# Clocks in the wild: biological rhythms of great tits and the environment

**Robyn Jade Womack**

BSc.

Submitted in fulfilment of the requirements of the Degree of Doctor of Philosophy

Institute of Biodiversity, Animal Health and Comparative Medicine

College of Medical, Veterinary and Life Sciences

University of Glasgow

May 2020

## Abstract

Biological clocks play a fundamental role in the physiological and behavioural processes of organisms. Internal timekeepers evolved to anticipate environmental changes, the most important of these being the geophysical light-dark cycle, to coordinate external changes with the timing of internal processes. Research into functions of the biological clock during captive studies has provided valuable insight into mechanisms by which clocks function, and how small environmental changes can affect the clock and its outputs. However, biological clocks have so far been understudied in ecology.

In this thesis, this gap in knowledge was addressed by placing studies of chronobiology into the context of the natural environment. A model species in avian ecology, the great tit (*Parus major*) was used to investigate biological rhythms in the wild at three levels; behaviour, transcripts and life histories. This thesis investigated how features of the natural environment shapes rhythms of behaviour and physiology in a wild animal, using experimental and observational approaches.

Differences in timing of individual rhythms, or chronotype, may provide wild animals with different consequences for fitness. In this thesis, individual behavioural rhythms of incubating great tits were quantified for birds in city and forest environments. There were strong effects of both the number of days to hatching and site on timing of incubation activities, where city birds rose earlier, and stayed out later, than forest birds. Maternal chronotype was then linked to fitness traits.

City birds face a number of new challenges in the urban habitat. The impacts of one feature of the urban habitat, artificial light at night, was tested using a forest nest box system. Nestling great tits were experimentally exposed to low-level artificial light at night, and aspects of condition and clock and immune gene transcripts were compared for nestlings under light at night and dark-night control. Nestlings under light at night treatment weighed less than control birds, and suppressive effects of light at night treatment were found for genes involved in the core pathways of the circadian clock and immune system. Time of day differences were also observed in transcript levels of genes.

Parasitic infections can cause consequences for fitness and reproductive success of wild birds. In this study, effects of infection with avian malaria parasites on nestling condition and immune system were investigated, at city and forest sites. The prevalence of *Leucocytozoon* parasites was higher at forest sites than city sites and increased with the

season. Infection had no suppressive effects on immune genes of nestlings, and no negative effects on condition were found.

In mammals, malaria is otherwise known as the “circadian disease” due to rhythmic development of parasites during their life cycle. In this study, host-parasite interactions with avian malaria parasites were investigated in the context of biological rhythms in wild great tits. Transcript levels of nestlings were determined by field sampling across a temporal profile and linked to infections with *Leucocytozoon* parasites. *Leucocytozoon* infection reduced overall transcript levels for circadian clock and immune gene targets, but did not alter the timing of expression.

This study ultimately demonstrated the importance of biological clocks for the ecology of great tits and provided important advances for studies of clocks in the wild.

# Table of Contents

# List of Figures

# List of Tables

## Acknowledgements

I am indebted to many people for their help during this project, whom I must thank. Firstly, I thank my supervisors Barbara Helm, Jane Robinson, Francesco Baldini and Peter O'Shaughnessy for their endless support and advice from the beginning to the end of the project, and for all the great lunches. My advisors, Michelle Bellingham and Dorothy McKeegan for their advice and input. I also thank Simon Babayan, Barbara Mable, Kathryn Elmer, Dan Haydon, Davide Dominoni and Luke Powell for all help during the project.

Secondly, I thank Christian Delles, Karine Pinel, Lesley Graham for their help in the use of the QuantiGene® Plex assay, and the Luminex® MAGPIX machine. Natascha Brinskelle-Schmal from ThermoFisher, and Margaret Mills, for their patience in giving us technical support during optimisation of the QuantiGene® Plex assay!

I thank the amazing technicians in IBAHCM: Kate Griffiths, Elizabeth Kilbride, Winnie Boner and Ana Monteiro, for patiently answering all my questions and for help with processing samples in the lab. I thank Nosrat Mirzai and the MVLS Bioelectronics team for building LED nest boxes used in this project, and Rona Brennan and Colin Adams for welcoming me at SCENE. I thank honours student Gillian O'Flaherty for the molecular sexing of the birds, and Barbara Mable for supervising this work. Pablo Capilla-Lasheras for developing the incR package used in this thesis, and MRes student Ciara McGlade for initial analyses of the iButton data.

I would like to thank Stewart "Stinky" White for the banter, early morning ringing sessions, and for taking me to hospital to have my "stookie" removed! Paul Jerem, and Ben the dog, for carrying out fieldwork while I was on crutches. Crinan Jarrett, Darren Talbot, Rachel Steenson, and all of the invaluable field volunteers over the years, for their hard work in the field tackling the rain, ticks, and biting midges of wild Scotland. The UofG Market Intelligence team: Elizabeth, Bo, James, Allison and Campbell, for helping me right through to the end. Lucy Cotgrove, Georgia Kirby, Matt Pace, Julie Miller, Hans Recknagel, and Megan Wright and Rosie Saxcoburg (my odd combination of friends), for their support and for keeping me grounded. Bedur Albalawi, my PhD sister (we did it!) for keeping me inspired. Allan MacRitchie, for his patience. Finlay Hind, for encouraging me to go for it. Finally, I thank Mum, Dad, Jamie, Shannon and Cody for their endless support and patience.

## Author Declaration

I declare that the work presented in this thesis is my own unless otherwise stated, and that no part of this thesis has been submitted for any other degree.

Signature _____

Printed name _____

## Contributions

The data in this thesis span three years and field seasons from 2016-2018. The following section provides a list of chapters in this thesis and the various contributions made by co-authors, students and collaborators.

**Chapter Three: Individual timing of incubation activities in city and forest birds, and implications for offspring fitness**

Data were collected for this chapter during field seasons 2016, 2017 and 2018. Concept and experimental design by Robyn Womack (RW), Barbara Helm (BH), Francesco Baldini (FB), Jane Robinson (JR) and Peter O'Shaughnessy (PO). Incubation activity and nestling condition data were collected by RW, BH and field volunteers during 2016 and 2017. Incubation activity data were collected by field volunteers and MRes student Ciara McGlade (CM) in 2018. Initial data sorting on incubation data was carried out by CM using incR package developed by Pablo Capilla-Lasheras (PC). Data analyses for this manuscript was carried out by RW. Manuscript was written by RW.

**Chapter Four**: **Effects of experimental exposure of low-level artificial light at night on circadian rhythms and health of wild nestlings**

Data and samples for this chapter were obtained during the field season in 2017. Concept and experimental design by RW, BH, FB, JR and PO. Data collection was carried out by BH, Paul Jerem (PJ) and field volunteers. Development of the QuantiGene® Plex RNA assay for use on avian blood samples was carried out by RW, and assays to determine transcript levels of immune and clock genes of samples were carried out by RW. Molecular sexing of samples was carried out by Gillian O'Flaherty (GO) under supervision of Barbara Mable (BM). Data analyses was carried out by RW. Manuscript was written by RW.

**Chapter Five: Avian malaria infection and condition in wild nestlings at city and forest sites**

Nestling data and samples for this chapter were obtained during field seasons in 2016 and 2017. Concept and experimental design by RW, BH, FB, JR and PO. Malaria PCR assays to determine infection prevalence were carried out by RW. Development of the QuantiGene® Plex RNA assay for use on avian blood samples was carried out by RW. Assays to determine transcript levels of immune genes of all samples were carried out by

RW. Molecular sexing of samples was carried out by GO under supervision of BM. Data analyses and manuscript writing was carried out by RW.

**Chapter Six: Malaria parasite effects on the circadian physiology of wild birds**

Nestling data and samples were obtained during field seasons in 2016 and 2017. Concept and experimental design by RW, BH, FB, JR and PO. Methodology to measure malaria infection intensity using qPCR was developed by PhD student Bedur Albalawi (BA) and FB. Malaria qPCRs to determine infection intensity were carried out by RW. Development of the QuantiGene® Plex RNA assay for use on avian blood samples was done by RW. Assays to determine transcript levels of immune and clock genes of all samples were carried out by RW. Molecular sexing of samples was carried out by GO and BM. Data analyses and writing of the manuscript was carried out by RW.

# Chapter One: General Introduction

Endogenous circadian rhythms of around 24 hours (from "*circa"* and *"dies",* meaning *"*about a day") are a core feature of animal physiology, vital for the normal function of rhythmic biological processes (Aschoff, 1965; Dunlap, 1999; Reppert and Weaver, 2002). Circadian clocks function to anticipate daily environmental changes and co-ordinate external factors with the internal behavioural and physiological processes of an organism, for example the releasing of enzymes to anticipate food intake (Stokkan et al., 2001).

Decades of research into biological clocks and their functions has elucidated the intricate workings of inner timekeeping mechanisms through *in vitro* and captive studies (Pittendrigh, 1954; Aschoff, 1967; Stephan & Zucker, 1972; Yamazaki et al. 1998; Vitaterna et al., 1999; Yoo et al., 2005), yet clocks remain understudied in evolutionary ecology. This thesis aimed to place existing work of clocks into a wider ecological context, by investigating biological rhythms in a wild model.

Birds are widespread, conspicuous and sensitive to habitat alterations, and therefore make great models for physiological studies in the wild. Much of avian behaviour is exhibited in rhythmic patterns, such as annual reproduction and migration behaviours, and daily foraging, singing and breeding activities. The avian system was therefore an ideal model to investigate ecological and evolutionary questions regarding biological clocks in an environmental context.

This review first outlines functions and mechanisms of the circadian clocks of mammals and birds, and then an appraisal of the literature concerning effects of environmental features on the timing of avian behaviour and physiology.

## 1.1 The circadian system: mechanisms and functions

Internal timekeeping mechanisms are ubiquitous throughout living organisms and are highly conserved, containing largely the same molecular components from cyanobacteria to mammals (Dunlap, 1999). In mammals, circadian systems are organised in a hierarchy of multiple circadian oscillators (Reppert and Weaver, 2002), with the suprachiasmatic nucleus (SCN) within the hypothalamic region of the brain acting as the master pacemaker, governing rhythms of "slave" oscillators in peripheral organs and tissues (Gaspar and Brown, 2015). Timing mechanisms between the SCN and peripheral clocks are very similar (Yagita et al., 2001), although the resulting temporal patterns of clock gene expression are often tissue specific (Pagani et al., 2010). Many processes of an organism are under circadian influence, such as the sleep/wake cycle (Saper et al., 2005), cycles of body temperature (Refinetti & Menaker, 1992), blood pressure (Pritchett & Reddy, 2015), metabolism (Karatsoreos et al., 2011), and cyclical release of hormones (Nicolaides et al., 2014).

In order to adapt to changing environmental conditions, circadian clock mechanisms must be actively synchronised to external cycles in a process known as entrainment (Pittendrigh, 1981). The light-dark cycle is the most potent environmental cue, otherwise known as zeitgeber, for clock synchronisation. However, it is also possible to entrain clocks to temperature cycles, or any environmental factor that fluctuates with consistent timing over the 24 h day (Pittendrigh, 1981).

### 1.1.1 Cogs of the circadian clock

Circadian studies have successfully characterised the molecular mechanisms behind the mammalian clock, since the first clock gene was discovered in mice (Vitaterna et al., 1994). In short, the mammalian clock is comprised of interconnecting core and stabilising loops, and overall functions as a transcriptional-translational feedback loop (Reppert and Weaver, 2002), (Figure 1.1.). The core loop is made up of positive elements CLOCK and BMAL1 and negative elements PER and CRY and functions as follows: CLOCK-BMAL1 heterodimers bind to specific E-box elements in target gene promoters, activating the rhythmic transcription of three period genes (*PER1, PER2, PER3*) and two cryptochrome genes (*CRY1, CRY2),* (Buhr and Takahashi, 2013; Gekakis et al., 1998). Negative elements PER and CRY proteins subsequently dimerize, inhibiting further transcriptional activity of

CLOCK and BMAL1 (Buhr and Takahashi, 2013; Reppert and Weaver, 2002). One full cycle of this loop is completed in just over 24 h, with positive element *BMAL1* expressed around 12 h out of phase with *PER* and *CRY* (Reppert and Weaver, 2002). It is this cycle of core clock genes within the SCN that governs biological cycles downstream in peripheral tissues and cells, driving rhythmic expression of proteins. Additional loops buffer and refine this molecular cycle, and link together the circadian and metabolic systems, with gene REVERBA (also known as NR1D1) at the interface, promoting transcription of *BMAL1* and *CLOCK* (Ueda et al., 2005).

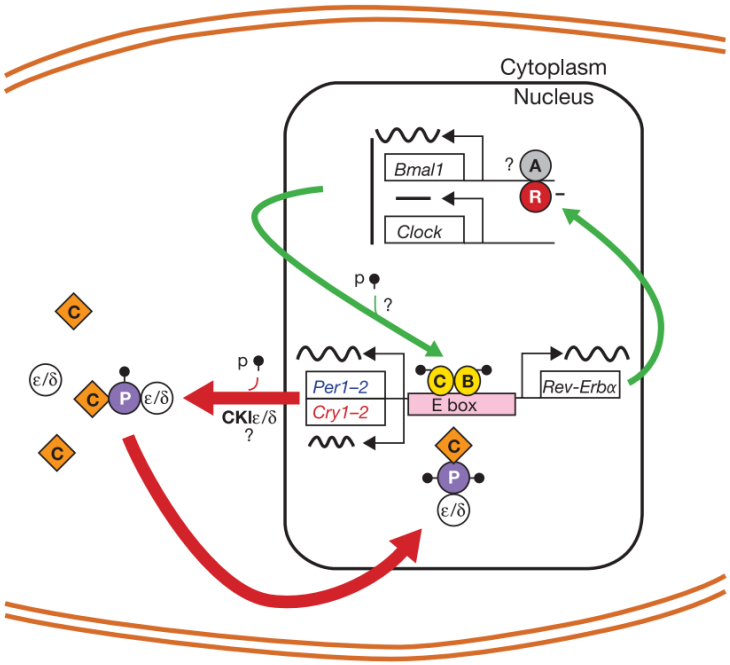



Figure 1.1. The current mammalian circadian clock model. From (Reppert and Weaver, 2002). The mammalian circadian clock consists of an interconnecting positive clock loop (green) and a negative loop (red). Positive elements *CLOCK* (C, yellow oval) and *BMAL1* (B, yellow oval) form heterodimers and facilitate the transcription of negative elements *PER* (purple circles) and *CRY* (C, orange diamond). *PER* and *CRY* are phosphorylated outside the nucleus, and within the nucleus the newly phosphorylated complex shuts down further transcription of *BMAL1* and *CLOCK*. During the positive loop of the clock, *REVERBA* (R, red circle) promotes transcription of *BMAL1* and *CLOCK*.

Circadian oscillators can become desynchronised in the absence of a strong zeitgeber for entrainment (Aschoff et al., 1967). In humans, air travel through multiple time zones, shift-work and sleep deprivation can all lead to desynchronization of peripheral oscillators to the SCN (Karatsoreos et al., 2011). Many homeostatic and immune functions are under circadian control (Scheiermann et al., 2013), and therefore chronic desynchronization of circadian oscillators to the SCN has been demonstrated to have severe adverse effects on

health. Experimental evidence on human and mouse demonstrates disrupting natural rhythms to have profound effects on the brain, metabolism and behaviour (Karatsoreos et al., 2011), metabolic dysregulation (Huang et al., 2011), mood disorders (McClung, 2013), inflammation (Preuss et al., 2008), and increasing incidences of cancer (Fonken and Nelson, 2014).

Following a phase shift of the clock, a stable relationship between peripheral circadian oscillators and the SCN must be re-established (Karatsoreos et al., 2011). Clocks can be re-entrained and normal rhythms restored by exposure to light-dark cycles via light-induced signals from the retina, which in turn modify expression of clock genes in the SCN (Schroeder and Colwell, 2013; Wright et al., 2013).

## 1.2 Avian clocks – mechanisms, functions and outputs

Although not as well characterised as the mammalian clock, current models of avian clock mechanisms are largely accepted. Three pacemakers make up the core avian clock mechanism, with the pineal gland rather than the SCN acting as "master pacemaker", along with additional core oscillators in the retina and hypothalamic region (Cassone, 2014; Gwinner and Brandstätter, 2001; Kumar et al., 2004), (Figure 1.2). These structures are thought to reinforce each other's rhythmicity in a "neuro-endocrine loop" that influences processes downstream (Cassone and Menaker, 1984), however the exact contribution of these three structures to avian circadian organisation has still yet to be defined (Karaganis et al., 2009).

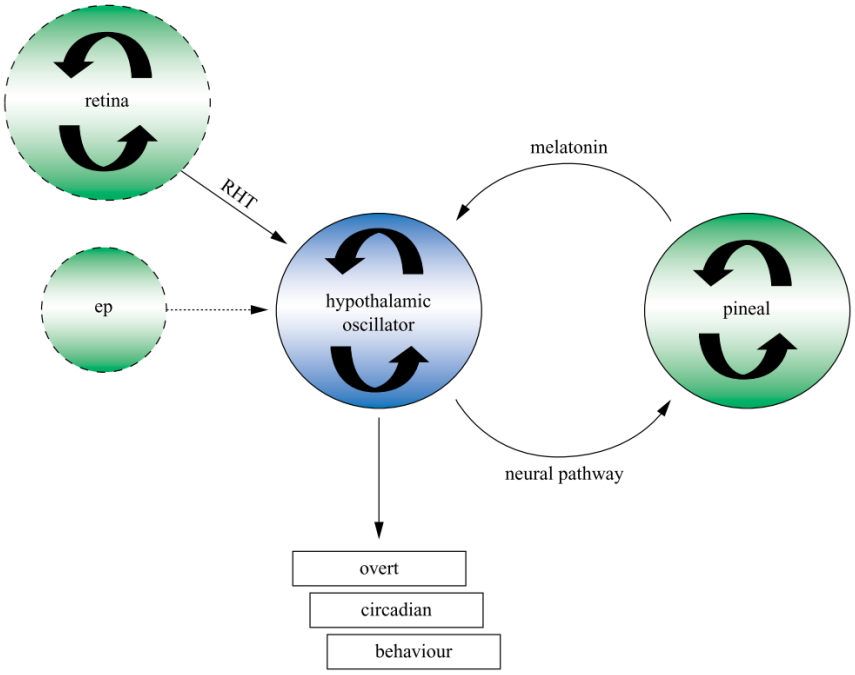



Figure 1.2. Components of the avian circadian clock. From (Gwinner and Brandstätter, 2001). Oscillators in the pineal, hypothalamus and retina contribute to avian circadian outputs in birds. Sites of photoreceptors include the retina and pineal (green), and encephalon (ep). RHT = retinohypothalamic tract.

Avian circadian clock genes are expressed in a similar fashion to those in the mammalian clock except for *PER1*, which is not present in avian genomes (Yasuo et al., 2003; Yoshimura et al., 2000). Molecular analyses of avian clock genes demonstrate *BMAL1* and *CLOCK* to be expressed during the late subjective day (in diurnal birds, twilight) and *PER2* and *PER3* expressed during subjective night to mid-subjective day (Cassone, 2014; Chong et al., 2003; Karaganis et al., 2009; Yoshimura et al., 2000).

### 1.2.1 The pineal gland and melatonin

Research into avian circadian systems has shown the pineal gland to be the major pacemaker in songbirds (Cassone, 2014; Wang et al., 2014). Pinealectomy studies, or surgical removal of the pineal gland, have revealed the pineal to be essential for sustaining activity rhythms during constant darkness (Gaston and Menaker, 1968), maintaining cycles of body temperature (Binkley et al., 1971), and daily patterns of singing behaviour (Wang et al., 2012). The pineal gland modulates rhythms by secretion of the hormone melatonin (Gwinner et al., 1997; Menaker and Zimmerman, 1976), which has downstream influences on patterns of behaviour. In addition to the control of circadian behaviour, pineal melatonin is possibly indirectly involved in the regulation of some seasonal behaviours (Gwinner, 2008), (Figure 1.3.).

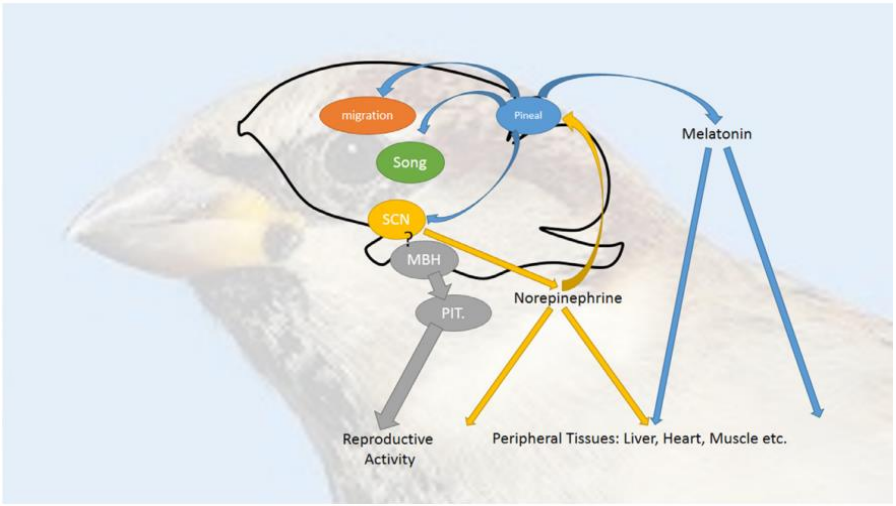



Figure 1.3. The avian pineal gland may have modifying effects on downstream rhythms of seasonal reproduction, migration and song behaviour. From Cassone (2014).

### 1.2.2 Avian photoreception and entrainment

For the circadian clock to entrain to external light-dark cycles, light information must first be received, transduced and translated. Unlike mammals, where the SCN receives all photic input via the retina, birds and other non-mammalian vertebrates possess extra-ocular photoreceptive structures (Cassone, 2014; Thakur and Kumar, 2015). Functional photoreceptors are present within the avian pineal gland and deep-brain regions, in addition to the retina (Thakur and Kumar, 2015). Rhythms of pineal melatonin can also be entrained by light entering the retina (Barrett and Underwood, 1991).

### 1.2.3 Development of avian rhythms

In avian embryos, transcripts of *BMAL1* and *CLOCK* are already present in the foregut at 48-52 hours old (Gonçalves et al., 2012). Oscillations of *BMAL1* are later present in the chick retina at eight days (De Lima et al., 2011) and in the liver at four days old (Zeman et al., 2009). Circadian rhythmicity of melatonin biosynthesis occurs in the pineal gland during the first day post-hatching (Zeman and Herichová, 2011) for both precocial and altricial species of birds (Zeman & Gwinner, 1993). The amplitude of this rhythm increases with age within the first two weeks post-hatching (Zeman & Gwinner, 1993; Van’t Hof and Gwinner, 1996).

## 1.3 Environmental influences on avian clocks

Many features within an organisms' environment can have influence on the timing of behaviour and physiology. In the following section, current knowledge of how several important environmental features influence the circadian rhythms of birds is reviewed.

### 1.3.1 Ambient light

The light-dark cycle is the most important synchronising cue for clock mechanisms (Pittendrigh, 1981). Given that birds possess photoreceptors in neural tissue and the pineal as well as in the retina (Thakur and Kumar, 2015), birds are arguably more light-sensitive than mammals. Previous captive studies on birds have shown rhythmic avian behaviours, such as song and activity, to be dictated by light and subsequent secretion of pineal melatonin (Wang et al., 2012).

Photoperiodic changes are important triggers for the seasonal biology of birds (Dawson et al., 2001; Gwinner, 2008). For example, exposure to long photoperiods can override other environmental cues to trigger physiological changes associated with reproduction (Dominoni et al., 2013b; Lambrechts and Perret, 2000). Light intensity can also influence perception of daylength; male European starlings (*Sturnus vulgaris*) exposed to low intensity light took longer to respond to photoperiodic changes than individuals under higher light intensities (Bentley et al., 1998).

Exposure to light during the subjective night can have particularly disruptive effects on rhythms (Fonken and Nelson, 2011). A captive study exposed great tits (*Parus major*) to five different intensities of nocturnal light and found strongest disruptive effects on activity patterns to occur with strongest light intensity (de Jong et al., 2016). This study also measured plasma melatonin concentrations and found a decrease in concentration with increasing intensity of light treatment. Light at night also disrupted rest and increased nocturnal activity in zebra finches (Alaasam et al., 2018). In great tits, dim light at night treatment shifted the onset of activity, but had no effect on circadian period length (Spoelstra et al., 2018).

### 1.3.2 Ambient temperatures

Birds and other animals possess a circadian rhythm of internal body temperature, which shows robust rhythmicity independent of environmental temperature oscillations, constant ambient temperatures and constant light (Merrow et al., 2005; Refinetti and Menaker, 1992). However, ambient temperature cycles can also entrain circadian rhythms (Pittendrigh, 1981). One of the fundamental features of the circadian clock is that rhythms are temperature-compensated, where the rate of the clock remains consistent during ambient temperature fluctuations that are physiologically permissive (Hastings, 2013; Pittendrigh, 1954).

Ambient temperature changes may have some influence on rhythms, however. A captive experiment by Lehmann et al., (2012) exposed great tits to temperatures of either 18 °C or 8 °C. Birds exposed to the higher temperature started their daily period of activity later, and terminated activity earlier in the day than those kept under 8 °C. Although observed shifts in daily activity were small, the authors suggest natural conditions where the temperature difference is larger than the 10 °C tested may have a larger effect on activity patterns (Lehmann et al., 2012). In another experiment, ambient temperature fluctuations influenced activity rhythms of Malachite sunbirds (*Nectarinia famosa*) directly by inducing torpor at low temperatures (Downs and Brown, 2002).

### 1.3.3 Food availability

Both the timing and the quality of food intake can affect biological rhythms in animals. Bird species follow daily patterns of foraging activity, usually peaking in the morning and the afternoon (Bednekoff and Houston, 1994), and can be entrained to feeding cycles under captive conditions (Hau and Gwinner, 1992). Timing of food intake acts as a zeitgeber in a process known as feeding-entrainment, coupling metabolic activity to molecular clocks of the SCN and liver (Stokkan et al., 2001). Mistimed or restricted feeding has been shown to decouple oscillators of metabolic and molecular clocks, causing health problems (Chabot and Menaker, 1992; Damiola et al., 2000). In addition, adequate nutrition is required for the generation of normal metabolic rhythms (Potter et al., 2016). An example of this from mouse models shows high-fat diets have been shown to alter period length and locomotory activity (Kohsaka et al., 2007).

### 1.3.4 Immunity, disease and parasites

Circadian clocks are closely linked to the immune system (Scheiermann et al., 2013). In birds, pineal melatonin regulates rhythms of innate immunity and seasonal immunity (Markowska et al., 2017). Diurnal changes in mRNA of interleukins *IL6* and *IL18* have been observed in chicken (*Gallus gallus domesticus*) leucocytes (Turkowska et al., 2013). Rhythmic oscillations of proinflammatory cytokines for inflammatory defences have been shown in the avian spleen, also regulated by melatonin (Naidu et al., 2010).

In their natural environment, birds are exposed to parasites and disease (Hamilton and Zuk, 1982). Parasites may directly impact on avian rhythms; for example, infestations with ectoparasites increased nocturnal preening activity and restlessness in chickens (Jacobs et al., 2019). Host-parasite interactions may also be complex, given the temporal fluctuations in host immunity (Martinez-Bakker and Helm, 2015). For example, malaria blood parasites (*Leucocytozoon, Haemoproteus* and *Plasmodium* species) exhibit rhythmicity during their life cycle within a host (Reece et al., 2017). Avian malaria species are also rhythmic (Gore et al., 1982), but interactions with the host circadian system have yet to be defined.

## 1.4 Measuring rhythms of individuals

### 1.4.1 Behaviour

In the absence of any environmental cues to entrain to, circadian clock mechanisms will "free-run" at a period length of approximately 24 h (Buhr and Takahashi, 2013). Endogenous circadian rhythms of individuals can be quantified by measurement of this free-running period length (or *tau*) under constant conditions such as constant light (LL) or dark (DD). Period length is highly repeatable in individuals and is usually determined either by periods of sleep and wake or by timing of locomotory activity, such as wheel-running in mice (Brown et al., 2005), or perching activity in birds (Underwood et al., 2001).

In the presence of cues in the natural environment, such as the light-dark cycle, individual timing in animals can be measured by quantifying rhythms of behavioural activity. Differences in behavioural timing can be used as a proxy for differences in endogenous rhythms, as the phase angle between the zeitgeber and activity timing depends on individual free-running period (Roenneberg et al., 2003).

Timing of individual activity patterns of animals can be quantified in their natural environment, given that the behaviour is repeatable and consistent. In birds, this can for example be timing of incubation behaviours (Bulla et al., 2016), sleep patterns (Stuber et al., 2015), and song (Naguib et al., 2019). Individual sleep-wake activity is often classified using the term "chronotypes", with early chronotypes (colloquially known as "larks") rising earlier than later "owl" chronotypes (Roenneberg et al., 2003).

### 1.4.2 Molecular mechanisms

Despite central oscillators of the circadian system in the SCN being synchronised by external cues, molecular clock mechanisms are cell autonomous, and rhythms can persist in isolated tissues. This allows for *in vitro* studies of circadian rhythms in biopsies from individual animals (Brown et al., 2005; Gaspar and Brown, 2015). In addition, the advance of microarray technologies has facilitated studies of cyclical gene expression, allowing for comparisons of clock mechanisms between species (Reddy, 2013). Since Harmer et al., (2000) mapped the circadian transcriptome in *Arabidopsis*, further studies have elucidated the circadian transcriptomes within avian tissues such as liver and heart (Zeman et al., 2009), brain (Karaganis et al., 2009), spleen (Naidu et al., 2010), ovary (Laine et al.,

2019), retina (Bailey et al., 2004), and pineal (Bailey et al., 2002). As in other species, expression of avian core clock genes appears to be tissue specific (Laine et al., 2019).

Despite advances in molecular methods facilitating studies of mechanisms of the circadian clock in many different species, the potential for these methods to be used in studies of wild animals has yet to be fulfilled.

## 1.5 Clocks in the wild: studies of daily rhythms of birds in the natural environment

Despite the importance of circadian rhythms for the physiology and behaviour of animals, relatively little is known about biological clocks in ecology (Kronfeld-Schor et al., 2013). In birds, observational differences in rhythms have been recorded at species' level by measurement of timing of behaviours such as arrival at a feeder (Fitzpatrick, 1997), timing of dawn song (Kempenaers et al., 2010; Naguib et al., 2019) or departure from nocturnal roosts (Graham et al., 2017; Hubálek, 2017).

For wild studies in individual birds, behavioural activity patterns are quantified to later infer rhythms. Usually, data loggers are used to measure activity patterns around the clock. One study used remote temperature sensors to record timing of incubation behaviours and infer activity patterns in different wader and seabird species (Bueno-Enciso et al., 2017; Bulla et al., 2016). Time–depth recorders have also been used to infer activity budgets of seabirds (Linnebjerg et al., 2014), or radio telemetry (Jansen et al., 1998; Keitt et al., 2004; Steiger et al., 2013). Another study investigated plasticity in clocks by quantifying sleep behaviours of individual roosting great tits from nest box camera footage (Stuber et al., 2015).

Observational studies of rhythms of individual birds can help to answer ecological questions on the function and modulations of clocks, such as how environmental changes influence timing. For example, overnight ambient temperatures influenced the start of song in male great tits, although the differences in song timing between individuals remained consistent (Naguib et al., 2019). In addition, the presence of artificial light at night influences the start of daily activity in passerines, with an earlier onset of activity dependent on the species (Kempenaers et al., 2010).

To date, few wild studies have explored the adaptive significance of behavioural timing. For example, in a study on male blue tits (*Cyanistes caeruleus*), individuals that rose earlier had higher success at extra-pair paternity (Poesel et al., 2006), suggesting that individual variation in timing may be under selection.

Experimental studies on individual rhythms can help to further understanding of how birds respond to environmental change. A nest box study investigating impacts of light at night on avian physiology and behaviour exposed free-living great tit nestlings to constant illumination at night, finding negative effects on sleep (Raap et al., 2016c). In addition,

another nest box study that experimentally increased nest temperatures caused birds to wake up approximately 30% more frequently during the night (Stuber et al., 2017).

### 1.5.1 Wild clocks in a model avian species; the great tit

The great tit (*Parus major*) is a model avian species and a widely studied passerine in ecology (Gosler, 1993; Lack, 1964). Great tits are a diurnal species, and show consistent daily rhythms in behaviours such as dawn and dusk song (Da Silva et al., 2015; Naguib et al., 2019), early morning feeding (Fitzpatrick, 1997) and incubation efforts (Bueno-Enciso et al., 2017). Intraspecific variation in rhythms have been observed for sleep behaviours in individuals (Stuber et al., 2016, 2015), individual timing of breeding (Graham et al., 2017), and nocturnal resting behaviours (Caorsi et al., 2019).

Great tits have been studied in the context of biological timing, and particularly effects of the environment on annual and daily cycles of behaviour. For example, the warming global climate has led to advancing vegetation phenology, and consequently, adaptive selection for earlier initiation of breeding activity in great tits (Visser et al., 1998).

Moreover, observations of behaviour of great tits as a response to environmental changes including an increase in vigilance time and reduction of feeding effort during periods of anthropogenic noise (Klett-Mingo et al., 2016). In addition, great tits under light at night have been shown to sing earlier in the morning (Kempenaers et al., 2010). Experimental studies on rhythms of wild great tits have shown sleep to be disrupted by light pollution (Raap et al., 2016c, 2015). In particular, white light exposure has been linked to an increase in nocturnal activity, decrease in oxalic acid levels (a marker for sleep debt), and an increase incidence of infection with avian malaria in adult great tits (Ouyang et al., 2017). Light pollution has also been linked with an increase in feeding rate of great tit parents during nestling provisioning (Titulaer et al., 2012).

## 1.6 Knowledge gaps

Previous research into biological rhythms of wild birds has provided evidence that individual activity patterns can be measured and quantified using logger technologies (Bulla et al., 2016) or behavioural observations (Stuber et al., 2015). However, classifying circadian phenotype into individual “chronotype” in a wild animal has rarely been done (Graham et al., 2017). In addition, despite the wealth of knowledge obtained from captive studies of avian molecular clock mechanisms, there has been no previous attempt to quantify circadian clocks at the molecular level in a wild bird, and few studies have investigated how individual differences in timing might relate to fitness of wild birds (Hau et al., 2017; Poesel et al., 2006).

Captive studies have demonstrated timing of cues such as light (de Jong et al., 2016) and food (Hau and Gwinner, 1992) to affect daily rhythms of birds. However, there is limited evidence into how such changes affect timing in birds in the natural environment, especially at the mechanistic level. Further research is needed into how rhythms of individuals are shaped by these environmental changes, such as the presence of light at night (Raap et al., 2016c; Stuber et al., 2017).

Birds face many pathogens in their natural environments (Ellis et al., 2014). Although timing of the avian immune system is under circadian control (Markowska et al., 2017), there has been no investigation into time of day differences of immune system activity in wild birds. Research is needed into how fitness challenges for birds, such as wild infections with parasites (van Riper et al., 1986), affect the host circadian and immune systems.

## 1.7 Aims of Thesis

The overarching aim of this thesis was to put studies of biological rhythms into the context of the organisms' natural environment. Specifically, the aims of this thesis were:

1. To further the understanding of how environmental factors influence timing in a wild animal
2. To investigate how changes in the environment, such as the city habitat and artificial light at night, affects timing in individuals at behavioural and transcript levels
3. To investigate consequences of wild infections with avian malaria parasites for host condition and immune and circadian physiology

In this thesis, the great tit was used as the study species, a common passerine widely used in ecological research (Gosler, 1993; Lack, 1964). Observational and experimental approaches were used to quantify individual rhythms of great tits at behavioural and molecular levels, using knowledge of circadian clock mechanisms and available genetic tools for the species (Laine et al., 2016; Santure et al., 2011). Many abiotic and biotic features can have influence on the clock, and therefore, two aspects of the environment were selected as the focus of chapters in this thesis: ambient light and host-parasite interactions (Figure 1.4.).

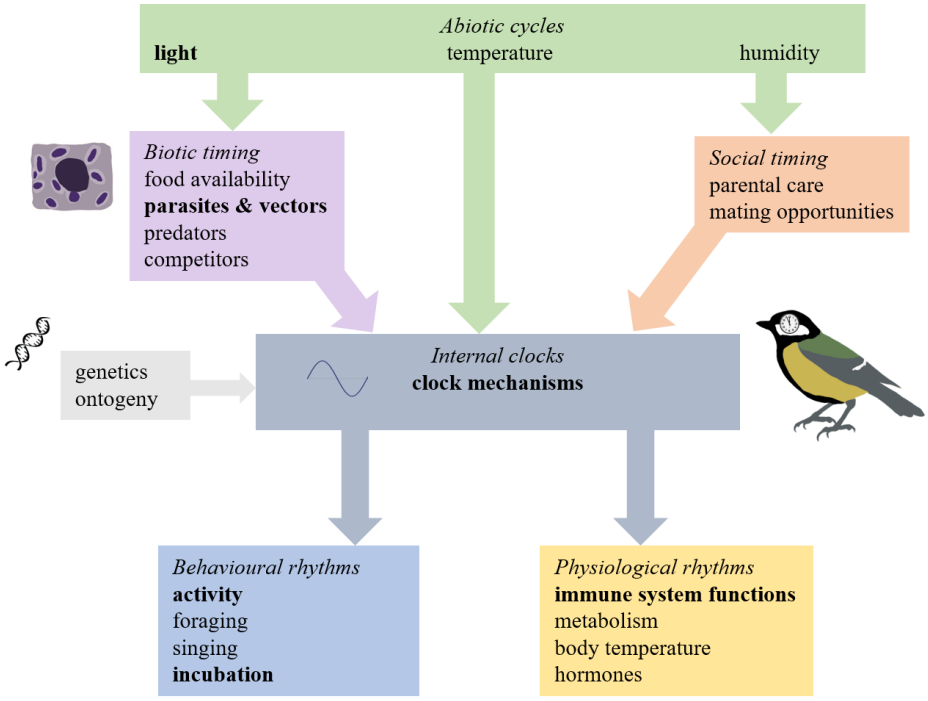



Figure 1.4. Influences of biotic and abiotic factors on internal clocks, and outputs. Factors studied in this thesis are highlighted in bold. Diagram adapted from (Helm et al., 2017).

## 1.8 Thesis outline

**Chapter Two** provided an overview of the General Methods used in the laboratory and field for data collection in this thesis, and an overview of the study species and nest box system.

**Chapter Three** of this thesis investigated differences in individual timing in great tits in city and forest habitats, using thermal sensors to quantify incubation behaviours. Individual "chronotype" was then determined from the timing of onset of activity of the mother inferred from incubation patterns. Chronotype of individual birds was then linked to parameters of condition in offspring, to determine whether differences in individual timing are important for the fitness of wild birds.

**Chapter Four** of this thesis investigated whether disruptive effects of artificial light at night on rhythms found in captive studies was reflected in wild birds. An experimental approach was used to investigate effects of low-level artificial light at night on nestlings. Molecular tools were used to quantify time of day differences in circadian clock transcripts, and transcripts of genes involved in the avian immune system. This chapter explored effects of exposure to artificial light on nestlings' clock, immune system and parameters of condition such as weight and haematocrit levels.

**Chapter Five** explored how wild infections with pathogens can affect condition of nestlings in city and forest habitats. This study took advantage of the high prevalence of *Leucocytozoon* avian malaria parasites in the study system (Capilla-Lasheras et al., 2017), to investigate fitness effects of infections on immune gene transcripts and condition of nestlings.

**Chapter Six** of this thesis investigated host-parasite effects on the circadian system of wild birds. In this study, transcript levels of circadian clock and immune genes were measured across a temporal profile to determine rhythmicity. Avian malaria (*Leucocytozoon* species) infections were also quantified and linked to rhythmicity in nestlings, to explore whether parasites impact on circadian and immune mechanisms in wild birds.

Finally, the **General Discussion** section provided an overview of the findings of this thesis, limitations of the studies undertaken, and contributions to the advancement of the fields of chronobiology and ecology.

# Chapter Two: General Methods

Data in this thesis were based on samples collected during three breeding seasons from April to June 2016-2018 in city and forest field sites in Scotland, United Kingdom. The following General Methods section outlines the field and laboratory methods that apply to chapters in this thesis.

## 2.1 Study species

In this thesis, the great tit (*Parus major*) was used as the study species. The great tit is a passerine species, widely distributed across Europe and Asia, and commonly sighted in oak woodlands and urban parks (Gosler, 1993). A small passerine, adult great tits weigh 13-21 g, and their plumage colouration consists of a black cap, nape and collar, white cheeks, yellow underparts and green and blue upperparts (Gosler, 1993). Male great tits are distinguishable from females by their larger size, and wider black belly-stripe (Norris, 1993). In juveniles, plumage areas which are white in adults are yellowish in colour, before their post juvenile-moult (Gosler, 1993). Brightness of feather colouration in great tits is dependent on diet and overall condition (Hõrak et al., 2001; Slagsvold and Lifjeld, 1985).

Great tits have a predominantly insectivorous diet (Perrins, 1991), and time their breeding activities to match peak caterpillar abundance (Noordwijk et al., 1995). Egg laying dates vary from April to May, and clutch size ranges from 7-12 eggs (Perrins and McCleery, 1989). Incubation of eggs is carried out by the female only, and lasts for approximately two weeks before hatching (Gosler, 1993; Haftorn, 1981).

Both parents are involved in nestling provisioning of prey such as caterpillars and spiders (Hinde, 2006; Naef-daenzer et al., 2016). Nestlings remain in the nest for 18-21 days until their full plumage has developed, finally fledging with a body weight of around 16-22 g (Gosler, 1993). The weight of nestlings at fledgling predicts their prospects for survival (Tinbergen and Boerlijst, 1990) and recruitment into the great tit population post-fledging (Both et al., 1999). Predators of the great tit include great spotted woodpeckers (*Picoides major*) or weasels (*Mustela nivalis*), (Curio and Onnebrink, 1995; Dunn, 1977), which commonly raid passerine nests. Sparrowhawks (*Accipiter nisus*) are also common predators of adult great tits and young fledglings (Götmark and Andersson, 2005).

Great tits are primarily cavity nesters (Gosler, 1993), however, they will readily nest in nest boxes. Nest boxes studies using great tits have facilitated studies of biological rhythms in ecology (Graham et al., 2017; Raap et al., 2015; Stuber et al., 2015). In this thesis, a nest

box system was used for observational and experimental studies on a breeding population of great tits and nestlings. This thesis also made use of the genetic tools available for great tits, including an annotated reference genome (Laine et al., 2016) and transcriptome (Santure et al., 2011; Watson et al., 2017).

## 2.2 Descriptions of field sites used

This study was conducted using an existing nest box system across two urban field sites and two forest field sites (Table 2.1.), which represent an urban (city) to rural (forest) gradient from Glasgow city to Loch Lomond and the Trossachs National Park, Scotland, United Kingdom.

Given the limited occupancy of great tits at nest boxes at city sites and resulting low numbers of nestlings, for this study, sites were classified as belonging to either city or forest categories to increase power of city and forest comparisons.

Table 2.1. Descriptions of study sites used in this thesis.

| | **Site** | | | |
|---|---|---|---|---|
| | **Cashel Native Forest (56º 6' N, 4º34' W)** | **Scottish Centre for Ecology and the Natural Environment (SCENE) (56º, 7.73N 4º 36.79W)** | **Garscube Campus (55º 9’ N, 4º31’ W)** | **Kelvingrove Park (55º52’ N, 4º17’ W)** |
| **Type** | Deciduous forest | Deciduous forest | Suburban parkland | Inner city parkland |
| **Site classification** | Forest | Forest | City | City |
| **Composition of tree species** | Oak (*Quercus* sp.), and birch (*Betula* sp.) | Oak (*Quercus* sp.) | Open land with abundance of introduced and native species | Parkland, introduced species and sparse birch and oak |
| **Total number of nest boxes at site (2016)** | 131 | 289 | 40 | 71 |

## 2.3 Nest box monitoring protocol

Throughout the breeding season (April-June), all nest boxes (woodcrete material, 260H x 170W x 180D, hole diameter 32 cm, supplier Schwegler, Germany) were checked at least weekly for signs of nest building activity and egg laying. Nests were identified as belonging to a great tit if eggs were >13mm in width (Van Noordwijk et al., 1981). Nest boxes were checked once every two days, two weeks after the estimated start of incubation to determine exact date of hatching (Haftorn, 1981). Except in the case of experimentally manipulated nest boxes in Chapter Four, where samples were taken on day eight, disturbance to nest boxes was avoided from hatch date until day thirteen post-hatching. On day thirteen, all nestlings within a brood were weighed and ringed for individual identification. Nest boxes were checked again after fledging (>21 days post-hatching) to determine the numbers and identities of any dead nestlings. In total, 177 great tit nest boxes were monitored during this study, 137 of these at forest sites, and 40 in the city (see Table 2.2. for breakdown by year and site).

