared to have an increase in their OXY levels by the end of the experiment, but this was not significant (Fig. 4.5, Table S3.5).

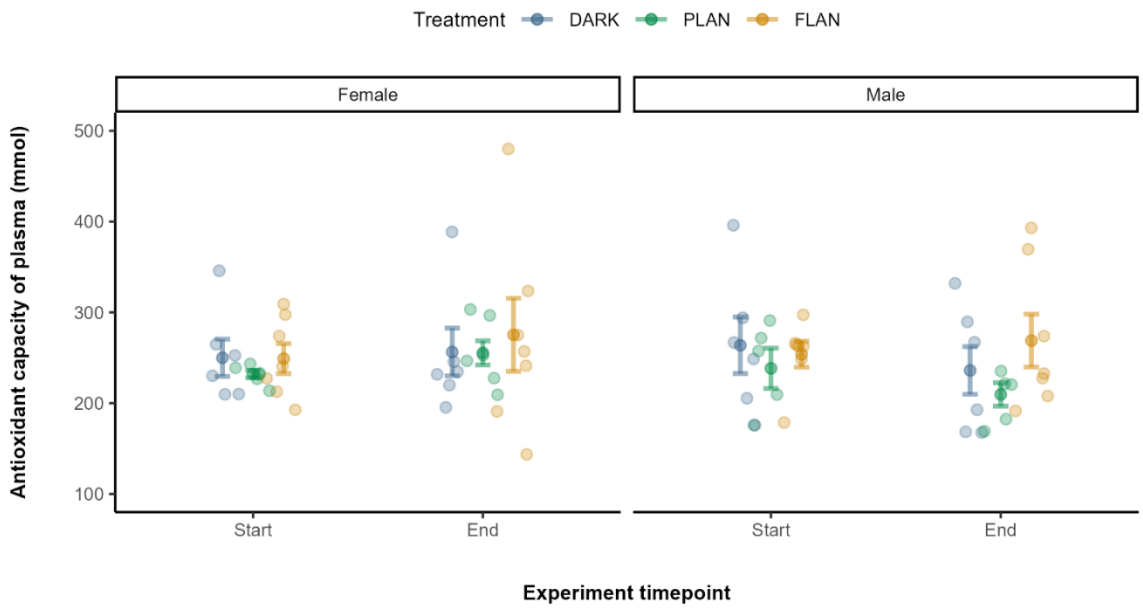

Figure 4.5: No clear relationship between ALAN and OXY levels. *The relationship between treatment groups and OXY in zebra finches at the start and end of the experiment. The DARK group are shown in blue; the PLAN group are shown in green, and the FLAN group are shown in yellow. The model residuals (without any plate effects) corrected for the mean OXY levels are shown the standard errors, the raw data points are also shown. Experiment timepoint is shown on the x axis and OXY is shown on the y axis. The separate panels represent the different sexes. Sample sizes range from n = 4-7.*

4.5 Discussion

It is well documented in the literature that organisms that are exposed to ALAN face both behavioural and physiological changes that can be detrimental to health. The results of this study show that, when exposed to full light at night birds are potentially experiencing circadian misalignment of glucose levels where the synchronisation between the circadian clock and external environmental cues are being disrupted. Birds exposed to FLAN also experienced accelerated telomere attrition. Interestingly, these effects of ALAN were not present when birds were exposed to PLAN. Neither ALAN treatment group had an effect on oxidative stress levels. These results could have important implications for the management of lighting regimes, as they suggest that turning off outdoor illumination after midnight may help reducing negative health and fitness consequences for songbirds. Future studies would apply this to other wildlife species.

4.5.1 The effect of artificial light at night on glucose levels.

Glucose levels have been shown to be under strong circadian control in several species (Kalsbeek, La Fleur and Fliers, 2014; Kumar Jha, Challet and Kalsbeek, 2015; Lobban, Downs and Brown, 2010; Remage-Healey and Romero, 2000). Glucose levels typically peak during active periods or when the organism is eating and are stored during rest phases to meet the metabolic demands throughout the day (Downs, Wellmann and Brown, 2010; Lobban, Downs and Brown, 2010; Remage-Healey and Romero, 2000). Our results clearly indicated that glucose levels in captive zebra finches experienced daily variation. Exposure to FLAN caused glucose levels to peak at 1am, in contrast to the PLAN and DARK groups, where peaks occurred at 8pm, followed by a decline. Therefore, exposure to PLAN did not appear to be enough to disrupt the circadian pattern of glucose. However, no significant pairwise difference was observed between the FLAN and PLAN groups at any of the sampling times, possibly due to low statistical power caused by a small sample size. There are limited studies that have investigated the effects of ALAN exposure on the circadian variation of glucose. It has been shown in both lab mice and captive zebra finches that exposure to ALAN can alter feeding behaviour and cause changes to activity-rest cycles, leading to them feeding and being active at unnatural times (Batra, Malik and Kumar, 2019; Fonken *et al.*, 2010). For example, diurnal captive zebra finches exposed to dim ALAN, were shown to forage at night, which resulted in elevated nighttime glucose levels, similar to our findings. (Batra, Malik and Kumar, 2019).

Lastly, when comparing glucose levels across treatment groups at similar sampling time, males in the DARK group showed a reduction in glucose levels over time which was not present in females. Individuals exposed to FLAN also experienced a decrease, but it was less pronounced. These differences could be caused by sex-specific digestive characteristics, as seen in Palestine sunbirds *(Cinnyris osea),* where males had a longer digestion time and lower digestive capacity than females (Markan *et al*., 2006). Studies investigating sex differences in glucose levels in birds show mixed results (Pouadjeu *et al.*, 2023; Remage-Healey and Romero, 2000; Tomasek *et al.*, 2019). One study found that glucose levels were higher in female free-living passerine birds (Tomasek *et al.*, 2019), while other studies found no evidence of sex differences in glucose levels in either captive starlings *(Sturnus vulgaris)* or free-living tropical passerine birds (Pouadjeu *et al.*, 2023; Remage-Healey and Romero, 2000). Variations in glucose levels over time may depend on many factors including sex, life stage and environment.

The findings in our current study suggest that comparing two static measurements of glucose over time provide little insights on the impact of ALAN on glucose levels. Instead, ALAN effects are only revealed when the circadian variation in glucose concentration is considered. There could be serious implications of circadian misalignment in blood glucose, as studies have shown that it can lead to metabolic abnormalities such as obesity, type 2 diabetes mellitus and cardiovascular disease (Kumar Jha, Challet and Kalsbeek, 2015; Mason *et al.*, 2020). Chronically high blood glucose levels in collared flycatchers *(Ficedula albicollis)* have been linked to a higher rate of mortality (Récapet *et al.*, 2016).

4.5.2 The impact of artificial light at night on telomere shortening over time.

We found a significant effect of FLAN exposure on telomere loss over the course of the experiment, but only when the PLAN treatment group was excluded from the model, suggesting that the lack of significance in the initial model was likely due to low sample size. PLAN exposure did not significantly affect telomere length and no significant pairwise differences were detected between treatment groups. There are limited studies that have investigated the effects of ALAN exposure on telomere loss and the studies that have been done show mixed results. Several studies report decreased telomere length over time (Liang *et al.*, 2011; Vardi-Naim *et al.*, 2022) while others show no effect (Grunst *et al.*, 2019; Ouyang *et al.*, 2017). For example, several studies show that when exposed to ALAN there is no impact on telomere loss in both nestling and adult songbirds (Grunst *et al.*, 2019; Ouyang *et al.*, 2017).

External stressors have been shown to negatively impact telomere length in various species, for example chronic noise exposure significantly reduced telomere length in nestling house sparrows *(Passer domesticus)* (Meillère *et al.*, 2015b). Another study found that great tit nestlings raised in urban habitats had shorter telomeres than those raised in rural habitats (Salmón *et al.*, 2016). These findings suggest that other urban stressors may influence telomere loss more than ALAN. The relationship we detected may be due to the study's duration, as previous studies that showed no effect of ALAN on telomere length were based in the field for a maximum of 15 days (Grunst *et al.*, 2019; Ouyang *et al.*, 2017). A six-month study on captive zebra finches also found no relationship between ALAN and telomere loss but this study used a lower light intensity (0.3 lux) compared to ours (Alaasam *et al.*, 2024).

An acceleration in telomere shortening could lead to negative consequences for the fitness and survival of the organism. Studies looking at bird species have shown that faster rates of

telomere shortening leads to increased mortality and reduced longevity (Barrett *et al.*, 2013; Boonekamp *et al.*, 2014; Salomons *et al.*, 2009).

4.5.3 The impact of artificial light at night on oxidative stress over time.

We found no evidence that ALAN affected oxidative stress levels over time in captive zebra finches. In females, MDA levels increased in the PLAN group but decreased in both the FLAN and DARK groups over the course of the experiment. For males, only the FLAN group showed an increase in their MDA levels, however these results were not significant. There was also no significant effect of ALAN on OXY levels, although the FLAN group exhibited an increase in OXY over the experiment when compared to the PLAN and DARK groups. These findings agree with studies that reported no effect of ALAN on oxidative stress levels (Czarnecka *et al.*, 2022; Dimovski and Robert, 2018; Raap *et al.*, 2016a). However, other studies suggest potential impacts of ALAN. For example, male rats exposed to constant light showed an increase in oxidative stress, marked by elevated MDA levels and lower antioxidant enzyme activity (Cruz *et al.*, 2003). Several oxidative stress biomarkers have also been shown to be under circadian control in the rat hippocampus for example rhythmic lipoperoxidation, catalase and GPX expression and activity (Navigatore Fonzo *et al.*, 2009). Catalase and MDA have also been shown to exhibit circadian rhythms in broiler chickens (Ayo *et al.*, 2018).

We observed sex differences in MDA levels between the start and end of the experiment. Males and females often differ in energy investment towards reproduction and traits that are under natural and sexual selection (Kokko and Jennions, 2008; Pape Møller and Thornhill, 1998), which may influence how they respond to environmental stressors (Owens and Hartley, 1998). Some studies have found sex-based differences in oxidative stress levels in response to stress (Costantini, 2018; Gomes, Vieira and da Silva, 2019; Kamper *et al.*, 2009). For example, in two species of Neotropical manakins, males experienced higher levels of oxidative damage than females when under environmental stress. However, other studies report no sex differences in oxidative stress levels (Costantini *et al.*, 2006; Costantini, Cardinale and Carere, 2007). This does highlight however that it may be important to consider sex differences when measuring oxidative stress levels in response to an environmental stressor.

In addition to an increase in MDA levels in FLAN males, birds in the FLAN group experienced accelerated telomere shortening. Previous studies suggest that increased

oxidative stress can increase rate of telomere attrition through DNA damage in the telomere sequence (Ahmed and Lingner, 2018; Coluzzi *et al.*, 2014; Kawanishi and Oikawa, 2004; Reichert and Stier, 2017). However, our analysis did not find any evidence that linked MDA levels to telomere shortening.

One of the main limitations in the current study was that we were constrained by a relatively small sample size, which means any interactions showing weak or marginal significance should be interpreted with caution. Further studies with larger sample sizes are needed to confirm the patterns identified in this study which would further strengthen our findings.

4.6 Conclusions.

Our results highlight that exposure to full ALAN impacts the circadian pattern of glucose in captive zebra finches, causing delayed glucose peaks compared to controls, which could lead to metabolic abnormalities and reduced fitness. Exposure to full ALAN also led to a faster rate of telomere shortening in zebra finches which may lead to an increased risk of mortality and reduced longevity. In contrast, partial night lighting did not produce these negative effects. However, limitations of this study include significant pairwise comparisons only between the full ALAN and the control (DARK) groups, a larger sample size might have shown stronger effects. A partial night lighting mitigation scheme in urban areas, where lights are switched off after midnight, could mitigate ALAN's negative effects on urban wildlife while maintaining the benefits of lighting for humans. This approach would also have economic benefits through reducing financial costs. ALAN sources in urban areas are also known to use up a huge amount of energy which would be reduced by introducing partial night lighting, contributing to reducing emissions. Our study contributes to solving the issue of light pollution by demonstrating that a partial light at night mitigation strategy could have the potential to reduce the negative impacts of ALAN.

4.7 Ethics statement

The experiment was approved by the Home Office under the project licence P6859F36E to Davide M Dominoni. All blood samples were taken by Personal licence holders.

4.8 Availability of data and materials

The data and code used in this analysis are available on Zenodo at [https://doi.org/10.5281/zenodo.15011283].

4.9 Funding

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4.11 Author contributions

Rachel Reid, Davide M Dominoni, Robert Gillespie and Jelle Boonekamp designed the study. Rachel Reid, Claire J Branston, Pablo Capilla Lasheras, Robert Gillespie and Davide M Dominoni collected data for the experiment. Rachel Reid, Christopher Mitchell, Neal J Dawson and Eleanor Duncan analysed the data in the lab. Rachel Reid conducted the data analysis. Rachel Reid wrote the initial draft of the manuscript with all contributing authors editing and revising the manuscript.

Chapter 5
Oxidative stress biomarkers show low within-individual repeatability, weakening trait associations: A meta-analysis in non-human organisms.

Rachel R Reid[1], Davide M Dominoni[1 *], Jelle Boonekamp[1]

[1]School of Biodiversity, One Health and Veterinary Medicine, Graham Kerr Building, University of Glasgow, Glasgow, G12 8QQ, UK

A version of this chapter will be submitted to The Proceedings of the National Academy of Sciences (PNAS).

5.1 Abstract

Markers of oxidative stress are widely used as biomarkers of health, ageing, and physiological stress. However, their reliability as biomarkers remains uncertain due to high intraindividual variation, obscuring associations with environmental conditions, lifestyle, and other physiological markers. This study presents the first meta-analysis assessing individual repeatability of oxidative stress markers across taxa, environments, and study designs, comprising of 123 repeat estimates over 17 oxidative stress measurement types, drawn from 22 studies. Our findings highlight that oxidative stress exhibits low individual repeatability (Intraclass correlation = 0.164). We observed substantial variation across studies, regardless of biomarker type, taxa, sex, study design, or environment. Additionally, most oxidative stress biomarkers were weakly correlated, emphasising that no single marker can be used as a universal indicator of oxidative status. Using individual-based simulations we show that to obtain sufficient statistical power, substantially larger samples sizes are required than the sample sizes that are typically used in this field. We also show that increasing the number of repeated samples from individuals can be a very efficient way of increasing statistical power, keeping samples sizes manageable. These findings highlight the need to untangle the causes of high intraindividual variation in oxidative stress to which end longitudinal sampling will be essential.

5.2 Introduction

Oxidative stress is a pervasive feature of life underpinning cellular senescence, ageing, and disease (López-Otín *et al.*, 2023). Oxidative stress occurs when prooxidants, inevitable by-products of aerobic energy metabolism, outnumber antioxidants (Costantini and Verhulst, 2009; Costantini *et al.*, 2010; Monaghan, Metcalfe and Torres, 2009). This imbalance initiates a chain of reactions with highly reactive substances causing DNA damage and alterations to membrane lipids and other macro-molecules (Von Zglinicki, 2002). On a systemic level, such cellular damage is widely believed to contribute to organismal senescence (Campisi, 2003; Chen, Hales and Ozanne, 2007; Pole, Dimri and Dimri, 2016) and elevated levels of oxidative stress are often associated with ageing related disease (Liguori *et al.*, 2018; Luo *et al.*, 2020; Reichert and Stier, 2017; Speakman *et al.*, 2015). Investigating the causes of individual variation in oxidative stress could provide crucial insight into the factors driving ageing. For example, elevated oxidative stress levels might reveal an unhealthy lifestyle if oxidative stress measurements link lifestyle factors to health. Given that health status (beyond obvious disease) is difficult to measure for the majority of the population, the utility of oxidative stress as biomarkers depends on their associations to other health markers such as telomere length. However, a recent meta-analysis showed the association between oxidative stress and telomere length is very poor ($r = 0.027$) raising the critical question of whether oxidative stress measurements accurately capture meaningful aspects of health (Armstrong and Boonekamp, 2023). The importance of this question cannot be overstated given the widespread use of oxidative stress measurements as health biomarkers in clinical and pharmaceutical research (Demirci-Çekiç *et al.*, 2022; Frijhoff *et al.*, 2015), ecotoxicology (Valavanidis *et al.*, 2006) and evolutionary ecology (Boonekamp, Mulder and Verhulst, 2018; Monaghan, Metcalfe and Torres, 2009; Smith, Nager and Costantini, 2016).

Telomeres are particularly susceptible to oxidative damage due to their high guanine content and the absence of robust DNA repair mechanisms (Ahmed and Lingner, 2018; Haussmann and Marchetto, 2010; Reichert and Stier, 2017). Accumulated telomere damage over time is associated with cellular senescence, ageing-related diseases and carcinogenesis (Ahmed and Lingner, 2018; Barnes, Fouquerel and Opresko, 2019; Houben *et al.*, 2008; Luo *et al.*, 2020). However, while pharmacological doses of oxidative stress have been conclusively shown to accelerate telomere attrition in cell cultures (Von Zglinicki, 2002), the relationship between naturally occurring oxidative stress and telomere

dynamics in vivo appears very low (Armstrong and Boonekamp, 2023; Boonekamp *et al.*, 2017). While the reason for this weak association between oxidative stress and telomeres in vivo remains unclear, it does not necessarily mean that oxidative stress does not contribute to telomere attrition in vivo. For example, if oxidative stress varies strongly within individuals, a single measurement may be insufficient to accurately capture an individual's oxidative profile. Nevertheless, the apparent oxidative stress could be casual in telomere attrition, but high intraindividual variation could present a 'detection issue', in particular when the intraindividual variation is unpredictable. This intraindividual variation could mask the relationship between telomere length and oxidative stress as well as obscuring correlations amongst different types of oxidative stress markers, as previously observed (Cohen *et al*., 2009).

Though oxidative stress is widely used in the fields of ecology, ecophysiology, biogerontology, clinical research, and biomedicine, there is limited information on the intraindividual repeatability of oxidative stress measurements, even when repeated measurements were collected. Substantial evidence suggests that oxidative stress can vary significantly across life stages (Alonso-Álvarez *et al.*, 2010; Cohen, Hau and Wikelski, 2008; Metcalfe and Alonso-Álvarez, 2010; Monaghan, Metcalfe and Torres, 2009), and environmental conditions (Cohen, Hau and Wikelski, 2008; Costantini and Bonadonna, 2010; Slos and Stoks, 2008; Van de Crommenacker *et al.*, 2011), as well as over relatively short time scales (Alonso-Álvarez *et al.*, 2010; Goldfarb *et al.*, 2014; Mallard *et al.*, 2020). For example, oxidative stress has been shown to fluctuate with environmental changes such as seasonality (Chainy, Paital and Dandapat, 2016; Rathwa *et al.*, 2017), predation risk (Slos and Stoks, 2008), and short-term variations in territory quality (Van de Crommenacker *et al.*, 2011). Moreover, life history like mating and reproductive efforts (Alonso-Álvarez *et al.*, 2010; Metcalfe and Alonso-Álvarez, 2010), along with high metabolic activities such as migration (Jenni-Eiermann *et al.*, 2014), can further contribute to these variations.

Despite the recognised variability in oxidative stress, few studies have quantified intraindividual variation, making it unclear how well single measurements of oxidative stress capture the oxidative stress 'profile' of the individual. For example, oxidative stress fluctuates naturally throughout an individual's lifespan (Beaulieu and Costantini, 2014) for reasons that remain largely unknown. Additionally, variability arises from tissue-specific differences and assay inconsistencies, which can obscure true biological variation (Hõrak

and Cohen, 2010; Speakman *et al.*, 2015). Reviews have highlighted these complexities, particularly when considering the weak correlations often observed between different oxidative stress markers, even within the same individual (Beaulieu and Costantini, 2014; Costantini *et al.*, 2010; Costantini, 2019; Hõrak and Cohen, 2010; Speakman *et al.*, 2015). This suggests that measuring a single oxidative stress marker or a single measurement in time may not reliably reflect broader oxidative status. Given this inherent variability, it is crucial to quantify the individual repeatability of oxidative stress measurements as this is an essential step to determine their biomarker potential. (Rudeck *et al.*, 2020). Few studies explicitly report the within-individual repeatability of oxidative stress. Some existing studies have shown moderate repeatability in specific oxidative stress markers, such as glutathione peroxidase ($r = 0.47$) in wild great tits *(Parus major)* across autumn and winter sampling periods (Norte *et al.*, 2008). Similarly in wild collared flycatchers *(Ficedula albicollis),* antioxidant defences ($r = 0.581$) exhibited moderate repeatability, whereas reactive oxygen metabolites were not repeatable ($r = 0.03$) (Récapet *et al.*, 2019). This highlights that repeatability can vary a lot depending on the marker used and/or the species being measured, driving a need for a comprehensive analysis on this.

Whilst the importance of establishing individual repeatability has been a key focus of research for several key physiological traits including metabolic rate (Nespolo and Franco, 2007), glucocorticoid hormones (Schoenemann and Bonier, 2018; Taff, Schoenle and Vitousek, 2018), cognitive performance (Cauchoix *et al.*, 2018), and telomere length (Kärkkäinen *et al.*, 2022), the individual repeatability of oxidative stress markers remains unclear. The increasing availability of longitudinal studies offers great opportunity to investigate the level of individual repeatability of oxidative stress markers for a wide range of species and identify factors contributing to intraindividual variation. Here, we present the first formal meta-analysis of individual repeatability of oxidative stress. By examining biological and methodological factors, we aim to identify the causes of variation in repeatability. We also evaluate the correlations among oxidative stress markers to determine if any systematic/conserved patterns exist. We subsequently explore the consequences of the observed low repeatability for study design and statistical power using individual based simulations and provide recommendations addressing these issues for future studies.