Table 2.2. Number of great tit nests monitored per field site and nestlings processed during this study (2016-2018).

| Site | Site Type | Year | Number of great tit broods monitored |
|---|---|---|---|
| **SCENE** | Forest | 2016 | 29 |
| | | 2017 | 32 |
| | | 2018 | 8 |
| **Cashel Forest** | Forest | 2016 | 31 |
| | | 2017 | 33 |
| | | 2018 | 4 |
| **Kelvingrove Park** | City | 2016 | 7 |
| | | 2017 | 11 |
| | | 2018 | 7 |
| **Garscube Estate** | City | 2016 | 3 |
| | | 2017 | 7 |
| | | 2018 | 5 |

## 2.4 Citronella treatment

During the 2017 breeding season, fifteen forest nest boxes were treated with citronella-based insect repellent as per Krams et al., (2013). According to Krams et al., (2013), inclusion of citronella treatment in their study nest boxes reduced the presence of avian malaria vectors by 80 %. In this study, citronella repellent was added to nest boxes with the aim of reducing abundance of avian malaria vectors (and therefore, avian malaria infections) in nest boxes used in Chapters Four and Five. Repellent consisted of 0.5 ml citronella oil, 200 mg carrageenan kappa, and 0.5 ml water. Four eppendorfs per nest box were first filled with citronella oil mixture and pierced with a 3 mm hole in the lid. One day prior to the expected hatch date entrance (two weeks after the start of incubation), these eppendorfs were fastened to the inner wall of nest box doors, 3 cm away from the nest box entrance.

## 2.5 Nestling processing and blood sampling

Ringing and measurements of all nestlings took place on day thirteen post-hatching during the 2016 and 2018 breeding seasons. In 2017, ringing and measurements of nestlings took place on day eight post-hatching, as part of the light at night study in Chapter Four. Blood sampling of all nestlings took place on day thirteen, and usually during the daylight hours. For some studies, blood sampling was carried out around the clock (see Chapters Four and Six).

During sampling, all nestlings of a brood were taken from the nest box and placed into a fabric drawstring bag, to avoid escapees. Nestlings were then individually processed as follows. Nestlings were ringed with an aluminium ring detailing individual identification number using rings obtained from the British Trust for Ornithology, and weighed using a portable balance (Spencer, 1976). Where tarsometatarsus (tarsus) measurements were required (Chapter Four and Five), the base of the metatarsals of each nestling were measured using dial callipers (Eck et al., 2011).

Blood sampling was only carried out on nestlings of a healthy weight (17-22 g) to reduce burden on nestlings. Prior to blood sampling, a cotton bud dipped in water was used to dampen feathers and expose the brachial wing vein, to increase accuracy of sampling. The brachial wing vein was then punctured following sampling protocol outlined in Owen (2011). The resulting blood bead was then collected using a heparinised microcapillary (Vitrex) and immediately pumped into an eppendorf containing 250 µl of RNA*later*® stabilization solution (Invitrogen) for RNA sampling, and into an eppendorf containing 500

µl 100% ETOH for DNA sampling. In total, a maximal amount of two 75 µl capillaries were collected per nestling. If this was not possible, samples were obtained for RNA and DNA from different siblings from the same nest. During the field season of 2017, some blood samples were collected for measurement of haematocrit levels (%), (see below section for details). On returning from the field, samples were placed into a refrigerator at 5 ºC for three days, before storage at -40 ºC.

## 2.6 Haematocrit measures

During the 2017 breeding season and for Chapter Four and Five, blood samples were taken for measurement of haematocrit, or the percentage volume of red blood cells compared to total blood volume, as a measure of nestling health (Ots et al., 1998). On day thirteen of life, blood samples were obtained from nestlings using the sampling protocol detailed above, taking ½ to ⅔ of a 75 µl heparinised haematocrit microcapillary (Vitrex) per nestling. Immediately following sampling, capillaries were sealed directly using a Cristaseal wax plate (Hawksley) and stored on wet ice for transport to the laboratory. Capillaries were placed into a centrifuge and spun for 5 minutes at 5000 RPM. Following this, capillaries were measured against a silver ruler (Figure 2.1.). Three measures were taken, starting at the wax end of the capillary each time:  1) end of the capillary to the seal wax plug; 2) end of the capillary to the packed red blood cell fraction; 3) end of the capillary to the end of the plasma region.

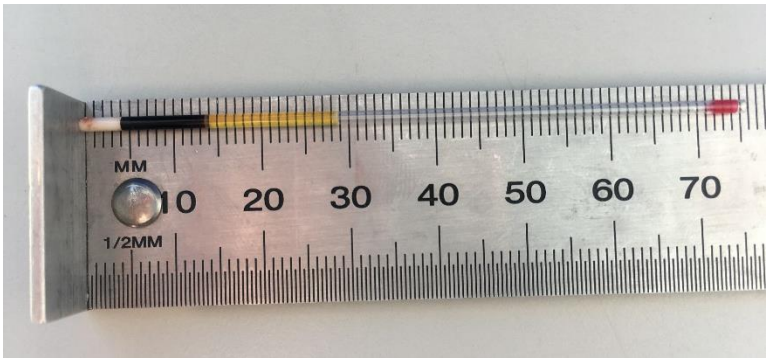



Figure 2.1. Measurement of plasma and packed red blood cell volume from a capillary containing great tit blood after centrifugation, to determine haematocrit levels (%)

Following these measurements, the length of the seal wax was subtracted from the total length of the sample (including the length of the red blood cell fraction), prior to calculation of haematocrit levels. Haematocrit levels (%) were then calculated as the length of the packed red blood cells, divided by the length of the total sample (red blood cells and

plasma). Each capillary was then scored with a glass cutter, and using a Pasteur pipette, pumped blood sample into an eppendorf containing 500 µl 100% ETOH. Blood samples were then placed into storage at -40 ºC.

## 2.7 Sample sizes and breakdown

In total, 297 nestlings were sampled during this study across the two sampling years (2016-2017). Of these samples, 259 were collected from forest sites, and 38 from city sites (see Table 2.3. by a breakdown by year and site).

Table 2.3. Number of nestlings sampled per site

| | **Site / Number of nestlings processed** | | | |
|---|---|---|---|---|
| **Year** | Cashel Forest | SCENE | Garscube | Kelvingrove Park |
| **2016** | 50 | 56 | 5 | 8 |
| **2017** | 97 | 56 | 13 | 12 |
| **2018** | No blood samples were obtained for this year | | | |

This study was conducted across three breeding seasons, and nestling data were used in more than one chapter of this thesis. Nestlings used as controls in Chapter Four were included in Chapters Three, Five and Six as part of the chronotype and malaria studies. However, nestlings exposed to low-level light at night treatment in Chapter Four were not included in any other chapter in this thesis.

Sample sizes for each chapter also differed due to the methods used within this thesis. For example, in Chapter Three, nestlings were only used in the chronotype and fitness analyses if iButton recordings were also obtained for incubating females of that brood. Similarly, nestlings were only used in analyses for Chapters Five and Six if samples had also been obtained for quantification of malaria infection and immune and clock (in the case of Chapter Six) gene transcripts. Samples in Chapter Five were taken during the noon hours, whereas samples in Chapter Six were taken at times throughout the day and night. A breakdown of number of nestlings used in analyses for each chapter is available in Table 2.4.

Table 2.4. Overview of number of nestlings from which data were collected for each chapter of this thesis.

| Chapter | Study Years | Number of Nestlings |
|---|---|---|
| Chapter Three | 2016, 2017, 2018 | 253 |
| Chapter Four | 2017 | 213 |
| Chapter Five | 2016, 2017 | 174 |
| Chapter Six | 2016, 2017 | 199 |

## 2.8 Ethical statement

All blood sampling of nestlings was carried out under the appropriate Home Office project licence held by Barbara Helm, and personal licences for avian sampling held by Robyn Womack and field technicians. Ringing of nestlings was carried out by persons holding appropriate licences obtained from the British Trust for Ornithology.

## 2.9 DNA extraction

In this thesis, genomic DNA samples were required for molecular sexing (Chapters Four and Five), for avian malaria prevalence testing (Chapter Five), and quantification of avian malaria infection (Chapter Six). Genomic DNA extraction was carried out on previously frozen (-40 ºC) whole blood samples stored in 500 µl of 100 % ETOH, using a commercial DNeasy whole blood extraction kit (Qiagen). Following extraction, DNA samples were eluted in 80 µl nuclease-free water. Successful extraction of DNA was confirmed by measuring 1.5 µl of each sample using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE).

## 2.10 Molecular sexing

For Chapters Four and Five, the sex of each individual nestling was determined by application of a PCR-based protocol as described in Griffiths et al., (1998). This method amplifies target avian genes CHD-W and CHD-Z. Two distinct bands in a well indicates presence of two products of different sizes and is said to be female (ZW). One distinct band in a well indicates presence of only one product and is said to be male (ZZ).

Molecular sexing work for this thesis was carried out by honours student Gillian O'Flaherty under the supervision of Elizabeth Kilbride and Prof. Barbara Mable.

Each reaction was carried out in volumes of 10 µl, and contained the following components: 1 µl buffer (Promega), 2 mM $MgCl_2$ (Promega), 0.8 mM each of forward and reverse primer of target genes, 0.16 mM dNTP, 0.375 unit Taq DNA Polymerase (Promega) and 5-10 mg/ µl of extracted DNA template. Primer sequences for CHD-W and CHD-Z were obtained from Griffiths et al., (1998).

PCR runs included a negative control of nuclease-free water and two positive control samples, one from a known female and one from a male. Samples were placed in a PCR machine (MJ Research Dyad™ Peltier Thermal Cycler) and amplified at 94 °C for 2 min, 49 °C for 45 s, 72 °C for 45 s, 94 °C for 30 s, step 2×29, 49 °C for 1 min, 72 °C for 5 min.

Samples were run on a 2% agarose gel stained with 10 µl SYBR safe along with a 100 bp marker, and bands were visualised using a Gel Documentation System (Bio-Rad). Any unclear results were repeated, along with one randomly selected sample from every ten successful samples, to test for potential error.

## 2.11 Avian malaria prevalence testing

To test for the presence of *Haemoproteus* / *Plasmodium* and *Leucocytozoon* parasite DNA within samples (Chapter Five), an existing nested polymerase chain reaction (PCR) protocol (Hellgren et al., 2006) was used. This protocol uses two primer sets, the first to amplify the 478 bp fragment of the mitochondrial cytochrome b, and the second to distinguish between *Plasmodium* or *Haemoproteus* and *Leucocytozoon* species (Table 2.5.). Positive controls were used from birds with known avian malaria infections evident from sequencing results, and negative controls where nuclease-free water was added to reactions in place of DNA template. Presence of DNA in negative samples was later checked for presence of an avian housekeeping gene, an 80 bp fragment of reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Table 2.5.). GAPDH primer sequences were obtained from Atema et al., (2013).

PCR reactions were performed in volumes of 20 µl, where each reaction contained 1.2 µM of each of the two primers, 10 µl GoTaq® universal PCR master mix (Promega), 5.6 µl nuclease free water and 2 µl genomic DNA. PCR reaction was amplified for 20 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s, and then a final extension at 72°C for 10 min.

Table 2.5. Primer sequences for nested PCR reactions.

| Parasite genera | Primer name | Forward sequence |
|---|---|---|
| **Initial PCR** | HaemNFI | 5'-CATATATTAAGAGAAZTATGGAG-3' |
| | HaemNR3 | 5'-ATAGAAAGATAAGAAATACCATTC-3’ |
| ***Haemoproteus/ Plasmodium*** | HaemF | 5’-ATGGTGCTTTCGATATATGCATG-3’ |
| | HaemR2 | 5’-GCATTATCTGGATGTGATAATGGT-3’ |
| ***Leucocytozoon*** | HaemFL | 5’-ATGGTGTTTTAGATACTTACATT-3’ |
| | HaemR2L | 5’-CATTATCTGGATGAGATAATGGZGC-3’ |
| **GAPDH** | FWD | 5’-TGTGATTTCAATGGTGACAGC-3’ |
| | REV | 5’-AGCTTGACAAAATGGTCGTTC-3’ |

A 2 µl aliquot of the products of the first reaction was further amplified with the same thermal profile, but for 35 cycles instead of 20 cycles. For *Haemoproteus/Plasmodium* screening, primers HaemF and HaemR2 were used. For *Leucocytozoon* screening, primers HaemFL and HaemR2L were used. Finally, 13 µl of each product from the second PCR rounds were loaded on a 1% agarose gel (0.5 x TBE; 54 g Tris Base, 27.5g boric acid and 4.65 g EDTA in 1 L distilled water), stained with SYBR Safe (ThermoFisher Scientific, California, USA) and visualised under ultraviolet light.

## 2.12 Quantifying avian malaria infection

To quantify malaria infection in young birds (Chapter Six), a sensitive qPCR protocol newly developed by PhD student Bedur Albalawi (2019) was used. This methodology compares quantities (gene copy number) of a reference bird gene and parasite DNA present in one avian DNA sample, to determine infection intensity using a standard curve created from serial dilution of plasmids. To create the serial dilutions for standard curves, the 18S gene for each of the two parasite genera present in the study system (*Leucocytozoon* and *Haemoproteus*) as well as a reference bird gene (GAPDH), were cloned via bacterial transformation (Albalawi, 2019).

Primers for the qPCR were designed based on the criteria that they were distinguishable between parasite genera *Leucocytozoon* and *Haemoproteus*, but identical among parasite species within each genera, based on alignments from existing sequencing among the study bird population (Table 2.6.). As *Plasmodium* species have not been previously detected in

the study system (Capilla-Lasheras et al., 2017), no primers were designed for *Plasmodium*. For the reference bird gene (GAPDH), existing primer sequences for great tit (Atema et al., 2013) were used. Primers were then tested for cross-reactivity, where samples known to be infected with *Leucocytozoon* were run using *Haemoproteus* primers, and vice versa (Albalawi, 2019).

Table 2.6. qPCR primer sequences used to measure malaria infection intensity.

| **Target Gene** | **Primer** | **Primer sequence** |
|---|---|---|
| ***Leucocytozoon*** | qLeucoF3 | 5'-GCTTCTATCGGTGAACTCTCGA-3' |
| **18S** | qLeucoR2 | 5'-TATTCTTTGCCTGGAGGTAATG-3' |
| ***Haemoproteus*** | qHaemoF2 | 5'-AGCTCACGCATCGCTTCTAA-3' |
| **18S** | qHaemoR1 | 5'-ATTTTCTTTGCCTGGAGGTTAC-3' |
| **GAPDH** | FWD | 5'-TGTGATTTCAATGGTGACAGC-3' |
| | REV | 5'-AGCTTGACAAAATGGTCGTTC-3' |

Each DNA sample was run in duplicates in a separate plate for each of the three target genes (*Haemoproteus*, *Leucocytozoon* and GAPDH). Serial dilutions of plasmid ($10^4$ – $10^0$) was included, as well as two reactions containing nuclease-free water in place of DNA template as negative controls.

The total volume of each qPCR reaction was 15 µl, with 5 µl (12ng/ µl) of DNA template. Reactions were run in MicroAmp Optical 96-well plates (0.1 ml, Applied Biosystems) on Stratagene Mx3000 (ThermoFisher), and contained 7.5 µl SYBR Select Master Mix (Applied Biosystems), and primer and nuclease-free water volumes outlined in Table 2.7.

Quality checks were performed on the data and any duplicates from individual birds with >0.5 cycle threshold (Ct) value were repeated. Amplification efficiency of each plate was calculated using: $E = -1+10^{(-1/slope)}$, where E = 1 indicates 100% efficiency, and values between 0.9-1.1 representing the acceptable range (Kubista et al., 2006). Following qPCR runs, the relative infection intensity of malaria parasites was calculated for each sample as: $x = 2^{-(\text{parasite Ct} - \text{GAPDH Ct})}$.

Table 2.7. Optimal primer concentrations for each target used in the malaria qPCR assay. From (Albalawi, 2019).

| Reagent | Target | | |
|---|---|---|---|
| | ***Haemoproteus*** | ***Leucocytozoon*** | ***GAPDH (100nM)*** |
| **SYBR Select Master Mix volume (Applied Biosystems)** | 7.5 µl | 7.5 µl | 7.5 µl |
| **Primer concentration (volume)** | 600/600 nM (1.8 µl) | 300/300 nM (0.9 µl) | 100/100 nM (0.3 µl) |
| **Nuclease-free water volume** | 0.7 µl | 1.6 µl | 2.2 µl |
| **Total volume** | 15 µl | 15 µl | 15 µl |
| **Annealing temperature (°C)** | 57 °C | 58 °C | 60 °C |

## 2.13 QuantiGene® Plex RNA assay

For quantification of transcript levels from RNA samples in Chapters Four, Five and Six, a platform for gene expression analysis, the QuantiGene® Plex RNA assay 2.0 (ThermoFisher), was used. QuantiGene® Plex is used as an alternative to RNA-seq and RT-qPCR, and was chosen for this study due to its ability to provide simultaneous readouts of relative gene expression of multiple target genes from a single sample (Gunawardana et al., 2019; Mills and Gallagher, 2016; Xue et al., 2013).

### 2.13.1 Overview of QuantiGene® Plex

The QuantiGene® Plex assay combined branched DNA amplification and multi-analyte profiling beads (xMAP®) to detect and quantify RNA targets in samples (Affymetrix, 2015). The workflow of the QuantiGene® Plex assay consisted of several stages, including sample preparation, target hybridisation, signal amplification and signal detection (Figure 2.2.), which took place across two days per assay run. On the first day of assay setup, samples were lysed and incubated overnight along with signal amplifiers. On the second day, there were several wash steps which aided signal amplification, followed by quantification of fluorescence signal of each target.

The QuantiGene® Plex assay worked as follows: custom designed gene probes for the target species were incubated along with blood samples containing RNA and xMAP® beads, and during wash steps on the second day of the assay workflow, created an amplification unit from RNA within the sample. Each amplification unit contained

hybridisation sites for Label Probes, which bound fluorescent protein Streptavidin-conjugated R-Phycoerythrin (SAPE). The fluorescence signal associated with individual Capture Beads was then read on a Luminex® MAGPIX flow cytometer and was reported as median fluorescence intensity (MFI). MFI was proportional to the number of target RNA molecules present in the sample (Affymetrix, 2015), and therefore was used as a proxy for gene transcript levels within the sample.

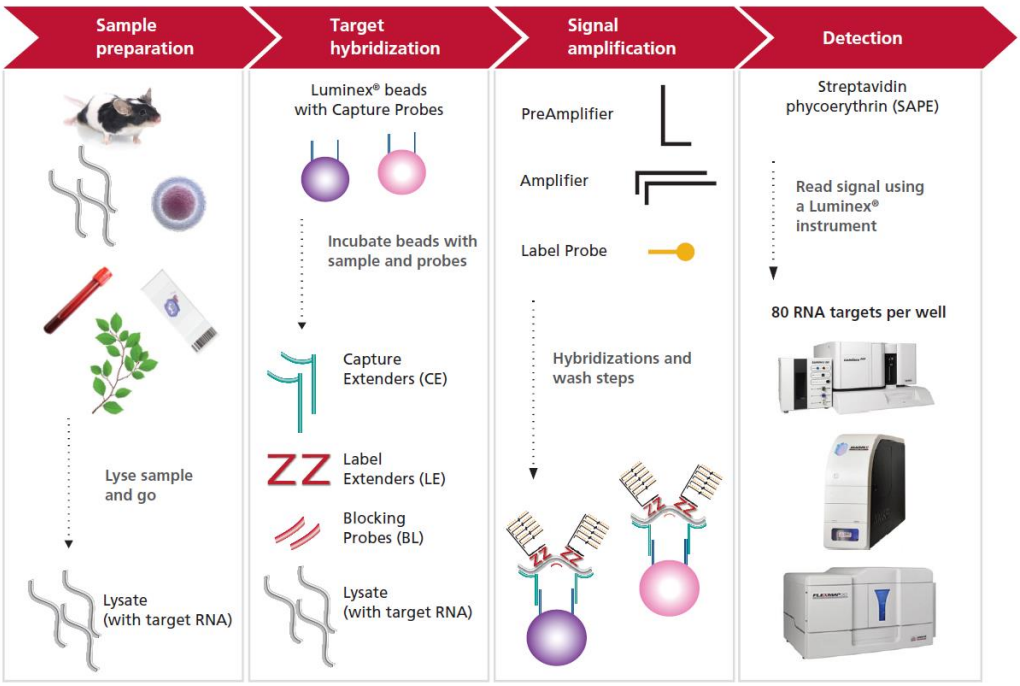



Figure 2.2. QuantiGene® Plex Assay workflow. Diagram obtained from Affymetrix, ThermoFisher (Affymetrix, 2015)

### 2.13.2 Gene targets

Eighteen probes were commissioned for circadian clock and immune gene targets of interest for great tits (*Parus major*) from ThermoFisher, using sequence information for great tit targets obtained from NCBI database (see Appendix 1). Additionally, probes for three housekeeping gene targets for great tits were commissioned. A list of gene targets, and the chapters in this thesis that they are included in, can be found in Table 2.8. An overview of all gene targets included in this thesis, and their main functions, is shown in Table 2.9.

Table 2.8. A list of gene targets used in this thesis, the gene type (Housekeeping, Clock or Immune targets), and the chapters that they are included in.

| **Gene target** | **Type** | Ch.4 | Ch. 5 | Ch. 6 |
|---|---|---|---|---|
| ***RPL19*** | Housekeeping | X | X | X |
| ***SDHA*** | Housekeeping | X | X | X |
| ***HMBS*** | Housekeeping | X | X | X |
| ***BMAL1*** | Clock | X | - | X |
| ***CLOCK*** | Clock | X | - | X |
| ***CRY1*** | Clock | X | - | X |
| ***CKA*** | Clock | - | - | X |
| ***CK1E*** | Clock | X | - | X |
| ***AANAT*** | Clock | X | - | X |
| ***PER2*** | Clock | X | - | X |
| ***REVERBA*** | Clock | X | - | X |
| ***LY86*** | Immune | X | X | X |
| ***TLR4*** | Immune | X | X | X |
| ***IL1*** | Immune | X | X | X |
| ***IL6*** | Immune | X | X | X |
| ***IGF1*** | Immune | X | X | X |
| ***PRKCA*** | Immune | X | X | X |
| ***GATA3*** | Immune | X | X | X |
| ***IKBA*** | Immune | X | X | X |
| ***NKRF*** | Immune | X | X | X |
| ***NRF2*** | Immune | X | X | X |

Table 2.9 An overview of gene targets used in this thesis and their main functions.

| Gene | Gene Type | Function | Citation(s) |
|---|---|---|---|
| **RPL19** | Housekeeping | Ribosomal Protein L19<br><br>This gene encodes a ribosomal protein of the 60S subunit. | (Olias et al., 2014) |
| **SDHA** | Housekeeping | Succinate Dehydrogenase Complex Flavoprotein Subunit A<br><br>This gene is involved in the mitochondrial respiratory chain. | (Olias et al., 2014) |
| **HMBS** | Housekeeping | Hydroxymethylbilane Synthase<br><br>This gene encodes a member of the hydroxymethylbilane synthase family | (Olias et al., 2014) |
| **BMAL1 / ARNTL** | Clock | Brain and Muscle ARNT-Like 1<br><br>Transcriptional activator which is a core component of the circadian clock. | (Reppert and Weaver, 2002; Shearman et al., 2000) |
| **CLOCK** | Clock | Circadian Locomotor Output Cycles Protein Kaput<br><br>This gene is involved in the regulation of the circadian clock. | (Gekakis et al., 1998; Reppert and Weaver, 2002; Shearman et al., 2000) |
| **CRY1** | Clock | Cryptochrome Circadian Regulator 1<br><br>This gene is involved in the core circadian oscillator. Upregulated by CLOCK/BMAL1 heterodimers. | (Kume et al., 1999; Reppert and Weaver, 2002) |
| **CKalpha** | Clock | Choline Kinase Alpha<br><br>CKa provides a molecular substrate for the actions of melatonin | (Reppert et al., 1995) |
| **CK1E** | Clock | Casein Kinase 1 Epsilon<br><br>This protein phosphorylates PER, involved in the circadian clock. | (Steinmeyer et al., 2012) |
| **AANAT** | Clock | Aralkylamine N-Acetyltransferase<br><br>Involved in melatonin biosynthesis, controls the day-night rhythm in melatonin production in the vertebrate pineal gland. | (Jin et al., 2011; Turkowska et al., 2014) |
| **REVERBa / NR1D1** | Clock | Nuclear Receptor Subfamily 1 Group D Member 1<br><br>Represses the circadian clock transcription factor BMAL1. | (Reppert and Weaver, 2002; Ueda et al., 2005) |
| **NKRF** | Immune | NF-Kappa-B-Repressing Factor<br><br>This gene mediates transcriptional repression of NFKB responsive genes which control production of cytokines. | (Lu et al., 2011) |
| **NRF2 / NFE2L2** | Immune | Nuclear Factor, Erythroid 2 Like 2<br><br>The encoded transcription factor regulates genes which contain antioxidant response elements; many of these genes encode proteins involved in response to injury and inflammation | (Kensler et al., 2007; Mills and Gallagher, 2016) |

| | | | |
|---|---|---|---|
| | | which includes the production of free radicals. | |
| **PER2** | Clock | Period Circadian Regulator 2<br><br>Involved in the core circadian clock loop. Upregulated by CLOCK/BMAL1 heterodimers. | (Reppert and Weaver, 2002) |
| **LY86** | Immune | Lymphocyte Antigen 86<br><br>Involved in the innate immune response to bacterial lipopolysaccharide (LPS) and cytokine production. | (Capilla-Lasheras et al., 2017) |
| **TLR4** | Immune | Toll Like Receptor 4<br><br>Plays a fundamental role in pathogen recognition and activation of innate immunity. | (Akira and Takeda, 2004; Vallance et al., 2017) |
| **IL1** | Immune | Interleukin 1 Beta<br><br>An important mediator of the inflammatory response, involved in cell proliferation, differentiation, and apoptosis. | (Akira and Takeda, 2004; Cuesta et al., 2016) |
| **IL6** | Immune | Interleukin 6<br><br>Involved in inflammation and the maturation of B cells. | (Cuesta et al., 2016; Mishra et al., 2019) |
| **IGF1** | Immune | Insulin Like Growth Factor 1<br><br>Similar to insulin in function, and is involved in mediating growth and development. | (Holzenberger et al., 2003; Lupu et al., 2001) |
| **PRKCA** | Immune | Protein Kinase C Alpha<br><br>Involved in cellular signaling pathways, and serve as receptors for tumour promoters. | (Nishikawa et al., 1997) |
| **GATA3** | Immune | GATA Binding Protein 3<br><br>A regulator of T-cell development and plays an important role in endothelial cell biology. | (Capilla-Lasheras et al., 2017; Wang et al., 2011) |
| **IKBa / NFKBIA** | Immune | NFKB Inhibitor Alpha<br><br>Inhibits NFKB, which is involved in inflammatory responses. | (Cabannes et al., 1999) |

### 2.13.3 Recommended settings for QuantiGene® Plex RNA assay using avian blood samples

As the QuantiGene® Plex RNA assay had not previously been run for passerine blood samples, assay protocols needed to be optimised for use in this thesis. Initial assay runs carried out using great tit whole blood samples resulted in low florescence signals for all targets, possibly due to the high viscosity of passerine blood preventing probes from binding to target RNA during the overnight incubation step. The next section outlined recommended settings for running of the QuantiGene® Plex RNA assay using passerine blood, after optimisation.

This methodology has been adapted from QuantiGene® Sample Processing Kit and QuantiGene® Plex RNA assay protocols (Affymetrix, 2015). Prior to assay setup, great tit lysates were prepared from blood samples previously frozen at -40°C and thawed on ice. Samples were subject to centrifugation for 3 min at 6000 rpm, and RNA*later*® stabilising solution was carefully removed from each sample using a pouring method and pipetting. Whole Blood Working Lysis Mixture was prepared using 1472 µl lysis mix (pre-warmed at 37°C), 2254 µl nuclease-free water and 92 µl proteinase K. From this, 84 µl of Whole Blood Working Lysis Mixture was added to 12 µl of the blood pellet of each sample in a 1.5 ml eppendorf. Samples were then briefly vortexed and placed in a shaking incubator at 60°C and at 250 RPM for one hour.

#### QuantiGene® Plex RNA assay setup (Day one)

Assays were prepared in a low-skirted 96 well plate (Invitrogen) and samples were all run in technical duplicates. Working Bead Mix was prepared by combining QuantiGene® Plex kit reagents (Affymetrix, 2015), in the order listed in Table 2.10. The final proportions of this mix were always 33% Lysis Mixture. Capture Beads were taken out of storage right before use and protected from light using foil. Once Capture Beads had been added to the Working Bead Mix, the mix was kept at room temperature to avoid freezing and shattering of beads.

Table 2.10. Reagents contained in Working Bead Mix for QuantiGene® Plex RNA assay.

| Order of addition | Reagent | 1 well (µl) | 96 wells (µl) |
|---|---|---|---|
| **1** | Nuclease-free Water | 2.6 | 250 |
| **2** | Lysis Mixture | 3.3 | 317 |
| **3** | Blocking Reagent | 1 | 96 |
| **4** | Proteinase K | 0.1 | 9.6 |
| **5** | Capture Beads (vortex 30 secs prior to adding) | 0.5 | 48 |
| **6** | Probe Set | 2.5 | 240 |
| **Total** | | 10 µl | 960 µl |

Each sample was diluted to 1:8 using Diluted Lysis Mixture (one volume of Lysis Mixture, plus two volumes of nuclease-free water, prepared fresh), where 35 µl of Diluted Lysis Mixture was added to each well. Lysed whole blood samples were removed from incubation and subjected to centrifugation at 6000 RPM for one minute. Working Bead Mix was vortexed for 10 seconds, then pipetted 10 µl into each well of the 96 well plate. Finally, 5 µl of the lysed supernatant of each sample was added into wells of the new plate. The total final volume of each well was 50 µl. For background control wells, 40 µl of diluted Lysis Mixture (one volume Lysis Mixture plus two volumes nuclease-free water) were added along with 10 µl Working Bead Mix. Plates were then sealed using a clear Pressure Seal by removal of the backing and placing seal onto the plate. The assay plate was then covered with aluminium foil and placed into a shaking incubator. Plate was incubated overnight at 54°C ± 1°C at 250 RPM, and any unused lysed samples were placed into storage at -80°C.

### QuantiGene® Plex® RNA assay setup (Day two)

On day two of the assay, the plate was removed from incubation and secured onto the Hand-Held Magnetic Plate Washer (Affymetrix) and then carefully unsealed. After one minute of acclimatisation, the assay plate was inverted into the sink to remove any sample not bound to magnetic Capture Beads. Using a multichannel pipette, 50 µl wash buffer (prepared using 300 µl Wash Buffer Component 1, 5 ml Wash Buffer Component 2 and 47.5 ml nuclease-free water) was added to each well. After 30 seconds, the plate was

inverted into the sink again. These wash steps were repeated twice more, for a total of three washes.

Next, 50 µl Pre-Amplifier solution was added to each well, before plate was resealed, removed from the hand-held magnet and placed into the shaking incubator at 50°C ± 1°C at 250 RPM for one hour. The three washes and hour of incubation at 50°C ± 1°C and 250 RPM steps were repeated in the same way, between additions of the following reagents in each well: 1) 50 µl Amplifier solution; 2) 50 µl Label Probe solution; 3) SAPE Working Reagent (prepared with 18 µl SAPE and 6000 µl SAPE Diluent). The last step was followed by a 30 minute incubation at room temperature and at 250 RPM. Wash steps were then repeated, using SAPE Wash Buffer in place of Wash Buffer. Finally, 130 µl SAPE Wash Buffer was added to each well and assay plate was shaken on a plate shaker at 750 rpm for 3 min, before immediate reading on the Luminex® MAGPIX instrument.

### Extraction and initial analyses of QuantiGene® data

Following reading of the assay plate on the Luminex® MAGPIX, Median Fluorescence Intensity (MFI) data were extracted into Microsoft Excel (2019). Data were visually inspected and assay wells were excluded from further analyses if the total Capture Bead count for that well did not exceed 50, as fluorescence was deemed too low for viable results (Thermofisher Technical Support, personal communication). To normalise fluorescence values, MFI values of each well were divided by the mean of the four background wells for each plate. MFI values were then normalised to housekeeping genes, by dividing the MFI value for each well by the geometric mean of the MFI of the three housekeeping genes (*RPL19, SDHA, HMBS*). Finally, the coefficient of variation for all technical duplicate wells was calculated. Technical duplicates with a coefficient of variation >35% were excluded from all further analyses, following advice from Thermofisher Technical Support.

# Chapter Three: Individual timing of incubation activities in city and forest birds, and implications for offspring fitness

## 3.1 Introduction

Circadian clocks are internal timekeepers regulating the behavioural and physiological rhythms of organisms with a period length of around 24 hours. Clocks are synchronised by daily changes within an organisms' environment, such as photoperiod and fluctuations in ambient temperature, by a process known as entrainment (Golombek and Rosenstein, 2010).

Individuals often differ in entrainment phase between internal clocks and the synchronising cues within their environment, leading to consistently advanced or delayed rhythms (Roenneberg et al., 2013). These differences in phase can give rise to various circadian phenotypes, where timing of activity differs relative to daylight. This is known as an individual's "chronotype" and is most obviously expressed by individual preference for timing of sleep and activity. "Early" chronotypes usually prefer to sleep earlier than "late" chronotypes (Roenneberg et al., 2003). Chronotype is a highly repeatable personality measure, and is usually classified by the midpoint of sleep (Roenneberg et al., 2003). Alternatively, the daily onset of timing of activity (wake-up time) has been used as a proxy for chronotype (Dominoni et al., 2013).

Individual chronotypes of animals have been documented through captive studies, and include fish, rodent and bird species (Amin et al., 2016; Labyak et al., 1997; Lehmann et al., 2012; Refinetti, 2006). In wild animals, chronotype is harder to study, given that it requires repeatable measurements of individual behaviour in relation to environmental cues, but individual chronotypes have been shown in mammals and birds (Graham et al., 2017; Ocampo-Garcés et al., 2006; Mueller et al., 2012).

For wild animals, many risks and opportunities, such as the presence of predators, food availability, and competition from conspecifics, depend on the time of day. Therefore, differences in individual chronotype may provide wild animals with different consequences for fitness, and may be under selection (Helm et al., 2017). Indeed, emerging evidence suggests that some differences in individual timing may prove advantageous. Experimental studies on passerines have demonstrated that individuals that rise earlier

often have higher reproductive success (Greives et al., 2015; Kempenaers et al., 2010; Poesel et al., 2006), suggesting that for blue tits and great tits, an earlier chronotype is beneficial. Other birds may benefit from extending their activity into the night (Russ et al., 2015). Given that the environmental opportunities as well as risks presented to wild animals are time-dependent, different chronotypes will have varied fitness consequences under different environmental conditions (Helm et al., 2017).

Environmental features that influence individual chronotype are becoming increasingly complex, as human changes to the natural habitat alter the selection pressures incurred on wild animals. Growing evidence suggests that human activity is having substantial effects on wild species' rhythms, including a global shift of activity into the night in response to human disturbance (Gaynor et al., 2018). Moreover, a recent study on the effects of climate change suggested that diurnal mammals may shift their activity into the night, as a buffer for temperature increases (Levy et al., 2019).

One dramatic change to the natural environment by humans is the presence of artificial light at night (ALAN). Exposure to ALAN has significant effects on timing of activity in wild birds, where species living in artificially-lit environments often begin their activity earlier in the morning (Da Silva et al., 2015; Kempenaers et al., 2010). These extensions of activity have also been found in experimental studies of ALAN exposure (de Jong et al., 2016; Raap et al., 2016c). Additionally, the presence of ALAN has been associated with an earlier onset of daily activity in urban birds, such as advanced timing of song in a number of passerine species (Kempenaers et al., 2010). Another study on city blackbirds (*Turdus merula*) noted an advanced onset of activity, but no association between light at night and the end of daily activity (Dominoni et al., 2014).

Differences in individual activity and chronotype arising from human changes to the environment may have fitness consequences for birds (Dominoni et al., 2016). One study on city birds revealed individuals living under ALAN rose earlier, and interestingly, had higher levels of extra-pair paternity than those without light at night in their territory (Kempenaers et al., 2010). It is therefore highly possible that human changes to the natural environment, such as the addition of ALAN, may alter the current selection pressures on activity patterns and individual chronotype in wild animals.

Aside from changes to chronotype, current research suggests that animals living in the city environment may be experiencing restless nights, possibly as a result of increased levels of ALAN (Alaasam et al., 2018; Ouyang et al., 2017). Living in the city environment may lead to disruption of natural circadian rhythms; a captive study revealed city blackbirds to

have less robust rhythms of behavioural activity than their forest conspecifics (Dominoni et al., 2013). Chronodisruption has huge negative implications for health (Dominoni, 2015; Preuss et al., 2008). Wild birds living in city environments therefore may, through chronic chronodisruption, experience a decline in their fitness. Evidently, there is pressing need for studies exploring the effects of the city environment on the activity rhythms, chronotype and fitness measures of wild birds.

One crucial avian life history stage where timing of behavioural activity is important for fitness is incubation, the process by which embryos receive an external supply of thermal energy for development (Deeming, 2002). During incubation, parents must maintain constantly high egg temperatures, as even short bouts of cooling can slow nestling development (Nord and Nilsson, 2011). Longer disruptions to incubation affect nestling prospects (Berntsen and Bech, 2016; Hepp et al., 2015). However, there is large variation in what is considered to be "normal" nest temperatures, due to parents periodically leaving the nest during the day to feed (Webb, 1987; Ar & Sidis, 2002). These nest "off-bouts" are interspersed between longer "on-bouts" of incubation on the nest (Cooper and Voss, 2013). Timing of the parent's first "off-bout" from the nest in the morning marks the onset of daily activity, and the last off-bout marks the end of daily activity (Gwinner et al., 2018). The length of incubation bouts vary, even within a species (Bulla et al., 2016).

During the incubation period, parents may alter behaviours depending on environmental conditions (Gwinner et al., 2018; Hainsworth et al., 1998). In the city environment, conditions are challenging for birds, with the presence of ALAN (Dominoni and Nelson, 2018), ambient noise (Klett-Mingo et al., 2016), pollutants (Isaksson et al., 2009) and reduced quality and availability of food (Pollock et al., 2017). Wild birds may alter their activity rhythms during incubation, to better suit the city environment. However, these changes in incubation patterns may have implications for the fitness of offspring, contributing to the overall decline of bird species associated with urbanisation (Sol et al., 2014).

## 3.2 Study aims and hypotheses

This study aimed to close the gap in knowledge on the chronotype and urban ecology of wild birds, by comparing daily timing and robustness of incubation activities of wild great tits living in city and forest environments. Furthermore, this study was unique in its aim to investigate the effects of differences in chronotype of wild great tits in city and forest environments on their reproductive success, and subsequent fitness, of offspring.

Increasingly affordable technologies such as thermal sensors and radio-frequency identification (RFID) readers have facilitated studies of incubation rhythms in the wild (Bulla et al., 2016; Graham et al., 2017). Thermal sensors can be placed into nests and behavioural activity can be inferred from the warmth of females while on the nest (Capilla-Lasheras, 2018). Great tits are uniparental intermittent incubators, with the full incubation period lasting for around thirteen days (Gosler, 1993; Haftorn, 1981), and therefore in this study it was possible to measure repeatable behavioural patterns of individual adult females for chronotyping. Individual chronotypes of females were determined by the onset of daily activity as in Dominoni et al., (2013), as morning timing has most relevance for avian ecology (Kacelnik and Krebs, 1983; Kempenaers et al., 2010; Poesel et al., 2006), and is highly repeatable in great tits (Stuber et al., 2015).

Timing of offset of activity was also measured in this study, to determine the duration of daily activity of each bird. Offset of activity was determined as the last daily on-bout to the nest. Nest attendance was measured as the percentage of time a bird is within a nest, assuming the female was in contact with eggs (Skutch, 1962). Lastly, night-time temperature variance was used as a measure for restlessness (Gwinner et al., 2018).

It was predicted that females incubating in the city environment would have less robust rhythms than forest conspecifics, due to the potential effects of ALAN on timing information from natural zeitgebers. This would give rise to differences in daily incubation activities in city birds, such as higher night-time restlessness (from sleep disturbance due to ALAN) and lower percentage of time spent in nest box. Furthermore, city birds were predicted to have an earlier onset of activity (chronotype), later offset of activity, and longer duration of daily activity.

Parameters of offspring fitness used in this study were hatching success, fledging success and nestling weight at day thirteen. The weight of nestlings was used as a fitness proxy given that heavier fledglings have a better chance of survival during the post-fledgling

period compared with lighter fledglings (Perrins, 1965). It was predicted that overall, city nestlings would have lower hatching success, weight and fledging success than forest counterparts as in previous studies in this system (Pollock et al., 2017). An association between chronotype of the mother, and the weight, hatching, and fledging success of her nestlings was expected. Given that in city environments, males with earlier chronotypes have higher reproductive success (Kempenaers et al., 2010), it was expected that nestlings whose mothers have an early chronotype would weigh more than those whose mother had a later chronotype. Likewise, it was expected that this effect was stronger in the city, where pressure to be early appears to be particularly high (Kempenaers et al., 2010; Dominoni et al., 2013). Ultimately, it was predicted that within each habitat, early birds relative to the others within the habitat do well and have better reproductive success.

## 3.3 Materials and Methods

### 3.3.1 Study sites

Data for this chapter were obtained during the breeding seasons (May-June) of 2016, 2017 and 2018 in free-living populations of nesting great tits. Field sites were existing forest nest box study systems located at the Scottish Centre for Ecology and the Natural Environment (SCENE; 56º 7.73'N, 4º 36.79'W), Cashel Forest (56°6'N, 4°34'W), and city sites at Kelvingrove Park, Glasgow (55°52' N, 4°17'W) and Garscube estate (55º 9'N, 4º31' W). All sites have been previously used in ecological studies of wild passerines (Pollock et al., 2017; Capilla-Lasheras et al., 2017). See General Methods for more information on study sites.

### 3.3.2 iButton temperature sensors

iButtons (DS1922L-F5, Thermochron) are small, cylindrical sensors using 1-Wire ® technology that can be pre-programmed to take temperature readings at equal intervals. In this study, iButtons were pre-programmed at a resolution of 0.0625 ºC and a sampling interval of three minutes to record within nest temperatures. Pre-programmed iButtons were covered with a small piece of material and wrapped together with a thin wire before insertion into great tit nests (Figure 3.1.). iButtons were inserted into nests containing three or more eggs prior to, or as close as possible to the start of, the incubation period. iButtons were placed carefully next to eggs and the wire thread through to the bottom of the nest and attached to a small 28.35 g fishing weight to secure its position within the box.

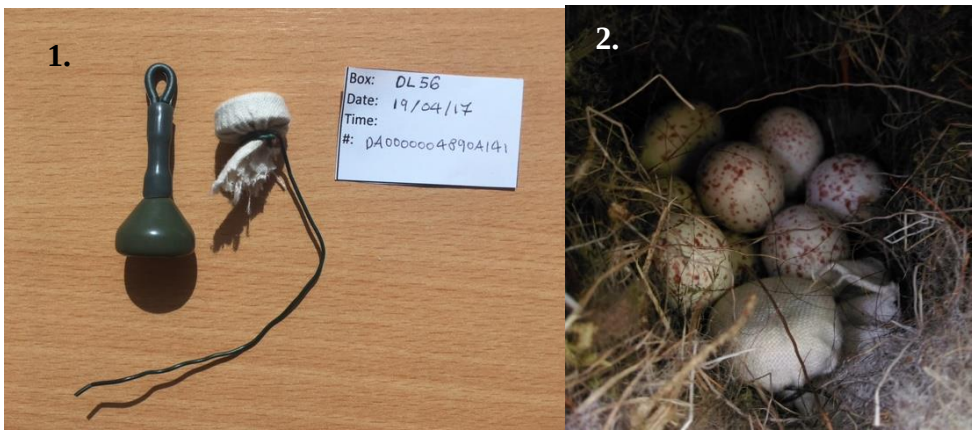



Figure 3.1. iButton setup for incubation data collection. 3.1.1. Pre-programmed iButton wrapped in material and attached to wire, 28.35 g fishing weight, label containing serial number, date and target nest box. 3.1.2. iButton positioned in nest, alongside eggs.

Additionally, two iButtons were pre-programmed to record ambient temperature data at each field site with a resolution of 0.5 ºC and sampling interval of five minutes, for direct reference to in-nest recordings during iButton data analyses. These iButtons were attached to the outside of nest boxes by taping them to the bottom of the box.

In years 2016 and 2017, replacement of iButtons took place weekly (due to limited data storage capacity on individual iButtons) to ensure that no gaps were left in the recordings. In 2018, iButtons were programmed to roll-over once data capacity was reached, and weekly readings were taken in the field using a TDHC Data Downloader (Thermodata Corporation, Milwaukee, WI). iButton data were downloaded as .csv files and read using OneWire Viewer software (Maxim Integrated, California). Sample sizes for the study are shown in Table 3.1.

Table 3.1. Sample sizes for total number of days of iButton recordings (and total number of nest boxes) per site and study year

| | Site | |
|---|---|---|
| **Year** | **City** | **Forest** |
| **2016** | 14 (4) | 218 (30) |
| **2017** | 104 (11) | 203 (34) |
| **2018** | 89 (12) | 104 (12) |

### 3.3.3 MET Office data

Environmental temperature data for Daily Mean Temperature (°C) were obtained for each year from the UK Met Office for an area nearby to forest field sites, Tyndrum (56°25'39.57"N, 4°42'18.20"W), and nearby to city sites, Bishopton (55°54'24.75"N, 4°30'11.20"W), to incorporate into statistical models.

### 3.3.4 Nestling processing

All nestlings (N = 253) were ringed for individual identification on day thirteen of life. Measurements were taken of nestling weight in all study years. To determine hatch dates, brood sizes and fledging success, all nest boxes were checked weekly during the breeding

season as part of a general nest box monitoring protocol (see General Methods for further details of nest box monitoring).