5.3 Methods

5.3.1 Literature search

We developed a search string using keywords derived from a review of the oxidative stress literature as follows:

(“Oxidative stress” OR "Antioxidant capacity" OR “Antioxidant*” OR "Oxidative damage") AND (“Repeated*” OR “Repeated measure” OR “Repeatability*” OR “Longitudinal*” OR “Longitudinal study” OR “Within Individual” OR “Within Individual study”) AND (“Wildlife*” OR “Wild animal” OR “Wild population” OR “Wild*” OR “Bird*” OR “Avian*” OR “Invertebrate*” OR “Fish*” OR “Herpetofauna*” OR “Vertebrate*” OR “Mammal*”) NOT (“Human*” OR “Woman*” OR “Women*” OR “Man*” OR “Children*” OR “Patients*” OR “People*” OR “Men*” OR “Child*”)

This search string was used on both the Web of Science core collection database (last search 7th of December 2023) and Scopus database (last search 4th of June 2024). After removing duplicates, we recorded the number of results for each iteration following the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) framework. The PRISMA diagram summarising the selection process is shown in Figure 5.1. We were able to include effect sizes from 22 studies (Bodey *et al.*, 2020; Cantón *et al.*, 2018; Christensen *et al.*, 2015; Cram, Blount and Young, 2015; Eikenaar *et al.*, 2020; Gormally *et al.*, 2019; Guindre-Parker *et al.*, 2013; Guindre-Parker and Rubenstein, 2018; Hau *et al.*, 2015; Herborn *et al.*, 2016; Kolling *et al.*, 2022; Marasco *et al.*, 2017; Noguera, Kim and Velando, 2017; Orquera-Arguero *et al.*, 2023; Récapet *et al.*, 2019; Rowe, Pierson and McGraw, 2015; Sauerwein *et al.*, 2020; Schull *et al.*, 2016; Skrip *et al.*, 2016; Těšický *et al.*, 2021; Velázquez- Viblanc *et al.*, 2018; Vitikainen *et al.*, 2016)

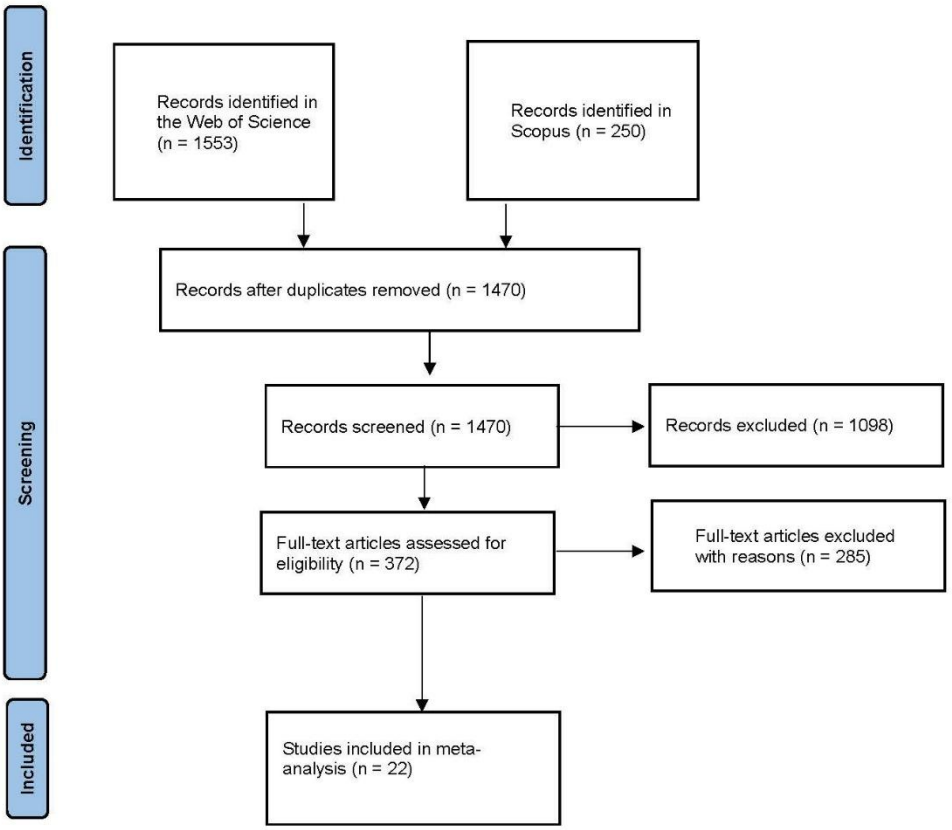

Figure 5.1: PRISMA diagram showing each step of the screening process.

5.3.2 Inclusion criteria

To be included in the meta-analysis, studies had to meet the following criteria:

1) Measured at least one biomarker of oxidative stress (oxidative damage or antioxidant defence).
2) Took at least two oxidative stress measurements from the same individual, reporting the amount of time elapsed between measurements.
3) Focused on non-human organisms.

4) Raw data was readily available.

We included both experimental and non-experimental studies, as well as research on wild, captive, and livestock species to keep the scope as broad as possible.

5.3.3 Screening process

We employed a two-step screening process to identify eligible studies, as repeat measurements were not always explicitly mentioned in abstracts. First, we used the MetaGear (Lajeunesse, 2016) package in R version 4.3.2 (R Core Team, 2024). to screen titles and abstracts, focusing on studies that measured oxidative stress in non-human organisms. Second, we reviewed the full text of articles flagged as relevant during the initial screening. At this stage, we examined the method section of each study to confirm that repeat oxidative stress measurements had been taken from the same individuals.

5.3.4 Data extraction

After screening, we extracted biological and methodological information from the relevant studies. The extracted data included the taxonomic class, species, sample size, (mean) number of oxidative stress samples per individual, (mean) time elapsed between samples, oxidative stress biomarker measured (oxidative damage, antioxidant defence), type of study (manipulation, non-manipulation), study length, and environment classed as the following four categories: wild (animals in their natural habitat), captive (animals kept in controlled environments), wild-captive (wild animals brought into captivity for the purposes of the experiment), or farm (livestock animals for agricultural purposes, including horses, cattle and sheep).

Oxidative stress biomarkers were categorised based on whether they measured antioxidant defences (enzymatic or non-enzymatic) or oxidative damage (lipids, proteins, DNA, or "unspecific damage" if multiple biomolecule types were affected) (refer to Table S4.1).

5.3.5 Repeatability

To calculate within-individual repeatability of oxidative stress measurements, we obtained the raw data from each study. For studies where raw data was not directly available, we contacted the authors. We estimated repeatability by determining the intraclass correlation coefficient (ICC) using ANOVAs based on equations 4 and 5 from Nakagawa and

Schielzeth, (2010) along with the standard error and 95% confidence intervals. This ANOVA-based method is recommended when repeatability is very low (<0.05) which was often the case in our dataset. In contrast, the linear mixed-effects model (LMM) approach tends to overestimate low repeatability values. While we used the ANOVA-based approach for our main analysis, we confirmed the consistency of the results with LMM-derived estimates (Fig. S4.1). Importantly, rather than calculating conditional repeatability by including covariates within the repeatability model itself, we stratified the data by relevant variables, specifically by sex, oxidative stress biomarker, and study type (manipulation vs. non-manipulation) to calculate separate ICC values for each subset. This approach allowed us to subsequently use these variables as moderators in the meta-analysis to explore potential sources of variation in repeatability. This yielded 123 effect sizes from 22 species across two taxonomic classes from 22 studies that were published between 2013 and 2023 (species listed in Table S4.2).

5.3.6 Pearson's correlation

When multiple oxidative stress biomarkers were measured within a study, we determined the respective pairwise relationships by calculating Pearson's correlation coefficients using the cor() function in R (R Core Team, 2024). We used these Pearson's correlations in an additional meta-analysis to evaluate the support for the among oxidative stress biomarker associations across studies.

5.3.7 Data analysis

All meta-analyses were conducted using a multi-level meta-analysis structure with the metafor package (Viechtbauer, 2010) in R version 4.3.2 (R Core Team, 2024). Prior to analysis, repeatability estimates (ICC) and Pearson's correlation values (r) were standardised using Fisher's Z transformation (Holtmann, Lagisz and Nakagawa, 2017). Corresponding sampling variances were calculated using the escalc() function in metafor (Viechtbauer, 2010). Model parameters were back transformed for easier interpretation in graphs and tables. First, we estimated the overall ICC for oxidative stress measures using an intercept-only model. Subsequently, we tested whether our moderators (study type, environment, taxonomic class, oxidative stress biomarker, average time between measurements (log transformed) and average number of samples (log transformed) explained the observed heterogeneity of effect sizes. Subset analyses were then performed to investigate if systematic differences occurred between oxidative damage and antioxidant

defence categories using the respective biomarker type as a moderator in each analysis. To explore relationships between oxidative stress biomarkers, a meta-analysis model was fitted using standardised Pearson's correlations as the response variable and biomarker pair as a moderator.

All models accounted for among-study and residual variation by including study ID and observation ID as random effects (Fernández-Castilla *et al.*, 2020; Dettori, Norvell and Chapman, 2022). Heterogeneity was estimated using the i2_ml() function in the OrchaRd package (Nakagawa *et al.*, 2023), which provided the total relative heterogeneity and the contribution of each random effect. Lastly, publication bias was assessed through two multi-level meta-regressions, with the square root of inverse sampling variance and mean-centred year of publication as moderators to test for small study effects and time lag bias respectively (Koricheva and Kulinskaya, 2019; Nakagawa *et al.*, 2022).

4.5.8 Simulation

We developed an individual-based simulation in R to examine the minimum sample sizes required to have sufficient statistical power (>80%) for detecting a correlation between oxidative stress and telomere length. We chose telomere length, because oxidative stress is widely thought of as one of the main causes of telomere attrition and yet a previous meta-analysis found that the correlation between telomere length and oxidative stress was very low (Armstrong and Boonekamp, 2023).

We simulated different scenarios of oxidative stress repeatability (i.e. ICC values) and the degree of causality (i.e. the extent to which oxidative stress is contributing to telomere attrition). To make the simulation biologically meaningful, we used starting values for telomere length, oxidative stress, and mortality that roughly reflect a population of great tit *(Parus major)* fledglings. Initially, we ran simulations assuming a full causal effect of oxidative stress on telomere attrition. However, we realised that full causality is unlikely, so we introduced various scenarios reflecting different degrees of causality. The extent of oxidative stress's influence on telomere attrition is a key factor that affects the statistical power to detect a correlation, making this an important consideration in the simulations.

Each simulation generated a population of great tit fledglings with a normal distribution of telomere length (mean = 30 kb, SD = 8kb) and oxidative stress (mean = 1500, SD = 450). Oxidative stress in the simulation was a unitless, arbitrarily scaled variable designed to

influence telomere loss. We then simulated annual survival, oxidative stress and telomere loss. We used a Gompertz function (Baseline mortality rate = 0.02, age-dependent increase = 0.15) to emulate realistic age-dependent mortality by non-specific causes. Individuals also died if their telomere length reached a critical limit (below 1000 bp), meaning that very short telomeres could cause mortality directly. However, most mortality was due to the Gompertz function and not telomere attrition. Each year an individual survived, they sustained oxidative damage determined by the following equation:

$$Damage = OS_{birth} + y^{*}(1-r)$$

Where r is repeatability and y˜a random value between -1500 and 4500 (with this range being somewhat arbitrary). Notably, if the repeatability is 1, the damage is invariably determined by the oxidative stress value that was determined at fledging. Vice-versa, if the repeatability parameter is set to 0, the resulting within-individual damage would show a low repeatability of ICC = 10% when using our starting parameter settings. Hence, the repeatability parameter does not directly reflect the actual intraclass correlation. We therefore determined the apparent ICC values that emerged from our simulations for our power calculations. Negative damage values were allowed in a small proportion of cases to reflect the occasional telomere elongation observed in a small portion of the population. Whether or not to include parameter space that would generate negative damage had no real impact on the correlation between oxidative stress and telomere length, or the associated statistical power.

We simulated different scenarios of repeatability ranging from ICC = 0.2 (representing the estimate based on our meta-analysis) to more optimistic scenarios of ICC = 0.4 and 0.9.

Telomere loss was generated annually as a function of oxidative stress and cell division using the following equation:

$$TL_{t+1} = TL_{t} - damage - cell\ division$$

Where TL stands for telomere length at age t, ‘damage’ is as defined above, and cell division is a random number from a normal distribution with a mean of 100 and SD of 20. This contribution of cell division is relatively small, as cell turnover is highest during the early developmental stages but then slows down at the age of fledging. We simulated different scenarios of causality for the effect of oxidative stress on telomere attrition

ranging from 30% (low), 60% (intermediate), to 100% (fully causal). Even in the fully causal scenario, some telomere loss was still attributed to cell division.

For each population we generated longitudinal data on within-individual oxidative stress and telomere trajectories. We determined the correlation between oxidative stress and telomere length at the median survival time of each population. This process was repeated for different combinations of population sample sizes (ranging from n = 10 to n = 350), repeatability scenarios, and causality scenarios. For each scenario, we computed the statistical power, and scenarios were repeated 1000 times to obtain precise power-sample size curves and 95% confidence intervals.

Finally, we investigated the impact of collecting repeated oxidative stress measurements within individuals on the statistical power for detecting a relationship between oxidative stress and telomere length. To this end we added a few additional scenarios regarding the number of oxidative stress measurements taken from each individual (ranging from 1-10).

5.4 Results

5.4.1 Individual repeatability of oxidative stress across taxa, sex, environment, study, and biomarker type

The overall ICC of oxidative stress measures was low (ICC [95% confidence interval, CI] = 0.164 [0.093, 0.233]; P < 0.001]; Table S4.3, Fig.5.2). There was significant heterogeneity among effect sizes $I^2 = 57.4$, which raises the question of what biological and methodological factors could underpin this variation. Taxonomic class explained a significant portion of study heterogeneity (QM = 17.752, df = 2, P < 0.001). Mammals exhibited slightly higher ICC values (ICC [95% confidence interval, CI] = 0.212 [0.058, 0.356]; P = 0.007; Table S4.3, Fig.5.2.) than birds (ICC [95% confidence interval, CI] = 0.155 [0.062, 0.245]; P = 0.012; Table S4.3, Fig.5.2.), but the difference was not significant (Estimate = 0.059, SE = 0.047, P = 0.526), and both groups showed a low repeatability. The low repeatability presents a major challenge as it limits the utility of oxidative stress measurements as biomarkers of health and environmental conditions. It is crucial therefore to investigate the causes of low repeatability. We conducted a series of moderator and subset analyses to investigate potential sources of variability with the data that were available to us.

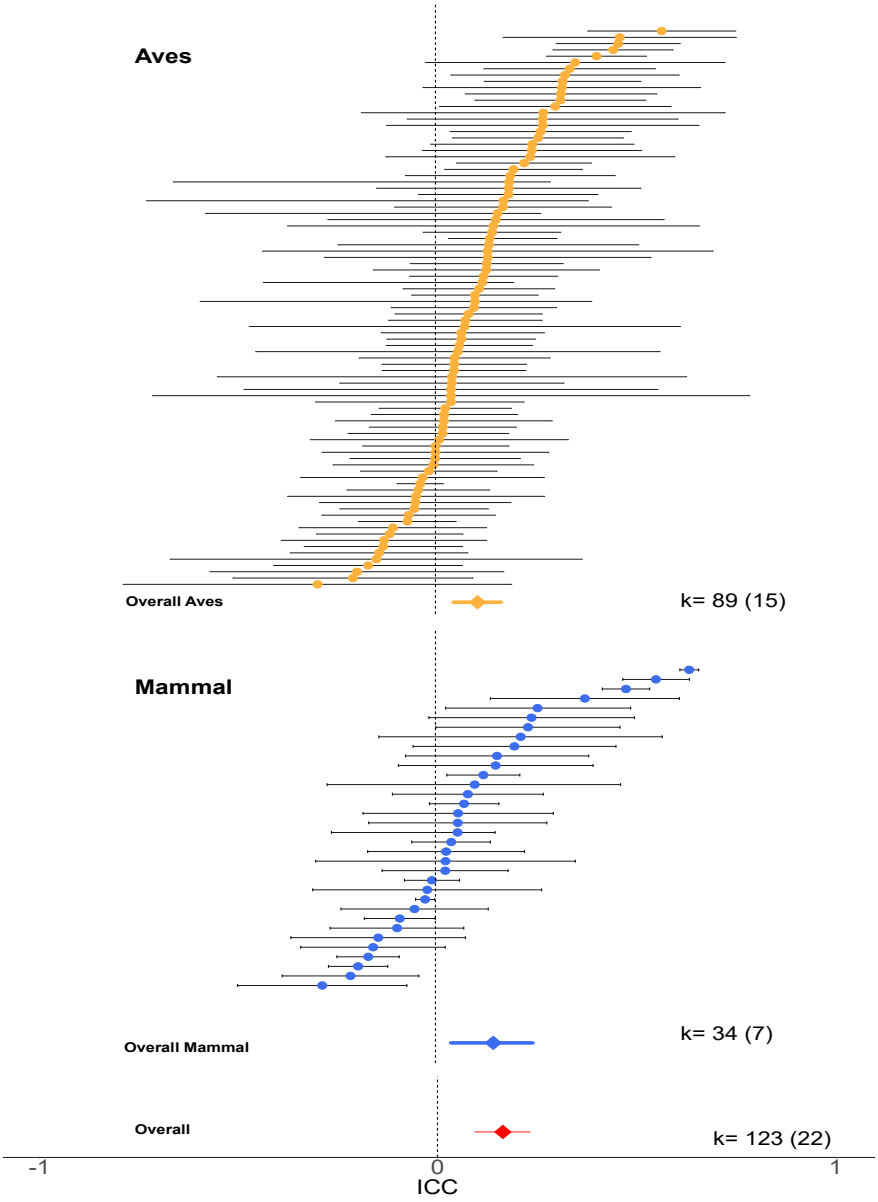

Figure 5.2: Caterpillar plot displaying individual effect sizes sorted from lowest to highest repeatability and split by taxonomic class. *Yellow points represent ICC values for the "Aves" class while blue points correspond to ICC values for the "Mammalia" class. The global ICC estimate for "Aves" is shown at the bottom of the Aves class facet, and the global estimate for "Mammalia" is shown similary. These estimates were extracted from the meta-model where taxanomic class was included as a moderator. ICC values are presented along with their 95% confidence intervals. The dashed line represents an ICC value of zero. The red diamond and error bars at the bottom of the plot represents the overall ICC value and its 95% confidence intervals from the intercept only model. The corresponding number of effect sizes is shown on the right side of the plot, with the number of studies indicated in parentheses.*

We compared study type, i.e., experimental vs. correlational, to determine if treatment effects on oxidative stress reduced their apparent individual repeatability. Such effect could occur if there exists individual variation in the response to treatment. Study type significantly reduced the observed heterogeneity (QM = 19.192, df = 2, P < 0.001). However, both groups did not differ significantly from each other (Estimate = -0.064, SE = 0.036, P = 0.372) (Fig. 5.3A). If anything, correlational studies showed a slightly lower repeatability overall (ICC [95% confidence interval, CI] = 0.146 [0.053, 0.230]; P = 0.002; Table S4.3, Fig.5.3A) compared to the experimental studies (ICC [95% confidence interval, CI] = 0.208 [0.091, 0.319]; P <0.001; Table S4.3, Fig.5.3A), contrary to what we predicted.

The moderator 'environment' significantly reduced the observed heterogeneity (QM = 19.789, df = 4, P = 0.001), but the moderator was not significant (QM = 4.79, df = 3, P = 0.188). Within the moderator levels, only farms showed a repeatability significantly above zero (ICC [95% confidence interval, CI] = 0.391 [0.178, 0.569]; P = 0.005; Table S4.3, Fig.5.3B). The other environmental classes showed very low repeatability's that were indistinguishable from zero: wild (ICC [95% confidence interval, CI] = 0.119 [-0.003, 0.239]; P = 0.057; Table S4.3, Fig.5.3B); wild captive (ICC [95% confidence interval, CI] = 0.165 [-0.052, 0.367]; P = 0.136; Table S4.3, Fig.5.3B); and captive-bred animals (ICC [95% confidence interval, CI] = 0.158 [-0.07, 0.371]; P = 0.173; Table S4.3, Fig.5.3B).

The moderator 'oxidative stress biomarker' had a significant impact on ICC (QM = 20.129, df = 2, P < 0.001), both oxidative damage and antioxidant defence had significant effects on repeatability, but there was no significant difference between them (Estimate = -0.016, SE = 0.032, P = 0.803). Oxidative damage biomarkers had slightly lower repeatability (ICC [95% confidence interval, CI] = 0.156 [0.056, 0.253]; P = 0.002; Table S4.3, Fig.5.3C) compared to antioxidant defence biomarkers (ICC [95% confidence interval, CI] 0.171 [0.082, 0.258]; P < 0.001]; Table S4.3, Fig.5.3C) although both groups exhibited low repeatability.

The moderator 'sex' had a significant effect on ICC (QM = 14.21, df = 2, P < 0.001), with both males and females showing significant effects on repeatability, with females showing slightly higher ICCs (ICC [95% confidence interval, CI] 0.167 [0.065, 0.266]; P = 0.001; Table S4.3, Fig.5.3D) compared to males (ICC [95% confidence interval, CI] 0.154 [0.03, 0.273]; P = 0.015; Table S4.3, Fig.5.3D). However, the difference between the groups was

not significant (Estimate = -0.013, SE = 0.039, P = 0.86) and both groups exhibited low repeatability.