### 3.3.5 Ethical statement

See General Methods for details of ringing licences.

### 3.3.6 Data analyses

Data analyses were carried out using R v. 3.4.4 (R Core Team, 2018). All statistical analyses were performed using packages *lme4* (Bates et al., 2014). Initial global models for response variables were linear-mixed models (LMM) with Gaussian error distribution. All global models included individual nest box as a random factor to account for repeated measures within the same nest box. Residuals were plotted and inspected to check assumptions of normality. Pairs of explanatory variables were also assessed for collinearity by calculating tolerance and variance inflation factors (VIF) and by visual inspection of pairwise plots, before statistical relevance was accepted.

Statistical relevance of fixed factors within all models was determined by dropwise model selection via Likelihood Ratio Testing using package *lmtest* (Zeileis & Hothorn, 2002), until the minimum adequate model (MAM) was found. Factors with a significance level of <5% were kept in the model. Site was always kept within the MAM regardless of significance.

### 3.3.7 Incubation timing analysis

Prior to statistical modelling, data recordings from iButtons were visualised using ggplot2 (Wickham, 2016). During data collection, some iButtons were pushed to the side of the nest box and did not successfully record within nest temperatures. Therefore, recordings that were identified by visual inspection to closely follow the pattern of environmental temperatures at the field site were removed from the dataset. This was done blind of site where recordings were taken. Analyses were restricted to up to sixteen days prior to the hatch date, and also after all eggs had been laid, as sporadic incubation behaviour may begin prior to clutch completion (Marasco and Spencer, 2015). In total, 732 days of iButton recordings were included in the final analysis, where 207 were taken from nest

boxes at urban sites and 525 from nest boxes at forest sites, using some data as a control group for another experiment in McGlade et al., (2020, *in prep*).

An R package, incR (Capilla-Lasheras, 2018; Gwinner et al., 2018), was used to determine parameters of incubation using iButton data. The incR package uses gradients of temperatures between the nest (around 35 ºC in incubating great tits) and the environment (between 5 and 15 ºC) to predict if a bird is currently sitting on the nest (Capilla-Lasheras, 2018). Using the incR algorithm, several parameters of incubation behaviour were determined for each nest box: first off-bout, last on-bout, duration of active day, and the percentage of time spent on the nest on a given day. To account for differences in photoperiod across the breeding season, activity onset (relative to sunrise) for each bird was calculated for each day of recordings by subtracting the timing of sunrise from the timing of the first off-bout on that day. Similarly, activity offset was determined for each day of recordings by subtracting the timing of sunset from the timing of the last on-bout that day. Timing information for sunrise and sunset at SCENE (56º 7.73’N, 4º 36.79’W) and Glasgow (55°52'N, 4°15'W) were obtained from *www.timeanddate.com*.

Duration of daily activity was calculated for each bird as the daily offset of activity subtracted by daily onset of activity. Percentage of time in nest box over a 24 hour period was calculated as the total time a bird was predicted by incR to be on the nest and in contact with eggs (Gwinner et al., 2018). Night-time nest temperature variance was used as a determinant for restlessness during incubation, as in Gwinner et al., (2018). Night-time restlessness was calculated from 22:00 – 03:00 GMT for each 24-hour period, with the assumption that all birds were within nest boxes during this time period.

Explanatory variables within initial global models for incubation activity include site as a two-level fixed factor (city/forest), the number of days before hatching, clutch size as the maximal number of eggs within a nest, recording date in April day format (where April 1$^{st}$ = day 1), and study year (2016/2017/2018). Mean ambient temperature was also included in global models as low temperatures may affect incubation bout length (Conway & Martin, 2000). The number of days before hatching was included in all models, both as a linear term and as a quadratic, as in (Gwinner et al., 2018). Additionally, interactions between the number of days before hatching and site were included in all models.

### 3.3.8 Analyses of nestling fitness data

To test whether chronotype predicts fitness of offspring, the mean activity onset of the mother was used as a proxy for chronotype. Then, chronotype of the mother was used as a predictor of nestling hatching success, weight at day thirteen, and fledging success. Nestling weight data were modelled using a Gaussian error distribution, with explanatory variables for study year (2016/2017/2018), hatch date (where April 1$^{st}$ = day 1), brood size, site (city/forest) and mother's chronotype (mean activity onset). Hatching success and fledging success data were modelled using Binomial error distributions, with additional explanatory variables for study year (2016/2017/2018), clutch size (brood size for fledging success), and mother's chronotype (mean activity onset). Nest box was included in all models as a random factor. An interaction was also included between site and chronotype in all models. Statistical relevance of fixed factors within all models was again determined by dropwise model selection via Likelihood Ratio Testing using the package *lmtest* (Zeileis & Hothorn, 2002).

## 3.4 Results

### 3.4.1 Timing of incubation activities

Over the duration of the study, onset of daily activity (relative to sunrise) ranged from 03:42 until 07:14. Onset of daily activity was not affected by the clutch size, recording date, mean ambient temperatures or study year (Table 3.3). The onset of daily activity became progressively later as incubation period progressed closer to hatching ($t_{2,732}$ = -2.15; P=0.024, LMM), (Figure 3.2.). This trend was stronger in forest birds than in city birds, as indicated by the significant interaction between site and days before hatching (Figure 3.2.; Table 3.3). Forest birds had an overall later onset of activity than city birds, where forest birds were estimated to leave their nest box 10 ± 3:55 minutes later than city birds (Table 3.3.).

Table 3.2. Minimum adequate models after likelihood ratio testing using incubation activity data. The global model for all incubation activity data was: Nest box + site + site*days before hatching$^2$ + site*days before hatching + days before hatching$^2$ + days before hatching + clutch size + mean temperature + recording date + study year. Nest box was included in all models as a random factor. Data were modelled using Gaussian error distribution. An interaction between terms is denoted by an asterisk (*). Addition of terms is denoted by a plus sign (+).

| Variable | Minimum Adequate Model (MAM) |
|---|---|
| **Onset of activity (relative to sunrise)** | Nest box + site + site*days before hatching² + site*days before hatching + days before hatching² + days before hatching + year |
| **Offset of activity (relative to sunset)** | Nest box + site + site*days before hatching² + site*days before hatching + days before hatching² + days before hatching + recording date + clutch size + year |
| **Duration of active day** | Nest box + site + site*days before hatching² + site*days before hatching + days before hatching² + days before hatching + year |
| **Percentage time in box** | Nest box + site + site*days before hatching² + site*days before hatching + days before hatching² + days before hatching + year |
| **Night time restlessness** | Nest box + site + site*days before hatching + days before hatching + year |

The offset of daily activity (relative to sunset) over the duration of the study ranged from 17:03 until 22:01. There were no effects of mean ambient temperatures or study year on offset of activity (Table 3.3). Offset of activity became progressively earlier as incubation period came closer to hatch date ($t_{2,708}$ = 4.07; P<0.001, LMM), and this trend was stronger in forest birds than in city birds as indicated by the significant interaction between site and days before hatching (Figure 3.2.; Table 3.3). Forest birds had an earlier offset of activity

than city birds, where forest birds were estimated to retire into nest boxes 10.35 (s.e. ± 06:59) minutes earlier than city birds (Table 3.3).

Offset of activity also was significantly affected by date of recording, where offset of activity was later as the season progressed ($t_{1,708}$ = -6.14, P<0.001, LMM), (Table 3.3).

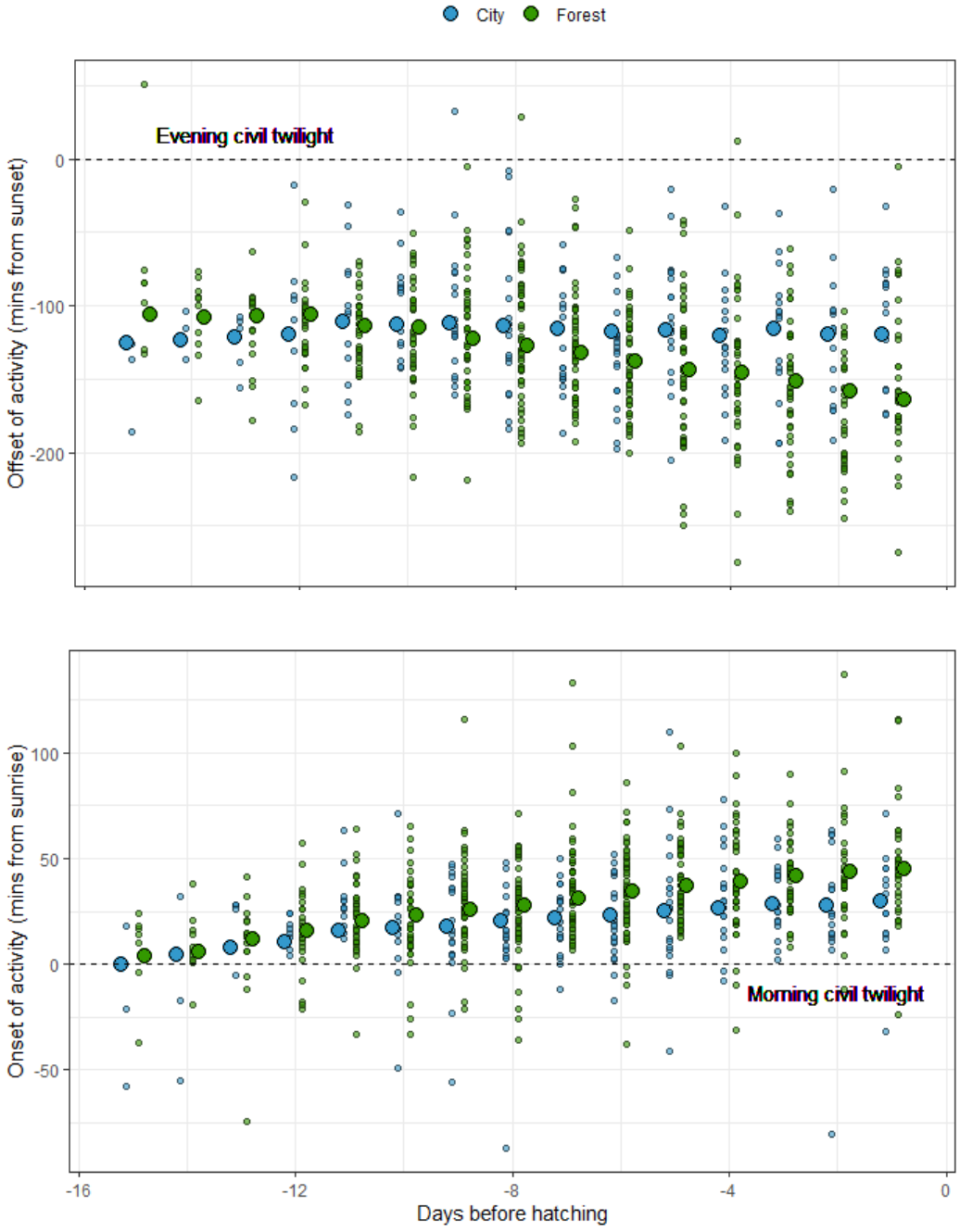



Figure 3.2. Offset (top) and onset (bottom) of activity in city (blue) and forest (green) birds, relative to sunrise and sunset and the number of days before hatching. Large dots represent the mean minimum adequate model predictions of activity onset/offset for that day. Dotted lines represent timing of morning and evening civil twilight, the time of day when the sun first rises in the morning or last sinks in the evening, respectively, below -6 º relative to the horizon.

Duration of activity across the study ranged from 660 minutes (11 hours) to 1011 minutes (16 hours 51 minutes). Duration of activity was not associated with mean ambient temperature, clutch size, recording date or study year (Table 3.3). As with onset and offset of activity, duration of activity was associated with the number of days before hatching ($t_{1,708}$= -6.03; P<0.001, LMM). The observed effect, a shortening of the active day closer to hatching, was stronger in forest birds than city birds as indicated by the significant interaction between site and days before hatching and steeper drop for forest birds (Figure 3.3.; Table 3.3). During early incubation, the active day was shorter in city birds, but this difference tended to reverse close to hatching (Figure 3.3.).

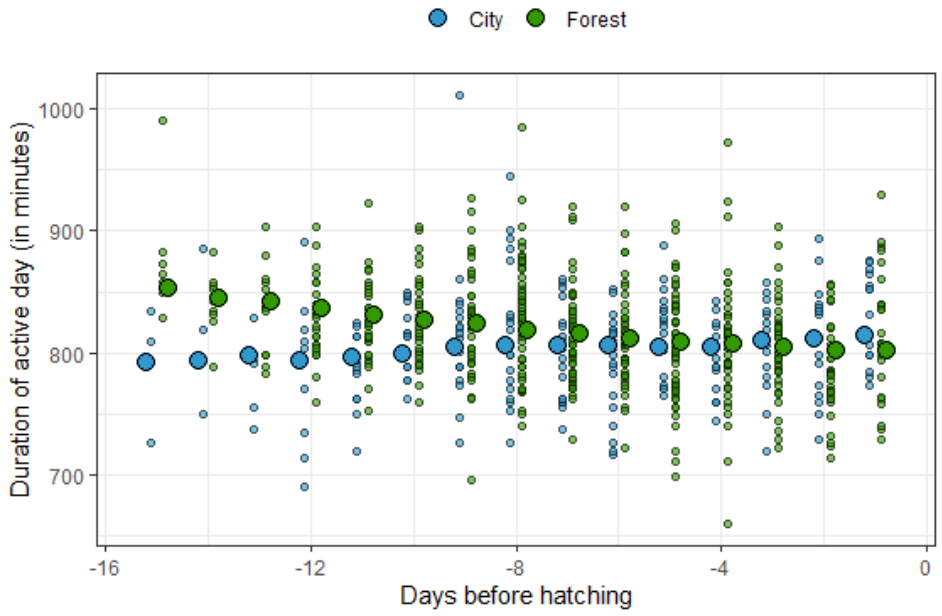



Figure 3.3. Duration of active day (minutes) and number of days before hatching in city (blue) and forest (green) birds. Large dots represent the mean minimum adequate model predictions of duration of activity for that day.

The percentage time within nest box ranged over the duration of the study from 40 % to 98.89 %. There was no effect of mean ambient temperature or recording date on percentage of time spent in nest box (Table 3.2.). Percentage time within nest box increased significantly as incubation progressed closer to hatching (linear: $t_{1,732}$ = 19.76; P<0.001, LMM; quadratic: $t_{1,732}$ = -17.85; P=0.006, LMM), (Figure 3.4.). During early incubation, city birds spent more time in nest boxes than forest birds, but this difference tended to disappear close to hatching (Figure 3.4.). When compared with 2016, the percentage of time within nest box was estimated to be 0.88 % (± 0.88) higher in 2017 and -2.02 % (± 1.05) lower in 2018 (Table 3.3). Moreover, females with a larger clutch size spent significantly longer in nest boxes than those with smaller clutches ($t_{1,732}$ = 2.00; P=0.041, LMM; Table 3.3).

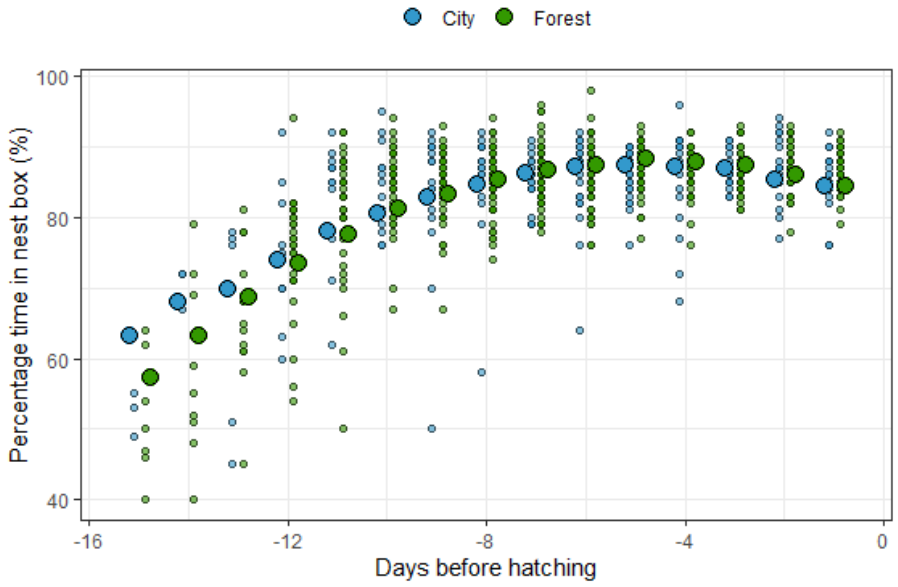



Figure 3.4. The percentage time in nest box (minutes) and number of days before hatching for city (blue) and forest (green) birds. Large dots represent the mean minimum adequate model predictions of percentage time spent in nest box for that day.

Night time nest temperature variance ranged over the duration of the study from 0.028 °C to 7.66 °C. There was no effect of hatch date or clutch size on night time temperature variance (Table 3.3). Forest birds had significantly higher levels of night time temperature variance than city birds ($t_{1,629}$ = 2.64; P=0.008, LMM), (Table 3.3). In addition to site, night time temperature variance was also significantly affected by mean ambient temperatures, date of recording and study year. When compared with 2016, night time temperature variance was estimated to be 0.14 °C (± 0.16) lower in 2017 and 0.63 °C (±

0.18) higher in 2018 (Table 3.3). Night time temperature variance also significantly decreased as the season progressed ($t_{1,629}$ = -3.73; P=0.002, LMM), but there was no interaction with site (Table 3.3).

Global model summaries for incubation activity data can be found in Table 3.4.

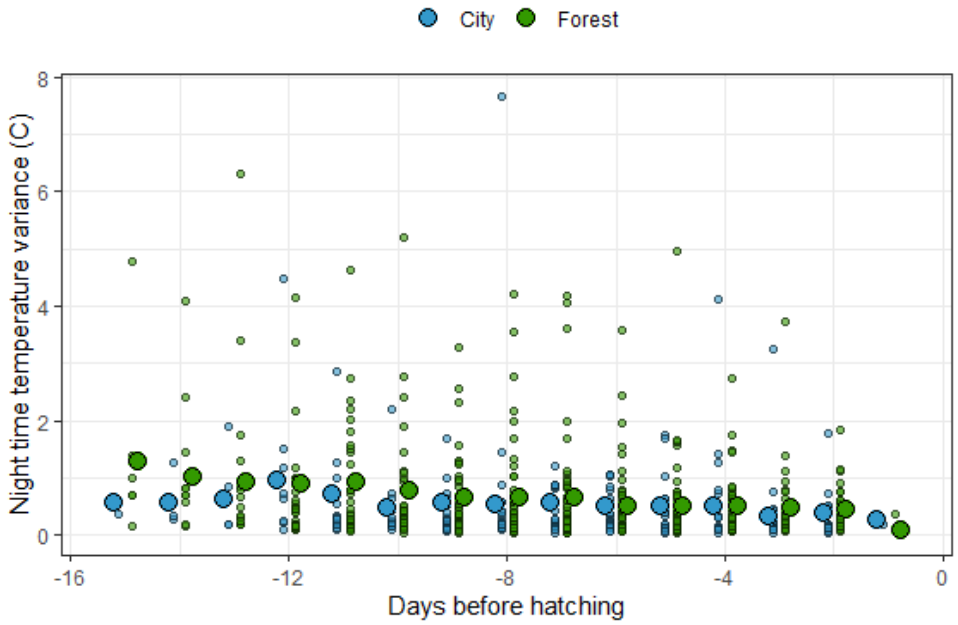



Figure 3.5. Night time temperature variance (restlessness) and the number of days before hatching for city (blue) and forest (green). Large dots represent the mean minimum adequate model predictions of night time temperature variance for that day.

Table 3.3. Minimum adequate model summaries from linear mixed models of incubation activity data. p-values shown here were obtained from likelihood ratio testing. Reference levels for intercept were: Year (2016), Site (City). An interaction between terms is denoted by an asterisk (*).

| **Variable** | **N** | **Estimate** | **s.e.m** | **d.f.** | ***t*-value** | ***p*-value LRT** |
|---|---|---|---|---|---|---|
| **Onset of activity** | 732 | | | | | |
| (Intercept) | | 39.61 | 3.55 | | 11.16 | |
| Site (Forest) | | 10.35 | 3.55 | 1 | 2.91 | |
| Days before hatching | | 2.87 | 0.27 | 1 | 10.57 | |
| Days before hatching² | | -41.70 | 19.41 | 2 | -2.15 | **0.024** |
| Site(City)*Days before hatching | | -91.79 | 46.67 | 2 | -1.96 | **0.037** |
| | | | | | | |
| **Offset of activity** | 708 | | | | | |
| (Intercept) | | -23.39 | 21.28 | | -1.10 | |
| Site(Forest) | | 10.35 | 6.98 | 1 | 1.48 | |
| Days before hatching | | -1.54 | 0.70 | 1 | -2.20 | |
| Date | | -2.87 | 0.47 | 1 | -6.14 | **<0.001** |
| Site(City)*Days before hatching | | 342.69 | 84.12 | 2 | 4.07 | **<0.001** |
| | | | | | | |
| **Duration of active day** | 708 | | | | | |
| (Intercept) | | 783.51 | 6.27 | | 125.02 | |
| Site(Forest) | | 11.58 | 5.81 | 1 | 1.99 | |
| Days before hatching | | -3.38 | 0.56 | 1 | -6.03 | |
| Site*Days before hatching | | 456.2 | 95.13 | 2 | 4.80 | **<0.001** |
| | | | | | | |
| **Percentage time in box** | 732 | | | | | |
| (Intercept) | | 89.69 | 1.73 | | 51.82 | |
| Site(Forest) | | -0.68 | 0.90 | 1 | -0.75 | 0.442 |
| Days before hatching | | 1.30 | 0.07 | 1 | 19.76 | |
| Days before hatching$^2$ | | -101.79 | 5.70 | 2 | -17.85 | |
| Clutch size | | 0.48 | 0.24 | 1 | 2.00 | **0.041** |
| Year(2017) | | 0.88 | 0.88 | 1 | 0.10 | **0.006** |
| Year(2018) | | -2.02 | 1.05 | 1 | -1.92 | **0.006** |
| | | | | | | |
| **Night time temperature variance** | 629 | | | | | |
| (Intercept) | | 1.79 | 0.37 | | 4.86 | |
| Site(Forest) | | 0.44 | 0.17 | 1 | 2.64 | **0.008** |
| Mean Temperature | | -0.03 | 0.01 | 1 | -2.54 | **0.012** |
| Date | | -0.03 | 0.01 | 1 | -3.73 | **0.002** |
| Year(2017) | | -0.14 | 0.16 | 2 | -0.85 | **<0.001** |
| Year(2018) | | 0.63 | 0.18 | 2 | 3.41 | **<0.001** |

Table 3.4. Global model summaries from linear mixed models of incubation activity data. p-values shown here were obtained from likelihood ratio testing. Reference levels for intercept were: Year (2016), Site (City). An interaction between terms is denoted by an asterisk (*).

| **Variable** | **N** | **Estimate** | **s.e.m** | **d.f.** | ***t*-value** | ***p*-value** |
|---|---|---|---|---|---|---|
| **Onset of activity** | 732 | | | | | |
| (Intercept) | | -2.50 | 15.63 | | -0.16 | |
| Site (Forest) | | 6.37 | 4.67 | 1 | 1.36 | |
| Days before hatching | | 1.32 | 0.51 | 1 | 2.60 | |
| Days before hatching² | | -61.39 | 36.01 | 2 | -1.70 | |
| Clutch size | | 0.20 | 1.01 | 1 | 0.20 | 0.843 |
| Mean temp | | 0.34 | 0.35 | 1 | 0.97 | 0.333 |
| Date | | 0.56 | 0.30 | 1 | 1.83 | **0.061** |
| Year (2017) | | 9.72 | 3.90 | 1 | 2.49 | **0.038** |
| Year (2018) | | 4.12 | 4.50 | 1 | 0.91 | **0.038** |
| Site*Days before hatching | | 105.26 | 48.01 | 2 | 2.19 | |
| Site*Days before hatching² | | 28.77 | 43.16 | 4 | 0.67 | **0.051** |
| | | | | | | |
| **Offset of activity** | 708 | | | | | |
| (Intercept) | | 3.67 | 26.30 | | 0.14 | |
| Site(Forest) | | 6.67 | 7.71 | 1 | 0.86 | |
| Days before hatching | | 1.77 | 0.91 | 1 | 1.95 | |
| Days before hatching² | | -73.98 | 63.73 | 2 | -1.16 | |
| Clutch size | | -1.81 | 1.66 | 1 | -1.10 | 0.259 |
| Mean temp | | -0.46 | 0.64 | | -0.71 | 0.479 |
| Date | | -2.51 | 0.51 | 1 | -4.91 | **<0.001** |
| Year (2017) | | 5.80 | 6.39 | 1 | 0.91 | 0.133 |
| Year (2018) | | -7.98 | 7.41 | 1 | -1.08 | 0.133 |
| Site*Days before hatching | | -350.69 | 86.70 | 2 | -4.04 | |
| Site*Days before hatching² | | 143.02 | 77.10 | 4 | 1.86 | 0.067 |
| | | | | | | |
| **Percentage time in box** | 732 | | | | | |
| (Intercept) | | 86.25 | 3.86 | | 22.32 | |
| Site(Forest) | | -1.40 | 1.11 | 1 | -1.26 | |
| Days before hatching | | 1.08 | 0.14 | 1 | 7.73 | |
| Days before hatching² | | -94.26 | 10.58 | 2 | -8.91 | |
| Clutch Size | | 0.47 | 0.23 | | 2.01 | **0.039** |
| Mean Temp | | -0.11 | 0.10 | | -1.11 | 0.273 |
| Date | | 0.09 | 0.08 | | 1.13 | 0.225 |
| Year(2017) | | 1.42 | 0.92 | 1 | 1.55 | **0.004** |
| Year(2018) | | -1.90 | 1.05 | 1 | -1.80 | **0.004** |
| Site*Days before hatching | | 18.58 | 13.84 | 2 | 1.34 | |
| Site*Days before hatching² | | -9.85 | 12.66 | 4 | -0.78 | 0.423 |

| Variable | N | Estimate | s.e.m | d.f. | *t*-value | *p*-value |
|---|---|---|---|---|---|---|
| **Duration of active day** | 708 | | | | | |
| (Intercept) | | 862.64 | 27.79 | | 31.04 | |
| Site(Forest) | | 13.48 | 8.01 | 1 | 1.68 | |
| Days before hatching | | 1.90 | 1.01 | 1 | 1.88 | |
| Days before hatching$^2$ | | -11.53 | 73.18 | 2 | -0.16 | |
| Clutch Size | | -1.42 | 1.70 | | -0.84 | 0.386 |
| Mean Temp | | -0.67 | 0.73 | | -0.92 | 0.366 |
| Date | | -0.45 | 0.54 | | -0.83 | 0.393 |
| Year(2017) | | -10.34 | 6.59 | 1 | -1.57 | 0.170 |
| Year(2018) | | -12.28 | 7.63 | 1 | -1.61 | 0.170 |
| Site*Days before hatching | | -479.22 | 98.45 | 2 | -4.87 | |
| Site*Days before hatching² | | 104.70 | 88.43 | 4 | 1.18 | 0.240 |
| | | | | | | |
| **Night time temperature variance** | 629 | | | | | |
| (Intercept) | | 2.89 | 0.63 | 1 | 4.57 | |
| Site(Forest) | | 0.62 | 0.19 | 1 | 3.20 | |
| Days before hatching | | 0.04 | 0.02 | 1 | 1.97 | |
| Days before hatching$^2$ | | 0.04 | 1.21 | 2 | 0.03 | |
| Clutch Size | | -0.08 | 0.04 | 1 | -1.85 | 0.058 |
| Mean Temp | | -0.04 | 0.01 | 1 | -3.03 | **0.002** |
| Date | | -0.04 | 0.01 | 1 | -2.97 | **0.003** |
| Year(2017) | | -0.15 | 0.16 | 1 | -0.90 | **<0.001** |
| Year(2018) | | 0.72 | 0.19 | 1 | 3.83 | **<0.001** |
| Site*Days before hatching | | -3.40 | 1.73 | 2 | -1.97 | |
| Site*Days before hatching$^2$ | | 1.05 | 1.47 | 4 | 0.71 | **0.047** |

### 3.4.2 Nestling fitness

Nestling weight was not significantly affected by brood size, hatch date, or study year (Table 3.5.). There was no significant interaction between site and chronotype of the mother (Figure 3.6), and effect sizes of later chronotype on nestling weight were very small (0.004 g ± 0.007), (Table 3.6). However, effects of site on nestling weight were evident, where forest nestlings weighed significantly more than nestlings at city sites ($t_{1,231}$ = 4.80; P<0.001, LMM), (Table 3.6). Forest nestlings were estimated to weigh 18.99 g (± 0.27) and city nestlings 17.70 g (± 0.26), (Table 3.6.).

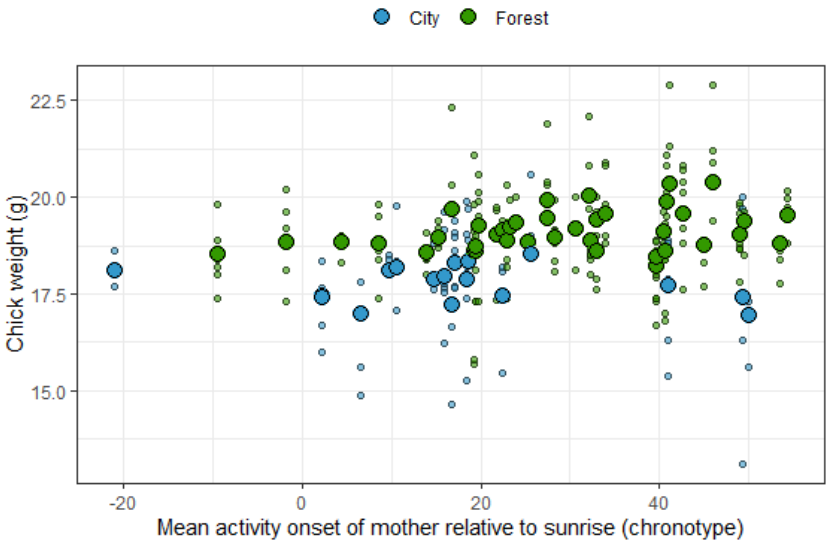



Figure 3.6. Nestling weight (g) at day thirteen and mean activity onset of mother (chronotype), for city (blue) and forest (green) nestlings. Large dots represent the minimum adequate model predictions of maternal chronotype for each nest box.

Hatching success overall was 80.96 % across the study period and all nest boxes. Hatching success was not affected by the study year, clutch size or the hatching date (Table 3.6.). In addition, hatching success was not significantly affected by chronotype of the mother ($z_{1,394}$ = -1.37; P=0.170, LMM), or the site ($z_{1,394}$ = 0.45; P=0.650, LMM).

Fledging success overall was 95.64 % across the study period and all broods. As with hatching success, fledging success was not significantly affected by study year, brood size, or the hatching date (Table 3.6.). Fledging success was also not significantly affected by chronotype of the mother ($z_{1,321}$ = 9.19; P=0.755, LMM), or site ($z_{1,321}$ = 250.16; P=0.768, LMM).

Global model summaries for nestling fitness traits can be found in Table 3.7.

Table 3.5. Global and minimum adequate models after likelihood ratio testing using nestling data. Nestling weight at day thirteen was modelled using Gaussian error distributions. Fledging success and hatching success data were modelled using Binomial error distributions. Nest box was included in all models as a random factor.

| Variable | Global Model | Minimum Adequate Model (MAM) |
|---|---|---|
| **Nestling weight** | Nest box + chronotype*site + chronotype + site + year + hatch date + brood size | Nest box + chronotype*site + chronotype + site + hatch date |
| **Hatching success** | Nest box + chronotype*site + site + chronotype + clutch size + hatch date + year | Nest box + chronotype + site |
| **Fledging success** | Nest box + chronotype + site + brood size + hatch date + year | Nest box + chronotype + site |

Table 3.6. Minimum adequate model summaries of nestling weight, hatching success and fledging success. Nestling weight data were modelled using Gaussian error distribution. Hatching success and Fledging success data were modelled using Binomial error distributions. The p-values shown here were obtained from likelihood ratio testing. Reference levels for intercept were: Site (City). An interaction between terms is denoted by an asterisk (*).

| **Variable** | **N** | **Estimate** | **s.e.m** | **d.f.** | ***t*-value** | ***p*-value LRT** |
|---|---|---|---|---|---|---|
| **Nestling weight** | 231 | | | | | |
| (Intercept) | | 17.70 | 0.26 | | 67.79 | |
| Chronotype | | 0.004 | 0.007 | 1 | 0.63 | 0.840 |
| Site (Forest) | | 1.29 | 0.27 | 1 | 4.80 | **<0.001** |
| | **N** | **Estimate** | **s.e.m** | **d.f.** | ***z*-value** | ***p*-value LRT** |
| **Hatching success** | 394 | | | | | |
| (Intercept) | | 2.01 | 0.42 | | 4.78 | |
| Chronotype | | -0.02 | 0.01 | 1 | -1.37 | 0.170 |
| Site (Forest) | | 0.19 | 0.42 | 1 | 0.45 | 0.650 |
| **Fledging success** | 321 | | | | | |
| (Intercept) | | 6.15 | 0.01 | | 3416.93 | |
| Chronotype | | 0.02 | 0.01 | 1 | 9.19 | 0.755 |
| Site (Forest) | | 0.45 | 0.01 | 1 | 250.16 | 0.768 |

Table 3.7. Global model summaries of nestling weight, hatching success and fledging success. Nestling weight data were modelled using Gaussian error distribution. Hatching success and Fledging success data were modelled using Binomial error distributions. The p-values shown here were obtained from likelihood ratio testing. Reference levels for intercept were: Site (City). An interaction between terms is denoted by an asterisk (*).

| **Variable** | **N** | **Estimate** | **s.e.m** | **d.f.** | ***t*-value** | ***p*-value LRT** |
|---|---|---|---|---|---|---|
| **Nestling weight** | 231 | | | | | |
| (Intercept) | | 20.75 | 1.58 | | 13.09 | |
| Chronotype | | -0.01 | 0.01 | 1 | -0.77 | |
| Site (Forest) | | 1.33 | 0.53 | 1 | 2.50 | |
| Year(2017) | | -0.57 | 0.34 | | -1.70 | 0.120 |
| Year(2018) | | -0.11 | 0.32 | | -0.34 | 0.120 |
| Hatch date | | -0.05 | 0.03 | 1 | -1.50 | 0.115 |
| Brood Size | | -0.08 | 0.07 | | -1.08 | 0.247 |
| Chronotype*Site | | 0.02 | 0.02 | 2 | 1.05 | 0.253 |
| | | | | | | |
| | **N** | **Estimate** | **s.e.m** | **d.f.** | ***z*-value** | ***p*-value LRT** |
| **Hatching success** | 394 | | | | | |
| (Intercept) | | 5.23 | 2.21 | | 2.37 | **0.018** |
| Site(Forest) | | 1.87 | 0.75 | 1 | 2.48 | **0.013** |
| Chronotype | | 0.01 | 0.02 | 1 | 0.62 | 0.534 |
| Clutch Size | | -0.07 | 0.11 | 1 | -0.63 | 0.530 |
| Hatch Date | | -0.08 | 0.05 | 1 | -1.73 | 0.083 |
| Year(2017) | | 0.04 | 0.49 | 1 | 0.09 | 0.930 |
| Year(2018) | | 0.29 | 0.42 | 1 | 0.68 | 0.493 |
| Site*Chronotype | | -0.04 | 0.02 | 2 | -1.54 | 0.123 |
| | | | | | | |
| **Fledging success** | 321 | | | | | |
| (Intercept) | | 5.23 | 2.21 | | 2.37 | **0.018** |
| Site (Forest) | | 1.87 | 0.75 | 1 | 2.48 | **0.013** |
| Chronotype | | 0.01 | 0.02 | 1 | 0.62 | 0.534 |
| Clutch Size | | -0.07 | 0.11 | 1 | -0.63 | 0.530 |
| Hatch Date | | -0.08 | 0.05 | 1 | -1.73 | 0.083 |
| Year(2017) | | 0.04 | 0.49 | 1 | 0.09 | 0.930 |
| Year(2018) | | 0.29 | 0.42 | 1 | 0.68 | 0.493 |
| Site*Chronotype | | -0.04 | 0.02 | 2 | -1.54 | 0.123 |

## 3.5 Discussion

### 3.5.1 Incubation patterns

In this Chapter, activity patterns of free-living incubating great tits were compared for city and forest habitats. There were strong effects of the number of days before hatching on the onset, percentage of time in the nest box and night time variance, as mothers spent more time in the nest box the closer to hatching date. Similar changes in parental behaviour during critical points of the embryonic developmental period have been shown in previous studies on incubation, such as a later onset of activity (Graham et al., 2017), and lower variance in night time temperatures (Gwinner et al., 2018) as hatch date becomes closer. This trend for changes in behaviour across the incubation period was stronger in the city site than the forest, as shown by significant interactions of site and the number of days before hatching in statistical models (Table 3.4.). This is evident in Figure 3.2., where city birds appeared to "break off" from forest birds halfway through the incubation period, starting their daily activity earlier and retiring later than forest birds. In addition, there was a shortening of active day in forest birds across the incubation period; a trend which was stronger than in city birds (Figure 3.3.).

As predicted, great tits incubating at city sites had earlier onsets of daily activity, and later offsets of daily activity overall, than forest birds (Figure 3.2.). These differences are consistent with previous studies of activity patterns of birds and urbanisation, with city birds rising earlier (Dominoni et al., 2013; Da Silva, Valcu and Kempenaers, 2015) and staying out later (Russ et al., 2015) than forest conspecifics. Curiously, earlier onset and later offset of activity for city birds did not translate to longer duration of active day for city birds in model predictions (Figure 3.3.). This result may require further analyses.

A possible cause for these observed site differences in timing of incubation activities may have been the high level of ALAN at city sites. Environmental light values extracted from a satellite map (VIIRS, 2019; *https://www.lightpollutionmap.info*) using global positioning system (GPS) co-ordinates of nest box locations used in the study revealed that mean light levels at city nest boxes were 55 lux, and forest nest boxes 0.44 lux. Previous research investigating the effects of ALAN on birds show that under artificially lit areas, birds are active earlier (Dominoni et al., 2014; Kempenaers et al., 2010; Kumar et al., 2018) and sometimes extend their activity later (Russ et al., 2015) than forest birds; trends which are reflected in the activity patterns of city birds in this study. The mechanisms behind these shifts in activity are currently unknown, but may be due to circadian disruption (de Jong et

al., 2018), or perception of longer daylength as shown for ALAN of as little as 0.3 lux (Dominoni et al., 2013a; Dominoni and Partecke, 2015).

In addition to ALAN, ambient noise in urban areas also has potential to disrupt normal activity patterns of wild birds (Slabbekoorn and Peet, 2003; Fuller, Warren and Gaston, 2007; Gil et al., 2015), although other authors argue that light is more important predictor of activity than noise (Dominoni et al., 2014). For this study, light and noise levels at city and forest sites were not included in statistical models but may have played a role in the observed significant differences between activity patterns of birds at the two habitats. Inclusion of environmental information on light and noise in further studies would help to untangle these effects.

It was predicted that rhythms in city birds would be less robust than in forest birds, and therefore a higher level of night time restlessness would be observed in city birds. This was predicted as per a previous captive study (Dominoni et al., 2013) and from experimental studies of wild birds (Ouyang et al., 2017; Raap et al., 2016b). Contrary to expectation, night time restlessness was higher in forest birds than in city birds (Figure 3.5.), possibly due to the abundance of nocturnal predators in forest sites such as weasels, martens (*Martes martes*) and owls (*Strix aluco*), (Dunn, 1977; Stuber et al., 2014). Although the observed differences in variability of egg temperature were small (0.18 ºC), this may be ecologically significant, as even small changes in temperature of embryos during incubation can slow development and affect nestling condition (Ardia et al., 2010; Berntsen and Bech, 2016).

Previous studies have shown ALAN exposure can disrupt sleep in wild birds (Raap et al., 2016c, 2015). Future wild studies measuring activity patterns in city birds ought to measure markers of sleep deprivation (Ouyang et al., 2017), or nocturnal melatonin concentrations (Dominoni et al., 2013) to find out whether city birds are indeed sleep deprived. Moreover, in-nest cameras could be used to quantify sleep behaviours as an addition to incubation patterns (Stuber et al., 2015).

A limitation of this study was the omission of day of year from statistical models of incubation activities. Changes in the environment across the season, such as an increase in day length, warmer temperatures (Avery & Krebs 1984; Kendeigh et al., 1969) and decreased rainfall (Radford et al., 2001) may affect timing of foraging activities. Given that city birds often lay earlier in the year than forest birds (Deviche & Davies 2014), these seasonal changes may have contributed to the observed differences between activity

patterns of city and forest birds. Inclusion of day of year in future analyses would therefore act as a control for differences in phenology between forest and city birds.

### 3.5.2 Chronotype and condition of offspring

This study was unique in investigating whether individual differences in timing could predict fitness traits of wild birds, by linking chronotype of the mother to hatching success, fledging success and nestling weight. Contrary to expectation, there were no effects of mother's chronotype on the parameters of chick fitness used in this study. Chronotype of the mother also had no significant effect on either hatching success or fledging success of nestlings, although both were higher at forest sites than city sites.

Many limiting resources within a birds' environment fluctuate with time of day (Helm et al., 2017), such as the availability of food (Bednekoff and Houston, 1994). Previous research carried out at the sites used in this study showed that food is limited in the city habitat (Pollock et al., 2017). Therefore, having an earlier chronotype may have provided a foraging advantage for great tits living in the city, both during incubation and nestling provisioning.

In this study, nestlings raised in the city habitat weighed less than those at forest sites, possibly due to this scarcity of food, and in accordance with previous research undertaken at these sites (Capilla-Lasheras et al., 2017; Pollock et al., 2017). Observed differences in nestling weight between sites may have ecological significance, as heavier nestlings have better prospects for survival and recruitment into the population post-fledging (Ringsby et al., 1998). Future studies might consider re-capturing study nestlings as adults, to investigate whether birds whose mother had an earlier chronotype are in better condition than those with later chronotypes.

### 3.5.3 Conclusions

This study provided no evidence to support the idea that differences in individual timing and chronotype have consequences for condition of offspring in wild birds. However, activity patterns of individual birds differed between city and forest habitats used in this study, which may have been due to the disruptive presence of ALAN on biological rhythms. In city environments where resources are already limited (Pollock et al., 2017), these differences in timing may give early individuals the edge for better survival of chicks or survival prospects post-fledging.

# Chapter Four: Effects of experimental exposure of low-level artificial light at night on circadian rhythms and health of wild nestlings

## 4.1 Introduction

Urban wildlife must navigate increasingly noisy, polluted, and fragmented habitats as the human population increases and cities continue to expand (McKinney, 2002). One predominant feature of the urban environment is ecological light pollution, defined as light emissions from sources such as streetlamps and buildings (Longcore and Rich, 2004).

Currently, the world is experiencing a loss of the night (Kyba et al., 2017), and therefore it is increasingly important to consider effects of ecological light pollution on wildlife living in artificially-lit environments. To date, artificial light at night (otherwise known as ALAN) has been shown to have observable effects on many wild animal groups, from a decrease in juvenile growth in amphibians (Dananay and Benard, 2018), to altered species composition of bat communities (Cravens and Boyles, 2019), and attraction of moth species to lamps (van Langevelde et al., 2011). Ecological light pollution, particularly artificial light at night, has therefore rapidly become a subject of ecological interest over the past decade.

A main suspected reason for potential harm of ALAN is through its effects on biological rhythms of wildlife and humans (Fonken and Nelson, 2011; Navara and Nelson, 2007). Ambient light-dark cycles are the most potent zeitgeber for endogenous timekeeping mechanisms (Pittendrigh, 1981). Diel rhythms of physiology and behavioural activity are tied strongly to light and darkness, for example by the timely release of the hormone melatonin, a key regulator of circadian rhythms (Dawson et al., 2001; Gwinner et al., 1997). Light at night has a suppressive effect on melatonin release (Navara and Nelson, 2007), providing one way in which ALAN has the potential to disrupt normal circadian rhythms.

For organisms living in artificially-lit environments, disruption of circadian rhythms by ALAN may have downstream effects on physiology and epidemiology. In mice, ALAN has been shown to increase metabolic disruption (Fonken and Nelson, 2014), and the likelihood of cancer (McFadden et al., 2014). Also in mice, dim light exposure disrupts expression of several clock genes in both the suprachiasmatic nucleus of the brain and

peripheral tissues (Fonken et al., 2013; Shuboni and Yan, 2010). Clocks are strongly tied to the immune system (Scheiermann et al., 2013) and therefore any circadian disruption has the potential to affect inflammatory and immune responses. Indeed, ALAN suppresses immune system function in hamsters (Bedrosian et al., 2013, 2011).

In birds, the potential of ALAN to disrupt rhythms is even greater than in mammals, given that birds possess deep-brain photoreceptors that are sensitive to light (Thakur and Kumar, 2015; Underwood et al., 2001). Captive studies have illuminated the adverse effects that ALAN exposure can have on avian physiology and health (Spoelstra and Visser, 2013). One study found an increase in corticosterone levels in adult great tits as a response to ALAN (Ouyang et al., 2015). Experimental exposure to light at night also results in shifts in the timing of behaviour, such as earlier onsets of daytime activity in Indian weaver birds (*Ploceus philippinus*; Kumar *et al.*, 2018) and also in great tits (de Jong et al., 2016).

Ecological studies on how ALAN affects avian rhythms are rare, but have so far demonstrated its presence to have similar effects to captive studies on behavioural timing, advancing onset of dawn singing in several passerine species in the wild (Da Silva et al., 2015; Kempenaers et al., 2010; Raap et al., 2015). Although it is well known that ALAN has effects on the timing of behaviours in wild birds, the mechanisms behind the observed shifts in activity timing as a result of exposure have yet to be elucidated. Furthermore, despite disruptive effects of ALAN on circadian rhythms in captive studies, and the detrimental effects of disruption on health (Fonken and Nelson, 2011), effects on the condition of wild birds have largely been unexplored. Therefore, there is a need for studies exploring effects of ALAN on both circadian rhythms and fitness of birds in the natural environment.

In ecological studies, the use of nest boxes allows for experimental manipulations such as the implementation of ALAN treatments, and provide valuable insights into effects on nestling birds. A previous nest box study exposing great tit nestlings to constant light for two successive nights found negative effects on nestling growth and increased oxidative stress (Raap et al., 2016c). Another study found nestlings exposed to ALAN for seven consecutive nights to have a decline in overall body condition (Grunst et al., 2019). However, these studies were limited in their approach, as the authors did not expose nestlings to ALAN for the entire growth period.

## 4.2 Study aims and hypotheses

To address current gaps in knowledge, in this Chapter, wild great tit nestlings were experimentally exposed to low-level artificial light at night from their hatching up until fledging to compare condition and health parameters of ALAN-raised nestlings with those raised under a dark night. Ecological studies of the effects of ALAN in urban environments are often limited by the presence of other environmental effects such as ambient noise, temperature and atmospheric pollutants (Dominoni, 2015). As such, it is difficult to untangle the effects of noise and light on birds, although a few ecological studies have done so (Caorsi et al., 2019; Casasole et al., 2017; de Jong et al., 2018). This study was conducted in a forest habitat in order to isolate the effects of ALAN and minimise confounding environmental factors such as urban noise.

Condition and health effects of being raised under ALAN were assessed using commonly used parameters of nestling condition, namely nestling weight and tarsus length (Eck et al., 2011), and fledging success. For information on health, blood samples were used to measure red blood cell count (haematocrit levels (%)) and to monitor transcript levels of relevant genes.

In this Chapter, the possibility of similar circadian disruption from ALAN exposure was addressed, by comparisons of transcript levels of seven core avian clock genes in ALAN-raised nestlings with those raised under dark nights. The following components of the molecular clock were included in this study: positive clock elements and transcriptional activators *BMAL1* (brain and muscle ARNT-like 1) and *CLOCK* (circadian locomotor output cycles kaput); negative clock elements and inhibitors *CRY1* (cryptochrome circadian regulator 1) and *PER2* (period circadian regulator 2); and accessory loop protein *REVERBA* (Kumar and Sharma, 2018; Yoshimura et al., 2000). Additional gene targets included *CK1Ɛ* (casein kinase 1), which catalyses phosphorylation of E4bp4, a light-inducible gene in the nestling pineal gland (Doi et al., 2004), and *AANAT* (aralkylamine N-acetyltransferase), the rate-limiting enzyme regulating melatonin synthesis (Bernard et al., 2002).

This study also addressed the possibility that ALAN affects immune system function in nestlings, by comparing transcript levels of eleven immune targets in ALAN-raised birds to those raised under dark nights. Targets included *LY86* (lymphocyte antigen 86) and *TLR4* (toll-like receptor 4) which are involved in anti-bacterial and anti-malarial responses (Medzhitov, 2001), and the type 2 transcription factor *GATA3* (GATA binding protein 3) involved in adaptive immunity (Wang et al., 2011). Additional immune targets included: *IL1* and *IL6* (interleukins 1 and 6) which are inflammatory response mediators (Klasing, 1998); *PRKCA* (protein kinase C alpha), an enzyme responsible for antiviral effects and cell growth regulation (Clemens and Elia, 1997); and *IKBA* (NF-kappa-B inhibitor alpha) and *NKRF* (NF-kappa-B repressing factor), which are two inhibitors of the immune response regulator NF-kappa-B (Cabannes et al., 1999; Nourbakhsh and Hauser, 1999). Finally, immune targets *NRF2* (nuclear factor erythroid 2) and *IGF1* (insulin like growth factor 1), which are both involved in resistance to oxidative stress and metabolic regulation (Holzenberger et al., 2003; Kensler et al., 2007) were included in this study. A table of the known functions of each gene used in this study is available in the General Methods section.

In order to capture fluctuations of transcript levels of circadian clock and immune system elements across a 24 hr day, the sampling protocol in this study was designed so that samples were obtained from nestlings during the day and at night.

Following previous studies that show a reduction of overall condition as a response to experimental exposure to ALAN (Grunst et al., 2019; Raap et al., 2016a, 2016b), it was expected that birds under ALAN treatment would have reduced condition, such as lower weight and haematocrit levels, shorter tarsi, and lower fledging success than control birds.

Previous studies have shown ALAN to disrupt rhythmic expression of clock genes (Bedrosian et al., 2013, 2011) and immunity (Mishra et al., 2019). In this study, it was expected that ALAN treatment would have significant downregulatory effects on the timing of expression of clock and immune genes, with an overall reduction in time of day differences.

In addition, ALAN has been shown to elevate (Raap et al., 2016b; Saini et al., 2019; Zhang et al., 2019), and also downregulate (Moore and Siopes, 2000) aspects of immune activity. In this study, it was expected that ALAN would have significant effects on the levels of immune genes, with target specific responses.

## 4.3 Materials and Methods

### 4.3.1 Study sites

Data for this chapter were obtained between April and June of 2017 at forest field sites on a free-living population of breeding great tits. Field sites were existing forest nest box study systems located at the Scottish Centre for Ecology and the Natural Environment (SCENE; 56º, 7.73’N, 4º 36.79’W), and Cashel Forest (56° 6'N, 4°34'W), and were previously used in ecological studies of wild passerines (Capilla-Lasheras et al., 2017; Pollock et al., 2017). To determine hatch dates, brood sizes and fledging success, all nest boxes were initially checked weekly and, closer to hatching, every second day as part of a general nest box monitoring protocol (see General Methods for further details of nest box monitoring).

### 4.3.2 Light at night treatment

Fifteen treatment nest boxes (Schwegler, Germany) contained one small light-emitting diode (LED) of broad-spectrum low intensity (1 lux) within the ceiling of the nest box. Lighting intensity was selected as the lowest intensity ALAN treatment to have a significant effect on great tit activity patterns as per de Jong *et al.*, (2016). Treatment (ALAN) and control nest boxes were assigned to nest boxes previously identified as containing great tit clutches. Seventeen nest boxes containing no LED (dark-night) were monitored as controls. Nest boxes were matched by synchronous hatch date. Treatment nest boxes were swapped with existing nest boxes, by careful removal of the whole nest (including eggs) and replacement into the nest box containing an LED. This occurred on the expected hatch date, which was calculated to be fourteen days after laying of the first egg as a conservative estimate of great tit incubation length. Lights were switched on in treatment nest boxes on the expected hatch date and were left on constantly until after nestling fledging. Similar levels of experimental disturbance occurred for all nest boxes within the study, where both treatment and control nest boxes were visited on the expected day of hatching, once on day eight of life (where hatch date = day 0 of life), and twice on the sampling day on day thirteen of life. On this day, nest boxes were visited during the midday hours (11:00-15:00 GMT) and then again during the hours surrounding midnight (23:00-02:00 GMT).

Fifteen of the nest boxes within this study (seven ALAN and eight control nest boxes) were treated with a citronella oil-based insect repellent as per (Krams *et al.*, (2013) as part of an avian malaria study (see General Methods section for more details). Citronella treatment was kept as a variable in all statistical models in this study.

### 4.3.3 Nestling processing

In total, 97 nestlings from fifteen nest boxes were subjected to ALAN treatment, and 116 nestlings from 17 nest boxes were under "dark-night" controls. All nestlings (N=213) were ringed for individual identification on day eight of life. Measurements of nestling weight and length of the tarsometatarsus bone (tarsus) were taken from each nestling. On day thirteen of life, nestlings were individually weighed again, and their tarsi measured as in Eck *et al.*, (2011). All nestlings were weighed during the day between (08:00 and 17:00 GMT) to reduce diurnal fluctuations in weight. To reduce burden of sampling on nestlings, no nestling was sampled twice on one day. Sampling of broods on day thirteen was split so that up to three of the nestlings within a brood were sampled during the day (10:00-15:00 GMT), and up to three others during the night (23:00-02:00 GMT). Table 4.1. summarises sample sizes obtained during the study.

Two blood samples of approximately 50 µl were obtained from each nestling via the brachial wing vein. During noon sampling, the first sample was taken up by a heparinised capillary, sealed using a wax plug and stored on ice for haematocrit level (%) readings (See General Methods for more details on protocol for haematocrit readings). After haematocrit readings were taken, this sample was removed from the capillary via pipette and stored in 0.5 ml 100% ETOH for molecular analyses. For night sampling, the first sample was taken up by heparinised capillary and immediately stored in 0.5 ml 100% ETOH for molecular analyses. Haematocrit level readings were not obtained for nestlings during night sampling given the difficulties of night sampling in the field. For both noon and night sampling, a second blood sample was obtained for each nestling via heparinised capillary and stored in 250 µl RNAlater® stabilising solution (Invitrogen) for analyses of gene transcript levels. The General Methods section provides further details on nestling blood sampling protocol and ethical licencing.

Table 4.1. Breakdown of number of samples obtained from ALAN treatment and control nest boxes by day. A maximum of six nestlings were sampled per nest on day thirteen.

| Treatment | Day Eight | Day Thirteen Noon 10:00-15:00 GMT | Day Thirteen Night 23:00-02:00 GMT |
|---|---|---|---|
| **ALAN** | 87 | 30 | 30 |
| **Control** | 105 | 43 | 29 |
| | | 73 | 59 |

### 4.3.4 Laboratory procedures

Haematocrit levels (%) were measured for noon samples, before pipetting out the remaining blood sample into 100% ETOH. Samples in 100% ETOH were frozen and stored at -40 °C. Genomic DNA was later extracted from these samples via DNAeasy Blood and Tissue Kit (Qiagen) and molecular sexing was carried out via PCR (See General Methods for details).

Measurement of Median Fluorescence Intensity units (as a proxy for transcript levels) for immune, clock and housekeeping gene targets outlined in Table 4.2. was carried out on all samples stored in *RNAlater*® using the QuantiGene® Plex RNA 2.0 assay (ThermoFisher). The QuantiGene® Plex RNA 2.0 assay is a platform that allows for simultaneous readouts of transcript levels of many target genes from a single blood sample using branched DNA signal amplification and multi-analyte profiling beads (Mills and Gallagher, 2016). The General Methods section provides further details on lab procedures carried out in this Chapter.

Table 4.2. Housekeeping, clock and immune gene targets used within this study. Primer information for gene targets can be found in the Appendix.

| Transcript targets | Type |
|---|---|
| *RPL19, SDHA, HMBS* | Housekeeping genes |
| *BMAL1, CLOCK, CRY1,CK1Ɛ, AANAT, PER2, REVERBA* | Circadian clock genes and melatonin synthesis |
| *LY86, TLR4, IL1, IL6, PRKCA, GATA3, IKBA, NKRF, NRF2, IGF1* | Immune genes |

### 4.3.5 Ethical Statement

See General Methods for details of ringing licences and blood sampling.

### 4.3.6 Statistical Analysis and Model Selection

All statistical analyses were performed using R v. 3.4.4 (R Core Team, 2018) and packages *lme4* (Bates et al., 2015). Data were visualised using ggplot2 (Wickham, 2014). Initial global models for response variables were linear-mixed models (LMM), including individual nest box as a random factor to account for repeated measures within the same nest box. Residuals were plotted and inspected to check assumptions of normality whilst modelling using LMMs. Pairs of explanatory variables were also assessed for collinearity by calculating tolerance and variance inflation factors (VIF) and also by visual inspection of pairwise plots, before statistical relevance was accepted.

ALAN treatment and control were included in all models as a two-level fixed factor. Fixed factors in all models included sex of the nestling (M/F) for sex differences, brood size, hatch date in April day format (where April 1$^{st}$ = 1) to account for seasonal changes, and citronella oil repellent (presence/absence). Nestling weight, tarsal length and haematocrit were modelled using a Gaussian distribution, and fledging success was modelled using a binomial generalized linear-mixed model (GLMM).

Median fluorescence intensity data obtained from the QuantiGene® Plex RNA assay 2.0 (Thermofisher) were normalised by division of the mean fluorescence of background wells and then dividing each sample by the geometrical mean of levels of housekeeping genes in Table 4.2, thus, transcript levels are expressed as relative levels to housekeeping genes. Transcript level data for all targets were then modelled using Gamma log-link function for analysis using LMMs. “Sample time” was included as a fixed two-level factor by “noon” for samples obtained during 10:00-15:00 GMT or “midnight” for samples obtained during 23:00-02:00 GMT. An interaction between ALAN treatment and sample time was also included in all gene models.

Statistical relevance of fixed factors within all models was determined by sequential model selection via Likelihood Ratio Testing using package *lmtest* (Zeileis and Hothorn, 2002), until the minimum adequate model (MAM) was found. Factors with a significance level of <5% were kept in the model. ALAN treatment was kept in final models regardless of significance to monitor effect size.

## 4.4 Results

### 4.4.1 Artificial light at night treatment and nestling health

No significant effects of ALAN treatment were observed during modelling of nestlings' body weight at day eight, tarsus length or haematocrit (Table 4.4.; Figures 4.1., 4.2., 4.3.). However, nestling weight at day thirteen was significantly affected by presence of ALAN treatment ($t_{1,129}$=-2.04: P=0.038, LMM), (Figure 4.1.2.). Body weight of control nestlings (20.53 g ± 0.94) was on average higher than nestlings under ALAN (19.68 g ± 0.42).

There was little variation in fledging success between treatment (97%) and control (99%) groups, and possibly because of this, no effect of ALAN treatment on fledging success was found ($z_{1,207}$=0.74, P=0.452, GLM). Hatch date significantly affected fledging success, however, with fledging success decreasing as the season progressed ($z_{1,207}$=-2.38: P=0.002, GLM).

There were no significant effects of brood size or hatch date on body weight (at day eight), or tarsus length of nestlings (Table 4.3.). However, brood size significantly affected body weight of nestlings at day thirteen ($t_{1,129}$ = -1.96: P = 0.045; LMM), where nestlings in larger clutches had lower body weight. The presence of citronella treatment had no significant effect on nestling body weight, tarsus length, or haematocrit levels on day eight or thirteen (Table 4.4.).

Sex differences in nestling morphology were evident in this study, with male nestlings significantly heavier than females both at day eight ($t_{1,153}$ = 2.66: P = 0.010; LMM) and at day thirteen ($t_{1,129}$ = 2.90: P=0.004; LMM). On day eight, male nestlings weighed 14.7 g (± 0.20) and females 14.17 g (± 0.25), (Table 4.4.). On day thirteen, males weighed 21.13 g (± 0.21) and females 20.53 g (± 0.94), (Table 4.4.). In addition, male nestlings had significantly longer tarsi than females at day thirteen ($t_{1,163}$ = 3.36: P<0.001; LMM), with tarsi of male nestlings 20.64 mm (± 0.10) and females 20.29 mm (± 0.21), (Table 4.4.).

Global model summaries for nestling health data can be found in Table 4.5.

Table 4.3. Final (MAM) linear-mixed models of nestling weight, tarsus length and haematocrit levels at day eight and thirteen. Sex was a two-level fixed factor (M/F). Hatch date was defined as the number of days from the 1st of April 2017, brood size was the number of nestlings within a nest box, ALAN treatment a two-level fixed factor (ALAN/Control), Citronella presence was a two-level fixed factor (Presence/Absence). The global model for fledging success was Nest box (random) + Hatch date + ALAN + Citronella. Global models for all other variables of nestling condition were: Sex + Nest box (random) + Brood size + Hatch date + ALAN + Citronella. Nest box was included in all models as a random factor. Addition of terms is denoted by a plus sign (+).

| Variable | ANOVA Minimum Adequate Model (MAM) |
|---|---|
| **Weight day eight** | Nest box + Sex + ALAN |
| **Weight day thirteen** | Nest box + Sex + ALAN + Brood size |
| **Tarsus length day eight** | Nest box + ALAN |
| **Tarsus length day thirteen** | Nest box + Sex + ALAN |
| **Haematocrit % day eight** | Nest box + Hatch date + Brood size + Sex + ALAN |
| **Haematocrit % day thirteen** | Nest box + ALAN |
| **Fledging success** | Nest box + Hatch date + ALAN |

Table 4.4. Minimum adequate model summaries from linear mixed models for nestling weight, tarsus length and haematocrit (%) at days eight (d8) and day thirteen (d13), and fledging success. All variables were modelled using a Gaussian distribution except for fledging success, which was modelled using Binomial distribution. Significance ($p<0.05$) were shown in bold and values were obtained from likelihood ratio testing. Reference levels for the intercept were: Sex (F), Treatment (Control), Citronella (Citronella present, 1).

| **Variable** | **N** | **Estimate** | **s.e.m** | **d.f.** | ***t*-value** | ***p*-value LRT** |
|---|---|---|---|---|---|---|
| **Weight d8** | 153 | | | | | |
| (Intercept) | | 14.17 | 0.25 | | 56.94 | |
| Sex (M) | | 0.53 | 0.20 | 1 | 2.66 | **0.010** |
| ALAN | | -0.22 | 0.34 | 1 | -0.65 | 0.504 |
| **Weight d13** | 129 | | | | | |
| (Intercept) | | 20.53 | 0.94 | | 21.89 | |
| Sex (M) | | 0.60 | 0.21 | 1 | 2.90 | **0.004** |
| Brood size | | -0.25 | 0.13 | 1 | -1.96 | **0.045** |
| ALAN | | -0.85 | 0.42 | 1 | -2.04 | **0.038** |
| **Tarsus d8** | 191 | | | | | |
| (Intercept) | | 17.59 | 0.31 | | 56.59 | |
| ALAN | | -0.50 | 0.45 | 1 | -1.10 | 0.251 |
| **Tarsus d13** | 163 | | | | | |
| (Intercept) | | 20.29 | 0.21 | | 95.57 | |
| Sex (M) | | 0.35 | 0.10 | 1 | 3.36 | **<0.001** |
| ALAN | | -0.05 | 0.31 | 1 | -0.14 | 0.876 |
| **Haematocrit % d8** | 142 | | | | | |
| (Intercept) | | 65.45 | 8.48 | | 7.72 | |
| Sex(M) | | 2.86 | 1.27 | 1 | 2.25 | **0.023** |
| Brood Size | | -1.00 | 0.40 | 1 | -2.48 | **0.013** |
| Hatch Date | | -0.41 | 0.17 | 1 | -2.34 | **0.018** |
| ALAN | | -0.74 | 1.35 | 1 | -0.55 | 0.575 |
| **Haematocrit % d13** | 45 | | | | | |
| (Intercept) | | 38.92 | 1.16 | | 33.52 | |
| ALAN | | 2.33 | 1.77 | 1 | 1.32 | 0.175 |
| | | | | | | |
| **Variable** | **N** | **Estimate** | **s.e.m** | **d.f.** | **z-value** | ***p*-value LRT** |
| **Fledging Success** | 207 | | | | | |
| (Intercept) | | 34.85 | 13.20 | | 2.64 | |
| Hatch date | | -0.59 | 0.25 | 1 | -2.38 | **0.002** |
| ALAN | | 1.34 | 1.82 | 1 | 0.74 | 0.452 |

Table 4.5. Global model summaries from linear mixed models for nestling weight, tarsus length and haematocrit (%) at days eight (d8) and day thirteen (d13), and fledging success. All variables were modelled using a Gaussian distribution except for fledging success, which was modelled using Binomial distribution. Significance ($p<0.05$) were shown in bold and values were obtained from likelihood ratio testing. Reference levels for the intercept were: Sex (F), Treatment (Control), Citronella (Citronella present, 1).

| **Variable** | **N** | **Estimate** | **s.e.m** | **d.f.** | ***t*-value** | ***p*-value LRT** |
|---|---|---|---|---|---|---|
| **Weight d8** | 153 | | | | | |
| (Intercept) | | 16.93 | 2.36 | | 7.17 | |
| Sex (M) | | 0.53 | 0.20 | 1 | 2.65 | **0.012** |
| Brood Size | | -0.06 | 0.12 | 1 | -0.54 | 0.548 |
| Hatch Date | | -0.05 | 0.05 | 1 | -0.96 | 0.302 |
| ALAN | | -0.13 | 0.38 | 1 | -0.34 | 0.695 |
| Citronella(0) | | -0.22 | 0.36 | 1 | -0.60 | 0.514 |
| **Weight d13** | 129 | | | | | |
| (Intercept) | | 24.49 | 3.38 | | 7.30 | |
| Sex (M) | | 0.60 | 0.21 | 1 | 2.92 | **0.004** |
| Brood Size | | -0.22 | 0.13 | 1 | -1.71 | 0.066 |
| Hatch Date | | -0.09 | 0.07 | 1 | -1.26 | 0.177 |
| ALAN | | -0.75 | 0.43 | 1 | -1.75 | 0.059 |
| Citronella(0) | | -0.06 | 0.43 | 1 | -0.14 | 0.087 |
| **Tarsus d8** | 191 | | | | | |
| (Intercept) | | 19.93 | 2.92 | | 6.82 | |
| Sex(M) | | 0.16 | 0.16 | 1 | 1.04 | 0.290 |
| Brood Size | | -0.11 | 0.14 | 1 | -0.73 | 0.423 |
| Hatch Date | | -0.03 | 0.06 | 1 | -0.44 | 0.626 |
| ALAN | | -0.43 | 0.47 | 1 | -0.92 | 0.319 |
| Citronella(0) | | -0.82 | 0.45 | 1 | -1.84 | 0.051 |
| **Tarsus d13** | 163 | | | | | |
| (Intercept) | | 24.24 | 2.52 | | 9.60 | |
| Sex(M) | | 0.35 | 0.10 | 1 | 3.34 | **<0.001** |
| Brood Size | | -0.11 | 0.10 | 1 | -1.09 | 0.237 |
| Hatch Date | | -0.07 | 0.05 | 1 | -1.29 | 0.166 |
| ALAN | | -0.04 | 0.32 | 1 | -0.11 | 0.888 |
| Citronella(0) | | 0.17 | 0.32 | 1 | 0.52 | 0.561 |
| **Haematocrit % d8** | 142 | | | | | |
| (Intercept) | | 65.39 | 8.52 | | 7.68 | |
| Sex(M) | | 2.96 | 1.29 | 1 | 2.29 | **0.020** |
| Brood Size | | -1.03 | 0.41 | 1 | -2.51 | **0.011** |
| Hatch Date | | -0.41 | 0.17 | 1 | -2.35 | **0.017** |
| ALAN | | -0.77 | 1.36 | 1 | -0.57 | 0.564 |
| Citronella(0) | | 0.65 | 1.27 | 1 | 0.51 | 0.600 |
| **Haematocrit % d13** | 45 | | | | | |
| (Intercept) | | 39.00 | 14.97 | | 2.60 | |
| Sex(M) | | 0.53 | 1.58 | 1 | 0.34 | 0.728 |
| Brood Size | | -0.55 | 0.71 | 1 | -0.77 | 0.381 |
| Hatch Date | | 0.07 | 0.31 | 1 | 0.24 | 0.729 |
| ALAN | | 2.49 | 2.07 | 1 | 1.20 | 0.185 |
| Citronella(0) | | -0.33 | 2.14 | 1 | -0.15 | 0.852 |

| Variable | N | Estimate | s.e.m | d.f. | z-value | *p*-value LRT |
|---|---|---|---|---|---|---|
| **Fledging Success** | 207 | | | | | |
| (Intercept) | | 34.42 | 13.29 | | 2.59 | **<0.001** |
| Hatch date | | -0.58 | 0.25 | 1 | -2.35 | **0.019** |
| ALAN | | 1.34 | 1.86 | 1 | 0.72 | 0.472 |
| Citronella(0) | | 0.25 | 1.12 | 1 | 0.22 | 0.824 |

4.1.1. 4.1.2.

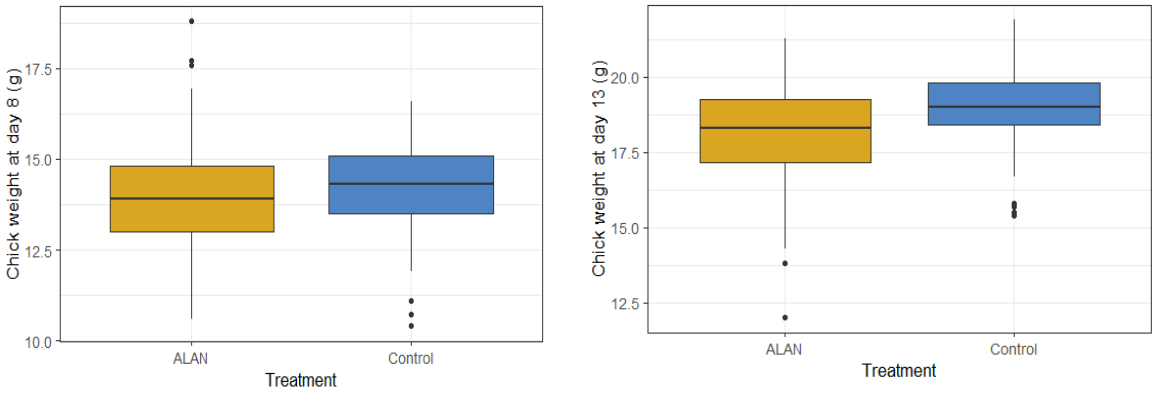



Figure 4.1. Artificial light at night and control treatment and nestling weight (g) at 4.1.1. Day eight and 4.1.2. Day thirteen. Box plots show the interquartile range of the raw data, where boxes above and below the medians (horizontal lines) show the first and third quartiles, respectively. Boxplot whiskers extend to 1.5 times the interquartile range. Dots represent outliers.

4.2.1. 4.2.2.

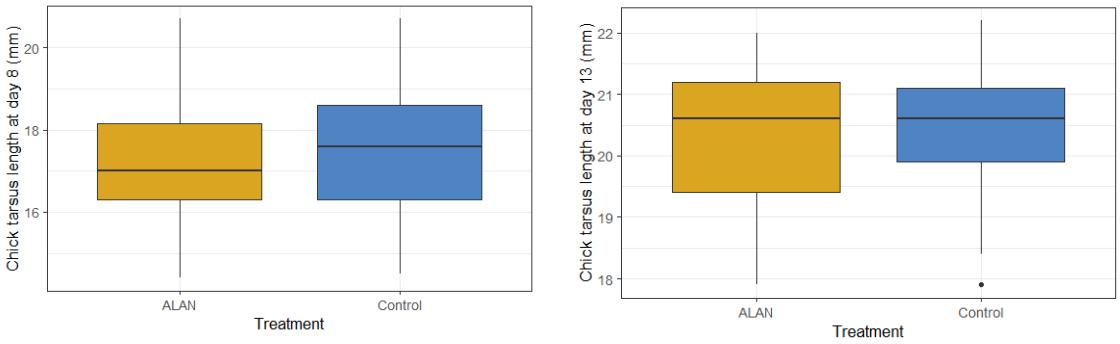



Figure 4.2. Artificial light at night and control treatment and tarsus length (mm) at 4.2.1. Day eight and 4.2.2. Day thirteen. Box plots show the interquartile range of the raw data, where boxes above and below the medians (horizontal lines) show the first and third quartiles, respectively. Boxplot whiskers extend to 1.5 times the interquartile range. Dots represent outliers.

4.3.1. 4.3.2.

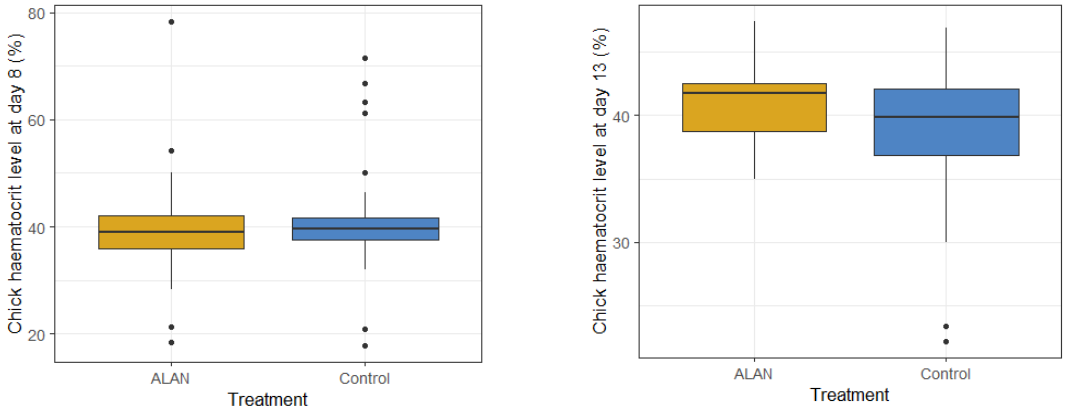



Figure 4.3. Artificial light at night and control treatment and haematocrit levels (%) at 4.3.1. Day eight and 4.3.2. Day thirteen. Box plots show the interquartile range of the raw data, where boxes above and below the medians (horizontal lines) show the first and third quartiles, respectively. Boxplot whiskers extend to 1.5 times the interquartile range. Dots represent outliers.

### 4.4.2 Artificial light at night treatment and gene transcript levels

Gene transcript levels (measured as median fluorescence intensity) were plotted for ALAN treatment in Figures 4.4. and 4.5. and outcomes of minimum adequate models listed in Table 4.6.

Transcript levels for target genes did not differ significantly between ALAN treatment and control groups, except for the clock gene *REVERBA* ($t_{1,93}$=-2.35: P=0.026, LMM), (Table 4.7). For this gene, transcript levels were significantly lower in ALAN birds (0.24 ± 0.32) than in control birds (0.51 ± 0.22), with a >2-fold difference overall (Figure 4.4.1.). Overall, no indication was found for interactions between time of day effects and ALAN treatment, and therefore this interaction term was dropped from minimum adequate models for all gene targets (Table 4.6).

Eleven (*IGF1, TLR4, IL1, IL6, PRKCA, GATA3, IKBA, BMAL1, CRY1, CK1Ɛ, AANAT*) out of seventeen target genes (both clock and immune) differed in transcript levels between day and night, with transcript levels lower at noon compared to midnight. These day-night differences ranged from a 1.13-fold change to 1.54-fold change (Figures 4.4., 4.5.). The remaining six gene targets (*LY86, REVERBA, CLOCK, NKRF, NRF2* and *PER2*) did not differ significantly in transcript levels between day and night (Figures 4.4., 4.5.).

Male nestlings had significantly lower transcript levels than females (ranging from a 1.14-fold change to 1.43-fold change) in eleven (*IGF1, TLR4, IL1, IL6, PRKCA, IKBA, BMAL1,*

*CRY1*, *CK1Ɛ*) clock and immune gene targets (Table 4.7). Male nestlings had significantly higher levels of *NKRF* and *NRF2* than females (Table 4.7). There was no evidence for effects of hatch date, brood size or citronella on transcript levels of target genes (Table 4.6.).

Global model summaries for clock and immune gene transcript data can be found in Table 4.8.

Table 4.6. Global and final (MAM) linear-mixed models of circadian clock and immune gene expression. SampleTime was a two-level factor (NOON/MIDNIGHT), ALAN treatment a two-level fixed factor (ALAN/Control) and ALAN*SampleTime a two-way interaction. All gene expression data were modelled using Gamma distribution except for *NRF2*, which was modelled using a Gaussian distribution. Global gene models were: Nest box (random) + Sample Time + Sex + ALAN + Brood size + Hatch date + Citronella + (Sample Time*ALAN). Nest box was always retained as a random factor. Addition of terms is denoted by a plus sign (+). Interaction of terms is denoted by an asterisk (*).

| Variable | Minimum Adequate Model (MAM) |
|---|---|
| ***LY86*** | Nest box + ALAN |
| ***REVERBA*** | Nest box + ALAN |
| ***IGF1*** | Nest box + Sample Time + Sex + ALAN |
| ***TLR4*** | Nest box + Sample Time + Sex + ALAN |
| ***IL1*** | Nest box + Sample Time + Sex + ALAN |
| ***IL6*** | Nest box + Sample Time + Sex + ALAN |
| ***PRKCA*** | Nest box + Sample Time + Sex + ALAN |
| ***GATA3*** | Nest box + Sample Time + ALAN |
| ***IKBA*** | Nest box + Sample Time + ALAN |
| ***BMAL1*** | Nest box + Sample Time + Sex + ALAN |
| ***CLOCK*** | Nest box + ALAN |
| ***CRY1*** | Nest box + Sample Time + Sex + ALAN |
| ***CK1Ɛ*** | Nest box + Sample Time + Sex + ALAN |
| ***AANAT*** | Nest box + Sample Time + Sex + ALAN |
| ***NKRF*** | Nest box + Sex + ALAN |
| ***NRF2*** | Nest box + Sex + ALAN |
| ***PER2*** | Nest box + ALAN |

Table 4.7. Minimum adequate model summaries of transcript levels of clock and immune targets from linear mixed models. Estimates are log-transformed. Significance ($p<0.05$) is emphasised in bold and values were obtained through likelihood ratio testing. Reference levels for the intercept were: Sex (F), SampleTime (Midnight), Treatment (Control).

| **Variable** | **N** | **Estimate** | **s.e.m** | **d.f.** | ***t*-value** | ***p*-value LRT** |
|---|---|---|---|---|---|---|
| ***LY86*** | 106 | | | | | |
| (Intercept) | | -1.55 | 0.002 | | -565.19 | |
| ALAN | | -0.03 | 0.002 | 1 | -11.16 | 0.853 |
| ***REVERBA*** | 93 | | | | | |
| (Intercept) | | -0.68 | 0.22 | | -3.13 | |
| ALAN | | -0.76 | 0.32 | 1 | -2.35 | **0.026** |
| ***IGF1*** | 93 | | | | | |
| (Intercept) | | -1.80 | 0.16 | | -11.26 | |
| Sex(M) | | -0.25 | 0.11 | 1 | -2.21 | **0.030** |
| Sample Time(Noon) | | -0.30 | 0.11 | 1 | -2.81 | **0.006** |
| ALAN | | 0.08 | 0.20 | 1 | -0.44 | 0.660 |
| ***TLR4*** | 96 | | | | | |
| (Intercept) | | -1.64 | 0.17 | | -9.68 | |
| Sex(M) | | -0.26 | 0.11 | 1 | -2.24 | **0.028** |
| Sample Time(Noon) | | -0.26 | 0.10 | 1 | -2.53 | **0.013** |
| ALAN | | -0.15 | 0.22 | 1 | -0.70 | 0.486 |
| ***IL1*** | 92 | | | | | |
| (Intercept) | | -1.64 | 0.14 | | -11.82 | |
| Sex(M) | | -0.23 | 0.10 | 1 | -2.24 | **0.027** |
| Sample Time(Noon) | | -0.35 | 0.10 | 1 | -3.49 | **0.001** |
| ALAN | | -0.23 | 0.16 | 1 | -1.43 | 0.172 |
| ***IL6*** | 88 | | | | | |
| (Intercept) | | -1.73 | 0.16 | | -10.63 | |
| Sex(M) | | -0.28 | 0.11 | 1 | -2.59 | **0.011** |
| Sample Time (Noon) | | -0.33 | 0.11 | 1 | -3.13 | **0.002** |
| ALAN | | -0.17 | 0.20 | 1 | -0.88 | 0.396 |
| ***PRKCA*** | 86 | | | | | |
| (Intercept) | | -1.85 | 0.18 | | -10.49 | |
| Sex(M) | | -0.30 | 0.12 | 1 | -2.51 | **0.014** |
| Sample Time(Noon) | | -0.34 | 0.11 | 1 | -3.03 | **0.003** |
| ALAN | | -0.07 | 0.21 | 1 | -0.34 | 0.734 |
| ***GATA3*** | 98 | | | | | |
| (Intercept) | | -1.84 | 0.13 | | -14.05 | |
| Sample Time(Noon) | | -0.17 | 0.09 | 1 | -1.86 | **0.033** |
| ALAN | | -0.10 | 0.17 | 1 | -0.59 | 0.561 |

| Variable | N | Estimate | s.e.m | d.f. | *t*-value | *p*-value LRT |
|---|---|---|---|---|---|---|
| ***IKBA*** | 102 | | | | | |
| (Intercept) | | -1.78 | 0.16 | | -10.99 | |
| Sex(M) | | -0.20 | 0.09 | 1 | -2.15 | **0.033** |
| Sample Time(Noon) | | -0.23 | 0.08 | 1 | -2.77 | **0.006** |
| ALAN | | -0.05 | 0.22 | 1 | -0.23 | 0.818 |
| | | | | | | |
| ***BMAL1*** | 94 | | | | | |
| (Intercept) | | -1.75 | 0.14 | | -12.65 | |
| Sex(M) | | -0.30 | 0.09 | 1 | -3.46 | **0.001** |
| Sample Time(Noon) | | -0.20 | 0.08 | 1 | -2.37 | **0.019** |
| ALAN | | -0.02 | 0.18 | 1 | -0.13 | 0.896 |
| | | | | | | |
| ***CLOCK*** | 101 | | | | | |
| (Intercept) | | -1.56 | 0.07 | | -22.39 | |
| ALAN | | -0.17 | 0.10 | 1 | -1.64 | 0.108 |
| | | | | | | |
| ***CRY1*** | 109 | | | | | |
| (Intercept) | | -0.86 | 0.08 | | -10.26 | |
| Sex(M) | | -0.12 | 0.06 | 1 | -2.02 | **0.046** |
| Sample Time(Noon) | | -0.12 | 0.05 | 1 | -2.22 | **0.028** |
| ALAN | | -0.14 | 0.10 | 1 | -1.38 | 0.180 |
| | | | | | | |
| ***CK1Ɛ*** | 93 | | | | | |
| (Intercept) | | -1.82 | 0.16 | | -11.27 | |
| Sex(M) | | -0.25 | 0.10 | 1 | -2.72 | **0.016** |
| Sample Time(Noon) | | -0.27 | 0.10 | 1 | -2.72 | **0.007** |
| ALAN | | -0.04 | 0.21 | 1 | -0.19 | 0.852 |
| | | | | | | |
| ***AANAT*** | 92 | | | | | |
| (Intercept) | | -1.83 | 0.16 | | -11.33 | |
| Sample Time(Noon) | | -0.23 | 0.11 | 1 | -2.21 | **0.013** |
| ALAN | | -0.19 | 0.22 | 1 | -0.85 | 0.369 |
| | | | | | | |
| ***NKRF*** | 108 | | | | | |
| (Intercept) | | 0.48 | 0.10 | | 4.88 | |
| Sex(M) | | 0.11 | 0.04 | 1 | 3.17 | **0.002** |
| ALAN | | -0.2 | 0.15 | 1 | -1.38 | 0.175 |
| | | | | | | |
| ***NRF2*** | 110 | | | | | |
| (Intercept) | | 5.75 | 0.35 | | 16.26 | |
| Sex(M) | | 1.07 | 0.21 | 1 | 4.99 | **<0.001** |
| ALAN | | -0.50 | 0.51 | 1 | -0.98 | 0.227 |
| | | | | | | |
| ***PER2*** | 105 | | | | | |
| (Intercept) | | -1.44 | 0.15 | | -9.35 | |
| ALAN | | -0.19 | 0.23 | 1 | -0.82 | 0.421 |

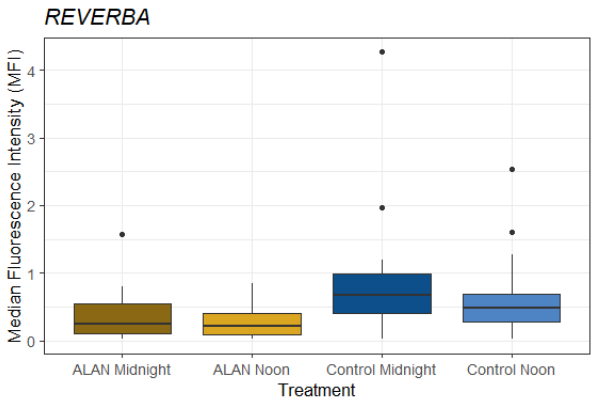
Table 4.8. Global model summaries of transcript levels of clock and immune targets from linear mixed models. Estimates are log-transformed. Significance ($p<0.05$) is emphasised in bold and values were obtained through likelihood ratio testing. Reference levels for the intercept were: Sex (F), SampleTime (Midnight), Treatment (Control).

| **Variable** | **N** | **Estimate** | **s.e.m** | **d.f.** | ***t*-value** | ***p*-value LRT** |
|---|---|---|---|---|---|---|
| ***LY86*** | 106 | | | | | |
| (Intercept) | | -2.09 | 1.26 | | -1.66 | 0.096 |
| SampleTime(Noon) | | -0.04 | 0.08 | 1 | -0.46 | 0.649 |
| Sex(M) | | -0.08 | 0.06 | 1 | -1.26 | 0.207 |
| ALAN | | 0.03 | 0.17 | 1 | 0.18 | 0.857 |
| Brood Size | | -0.02 | 0.05 | 1 | -0.37 | 0.710 |
| Citronella(0) | | 0.12 | 0.15 | 1 | 0.81 | 0.420 |
| Hatch Date | | 0.01 | 0.03 | 1 | 0.53 | 0.597 |
| SampleTime*ALAN | | -0.15 | 0.11 | 2 | -1.27 | 0.203 |
| ***REVERBA*** | 93 | | | | | |
| (Intercept) | | 2.17 | 2.57 | | 0.85 | 0.397 |
| SampleTime(Noon) | | -0.16 | 0.21 | 1 | -0.80 | 0.424 |
| Sex(M) | | -0.28 | 0.16 | 1 | -1.76 | 0.078 |
| ALAN | | -0.60 | 0.36 | 1 | -1.66 | 0.097 |
| Brood Size | | -0.06 | 0.09 | 1 | 0.59 | 0.555 |
| Citronella(0) | | 0.05 | 0.31 | 1 | 0.17 | 0.863 |
| Hatch Date | | -0.06 | 0.05 | 1 | -1.19 | 0.236 |
| SampleTime*ALAN | | -0.02 | 0.29 | 2 | -0.71 | 0.481 |
| ***IGF1*** | 93 | | | | | |
| (Intercept) | | -4.02 | 1.58 | | -2.54 | **0.011** |
| SampleTime(Noon) | | -0.27 | 0.14 | 1 | -1.88 | 0.060 |
| Sex(M) | | -0.25 | 0.12 | 1 | -2.15 | **0.031** |
| ALAN | | -0.10 | 0.23 | 1 | -0.45 | 0.652 |
| Brood Size | | -0.03 | 0.06 | 1 | -0.45 | 0.650 |
| Citronella(0) | | -0.07 | 0.19 | 1 | -0.36 | 0.718 |
| Hatch Date | | 0.05 | 0.03 | 1 | 1.52 | 0.127 |
| SampleTime*ALAN | | -0.08 | 0.21 | 2 | -0.36 | 0.717 |
| ***TLR4*** | 96 | | | | | |
| (Intercept) | | -3.55 | 1.78 | | -2.00 | 0.046 |
| SampleTime(Noon) | | -0.21 | 0.14 | 1 | -1.48 | 0.140 |
| Sex(M) | | -0.26 | 0.12 | 1 | -2.26 | **0.024** |
| ALAN | | -0.13 | 0.25 | 1 | -0.53 | 0.599 |
| Brood Size | | -0.02 | 0.06 | 1 | -0.25 | 0.805 |
| Citronella(0) | | -0.08 | 0.21 | 1 | -0.36 | 0.719 |
| Hatch Date | | 0.04 | 0.04 | 1 | 1.15 | 0.251 |
| SampleTime*ALAN | | -0.12 | 0.21 | 2 | -0.58 | 0.564 |
| ***IL1*** | 92 | | | | | |
| (Intercept) | | -2.45 | 1.35 | | -1.81 | 0.070 |
| SampleTime(Noon) | | -0.25 | 0.14 | 1 | -1.86 | 0.063 |
| Sex(M) | | -0.23 | 0.10 | 1 | -2.25 | **0.025** |
| ALAN | | -0.14 | 0.21 | 1 | -0.69 | 0.489 |
| Brood Size | | -0.01 | 0.05 | 1 | -0.11 | 0.911 |
| Citronella(0) | | 0.07 | 0.17 | 1 | 0.44 | 0.660 |
| Hatch Date | | 0.02 | 0.03 | 1 | 0.56 | 0.573 |
| SampleTime*ALAN | | -0.17 | 0.20 | 2 | -0.87 | 0.384 |