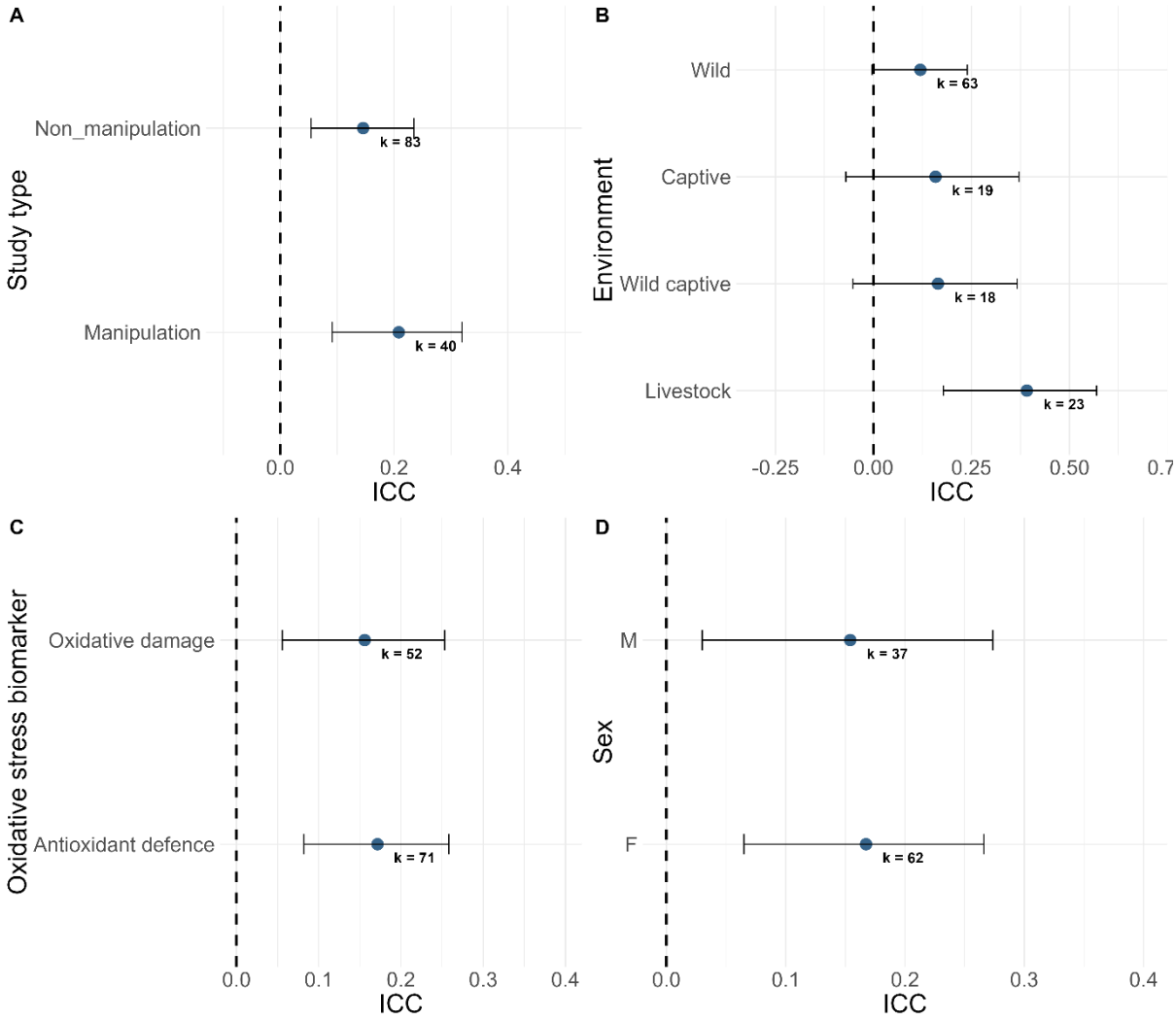

Figure 5.3: Within-individual repeatability of oxidative stress measures for each moderator level. *Each panel represents a different moderator: A) study type, B) environment, C) oxidative stress biomarker, D) sex. Model estimates are shown along with the 95% confidence intervals. The dashed line indicates an ICC value of zero. The moderator is shown on the y axis, and ICC values (back transformed from Fisher's Z values) are plotted on the x-axis.*

5.4.2 Does the number of repeated measurements and time elapsed between measurements affect the within-subject repeatability of oxidative stress?

We hypothesised that time elapsed between repeated measurements would negatively affect repeatability, while the number of samples would positively influence it. Contrary to expectation, an increase in the time between measurements resulted in a small but significant positive effect on ICC (slope estimate [95% confidence interval, CI] =0.022 [0.006, 0.038]; P = 0.008; Table S4.3, Fig.5.4A).

In line with our expectations, repeatability increased with the average number of samples per individual (slope estimate [95% confidence interval, CI] = 0.097 [0.064, 0.130]; P < 0.001; Table S4.3, Fig.5.4B).

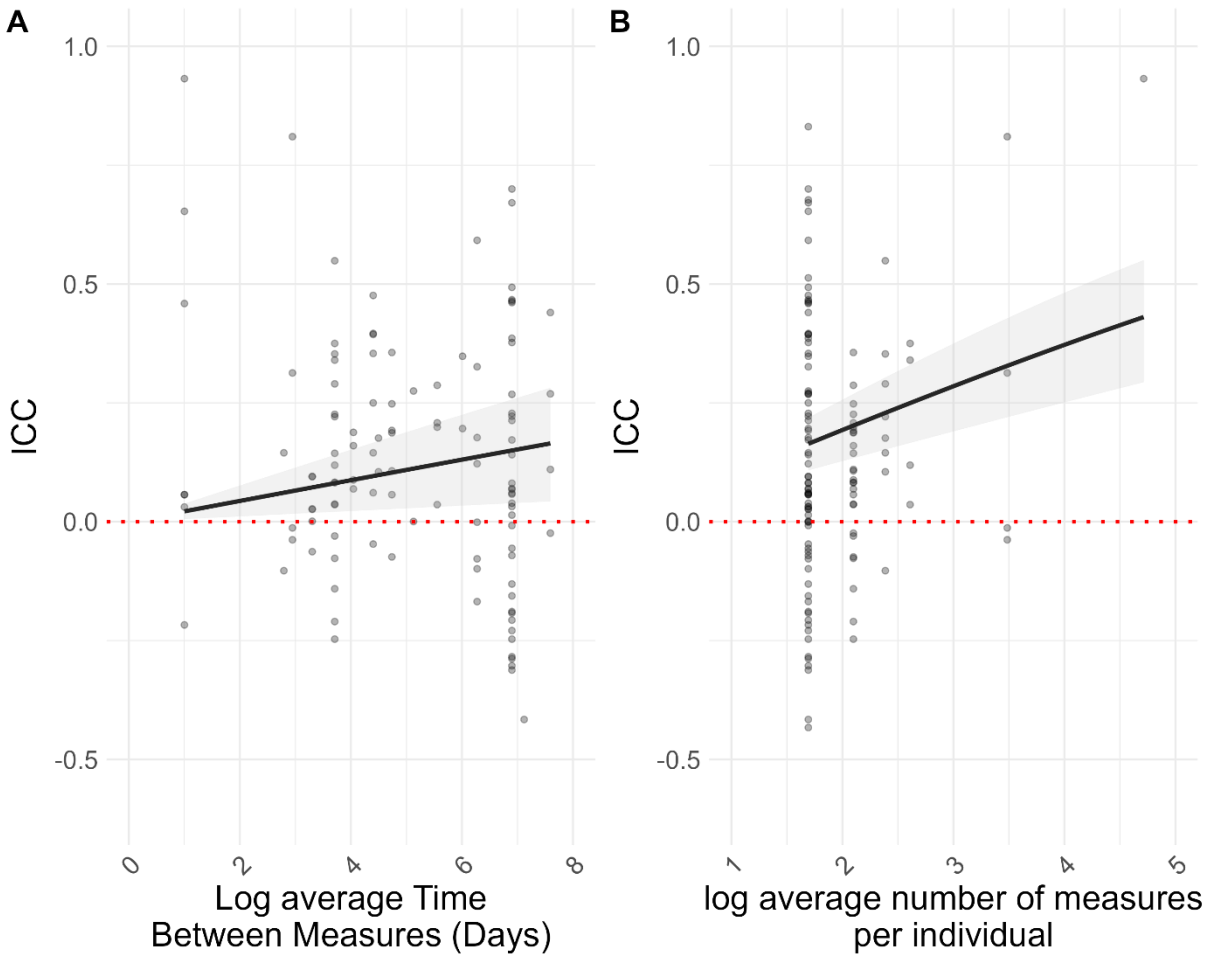

Figure 5.4: Within-individual repeatability of oxidative stress measures for different moderators*. A) the average time between measures (log transformed), and B) the average number of measures per individual (log transformed). The red dotted line indicates where ICC equals zero. The regression line represents predicted values from the model output, with the grey ribbon showing the associated 95% confidence intervals. The grey points represent the raw data points. Moderators are shown on the x axis, and ICC values is shown on the y axis.*

5.4.3 Is any particular oxidative stress marker sub-type showing higher individual repeatability?

The moderator “type of oxidative damage” was shown to have a significant effect on ICC (QM = 16.036, df = 4, P = 0.003). Lipid peroxidation was shown to have a significant effect on repeatability and exhibited the highest albeit still low ICC (ICC [95% confidence interval, CI] = 0.368 [0.182, 0.529]; P < 0.001]; Table S4.4, Fig.5.5A). Other damage types, showed low repeatability, with DNA damage (ICC [95% confidence interval, CI] = 0.135 [-0.224, 0.461]; P = 0.464; Table S4.4, Fig.4A), protein damage (ICC [95% confidence interval, CI] = -0.068 [-0.346, 0.220]; P = 0.646; Table S4.4, Fig.5.5A) and nonspecific damage (ICC [95% confidence interval, CI] = 0.059 [-0.137, 0.252]; P = 0.59; Table S4.4, Fig.5.5A) contributing little to within-individual repeatability.

The moderator “type of antioxidant defence” was found to have a significant effect on ICC (QM = 13.985, df = 2, P < 0.001). In the antioxidant defence subset, enzymatic biomarkers showed slightly higher ICC (ICC [95% confidence interval, CI] = 0.204 [0.081, 0.320]; P = 0.001; Table S4.4, Fig.5.5B) compared to non-enzymatic defence biomarkers (ICC [95% confidence interval, CI] = 0.136 [0.042, 0.228]; P = 0.005; Table S4.4, Fig.5.5B). Both categories significantly affected repeatability, but the differences between them were not significant (Estimate = -0.069, SE = 0.033, P = 0.296), with both showing low overall repeatability.

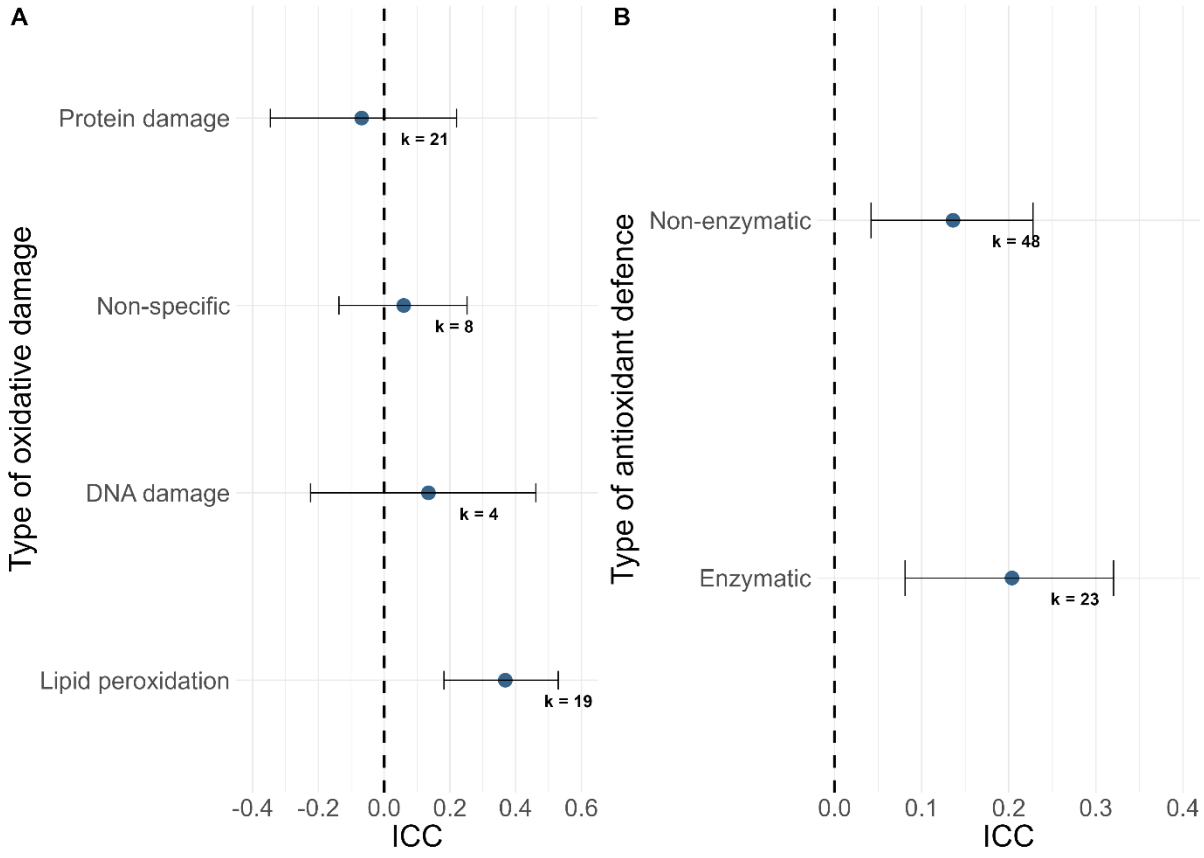

Figure 5.5: Within-individual repeatability of oxidative stress measures for "oxidative damage" and "antioxidant defence" subgroups. *This orchard plot displays the model output for the moderators: A) type of oxidative damage and B) type of antioxidant defence. Model estimates are presented alongside their 95% confidence intervals. The dashed line indicates where ICC equals zero. Moderator levels are shown on the y axis, and ICC values (back transformed from Fisher's Z) are displayed on the x axis.*

5.4.4 Are biomarkers of oxidative stress correlated?

Oxidative stress markers are often weakly correlated (Cohen and Mcgraw, 2009; Hõrak and Cohen, 2010). Our meta-analysis revealed that oxidative stress markers generally exhibit low within-individual repeatability. This raises questions about their suitability for characterising an individual's oxidative stress physiology. For instance, with an average repeatability of around 20%, the maximum expected correlation between markers would be r = 0.04, indicating a very weak relationship. This low repeatability across various markers may explain the previously reported weak correlations between oxidative stress markers, as well as their weak associations with other traits such as telomere length, and by extension, environmental and lifestyle factors.

Our meta-analysis presents a unique opportunity to test the correlations amongst different biomarkers of oxidative stress. Using the raw data available to us, we could estimate the correlations between protein carbonyls – superoxide dismutase (SOD), malondialdehyde (MDA) – antioxidant capacity (OXY), OXY - SOD, MDA - SOD, and reactive oxygen metabolites (dROMS) - OXY (Fig. 5.6). Our meta-analysis of these correlations reveals that most of them were very weak and not statistically significant (Fig. 5.6). However, there was a weak but significant positive correlation between dROMs and OXY (r [95% confidence interval, CI] = 0.250 [0.091, 0.396]; Table S4.5, Fig.5.6). This finding is interesting because the ICC values for dROMs and OXY (0.107 and 0.133) would predict a correlation of r = 0.014. This suggests that while these biomarkers are poorly repeatable over time, they still show a modest relationship with each other.

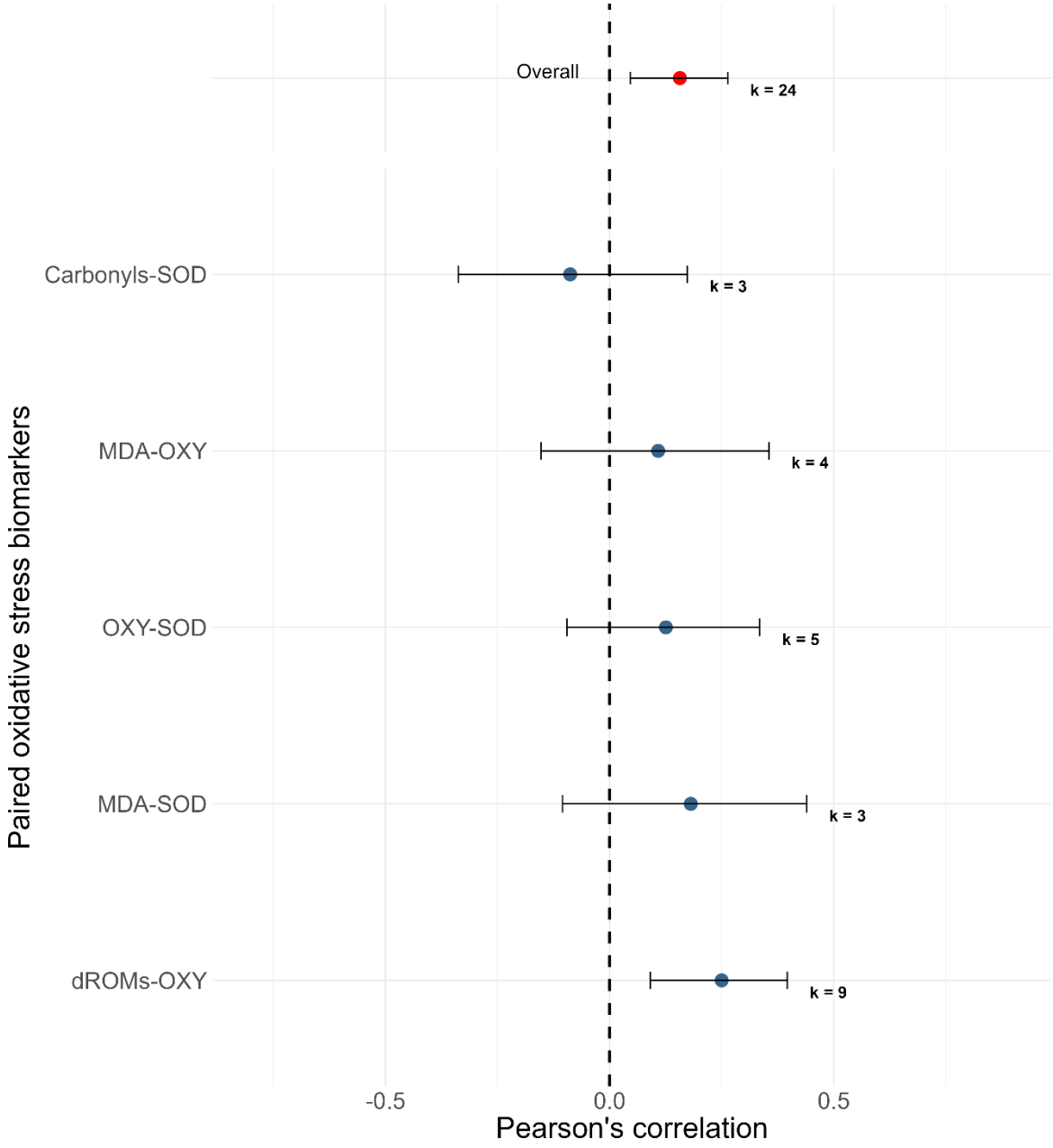

Figure 5.6: Pearson's correlation for paired oxidative stress biomarkers. *The model estimates are shown along with 95% confidence intervals. The dashed line indicates where Pearson's correlation is zero. The paired oxidative stress biomarkers are shown on the y axis and Pearson's correlation (back transformed from Fisher's Z) is shown on the x axis. The red point indicates the model estimate for the intercept-only model along with the 95% confidence intervals.*

Our meta-analysis demonstrates that oxidative stress measures exhibit low individual repeatability. This key finding raises concerns about the reliability of oxidative stress markers and their utility as biomarkers of health. The intraindividual variation limits our ability to detect meaningful patterns in the data and reduces statistical power. To address

this issue, we conducted an individual-based simulation study to investigate how repeatability affects the sample size required to achieve sufficient statistical power regarding detecting a relationship between oxidative stress and telomere length. We simulated several scenarios, with different assumptions regarding the effect of oxidative stress on telomere attrition.

5.4.5 Sample size required to detect a correlation between telomere length and oxidative stress

Our simulations show that at low oxidative stress ICC values (0.2), a substantially larger sample size is required to achieve >80% statistical power than what is typically considered in ecophysiology research. In fact, there were only 16 out of 37 studies from a previous meta-analysis on the association between oxidative stress and telomere length that yielded a sample size above n = 100 (Armstrong and Boonekamp, 2023), the threshold revealed by our simulation under the assumption that oxidative stress is fully causal in telomere attrition. Clearly, even larger sample sizes are required when the true causality of oxidative stress is reduced (Fig. 5.7).

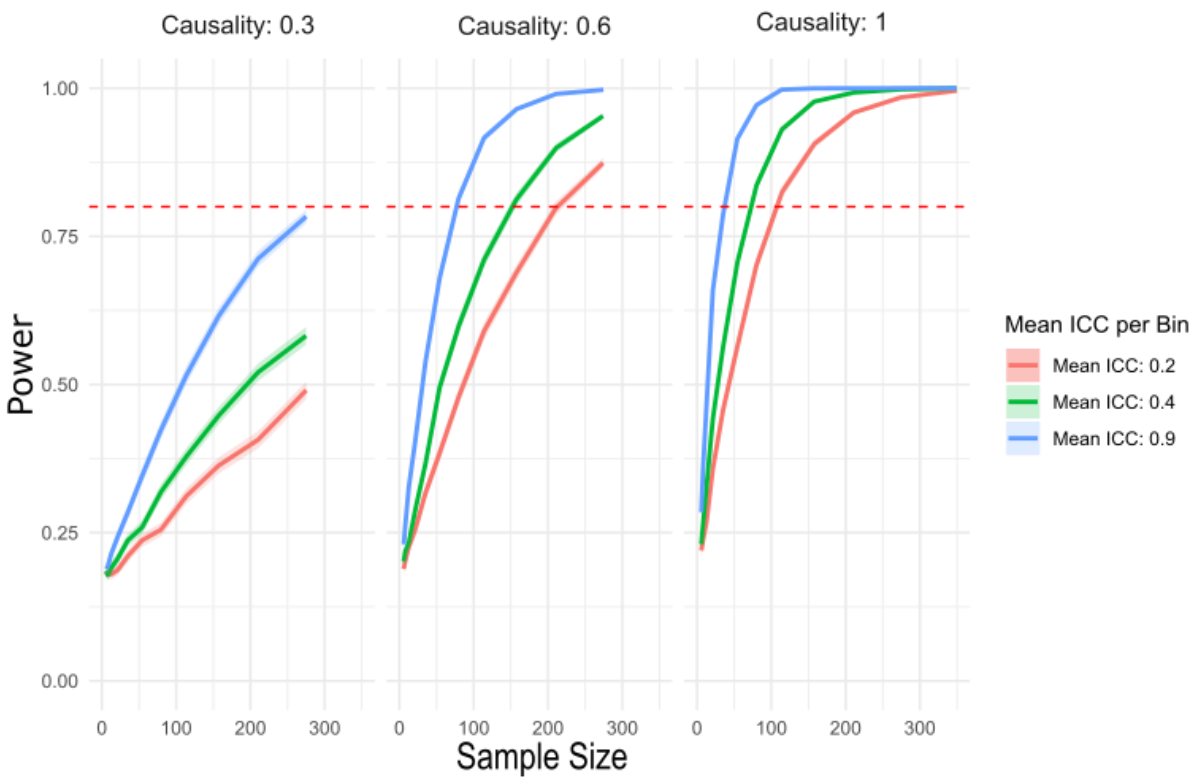

Figure 5.7: Simulated power of telomere length and oxidative stress correlations across different sample sizes, causality levels and individual repeatability of oxidative stress values*. The coloured lines represent the mean value of each repeatability (ICC) group that was simulated. Shaded areas show the 95% confidence intervals. The red dashed line marks the 80% statistical power threshold. The panels differentiate the different causality strengths between oxidative stress and telomere length. Where the coloured line crosses the 80% threshold this would indicate the minimum sample size required to have this level of power to detect the relationship between telomere length and oxidative stress.*

When simulating a scenario where oxidative stress ICC is low (0.2) and the causality between oxidative stress and telomere length is intermediate (0.6), we find that increasing the number of repeated samples per individual substantially reduces the minimum total sample size required to achieve >80% power. However, the power improvements show diminishing returns and taking more than five measurements per individual shows negligible improvements (Fig.5.8). Taking 3-4 measures per individual reduces the required sample size for 80% power by 25-30%.