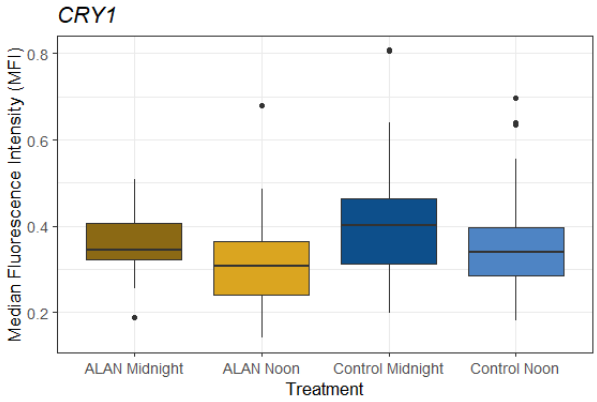
| Variable | N | Estimate | s.e.m | d.f. | *t*-value | *p*-value LRT |
|---|---|---|---|---|---|---|
| ***IL6*** | 88 | | | | | |
| (Intercept) | | -3.40 | 1.55 | | -2.19 | 0.029 |
| SampleTime(Noon) | | -0.22 | 0.15 | 1 | -1.51 | 0.131 |
| Sex(M) | | -0.31 | 0.11 | 1 | -2.76 | **0.006** |
| ALAN | | -0.11 | 0.23 | 1 | -0.46 | 0.643 |
| Brood Size | | -0.02 | 0.06 | 1 | -0.42 | 0.675 |
| Citronella(0) | | -0.07 | 0.19 | 1 | -0.37 | 0.711 |
| Hatch Date | | 0.04 | 0.03 | 1 | 1.17 | 0.240 |
| SampleTime*ALAN | | -0.23 | 0.21 | 2 | -1.09 | 0.278 |
| ***PRKCA*** | 86 | | | | | |
| (Intercept) | | -4.00 | 1.69 | | -2.37 | 0.018 |
| SampleTime(Noon) | | -0.30 | 0.15 | 1 | -1.95 | 0.051 |
| Sex(M) | | -0.29 | 0.12 | 1 | -2.44 | **0.015** |
| ALAN | | -0.09 | 0.25 | 1 | -0.35 | 0.730 |
| Brood Size | | -0.05 | 0.06 | 1 | -0.81 | 0.416 |
| Citronella(0) | | -0.11 | 0.20 | 1 | -0.53 | 0.599 |
| Hatch Date | | 0.05 | 0.03 | 1 | 1.50 | 0.134 |
| SampleTime*ALAN | | -0.09 | 0.22 | 2 | -0.39 | 0.695 |
| ***GATA3*** | 98 | | | | | |
| (Intercept) | | -3.05 | 1.45 | | -2.11 | 0.035 |
| SampleTime(Noon) | | -0.16 | 0.13 | 1 | -1.24 | 0.214 |
| Sex(M) | | -0.16 | 0.10 | 1 | -1.61 | 0.108 |
| ALAN | | -0.10 | 0.21 | 1 | -0.47 | 0.635 |
| Brood Size | | -0.01 | 0.05 | 1 | -0.23 | 0.819 |
| Citronella(0) | | -0.02 | 0.18 | 1 | -0.13 | 0.897 |
| Hatch Date | | 0.03 | 0.03 | 1 | 0.94 | 0.347 |
| SampleTime*ALAN | | -0.08 | 0.19 | 2 | -0.43 | 0.669 |
| ***IKBA*** | 102 | | | | | |
| (Intercept) | | -4.77 | 1.69 | | -2.82 | 0.005 |
| SampleTime(Noon) | | -0.18 | 0.12 | 1 | -1.54 | 0.125 |
| Sex(M) | | -0.20 | 0.09 | 1 | -2.07 | 0.038 |
| ALAN | | -0.07 | 0.23 | 1 | -0.28 | 0.777 |
| Brood Size | | -0.01 | 0.06 | 1 | -0.20 | 0.845 |
| Citronella(0) | | -0.01 | 0.20 | 1 | -0.03 | 0.977 |
| Hatch Date | | 0.06 | 0.03 | 1 | 1.81 | 0.070 |
| SampleTime*ALAN | | -0.10 | 0.16 | 2 | -0.61 | 0.541 |
| ***BMAL1*** | 94 | | | | | |
| (Intercept) | | -2.62 | 1.44 | | -1.81 | 0.070 |
| SampleTime(Noon) | | -0.14 | 0.12 | 1 | -1.17 | 0.242 |
| Sex(M) | | -0.31 | 0.09 | 1 | -3.54 | <0.001 |
| ALAN | | 0.03 | 0.20 | 1 | 0.16 | 0.876 |
| Brood Size | | -0.02 | 0.05 | 1 | -0.33 | 0.742 |
| Citronella(0) | | -0.11 | 0.18 | 1 | -0.62 | 0.537 |
| Hatch Date | | 0.02 | 0.03 | 1 | 0.69 | 0.489 |
| SampleTime*ALAN | | -0.13 | 0.16 | 2 | -0.81 | 0.419 |
| ***CLOCK*** | 101 | | | | | |
| (Intercept) | | -0.69 | 0.30 | | -2.27 | 0.023 |
| SampleTime(Noon) | | -0.07 | 0.08 | 1 | -0.91 | 0.362 |
| Sex(M) | | -0.12 | 0.06 | 1 | -2.10 | 0.036 |
| ALAN | | -0.17 | 0.12 | 1 | -1.40 | 0.161 |
| Brood Size | | -0.02 | 0.03 | 1 | -0.79 | 0.428 |
| Citronella(0) | | 0.04 | 0.10 | 1 | 0.36 | 0.715 |
| Hatch Date | | -0.01 | 0.01 | 1 | -3.10 | 0.002 |
| SampleTime*ALAN | | -0.02 | 0.11 | 2 | -0.22 | 0.829 |

| Variable | N | Estimate | s.e.m | d.f. | *t*-value | *p*-value LRT |
|---|---|---|---|---|---|---|
| ***CRY1*** | 109 | | | | | |
| (Intercept) | | 0.36 | 0.24 | | 1.46 | |
| SampleTime(Noon) | | -0.05 | 0.03 | 1 | -1.54 | |
| Sex(M) | | -0.04 | 0.02 | 1 | -1.62 | 0.103 |
| ALAN | | -0.05 | 0.04 | 1 | -1.23 | |
| Brood Size | | 0.01 | 0.01 | 1 | 0.77 | 0.374 |
| Citronella(0) | | -0.01 | 0.03 | 1 | -0.41 | 0.638 |
| Hatch Date | | <0.01 | 0.01 | 1 | 0.14 | 0.912 |
| SampleTime*ALAN | | <0.01 | 0.05 | 2 | -0.04 | 0.955 |
| ***CK1Ɛ*** | 93 | | | | | |
| (Intercept) | | -4.65 | 1.59 | | -2.93 | 0.003 |
| SampleTime(Noon) | | -0.20 | 0.13 | 1 | -1.49 | 0.137 |
| Sex(M) | | -0.25 | 0.10 | 1 | -2.49 | 0.013 |
| ALAN | | -0.03 | 0.23 | 1 | -0.12 | 0.908 |
| Brood Size | | -0.02 | 0.06 | 1 | -0.40 | 0.692 |
| Citronella(0) | | -0.04 | 0.19 | 1 | -0.19 | 0.850 |
| Hatch Date | | 0.06 | 0.03 | 1 | 1.88 | 0.061 |
| SampleTime*ALAN | | -0.14 | 0.20 | 2 | -0.74 | 0.461 |
| ***AANAT*** | 92 | | | | | |
| (Intercept) | | -4.35 | 1.75 | | -2.49 | 0.013 |
| SampleTime(Noon) | | -0.21 | 0.14 | 1 | -1.46 | 0.145 |
| Sex(M) | | -0.21 | 0.12 | 1 | -1.78 | 0.075 |
| ALAN | | -0.20 | 0.25 | 1 | -0.82 | 0.411 |
| Brood Size | | -0.02 | 0.06 | 1 | -0.37 | 0.715 |
| Citronella(0) | | -0.05 | 0.21 | 1 | -0.23 | 0.816 |
| Hatch Date | | -0.06 | 0.04 | 1 | 1.58 | 0.113 |
| SampleTime*ALAN | | -0.11 | 0.21 | 2 | -0.55 | 0.585 |
| ***NKRF*** | 108 | | | | | |
| (Intercept) | | -4.35 | 1.75 | | -2.49 | 0.013 |
| SampleTime(Noon) | | -0.21 | 0.14 | 1 | -1.46 | 0.145 |
| Sex(M) | | -0.21 | 0.12 | 1 | -1.78 | 0.075 |
| ALAN | | -0.20 | 0.25 | 1 | -0.82 | 0.411 |
| Brood Size | | -0.02 | 0.06 | 1 | -0.37 | 0.715 |
| Citronella(0) | | -0.05 | 0.21 | 1 | -0.23 | 0.816 |
| Hatch Date | | 0.06 | 0.04 | 1 | 1.58 | 0.113 |
| SampleTime*ALAN | | -0.11 | 0.21 | 2 | -0.55 | 0.585 |
| ***NRF2*** | 110 | | | | | |
| (Intercept) | | 14.67 | 3.72 | | 3.95 | |
| SampleTime(Noon) | | 0.03 | 0.28 | 1 | 0.09 | |
| Sex(M) | | 1.02 | 0.22 | 1 | 4.65 | <0.001 |
| ALAN | | 0.07 | 0.52 | 1 | 0.13 | |
| Brood Size | | 0.26 | 0.14 | 1 | 1.92 | 0.040 |
| Citronella(0) | | 0.26 | 0.45 | 1 | 0.58 | 0.518 |
| Hatch Date | | -0.22 | 0.08 | 1 | -2.93 | 0.003 |
| SampleTime*ALAN | | -0.39 | 0.41 | 2 | -0.97 | 0.306 |
| ***PER2*** | 105 | | | | | |
| (Intercept) | | -4.19 | 1.82 | | -2.30 | 0.021 |
| SampleTime(Noon) | | -0.07 | 0.11 | 1 | -0.63 | 0.531 |
| Sex(M) | | -0.13 | 0.09 | 1 | -1.47 | 0.142 |
| ALAN | | -0.24 | 0.24 | 1 | -1.01 | 0.314 |
| Brood Size | | <0.01 | 0.07 | 1 | -0.07 | 0.943 |
| Citronella(0) | | -0.13 | 0.22 | 1 | -0.61 | 0.545 |
| Hatch Date | | 0.06 | 0.04 | 1 | 1.64 | 0.101 |
| SampleTime*ALAN | | -0.07 | 0.16 | 2 | -0.40 | 0.686 |

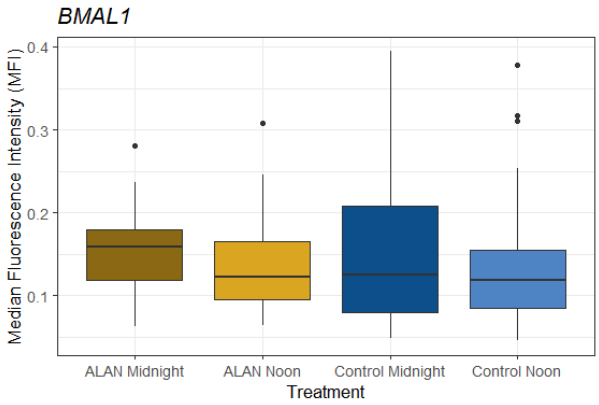
4.4.1.



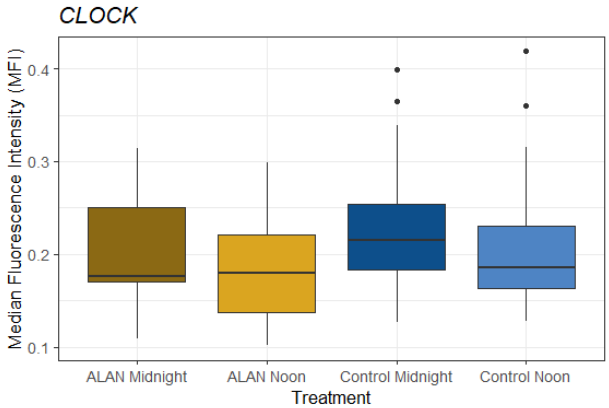

4.4.2.



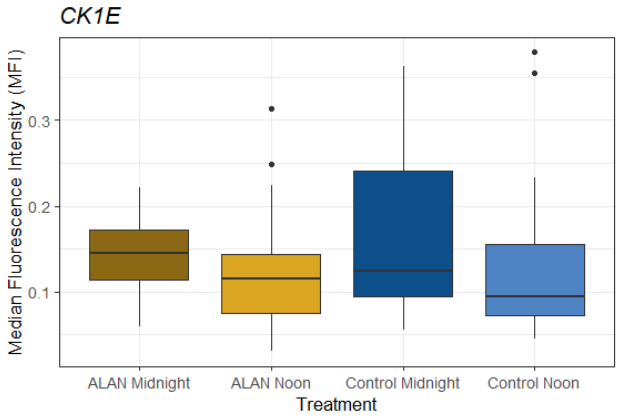

4.4.3.



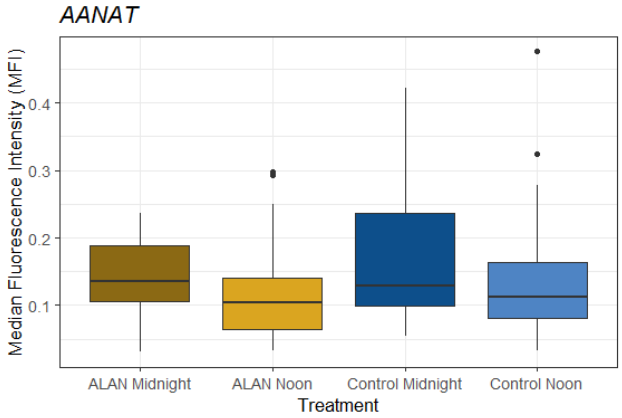

4.4.4.



4.4.5.




4.4.6.




4.4.7.

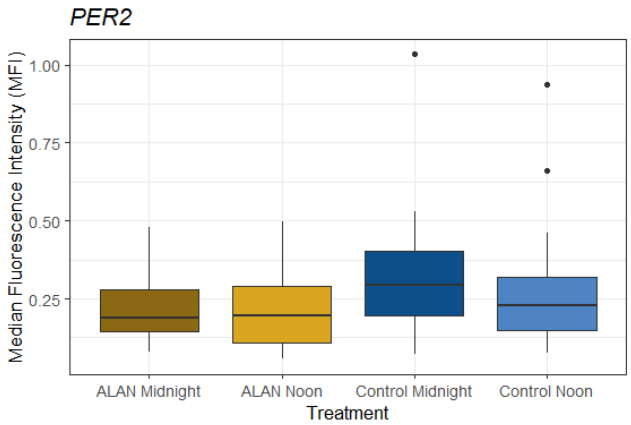
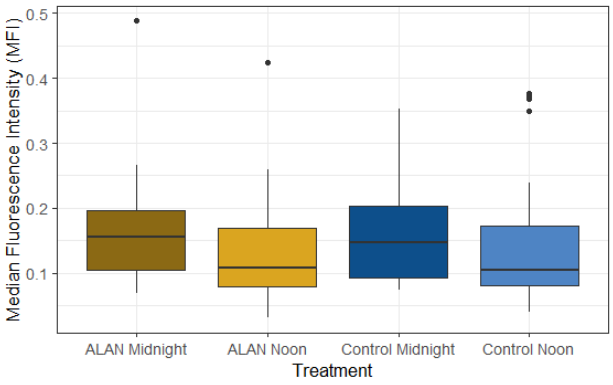



Figure 4.4. Artificial light at night and control treatment and transcript levels of circadian clock gene targets. 1. *REVERBA*, 2. *CRY1*, 3. *BMAL1*, 4. *CLOCK*, 5. *CK1E*, 6. *AANAT*, 7. *PER2*.

Colours indicate treatment and time of day of sampling: ALAN Midnight (Dark yellow), ALAN Noon (Light yellow), Control Midnight (Dark blue), Control Noon (Light blue). Data are shown by sample time at NOON (10:00-15:00 GMT) and NIGHT (23:00-02:00 GMT). Transcript levels are measured in Median Fluorescence Intensity Units (MFI). Box plots show the interquartile range of the raw data, where boxes above and below the medians (horizontal lines) show the first and third quartiles, respectively. Boxplot whiskers extend to 1.5 times the interquartile range, and dots represent outliers.

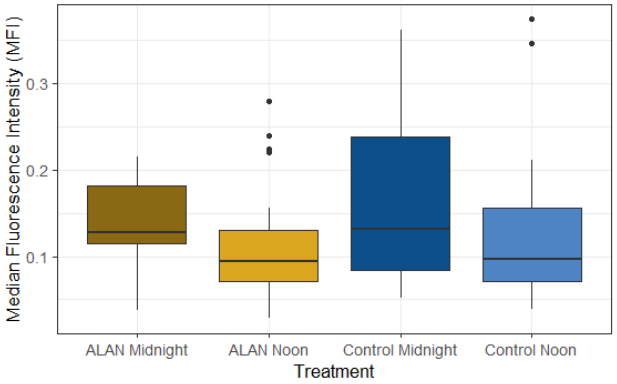
4.5.1.

*IKBA*



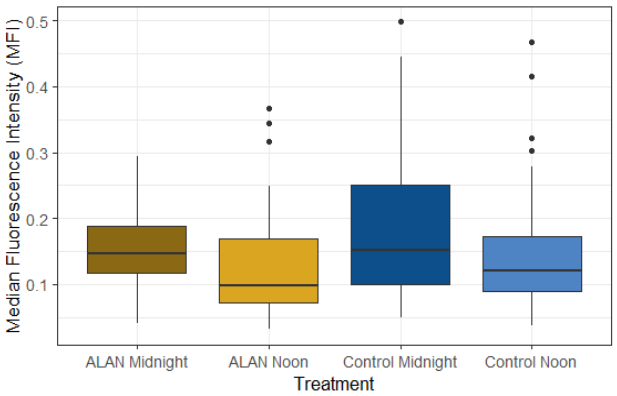

4.5.2.

*IGF1*



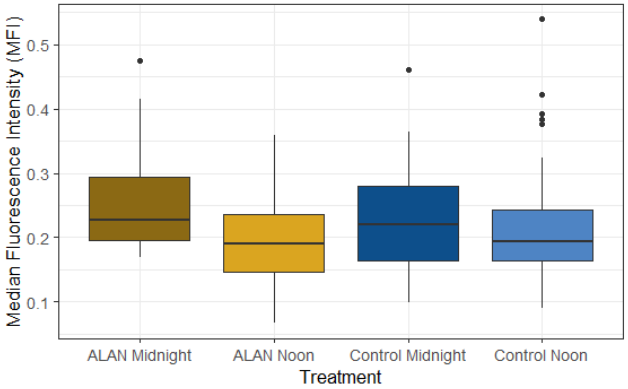

4.5.3.

*TLR4*



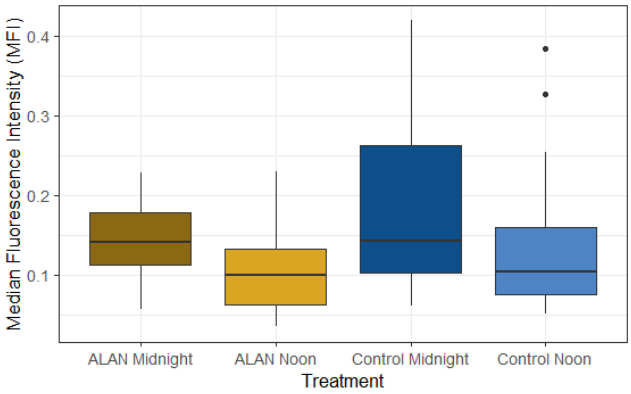

4.5.4.

*LY86*



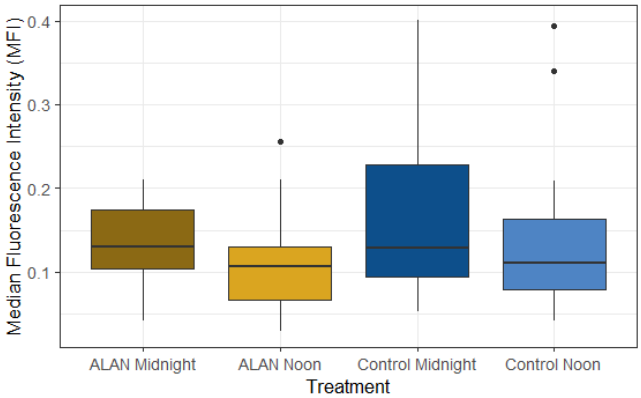
4.5.5.

*IL1*



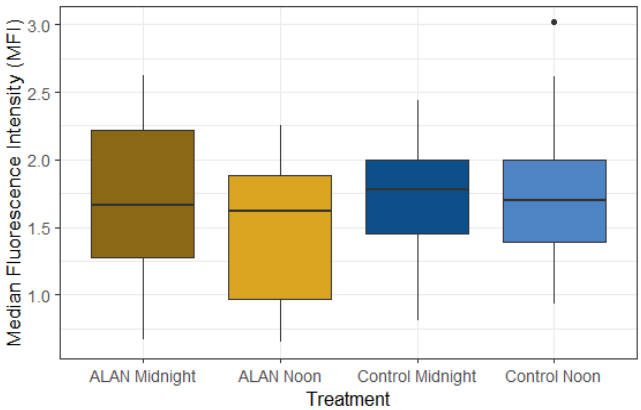

4.5.6.

*IL6*



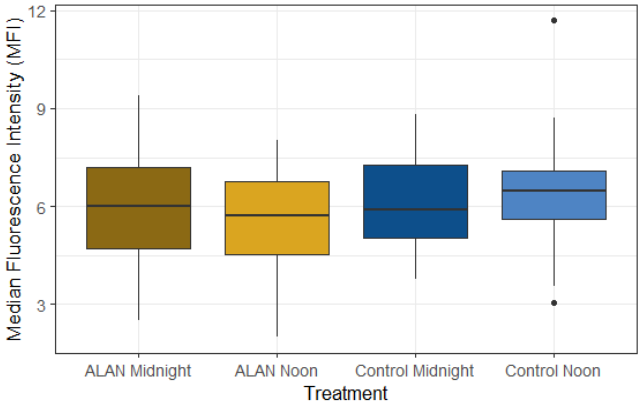

4.5.7.

*NKRF*

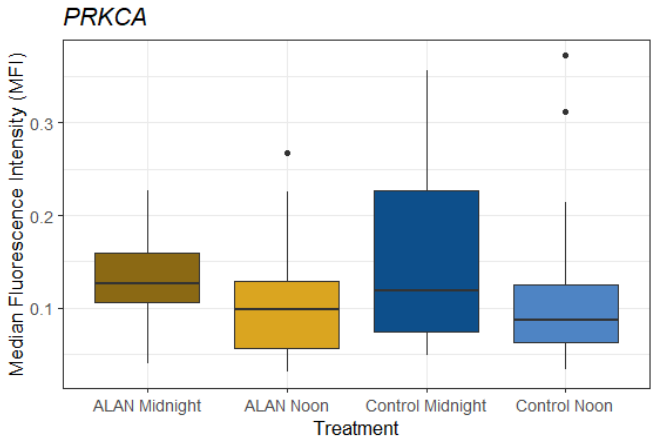



4.5.8.

*NRF2*



4.5.9.




4.5.10

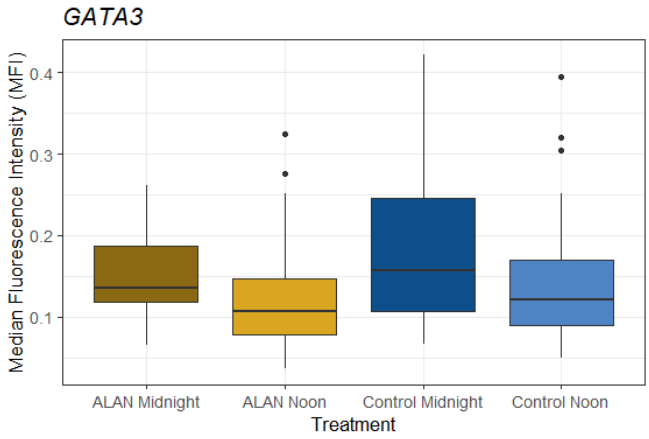



Figure 4.5. Artificial light at night (ALAN) and control treatments and transcript levels of immune gene targets. 1. *IKBA*, 2. *IGF1*, 3. *TLR4*, 4. *LY86*, 5. *IL1*, 6. *IL6*, 7. *NKRF*, 8. *NRF2*, 9. *PRKCA*, 10. *GATA3*.

Colours indicate treatment and time of day of sampling: ALAN Midnight (Dark yellow), ALAN Noon (Light yellow), Control Midnight (Dark blue), Control Noon (Light blue). Data are shown by sample time at NOON (10:00-15:00 GMT) and NIGHT (23:00-02:00 GMT). Transcript levels are measured in Median Fluorescence Intensity Units (MFI). Box plots show the interquartile range of the raw data, where boxes above and below the medians (horizontal lines) show the first and third quartiles, respectively. Boxplot whiskers extend to 1.5 times the interquartile range, and dots represent outliers.

## 4.5 Discussion

### 4.5.1 Effects of ALAN on nestling condition

In a previous experimental nest box study on great tits, exposure to ALAN halted nestling growth (Raap et al., 2016a). Here, ALAN treatment did not halt growth, yet nestlings under treatment were significantly lighter than control birds at day thirteen. This difference in result between the two studies may be explained by experimental design; Raap et al., (2016a) exposed nestlings to ALAN treatment for two consecutive nights on days thirteen to fifteen, whereas in this study, nestlings were exposed for a longer period during the nestling growth phase. Nestlings in this study were also weighed on day thirteen, which was the beginning of light treatment in Raap et al., (2016a). It is possible that effects of ALAN depend on stage of growth, where exposure prior to day thirteen of life has more impact on nestling weight than after day thirteen. The light intensity of ALAN treatment used by Raap et al., (2016a) was also three times (3 lux) that used in this study (1 lux), and therefore resulting effects on nestling weight may have been stronger. Given that small reductions in nestling weight have been demonstrated to negatively affect prospects for recruitment into the population post-fledging (Both et al., 1999) and for survival (Ringsby et al., 1998), observed weight differences as a consequence of ALAN exposure may have significant negative impacts on avian life histories.

The tarsus length and fledging success of nestlings was not affected by ALAN treatment as per a previous experimental study on great tit nestlings (Grunst et al., 2019). The lack of observable effect on fledgling was perhaps due to high level of fledging success overall at the study site. Sex effects on both the weight and tarsus length of nestlings were as expected, as male great tits are structurally larger than females (Gosler, 1993).

Nestlings under ALAN treatment did not have significantly different levels of haematocrit to control birds. Haematocrit levels in birds are highly variable, and may have been affected by many features at study sites, such as ambient temperatures (Fair et al., 2007).

### 4.5.2 Effects of ALAN on transcript levels of clock and immune genes

Previous studies on effects of ALAN on gene expression have shown either downregulations of clock and immune genes (Bedrosian et al., 2013, 2011), or an elevation of aspects of immune activity (Raap et al., 2016b; Saini et al., 2019). Here, it was expected that transcript levels of immune and clock genes would be altered by ALAN. Suppressive effects were found for *REVERBA*, a gene involved in the core pathway of the circadian clock as part of the *REVERBA/ROR* response element, which generates expression of *BMAL1*, and in antiphase to *PER2* (Ueda et al., 2002). It is not clear in this study why *REVERBA* was suppressed by ALAN more than in circadian clock genes of the same pathway, such as *BMAL1*. However, given that *REVERBA* plays an important role in glucose homeostasis (Yin et al., 2007), lipid metabolism (Raspe et al., 2002) and adipogenesis (Fontaine et al., 2003), its observed suppression by ALAN may have negative impacts on nestling physiology.

For most genes, transcripts levels significantly differed between day and night. However, ALAN treatment had no interaction with time of day differences in transcript levels, and therefore in this study there was no evidence of circadian disruption by ALAN. It may be that the low intensity of light used in this study (1 lux) may not have been sufficiently bright to disrupt rhythmic transcript levels of genes. In a study on zebra finches, (*Taeniopygia guttata*), the rhythmic expression of pro and anti-inflammatory cytokines was lost under constant bright light exposure, but not under dim light of 3 lux (Mishra et al., 2019). In another study on Siberian hamsters (*Phodopus sungorus*), dim light at night (5 lux) reduced peaks of daily expression of circadian clock proteins *PER2* and *BMAL1* in the hypothalamus and SCN (Bedrosian et al., 2013). In both studies, dim light treatment was of higher intensity than the ALAN treatment used here. Given that avian responses to ALAN are light-intensity dependent for behaviour (de Jong et al., 2016) reproductive biology (Dominoni et al., 2018; Zhang et al., 2019) and the endocrine system (Ouyang et al., 2018), it is likely that transcript level responses are also dependent on the intensity of light. Future studies investigating the effects of ALAN on the rhythmicity of avian immune and circadian clock systems should incorporate varying intensities of light treatment to elucidate whether transcript level responses are light intensity dependent.

In this study, there appeared to be no difference in how positive clock elements (e.g. *CLOCK, BMAL1*) were affected by ALAN treatment, compared with negative clock elements (*PER2, CRY1*). Given that Bedrosian et al., (2013) found ALAN treatment to reduce peaks of proteins found in both the positive circadian clock loop (*BMAL1*) and

negative clock loop (*PER2*), it is possible that ALAN may affect all aspects of the circadian clock in a similar fashion. Indeed, in this study, all circadian clock gene targets (*REVERBA, BMAL1, CLOCK, CRY1, CK1Ɛ, AANAT, PER2*) were downregulated by ALAN treatment, although all except *REVERBA* were non-significant effects.

This study makes use of samples taken during two time windows, at noon and at midnight. A potential limitation of this was that "noon" in this case was not exactly "noon" but a time window of a few hours (10:00-15:00). The nature of sampling transcript levels within these two time windows meant that the peak of expression of several genes (e.g. *LY86, REVERBA, CLOCK, NKRF, NRF2*) may have occurred for example at 18:00, and therefore the phase would have been missed. As a result, the circadian profile of these genes is unknown. Therefore, it is not possible to say that the lack of day-night differences in levels of these gene targets is due to circadian disruption. This gap in view of the "true" circadian profiles may also explain why circadian clock genes involved in the positive feedback loop (such as *BMAL1* and *CLOCK*) did not appear to be in the opposite phase to negative clock elements (*PER2, CRY1*), as both elements may have been in "mid-phase" during sampling. Future studies should incorporate a time profile approach (as in Chapter Six), taking blood samples from nestlings throughout the day and night in order to capture fluctuations in transcript levels of each circadian gene across 24 hr.

For many immune genes in this study, there were significant effects of sex, where female nestlings had higher transcript levels for many genes (*IGF1, TLR4, IL1, IL6, PRKCA, IKBA*) than males. Previous research of immunity in free-living juvenile birds has shown male nestlings to be less immunocompetent than females (Fargallo et al., 2002; Tschirren et al., 2003). These sex-specific differences in immunocompetence of nestlings are often explained by the modulating effects of sex hormones (Gaillard and Spinedi, 1998). Additionally, levels of several clock gene transcripts (*BMAL1, CRY1, CK1E*) also appeared to be lower in male nestlings than in female nestlings. In songbird species, there is a sexual dimorphism in the number of melatonin binding sites in telencephalic region of the brain (Aste et al., 2001). Given the role of melatonin in regulation of the avian circadian system (Gwinner et al., 1997), this dimorphism may explain observed differences in levels of circadian transcripts between male and female nestlings in this study.

A key strength of this study was that it was undertaken in the natural environment. However, the wild nature of this study may have made it difficult to capture transcript level responses to ALAN, given that gene expression in wild animals is highly variable in comparison to captive model species (Alvarez et al., 2015). Effects of ALAN were most

conspicuous for *REVERBA*, which interestingly, was one of the few genes in this study without an effect of time of day or sex. For this gene, there may have been less noise from other factors in statistical models, and therefore the effects of ALAN were more evident.

### 4.5.3 Conclusions

Ultimately, in this study, there was no indication that nestlings under ALAN treatment were in poor condition or had reduced fledging success. However, nestlings under ALAN treatment did weigh less than control birds, which may have fitness consequences post-fledging. This study also provided evidence that even low-levels of light at night may suppress elements of the avian circadian clock mechanism. This was an important new addition to previous studies that have shown shifts in activity (de Jong et al., 2016; Spoelstra et al., 2018) and physiology (Raap et al., 2016b; Saini et al., 2019) as a response to experimental doses of ALAN. Suppression of clock system elements may have consequences for wild birds, particularly during fitness challenges such as parasitic infection.

Finally, a novel aspect of this study was that it showed that it was possible to capture time of day differences in transcript levels of genes involved in circadian and immune pathways in wild animals. This study opened exciting avenues for further research into the effects of light at night on wildlife, and for field chronobiology.

# Chapter Five: Avian malaria infection and condition in wild nestlings at city and forest sites

## 5.1 Introduction

Parasites impose significant costs for the fitness and reproduction of wild birds (Hamilton and Zuk, 1982). Avian malaria is caused by Haemosporidian parasites belonging to genera *Leucocytozoon*, *Haemoproteus* and *Plasmodium*, and is globally distributed except for Antarctica. Given their wide distribution and ability to infect bird species from every order examined to date (Lapointe et al., 2012), avian malaria parasites provide ideal systems for studies of host-parasite interactions and life history trade-offs (Christe et al., 2012; Coon et al., 2016; Marzal et al., 2005). Molecular techniques to determine the presence or absence of parasites in samples of DNA obtained from blood (Hellgren et al., 2006), or direct quantification of parasites from examination of blood smears (Garnham, 1966), have facilitated ecological studies on the impacts of avian malaria infections on wild birds.

All avian malaria parasites have similar life cycles, as shown in Figure 5.1., (Atkinson and Van Riper, 1991). Dipteran vectors (e.g. *Culex, Aedes* and *Culiseta* mosquitoes, *Simuliidae* blackflies and *Culicoides* biting midges) introduce infective-stage sporozoites to the avian host via blood meal. Following successful transmission, parasites migrate to liver cells of the host, and mature into schizonts, which then rupture and release merozoites into the host bloodstream. Merozoites then infect circulating host blood cells and undergo further asexual replication, maturing into schizonts that rupture host cells and release new merozoites. Gametocytes then develop inside host erythrocytes and are taken up by blood feeding insect vectors. The gametocytes then reproduce sexually in the vectors' gut (Beier, 1998), producing infective sporozoites that migrate to salivary glands to transmit to another host.

Hosts experience high levels of parasitaemia during the acute stages of infection, and this is when most pathologies manifest (Williams, 2005). Most early-stage infections result in a launch of host defences via innate, non-specific immunity, with a large number of immune system processes activated during peak parasitaemia, and a decline in activity shortly after the acute phase of infection (Videvall et al., 2015). Following the acute phase, infections may be cleared, or else pass into a chronic stage, which can last for months or years before

the host immune system is able to clear the infection, or persist within the host throughout their lifetime (Bishop et al., 1938).

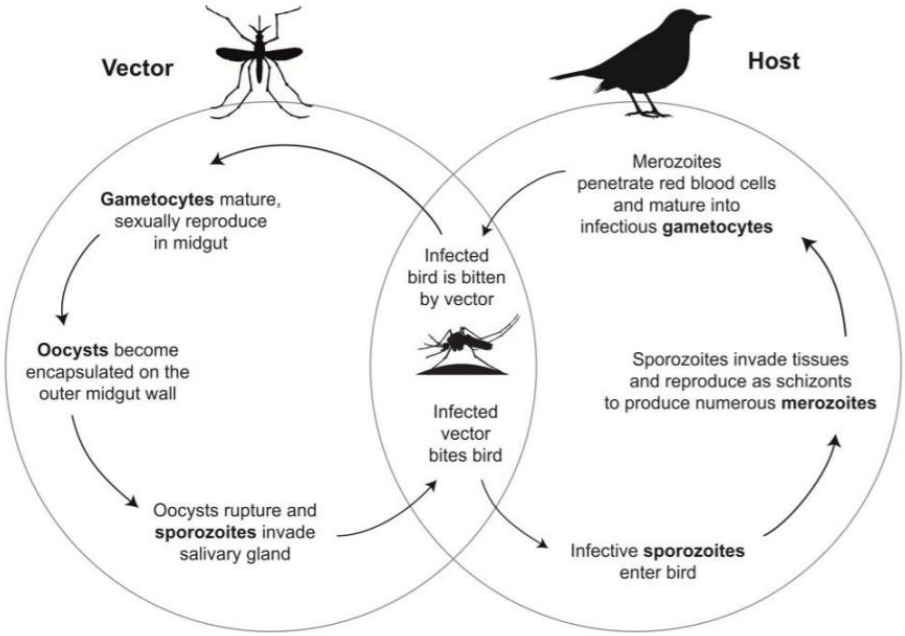



Figure 5.1. Avian malaria parasites life cycle. Diagram adapted from (Atkinson and Van Riper, 1991).

Effects of avian malaria on the host can range from asymptomatic, life-long infections (Bensch et al., 2007) to debilitating effects of acute infection such as anaemia (Palinauskas et al., 2015). In extreme cases, acute infection can result in cerebral ischaemia and mortality (Atkinson et al., 1995; Ilgunas et al., 2019). Responses to infection with avian malaria vary depending on the parasite lineage (Ricklefs and Fallon, 2002), host species (Ilgunas et al., 2019) and degree to which parasites and host are co-evolved (Jenkins et al., 2015). Infections in previously malaria-naïve birds can cause population level impacts, such as the case of parasite *Plasmodium relictum*, which has caused declines and extinctions of endemic bird species on the islands of Hawaii (Atkinson et al., 1995; van Riper et al., 1986), and the deaths of captive non-native birds in zoos (Brossy, 1992). Low-intensity chronic infections may have negative consequences in terms of reproductive success and lifespan (Asghar et al., 2015, 2011; Knowles et al., 2010a). However, other studies have reported no effects of chronic infection on avian fitness (Hahn et al., 2018; Kilpatrick et al., 2006). In wild studies, malaria infection presents a trade-off for host life histories. Studies investigating effects of *Leucocytozoon* infection on life history traits have shown both adverse effects of infection on parental condition and reproductive success (Merino et al., 2000), and an increase in reproductive success (Pigeault et al., 2018).

Many environmental factors affect the abundance and distribution of malaria vectors within a habitat, including seasonal changes and proximity to bodies of water (Chahad-Ehlers et al., 2018; Cosgrove et al., 2008; De Aguilar et al., 2018; Krama et al., 2015). The larvae of different vector families have different developmental needs; for example mosquitoes develop in standing water (Townroe and Callaghan, 2014), while blackfly larvae require clean running water in order to filter feed (Carlsson, 1967; Craig and Chance, 1982). Urban environments typically provide a less ideal habitat for vectors (Bradley and Altizer, 2007; Townroe and Callaghan, 2014), and therefore corresponding lower vector densities in cities may reduce the risk of avian malaria infection for urban bird populations (Bailly et al., 2016; Ferraguti et al., 2016). In an urban study on blackbirds (*Turdus merula*), there were large reductions in the prevalence of *Haemoproteus* and *Plasmodium* when compared with forest sites (Evans et al., 2009). However, prevalence of *Plasmodium* in a suburban population of house sparrows was recently shown to be 74%, a higher level than any population of wild birds in Northern Europe (Dadam et al., 2019), likely driven by the increase in vector abundance and environmental conditions favouring mosquito reproduction.

In the city environment, birds must contend with poorer food availability and quality than their forest conspecifics (Pollock et al., 2017), and an increase in environmental pollutants (Isaksson et al., 2009). Previous studies on malaria infections in city birds have indicated infected birds to have reductions in plumage colouration (Hõrak et al., 2001; Jacquin et al., 2011), a lower tolerance for pollutants (Bichet et al., 2013), higher leukocyte counts (Fokidis et al., 2008) and reductions in body condition (Jiménez-Peñuela et al., 2019). Immune responses to infections are energetically and nutritionally costly (Lochmiller and Deerenberg, 2000). City birds may be in poorer condition and have significantly reduced capacity for resisting infections and increased related fitness costs than forest birds. In addition, sleep deprivation and increased stress from exposure to light at night may affect city birds' ability to resist infections (Ouyang et al., 2017).

To date, many ecological studies comparing effects of avian malaria infection on city and forest birds have focused on chronic infections in adult birds (Bichet et al., 2013; Hõrak et al., 2001). However, malaria infections can occur in nestlings (Capilla-Lasheras et al., 2017; Krams et al., 2013a; Pollock et al., 2017). In a study on great tits at a forest site, nestlings infected with malaria parasites had lower survival rates and lower levels of haemoglobin (Krams et al., 2013a). Given that city birds may have reduced capacity to

resist infection than their forest conspecifics, and early infections may have consequences for life histories, it is important to consider the effects of acute malaria infection on urban nestling birds.

## 5.2 Study aims and hypotheses

The aim of this study was firstly to quantify prevalence of avian malaria infections in great tit nestlings at two city and two forest sites. An existing nest box system was used (Capilla-Lasheras et al., 2017; Pollock et al., 2017; Woodford et al., 2018), and the study was repeated over two years to account for between-year variation in vector and parasite abundance.

The second aim of this study was to investigate impacts of acute infection with avian malaria on nestling condition. To do this, the condition of infected and non-infected individuals was determined using common parameters for nestling condition such as weight and tarsal length (Eck et al., 2011), and fledging success. To determine pathology associated with infection, such as anaemia, red blood cell count (haematocrit levels) were measured as in Marzal et al., (2008).

Previous studies have captured responses of the avian immune system to acute infections under captive conditions (Videvall et al., 2015). However, to date, there have been few investigations of immune systems responses to malaria infections in the wild (Capilla-Lasheras et al., 2017). This study aimed to capture responses to acute infection by comparing transcript levels of genes involved in the inflammatory and immune responses in infected and non-infected individuals. Gene targets included *LY86* (lymphocyte antigen 86) and *TLR4* (toll-like receptor 4) which are involved in anti-bacterial and anti-malarial responses (Medzhitov, 2001), and the type 2 transcription factor *GATA3* (GATA binding protein 3) involved in adaptive immunity (Wang et al., 2011), *IL1* and *IL6* (interleukins 1 and 6) which are inflammatory response mediators (Klasing, 1998).

Additional gene targets involved in growth, oxidative stress and metabolism were included to investigate differences in transcript levels between city and forest birds. *PRKCA* (protein kinase C alpha), an enzyme responsible for antiviral effects and cell growth regulation (Clemens and Elia, 1997); and *IKBA* (NF-kappa-B inhibitor alpha) and *NKRF* (NF-kappa-B repressing factor), which are two inhibitors of the immune response regulator NF-kappa-B (Cabannes et al., 1999; Nourbakhsh and Hauser, 1999). Finally, immune targets *NRF2* (nuclear factor erythroid 2) and *IGF1* (insulin like growth factor 1) which are both involved in resistance to oxidative stress and metabolic regulation (Holzenberger et al., 2003; Kensler et al., 2007) were included in this study.

In this study, it was predicted that there would be lower overall prevalence of avian malaria at city sites than at forest sites, following previous studies (Evans et al., 2009; Jiménez-Peñuela et al., 2019). Nestlings infected with malaria were predicted to have significantly reduced condition, such as lower body weight and shorter tarsi, as resources are allocated to resisting infections instead of growth. This effect was expected to be stronger in city birds, where conditions are generally poorer (Pollock et al., 2017). To test this, an interaction between site and malaria infection was included in statistical models.

Fledging success of infected individuals was predicted to be lower than non-infected individuals, as in a previous study on malaria infection in great tit nestlings (Krams et al., 2013a). Given that anaemia is a common pathology of avian malaria infection (Palinauskas et al., 2015), it was also predicted that infected birds would have significantly reduced haematocrit levels (Krams et al., 2013a).

It was predicted that overall transcript levels of immune and inflammatory targets would be elevated for infected individuals at both city and forest sites, as in a previous transcriptome study (Videvall et al., 2015). Specifically, gene targets such as *GATA3* and *TLR4* were expected to be elevated in nestlings in city sites as in (Capilla-Lasheras et al., 2017). In addition, it was predicted that transcript levels of oxidative stress associated immune targets such as *NRF2* and *IGF1* would be higher for city nestlings than for their conspecifics in the forest (Isaksson et al., 2009; Watson et al., 2017).

## 5.3 Materials and Methods

### 5.3.1 Study sites

Data for this chapter were obtained between April and June of 2016 and 2017 at forest and city sites on free-living populations of breeding great tits. Field sites were existing forest nest box study systems located at the Scottish Centre for Ecology and the Natural Environment (SCENE; 56º 7.73’N, 4º 36.79’W), Cashel Forest (56° 6'N, 4°34'W), and city sites at Kelvingrove Park, Glasgow (55°52’ N, 4°17’W) and Garscube estate (55º 9’N, 4º31’ W). These sites were previously used in ecological studies of wild passerines and malaria vectors (Capilla-Lasheras et al., 2017; Pollock et al., 2017; Woodford et al., 2018). To determine hatch dates, brood sizes and fledging success, all boxes were initially checked weekly and, closer to hatching, every second day as part of a general nest box monitoring protocol (see General Methods for further details of nest box monitoring).

### 5.3.2 Nestling processing

All nestlings (N = 174) were ringed for individual identification on day thirteen of life. Measurements were taken of nestling weight and length of the tarsometatarsus bone (tarsus) from each nestling. Samples were only taken during the noon hours (11:00-15:00 GMT) to exclude the possibility of potential circadian effects on genes. From each nestling, two blood samples of approximately 50 µl were obtained via the brachial wing vein as in Owen (2011). The first was taken up by a heparinised capillary for haematocrit readings (in the study year 2017), sealed using a wax plug and stored on ice until return to the laboratory. A second blood sample was obtained and stored in 250 µl RNAlater® stabilising solution (Invitrogen) for molecular analyses. A breakdown of number of RNA samples collected for this study can be found in Table 5.1. See General Methods section for further details on the nestling blood sampling protocol and ethical licencing.