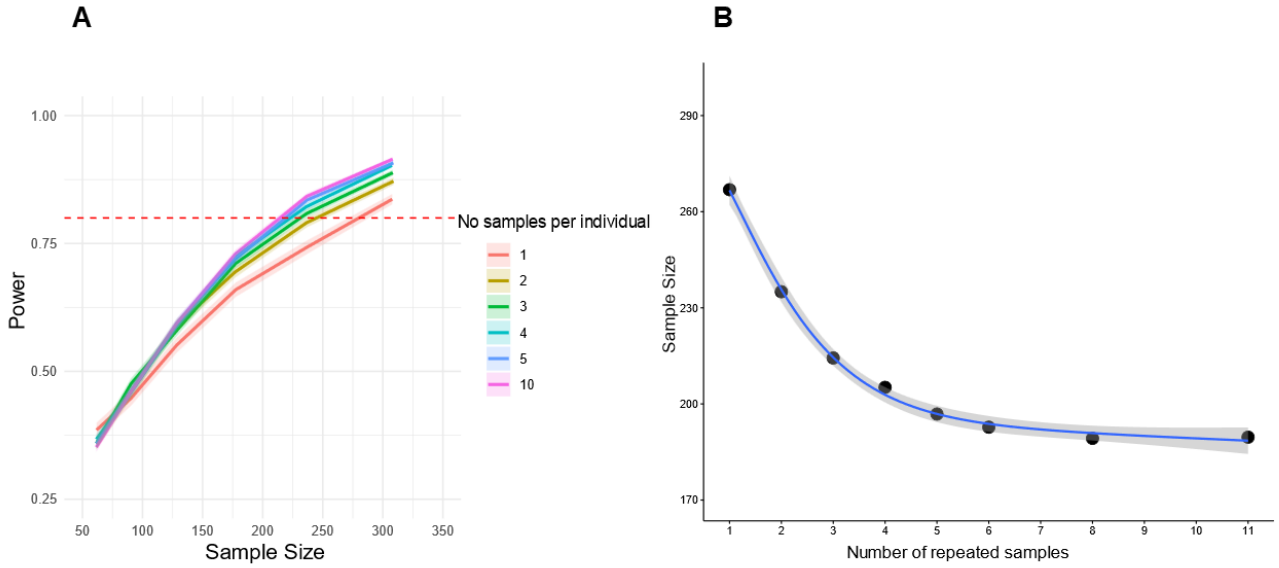

Figure 5.8: Relationship between sample size and repeated measures needed to achieve statistical power above 80% for detecting a correlation between telomere length and oxidative stress, using a scenario where the oxidative stress individual repeatability is low (ICC = 0.2) and causality of the oxidative stress effect on telomere length is intermediate (0.6). *A) the different colours represent the number of repeated measures per individual. The red dashed line marks the 80% power threshold. Shaded contours reflect the 95% confidence intervals. B) the points show the mean sample size required at each number of oxidative stress samples per individual to achieve >80% power to detect the correlation between telomere length and oxidative stress. The curve merely illustrates the trend.*

5.5 Discussion

The within-individual repeatability (ICC) of oxidative stress measurements over time is crucial for understanding the dynamics of physiological traits and the factors driving these changes. Oxidative stress varies both in the short and long term due to various factors, including natural variation over an organism's lifespan and environmental fluctuations (Alonso-Álvarez *et al.*, 2010; Beaulieu and Costantini, 2014; Chainy, Paital and Dandapat, 2016; Metcalfe and Alonso-Álvarez, 2010). Despite this variability, oxidative stress markers are widely used as indicators of physiological condition and as predictors of survival and fitness. However, many studies do not explicitly report the within-individual

repeatability of oxidative stress markers, and no comprehensive analysis has yet evaluated whether these markers remain consistent over time within individuals, which is required from a biomarker-utility perspective.

In this study, we conducted a meta-analysis to assess the within-individual repeatability of oxidative stress markers, investigating the potential biological and methodological factors that may influence this relationship. Furthermore, we performed a simulation analysis to estimate the minimum sample size required to achieve sufficient statistical power (>80%) for detecting the relationship between oxidative stress and telomere length when repeatability is low.

5.5.1 Within-individual repeatability of oxidative stress and the impacts of moderators

Our meta-analysis revealed that oxidative stress exhibits low repeatability (ICC = 0.164) and yet high heterogeneity across studies. While repeatability did not significantly differ between taxonomic classes, we observed that birds exhibited lower repeatability than mammals, although both groups showed overall low repeatability. Variation within each group suggests life-history strategies may influence oxidative stress responses. For instance, physiological adaptations that allow organisms to cope with environmental fluctuations, such as hibernation and migration, are closely linked to metabolism and could drive seasonal variation in oxidative stress (Chainy, Paital and Dandapat, 2016). Migratory birds often experience oxidative stress during endurance flights, with European robins *(Erithacus rubecula)* exhibiting higher oxidative damage during nocturnal flight than daytime rest (Jenni-Eiermann *et al.*, 2014). On the other hand, in brent geese *(Branta bernicla)*, a long-lived migratory species, lipid damage and non-enzymatic antioxidants remained stable over time, whereas superoxide dismutase levels were inconsistent (Bodey *et al.*, 2019). Whilst these life history related factors could all contribute to the observed heterogeneity, they do not provide satisfying explanations for the overwhelming pattern of low individual repeatability.

We hypothesised that oxidative stress markers would exhibit higher individual repeatability in captive animals, given the stable conditions that minimise environmental stressors such as predation, temperature and food scarcity (Beaulieu, 2016; Calisi and Bentley, 2009; Costantini, 2022). Contrary to our expectations, we found no significant differences in repeatability between wild, captive and wild-captive groups, with all groups exhibiting low

repeatability. This may be due to captivity introducing its own physiological variability, for example, captive zebra finches *(Taeniopygia guttata)* are less genetically diverse, heavier, and less active than wild individuals, which could increase oxidative stress variability (Beaulieu, 2016). In contrast, livestock exhibited higher albeit still low repeatability. This may reflect management practices aimed at optimising performance and productivity. For instance, frequent antioxidant supplementation in livestock could reduce oxidative stress variability (Miller, Brzezinska-Slebodzinska and Madsen, 1993; Ponnampalam *et al.*, 2022). Additionally, higher sampling rates in livestock (3 to 41 repeat measurements per individual) may have contributed to the observed higher ICC. Overall, our findings indicate that oxidative stress biomarkers show considerable variability within individuals, regardless of the environment. However, we do not understand the source of this variability, which is a key problem, as understanding the source of variability would allow us to control for it thereby increasing conditional repeatability.

Our analysis revealed that both oxidative damage and antioxidant defence biomarkers showed low within-individual variation, with no significant difference between groups. We had expected antioxidant defences to be more repeatable than oxidative damage measures due to the short-lived nature of ROS (Ito, Sono and Ito, 2019). Previous research has shown that in free-living adult collared flycatchers, antioxidants were repeatable within individuals, whereas reactive oxygen metabolites were not (Récapet *et al.*, 2019). However, antioxidants are highly sensitive to external stressors and are directly influenced by the level of circulating ROMs. As such they can fluctuate significantly within and among individuals over time (Costantini *et al.*, 2010). This sensitivity may help explain the overall low repeatability observed in our study, emphasising the dynamic nature of the oxidative stress system. Surprisingly, studies involving manipulations showed slightly higher repeatability than non-manipulation studies, contrary to our expectation that manipulations would increase variability due to additional stressors or physiological changes. This pattern may reflect the consistent effects of controlled manipulations, such as dietary interventions, which can reduce variation. In contrast, non-manipulated studies may have experienced greater natural variability, contributing to the higher observed variation in this group.

We hypothesised that sex might contribute to variation in oxidative stress responses due to differences in male and female life histories. For instance, many female birds experience higher oxidative stress during the breeding season, attributed to the energetic costs of egg

production and incubation (Williams, 2005; Gomes, Vieira and da Silva, 2019). Conversely, in Florida scrub jays *(Aphelocoma coerulescens),* males were shown to invest more in reproductive effort than females, leading to higher oxidative damage in males during this period (Heiss and Schoech, 2012). However, our study found no significant difference in repeatability of oxidative stress between males and females. This suggests that, despite potential sex-based differences in reproductive investment and energetic demands, both sexes may exhibit high temporal variation in oxidative stress. Our findings align with previous studies showing no consistent sex differences in oxidative physiology across bird species (Costantini and Bonadonna, 2010; Vincze *et al.*, 2022). Overall, the low repeatability observed in both sexes suggests that oxidative stress is highly dynamic, influenced by factors beyond biological differences.

Oxidative stress is known to fluctuate over time due to various factors, including seasonality and environmental changes (Chainy, Paital and Dandapat, 2016; Lemieux *et al.*, 2019; Raja-Aho *et al.*, 2012; Van de Crommenacker *et al.*, 2011). We therefore expected oxidative stress markers to show low repeatability over time. However, our analysis revealed a weak but significant positive relationship between the time elapsed between successive measures and repeatability. One plausible explanation for this result is that individuals develop more stable oxidative profiles over longer periods, potentially due to consistent individual differences in physiology, or behaviour as organisms establish relatively stable metabolic or life-history strategies (Burger, Hou and Brown, 2019; Varpe, 2017). Alternatively, in many longitudinal field studies, repeated sampling often occurs at the same time each year for example during the breeding season. This temporal alignment may reduce environmental noise such as temperature fluctuations, or seasonal variation in diet which may increase the similarity of repeated measures even over long internals (Žaja *et al.,* 2016; Chainy, Paital and Dandapat, 2016). Acute stressors, such as immune challenges, can also drive rapid short-term changes in oxidative stress levels. For example, short-tailed fruit bats *(Carollia perspicillata)* showed an increase in oxidative stress following an inflammatory challenge (Schneeberger, Czirjk and Voigt, 2013).

Additionally, as predicted, we observed a positive relationship between repeatability and the number of successive samples taken per individual. More frequent sampling likely captures consistent patterns and reduces the influence of environmental fluctuations and measurement noise (Biro and Stamps, 2015). Together, these findings highlight the

importance of repeated measures over varying time intervals to understand oxidative stress variability.

5.5.2 The effect of the type of oxidative stress biomarker on repeatability

Our results suggest that the type of oxidative damage biomarker used influences repeatability. Among the markers examined, only lipid peroxidation showed a significant effect on ICC, showing a higher albeit still low repeatability compared to the other biomarkers. DNA damage, protein damage and nonspecific oxidative damage all displayed low repeatability, with protein and nonspecific damage showing near-zero repeatability. This aligns with our expectations, as ROS are short-lived, highly reactive and encompass diverse molecules with varying interactions (Murphy *et al.*, 2022). Lipid peroxidation however is widely used in clinical studies as a biomarker of disease risk and progression (Ito, Sono and Ito, 2019; Niki, 2014). Although measuring oxidative damage can be methodologically challenging due to the instability of ROS, lipid peroxidation can be assessed with high reliability using high-performance liquid chromatography (HPLC). However, simpler and faster assays used to measure lipid peroxidation tend to be less accurate, which could contribute to greater variability in this biomarker (Ito, Sono and Ito, 2019; Katerji, Filippova and Duerksen-Hughes, 2019).

For antioxidant defence biomarkers, enzymatic antioxidants showed slightly higher repeatability than non-enzymatic antioxidants, although both exhibited low repeatability overall, with no significant difference between them. This is expected, given that antioxidants are highly variable and sensitive to multiple stressors (Costantini *et al.*, 2010). Non-enzymatic antioxidants, such as uric acid and carotenoids, may exhibit even lower repeatability due to the influence of diet type and seasonal fluctuations (Cohen, Klasing and Ricklefs, 2007). Furthermore, the diverse nature of antioxidants, each with distinct chemical properties and interactions with ROS, means that direct comparisons are challenging (Murphy *et al.*, 2022). Variability in results across different measurement techniques further complicates comparisons between antioxidants, as the performance of assays may differ (Bartosz, 2010).

5.5.3 Do biomarkers of oxidative stress correlate with each other?

Our analysis revealed that most oxidative stress markers had very weak correlations with each other. However, we found a weak but significant positive correlation between reactive

oxygen metabolites and antioxidant capacity. This lack of strong correlation between markers is well-documented in the literature (Christensen *et al.*, 2015; Costantini, 2019; Sepp *et al.*, 2012b) and could be due to high intraindividual variation masking associations between biomarkers, as highlighted in the overall low repeatability across oxidative stress biomarkers. These findings highlight the need to measure multiple oxidative stress biomarkers before and during experiments to better assess oxidative status, however this approach is rarely implemented (Costantini, 2019; Hõrak and Cohen, 2010). Each assay captures a specific biochemical process, emphasising the importance of considering both ecological and experimental context when interpreting results. Relying on a single marker as a universal index of oxidative stress can be misleading (Christensen *et al.*, 2015; Hõrak and Cohen, 2010; Ito, Sono and Ito, 2019; Sepp *et al.*, 2012b). The positive correlation between reactive oxygen metabolites and antioxidant capacity observed in our study supports findings from other studies, though the strength of this relationship can vary depending on experimental conditions and environmental differences between years (Récapet *et al.*, 2019). This suggests individuals experiencing high oxidative damage may also exhibit elevated antioxidant defences, which is likely an adaptive response. Both biomarkers showed low within-individual repeatability, highlighting that their correlation likely reflects an underlying physiological connection rather than being influenced by repeatability.

5.5.4 Sample size required to detect a relationship between oxidative stress and telomere length at a low repeatability

Using a simulation, we demonstrated that when oxidative stress has a low within-individual ICC, a significantly higher sample size is required to achieve greater than 80% statistical power in detecting the relationship between oxidative stress and telomere length. In contrast, studies with higher ICC values require fewer individuals to reach the same power. This relationship is further influenced by the degree of causality between oxidative stress and telomere loss. When the effect of oxidative stress on telomere loss is weak, a low ICC exacerbates the challenges of detecting an association, even with a larger sample size. This is expected, as low ICC reflects high levels of within-individual variability, which introduces random noise making it harder to detect effects, thus requiring a larger sample size. As ICC increases, a larger proportion of the variation in telomere length can be attributed to individual differences rather than random noise, making detection of relationships more feasible.

Oxidative stress is widely regarded as an important measure in the context of ageing, ageing related diseases and telomere attrition, therefore is often used to infer future fitness and survival in many organisms, including humans (Ahmed and Lingner, 2018; Beaulieu and Costantini, 2014; Luo *et al.*, 2020; Yadav and Maurya, 2022). However, the relationship between oxidative stress and telomere attrition is not always clear (Beaulieu and Costantini, 2014; Boonekamp *et al.*, 2017). Our simulation highlights the importance of interpreting findings cautiously, especially when oxidative stress measures have low repeatability and sample sizes are small. It emphasizes the need for calculating repeatability and ensuring sufficiently large sample sizes to reliably detect meaningful relationships between oxidative stress and telomere length. Although individual variation in oxidative stress can be zero, it can still have a casual effect on telomere loss. This is important as oxidative stress therefore remains an important marker to measure and study. The main problem the high intraindividual variation of oxidative stress provides is it causes a detection issue where it is challenging to detect relationships with other markers which raises the question on how we deal with the data.

We also demonstrate that increasing the number of repeated samples per individual, reduces the minimum sample size required to achieve high statistical power in detecting a relationship between telomere length and oxidative stress. This underscores the importance of including repeated measures to better capture within-individual variability. Once the number of repeat samples reaches five per individual, the required sample size does not decrease significantly with further increases in repeat samples. Therefore, using more than three repeat measures per individual will likely not increase statistical power.

5.6 Conclusion

In conclusion, our study reveals that oxidative stress exhibits low within-individual repeatability across various taxa, study types, environments, biomarkers and sexes. This low repeatability, coupled with the lack of significant correlations between most oxidative stress markers, raises concerns about their reliability in charactering an individual's oxidative physiology. It also highlights the need to measure multiple markers to gain a comprehensive understanding of the whole system, as no single marker of oxidative stress can serve as a universal index.

Additionally, we demonstrate that increasing the number of repeated measurements improves repeatability, highlighting the importance of taking multiple measurements of the

same individual across various timescales to capture accurate oxidative profiles. Our simulation further emphasises the challenges of using oxidative stress markers to investigate relationships with other traits, such as telomere length. Low repeatability significantly increases the sample size required to detect relationships between oxidative stress and telomere length, which poses difficulties for studies with ethical or technical constraints on sample size. Therefore, it is critical to understand the repeatability of the oxidative stress marker being measured and to calculate the minimum sample size required to reliably capture these relationships.

Finally, we show that increasing the number of samples taken per individual can reduce the minimum sample size required to achieve high statistical power. Therefore, researchers should aim to take at least 4-5 samples from each individual over time to reduce the overall sample size required when investigating the relationship between oxidative stress and telomere length. These findings question the use of oxidative stress markers as consistent indicators of physiological change, particularly when investigating long-term outcomes like telomere attrition. Therefore, it is crucial that these biomarkers be interpreted with caution and that care is taken when utilising these biomarkers in a study.

5.7 Availability of data and materials

The code and data used in this study will be made available online following peer review at the time when the manuscript is accepted for publication

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5.10 Author contributions

Rachel Reid, Davide M Dominoni and Jelle Boonekamp conceived of and designed the study. Rachel Reid and Jelle Boonekamp developed the simulation. Rachel Reid ran the analysis. Rachel Reid drafted the initial manuscript with edits from both Davide M Dominoni and Jelle Boonekamp. All authors read and approved the final version.

Chapter 6
General discussion

6.1 Key findings from the thesis

This study investigated the relationship between urbanisation and avian health on a global scale, identifying key drivers behind this relationship including species-specific traits, life stage, biomarker type, and the degree of urbanisation. I then narrowed my focus to artificial light at night (ALAN) as a specific anthropogenic stressor, assessing its impact on several health biomarkers in birds including body condition, telomere shortening, oxidative stress, corticosterone, and metabolic disruption, through both field and captive experiments. Finally, I explored the reliability of oxidative stress biomarkers by assessing their within-individual repeatability through a meta-analysis and highlighting the potential challenges this may cause when detecting associations with other physiological traits using individual simulations.

6.1.1 Urbanisation and avian health

Using a phylogenetically controlled meta-analysis, I found no overall relationship between urbanisation and avian health. However, when urbanisation was treated as a continuous variable, health deteriorated with increasing urbanisation. This suggests that urban environments differ significantly, and a more refined, quantitative approach to define urbanisation is needed to capture this variability. Cities vary significantly in their abiotic and biotic factors, this includes pollution levels, green space availability, food provisioning, and human population density (Abbaspour *et al.*, 2015; Duh *et al.*, 2008; Gallaway, Olsen and Mitchell, 2010; Gong *et al.*, 2012; Mathew, Khandelwal and Kaul, 2016; Nowak *et al.*, 2005; Potgieter *et al.*, 2019; Theimer *et al.*, 2015), all of which likely influence species health responses. A scoring system that incorporates this heterogeneity could offer more precise insights into how urbanisation impacts wildlife health.

Further exploration revealed the effects of urbanisation on health were context-dependent, varying depending on the specific biomarker assessed, with some effects detected only in certain life stages. For instance, adult birds showed a lower ectoparasite burden in urban areas, likely due to reduced ectoparasite survival in urban microclimates (Brennan *et al.*, 2023) and increased time for self-maintenance behaviours, such as preening, facilitated by

supplemental food sources reducing the need to forage (Villa *et al.*, 2016). Conversely, nestlings in urban environments exhibited lower body condition, potentially due to lower food availability and the lack of essential nutrients acquired in their diet which are needed for growth and development (Catto *et al.*, 2021; Heiss, Clark and Mcgowan, 2009; Sinkovics *et al.*, 2021; Grames *et al.*, 2023). This effect was not observed in adults, who can exploit additional resources in urban habitats, unlike nestlings (Catto *et al.*, 2021). The relationship between urbanisation and body condition were less impactful for generalist species, likely due to their greater behavioural flexibility. In contrast, specialist species, such as terrestrial birds, may be more vulnerable to urbanisation due to behavioural constraints (including foraging and nesting behaviours) that limit their ability to adapt to changing conditions (Lakatos *et al.*, 2022; Marques *et al.*, 2021). These findings emphasise the importance of considering the degree of urbanisation, species-specific traits and life stage when assessing the impacts of urbanisation on health. Understanding the drivers behind negative impacts of urbanisation as well as the species that are more vulnerable will allow more the development of more focused and precise mitigation strategies to combat this. It is important to also understand the specific stressors that are behind the negative impacts on health in urban areas including ALAN.