Table 5.1. Number of RNA samples collected for 2016 and 2017 at city and forest sites for analyses of transcript levels of immune target genes in this Chapter.

| Study year | Site | Number of RNA samples obtained |
|---|---|---|
| 2016 | City | 12 |
| 2016 | Forest | 51 |
| 2017 | City | 24 |
| 2017 | Forest | 41 |

As part of a vector study in 2017, eleven nest boxes were treated with a citronella oil-based insect repellent as per Krams et al., (2013), (see General Methods section). Initial modelling of the dataset via a generalised linear mixed-model revealed citronella treatment to have no significant effect on the prevalence of *Leucocytozoon* parasites in nestlings (GLM; $z_{1,78}$=-0.68: P=0.488), and therefore citronella was dropped from further analyses to pool 2017 and 2016 datasets. In addition, nestlings from nest boxes that were experimentally exposed to artificial light at night treatment (in Chapter Four) were excluded from this study (See General Methods section for a breakdown of sample sizes for each chapter).

### 5.3.3 Ethical Statement

See General Methods for details of ringing licences and blood sampling.

### 5.3.4 Laboratory procedures

Haematocrit readings were taken as described in Chapter Four (See General Methods for more details on protocol for haematocrit readings). After haematocrit readings were taken, this sample was removed from the capillary via pipette and stored in 0.5 ml 100% ETOH for molecular analyses. Samples in 100% ETOH were frozen at -40°C. Genomic DNA was later extracted from these samples via DNAeasy Blood and Tissue Kit (Qiagen) and molecular sexing was carried out via PCR (See General Methods for details).

Measurement of Median Fluorescence Intensity units (as a proxy for transcript levels) for immune and housekeeping gene targets outlined in Table 5.2. was carried out on all samples stored in *RNAlater*® using the QuantiGene® Plex RNA 2.0 assay (ThermoFisher). The QuantiGene® Plex RNA 2.0 assay is a platform that allows for simultaneous readouts of transcript levels of many target genes from a single blood sample using branched DNA

signal amplification and multi-analyte profiling beads (xMAP®). The General Methods section provides further details on lab procedures carried out in this Chapter.

Table 5.2. Housekeeping and immune gene targets used within this study. See General Methods section for more details on gene functions, and Appendix 1 for primer information.

| Transcript targets | Type |
|---|---|
| *RPL19, SDHA, HMBS* | Housekeeping genes |
| *LY86, TLR4, IL1, IL6, PRKCA, GATA3, IKBA, NKRF, NRF2, IGF1* | Immune genes |

### 5.3.5 Prevalence testing for *Leucocytozoon*

A nested PCR method was used in this study to test for the presence of *Leucocytozoon*, *Haemoproteus* and *Plasmodium* DNA within avian DNA samples. This PCR method was outlined in the General Methods section.

Four birds were found to be infected with *Haemoproteus* and were excluded from the rest of this study due to small sample size. One bird was infected with both *Haemoproteus* and *Leucocytozoon* and was also excluded from this study. No birds were infected with *Plasmodium*.

### 5.3.6 Statistical Analysis and Model Selection

All statistical analyses were performed using R v. 3.4.4 (R Core Team, 2018) and packages *lme4* (Bates et al., 2015). Data were visualised using ggplot2 (Wickham, 2014). All global models included individual nest box as a random factor to account for repeated measures within the same nest box. Residuals were plotted and inspected to check assumptions of normality prior to modelling using linear mixed models (LMMs). Pairs of explanatory variables were also assessed for collinearity by calculating tolerance and variance inflation factors (VIF) and also by visual inspection of pairwise plots, before statistical relevance was accepted.

*Leucocytozoon* prevalence data were modelled using a binomial distribution, (1 = *Leucocytozoon* detected, 0 = no *Leucocytozoon* detected). The global generalised linear mixed model for *Leucocytozoon* prevalence included the following explanatory variables: nestling sex (M/F); study site (Forest/City site); brood size; hatch date (to look for seasonal differences in infections); and study year (2016/2017).

Data for nestling weight, haematocrit level and tarsus length were modelled using linear mixed models and Gaussian distributions. Global models included the following explanatory variables: *Leucocytozoon* infection (two-level fixed factor 0/1), sex (M/F), site (City/Forest), brood size, hatch date. All models included an interaction between *Leucocytozoon* infection and site. This interaction remained in the model regardless of significance.

Additionally, the model for nestling weight included study year (2016/2017). Site (City/Forest) was removed from the global model for haematocrit level, as there was only one infected city bird in the haematocrit dataset, and therefore insufficient power for the model to run. For weight, tarsus length and *Leucocytozoon* prevalence models, an interaction between site and hatch date was included, as city birds tend to nest earlier in the year than forest birds (Deviche and Davies, 2014). Interactions between year and hatch date and year and site were included in global models to account for year to year variation in infections.

Median fluorescence intensity data (as a proxy for transcript levels of genes) were obtained from the QuantiGene® Plex RNA assay 2.0 (Thermofisher). These data were normalised by division of the mean fluorescence of background wells and then dividing each sample by the geometrical mean of levels of housekeeping genes in Table 5.2, thus, transcript levels are expressed as relative levels to housekeeping genes. Transcript level data for all targets were then modelled using LMMs and Gamma distribution with log-link function. Global models for transcript level data contained the same explanatory variables as the model for nestling weight.

Statistical relevance of fixed factors within all models was determined by sequential model selection via Likelihood Ratio Testing using package *lmtest* (Zeileis and Hothorn, 2002), until the minimum adequate model (MAM) was found. Factors with a significance level of <5% were kept in the model. *Leucocytozoon* infection and site (City/Forest) was kept in final models regardless of significance.

## 5.4 Results

### 5.4.1 *Leucocytozoon* prevalence

In total, 174 nestlings were sampled and tested for presence of *Leucocytozoon* parasites. *Leucocytozoon* prevalence was significantly higher at forest sites than at city sites (GLM, $z_{1,174} = 2.00$; P=0.035). In 2016, *Leucocytozoon* prevalence at forest sites was 83.9% and city sites 33.33%. In 2017, *Leucocytozoon* prevalence was much lower than the previous year, with prevalence at forest sites 36.07% and city sites 11.76%, (Figure 5.2.).

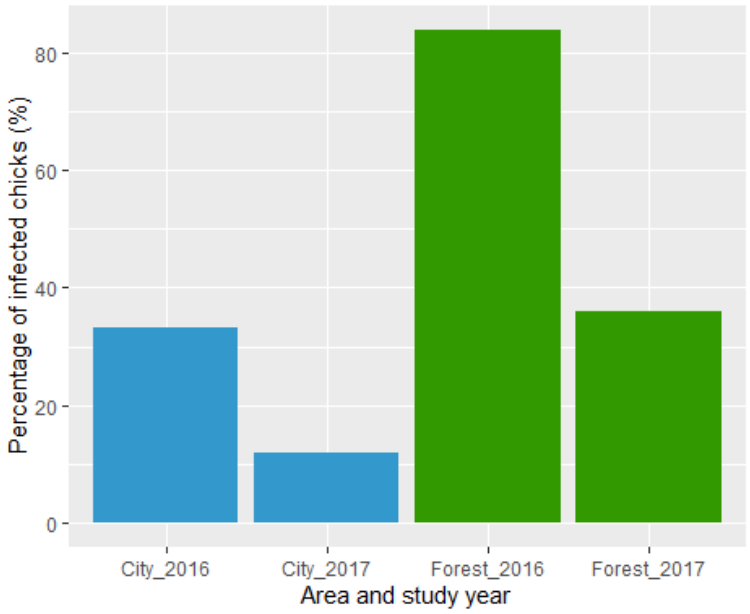



Figure 5.2. *Leucocytozoon* infection prevalence (%) at city (blue) and forest (green) sites for 2016 and 2017.

In addition, *Leucocytozoon* prevalence increased along with hatch date, and this was more evident in 2016 than in 2017 (GLM, $z_{1,174}$=0.98, P=0.039), (Figure 5.3.). There was no effect of sex or brood size on prevalence of *Leucocytozoon* in nestlings (Table 5.3.).

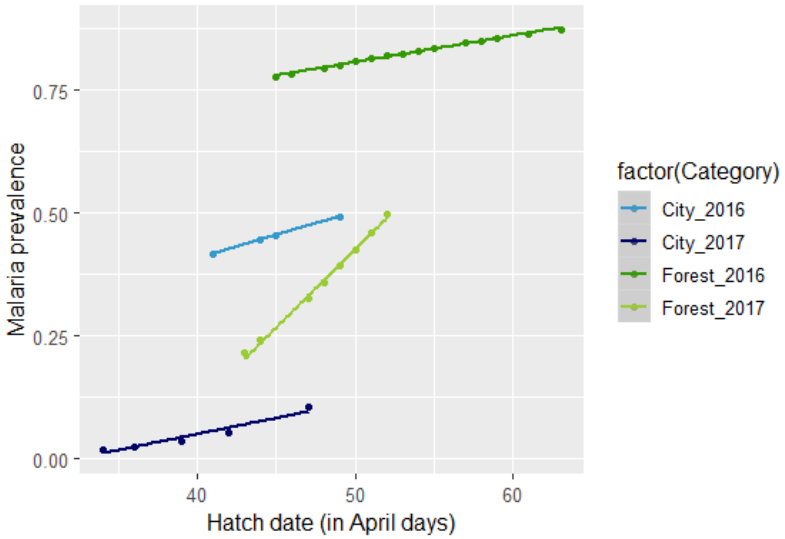



Figure 5.3. *Leucocytozoon* prevalence and hatch date (where April 1st=1) at city (blue) and forest sites (green) for 2016 and 2017. Prevalence data were obtained for each site and year using the minimum adequate model for *Leucocytozoon* prevalence.

The interaction between *Leucocytozoon* infection and site was non-significant in all models (Table 5.4). In addition, there was no effect of *Leucocytozoon* infection on the weight of nestlings (LMM; $t_{1,174}$=0.43: P=0.055), (Figure 5.4.), or their tarsus length (LMM; $t_{1,67}$=1.13: P=0.080), (Figure 5.5.). Effects of *Leucocytozoon* infection on haematocrit levels were also not significant (LMM: $t_{1,30}$=-0.81: P=0.966). Fledging success was very high at both forest (94 %) and city sites (92 %), and due to little variation between sites, the effect of *Leucocytozoon* infection on fledging was not included in models.

Sex differences in morphology were apparent, where male nestlings were significantly heavier than females (LMM; $t_{1,174}$=4.84, P<0.001), with mean weight of males 19.45 g (s.e. ± 0.88) and females 18.6 g (± 0.71). In addition, nestlings at forest sites were significantly heavier than city nestlings (LMM; $t_{1,174}$=4.93, P<0.001), where forest nestlings were 22.26 g (s.e. ± 0.48) and city nestlings 20.34 g (s.e. ± 1.27). There were no effects of brood size, hatch date or study year on the weight of nestlings (Table 5.3.).

The tarsus length and haematocrit levels of nestlings were not affected by either the brood size or hatch date (Table 5.3.). Male nestlings had significantly longer tarsi than females

(LMM; $t_{1,67}$=3.55, P=0.002), where mean male tarsi were 20.01 mm (s.e. ± 0.14), and females 19.53 mm (s.e. ± 0.37). Differences in tarsi length of nestlings at city and forest sites were not significant (LMM; $t_{1,174}$=1.77, P=0.098), (Figure 5.5.).

Global model summaries for nestling data are shown in Table 5.5.

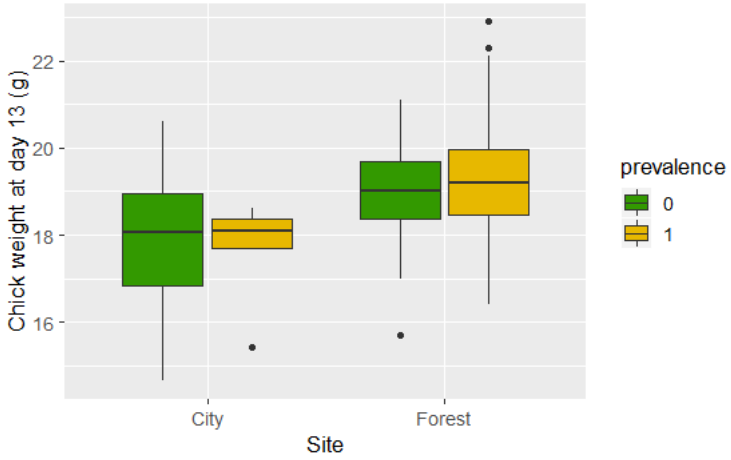



Figure 5.4. Nestling weight (g) at day thirteen and *Leucocytozoon* infection prevalence at city and forest sites. *Leucocytozoon* infection prevalence is indicated by colour: No infection (green), Infection present (yellow). Box plots show the interquartile range of the raw data, where boxes above and below the medians (horizontal lines) show the first and third quartiles, respectively. Boxplot whiskers extend to 1.5 times the interquartile range. Dots represent outliers.

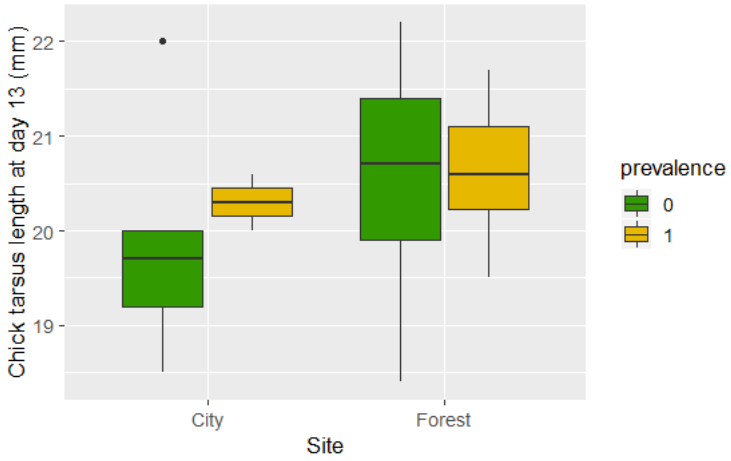



Figure 5.5. Tarsus length (mm) at day thirteen and *Leucocytozoon* infection prevalence at city and forest sites. *Leucocytozoon* infection prevalence is indicated by colour: No infection (green), Infection present (yellow). Box plots show the interquartile range of the raw data, where boxes above and below the medians (horizontal lines) show the first and third quartiles, respectively. Boxplot whiskers extend to 1.5 times the interquartile range. Dots represent outliers.

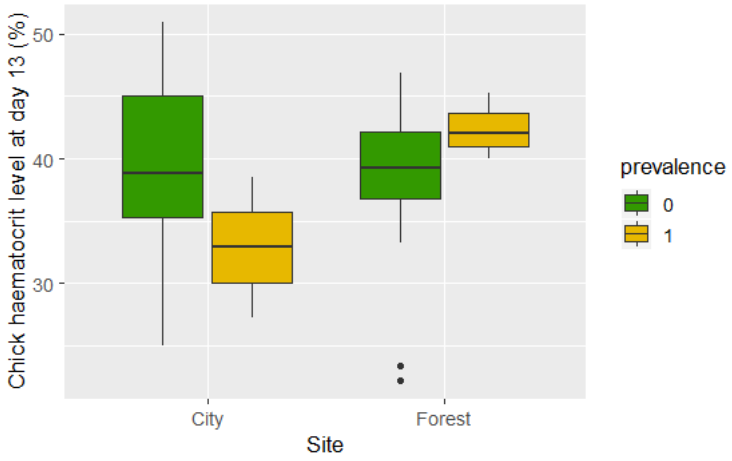



Figure 5.6. Haematocrit levels (%) at day thirteen and *Leucocytozoon* infection prevalence at city and forest sites. *Leucocytozoon* infection prevalence is indicated by colour: No infection (green), Infection present (yellow). Box plots show the interquartile range of the raw data, where boxes above and below the medians (horizontal lines) show the first and third quartiles, respectively. Boxplot whiskers extend to 1.5 times the interquartile range. Dots represent outliers.

Table 5.3. Global and minimum adequate models. *Leucocytozoon* infection and site (City/Forest) were always kept in the model, regardless of significance. *Leucocytozoon* prevalence was modelled using a Binomial distribution (0=no *Leucocytozoon* detected, 1=*Leucocytozoon* detected). Nestling weight, tarsus length and haematocrit data were modelled using Gaussian distributions. Nest box was always included as random factor. Addition of terms is denoted by a plus sign (+). Interaction of terms is denoted by an asterisk (*).

| Variable | Global model | Final MA model |
|---|---|---|
| ***Leucocytozoon* prevalence** | Nest box + sex + brood size + site*hatch date + year*hatch date + year*site | Nest box + site + year*hatch date |
| **Nestling weight** | Nest box + infection + sex + brood size + hatch date*year + year*site + site*hatch date + site*infection | Nest box + sex + site*infection |
| **Tarsus length (2017 only)** | Nest box + infection + sex + brood size + site*hatch date + site*infection | Nest box + sex + site*infection |
| **Haematocrit % (2017 only)** | Nest box + infection + sex + brood size + hatch date + site*infection | Nest box + infection + hatch date + site*infection |

Table 5.4. Minimum adequate model summaries. Estimates and standard error values for immune gene transcript levels are log transformed. Intercept was as follows: Site (City), Year (2016), Sex (F), Infection (0). Interaction of terms is denoted by an asterisk (*).

| **Variable** | **N** | **Estimate** | **s.e.m** | **d.f.** | **z-value** | ***p*-value** |
|---|---|---|---|---|---|---|
| ***Leucocytozoon* prevalence** | 174 | | | | | |
| (Intercept) | | -1.90 | 2.96 | 1 | -0.64 | |
| Site | | 1.42 | 0.71 | 1 | 2.00 | **0.035** |
| Hatch date | | 0.04 | 0.06 | 1 | 0.63 | |
| Year | | -6.89 | 5.22 | 1 | -1.32 | |
| Year*Hatch date | | 0.10 | 0.11 | 2 | 0.98 | **0.039** |
| | | | | | | |
| **Variable** | **N** | **Estimate** | **s.e.m** | **d.f.** | **t-value** | ***p*-value** |
| **Nestling weight** | 174 | | | | | |
| (Intercept) | | 20.34 | 1.27 | | 15.98 | |
| Infection (1) | | 0.29 | 0.66 | 1 | 0.43 | 0.055 |
| Sex (M) | | 0.86 | 0.17 | 1 | 4.93 | **<0.001** |
| Site (Forest) | | 1.92 | 0.48 | 1 | 4.02 | **<0.001** |
| Hatch Date | | -0.07 | 0.03 | 1 | -2.44 | **0.014** |
| Infection*Site | | 0.10 | 0.68 | 2 | 0.15 | 0.884 |
| | | | | | | |
| **Tarsus length (2017 only)** | 67 | | | | | |
| (Intercept) | | 19.53 | 0.37 | | 52.74 | |
| Infection (1) | | 0.56 | 0.49 | 1 | 1.13 | 0.080 |
| Sex (M) | | 0.48 | 0.14 | 1 | 3.55 | **<0.001** |
| Site (Forest) | | 0.73 | 0.41 | 1 | 1.77 | 0.098 |
| Infection*Site | | -0.35 | 0.52 | 2 | -0.68 | 0.472 |
| **Haematocrit % (2017 only)** | 30 | | | | | |
| (Intercept) | | 68.69 | 15.87 | | 15.87 | |
| Infection (1) | | -0.46 | 4.87 | 1 | 4.87 | 0.696 |
| Hatch Date | | -0.79 | 0.41 | 1 | 0.41 | **0.049** |
| Site | | 7.20 | 4.59 | 1 | 4.59 | 0.058 |
| Infection*Site | | 2.59 | 6.29 | 2 | 6.29 | 0.601 |

Table 5.5. Global model summaries. Estimates and standard error values for immune gene transcript levels are log transformed. Intercept was as follows: Site (City), Year (2016), Sex (F), Infection (0). Interaction of terms is denoted by an asterisk (*).

| **Variable** | **N** | **Estimate** | **s.e.m** | **d.f.** | **z-value** | ***p*-value** |
|---|---|---|---|---|---|---|
| ***Leucocytozoon* prevalence** | 174 | | | | | |
| (Intercept) | | -10.11 | 9.43 | 1 | -1.07 | |
| Sex(M) | | 0.44 | 0.39 | | 1.12 | 0.264 |
| Site(Forest) | | 12.86 | 9.52 | 1 | 1.35 | 0.177 |
| Brood Size | | -0.10 | 0.13 | 1 | -0.75 | 0.454 |
| Hatch date | | 0.21 | 0.20 | 1 | 1.04 | 0.296 |
| Year(2017) | | -9.06 | 6.07 | 1 | -1.49 | 0.135 |
| Site*Hatch date | | -0.22 | 0.20 | 2 | -1.10 | 0.270 |
| Site*Year | | 0.21 | 0.13 | 2 | 1.60 | 0.110 |
| Year*Hatch Date | | -3.14 | 1.48 | 2 | -2.12 | **0.034** |
| | | | | | | |
| **Variable** | **N** | **Estimate** | **s.e.m** | **d.f.** | **t-value** | ***p*-value** |
| **Nestling weight** | 174 | | | | | |
| (Intercept) | | 26.88 | 5.19 | | 5.18 | |
| Infection (1) | | 0.32 | 0.70 | 1 | 0.46 | |
| Sex (M) | | 0.89 | 0.18 | 1 | 4.95 | **<0.001** |
| Site (Forest) | | -3.17 | 5.16 | 1 | -0.61 | |
| Brood Size | | -0.09 | 0.10 | 1 | -0.95 | 0.310 |
| Hatch Date | | -0.19 | 0.11 | 1 | -1.72 | |
| Year(2017) | | -5.96 | 4.23 | 1 | -1.41 | |
| Hatch Date*Year | | 0.10 | 0.09 | 2 | 1.13 | 0.214 |
| Site*Year | | 0.95 | 1.05 | 2 | 0.91 | 0.318 |
| Infection*Site | | 0.05 | 0.74 | 2 | 0.07 | 0.953 |
| Site*Hatch Date | | 0.10 | 0.11 | 2 | 0.92 | 0.306 |
| | | | | | | |
| **Tarsus length (2017 only)** | 67 | | | | | |
| (Intercept) | | 18.97 | 3.54 | | 5.35 | |
| Infection (1) | | 0.48 | 0.57 | 1 | 0.85 | |
| Sex (M) | | 0.48 | 0.14 | 1 | 3.44 | **<0.001** |
| Site (Forest) | | 5.86 | 5.35 | 1 | 1.10 | |
| Brood Size | | -0.05 | 0.12 | 1 | -0.46 | 0.601 |
| Hatch Date | | 0.02 | 0.09 | 1 | 0.23 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Infection*Site | | -0.26 | 0.59 | 2 | -0.44 | 0.634 |
| Site*Hatch Date | | -0.11 | 0.13 | 2 | -0.85 | 0.346 |
| | | | | | | |
| **Haematocrit % (2017 only)** | 37 | | | | | |
| (Intercept) | | 66.38 | 18.75 | | 3.54 | |
| Infection (1) | | -0.22 | 4.99 | 1 | -0.04 | |
| Sex(M) | | 0.16 | 2.41 | 1 | 0.07 | 0.912 |
| Brood Size | | 0.39 | 1.20 | 1 | 0.33 | 0.687 |
| Hatch Date | | -0.77 | 0.44 | 1 | -1.74 | 0.049 |
| Site(Forest) | | 6.22 | 5.77 | 1 | 1.08 | |
| Infection*Site | | 2.11 | 6.54 | 2 | 0.32 | 0.636 |

### 5.4.2 Transcript levels of immune genes

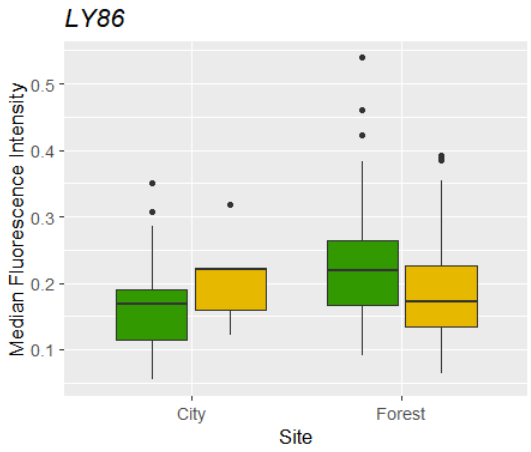
Infection with *Leucocytozoon* did not significantly affect the transcript levels of any of the immune targets tested in this study (Table 5.5.; Figure 5.7.). Moreover, the interaction between *Leucocytozoon* infection and site was found to be non-significant for all immune gene models (Table 5.6). No support was found for effects of brood size on transcript levels of all immune targets (Table 5.6.). For immune targets *IL1, PRKCA,* transcript levels increased along with later hatch date (Table 5.7.), and this trend was stronger in forest birds than city birds. For *NKRF*, transcript levels decreased as the season progressed (Table 5.7.).

Transcript levels of immune genes at city and forest sites did not differ significantly for many of the targets tested (Table 5.6.). However, transcript levels of immune targets *IL1, PRKCA*, *NKRF* and *NRF2* were significantly higher at forest sites than at city sites (Table 5.7.). Notably, levels of *NKRF* were found to be lower by 33 % in city birds than in forest birds (LMM; $t_{1,157}$=3.66: P<0.001), (Figure 5.7.9.). The same was true for *NRF2,* (LMM; $t_{1,156}$=2.75: P=0.006), where transcript levels in city birds were 18 % lower than those of forest birds (Figure 5.7.10.).

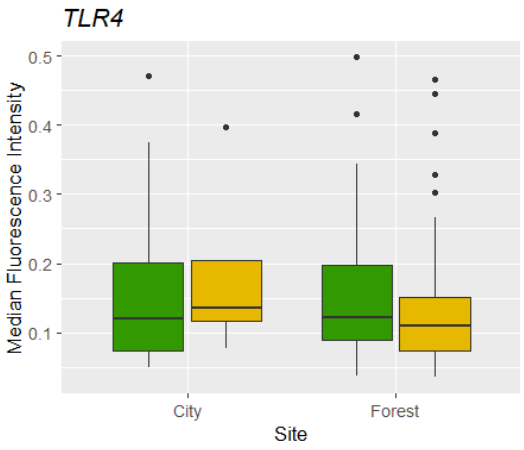
No support was found for effects of study year on the transcript levels of immune targets except for *IL1* (Table 5.6.), levels of which were significantly higher in birds in 2017 than in 2016 (LMM; $t_{1,136}$=2.41: P=0.016). Similarly, no support was found for effect of sex on transcript levels aside from *NRF2,* with males estimated to have higher transcript levels than female birds (LMM; $t_{1,156}$=2.75: P=0.006).

Global model summaries for immune gene data can be found in Table 5.8.

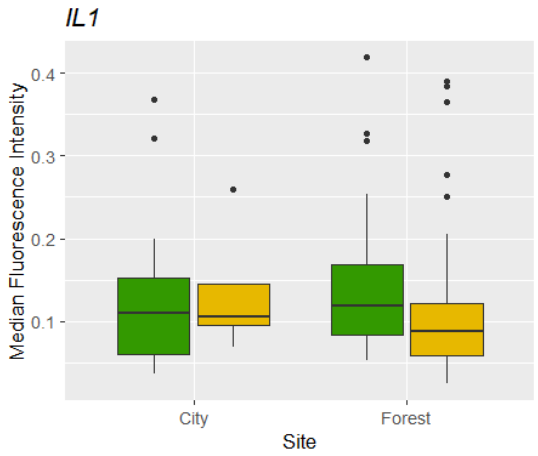

5.8.1.



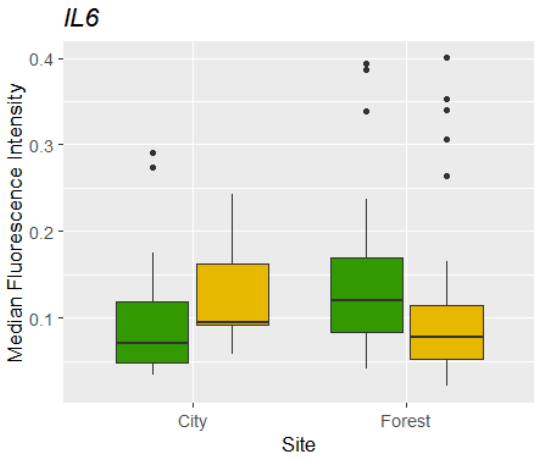

5.8.2.



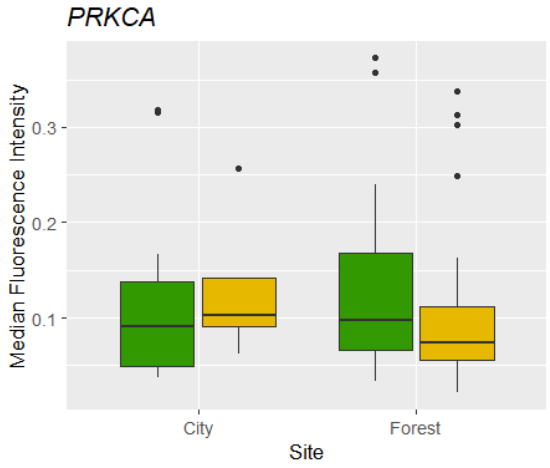

5.8.3.



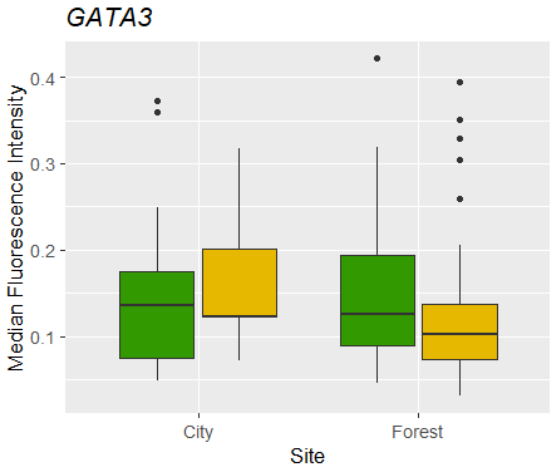

5.8.4.



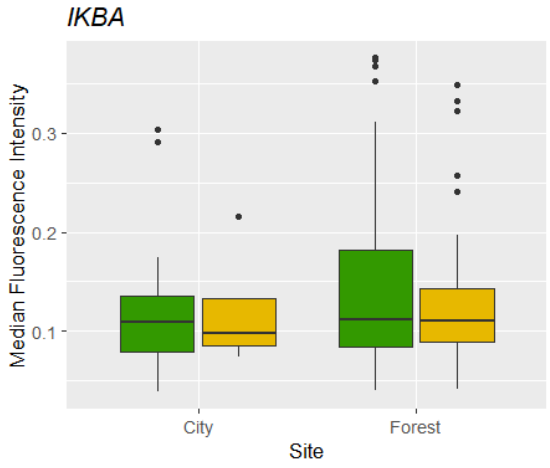

5.8.5.



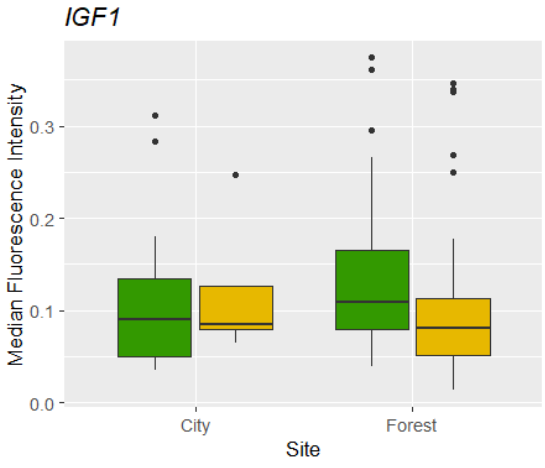

5.8.6.



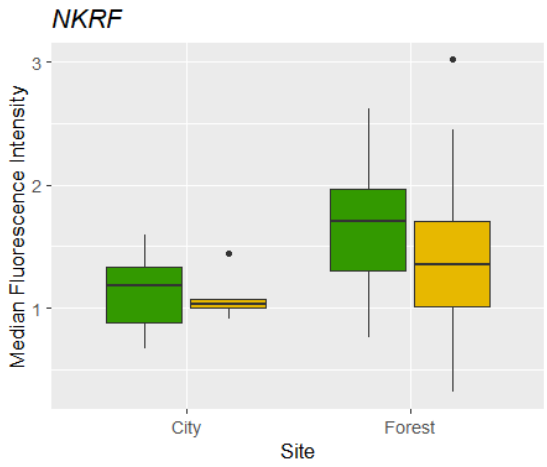
5.8.7.




5.8.8.




5.8.9.




5.8.10.

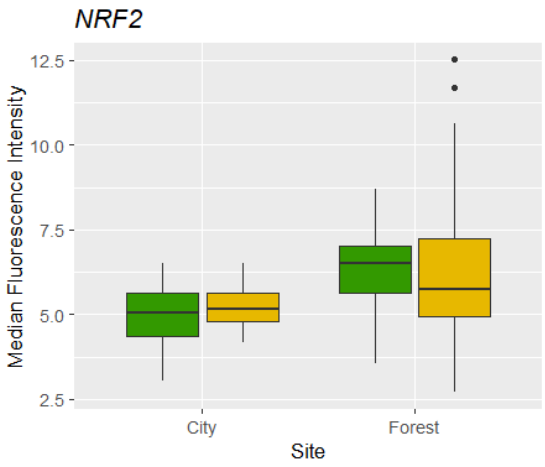



Figure 5.8. *Leucocytozoon* infection prevalence (where green = no infection, yellow = infection) and transcript levels (median fluorescence intensity) for immune gene targets 1) *LY86*, 2) *TLR4*, 3) *IL1*, 4) *IL6*, 5) *PRKCA*, 6) *GATA3*, 7) *IKBA*, 8) *IGF1*, 9) *NKRF*, 10) *NRF2*. Data shown are empirical values obtained from QuantiGene® Plex RNA assay and shown in Median florescence intensity units (MFI). Box plots show the interquartile range of the raw data, where boxes above and below the medians (horizontal lines) show the first and third quartiles, respectively. Boxplot whiskers extend to 1.5 times the interquartile range. Dots represent outliers.

Table 5.6. Global and minimum adequate models. Nest box was always included as random factor. An interaction between *Leucocytozoon* infection and site (City/Forest) was always kept in the model, regardless of significance. Immune transcript data were modelled using Gamma distributions with log link function. Addition of terms is denoted by a plus sign (+). Interaction of terms is denoted by an asterisk (*).

| Variable | Global model | Final MA model |
|---|---|---|
| ***LY86*** | Nest box + infection + sex + brood size + hatch date*year + site*hatch date + infection*site | Nest box + infection*site |
| ***TLR4*** | Nest box + infection + sex + brood size + hatch date*year + site*hatch date + infection*site | Nest box + infection*site |
| ***IL1*** | Nest box + infection + sex + brood size + hatch date*year + site*hatch date + infection*site | Nest box + infection*site |
| ***IL6*** | Nest box + infection + sex + brood size + hatch date*year + site*hatch date + infection*site | Nest box + infection*site |
| ***PRKCA*** | Nest box + infection + sex + brood size + hatch date*year + site*hatch date + infection*site | Nest box + infection*site |
| ***GATA3*** | Nest box + infection + sex + brood size + hatch date*year + site*hatch date + infection*site | Nest box + infection*site |
| ***IKBA*** | Nest box + infection + sex + brood size + hatch date*year + site*hatch date + infection*site | Nest box + infection*site |
| ***IGF1*** | Nest box + infection + sex + brood size + hatch date*year + site*hatch date + infection*site | Nest box + infection*site |
| ***NKRF*** | Nest box + infection + sex + brood size + hatch date*year + site*hatch date + infection*site | Nest box + infection*site + hatch date |
| ***NRF2*** | Nest box + infection + sex + brood size + hatch date*year + site*hatch date + infection*site | Nest box + sex + infection*site |

Table 5.7. Model summaries for MAMs. Estimates and standard error values for immune gene transcript levels are log-transformed. Reference level for the intercept: Site (City), Year (2016), Sex (F), Infection (0).

| **Variable** | **N** | **Estimate** | **s.e.m** | **d.f.** | **t-value** | ***p*-value LRT** |
|---|---|---|---|---|---|---|
| ***LY86*** | 155 | | | | | |
| (Intercept) | | -1.86 | 0.13 | | -14.06 | |
| Infection (1) | | 0.20 | 0.24 | 1 | 0.84 | 0.403 |
| Site (Forest) | | 0.28 | 0.15 | 1 | 1.83 | 0.068 |
| Infection*Site | | -0.31 | 0.25 | 2 | -1.23 | 0.217 |
| ***TLR4*** | 138 | | | | | |
| (Intercept) | | -1.94 | 0.18 | | -10.68 | |
| Infection (1) | | -0.07 | 0.44 | 1 | -0.16 | 0.874 |
| Site (Forest) | | 0.02 | 0.21 | 1 | 0.08 | 0.940 |
| Infection*Site | | -0.13 | 0.46 | 2 | -0.28 | 0.778 |
| ***IL1*** | 136 | | | | | |
| (Intercept) | | -5.90 | 1.33 | | -4.42 | |
| Infection (1) | | -0.42 | 0.43 | 1 | -0.97 | 0.331 |
| Site (Forest) | | 3.82 | 1.49 | 1 | 2.57 | **0.010** |
| Hatch Date | | 0.08 | 0.03 | 1 | 2.71 | **0.007** |
| Year(2017) | | 0.40 | 0.17 | 1 | 2.41 | **0.016** |
| Infection*Site | | 0.35 | 0.44 | 2 | 0.79 | 0.432 |
| Site*Hatch Date | | -0.09 | 0.03 | 2 | -2.67 | **0.008** |
| ***IL6*** | 130 | | | | | |
| (Intercept) | | -2.40 | 0.19 | | -12.71 | |
| Infection (1) | | 0.22 | 0.35 | 1 | 0.64 | 0.523 |
| Site (Forest) | | 0.26 | 0.22 | 1 | 1.15 | 0.251 |
| Infection*Site | | -0.47 | 0.37 | 2 | -1.25 | 0.210 |
| ***PRKCA*** | 128 | | | | | |
| (Intercept) | | -5.12 | 1.35 | | -3.78 | |
| Infection (1) | | -0.40 | 0.44 | 1 | -0.90 | 0.366 |
| Site (Forest) | | 3.29 | 1.64 | 1 | 2.00 | **0.045** |
| Hatch Date | | 0.07 | 0.03 | 1 | 2.10 | **0.036** |
| Infection*Site | | 0.24 | 0.46 | 2 | 0.53 | 0.595 |
| Site*Hatch Date | | -0.08 | 0.04 | 2 | -2.07 | **0.038** |
| ***GATA3*** | 142 | | | | | |
| (Intercept) | | -2.05 | 0.16 | 1 | -12.95 | |
| Infection (1) | | 0.10 | 0.31 | 1 | 0.34 | 0.735 |
| Site (Forest) | | 0.01 | 0.19 | 1 | 0.03 | 0.977 |
| Infection*Site | | -0.25 | 0.32 | 2 | -0.78 | 0.434 |

| **Variable** | **N** | **Estimate** | **s.e.m** | **d.f.** | **t-value** | ***p*-value LRT** |
|---|---|---|---|---|---|---|
| ***IKBA*** | 148 | | | | | |
| (Intercept) | | -2.18 | 0.15 | | -14.77 | |
| Infection (1) | | -0.13 | 0.34 | 1 | -0.37 | 0.710 |
| Site (Forest) | | 0.10 | 0.17 | 1 | 0.58 | 0.563 |
| Infection*Site | | 0.07 | 0.35 | 2 | 0.19 | 0.851 |
| ***IGF1*** | 135 | | | | | |
| (Intercept) | | -2.26 | 0.19 | | -11.72 | |
| Infection (1) | | -0.05 | 0.43 | 1 | -0.10 | 0.917 |
| Site (Forest) | | 0.07 | 0.22 | 1 | 0.32 | 0.746 |
| Infection*Site | | -0.17 | 0.45 | 2 | -0.39 | 0.697 |
| ***NKRF*** | 157 | | | | | |
| (Intercept) | | 1.04 | 0.43 | | 2.43 | |
| Infection (1) | | 0.17 | 0.18 | 1 | 0.95 | 0.342 |
| Site (Forest) | | 0.58 | 0.16 | 1 | 3.66 | **<0.001** |
| Hatch Date | | -0.02 | 0.01 | 1 | -2.38 | **0.017** |
| Infection*Site | | -0.23 | 0.19 | 2 | -1.25 | 0.212 |
| **Variable** | **N** | | | | | |
| ***NRF2*** | 156 | | | | | |
| (Intercept) | | 1.52 | 0.08 | | 18.48 | |
| Infection (1) | | 0.17 | 0.16 | 1 | 1.02 | 0.310 |
| Sex (M) | | 0.10 | 0.03 | 1 | 2.95 | **0.003** |
| Site (Forest) | | 0.26 | 0.09 | 1 | 2.75 | **0.006** |
| Infection*Site | | -0.19 | 0.17 | 2 | -1.12 | 0.261 |

Table 5.8. Global model summaries. Estimates and standard error values for immune gene transcript levels are log-transformed. Reference level for the intercept: Site (City), Year (2016), Sex (F), Infection (0).

| **Variable** | **N** | **Estimate** | **s.e.m** | **d.f.** | **t-value** | ***p*-value LRT** |
|---|---|---|---|---|---|---|
| ***LY86*** | 155 | | | | | |
| (Intercept) | | -5.49 | 1.91 | | -2.87 | |
| Infection (1) | | 0.07 | 0.24 | 1 | 0.29 | 0.774 |
| Sex(M) | | 0.04 | 0.06 | 1 | 0.71 | 0.477 |
| Site (Forest) | | 3.79 | 1.82 | 1 | 2.08 | **0.037** |
| Brood Size | | 0.01 | 0.04 | 1 | 0.33 | 0.738 |
| Hatch Date | | 0.08 | 0.04 | 1 | 1.90 | 0.057 |
| Year(2017) | | 1.98 | 1.67 | 1 | 1.19 | 0.235 |
| Infection*Site | | -0.16 | 0.25 | 2 | -0.64 | 0.524 |
| Hatch Date*Year | | -0.04 | 0.03 | 2 | -1.12 | 0.264 |
| Site*Hatch Date | | -0.08 | 0.04 | 2 | -1.99 | **0.046** |
| ***TLR4*** | 138 | | | | | |
| (Intercept) | | -5.77 | 2.61 | | -2.21 | |
| Infection (1) | | -0.48 | 0.46 | 1 | -1.05 | 0.295 |
| Sex(M) | | -0.10 | 0.10 | 1 | -0.99 | 0.322 |
| Site (Forest) | | 2.71 | 2.45 | 1 | 1.11 | 0.269 |
| Brood Size | | 0.05 | 0.05 | 1 | 0.98 | 0.329 |
| Hatch Date | | 0.09 | 0.06 | 1 | 1.51 | 0.130 |
| Year(2017) | | 0.40 | 2.19 | 1 | 0.18 | 0.855 |
| Infection*Site | | 0.32 | 0.47 | 2 | 0.68 | 0.495 |
| Hatch Date*Year | | 0.01 | 0.05 | 2 | -0.09 | 0.931 |
| Site*Hatch Date | | -0.07 | 0.05 | 2 | -1.31 | 0.191 |
| ***IL1*** | 136 | | | | | |
| (Intercept) | | -6.56 | 2.34 | | -2.80 | |
| Infection (1) | | -0.47 | 0.43 | 1 | -1.11 | 0.268 |
| Sex(M) | | -0.12 | 0.09 | 1 | -1.30 | 0.194 |
| Site (Forest) | | 4.24 | 2.18 | 1 | 1.95 | 0.052 |
| Brood Size | | 0.04 | 0.05 | 1 | 0.78 | 0.433 |
| Hatch Date | | 0.10 | 0.05 | 1 | 1.91 | 0.056 |
| Year(2017) | | 1.08 | 1.97 | 1 | 0.55 | 0.585 |
| Infection*Site | | 0.41 | 0.44 | 2 | 0.93 | 0.350 |
| Hatch Date*Year | | -0.01 | 0.04 | 2 | -0.36 | 0.717 |
| Site*Hatch Date | | -0.10 | 0.05 | 2 | -1.20 | **0.035** |

| Variable | N | Estimate | s.e.m | d.f. | t-value | *p*-value LRT |
|---|---|---|---|---|---|---|
| ***IL6*** | 130 | | | | | |
| (Intercept) | | -4.21 | 2.43 | | -1.73 | |
| Infection (1) | | 0.13 | 0.35 | 1 | 0.36 | 0.720 |
| Sex(M) | | -0.12 | 0.10 | 1 | -1.19 | 0.236 |
| Site (Forest) | | 1.65 | 2.26 | 1 | 0.73 | 0.465 |
| Brood Size | | 0.01 | 0.05 | 1 | -0.08 | 0.932 |
| Hatch Date | | 0.04 | 0.05 | 1 | 0.84 | 0.400 |
| Year(2017) | | -0.60 | 2.09 | 1 | -0.29 | 0.773 |
| Infection*Site | | -0.30 | 0.37 | 2 | -0.81 | 0.418 |
| Hatch Date*Year | | 0.02 | 0.04 | 2 | 0.47 | 0.635 |
| Site*Hatch Date | | -0.04 | 0.05 | 2 | -0.76 | 0.449 |
| ***PRKCA*** | 128 | | | | | |
| (Intercept) | | -6.40 | 2.67 | | -2.40 | |
| Infection (1) | | -0.44 | 0.45 | 1 | -0.98 | 0.328 |
| Sex(M) | | -0.13 | 0.10 | 1 | -1.28 | 0.202 |
| Site (Forest) | | 3.55 | 2.50 | 1 | 1.42 | 0.156 |
| Brood Size | | 0.01 | 0.05 | 1 | 0.15 | 0.882 |
| Hatch Date | | 0.10 | 0.06 | 1 | 1.65 | 0.099 |
| Year(2017) | | 0.85 | 2.23 | 1 | 0.38 | 0.702 |
| Infection*Site | | 0.34 | 0.47 | 2 | 0.73 | 0.468 |
| Hatch Date*Year | | -0.01 | 0.05 | 2 | -0.27 | 0.785 |
| Site*Hatch Date | | -0.09 | 0.06 | 2 | -1.56 | 0.118 |
| ***GATA3*** | 142 | | | | | |
| (Intercept) | | -5.11 | 2.13 | | -2.40 | |
| Infection (1) | | -0.04 | 0.30 | 1 | -0.14 | 0.893 |
| Sex(M) | | 0.02 | 0.08 | 1 | 0.25 | 0.801 |
| Site (Forest) | | 2.72 | 1.99 | 1 | 1.37 | 0.170 |
| Brood Size | | 0.02 | 0.04 | 1 | 0.46 | 0.643 |
| Hatch Date | | 0.07 | 0.05 | 1 | 1.46 | 0.146 |
| Year(2017) | | 0.54 | 1.83 | 1 | 0.29 | 0.769 |
| Infection*Site | | -0.06 | 0.32 | 2 | -0.18 | 0.854 |
| Hatch Date*Year | | -0.01 | 0.04 | 2 | -0.14 | 0.890 |
| Site*Hatch Date | | -0.07 | 0.04 | 2 | -1.50 | 0.133 |
| ***IKBA*** | 148 | | | | | |
| (Intercept) | | -3.73 | 2.16 | | -1.73 | |
| Infection (1) | | -0.33 | 0.36 | 1 | -0.92 | 0.356 |
| Sex(M) | | -0.06 | 0.07 | 1 | -0.86 | 0.388 |
| Site (Forest) | | 1.58 | 2.05 | 1 | 0.77 | 0.442 |
| Brood Size | | 0.03 | 0.04 | 1 | 0.58 | 0.559 |
| Hatch Date | | 0.04 | 0.05 | 1 | 0.76 | 0.447 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Year(2017) | | -0.28 | 1.84 | 1 | -0.15 | 0.881 |
| Infection*Site | | 0.29 | 0.37 | 2 | 0.80 | 0.426 |
| Hatch Date*Year | | 0.01 | 0.04 | 2 | 0.21 | 0.831 |
| Site*Hatch Date | | -0.04 | 0.05 | 2 | -0.84 | 0.402 |
| ***IGF1*** | 135 | | | | | |
| (Intercept) | | -5.40 | 2.52 | | -2.15 | |
| Infection (1) | | -0.41 | 0.46 | 1 | -0.89 | 0.371 |
| Sex(M) | | -0.13 | 0.10 | 1 | -1.30 | 0.192 |
| Site (Forest) | | 2.57 | 2.33 | 1 | 1.10 | 0.270 |
| Brood Size | | 0.02 | 0.05 | 1 | 0.42 | 0.675 |
| Hatch Date | | 0.07 | 0.05 | 1 | 1.32 | 0.188 |
| Year(2017) | | 0.18 | 2.09 | 1 | 0.08 | 0.932 |
| Infection*Site | | 0.27 | 0.48 | 2 | 0.56 | 0.579 |
| Hatch Date*Year | | 0.01 | 0.04 | 2 | 0.09 | 0.931 |
| Site*Hatch Date | | -0.06 | 0.05 | 2 | -1.24 | 0.217 |
| ***NKRF*** | 157 | | | | | |
| (Intercept) | | -1.40 | 1.45 | | -0.97 | |
| Infection (1) | | 0.13 | 0.18 | 1 | 0.71 | 0.478 |
| Sex(M) | | 0.04 | 0.05 | 1 | 0.88 | 0.379 |
| Site (Forest) | | 2.50 | 1.40 | 1 | 1.79 | 0.074 |
| Brood Size | | -0.01 | 0.03 | 1 | -0.16 | 0.870 |
| Hatch Date | | 0.03 | 0.03 | 1 | 0.87 | 0.382 |
| Year(2017) | | 1.88 | 1.27 | 1 | 1.48 | 0.138 |
| Infection*Site | | -0.18 | 0.19 | 2 | -0.97 | 0.332 |
| Hatch Date*Year | | -0.04 | 0.03 | 2 | -1.36 | 0.173 |
| Site*Hatch Date | | -0.04 | 0.03 | 2 | -1.38 | 0.168 |
| ***NRF2*** | 156 | | | | | |
| (Intercept) | | 1.19 | 1.07 | | 1.11 | |
| Infection (1) | | 0.19 | 0.17 | 1 | 1.13 | 0.260 |
| Sex(M) | | 0.10 | 0.03 | 1 | 2.94 | **0.003** |
| Site (Forest) | | 0.74 | 1.05 | 1 | 0.71 | 0.478 |
| Brood Size | | 0.01 | 0.03 | 1 | 0.01 | 0.997 |
| Hatch Date | | 0.01 | 0.02 | 1 | 0.24 | 0.813 |
| Year(2017) | | 0.93 | 0.96 | 1 | 0.97 | 0.334 |
| Infection*Site | | -0.22 | 0.17 | 2 | -1.24 | 0.216 |
| Hatch Date*Year | | -0.02 | 0.02 | 2 | -1.01 | 0.314 |
| Site*Hatch Date | | -0.01 | 0.02 | 2 | -0.36 | 0.716 |

## 5.5 Discussion

### 5.5.1 *Leucocytozoon* prevalence

Malaria prevalence in this study was substantially higher at forest sites than at city sites, which is consistent with previous studies of avian malaria and urbanisation (Evans et al., 2009; Jiménez-Peñuela et al., 2019). Of the infected birds in this study, most infections were of *Leucocytozoon* species, four were of *Haemoproteus*, and one bird was co-infected with *Haemoproteus* and *Leucocytozoon.* No birds were found to be infected with *Plasmodium.*

In this study, parasites were detected from sampling circulating host blood. For *Leucocytozoon* parasites, the pre-patent stage, where parasites are present in the host liver but not yet in blood, is 5-7 days after introduction of parasites to the host (Eide and Fallis, 1972; Kocan and Clark, 1966). For *Haemoproteus* parasites, the pre-patent period is around 12-14 days (Fallis and Bennett, 1961) and for *Plasmodium* parasites it is 4-12 days (Ilgunas et al., 2019). Nestlings in this study may therefore have been harbouring *Haemoproteus* and *Plasmodium* infections, but levels of parasitaemia were not at a detectable level at day thirteen.