6.1.2 Impacts of artificial light at night on avian health

Chapters 3 and 4 of this thesis focused on ALAN as a key anthropogenic stressor. In great tit *(Parus major)* nestlings, ALAN exposure did not have a clear negative effect on oxidative stress or feather corticosterone levels, but it did lead to lower body condition which aligns with previous studies (Ferraro, Le and Francis, 2020; Raap *et al.*, 2016a). This effect could be due to several reasons, ALAN may contribute to increased nocturnal begging behaviour in the nestlings (Raap, Pinxten and Eens, 2016), diverting energy away from important processes such as growth and metabolism (Soler *et al.*, 2014). Additionally, ALAN could disrupt the sleep patterns of adult birds, thereby reducing the efficiency of parental care (Injaian, Taff and Patricelli, 2018; Injaian *et al.*, 2021; Raap, Pinxten and Eens, 2016). These findings offer mechanistic insights into the negative effects of urbanisation on nestling body condition observed in the meta-analysis. This is an important relationship to consider as birds that fledge the nest in poor condition have been shown to have less chance to survive until adulthood which could end up having population-level effects such as disrupted population dynamics and lower reproductive success within the population (Naef-Daenzer, Widmer and Nuber, 2001).

In captive zebra finches *(Taeniopygia guttata)*, ALAN exposure did not significantly affect oxidative stress levels over time. However, it did alter circadian glucose rhythms, with birds exposed to full ALAN showing peak glucose levels at 1am, in comparison to the controls and the birds exposed to partial ALAN where glucose levels peaked at 8pm. Although limited, previous studies have shown that ALAN exposure can disrupt feeding behaviour and activity-rest cycles, leading to elevated glucose levels during the night (Batra, Malik and Kumar, 2019; Batra *et al.*, 2020; Fonken *et al.*, 2010). This disruption could lead to several metabolic abnormalities including obesity, type 2 diabetes, and cardiovascular disease which has been shown in rodent model systems (Kumar Jha, Challet and Kalsbeek, 2015; Mason *et al.*, 2020). Furthermore, birds exposed to full ALAN exhibited increased telomere attrition, which is linked to increased mortality and reduced longevity (Barrett *et al.*, 2013; Boonekamp *et al.*, 2014; Salomons *et al.*, 2009). While some studies have found no impact of ALAN on telomere shortening (Grunst *et al.*, 2019; Ouyang *et al.*, 2017), these studies typically used short-term exposure to ALAN. One study lasted a duration of six months and still did not find a relationship; however, they used low light intensity of 0.3 lux compared to the 5-lux used in our experiment (Alaasam *et al.*, 2024). Therefore, the intensity and duration of ALAN exposure may be drivers behind this relationship, highlighting the need for further research. Therefore, our findings indicate that long-term exposure to ALAN can have negative physiological consequences for birds potentially impacting their fitness and survival prospects.

A key finding of the captive study was that birds exposed to partial ALAN did not show the negative physiological effects observed under full ALAN. This suggests that implementing partial-night lighting schemes, where lights are switched off during certain hours, could help mitigate ALAN's impact on wildlife while still meeting human needs. This approach could also offer economic and environmental benefits by reducing energy consumption and contributing to the reduction of emissions (Gallaway, Olsen and Mitchell, 2010; Pagden, Ngahane and Amin, 2020). Other studies have shown mixed results on the benefits of a partial light at night mitigation scheme in urban environments. For example, partial light at night appeared to make no difference in the activity patterns of bat species (Azam *et al.*, 2015) however it did have positive effects on behavioural biological rhythms in *Crassostrea gigas* oysters (Botté *et al.*, 2023). This highlights that much more research has to be conducted on this scheme as the benefits may depend on the environment, the species, as well as the time the lights are turned off.

6.1.3 Reliability of oxidative stress biomarkers

The final chapter of this thesis investigated the within-individual repeatability of oxidative stress markers using a meta-analysis approach. Oxidative stress biomarkers showed low repeatability overall, with considerable variability across studies. Repeatability was low across all moderator levels including taxonomic class, sex, environment, experiment type, and the type of oxidative stress biomarker used. This is not unexpected as oxidative stress has been shown to fluctuate on both short and long-term timescales due to a variety of factors including environmental fluctuations such as seasonality and habitat quality (Chainy, Paital and Dandapat, 2016; Van de Crommenacker *et al.*, 2011; Rathwa *et al.*, 2017), species-specific behaviours including courtship displays and reproductive investment (Alonso-Álvarez *et al.*, 2010; Metcalfe and Alonso-Álvarez, 2010), as well as natural variation that occurs over an individual's lifespan (Beaulieu and Costantini, 2014). Variability can also arise due to tissue-specific differences as well as assay inconsistencies (Hõrak and Cohen, 2010; Speakman *et al.*, 2015). There was a positive relationship between repeatability and the number of successive samples taken per individual. Frequent sampling captures more consistent patterns, reducing random noise and improving accuracy (Biro and Stamps, 2015). This finding underscores the importance of repeated measurements per individual to enhance repeatability and ensure robust results. This high intraindividual variation could present a detection issue challenge, this variation could therefore mask the relationship between oxidative stress and other physiological traits such as telomere length, as well as obscuring correlations between different types of oxidative stress markers.

The analysis also revealed that when Pearson's correlation was calculated for paired oxidative stress biomarkers, most oxidative stress markers correlated weakly with each other. This was not an unexpected result, as several reviews have discussed the weak correlations often observed between different oxidative stress markers (Beaulieu and Costantini, 2014; Costantini *et al.*, 2010; Costantini, 2019; Hõrak and Cohen, 2010; Speakman *et al.*, 2015). This emphasises that a single oxidative stress marker may not reliably reflect an individual's overall oxidative status. Consequently, ecological studies should ideally measure multiple oxidative stress markers simultaneously and repeatedly over time to gain a more accurate assessment of oxidative status.

A simulation conducted as part of this analysis demonstrated that when oxidative stress biomarkers exhibit low within-individual repeatability, a larger sample size is required to achieve sufficient statistical power (>80%) to detect associations between oxidative stress and telomere length. In contrast, studies with a higher oxidative stress repeatability require a smaller sample size to achieve the same statistical power. This relationship is further influenced by the causality between oxidative stress and telomere length in a given study, if oxidative stress has a low influence on telomere loss, then this relationship will be extremely hard to detect at a low oxidative stress repeatability even with a large sample size. Low repeatability introduces random variation, which makes it more difficult to detect patterns in the data. Moreover, increasing the number of repeated measures per individual reduces the minimum sample size required for high statistical power, showing that the optimal number of repeats per individual was five. Given the widespread use of oxidative stress as a measure of health, ageing, and fitness, especially in relation to telomere attrition (Ahmed and Lingner, 2018; Beaulieu and Costantini, 2014; Luo *et al.*, 2020; Yadav and Maurya, 2022), these findings highlight the importance of calculating and understanding the within-individual repeatability of oxidative stress measures used within a study. The variability observed in oxidative stress biomarkers highlights the need for careful interpretation of results, particularly when inferring the impacts of oxidative stress on fitness and survival.

6.2 Challenges, limitations and next steps.

This thesis did come across certain challenges and limitations that must be taken into consideration for future research. The meta-analysis on urbanisation and avian health in chapter 2 was constrained by a limited sample size when quantifying the degree of urbanisation for each study location. The approach used to calculate the degree of urbanisation required precise geographic coordinates to assess urbanisation levels within various buffer zones, but this data was available only for a subset of studies. A larger sample size may have strengthened the observed relationships and uncovered hidden patterns. This highlights the need for future research to report the exact study site coordinates to improve cross-study comparisons.

Additionally, the dataset exhibited biases in both study species and geographic coverage. For example, most studies measuring ectoparasites focused on ticks and primarily used blackbirds *(Turdus merula)* as a model species within Europe and North America.

Expanding research to a wider range of species and geographic regions, particularly in the Southern Hemisphere, is crucial for a more representative understanding of the effects of urbanisation on bird species. Furthermore, most studies were conducted at temperate latitudes, limiting our ability to generalise findings. Health responses to urbanisation may vary with climatic factors such as temperature and rainfall (Nunn *et al.*, 2005; Simonis *et al.*, 2023). There is a need for further studies to be conducted in tropical megacities where the rate of urbanisation is rapidly increasing, addressing these geographic gaps is essential for a more comprehensive understanding of the relationship between urbanisation and avian health.

The field experiment which investigated the impacts of ALAN on the health of great tit nestlings (Chapter 3) was limited to a single breeding season, preventing an assessment of interannual variability. Multi-year studies would help account for environmental fluctuations, such as weather conditions, which could influence health biomarkers. However, many prior studies on ALAN have also relied on single-season data (Dominoni *et al.*, 2021; Grunst *et al.*, 2019; Grunst *et al*., 2020; Raap *et al.*, 2016a, Raap *et al.,* 2017b) and collectively, these studies contribute to a growing body of evidence that can be synthesised through a future meta-analysis on the topic. Nevertheless, future research should incorporate a muti-year approach to improve the robustness of findings. Another limitation of this experiment was the inability to determine the precise mechanism underlying the observed reduction in nestling body condition. ALAN could be affecting nestlings directly (e.g increased nocturnal begging and energy expenditure) or indirectly (e.g disrupting parental sleep and provisioning efficiency). Future studies could use nest box cameras to monitor both nocturnal activity and feeding rates to disentangle these mechanisms. Understanding the mechanism behind the relationship between ALAN and body condition could be crucial to develop precise strategies for habitat management to mitigate these negative effects.

The captive experiment in chapter 4 faced several challenges including statistical power limitations. The significant effect of full ALAN on telomere attrition was only detected when the partial ALAN treatment group was removed from the model, suggesting that the sample size may have been too small to detect subtle effects when all three groups were included. With a sample size of 44 zebra finches split across three treatment groups, statistical power was reduced. Future studies should replicate these findings with larger sample sizes to confirm the patterns observed. Additionally, while captive experiments

allow control over environmental variables allowing for more confidence in the results obtained, there are concerns over whether results can be generalised to wild populations, where individuals experience fluctuating environmental conditions as well as other challenges. To strengthen the finding that partial-night lighting can mitigate the effects of ALAN, it would be valuable to test this mitigation strategy in more ecologically realistic scenarios using multiple species.

The meta-analysis assessing within-individual repeatability in oxidative stress markers in chapter 5 faced some key limitations. One primary constraint was the relatively small sample size, as we were only able to extract raw data from 22 studies. Identifying suitable literature for inclusion was challenging, as studies that measured oxidative stress multiple times in the same individual did not always explicitly state this in the abstract. As a result, considerable effort was required to ensure papers met the inclusion criteria. To address this challenge, I developed a robust search string to capture all relevant studies and implemented a two-step screening process. First, abstracts were scanned for key inclusion criteria (i.e confirmation that oxidative stress was measured), and then full texts were examined to confirm that successive samples have been taken from the same individuals. Additionally, I searched two databases to maximise the scope of our literature search. Despite these efforts, it is possible that some relevant studies were missed. Another significant limitation in chapter 5 was the availability of raw data, which was essential for calculating repeatability in a standardised way. Older studies, in particular, lacked accessible raw data which meant our meta-analysis was largely restricted to studies published after 2013. This highlights the importance of data archiving in ecological research to facilitate future meta-analysis and cross-study comparisons.

Given that oxidative stress biomarkers were used in both the captive and field experiments to assess the impacts of ALAN, the findings from the meta-analysis raise concerns about their reliability as health indicators. In hindsight, had this meta-analysis been conducted before the ALAN experiments, it would have provided valuable considerations regarding the suitability of oxidative stress as a biomarker in these studies. Specifically, the field study in chapter 3 relied on a single oxidative stress measurement at one time point with a limited sample size. The meta-analysis demonstrated that oxidative stress markers have low within-individual repeatability and that a significantly larger sample size as well as repeated measures per individual are essential to detect meaningful patterns. Given these constraints, oxidative stress may not have been the most appropriate biomarker for

assessing ALAN's effects. The meta-analysis also highlighted the weak correlations between different biomarkers of oxidative stress; this emphasises that optimally I should have measured multiple markers of both oxidative damage and antioxidant defence to provide a more robust assessment of oxidative status. High intraindividual variation could explain why I could find no clear impacts of ALAN on oxidative stress in either experiment, or why I found no associations between the different physiological markers.

6.3 The big picture

6.3.1 Why should we care?

Urbanisation is accelerating at an unprecedented rate globally, delivering undeniable benefits such as technological advancements, economic development, productivity growth, and enhanced societal infrastructure (Biłozor and Cieślak, 2021; Brunt and García-Peñalosa, 2022). With over 55% of the global population currently living in cities and projections estimating that this may reach 70% by 2050, the demand for urban space is further intensifying (Biłozor and Cieślak, 2021). Yet unfortunately, this expansion comes at a substantial ecological cost. Urban environments increasingly expose wildlife to novel anthropogenic stressors, including air pollution, noise pollution, and artificial light at night (ALAN) (Abbaspour *et al.*, 2015; Cárdenas Rodríguez, Dupont-Courtade and Oueslati, 2016; Chepesiuk, 2009; Manisalidis *et al.*, 2020; Yang *et al.*, 2020). These stressors have been shown to negatively impact both human and wildlife health, influencing physiological, behavioural, and psychological well-being (Abbaspour *et al.*, 2015; Ciach and Fröhlich, 2017; Gaston *et al.*, 2013; Gurjar *et al.*, 2010; Grubisic *et al.*, 2019; Manisalidis *et al.*, 2020; Raap, Pinxten and Eens, 2015).

Of the many urban stressors, ALAN is especially pervasive yet is often underestimated. ALAN is deeply integrated into modern society, improving human safety, productivity, and social activity (Hölker *et al.*, 2021). However, it disrupts natural light-dark cycles that are fundamental to the regulation of many biological and ecological processes, and this can affect species at multiple levels of the ecosystem (Dominoni, Quetting and Partecke, 2013; Gaston and Sánchez De Miguel, 2022; De Jong *et al.*, 2016; Robert *et al.*, 2015). The urgency of addressing ALAN's ecological consequences was discussed at the World Biodiversity Forum in Davos, Switzerland in 2020, where it was emphasised that there is a critical need for a multidisciplinary approach to understand and mitigate the effects of ALAN (Hölker *et al.*, 2021).

A critical concern is that ALAN is often introduced into the environments at times, wavelengths, and intensities that are ecologically unnatural, which can lead to substantial alternations in the biological rhythms of species and their ecological interactions (Gaston *et al.*, 2015; Kyba *et al.*, 2017). The shift to LED technology, has been beneficial in being more energy efficient, however this has likely intensified the negative impacts of ALAN, emitting high levels of visible light that escape detection by current satellite sensors. Some estimates suggest global increases in visible-spectrum radiance may be as high as 270%, with some regions experiencing rises of up to 400% (de Miguel *et al.*, 2021). These changes are not only poorly quantified but also pose a risk of driving long-term physiological disruptions with significant ecological impacts, the consequences of which we do not yet fully understand (Hölker *et al.*, 2021).

Birds have been widely regarded as reliable bioindicators of environmental health (Francis, 2017; Mekonen, 2017). My research has demonstrated that ALAN exposure negatively affects avian health, including lower body condition in great tit nestlings, disrupted metabolic rhythms, and increased telomere attrition in adult zebra finches. Each of these impacts has the potential to cause long-term implications for survival and fitness (Heidinger *et al.*, 2012; Guan *et al.*, 2022; Ledford, 2012; Mason *et al.*, 2020; Versteeg *et al.*, 2016). While these findings focus on birds, they likely extend to a broader range of taxa (Francis, 2017; Mekonen, 2017). Given our limited understanding of ALAN's cumulative impacts, expanding research into its underlying mechanisms and the development of evidence-based mitigation strategies is essential for balancing human development with biodiversity conservation.

As urbanisation intensifies, evaluating its effects on wildlife health and fitness becomes more critical. This requires biologically meaningful, reliable and repeatable health biomarkers. A key focus of this thesis was evaluating the within-individual repeatability of oxidative stress markers, which are commonly used in ecological studies (Herrera-Dueñas *et al.*, 2017; Kennany and Badrany, 2007; Metcalfe and Alonso-Álvarez, 2010; Tkachenko and Kurhaluk, 2013). However, results revealed low within-individual repeatability, introducing substantial random variation to measures. This high intraindividual variability can obscure relationships between oxidative stress and other physiological traits including telomere length, and this can make it extremely challenging to detect meaningful biological patterns. It's important to note that a lack of repeatability does not necessarily imply the absence of a relationship with other traits, rather it reflects a detection issue.

Despite these limitations, oxidative stress remains a valuable marker, particularly given its role in ageing and disease (Barnes, Fouquerel and Opresko, 2019; Luo *et al.*, 2020; Pole, Dimri and Dimri, 2016). Therefore, it is essential for future research to refine experimental designs and interpret these markers with caution. This includes increasing sample sizes, incorporating repeated measures, and exploring multi-biomarker approaches to better capture an individual's oxidative status and improve biological inference.

6.3.2 Remaining knowledge gaps

How do we accurately quantify urbanisation?

The meta-analysis in chapter 2 revealed that avian health declines with increasing urbanisation only when urbanisation is quantified as a continuous variable. In contrast, no such pattern was found when urbanisation was treated as a binary categorical variable, which is an approach still widely used in the literature. This highlights the need for more nuanced and standardised measures that reflect heterogeneity of urban environments, otherwise we risk underestimating or misinterpreting the ecological consequences of urbanisation. However, the question remains, how can we universally define and measure urbanisation so that we can allow for cross-study comparisons without oversimplifying complex urban gradients? Several studies have explored methods for quantifying urbanisation, such as incorporating landscape metrics as well as demographic and physical characteristics into the calculation (Hahs and McDonnell, 2006; Inostroza *et al.*, 2019; Salmón *et al.*, 2018b). It is also increasingly important that future urban scoring systems include diverse environmental stressors such as light and noise pollution, temperature fluctuations, and human population density as these will also impact wildlife health individually or simultaneously. Developing such comprehensive metrics will be challenging and will likely require a collaborative, interdisciplinary effort across research groups, but this could have the benefit of allowing for easier cross-study comparisons and the development of a more robust evidence base.

What are the interactive effects of novel anthropogenic stressors?

Wildlife in urban environments are rarely exposed to a single anthropogenic stressor. Instead, animals will typically face multiple stressors simultaneously. Despite this, most studies such as the ones included in this thesis tend to examine anthropogenic stressors such as ALAN, noise pollution, chemical pollutants, and anthropogenic food availability in

isolation (Grunst *et al*., 2019; Injaian, Poon and Patricelli, 2018; Injaian, Taff and Patricelli, 2018; Injaian *et al*., 2021; Raap, Pinxten and Eens, 2018; Robert *et al*., 2015; Sweet *et al*., 2022; Ziegler *et al*., 2021). Studies that do explore combined stressors have often focused on ALAN and anthropogenic noise, with findings suggesting that interactive effects can be more severe than those of individual stressors. These include amplified effects on health, behaviour, and activity patterns in bird species (Dominoni *et al.*, 2020; Ferraro, Le and Francis, 2020; Wilson *et al.*, 2021). The effects appear to be both species and habitat dependent, highlighting the complexity of ecological responses. To better simulate real world conditions, future research should adopt integrated approaches that assess the cumulative effects of multiple anthropogenic stressors such as ALAN, noise, temperature fluctuations, chemical pollutants, and food availability on wildlife health and fitness across a range of taxa and environments.

What are the long-term consequences of exposure to artificial light at night?

Most existing studies, including the studies presented in this thesis, focus on the short-term effects of ALAN on wildlife health and fitness. However, the chronic and cumulative effects of ALAN over an individual's lifespan are rarely studied and as a result they are poorly understood. This knowledge gap is particularly concerning given that some negative effects may be delayed and therefore are not detected in short-term studies. The few existing longitudinal studies that span multiple years are not individual based, limiting their ability to track cumulative effects across lifespans (Li *et al.*, 2024). True individual-based longitudinal studies are needed to investigate how persistent ALAN exposure may influence survival, reproduction, ageing, and evolutionary responses and cause potential population-level ecological consequences.

What is the best approach to mitigating the effects of artificial light at night?

With the increasing prevalence of ALAN, and the mounting evidence of its harmful effects on wildlife, the development of effective mitigation strategies is urgent. Several approaches have been tested throughout the literature, including modifying the wavelength of artificial lighting which has shown some promising results. For example, bat species tend to avoid white and green light but are more tolerant of red light (Spoelstra *et al.*, 2017). Similarly, adult great tits exposed to white light exhibited higher nocturnal activity, greater sleep debt and had a higher risk of malaria infection compared to those exposed to red or green light (Ouyang *et al.*, 2017). Lowering light intensity may also help reduce

disruptions. In great tits, increasing light intensity advanced the onset and delayed the offset of daily activity. However, effects persisted even at low intensities, raising concerns about the efficiency of this strategy alone (De Jong *et al.*, 2016).

Partial night lighting was the strategy tested in this thesis showing potential benefits, however it remains understudied and results in the literature have shown mixed results. For example, partial night lighting was shown to benefit oyster species and reduced negative effects on biological rhythms (Botté *et al.*, 2023). However, partial night lighting showed no impacts on the activity levels of several bat species (Azam *et al.*, 2015; Day *et al.*, 2015). Therefore, the success of this strategy likely depends on the species, habitat type, and timing of light reduction. Scheduled lighting systems can be dimmed or turned off automatically and can be tailored to specific locations, for example the use of stricter lighting controls in important conservation zones. Importantly, these types of systems have also been associated with significant energy savings (Erguzel *et al.*, 2017).

Despite these many options, no single mitigation approach has proven universally effective. There is a clear need for more comparative, field-based studies assessing the ecological outcomes of different strategies across various species and environments. For instance, one study showing that flying shearwaters *(Puffinus puffinus)* were less repelled by red light compared to green or blue light and were less affected by shorter durations of lighting as well as lower intensity lighting (Syposz *et al.*, 2021). More research of this kind is essential to identify context-specific solutions and inform policy implementation with evidence.

How can we address the low repeatability of oxidative stress markers?

This thesis demonstrated that oxidative stress markers often show low within-individual repeatability, which can significantly limit the statistical power of studies investigating associations with traits like telomere length. While increasing sample sizes and the number of repeated measures per individual can partially address this issue, more work is needed to improve the robustness of oxidative stress metrics. Despite their low repeatability, oxidative stress markers remain important indicators, particularly in studies of ageing and ageing-related diseases (Barnes, Fouquerel and Opresko, 2019; Chen, Hales and Ozanne, 2007; Luo *et al.*, 2020; Marasco *et al.*, 2017; Pole, Dimri and Dimri, 2016). The challenge therefore lies not in their relevance, but in the difficulty of detecting relationships when intraindividual variability is high.

It is therefore important for future studies to work towards addressing this, for example by testing the simulation outcomes shown in this thesis in real-time, examining how repeatability varies with different sampling frequencies and sample sizes. There could also be a push for studies to adopt a multi-biomarker approach, using multiple indicators of both oxidative damage and antioxidant defence across different tissue types to improve reliability of capturing oxidative status. By improving methodological consistency and study design, biologically meaningful patterns can be better detected and the use of oxidative stress as a tool for ecological health measures can be strengthened. It is worth noting that the issue of intraindividual variation is likely not unique to oxidative stress markers but may affect other labile physiological markers as well. As such, it is important for future studies to assess the within-individual repeatability of all physiological measures used in their analysis for a more accurate interpretation of the findings.

6.3.3 Scope for innovative future research

Longitudinal urban studies

There is an urgent need for long-term, individual based studies that follow wildlife throughout their lifetimes across varying levels of urbanisation. Tracking animals from hatching to adulthood could provide invaluable insights into the cumulative effects of urbanisation on wildlife health, survival, reproduction, and fitness outcomes. A global collaborative effort could enhance this by facilitating large scale tracking of wildlife across diverse regions, including understudied areas such as tropical megacities (Smit, 2021). It has been noted in ecological reviews that several fundamental questions in ecology and evolutionary biology can only be answered through long-term, individual-based data that track the life histories of recognisable individuals (Clutton-Brock and Sheldon, 2010).These long-term studies would reply on a variety of methods such as repeated field sampling through the repeated recapturing of individuals across their lifetime (Millon *et al.*, 2019; Péron *et al.*, 2010) or the use of biologging devices for remote monitoring. Biologging technologies, like GPS trackers, accelerometers and heart rate monitors could be combined on one device to allow for continuous monitoring of an individual's health, behaviour, and environmental interactions (Gould *et al.*, 2024; Wilmers *et al.*, 2015). This type of data would provide critical information on the impacts of urbanisation on different aspects of wildlife health and fitness over time as well as providing invaluable information on its role in shaping population dynamics. If connected globally, these efforts could go a

long way in bridging significant knowledge gaps and allow for understudied areas to be prioritised.

Investigating long-term impacts of artificial light at night exposure on wildlife

A key urban stressor that requires detailed, long-term individual-based studies is ALAN. While short-term studies such as the ones in this thesis have provided valuable insights into the immediate responses to ALAN, they often fail to reveal its long-term effects or population-level consequences. There is a pressing need for multi-generational studies that track the physiological and behavioural adaptations of wildlife exposed to chronic ALAN.

For example, while several studies (including this thesis) have examined the effects of ALAN exposure on telomere dynamics, results have been mixed which is potentially due to variations in light intensity, exposure duration, or life stage. To date, no study has tracked telomere loss across the entire lifespan of wild individuals exposed to ALAN, therefore we have a limited understanding of the role of ALAN in senescence and longevity of wild organisms. Although such studies are difficult to conduct in wild populations, they are feasible. For example, individuals in urban areas could be fitted with light loggers and trackers and be recaptured at various intervals across their lifespan to monitor cumulative light exposure and measure telomere attrition alongside other health and fitness markers. Technology is rapidly advancing our ability to remotely monitor populations and continuously measure aspects of an individual's state (Gould *et al.*, 2024; Wilmers *et al.*, 2015). Alternatively, controlled captive studies on model organisms could be used to manipulate the levels of ALAN and limit confounding variables. For example, muti-generational experiments on field crickets, could offer insights into potential transgenerational effects under regulated conditions (Durrant, Green and Jones, 2020).

A global urban wildlife health database

To facilitate data synthesis and the ability to detect patterns globally, a centralised database of urban wildlife health metrics should be established. This database would allow researchers across the world to contribute to and access standardised data, improving the comparability and reproducibility of ecological studies. This dataset could include: the study location and coordinates, degree and method of urbanisation measurement, species traits and life stage, type of biomarker or trait measured (Physiological, behavioural,

fitness), results and sample sizes. This resource would allow for future comprehensive meta-analysis, identification of species-specific vulnerabilities, detection of urban biodiversity hotspots, and estimation of urbanisation thresholds beyond which negative impacts on wildlife health and fitness escalate. Current examples of successful databases include WILDbase, which compiles data based on zoonotic pathogens in European urban wildlife (De Cock *et al.*, 2024) and the Global Biodiversity Information Facility which allows access to specimen data from a collection of databases on biological surveys and collections (Yesson *et al.*, 2007). There are also databases that currently exist to compile information on specific wildlife traits including HormoneBase (Vitousek *et al.*, 2018) and Amniote life-history database (Myhrvold *et al.*, 2015). Therefore, a challenge of creating a centralised database would be to integrate existing knowledge first.

Access to this type of centralised dataset would also allow for the development of simulations or predictive models that can identify which species are more vulnerable to urbanisation and why. These models could highlight key traits linked to sensitivity to urbanisation and inform targeted mitigation strategies that could prioritise high risk species. Additionally, by compiling diverse health biomarkers from different studies, the database could support the exploration of more complex multivariate relationships across health, fitness, and urbanisation, allowing us to overcome the logistical and ethical constraints of collecting multiple biomarkers that individual studies often face.

Collaboration and multidisciplinary approaches to research

One of the most effective ways to enhance sample sizes, broaden scope, and increase the ecological meaningfulness of findings is through greater collaboration and interdisciplinary research. Bridging gaps between ecological science, public policy, and urban planning is essential for implementing real-world solutions to reduce the negative impacts of urbanisation. The multi-faceted nature of urban environmental stressors requires a push towards integrative approaches (Butt and Dimitrijević, 2022; Karvonen *et al.*, 2021; Kapucu *et al.*, 2024). Collaboration between researchers specialising in ecology, chronobiology, physiology, data science, engineering, and environmental policy could allow for a more comprehensive understanding of urbanisation and allow us to design sustainable urban spaces that drive both human benefits and benefits for biodiversity, this would also allow for more effective advocation towards implementing evidence-based change.

Appendix 1 - Supplementary materials for chapter 2

Table S1.1: Health traits included in the meta-analysis along with their assigned directionality. An explanation of why each health trait was assigned the directionality is also included.

Class	Health Trait	Directionality
Physiological	Antioxidant capacity	Positive An increase in antioxidant capacity is associated with an increased level of defence against oxidative stress which would have a positive effect on health. It should be noted that an increase in antioxidant capacity could also indicate an increase in oxidative stress.
	Oxidative stress	Negative An increase in oxidative stress can lead to damage at the cellular level leading to poor effects on health
	Blood sugar	Negative An increase in blood sugar levels can have negative implications for health including hyperglycaemia and can indicate a high metabolic rate.
	Unsaturated fatty acids	Positive Unsaturated fatty acids are beneficial fats often obtained in the diet which are known to aid in many different physiological functions.
	Vitamins and carotenoids	Positive Molecules and naturally occurring pigments that are beneficial to aid in many different functions such as immune response and antioxidant defence.
	Haematological index	Positive An increase in haemoglobin and haematocrit in the blood is required for metabolic function.
	Immune response	Positive An increase in immune response signifies the organism's ability to fight infection. It has to also be noted that an increase in immune response also points to the organism being exposed to infection.

	Corticosterone concentration	Negative An increase in Corticosterone can indicate that the organism has been exposed to stress which can have negative implications for health.
	Telomere length	Positive A shorter telomere length can lead to wide range of health conditions and is also linked to shortened lifespan.
	Breathe rate	Negative Breathe rate is a measure of stress response, high stress response can lead to negative implications on health.
Morphological	Body condition	Positive A decline in body condition may mean that an organism has reduced health which can impact overall fitness and survival
	Plumage condition	Positive A decline in plumage condition may mean that an organism has reduced health can impact overall fitness and survival.
Parasitism	Endoparasites	Negative Endoparasite burden can use up host resources and damage host tissues.
	Ectoparasites	Negative Ectoparasite burden can use up the hosts resources and damage host tissues.
Disease	Avian Influenza virus	Negative Avian influenza can have many impacts on health including respiratory distress and lead to death in many cases.
	Avian Pox virus	Negative Avian pox symptoms can include tumour like growths which may hinder vision and prevent the bird from being able to eat.

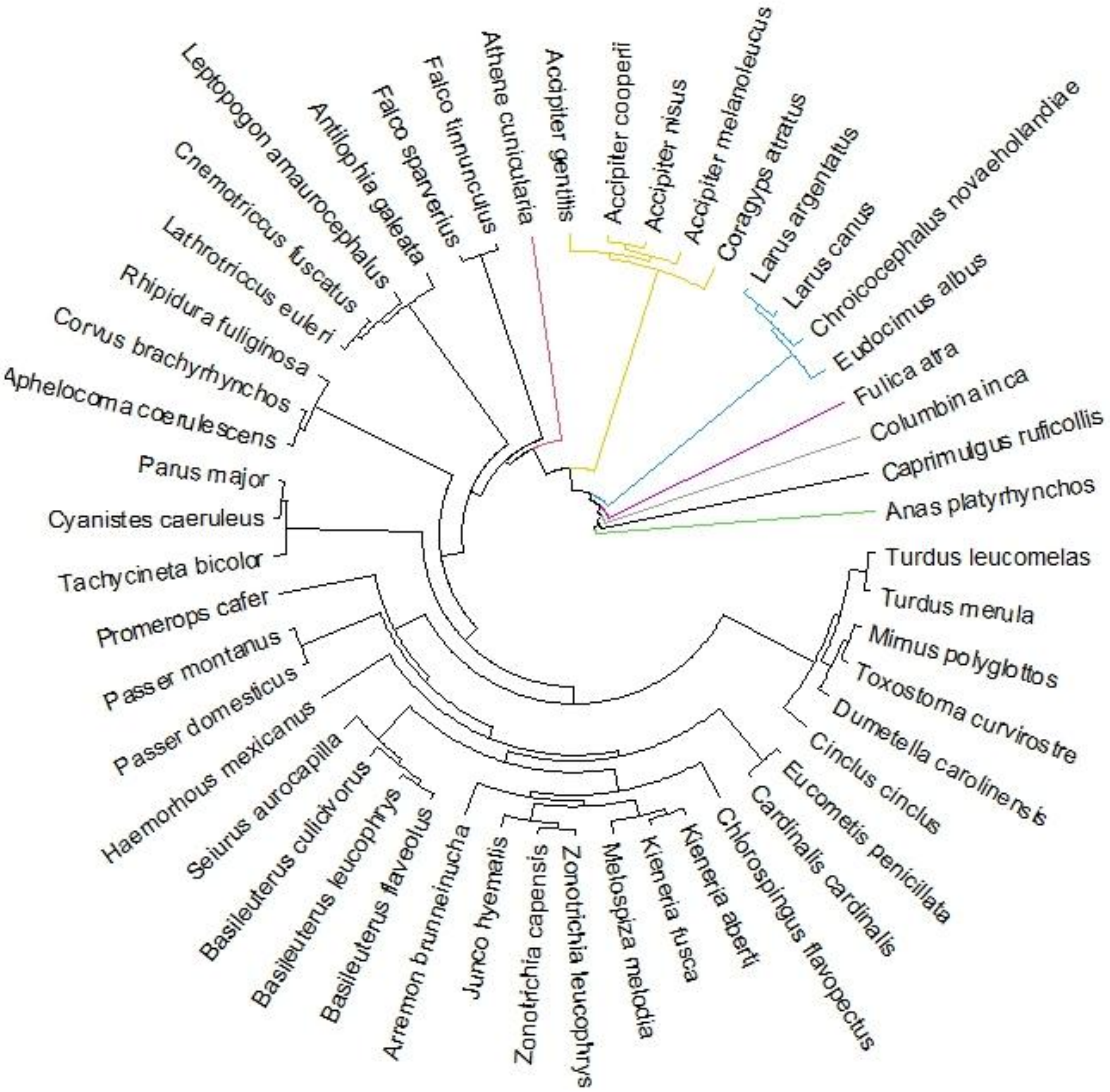

Figure S1.1. Phylogenetic tree of all species included in the meta-analysis.

Table S1.2: Levels of each moderator with a description of each category; the number of effect sizes are shown in brackets next to each category.

Moderator	Category	Description
Health Trait	Vitamins and carotenoids (11)	Measures of naturally occurring pigments that can aid in many physiological processes.
	Unsaturated fatty acids (17)	Measures of a variety of essential fatty acids that aid with normal physiological function.
	Telomere length (7)	Measures of telomere length which protect the ends of chromosomes and are linked to a wide range of diseases when shortened.
	Breathe rate (20)	Measures of breath rate which is a proxy of stress response as they will increase breath rate as they become more stressed.
	Corticosterone concentration (58)	Measures of Corticosterone which is a glucocorticoid involved in many physiological processes.
	Plumage condition (50)	Looking at the structure and colouration of feathers in birds as this will reflect their health.
	Oxidative stress (22)	An excess of free radicals in the cells that can cause tissue and cellular damage. Measures include biomarkers of oxidative stress.

	Immune response (85)	The bodies response to exposure to infection and disease. Measures include specific immune pathways.
	Haematological (44)	Looking at the health of blood, includes measures of Haemoglobin and Haematocrit where an imbalance can lead to blood diseases.
	Endoparasites (140)	Parasites that occur inside the body of the host including in the blood and gastrointestinal tract.
	Ectoparasites (8)	Parasites that occur outside of the body of the host including on the skin.
	Blood sugar (25)	Sugars that can be measured in the blood and obtained in the diet which are linked to a range of physiological processes.
	Avian pox virus (12)	A disease that causes lesions to appear over the body and can be easily spread between bird populations.
	Avian influenza virus (6)	A disease that can cause severe respiratory distress.
	Antioxidant capacity (30)	The ability to deal with oxidative stress, includes measures of specific antioxidants.
		The morphological appearance of the bird, this

	Body condition (109)	can include their body mass.
Life stage	Nestling (113) Adult (559)	The bird is less than 1 year of age. The bird is 1 year of age or over.
Primary Behaviour	Terrestrial (217) Insessorial (252) Generalist (131) Aquatic (2) Aerial (12)	Species spends most of its time on the ground. Species spends most of its time perching above ground. Spends time in different lifestyle classes. Species spends most of its time sitting on water. Species spends most of times time in flight.
Migratory Behaviour	Sedentary (567) Partial migration (32) Migratory (44)	The population does not migrate. Minority of the population migrates long distances or most will undergo short distance migrations. Majority of the population will undergo long distance migrations.
Trophic niche	Vertivore (33)	Obtains at least 60% of resources from vertebrate animals in terrestrial systems.

	Scavenger (3) Omnivore (159) Insectivore (239) Granivore (194) Frugivore (2) Aquatic predator (8) Aquatic herbivore (5)	Obtains at least 60% of resources from carrion, offal, or refuse. Species using multiple niches, within or across trophic levels, in relatively equal proportions. Obtains at least 60% of food resources from invertebrates in terrestrial systems. Obtains at least 60% of food resources from nuts or seeds. Obtains at least 60% of food from fruit. Obtains at least 60% of food resources from vertebrate and invertebrate animals in aquatic systems. Obtains at least 60% of food resources from plant materials in aquatic systems.
Maximum life span	Continuous	The average maximum lifespan of a species.
Latitude	Continuous	A coordinate that specifies the north-south position of a point on the surface of the earth.

Table S1.3: The Akaike Information Criterion for each meta-analytic mixed-effect models looking with urban score as a moderator at each buffer radius where urban score was extracted around the coordinates used for each location.

Buffer Radius	AIC value
250	2708.402
500	2707.491
750	2707.064
1000	**2706.99**
1250	2707.606
1500	2709.117
1750	2709.478
2000	2709.249
2250	2709.016
2500	2709.165
2750	2709.202
3000	2709.297
3250	2709.338
3500	2709.442
3750	2709.695
4000	2709.956
4250	2715.631
4500	2715.324
4750	2715.089
5000	2715.454

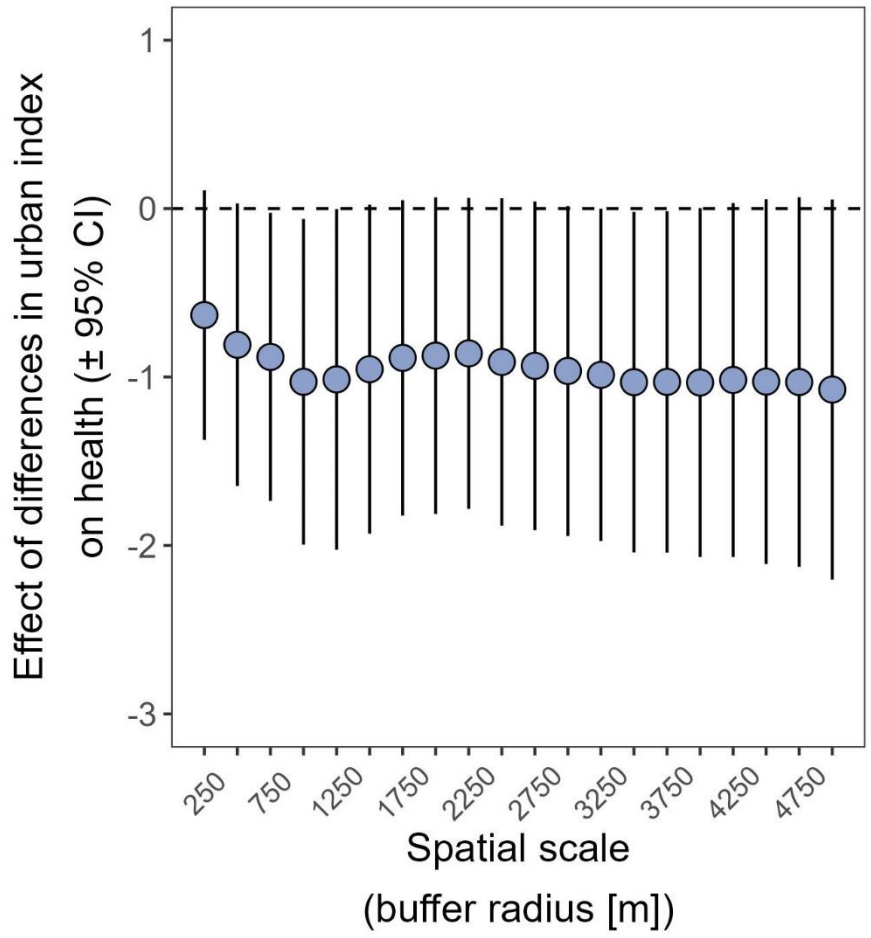

Figure S1.2: The figure above shows the effect of differences in urban score on health at different spatial scales (the area of the circular area centred at each study location and over which urban score was calculated. The points represent mean model estimates with confidence intervals.

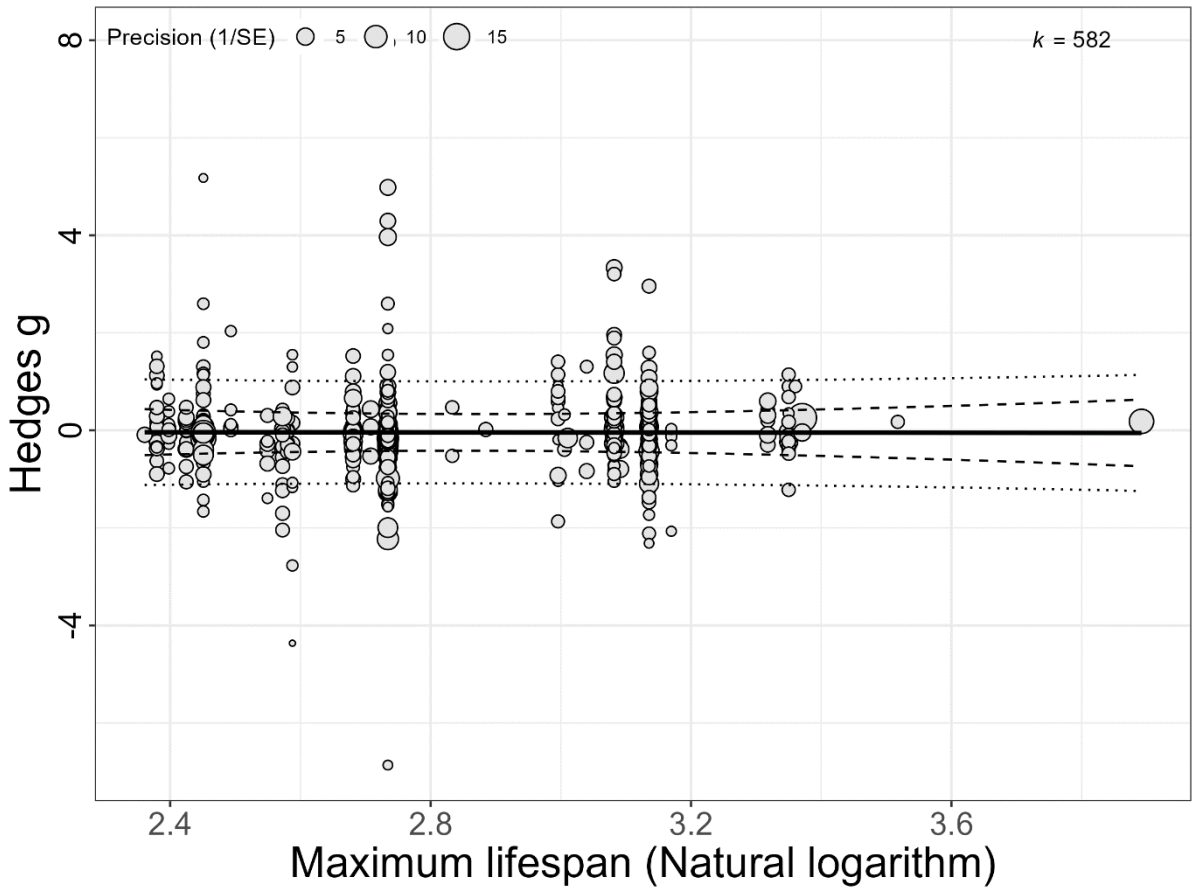

Figure S1.3: Maximum species lifespan does not influence the relationship between urbanisation and avian health. The individual effect sizes are scaled by their precision which is (1/SE) the more precise the effect size is the larger the circle. The solid line represents the model estimate with the dashed lines representing the 95% confidence intervals and the dotted lines representing the 95% prediction intervals. K shows the total number of effect sizes where maximum lifepan was known.