During this study, the overall prevalence of *Leucocytozoon* detected in the population differed between years; prevalence was much higher in 2016 than in 2017. *Leucocytozoon* prevalence also increased during the season, and this effect was strongest in 2016. A study conducted in the forest habitat used in this study observed seasonal variation in blackfly (genus *Simulium*) abundance and species diversity (Woodford et al., 2018). Therefore, it is likely that the increase in *Leucocytozoon* prevalence in great tit nestlings across the season was due to fluctuating abundance of blackfly vectors, or perhaps individuals hatching later were more susceptible to infection. A reduction in food abundance, or a deterioration of food quality, may also have contributed to the increase in infections across the season. In the city environment especially, parents may have stayed away longer to forage, or else have been in a worse state, leaving nestlings susceptible to infections.

In this study, *Leucocytozoon* prevalence was higher at forest sites than at city sites, and this difference in prevalence was conserved across years. Blackflies require lotic habitats for their larval development (Crosskey, 1990). It may be that city sites used in this study were less suitable habitats for black fly vectors, although abundance of vectors was not measured in this study. Indeed, prevalence of *Leucocytozoon* was very low in a study on

house sparrows at a city site (Dadam et al., 2019). Future studies of avian malaria and urbanisation should consider quantifying vector species abundance at study sites.

Previous studies report sex bias in infection, where males are more likely to be infected than females (Calero-Riestra and Garcia, 2016; Jenkins et al., 2015), although other authors have reported no sex bias in infection (Dunn et al., 2011). In this study, no sex bias in infection was found.

### 5.5.2 *Leucocytozoon* infection and nestling condition

This study found no evidence of effects of malaria infection on nestling condition, in contrast to previous studies that found a reduction in condition (Calero-Riestra and Garcia, 2016) and fledging success (Krams et al., 2013a). Male nestlings were heavier and had longer tarsi than females, which was expected, due to male great tits being structurally larger than females (Gosler, 1993). City birds also weighed significantly less than forest birds; however, there was no evidence that *Leucocytozoon* infection affected condition more in city birds than forest birds.

Contrary to expectation, haematocrit levels of nestlings were not significantly affected by presence of *Leucocytozoon* infection. Similarly, in a study on four songbird species, *Leucocytozoon* infection did not have an effect on body condition or haematocrit levels (Granthon and Williams, 2017). *Leucocytozoon* parasites infect host thrombocytes instead of red blood cells (Zhao et al., 2015), and therefore may not cause anaemias to the same degree as *Plasmodium* or *Haemoproteus* species. Moreover, many features in the natural environment as well as physiological condition can cause variation in haematocrit levels in wild birds (Fair et al., 2007).

Fledging success was high during both years at both sites in this study, and therefore acute *Leucocytozoon* infection in this system did not seem to affect fledging. The effects of *Leucocytozoon* infection on condition may be more evident later in life, and therefore future studies might consider recapturing nestlings after fledging to measure impacts of chronic infections on health.

### 5.5.3 *Leucocytozoon* infection and immune gene transcripts

In this study, it was expected that transcript levels of immune gene targets would be elevated in birds infected with *Leucocytozoon*. However, infection with *Leucocytozoon* did

not appear to affect the transcript levels of immune genes studied. In a previous study on siskins (*Carduelis spinus*), transcripts of many immune processes were elevated in response to *Plasmodium* infection (Videvall et al., 2015). Given that adaptive immune responses to infection vary even in closely related bird species (Lee et al., 2006), great tits may respond differently and varying in their susceptibility to infections. Furthermore, some birds may have been tolerant, or else resistant to infection (Palinauskas et al., 2008).

In addition, the nature of *Leucocytozoon* parasites to primarily infect host thrombocytes (Zhao et al., 2015), rather than erythrocytes (like *Plasmodium* and *Haemoproteus* parasites), may have influenced the detection of effects on the host immune system in this study.

### 5.5.4 Immune transcripts at city and forest sites

Following previous studies, it was predicted that transcript levels of oxidative stress associated immune targets would be higher for city nestlings than for their conspecifics in the forest (Isaksson et al., 2009; Watson et al., 2017). However, contrary to expectation, transcript levels of immune response elements *NRF2* and *NKRF* were significantly higher in forest birds than in city birds. Forest birds may have had other infections not quantified in this study, causing higher transcript levels of these two genes.

Higher levels of *TLR4* and *GATA3* were found in city birds than forest birds in a previous study (Capilla-Lasheras et al., 2017), however, these differences in transcript levels between sites were not reflected here. Ultimately, there was no evidence in this study to support that city birds have higher transcript levels of immune genes than forest conspecifics.

### 5.5.5 Conclusions and future studies

This study made use of a platform for measuring transcript levels of many genes from a single sample, the QuantiGene® Plex assay. Given that this assay had not been used before for passerine samples, transcript data in this study were noisy overall. Large variation in transcript data may have increased difficulties with detecting signals and responses to *Leucocytozoon* infection (See General Discussion for more details). Additionally, the qPCR method used to detect parasite infections from DNA was sensitive, and therefore nestlings that identified as malaria-positive may have had very low infection intensity. This

may be why there were small differences in immune gene transcript between infected and non-infected individuals. Further studies could quantify the intensity of infection with malaria parasites, rather than prevalence.

A further limitation of this study was that given that this study took place in the natural environment, the time that nestlings were bitten was unknown. This study included samples taken during the noon hours, but not at night, and therefore may have captured just one phase of the parasite life cycle. It is possible that at the time of sampling, erupted parasites during the acute blood stage of parasitaemia had already escaped into host cells, or else had been cleansed by the host spleen. Thus, the levels of parasitaemia in birds in this study may have been underestimated. Given the “circadian nature” of malaria parasites (Mideo et al., 2013), future studies ought to capture the full time profile of parasitaemia and host rhythmicity, instead of solely at the hours surrounding noon.

# Chapter Six: Malaria parasite effects on the circadian physiology of wild birds

## 6.1 Introduction

Parasites are ubiquitous in natural environments and present many challenges for behaviour and physiology of their hosts (Hamilton and Zuk, 1982). During their life cycles, parasites must overcome barriers to transmission, survival and replication. Conversely, the host must actively combat infections, compensate for incurred damage, or tolerate them. Ecological interactions between host and parasite are complex, and selective pressures on fitness drive co-evolutionary relationships (Anderson and May, 1982). For many of these interactions, timing is crucial.

Many parasites show seasonal (circannual) or diel (circadian) traits during their development (Martinez-Bakker and Helm, 2015; Westwood et al., 2019). Endogenous timekeeping is advantageous as organisms can anticipate changes in the natural environment and synchronise internal biological rhythms accordingly. One of the most extensively studied host-parasite interactions in the context of rhythms are those of malaria parasites (*Plasmodium, Haemoproteus* and *Leucocytozoon* species) and their vertebrate hosts. Malaria infections typically give rise to symptoms of periodic fevers in their host, with a cycle length of 24 hours or multiples of 24 hours depending on the species of parasite (Kwiatkowski and Greenwood, 1989). This is a consequence of cycles of asexual replication within a host during the blood stages of parasite development. Individual merozoites invade host red blood cells, replicate and then divide to become schizonts (Reece et al., 2017). Subsequently, mature schizonts exhibit bursting behaviour, releasing merozoites into the host bloodstream (Figure 6.1.). Bursting behaviour in parasite species such as *Plasmodium chabaudi* occurs synchronously, and usually beginning at a specific time of the day (Mideo et al., 2013).

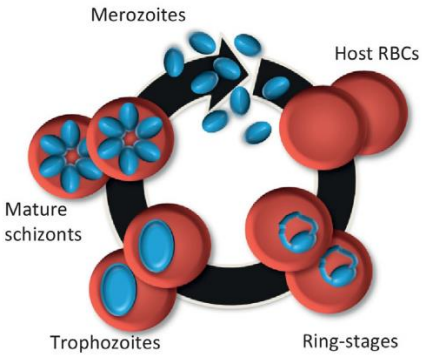



Figure 6.1. Conceptual diagram of development of malaria parasites within host blood cells. From (Mideo et al., 2013)

Rhythmic properties of malaria parasites have so far been documented in humans, mice and in birds (O'Donnell et al., 2013; Pigeault et al., 2015). Studies documenting the periodicity of malaria parasites have used blood smears collected at intervals over a period of 24 hours to estimate abundance of parasites by visual inspection (Gore et al., 1982; Roller and Desser, 1973), or determined abundance through molecular analyses (Hellgren et al., 2006).

### 6.1.1 Vector-host-parasite clock interactions

Given the rhythmic nature of both parasites and host, evolution has selected different rhythmic strategies for parasites. Selective advantages for malaria parasites in synchronising with rhythms of their hosts have driven evolutionary relationships of parasite clocks at each stage of their life cycle (Reece et al., 2017). For example, parasites exploit diel rhythms of insect vector biting behaviour for optimal transmission to their vertebrate hosts (Pigeault et al., 2018; Rund et al., 2016; Schneider et al., 2018).

Once parasites have successfully invaded a host, they must avoid elimination by host immune system defences (Reece et al., 2017). To avoid attack by the host immune system, it is thought that rhythmic bursting behaviour of mature schizonts is timed to occur when host defences are low (Mideo et al., 2013). In addition, the synchronous bursting of schizonts releases a high number of parasites at once, overwhelming the host immune system with sheer numbers (Reece et al., 2017). Mismatch of synchronicity with the host is costly for parasites; studies on experimental mismatch of *Plasmodium chabaudi* to mouse host results in reduced virulence and a two-fold cost for production and replication (O'Donnell et al., 2013, 2011).

Circadian rhythms in host immune system activity are well documented in humans (Labrecque and Cermakian, 2015; Scheiermann et al., 2013), and in birds (Markowska et al., 2017). Host rhythms can become disrupted in response to infections. For example, infection with *Plasmodium chabaudi* in mice disrupted rhythms in locomotor activity and body temperature, causing lethargy and hypothermia (Prior et al., 2019), although it was unclear whether this disruption was reflected in core circadian oscillators. In birds, malaria infections have been shown to cause significant shifts in the blood transcriptome (Videvall et al., 2015), however it is unknown whether malaria parasites affect circadian rhythms of their avian hosts.

### 6.1.2 Malaria parasite effects on avian hosts

Avian malaria parasites are globally distributed, occurring in most bird species (Bennett et al., 1994) and are highly prevalent in wild populations (Bensch et al., 2000; Lapointe et al., 2012). Pathologies of avian malaria infections vary from periodic fever, anaemia and mortality (Atkinson et al., 1995; Williams, 2005) to asymptomatic chronic infections (Bensch et al., 2007). Responses to infection with avian malaria vary depending on the parasite lineage (Ricklefs and Fallon, 2002), and bird species (Ilgunas et al., 2019).

Similar to infections with human and mouse malaria, avian malaria parasites show circadian periodicity during their development (Gore et al., 1982; Roller and Desser, 1973; Wolfson, 1936). Time of day effects occur in both acute and chronic infections, with periodic peaks of parasitaemia occurring in the late afternoon in some *Plasmodium* and *Leucocytozoon* species (Pigeault et al., 2018; Roller and Desser, 1973).

Despite the effects that malaria infections can have for fitness of wild birds (Atkinson et al., 1995; Krams et al., 2013b), host-parasite interactions in natural avian populations have not yet been studied in the context of rhythms. Given the complex evolutionary relationships that occur between malaria parasites and their hosts (Mideo et al., 2013; Prior et al., 2019; Reece et al., 2017), it is important to consider timing effects of parasites in ecological studies of avian malaria infections. This study aimed to address this gap in knowledge by investigating whether acute infection with avian malaria influences host circadian physiology, and in particular, the rhythmic expression of biological clock and immune system components in wild birds.

## 6.2 Study aims and hypotheses

The aim of this study was to test for differences in transcript levels of circadian clock and immune genes in wild passerine nestlings naturally infected with *Leucocytozoon* malaria parasites. Specifically, this study investigated three ways that parasites might affect rhythms of their avian hosts, by asking the following:

1) Does acute infection with *Leucocytozoon* result in any changes in host rhythmicity of genes involved in the circadian clock?

2) Does acute infection result in changes in the timing of peak levels of circadian clock gene transcripts, indicating that rhythmic effects of *Leucocytozoon* are clock-mediated?

3) Are transcripts of immune genes in infected birds upregulated or downregulated by presence of *Leucocytozoon* parasites during acute infection?

In a recent study on *P. chabaudi* infected mice, night peaks of locomotory activity were reduced and body temperature rhythms disrupted in malaria-infected individuals (Prior et al., 2019). It is unknown whether this disruption in activity and body temperature rhythms was reflected in core circadian clock oscillators of mice. However, behaviour is linked to rhythms in the SCN (van Oosterhout et al., 2012). Therefore, it was expected that in this study, rhythmicity of transcript levels of genes involved in the circadian clock would be disrupted in infected birds, compared to non-infected birds.

In (Prior et al., 2019), infected mice were active earlier at night, indicating a phase shift in host rhythms by parasites. Great tits are diurnal (Gosler, 1993), and therefore in contrast to nocturnally active mice, it was expected that infected birds may experience a phase shift, to be earlier in the morning. This shift may be seen as a change in peak timing of transcript levels of circadian clock transcripts.

Acute avian malaria infection has previously been shown to alter the transcriptome of birds by elevating immune transcripts (Videvall et al., 2015). Reductions of immune transcripts in infected birds may occur if birds are immunocompromised, or otherwise in a poor state (Kwon et al., 2008; Meitern et al., 2014). A previous experiment carried out at the field site used in this study showed no association with immune gene transcripts levels and avian malaria infections (Capilla-Lasheras et al., 2017), however, this study had low sample sizes and did not account for host rhythmicity.

In this study, it was expected that there may be changes in transcript levels by either elevation or suppression of immune system components in infected birds compared to non-infected birds. As the immune system of birds is closely tied to the circadian clock (Markowska et al., 2017), changes in the timing of expression of immune transcripts, such as differences in acrophase or amplitude, were also expected.

## 6.3 Materials and Methods

### 6.3.1 Study approaches

Rhythmic fluctuations of circadian clock and immune gene transcripts can be determined from blood (Archer et al., 2014). In this study, wild nestlings were blood-sampled throughout the day and night, to capture diel expression of clock and immune gene transcripts.

For analyses of malaria infection on clock gene transcript levels, the following components of the molecular clock were included in this study: positive clock elements and transcriptional activators *BMAL1* (brain and muscle ARNT-like 1) and *CLOCK* (circadian locomotor output cycles kaput); negative clock elements and inhibitors *CRY1* (cryptochrome circadian regulator 1) and *PER2* (period circadian regulator 2); and accessory loop protein *REVERBα* (Kumar and Sharma, 2018; Yoshimura et al., 2000). Additional gene targets included *CK1Ɛ* (casein kinase 1), which catalyses phosphorylation of *E4BP4*, a light-inducible gene in the nestling pineal gland (Doi et al., 2004) and *AANAT* (aralkylamine N-acetyltransferase), an enzyme regulating melatonin synthesis (Bernard et al., 2002).

In addition to core avian clock genes, this study aimed to investigate circadian effects of *Leucocytozoon* infection on transcript levels of immune genes across a time profile. Immune gene targets included *LY86* (lymphocyte antigen 86) and *TLR4* (toll-like receptor 4) which are involved in anti-bacterial and anti-malarial responses (Medzhitov, 2001), and the type 2 transcription factor *GATA3* (GATA binding protein 3) involved in adaptive immunity (Wang et al., 2011). Additional immune targets included: *IL1* and *IL6* (interleukins 1 and 6) which are inflammatory response mediators (Klasing, 1998); *PRKCA* (protein kinase C alpha), an enzyme responsible for antiviral effects and cell growth regulation (Clemens and Elia, 1997); and *IKBA* (NF-kappa-B inhibitor alpha) and *NKRF* (NF-kappa-B repressing factor), which are two inhibitors of the immune response regulator NF-kappa-B ((Cabannes et al., 1999; Nourbakhsh and Hauser, 1999). Finally, immune targets *NRF2* (nuclear factor erythroid 2) and *IGF1* (insulin like growth factor 1) which are both involved in resistance to oxidative stress (Kensler et al., 2007; Holzenberger, 2003 respectively) were included in this study.

Gene transcript data in this study were first fitted to a cosine curve to determine if transcript levels were circadian across the sampling time profile. If transcript levels of

genes were circadian, parameters of the cosine curve, amplitude and acrophase (Figure 6.2.) were determined for both malaria infected and non-infected birds. The amplitude and acrophase of circadian transcripts were then compared for infected and non-infected birds, to determine if *Leucocytozoon* parasites influence rhythmic expression of clock and immune genes in their host.

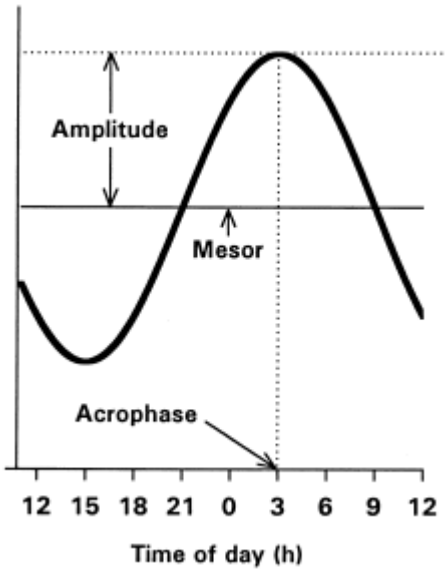



Figure 6.2. Schematic representation of acrophase, mesor and amplitude of a circadian rhythm. Amplitude is one half of the maximal fluctuation between trough and peak. Acrophase is the location of the peak with respect to reference time. Mesor is the midline, or rhythm-adjusted average. Figure obtained from Hayano et al., (1998).

### 6.3.2 Study sites

Data for this chapter were obtained between April and June of 2016 and 2017 at forest sites on free-living populations of breeding great tits. Field sites were existing forest nest box study systems located at the Scottish Centre for Ecology and the Natural Environment (SCENE; 56º, 7.73’N, 4º 36.79’W) and Cashel Forest (56° 6'N, 4°34'W). These sites were previously used in ecological studies of wild passerines and avian malaria (Capilla-Lasheras et al., 2017; Pollock et al., 2017; Woodford et al., 2018). To determine hatch dates, all boxes were initially checked weekly and, closer to hatching, every second day as part of a general nest box monitoring protocol (see General Methods for further details of nest box monitoring). Nestlings used in ALAN experiment in Chapter Four were excluded from this study.

### 6.3.3 Nestling processing

All nestlings (N=199) in this study were ringed for individual identification on day thirteen of life. Sampling was carried out at timepoints distributed throughout the day and night on day thirteen (Figure 6.3.). Sampling was undertaken where possible close to the six hour intervals of the day commonly used in circadian biology (06:00, 12:00, 18:00 and 00;00), with the aim of creating a profile of rhythmic expression of transcript levels across a 24 hr day. Due to constraints of sampling nestlings in the field from multiple nest boxes, samples were not taken "on the hour". Therefore, for ease of statistical analysis, sample time was rounded up or down to the nearest hour (e.g. sample taken at 12:30 was rounded to 13:00).

To reduce burden on each nestling, individuals were sampled once, which therefore enabled spread of sampling of a given brood over different times of day. During sampling, two blood samples of ~50 µl were obtained from each nestling via the brachial wing vein. The first was taken by a heparinised capillary and stored in 0.5 ml 100% ETOH for quantification of malaria parasites. A second blood sample was obtained and stored in 250 µl RNAlater® stabilising solution (Invitrogen) for analyses of gene transcript levels. See General Methods section for further details on nestling blood sampling protocol.

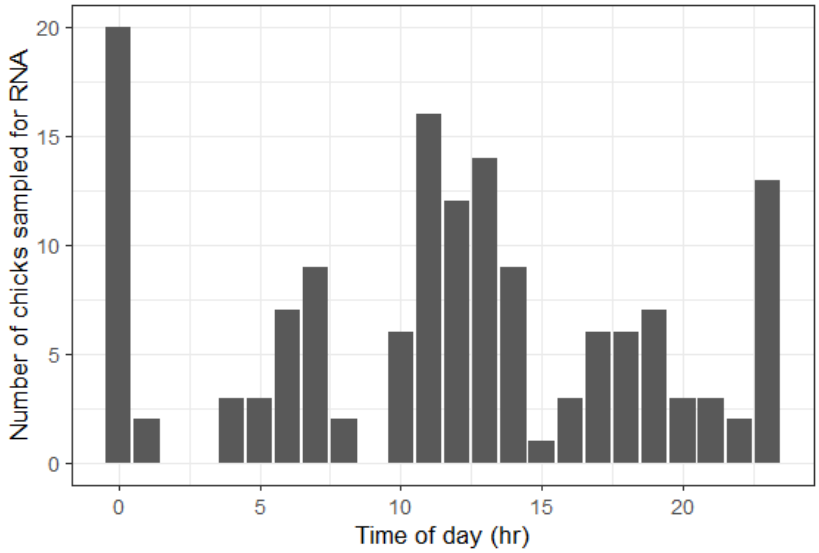



Figure 6.3. Sample time distribution of RNA samples taken for this study. Samples were taken across the breeding seasons in two years (2016 and 2017).

### 6.3.4 Ethical Statement

See General Methods section for details of ringing licences and blood sampling.

### 6.3.5 Laboratory procedures

*Determining transcript levels of clock and immune genes*

Samples in 100% ETOH were frozen and stored at -40 °C. Genomic DNA was later extracted from these samples via DNAeasy Blood and Tissue Kit (Qiagen). Measurement of Median Fluorescence Intensity (as a proxy for transcript levels) for immune and clock gene targets outlined in Table 6.1. was carried out on all samples stored in *RNAlater*® using the QuantiGene® Plex RNA assay 2.0 (ThermoFisher). See General Methods section for further details on the QuantiGene® Plex RNA assay procedure.

Table 6.1. Housekeeping, clock and immune gene targets used within this study.

| Transcript targets | Type |
|---|---|
| *RPL19, SDHA, HMBS* | Housekeeping genes |
| *BMAL1, CLOCK, CRY1 ,CK1ε, AANAT, PER2, REVERBA* | Circadian clock genes and melatonin synthesis |
| *LY86, TLR4, IL1, IL6, PRKCA, GATA3, IĸBA, NĸRF, NRF2, IGF1* | Immune genes |

*Measuring infection intensity of Leucocytozoon parasites*

All avian DNA samples were run using a sensitive qPCR protocol for quantifying malaria infection in young birds developed by another PhD student (Albalawi, 2019). See General Methods section for full details of malaria qPCR protocol.

### 6.3.6 Statistical Analysis and Model Selection

All statistical analyses were performed using R v. 3.4.4 (R Core Team, 2018), and packages *lme4* (Bates et al., 2015). Data were visualised using ggplot2 (Wickham, 2014). Initial global models for response variables were generalised linear-mixed models (GLM), including individual nest box as a random factor to account for repeated measures within the same nest box. Residuals were plotted and inspected to check assumptions of normality whilst modelling using GLMs. Pairs of explanatory variables were also assessed for collinearity by calculating tolerance and variance inflation factors (VIF) and also by visual inspection of pairwise plots, before statistical relevance was accepted.

Based on the number of *Leucocytozoon* parasite gene copies (as a proxy for number of individual parasites) detected in the sample, infections were quantified into categories.

From visual inspection of the distribution of the data, two main infection groups were identified. These were: low levels of infection (≤1% of blood cells infected) and high levels of infection (>1% of blood cells infected). Additionally, a category was included where no parasites/infection was detected in the sample. Each sample was then assigned to the mean of the infection group and entered into models as a numeric explanatory variable (where no infection=1, low infection=2 and high infection=3), to preserve ordinal categories.

Median fluorescence intensity data (as a proxy for transcript levels of genes) were obtained from the QuantiGene® Plex RNA assay 2.0 (Thermofisher). These data were normalised by division of the mean fluorescence of background wells and then dividing each sample by the geometric mean of levels of housekeeping genes in Table 6.1., thus, transcript levels are expressed as relative levels to housekeeping genes. Transcript data were modelled using a Gamma distribution with log-link function. Time of day information was converted to decimal time, then to radians, and then entered into the model as an explanatory variable in its components of sine(time) and cosine(time). Global models included interactions between infection and sin(time), and also infection and cos(time). Additional explanatory variables included hatch date in April days (where April $1^{st}$ = 1) and study year (2016/2017). Nest box was included in all models as a random factor.

Statistical relevance of fixed factors within all models was determined by sequential model selection via Likelihood Ratio Testing using package *lmtest* (Zeileis & Hothorn, 2002), until the minimum adequate model (MAM) was found. Factors with a significance level of <5% were kept in the model. Infection and sine and cosine were kept in the model regardless of significance.

Cosinor analyses were carried out using model outputs of genes found to be rhythmic in initial models (where sine or cosine were significant). The amplitude of rhythms of transcript levels was calculated as the square root of the sum of sine and cosine coefficients: $\text{sqrt}(\text{sine}^2 + \text{cosine}^2$. The acrophase was determined as arctan(- sine / cosine).

## 6.4 Results

In total, infection with *Leucocytozoon* parasites was quantified in 158 nestlings. Out of those sampled, 61 nestlings had no detectable infection, 68 had low-level infection (≤1%) and 29 had high levels of infection (>1%).

### 6.4.1 Clock gene transcripts

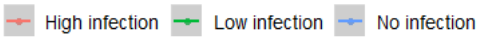
For all clock genes analysed in this study, there was no significant interaction between sine and cosine and *Leucocytozoon* infection (Table 6.2.). Moreover, transcript levels of clock genes did not fit to either sine or cosine curves, and therefore there was no support for rhythmicity (Figure 6.4). Transcript levels of clock genes were also not significantly affected by either hatch date or study year (Table 6.2.).

High levels of infection with *Leucocytozoon* significantly reduced the levels of transcripts of three clock genes: *AANAT* ($t_{1,116}$=-2.62, P=0.009; LMM), *CLOCK* ($t_{1,131}$=-4.04, P<0.001; LMM), and *CRY1* ($t_{1,138}$=-2.06, P=0.039; LMM), (Table 6.2). For *AANAT*, transcript levels in infected birds were 24 % lower compared with non-infected birds (Figure 6.4.2.). For *CLOCK*, transcript levels of infected birds were 16 % lower than non-infected birds (Figure 6.6.4.). Finally, transcript levels for *CRY1* were 9 % lower in infected birds (Figure 6.4.8.). In addition, reductive effects of high levels of *Leucocytozoon* infection on transcript levels tended towards significance for *PER2* ($t_{1,139}$=-1.94, P=0.068; LMM), (Figure 6.4.5).

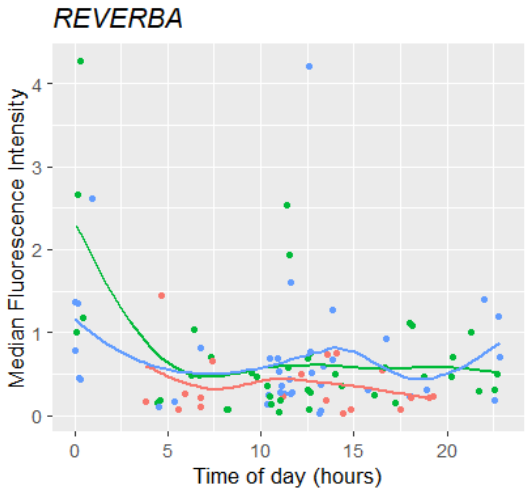
Table 6.2. Model summaries for circadian clock gene targets. Estimates shown here were log-transformed. Global models for all targets included: Nest box + infection * sine(time) + infection * cosine(time) + hatch date + study year. Nest box was included in all models as a random factor. Sine and cosine were tested together during likelihood ratio testing.

| **Variable** | **N** | **Estimate** | **s.e.m** | **d.f.** | ***t*-value** | ***p*-value LRT** |
|---|---|---|---|---|---|---|
| ***REVERBA*** | 112 | | | | | |
| (Intercept) | | 2.10 | 1.34 | | 1.56 | |
| Infection(Low) | | 0.01 | 0.19 | 1 | 0.07 | 0.945 |
| Infection(High) | | -0.26 | 0.23 | 1 | -1.12 | 0.262 |
| sin(Time) | | -0.11 | 0.28 | 1 | -0.38 | 0.704 |
| cos(Time) | | 0.22 | 0.16 | 1 | 1.35 | 0.177 |
| Hatch date | | -0.06 | 0.03 | 1 | -2.10 | 0.055 |
| Infection(Low)*sin(Time) | | 0.06 | 0.33 | 2 | 0.17 | 0.867 |
| Infection(High)*sin(Time) | | 0.49 | 0.37 | 2 | 1.33 | 0.183 |
| Infection(Low)*cos(Time) | | -0.04 | 0.22 | 2 | -0.18 | 0.856 |
| Infection(High)*cos(Time) | | 0.01 | 0.33 | 2 | 0.04 | 0.967 |
| ***AANAT*** | 116 | | | | | |
| (Intercept) | | -1.30 | 0.92 | | -1.42 | |
| Infection(Low) | | -0.18 | 0.12 | 1 | -1.46 | 0.145 |
| Infection(High) | | -0.43 | 0.16 | 1 | -2.62 | **0.009** |
| sin(Time) | | -0.05 | 0.19 | 1 | -0.26 | 0.792 |
| cos(Time) | | 0.07 | 0.11 | 1 | 0.66 | 0.510 |
| Hatch date | | -0.01 | 0.02 | 1 | -0.79 | 0.431 |
| Infection(Low)*sin(Time) | | 0.08 | 0.22 | 2 | 0.37 | 0.709 |
| Infection(High)*sin(Time) | | 0.18 | 0.25 | 2 | 0.72 | 0.472 |
| Infection(Low)*cos(Time) | | 0.02 | 0.15 | 2 | 0.11 | 0.913 |
| Infection(High)*cos(Time) | | -0.07 | 0.23 | 2 | -0.31 | 0.760 |
| ***CK1E*** | 126 | | | | | |
| (Intercept) | | -1.34 | 0.77 | | -1.74 | |
| Infection(Low) | | -0.04 | 0.12 | 1 | -0.35 | 0.724 |
| Infection(High) | | -0.20 | 0.14 | 1 | -1.41 | 0.160 |
| sin(Time) | | -0.15 | 0.17 | 1 | -0.90 | 0.367 |
| cos(Time) | | -0.04 | 0.10 | 1 | -0.39 | 0.693 |
| Hatch date | | -0.02 | 0.01 | 1 | -1.03 | 0.303 |
| Infection(Low)*sin(Time) | | 0.19 | 0.19 | 2 | 0.96 | 0.338 |
| Infection(High)*sin(Time) | | 0.25 | 0.22 | 2 | 1.14 | 0.253 |
| Infection(Low)*cos(Time) | | 0.18 | 0.14 | 2 | 1.27 | 0.203 |
| Infection(High)*cos(Time) | | 0.01 | 0.19 | 2 | 0.05 | 0.958 |
| ***CLOCK*** | 131 | | | | | |
| (Intercept) | | -0.75 | 0.46 | | -1.63 | 0.104 |
| Infection(Low) | | -0.12 | 0.06 | 1 | -1.93 | 0.054 |
| Infection(High) | | -0.34 | 0.08 | 1 | -4.04 | **<0.001** |
| sin(Time) | | -0.07 | 0.09 | 1 | -0.73 | 0.466 |
| cos(Time) | | 0.00 | 0.05 | 1 | 0.02 | 0.987 |
| Hatch date | | -0.02 | 0.01 | 1 | -1.83 | 0.067 |
| Infection(Low)*sin(Time) | | 0.19 | 0.11 | 2 | 1.67 | 0.094 |
| Infection(High)*sin(Time) | | 0.04 | 0.12 | 2 | 0.35 | 0.725 |
| Infection(Low)*cos(Time) | | 0.07 | 0.07 | 2 | 0.98 | 0.328 |
| Infection(High)*cos(Time) | | 0.04 | 0.10 | 2 | 0.35 | 0.728 |
| ***PER2*** | 139 | | | | | |
| (Intercept) | | -2.16 | 1.11 | | -1.95 | |
| Infection(Low) | | -0.04 | 0.09 | 1 | -0.41 | 0.683 |
| Infection(High) | | -0.23 | 0.13 | 1 | -1.83 | 0.068 |
| sin(Time) | | 0.02 | 0.16 | 1 | 0.10 | 0.920 |
| cos(Time) | | -0.02 | 0.07 | 1 | -0.30 | 0.765 |
| Hatch date | | 0.02 | 0.02 | 1 | 0.75 | 0.454 |
| Infection(Low)*sin(Time) | | -0.03 | 0.18 | 2 | -0.16 | 0.874 |
| Infection(High)*sin(Time) | | 0.15 | 0.20 | 2 | 0.74 | 0.460 |
| Infection(Low)*cos(Time) | | 0.13 | 0.11 | 2 | 1.14 | 0.253 |

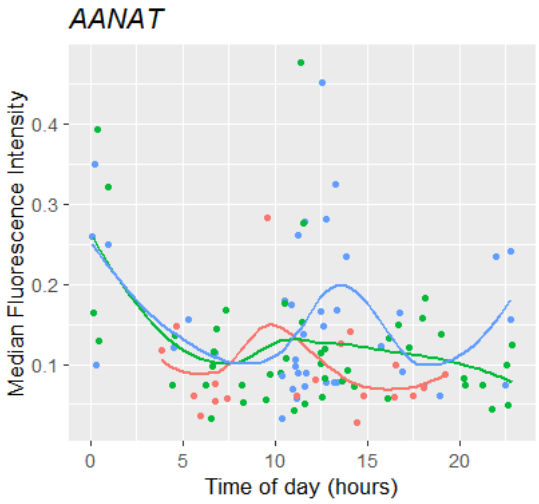
| | | | | | | |
|---|---|---|---|---|---|---|
| Infection(High)*cos(Time) | | 0.15 | 0.16 | 2 | 0.96 | 0.338 |
| ***BMAL1*** | 125 | | | | | |
| (Intercept) | | -1.43 | 0.77 | | -1.85 | |
| Infection(Low) | | -0.15 | 0.10 | 1 | -1.50 | 0.135 |
| Infection(High) | | -0.20 | 0.13 | 1 | -1.45 | 0.146 |
| sin(Time) | | -0.05 | 0.15 | 1 | -0.30 | 0.761 |
| cos(Time) | | -0.03 | 0.07 | 1 | -0.48 | 0.633 |
| Hatch date | | -0.01 | 0.01 | 1 | -0.80 | 0.424 |
| Infection(Low)*sin(Time) | | 0.08 | 0.17 | 2 | 0.45 | 0.652 |
| Infection(High)*sin(Time) | | 0.08 | 0.20 | 2 | 0.39 | 0.697 |
| Infection(Low)*cos(Time) | | 0.15 | 0.11 | 2 | 1.32 | 0.187 |
| Infection(High)*cos(Time) | | 0.25 | 0.17 | 2 | 1.50 | 0.134 |
| ***CKA*** | 142 | | | | | |
| (Intercept) | | 0.82 | 0.41 | | 2.00 | |
| Infection(Low) | | -0.02 | 0.04 | 1 | -0.52 | 0.603 |
| Infection(High) | | 0.00 | 0.05 | 1 | 0.02 | 0.986 |
| sin(Time) | | 0.02 | 0.06 | 1 | 0.28 | 0.779 |
| cos(Time) | | 0.00 | 0.03 | 1 | -0.11 | 0.910 |
| Hatch date | | -0.01 | 0.01 | 1 | -1.56 | 0.118 |
| Infection(Low)*sin(Time) | | -0.04 | 0.07 | 2 | -0.55 | 0.579 |
| Infection(High)*sin(Time) | | -0.05 | 0.08 | 2 | -0.65 | 0.514 |
| Infection(Low)*cos(Time) | | 0.01 | 0.05 | 2 | 0.27 | 0.785 |
| Infection(High)*cos(Time) | | 0.10 | 0.07 | 2 | 1.46 | 0.143 |
| ***CRY1*** | 138 | | | | | |
| (Intercept) | | 2.00 | 0.045 | | -1.40 | |
| Infection(Low) | | -0.52 | 0.603 | 1 | -0.96 | 0.340 |
| Infection(High) | | 0.02 | 0.986 | 1 | -2.06 | **0.039** |
| sin(Time) | | 0.28 | 0.779 | 1 | -0.76 | 0.449 |
| cos(Time) | | -0.11 | 0.910 | 1 | -0.11 | 0.910 |
| Hatch date | | -1.56 | 0.118 | 1 | -0.34 | 0.733 |
| Infection(Low)*sin(Time) | | -0.55 | 0.579 | 2 | 0.25 | 0.801 |
| Infection(High)*sin(Time) | | -0.65 | 0.514 | 2 | 1.45 | 0.148 |
| Infection(Low)*cos(Time) | | 0.27 | 0.785 | 2 | 1.32 | 0.186 |
| Infection(High)*cos(Time) | | 1.46 | 0.143 | 2 | 0.79 | 0.428 |



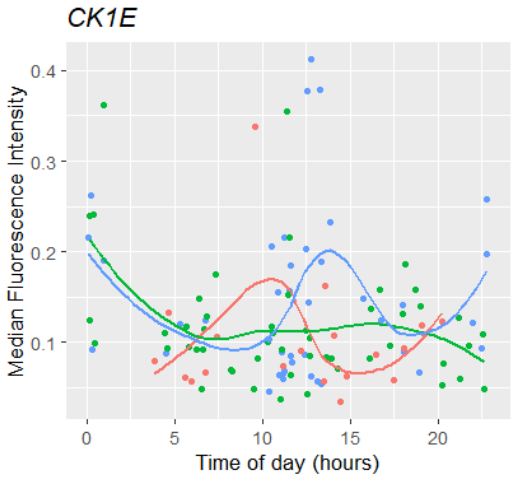

6.4.1.



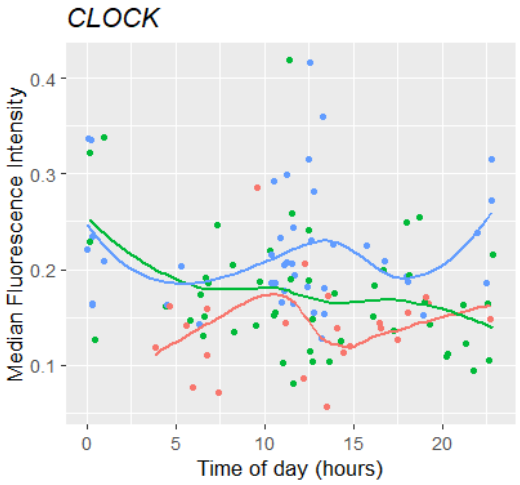

6.4.2.



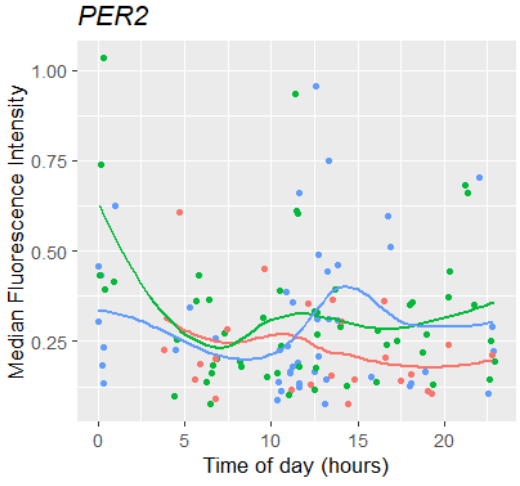

6.4.3.