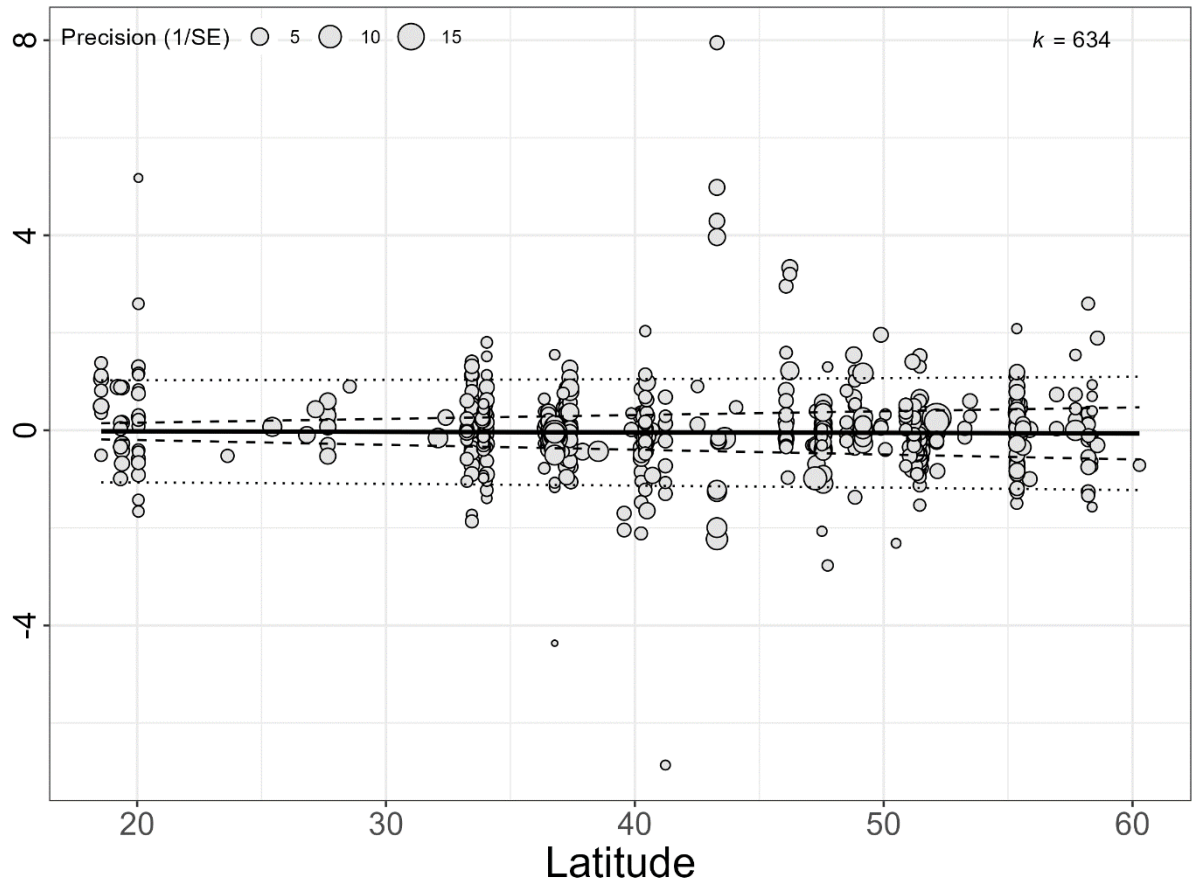

Figure S1.4: Latitude does not influence the relationship between urbanisation and avian health. The individual effect sizes are scaled by their precision which is (1/SE) the more precise the effect size is the larger the circle. The solid line represents the model estimate with the dashed lines representing the 95% confidence intervals and the dotted lines representing the 95% prediction intervals. K shows the total number of effect sizes.

Table S1.4: Summary of the meta-analytic mixed-effect models. Bold estimates indicate confidence intervals (CI) that do not overlap zero. k is the number of effect sizes per level, and n is the number of studies. For the "intercept-only model", i.e., meta-analytic mean, the associated heterogeneity (I^2) explained for each random factor and the whole model is shown.

	Estimate [95%CI]	k	n
Intercept only model *I2 total=89.03* *I2 Obs ID=66.92* *I2 Study=18.28* *I2 Phylogeny=4.43*	0.0276[-0.119,0.254]	644	112
Health trait			
Antioxidant capacity	-0.063[-0.612,0.486]	30	8
Avian Influenza virus	0.2031[-0.67,1.096]	6	2
Avian Pox virus	-0.117[-1.087,0.852]	12	1
Blood sugar	-0.227[-0.871,0.418]	25	10
Body condition	-0.279[-0.702,0.143]	109	41
Ectoparasites	**1.728[0.963,2.493]**	8	3
Endoparasites	0.126[-0.406,0.659]	140	19
Haematological index	-0.202[-0.854,0.451]	44	15
Immune response	0.097[-0.411,0.605]	85	16
Oxidative stress	-0.084[-0.719, 0.552]	22	7

Plumage condition	0.037[-0.762,0.835]	50	7
Corticosterone conc	-0.042[-0.442,0.368]	58	26
Breathe rate	-0.318[-1.044,0.0407]	20	3
Telomere length	-0.137[-0.824,0.549]	7	3
Unsaturated fatty acids	-0.058[-0.893, 0.777]	17	2
Vitamins and carotenoids	0.057[-0.652, 0.766]	11	3
Migratory Behaviour			
Partial migration	-0.107[-0.656, 0.442]	32	12
Sedentary	0.037[-0.532, 0.459]	567	88
Trophic behaviour			
Carnivore	-0.006[-1.163, 1.15]	28	6
Frugivore	0.37[-1.073, 1.813]	2	1
Granivore	0.133[-0.884, 1.151]	194	29
Insectivore	0.186[-0.877, 1.249]	239	32
Omnivore	0.177[-0.895, 1.25]	159	32
Scavenger	-0.285[-2.026, 1.456]	3	1
Aquatic predator	0.507[-0.629, 1.643]	8	4
Primary behaviour			
Aquatic	0.443[-0.988, 1.874]	2	1
Generalist	-0.112[-0.87, 0.646]	131	26
Insessorial	-0.01[-0.874, 0.856]	252	45

Terrestrial	-0.139[-1.062, 0.783]	217	36
Lifestage			
Adult	0.191[-1.079, 1.46]	559	90
Nestling	-0.132[-1.418, 1.154]	113	30
Latitude	-0.005[-0.02, 0.01]	644	112
Lifespan	0.011[-0.565, 0.543]	592	99

Table S1.5: Summary of the meta-analytic mixed-effect models for data subset by "nestling" life stage. Bold estimates indicate confidence intervals (CI) that do not overlap zero. k is the number of effect sizes per level, and n is the number of studies.

	Estimate [95%CI]	K	N
Health trait *I2 total=79.81* *I2 Study ID=62.92* *I2 Phylogeny=16.89*			
Antioxidant capacity	-0.280[-0.894, 0.333]	7	3
Avian Influenza virus	-0.093[-0.990, 0.804]	1	1
Blood sugar	-0.196[-0.69, 0.297]	18	5
Body condition	**-0.499[-0.901, -0.097]**	24	10
Endoparasites	-0.301[-0.811, 0.209]	8	4
Haematological index	-0.235[-0.795, 0.324]	33	7
Immune response	0.0785[-0.534, 0.691]	8	3
Oxidative stress	0.151[-0.12, 0.923]	2	2
Steroid hormones	-0.387[-0.917, 0.142]	5	4
Telomere length	-0.275[-1.163, 0.612]	1	1

Table S1.6: Summary of the meta-analytic mixed-effect models for data subset by "adult" life stage. Bold estimates indicate confidence intervals (CI) that do not overlap zero. k is the number of effect sizes per level, and n is the number of studies.

	Estimate [95%CI]	K	N
Health trait			
I2 total=79.81			
I2 Study ID=62.92			
I2 Phylogeny=16.89			
Antioxidant capacity	0.005[-0.6, 0.610]	23	5
Avian Influenza virus	0.214[-0.701, 1.129]	5	1
Blood sugar	-0.53[-1.064, 0.005]	7	6
Body condition	-0.087[-0.466, 0.292]	85	33
Ectoparasites	**1.728[0.972, 2.484]**	8	3
Endoparasites	0.127[-0.435, 0.688]	122	15
Haematological index	-0.048[-0.549, 0.453]	11	8
Immune response	0.098[-0.419, 0.616]	77	14
Oxidative stress	-0.102[-0.756, 0.552]	20	5
Plumage condition	0.031[-0.752, 0.826]	50	7
Corticosterone concentration	0.013[-0.403, 0.43]	53	22
Breathe rate	-0.318[-1.034, 0.398]	20	3
Telomere length	-0.071[-0.846, 0.703]	6	2
Unsaturated fatty acids	-0.058[-0.884, 0.768]	17	2

Vitamins and carotenoids	0.057[-0.647, 0.761]	11	3

Table S1.7: Summary of the meta-analytic mixed-effect models for data subset by "nestling" life stage and "body condition" health biomarker. Bold estimates indicate confidence intervals (CI) that do not overlap zero. k is the number of effect sizes per level, and n is the number of studies.

	Estimate [95%CI]	K	N
Trophic niche			
Aquatic predator	-0.091[-2.421, 2.238]	1	1
Carnivore	-0.308[-2.469, 1.853]	5	2
Granivore	-0.946[-3.172, 1.28]	3	2
Insectivore	-0.466[-2.635, 1.703]	14	4
Omnivore	-0.141[-2.435, 2.153]	1	1
Primary behaviour			
Aerial	**-2.070[-3.866, -0.274]**	1	1
Generalist	-0.515[-0.804, 0.501]	5	2
Insessorial	-0.449[-0.914, 0.016]	15	5
Terrestrial	**-0.946[-1.685, -0.207]**	3	2

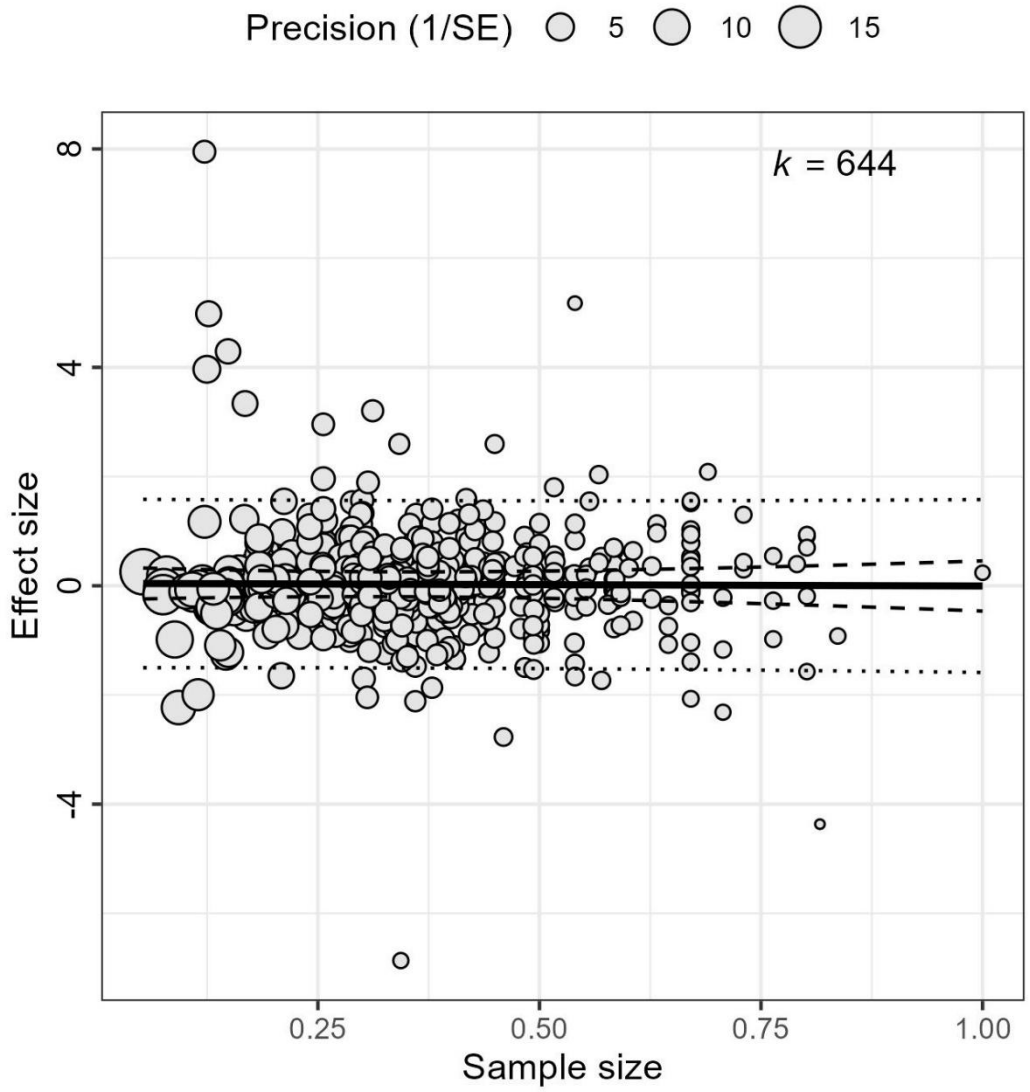

Figure S1.5: This bubble plot shows the impacts of small study size on the size of the effect found in the study using the full dataset which helps us understand is publication bias is present. The sample size of the study which has been transformed using the square root of the inverse of the sample size can be seen on the x asis with the effect size on the y axis. The individual effect sizes are scaled by their precision. The solid line represents the model estimate with the dashed line representing the 95% confidence intervals and the dotted lines representing the 95% prediction intervals.

Appendix 2 - Supplementary materials for chapter 3

Table S2.1: Number of control and experimental boxes at each study location along with the coordinates of each location.

Site name	Location		No of experimental boxes	No of control boxes
	Latitude	Longitude		
Cashel	56.1093	-4.5779	3	2
Drymen Forest	56.0728	-4.4526	1	2
Killearn Forest	56.0410	-4.3881	2	1
Strathblane Forest	55.9887	-43881	1	1
Mugdock Park	55.9624	-4.3179	1	2
Sallochy	56.1238	-4.6008	3	4
SCENE	56.1299	-4.6171	3	3

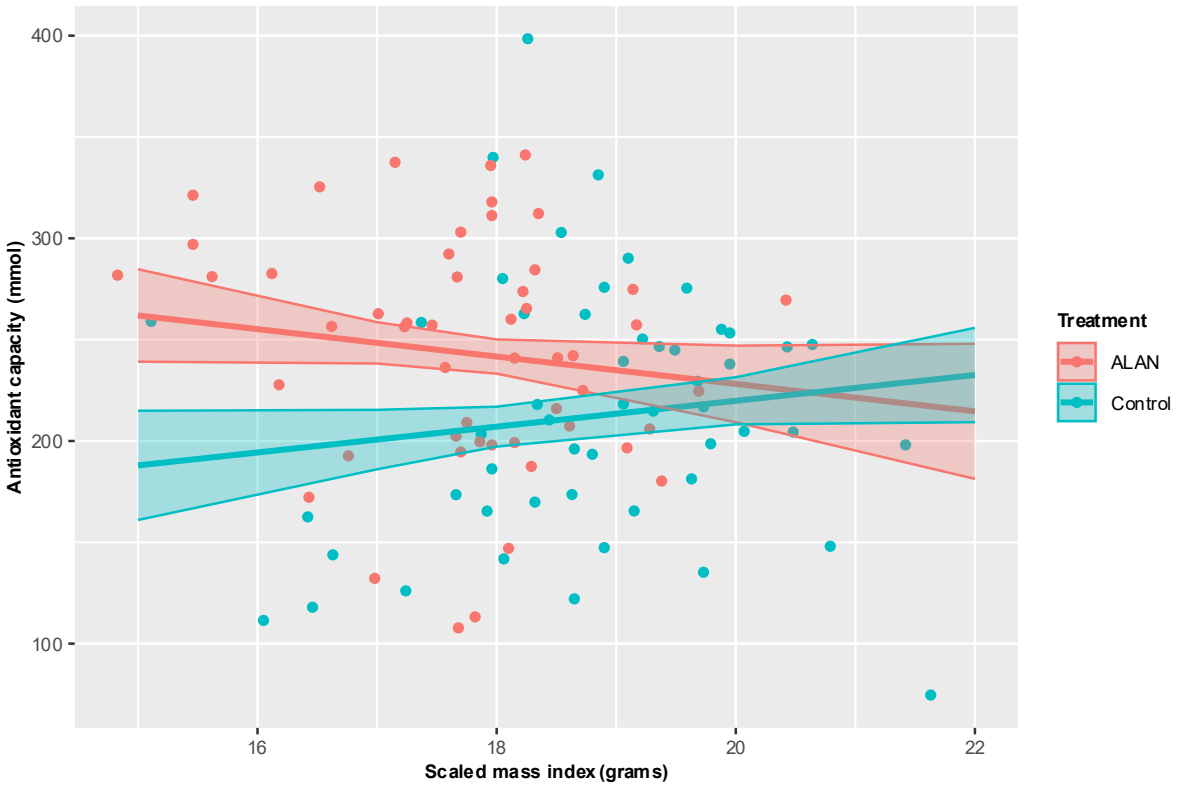

Figure S2.1: OXY affected by SMI and treatment group. This effect plot shows the relationship between antioxidant capacity of plasma (OXY) and scaled mass index (SMI) in the different treatment groups. The raw data points (blue represent control group and red represents the ALAN group) are shown along with solid regression lines (blue represents the control group and red represents the ALAN group) which was calculated using the predicted values from the linear mixed-effects model. The shaded ribbon around regression line shows the 95% confidence intervals.

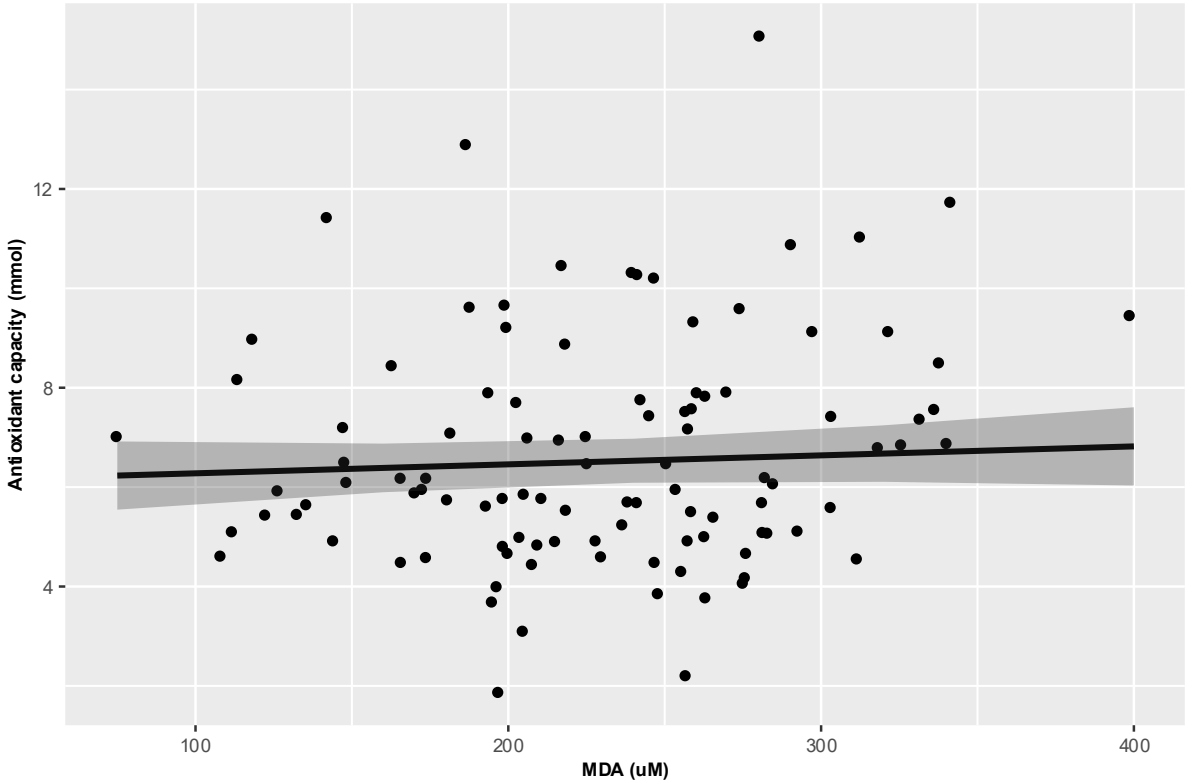

Figure S2.2: MDA levels not dependent on OXY levels. The scatterplot shows the relationship between MDA levels and OXY across all individuals. The raw data points are shown along with a solid regression line which was calculated using the predicted values from the linear mixed-effects model. The shadow around the regression line shows the 95% confidence intervals calculated for the predicted values.

Table S2.2: The results of the post hoc analysis showing the pairwise comparisons of treatment and sex and their impact on MDA levels. P-values were adjusted for multiple comparisons using Tukey's Honest Significant Difference method.

	Est	SE	P	Ci low	Ci Upp
Control F – ALAN F	1.101	0.755	0.471	-0.910	3.11
ALAN F – ALAN M	-0.193	0.610	0.989	-1.791	1.40
ALAN F – Control M	0.528	0.803	0.912	-1.600	2.66
Control F – ALAN M	0.907	0.734	0.608	-1.052	2.87
Control F – Control M	1.629	0.656	0.069	-0.088	3.35
Control M – ALAN M	-0.721	0.783	0.793	-2.799	1.36

Appendix 3: Supplementary materials for chapter 4.

Table S3.1: The output of the linear mixed-effects model testing the effect of fixed effects on the circadian variation of glucose with the values from likelihood ratio tests. The output shows the model estimate, standard error, 95% confidence intervals, degrees of freedom and P values. The fixed effects can be seen in bold, and any factor levels are shown in italics. A SE = Standard Error, CI = Confidence Interval. Significant P values are indicated by *.

Fixed effect	Estimate	SE[A]	95% CI[A]	χ^2	df	p-value
Intercept	16	7.529	1.5, 31			
Weight	-0.06	0.277	-0.61, 0.48	0.08	1	0.774
Treatment						
DARK	—	—	—			
FLAN	-0.23	2.312	-4.8, 4.3			
PLAN	2.9	2.486	-2.0, 7.7			
Sex						
Female	—	—	—			
Male	2.0	2.505	-2.9, 6.9			
Time of day						
1am	—	—	—			
1pm	-3.5	2.232	-7.9, 0.84			
6am	-1.4	2.509	-6.3, 3.5			
8pm	3.5	2.611	-1.7, 8.6			
Group size	-1.0	0.986	-2.9, 0.92	1.61	1	0.205
Treatment * Sex * Time of day				31.90	17	**0.015***
*DARK * Female * 1am*	2.5	4.847	-7.0, 12			
*FLAN * Female * 1am*	8.1	4.849	-1.4, 18			
*PLAN * Female * 1am*	3.2	3.504	-3.6, 10			
*DARK * Male * 1am*	-1.4	3.961	-9.2, 6.4			
*FLAN * Male * 1am*	4.9	3.516	-2.0, 12			
*DARK * Female * 1pm*	4.7	4.842	-4.8, 14			
*FLAN * Female * 1pm*	8.2	4.973	-1.5, 18			

*PLAN * Female * 1pm*	3.5	3.582	-3.5, 11			
*DARK * Male * 1pm*	-0.16	3.964	-7.9, 7.6			
*FLAN * Male * 1pm*	6.6	3.508	-0.27, 13			
*DARK * Female * 6am*	0.08	4.273	-8.3, 8.5			
*FLAN * Female * 6am*	3.5	4.071	-4.4, 12			
*PLAN * Female * 6am*	-1.6	2.527	-6.6, 3.3			
*DARK * Male * 6am*	5.1	2.526	0.11, 10			
*FLAN * Male * 6am*	2.9	2.475	-2.0, 7.7			
*DARK * Female * 8pm*	3.0	3.396	-3.6, 9.7			
*FLAN * Female * 8pm*	2.8	3.146	-3.4, 9.0			
[A] SE = Standard Error, CI = Confidence Interval						

Table S3.2: The output of the post hoc analysis for pairwise comparison between the treatment groups at each sex level and time of day level when investigating the impact of treatment group, sex and time of day on the circadian variation of glucose. P-values were adjusted for multiple comparisons using Tukey's Honestly Significant Difference method.

Contrast	Sex	Timepoint	Est	SE	Cl.lower	Cl.upper	P value
DARK-FLAN	Female	1pm	-3.336	2.62	-9.68	3.010	0.417
DARK-PLAN	Female	1pm	-1.694	2.50	-7.76	4.367	0.777
FLAN-PLAN	Female	1pm	1.642	2.81	-5.16	8.448	0.829
DARK-FLAN	**Male**	**1pm**	**-6.535**	**2.59**	**-12.85**	**-0.224**	**0.041**
DARK-PLAN	Male	1pm	-3.013	2.78	-9.76	3.738	0.528
FLAN-PLAN	Male	1pm	3.522	2.56	-2.70	9.748	0.363
DARK-FLAN	Female	6am	-3.215	2.46	-9.21	2.782	0.400
DARK-PLAN	Female	6am	-1.164	2.32	-6.79	4.461	0.870
FLAN-PLAN	Female	6am	2.051	2.43	-3.87	7.967	0.678
DARK-PLAN	Male	6am	2.401	2.18	-2.90	7.700	0.519
DARK-PLAN	Male	6am	2.208	2.37	-3.56	7.980	0.623
FLAN-PLAN	Male	6am	-0.193	2.23	-5.63	5.241	0.996
DARK-FLAN	**Female**	**1am**	**-5.364**	**2.35**	**-11.08**	**0.347**	**0.07**
DARK-PLAN	Female	1am	-3.572	2.37	-9.35	2.210	0.299
FLAN-PLAN	Female	1am	1.792	2.44	-4.14	7.726	0.744
DARK-FLAN	Male	1am	-6.060	2.59	-12.37	0.251	0.062

DARK-PLAN	Male	1am	-4.250	2.77	-10.99	2.492	0.286
FLAN-PLAN	Male	1am	1.810	2.58	-4.45	8.075	0.763
DARK-FLAN	Female	8pm	0.469	2.44	-5.48	6.419	0.98
DARK-PLAN	Female	8pm	0.183	2.31	-5.44	5.801	0.997
FLAN-PLAN	Female	8pm	-0.287	2.42	-6.19	5.620	0.992
DARK-FLAN	Male	8pm	0.234	2.32	-5.39	5.857	0.994
DARK-PLAN	Male	8pm	-2.854	2.49	-8.91	3.204	0.492
FLAN-PLAN	Male	8pm	-3.088	2.50	-9.15	2.970	0.438

Table S3.3: The output of the model testing the effect of fixed effects on the glucose levels with the values from likelihood ratio tests. The output shows the model estimate, standard error, 95% confidence intervals, degrees of freedom and P values. The fixed effects can be seen in bold, and any factor levels are shown in italics. A SE = Standard Error, CI = Confidence Interval. Significant P values are indicated by *.

Fixed effect	Estimate	SE[A]	95% CI[A]	χ^2	df	p-value
Intercept	14	4.850	4.8, 24			
Weight	-0.04	0.261	-0.55, 0.47	0.00	1	0.990
Treatment				1.68	2	0.431
DARK	—	—	—			
FLAN	1.4	1.116	-0.80, 3.6			
PLAN	0.76	1.012	-1.2, 2.7			
Sex				0.00	1	0.952
Female	—	—	—			
Male	0.04	0.915	-1.8, 1.8			
Experiment timepoint				0.07	1	0.797
End	—	—	—			
Start	-0.14	0.632	-1.4, 1.1			
Group size	0.35	0.585	-0.80, 1.5	0.45	1	0.503
[A] SE = Standard Error, CI = Confidence Interval						

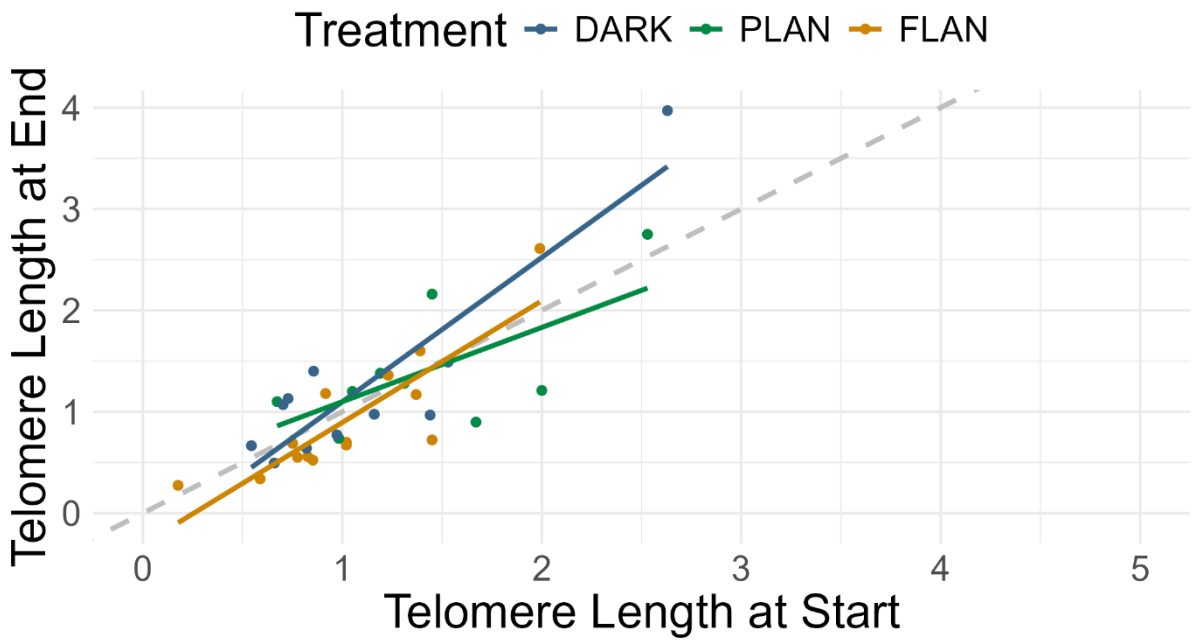

Figure S3.1: Telomere length for each individual at the start of the experiment plotted against telomere length at the end of the experiment. The grey dotted line plotted through zero represents y = x. The different coloured regression lines represent the different treatment groups (DARK, PLAN, FLAN). Telomere loss is shown by the amount of data points that lie beneath the y = x line.

Table S3.4: The output of the linear mixed-effects model testing the effect of fixed effects on MDA levels with the values from likelihood ratio tests. The output shows the model estimate, standard error, 95% confidence intervals, degrees of freedom and P values. The fixed effects can be seen in bold, and any factor levels are shown in italics. A SE = Standard Error, CI = Confidence Interval. Significant P values are indicated by *.

Fixed effect	Estimate	SE[A]	95% CI[A]	χ^2	df	p-value
Intercept	-0.89	4.024	-8.8, 7.0			
Weight	0.39	0.246	-0.09, 0.87	2.72	1	0.099
Treatment				0.23	2	0.891
DARK	—	—	—			
FLAN	0.32	0.949	-1.5, 2.2			
PLAN	-0.11	0.963	-2.0, 1.8			
Sex				5.49	1	**0.019***
Female	—	—	—			
Male	-1.7	0.743	-3.2, -0.24			
Experiment timepoint				0.93	1	0.335
End	—	—	—			
Start	0.68	0.734	-0.76, 2.1			

[A] SE = Standard Error, CI = Confidence Interval

Table S3.5: The output of the linear mixed-effects model testing the effect of fixed effects on OXY levels with the values from likelihood ratio tests. The output shows the model estimate, standard error, 95% confidence intervals, degrees of freedom and P values. The fixed effects can be seen in bold, and any factor levels are shown in italics. A SE = Standard Error, CI = Confidence Interval. Significant P values are indicated by *.

Fixed effect	Estimate	SE[A]	95% CI[A]	χ^2	df	p-value
Intercept	37	92.037	-144, 217			
Weight	13	4.917	3.4, 23	6.99	1	**0.008***
Treatment				1.08	2	0.583
DARK	—	—	—			
FLAN	-13	21.700	-56, 29			
PLAN	-20	20.499	-60, 21			
Sex				0.10	1	0.746
Female	—	—	—			
Male	-5.2	18.499	-41, 31			
Experiment timepoint				0.71	1	0.400
End	—	—	—			
Start	-14	14.391	-42, 14			
Group_size	3.8	11.754	-19, 27	0.15	1	0.694

[A] SE = Standard Error, CI = Confidence Interval

Appendix 4 – Supplementary materials for chapter 5

Table S4.1: List of oxidative stress biomarkers used in the meta-analysis alongside the type of damage or defence; the corresponding sample sizes are also listed.

Measure type – full name	Acronym	Overall Biomarker	Type of damage or defence	Sample size
8-hydroxyl-2'-deoxyguanosine	8OHdG	Oxidative damage	DNA damage	4
Advanced Oxidation Protein Products	AOPP	Oxidative damage	Protein damage	2
Malondialdehyde	MDA	Oxidative damage	Lipid peroxidation	12
Peroxidation index	Peroxidation index	Oxidative damage	Lipid peroxidation	2
Protein carbonyls	Protein carbonyls	Oxidative damage	Protein damage	6
Reactive Oxygen Metabolites	ROMs	Oxidative damage	Widespread damage	19
Thiobarbituric aid reactive substances	TBARs	Oxidative damage	Lipid peroxidation	5
Total oxidative burst responsiveness	TOBR	Oxidative damage	Widespread damage	2
Catalase	Catalase	Antioxidant defence	Enzymatic defence	2
Glutathione peroxidase	GPX	Antioxidant defence	Enzymatic defence	8
Reduced glutathione	GSH	Antioxidant defence	Non-enzymatic defence	2

Total antioxidant capacity of plasma	OXY	Antioxidant defence	Non-enzymatic defence	30
Plasma carotenoids	Plasma carotenoids	Antioxidant defence	Non-enzymatic defence	4
Superoxide dimutase	SOD	Antioxidant defence	Enzymatic defence	13
Thiols	Thiols	Antioxidant defence	Non-enzymatic defence	2
Uric acid	UA	Antioxidant defence	Non-enzymatic defence	8
Vitamin E	Vitamin E	Antioxidant defence	Non-enzymatic defence	2

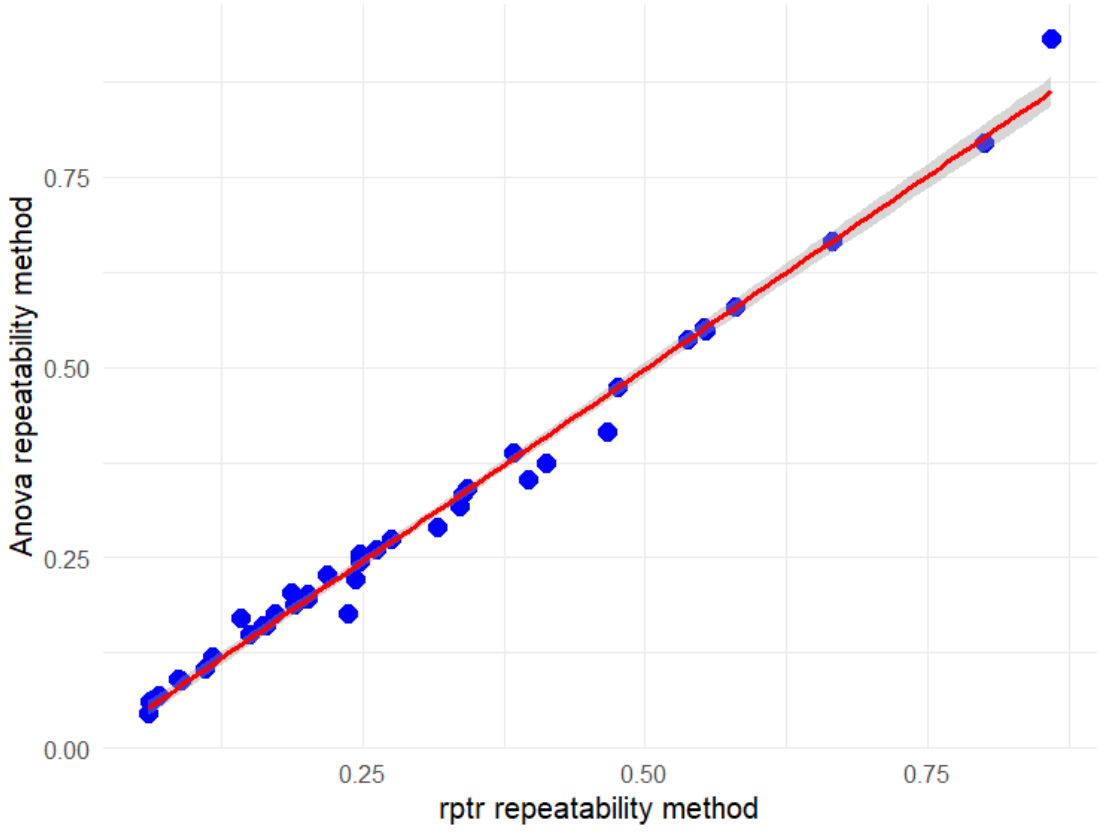

Figure S4.1: Correlation between the LMM and ANOVA methods of calculating repeatability. The blue points show the repeatability values and the red regression lines represents the correlation between the two methods, the shaded area around the line represents the 95% confidence intervals.

Table S4.2: List of the study species used in the meta-analysis alongside the type of environment the study took place in alongside the corresponding sample size.

Study species Latin name	Common name	Taxa	Environment	Sample size
Anas acuta	Northern Pintail	Aves	Captive	2
Anas platyrhynchos	Mallard Duck	Aves	Captive	2
Aptenodytes patagonicus	King Penguin	Aves	Wild Captive	4
Bos Taurus	Cattle	Mammal	Livestock	11
Branta bernicla	Light bellied Brent Geese	Aves	Wild	8
Bubalus bubalus	Buffalo	Mammal	Livestock	8
Equus caballus	Horse	Mammal	Livestock	4
Ficedula albicollis	Collared Flycatcher	Aves	Wild	8
Lamprotornis nitens	Glossy Starling	Aves	Wild	2
Lamprotornis superbus	Superb Starling	Aves	Wild	4
Larus michahellis	Yellow-legged Gull	Aves	Wild	6
Mungos mungo	Banded Mongoose	Mammal	Wild	1
Oenanthe oenanthe	Wheatear	Aves	Wild Captive	6
Ovis aries	Sheep	Mammal	Livestock	8
Parus major	Great tit	Aves	Wild	10
Passer domesticus	House Sparrow	Aves	Wild Captive	2
Phalacrocorax aristotelis	Shag	Aves	Wild	4
Plectrophenax nivalis	Snow Bunting	Aves	Wild	2
Plocepasser mahali	White-browed Sparrow Weaver	Aves	Wild	8
Taeniopygia castanotis	Zebra Finches	Aves	Captive	15
Turdus merula	Blackbird	Aves	Wild Captive	6
Urocitellus columbianus	Columbian Ground Squirrel	Mammal	Wild	2

Table S4.3: The model outputs for all moderators used on the full dataset. Bold estimates indicate confidence intervals (CI) that do not overlap zero. k is the number of effect sizes per level, and n is the number of studies. The values in the table have been back transformed from Fisher's Z to ICC.

	Estimate [95%CI]	**k**	**n**
Intercept only model	**0.164 [0.093, 0.233]**	123	22
I2 total= 57.399			
I2 Obs ID= 51.295			
I2 Study= 6.104			
Log mean time between measures (days)	0.022 [0.006, 0.038]	65	21
Log mean number of samples per individual	**0.097 [0.064, 0.130]**	68	22
Taxa			
Aves	**0.155 [0.062, 0.245]**	89	15
Mammalia	**0.212 [0.058, 0.356]**	34	7
Environment			
Livestock	**0.391 [0.178, 0.569]**	23	4
Wild	0.119 [-0.003, 0.239]	63	11
Captive	0.158 [-0.07, 0.371]	19	3

Wild captive	0.165 [-0.052, 0.367]	18	4
Overall Biomarker			
Antioxidant defence	**0.171 [0.082, 0.258]**	71	20
Oxidative damage	**0.156 [0.056, 0.253]**	52	20
Study Type			
Manipulation	**0.208 [0.091, 0.319]**	40	12
Non-manipulation	**0.146 [0.053, 0.230]**	83	18
Sex			
Female	0.167 [0.065, 0.266]	62	16
Male	0.154 [0.03, 0.273]	37	12

Table S4.4: The model output for the subgroup analysis on both "Oxidative damage" and "Antioxidant defence" subgroups. Bold estimates indicate confidence intervals (CI) that do not overlap zero. k is the number of effect sizes per level, and n is the number of studies. The values in the table have been back transformed from Fisher's Z to ICC.

	Estimate [95%CI]	**k**	**n**
Type of oxidative damage			
DNA damage	0.135 [-0.224, 0.461]	4	2
Protein damage	-0.068 [-0.346, 0.220]	8	4
Lipid peroxidation	**0.368 [0.182, 0.529]**	19	10
Widespread	0.059 [-0.137, 0.252]	21	10
Type of antioxidant defence			
Enzymatic	**0.204 [0.081, 0.320]**	23	9
Non-enzymatic	**0.136 [0.042, 0.228]**	48	20

Table S4.5: The model output for the subgroup analysis on both "Oxidative damage" and "Antioxidant defence" subgroups. Bold estimates indicate confidence intervals (CI) that do not overlap zero. k is the number of effect sizes per level, and n is the number of studies. The values in the table have been back transformed from Fisher's Z to ICC.

	Estimate [95%CI]	**k**	**n**
Type of oxidative damage			
DNA damage	0.135 [-0.224, 0.461]	4	2
Protein damage	-0.068 [-0.346, 0.220]	8	4
Lipid peroxidation	**0.368 [0.182, 0.529]**	19	10
Widespread	0.059 [-0.137, 0.252]	21	10
Type of antioxidant defence			
Enzymatic	**0.204 [0.081, 0.320]**	23	9
Non-enzymatic	**0.136 [0.042, 0.228]**	48	20

Table S4.6: The model output for Pearson's correlation between paired oxidative stress biomarkers. Bold estimates indicate confidence intervals (CI) that do not overlap zero. k is the number of effect sizes per level, and n is the number of studies. The values in the table have been back transformed from Fisher's Z to Pearson's correlation.

	Estimate [95%CI]	**k**	**n**
Intercept only model ***I2 total= 87.298*** ***I2 Obs ID= 73.026*** ***I2 Study= 14.272***	**0.157 [0.046, 0.264]**	24	14
Oxidative stress pair			
Protein Carbonyls – SOD	-0.088 [-0.338, 0.173]	3	3
dROMs-OXY	**0.250 [0.091, 0.396]**	9	8
MDA-OXY	0.108 [-0.153, 0.355]	4	4
MDA-SOD	0.181 [-0.105, 0.439]	3	3
OXY-SOD	0.126 [-0.095, 0.335]	5	5

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