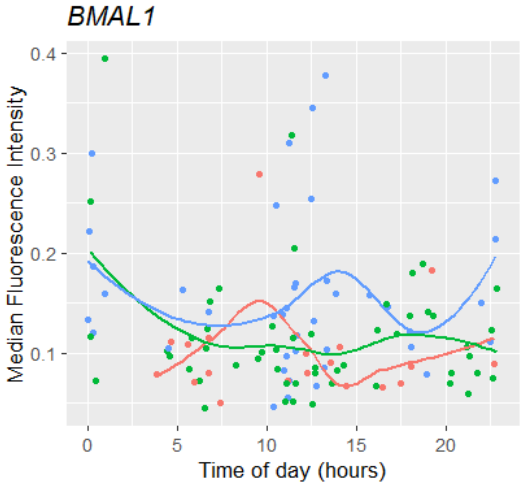

6.4.4.




6.4.5.




6.4.6.



6.4.7. 6.4.8.

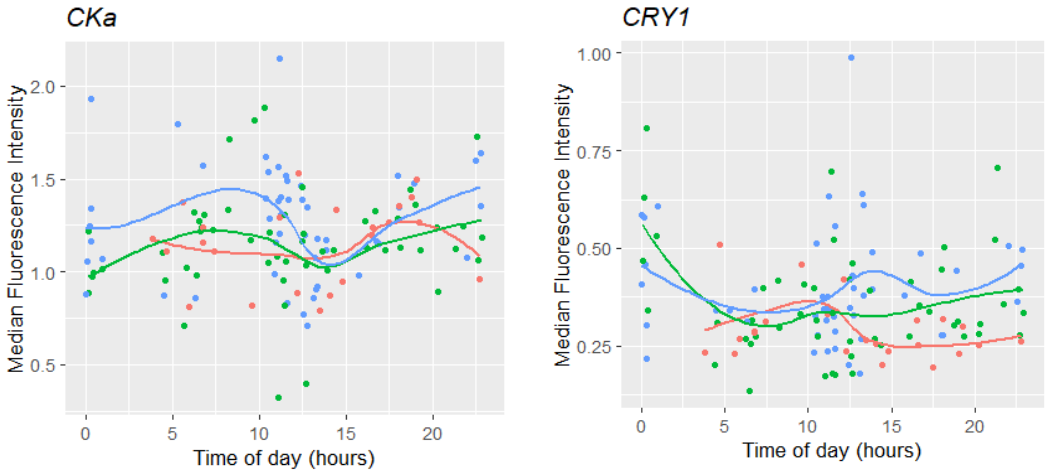



Figure 6.4. Transcript levels of circadian clock gene targets for infection with *Leucocytozoon* parasites, at different times of day: 1) *REVERBA* 2) *AANAT* 3) *CK1E* 4) *CLOCK* 5) *PER2* 6) *BMAL1* 7) *CKA* 8) *CRY1*. Colours indicate intensity of infection with Leucocytozoon parasites: No infection (blue), low-level infection (green) and high-level infection (red). Data shown are empirical values obtained from QuantiGene® Plex RNA assay and are shown in Median florescence intensity units (MFI). Data were fitted with Loess curves.

### 6.4.2 Immune gene transcripts

For all immune genes studied, there was no significant interaction between sine and cosine and *Leucocytozoon* infection (Table 6.3). Separate to infection, immune genes studied did not fit to either sine or cosine curves, and therefore there was no support for rhythmicity in any of these genes (Figure 6.5). Hatch date or study year did not affect transcript levels of any of the immune genes studied (Table 6.3).

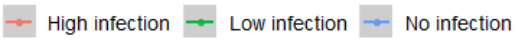
High-level infection with *Leucocytozoon* significantly reduced transcript levels in most of the immune genes studied: *IL1, TLR4, NRF2, GATA3, IL6, LY86, NKRF, PRKCA* and *IGF1* (Table 6.3, Figure 6.5.). Fold changes in transcript levels of these genes between infected birds (with high level of infection) and non-infected birds ranged from 0.88 to 0.05.

Table 6.3. Model summaries for immune gene transcripts. Estimates shown here are log-transformed. Global models included: Nest box + infection * sine(time) + infection * cosine(time) + hatch date + study year. Nest box was included in all models as a random factor. Sine and cosine were tested together during likelihood ratio testing.

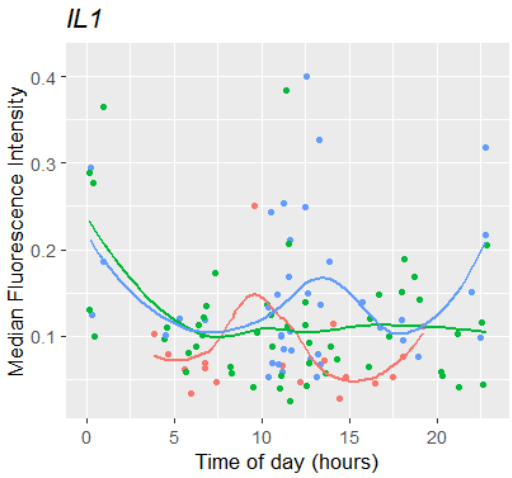
| **Variable** | **N** | **Estimate** | **s.e.m** | **d.f.** | ***t*-value** | ***p*-value LRT** |
|---|---|---|---|---|---|---|
| ***IL1*** | 120 | | | | | |
| (Intercept) | | -0.59 | 0.87 | | -0.68 | 0.494 |
| Infection(Low) | | -0.11 | 0.11 | 1 | -0.97 | 0.331 |
| Infection(High) | | -0.53 | 0.16 | 1 | -3.39 | **0.001** |
| sin(Time) | | -0.01 | 0.17 | 1 | -0.07 | 0.943 |
| cos(Time) | | 0.07 | 0.09 | 1 | 0.81 | 0.417 |
| Hatch date | | -0.03 | 0.02 | 1 | -1.71 | 0.088 |
| Infection(Low)*sin(Time) | | 0.08 | 0.20 | 2 | 0.41 | 0.684 |
| Infection(High)*sin(Time) | | 0.17 | 0.23 | 2 | 0.75 | 0.451 |
| Infection(Low)*cos(Time) | | 0.11 | 0.13 | 2 | 0.82 | 0.411 |
| Infection(High)*cos(Time) | | -0.09 | 0.21 | 2 | -0.41 | 0.682 |
| | | | | | | |
| ***TLR4*** | 115 | | | | | |
| (Intercept) | | -1.65 | 0.91 | | -1.82 | 0.068 |
| Infection(Low) | | -0.24 | 0.12 | 1 | -2.00 | **0.045** |
| Infection(High) | | -0.41 | 0.16 | 1 | -2.56 | **0.011** |
| sin(Time) | | -0.09 | 0.20 | 1 | -0.44 | 0.657 |
| cos(Time) | | 0.06 | 0.08 | 1 | 0.71 | 0.475 |
| Hatch date | | 0.00 | 0.02 | 1 | -0.19 | 0.849 |
| Infection(Low)*sin(Time) | | 0.11 | 0.22 | 2 | 0.47 | 0.636 |
| Infection(High)*sin(Time) | | 0.27 | 0.25 | 2 | 1.09 | 0.277 |
| Infection(Low)*cos(Time) | | 0.03 | 0.13 | 2 | 0.21 | 0.833 |
| Infection(High)*cos(Time) | | -0.02 | 0.21 | 2 | -0.10 | 0.923 |
| ***IKBA*** | 132 | | | | | |
| (Intercept) | | -1.45 | 0.77 | | -1.89 | 0.059 |
| Infection(Low) | | -0.04 | 0.09 | 1 | -0.45 | 0.650 |
| Infection(High) | | -0.11 | 0.12 | 1 | -0.87 | 0.383 |
| sin(Time) | | -0.13 | 0.14 | 1 | -0.96 | 0.339 |

| | | | | | | |
|---|---|---|---|---|---|---|
| cos(Time) | | -0.03 | 0.07 | 1 | -0.44 | 0.661 |
| Hatch date | | -0.01 | 0.01 | 1 | -0.80 | 0.425 |
| Infection(Low)*sin(Time) | | 0.16 | 0.16 | 2 | 0.96 | 0.336 |
| Infection(High)*sin(Time) | | 0.17 | 0.18 | 2 | 0.98 | 0.328 |
| Infection(Low)*cos(Time) | | 0.16 | 0.11 | 2 | 1.49 | 0.137 |
| Infection(High)*cos(Time) | | 0.03 | 0.16 | 2 | 0.17 | 0.864 |
| ***NRF2*** | 141 | | | | | |
| (Intercept) | | 1.82 | 0.46 | | 3.92 | |
| Infection(Low) | | 0.03 | 0.04 | 1 | 0.66 | 0.508 |
| Infection(High) | | -0.09 | 0.05 | 1 | -1.58 | 0.114 |
| sin(Time) | | 0.03 | 0.06 | 1 | 0.43 | 0.668 |
| cos(Time) | | 0.00 | 0.03 | 1 | 0.17 | 0.866 |
| Hatch date | | 0.00 | 0.01 | 1 | -0.01 | 0.989 |
| Infection(Low)*sin(Time) | | -0.10 | 0.07 | 2 | -1.32 | 0.186 |
| Infection(High)*sin(Time) | | -0.03 | 0.08 | 2 | -0.36 | 0.719 |
| Infection(Low)*cos(Time) | | 0.02 | 0.05 | 2 | 0.47 | 0.636 |
| Infection(High)*cos(Time) | | 0.11 | 0.07 | 2 | 1.58 | 0.113 |
| ***GATA3*** | 126 | | | | | |
| (Intercept) | | -1.04 | 0.76 | | -1.38 | |
| Infection(Low) | | -0.14 | 0.10 | 1 | -1.37 | 0.171 |
| Infection(High) | | -0.42 | 0.14 | 1 | -3.02 | **0.003** |
| sin(Time) | | 0.02 | 0.16 | 1 | 0.10 | 0.920 |
| cos(Time) | | 0.08 | 0.08 | 1 | 1.01 | 0.310 |
| Hatch date | | -0.02 | 0.01 | 1 | -1.19 | 0.232 |
| Infection(Low)*sin(Time) | | 0.01 | 0.19 | 2 | 0.04 | 0.968 |
| Infection(High)*sin(Time) | | 0.13 | 0.21 | 2 | 0.61 | 0.541 |
| Infection(Low)*cos(Time) | | 0.04 | 0.12 | 2 | 0.34 | 0.734 |
| Infection(High)*cos(Time) | | -0.08 | 0.19 | 2 | -0.42 | 0.678 |
| ***IL6*** | 115 | | | | | |
| (Intercept) | | -1.16 | 0.87 | | -1.34 | |
| Infection(Low) | | -0.19 | 0.12 | 1 | -1.57 | 0.116 |
| Infection(High) | | -0.59 | 0.17 | 1 | -3.43 | **0.001** |
| sin(Time) | | -0.07 | 0.20 | 1 | -0.35 | 0.725 |
| cos(Time) | | 0.00 | 0.09 | 1 | 0.02 | 0.986 |
| Hatch date | | -0.02 | 0.02 | 1 | -1.01 | 0.311 |
| Infection(Low)*sin(Time) | | 0.10 | 0.23 | 2 | 0.41 | 0.681 |
| Infection(High)*sin(Time) | | 0.23 | 0.26 | 2 | 0.86 | 0.392 |
| Infection(Low)*cos(Time) | | 0.20 | 0.14 | 2 | 1.41 | 0.159 |
| Infection(High)*cos(Time) | | -0.06 | 0.22 | 2 | -0.27 | 0.788 |
| | | | | | | |
| ***LY86*** | 139 | | | | | |
| (Intercept) | | -1.16 | 0.70 | | -1.65 | |
| Infection(Low) | | -0.09 | 0.07 | 1 | -1.20 | 0.231 |
| Infection(High) | | -0.22 | 0.10 | 1 | -2.25 | **0.025** |
| sin(Time) | | -0.07 | 0.11 | 1 | -0.59 | 0.554 |
| cos(Time) | | -0.01 | 0.05 | 1 | -0.16 | 0.875 |
| Hatch date | | -0.01 | 0.01 | 1 | -0.56 | 0.579 |
| Infection(Low)*sin(Time) | | 0.05 | 0.13 | 2 | 0.39 | 0.693 |
| Infection(High)*sin(Time) | | 0.04 | 0.15 | 2 | 0.27 | 0.787 |
| Infection(Low)*cos(Time) | | 0.13 | 0.08 | 2 | 1.54 | 0.125 |
| Infection(High)*cos(Time) | | 0.01 | 0.12 | 2 | 0.12 | 0.903 |
| | | | | | | |
| ***NKRF*** | 141 | | | | | |
| (Intercept) | | 1.61 | 0.62 | | 2.58 | |
| Infection(Low) | | 0.02 | 0.05 | 1 | 0.34 | 0.735 |
| Infection(High) | | -0.36 | 0.07 | 1 | -5.43 | **0.001** |
| sin(Time) | | -0.07 | 0.07 | 1 | -0.92 | 0.360 |
| cos(Time) | | 0.00 | 0.04 | 1 | -0.02 | 0.986 |
| Hatch date | | -0.02 | 0.01 | 1 | -1.95 | 0.052 |
| Infection(Low)*sin(Time) | | -0.05 | 0.09 | 2 | -0.51 | 0.607 |
| Infection(High)*sin(Time) | | 0.03 | 0.10 | 2 | 0.31 | 0.758 |
| Infection(Low)*cos(Time) | | -0.01 | 0.06 | 2 | -0.16 | 0.870 |
| Infection(High)*cos(Time) | | 0.10 | 0.08 | 2 | 1.22 | 0.222 |

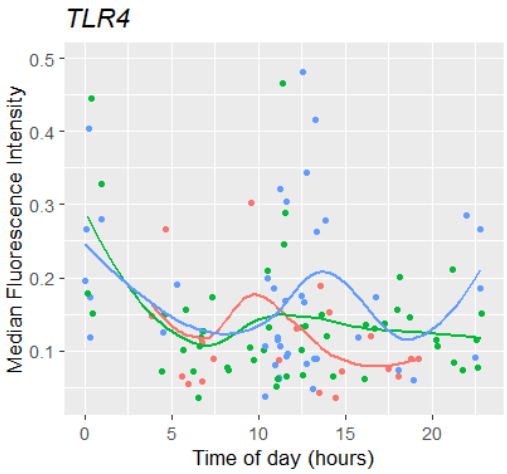
| Variable | N | Estimate | s.e.m | d.f. | t-value | *p*-value LRT |
|---|---|---|---|---|---|---|
| ***PRKCA*** | 113 | | | | | |
| (Intercept) | | -1.50 | 0.87 | | -1.72 | |
| Infection(Low) | | -0.16 | 0.13 | 1 | -1.26 | 0.207 |
| Infection(High) | | -0.47 | 0.16 | 1 | -2.85 | **0.004** |
| sin(Time) | | -0.12 | 0.19 | 1 | -0.61 | 0.540 |
| cos(Time) | | 0.12 | 0.09 | 1 | 1.31 | 0.190 |
| Hatch date | | -0.01 | 0.02 | 1 | -0.75 | 0.454 |
| Infection(Low)*sin(Time) | | 0.12 | 0.22 | 2 | 0.56 | 0.578 |
| Infection(High)*sin(Time) | | 0.27 | 0.25 | 2 | 1.08 | 0.282 |
| Infection(Low)*cos(Time) | | 0.02 | 0.15 | 2 | 0.16 | 0.875 |
| Infection(High)*cos(Time) | | -0.11 | 0.22 | 2 | -0.53 | 0.595 |
| | | | | | | |
| ***IGF1*** | 122 | | | | | |
| (Intercept) | | -1.26 | 0.83 | | -1.52 | |
| Infection(Low) | | -0.17 | 0.12 | 1 | -1.44 | 0.151 |
| Infection(High) | | -0.54 | 0.16 | 1 | -3.28 | **0.001** |
| sin(Time) | | -0.07 | 0.19 | 1 | -0.38 | 0.707 |
| cos(Time) | | 0.04 | 0.09 | 1 | 0.44 | 0.661 |
| Hatch date | | -0.02 | 0.02 | 1 | -1.00 | 0.319 |
| Infection(Low)*sin(Time) | | 0.07 | 0.22 | 2 | 0.31 | 0.755 |
| Infection(High)*sin(Time) | | 0.20 | 0.25 | 2 | 0.78 | 0.434 |
| Infection(Low)*cos(Time) | | 0.17 | 0.14 | 2 | 1.16 | 0.244 |
| Infection(High)*cos(Time) | | 0.02 | 0.21 | 2 | 0.10 | 0.923 |



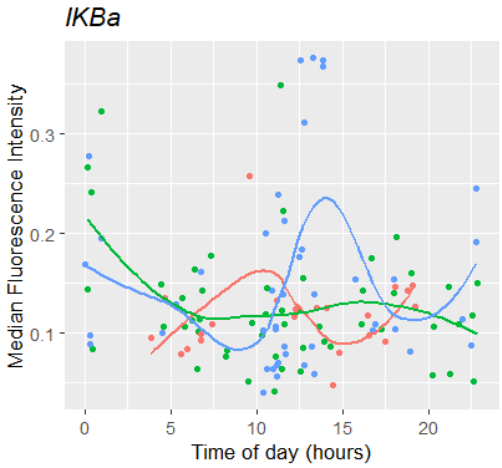

6.5.1.



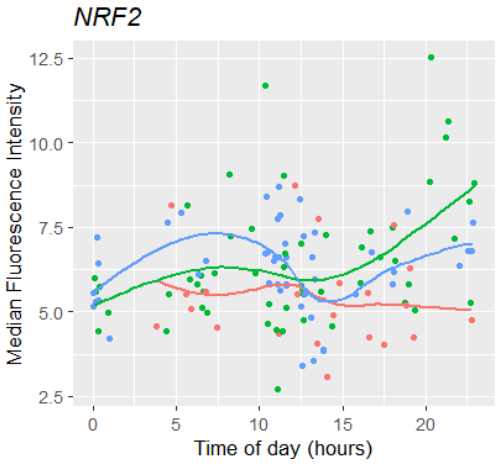

6.5.2.



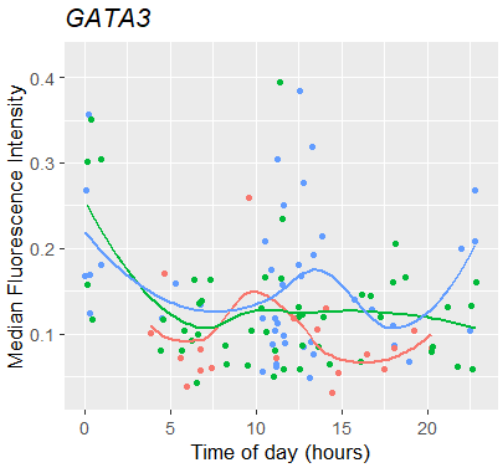

6.5.3.



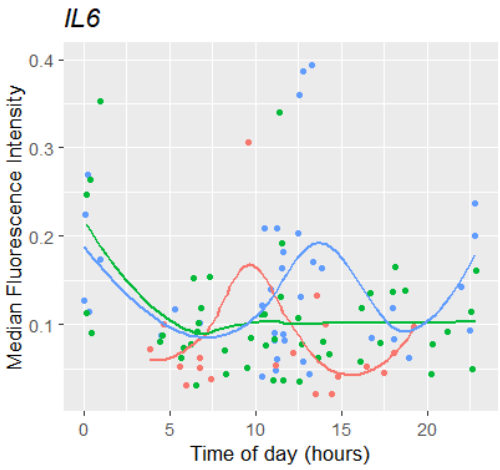

6.5.4.



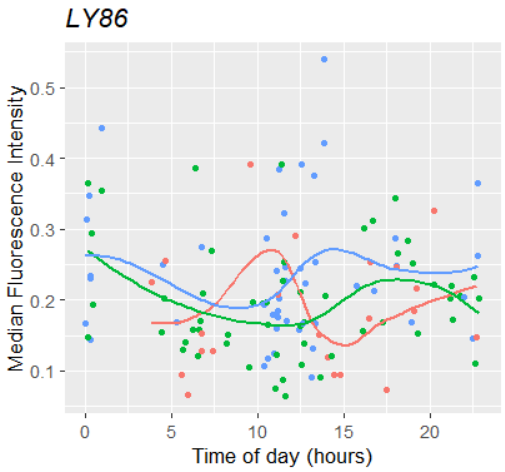

6.5.5.



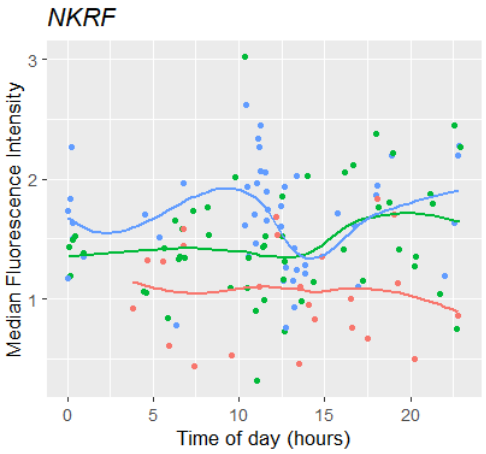

6.5.6.



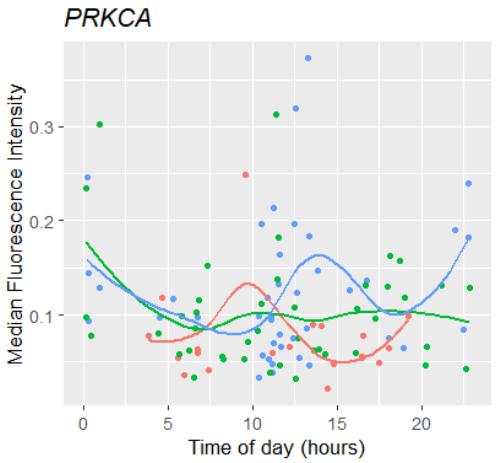
6.5.7.




6.5.8.




6.5.9.




6.5.10.

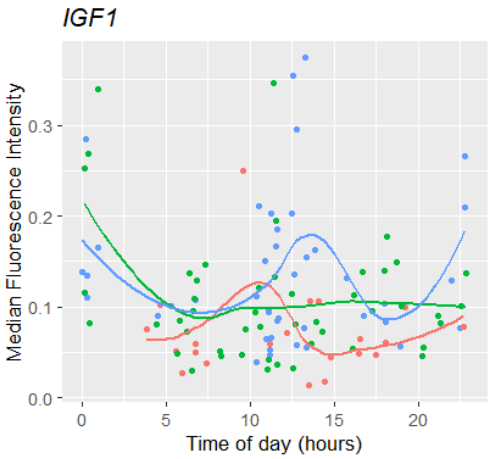



Figure 6.5. Transcript levels of immune gene targets for targets for infection with *Leucocytozoon* parasites, at different times of day. 1) *IL1* 2) *TLR4* 3) *IKBA* 4) *NRF2* 5) *GATA3* 6) *IL6* 7) *LY86* 8) *NKRF* 9) *PRKCA* 10) *IGF1*. Colours indicate intensity of infection with Leucocytozoon parasites: No infection (blue), low-level infection (green) and high-level infection (red). Data shown are empirical values obtained from QuantiGene® Plex RNA assay and are shown in Median florescence intensity units (MFI). Data were fitted with Loess curves.

## 6.5 Discussion

### 6.5.1 Rhythmicity in gene transcript levels

This study aimed to detect rhythmic transcripts in nestling great tits for clock and immune gene targets, for the first time in a wild animal. However, there was no support for rhythmicity for the circadian clock and immune genes studied.

Rhythmicity may not have been observed for transcript levels of gene targets in this study for a number of reasons. Firstly, nest disturbances during blood sampling may have influenced circadian gene expression, by way of sleep interruptions (Möller-Levet et al., 2013; Archer et al., 2014), or altered rhythms as a response to stress (Weibel et al., 2002). Furthermore, the nature of outdoor sampling introduces elements such as variations in ambient temperature. Although circadian mechanisms are largely temperature compensated (Merrow et al., 2005; Pittendrigh 1954), changing thermal conditions have nevertheless previously been shown to directly influence timing of activity patterns (Lehmann et al., 2012; Vivanco et al., 2010). Temperature variations therefore may have also played a role in increasing individual variation in circadian gene expression in this study.

In the two previous Chapters, this study made use of a platform for measuring transcript levels, the QuantiGene® Plex assay. Although previously used to measure transcripts in chickens (Gunawardana et al., 2019), this assay had not been used before for passerine samples. Therefore, the assay was not perfectly optimised for use on passerine blood samples, which may have contributed to the high levels of variation in transcript data, and why rhythmicity was detected in only two genes.

Finally, the use of blood samples instead of tissue may have reduced capacity to capture rhythmic expression of genes in this study. Rhythmic expression of genes can be detected from blood samples in humans (Archer et al., 2014). In birds, day-night differences in levels of immune genes have been shown in blood leucocytes of chickens (Turkowska et al., 2013), and previous studies has observed immune gene profiles from passerine blood (Meitern et al., 2014; Watson et al., 2017). However, there can be marked differences in the timing and level of expression of circadian gene transcripts between tissue types (Karaganis et al., 2009). The possibility remains the combined effect of noisy data from the QuantiGene® Plex assay, and low signal from the use of blood samples (in place of other tissues) masked the detection of rhythmicity in most genes in this study.

### 6.5.2 Effects of infection with *Leucocytozoon* parasites on circadian clock and immune transcript levels

This study investigated the effects of *Leucocytozoon* infection on the rhythmic expression of clock and immune transcript levels in wild nestlings. Transcript levels for many immune genes appeared to be significantly suppressed by *Leucocytozoon* infection. As discussed in Chapter Five, *Leucocytozoon* parasites primarily infect host thrombocytes (Zhao et al., 2015), and therefore may either directly infect cells that are expressing immune transcripts, or cause indirect effects as a consequence of activating T-cells responding to infected thrombocytes. In this study, *Leucocytozoon* parasites may have directly infected and destroyed host thrombocytes, resulting in low levels of immune gene transcripts.

Curiously, many circadian clock gene transcripts also appeared to be suppressed by high infection. Circadian transcripts are produced by thrombocytes (Ando et al., 2009; Turkowska et al., 2013), as well as erythrocytes (Archer et al., 2014). Therefore, circadian transcripts may have been suppressed by *Leucocytozoon* in a similar way to immune gene transcripts, by destruction of host thrombocytes.

### 6.5.3 Conclusions and future studies

This study provided the first attempt to quantify rhythmic transcripts in birds within the natural environment. However, in this study, no support for rhythmicity was found for the circadian clock and immune gene targets studied.

This study showed infection with *Leucocytozoon* parasites to significantly suppress transcript levels of genes involved in the circadian clock and immune system of wild nestlings. Suppression of the immune system may leave birds susceptible to further infections (Gonzalez et al., 1999; Nordling et al., 1998), and bring negative consequences for life histories in terms of reduced body condition (Marzal et al., 2008b), and reproductive fitness (Hamilton and Zuk, 1982).

The assay used in this study to quantify malaria infections was able to distinguish between infections with *Leucocytozoon*, or other parasite genera such as *Haemoproteus*, but it could not identify parasites at species level (Albalawi, 2019). Given that different lineages and species of malaria parasites have different rhythmic properties (Prior et al., 2019; Rivero and Gandon, 2018), future studies might take the parasite species into consideration when investigating impacts on host rhythms.

In addition, the time and day that nestlings were bitten could not be determined in this study. As such, *Leucocytozoon* parasites may have been in different developmental stages in host blood (e.g. liver stage), and therefore, the diel profile of *Leucocytozoon* parasite bursting activity could not be identified. Future studies should determine the diel stages of *Leucocytozoon* life cycle, by using blood smears to visually identify different developmental stages of parasites. This would help to further elucidate host-parasite interactions between *Leucocytozoon* parasites and avian circadian physiology.

An additional statistical analysis, running models for gene transcripts that were expected to shift in the same direction together (e.g. positive clock elements in the same pathway *CLOCK* and *BMAL1*), may provide further insight into effects of *Leucocytozoon* infection on gene pathways. In these models, gene transcripts should be treated as fixed effects, and cosine and sine curves fitted across all genes.

# Chapter Seven: General Discussion

Despite the growing evidence that robust circadian rhythms are important for physiology, studies of biological rhythms of animals in their natural environments remain rare. This thesis aimed to put studies of rhythms into an ecological context. Observational and experimental approaches were used to investigate daily rhythms in a wild bird, the great tit, at three levels: behaviour, gene transcripts, and life histories.

In this thesis, individual differences in behavioural timing were quantified using incubation behaviour. These differences in timing were then linked to fitness traits and put into the context of living in the evolutionarily novel city environment. Furthermore, impacts of a key feature of the city environment, artificial light at night, were investigated in the context of biological rhythms and condition of nestling great tits. Effects of infection with a widespread parasitic disease, avian malaria, on the immune system and condition of nestlings in city and forest habitats were then investigated. Finally, interactions of host clocks and avian malaria, a parasite known to be explicitly linked to biological rhythms, were explored in wild nestlings.

Altogether, this thesis provided new insights into how an animals' environment shapes clocks in the wild, with advances for both the fields of chronobiology and avian ecology research.

## 7.1 Advancements in avian ecology

This thesis provided further understanding of how biological rhythms of wild great tits are shaped by the environment. Previous literature has shown rhythms in city birds to differ from their forest counterparts in two ways: timing of daily activity (Kempenaers et al., 2010), and period length of endogenous clocks (Dominoni et al., 2013). In Chapter Three of this thesis, individual incubation rhythms were quantified for city and forest birds. There were clear differences in the onset and offset of daily activity between incubating city and forest birds, where city birds arose earlier and retired later than forest conspecifics.

Differences in behavioural timing between city and forest birds may have implications for fitness, given that important aspects of the environment, such as the availability of food, are time-dependent (Helm et al., 2017). As food can be scarce in the city habitat (Pollock et al., 2017), or of poorer quality (Isaksson & Andersson, 2007), the pressure on city birds

to rise earlier may be even greater than in the forest. This may create a trade-off for nestling parents, where the benefits of rising earlier to meet food demand for the energetic costs of incubation (Visser & Lessels, 2001), or nestling provisioning (Naef-Daenzer et al., 2000), will be balanced with the costs of sleep deprivation.

In addition, city sites used in Chapter Three had high levels of ALAN compared to forest sites, where mean light levels at city nest boxes were 55 lux, and forest nest boxes 0.44 lux. In a nest box study on great tits, ALAN increased begging behaviour of nestlings during the night (Raap et al., 2016c). Any reductions in sleep from rising earlier, for foraging opportunities, and through sleep disruption from light at night or begging offspring (Raap et al., 2016c) may mean that city birds have reduced energy (Laposky et al., 2008) to provide for nestlings. Given that city nestlings often weigh less than their forest conspecifics (Pollock et al., 2017), any reductions in provisioning by parents might have negative implications for chick survival (Both et al., 1999).

Site-level differences may have also played a role in the observed differences in activity timing between forest and city birds. For example, ambient temperatures are usually higher in city areas than surrounding areas, due to urban heat-island effects (Oke, 1982; Zhang et al., 2010). Warmer ambient temperatures influence the phenology of vegetation and insect food that breeding birds rely on (Deviche & Davies, 2014), and consequently, warming ambient temperatures influence great tits to breed earlier (Schaper et al., 2017). Great tit individuals that have earlier seasonal breeding have been shown to also have an earlier chronotype (Graham et al., 2017), which may help to explain earlier rising of city birds in this thesis. Intraspecific competition for food resources may also be higher in the city, where there are higher densities of bird populations (Partecke, 2014), which may increase pressure for city birds to rise earlier and be competitive.

Many studies have already shown the detrimental effects of ALAN on the condition of wild birds (Kernbach et al., 2018; Raap et al., 2016c). However, this thesis provided further evidence for negative effects of ALAN on wild bird fitness, and also novel insight into how ALAN may impact on mechanisms of the avian circadian clock in wild birds, by suppression of the core clock gene *REVERBA* (Chapter Four). This thesis also provided further evidence that ALAN exposure affects the condition of birds, given that nestlings exposed to low-level light at night treatment in Chapter Four were lighter than those under dark night. Experimental exposure to ALAN did not affect fledging success, however, small differences in nestling weight can have negative effects on survival and recruitment to the general population post-fledging (Both et al., 1999). From the findings of this thesis,

it is proposed that wild birds may be affected by ALAN in two ways – by directly impacting on circadian clock mechanisms of nestlings (Chapter Four), or indirectly on the behavioural rhythms of parents (Chapter Three).

Biological rhythms play an important role in regulation of the avian immune system (Markowska et al., 2017; Scheiermann et al., 2013). If natural rhythms of birds are disrupted as a result of ALAN, birds living in a city environment under light pollution may be immunocompromised, and therefore more vulnerable to disease (Kernbach et al., 2018). However, in Chapter Five, prevalence of *Leucocytozoon* parasites was significantly lower at city sites than at forest sites. Immune responses of avian hosts to parasites may be mediated by hormones such as corticosterone, the dominant stress steroid in birds (Romero, 2004). In some bird species, levels of corticosterone are elevated under urban conditions (Martin & Boruta, 2014). In a study on red-winged blackbirds (*Agelaius phoeniceus*), a higher endogenous concentration of circulating glucocorticoids was associated with reduced costs of infection with *Plasmodium* and *Haemoproteus* parasites, which indicated higher levels of tolerance (Schoenle et al., 2018). The observed lower prevalence of *Leucocytozoon* at city sites may have been due to increased tolerance in urban great tits. Future studies might explore how stress hormones such as corticosterone affects disease transmission and tolerance of birds in urban environments.

Additionally, the lower rate of infection with *Leucocytozoon* at city sites may be due to a lower abundance of insect vectors. *Leucocytozoon* vectors were not quantified in this study, yet abundance of another vector, the biting midge (*Culicoides*), was higher at forest sites than at city sites in previous years (Heather Ferguson, personal communication). Introductions of avian malaria parasites have proved damaging to naïve ecosystems (Atkinson et al., 1995). If avian malaria vectors continue to move into urban areas through landscape alterations or climate change, city birds may have a reduced ability to cope with acute infections (Dadam et al., 2019; Jiménez-Peñuela et al., 2019).

In this thesis, *Leucocytozoon* infection did not have a significant effect on nestling condition (Chapter Five). However, infections with *Leucocytozoon* significantly suppressed transcript levels of many immune genes in Chapters Five and Six. This may have been due to *Leucocytozoon* parasites infecting thrombocytes that are involved in production of immune responses to pathogens (Zhao et al., 2015). A suppressed immune system as a result of infection with *Leucocytozoon* may leave nestlings susceptible to further infections and also may have consequences for life histories (Hamilton and Zuk, 1982).

## 7.2 Advances for field chronobiology

This thesis provided new insights for chronobiology for quantifying rhythms of individual animals in their natural environments. In Chapter Three, individual chronotype was quantified for adult female birds from timing of repeatable behavioural activities during incubation. Patterns of incubation activity had been measured before in wild birds (Bulla et al., 2016; Graham et al., 2017), but chronotype had previously not been compared between city and forest habitats. Furthermore, uniquely in this study, individual chronotype was linked to fitness traits, although the effects of chronotype were non-significant. The results of this study add to the current debate in chronobiology that differences in individual timing are under selection (Helm and Womack, 2018; Kempenaers et al., 2010; Poesel et al., 2006).

In addition to behavioural rhythms, another main aim of this thesis was to quantify temporal changes in circadian clock gene transcripts as a proxy for internal clock mechanisms. Previously, this had not been done for a wild animal. In Chapter Four, time of day differences in transcript levels in great tit nestlings were quantified for many of the clock and immune gene targets studied. This thesis provided initial tentative evidence that it is possible to quantify biological rhythms both at the behavioural and transcript levels, which paves the way for future explorations of circadian rhythms of birds and other wildlife in chronobiology studies.

## 7.3 Challenges of measuring biological rhythms in wild birds

### 7.3.1 Measuring transcript levels from RNA

A main aim of this thesis was to quantify individual rhythms at the transcript level in a wild animal. To do this, a platform for gene expression analysis, the QuantiGene® Plex assay, was used to measure transcript levels of avian clock and immune genes from whole blood samples. The advantage of using the QuantiGene® Plex assay for this study was the ability to simultaneously measure transcript levels of all desired gene targets from one small blood sample. This reduced the burden of taking multiple blood samples from nestlings, and the analytic burden of a large series of qPCRs.

This assay has previously been used for samples collected from many species from fish (Mills and Gallagher, 2016), and mice (Song et al., 2015) to insects (Tan et al., 2016). However, avian samples had so far been limited to tissue from domestic chickens

(Gunawardana et al., 2019; Xue et al., 2013). Consequently, the assay had to be optimised for use on passerine blood samples for this thesis. Initial runs of the assay on great tit samples produced data with low fluorescence signal. Therefore, steps were taken to optimise the assay efficiency, such as increasing the time taken for initial digestion of the samples, or diluting samples to increase likelihood of probe binding to target RNAs. Following optimisation, there was marked improvement on the level of fluorescence signal from previous runs, however signal remained low overall for many of the gene targets.

For qPCR, the current standard method of quantifying gene transcript levels, it is often the case that lowly expressed genes show more variable results. In a similar way, the low fluorescence signal resulting from QuantiGene® Plex assay may have contributed to the noisiness of gene expression data in this thesis, and the lack of rhythmicity observed in circadian clock gene transcripts in Chapter Six aside from *REVERBA*.

In this thesis, the QuantiGene® Plex assay facilitated quantification of circadian transcript levels in a wild animal, for the first time. However, despite the optimisation steps taken to improve signal, fluorescence remained low. Future studies might further optimise the assay protocols for use of the QuantiGene® Plex on passerine blood samples.

### 7.3.2 Sampling distributions

This thesis provided support for taking samples across a 24 hr day during ecological studies of clocks. Inclusion of night time samples in Chapter Six showed effects of *Leucocytozoon* infection on several genes studied (*IGF1, PRKCA, NKRF, IL1, IL6, LY86, GATA3, TLR4*) not seen during day time sampling in Chapter Five, and therefore differences in results between chapters may be explained by temporal fluctuations of these genes across a day. This thesis highlighted the challenge for measuring temporal differences in gene transcripts, as limiting sampling to just a few timepoints resulted in a snapshot of genes, without knowing when the daily peak occurred.

However, in Chapter Four, transcript levels of many genes (*IGF1, TLR4, IL1, IL6, PRKCA, GATA3, IKBA, BMAL1, CLOCK, CK1E, AANAT*) did show time of day differences in transcript levels when comparing noon and midnight. These same genes did not show up as rhythmic during analysis of the full time profile in Chapter Six. As samples for Chapter Four were collected during the 2017 field season, and in Chapter Six were collected across two seasons (2016 and 2017), it may be that differences in years and distribution explains these discrepancies. Furthermore, in Chapter Six, sample size for several timepoints across

the time profile were often limited in number to <5 samples. An increase in samples for each of these timepoints would have improved the power of analysis, and therefore increased likelihood of detection of rhythmicity in all gene targets.

### 7.3.3 Rhythmicity

In Chapter Six, it was expected that temporal expression of gene transcripts of the core circadian clock (e.g. *BMAL1, CLOCK*) would be rhythmic. Unexpectedly, no genes out of those studied appeared to be rhythmic. This may be due to the issues of noisy transcript data from QuantiGene® Plex discussed earlier. However, another potential reason behind the lack of observed rhythmicity in circadian clock transcripts is differences in expression between blood and tissues. In this thesis, whole blood samples were used in place of tissue, to minimise impact on nestling welfare. Circadian transcripts have previously been detected in human blood (Archer et al., 2014; O'Neill and Reddy, 2011). In birds, rhythmic expression of circadian transcripts have been found in tissue samples such as ovaries (Laine et al., 2019), brain, liver, heart (Karaganis et al., 2009), spleen (Naidu et al., 2010) and retina (Bailey et al., 2002). Studies using bird blood for analyses of rhythmic gene transcripts are rarer, although one study using blood leucocytes of chickens observed day night differences in levels of immune genes *IL-6* and *IL-18* (Turkowska et al., 2013). Furthermore, great tit whole blood samples have been previously used for transcriptomics (Videvall et al., 2015; Watson et al., 2017). However, the possibility remains that circadian transcripts are not expressed at a detectable level in avian blood, or else are lowly expressed compared to other tissues. Wild studies ought to explore other possibilities of non-lethal sampling for quantifying rhythms, such as the use of fibroblasts (Gaspar and Brown, 2015).

Altogether, this thesis provided new insights into how molecular tools can be used to quantify rhythms in wild birds, and pioneered application of a new method for measuring circadian gene transcripts from avian blood.

## 7.4 Suggestions for future directions

### 7.4.1 Further directions

This thesis showed evidence for disruption of natural rhythms as a consequence of living in the city environment in Chapter Three. Also, in Chapter Four, nestlings under ALAN

treatment weighed less than those not exposed to ALAN. Previous nest box studies on the effects of ALAN exposure on great tits have shown a reduction in sleep behaviours (Raap et al., 2016c), possibly as a result of circadian disruption. To investigate whether this disruption is contributing to reduced sleep, future studies ought to test for markers of sleep deprivation in city birds or birds experimentally exposed to ALAN.

As discussed in Chapter Four, the intensity of light used in this study (1 lux) may not have been sufficiently bright to disrupt rhythmic transcript levels of genes. Avian responses to ALAN are light-intensity dependent for behaviour (de Jong et al., 2016) reproductive biology (Dominoni et al., 2018; Zhang et al., 2019) and the endocrine system (Ouyang et al., 2018), and therefore it is likely that transcript level responses are also dependent on the intensity of light. Future studies that investigate effects of ALAN on avian immune and circadian clock systems should incorporate varying intensities of light treatment (as in de Jong et al., (2016)), to elucidate whether transcript level responses are light-intensity dependent.

In this thesis, environmental features such as ambient noise and light were not quantified for study sites. Previous ecological studies have shown ambient noise to have impact on rhythms of activity (Fuller et al., 2007; Gil et al., 2015), and others suggest that light has more of an impact than noise (Dominoni et al., 2014). Future studies should quantify environmental features and include these in analyses, to untangle effects of light and noise on rhythms and fitness.

A limiting factor that should also be considered is the number of study sites (two city sites and two forest sites) used in this thesis. Given that the nest box occupancy rate of great tits at all study sites was low, the decision was made to compile data from both urban sites within Glasgow and treat these as “city” and the two forest sites within Loch Lomond National Park as “forest”. This increased the sample size for forest and city comparisons throughout the thesis but may have also introduced issues with pseudo replication. For example, it may be that nestlings at Kelvingrove Park react to light at night exposure in a similar way to each other, and differently from birds in the other city site, Garscube Estate. Kelvingrove Park therefore may be unrepresentative of an “urban site”. Future studies might consider including more city and forest study sites, and treating these as separate sites within analyses, to reduce possibility of pseudo replication. Additionally, for each site, the degree to which the habitat is urbanised should be quantified, to allow for comparisons between city sites with other avian urban ecology studies.

Furthermore, a focus of this thesis was on nestlings, an important life history stage. From Chapter Four, there was some evidence that ALAN exposure had a cumulative effect on the weight of nestlings. A suggestion for future studies exposing nestlings to ALAN is to re-capture ALAN treatment birds as adults, to see if later life history stages are affected. A similar re-capture study could be carried out on nestlings that were found to be infected with *Leucocytozoon* (Chapters Five and Six), to investigate whether adult birds were able to clear infections, or to explore effects of chronic malaria infection on fitness as in Knowles, Palinauskas and Sheldon, (2010).

In Chapter Six, temporal expression of immune and clock gene transcripts were quantified in nestlings. However, this study did not take into account the temporal activities of malaria parasites themselves. In future studies, the time profile of infection intensity with *Leucocytozoon* parasites ought to be quantified using blood smears, to better understand life cycle of parasites before capturing their rhythmicity. This temporal profile can then be matched to that of clock gene transcripts of infected birds, to further investigate temporal host-parasite interactions.

### 7.4.2 Further analyses

Statistical analyses within this thesis may be improved by carrying out the following additional steps. Inclusion of coefficients of variance for the random effect of nest box in all statistical models would provide insight into between-nest effects of treatment. For example, analyses of nest box variance in Chapter Four may provide information on whether nest boxes (families) are all equally affected by ALAN. Assessing significance of this random effect of nest box within models would also inform how much within treatment variance is explained by individual differences in e.g. nestling weight.

Additionally, inclusion of forest plots (such as that in Videvall et al., 2015) would be beneficial in Chapters Four and Six. This would help to better visualise the size and direction of the effects of each variable in model (e.g. sex, brood size, hatch date) on clock and immune gene transcripts, and also the direction of their responses to ALAN treatment, or *Leucocytozoon* infection.

## 7.5 Conclusions

Ultimately, this thesis provided new insights into how biological rhythms of wild birds can be shaped by their environment, at behavioural and transcript levels, and how differences in individual timing may have consequences for life histories. This thesis provided evidence for disruptive effects of artificial light at night on elements of the circadian clock and condition of wild birds, and evidence for differences in timing of activity of birds living in the city habitat. In an increasingly urbanised world, it is important to understand the effects of human activity on wildlife, and to take steps to mitigate the negative impacts of artificial light. Although this thesis focused on one model species in ecology, the great tit, the findings and methods used in this thesis may have wider applications for future studies of wild clocks in other species.

# Appendix 1

## QuantiGene® Plex RNA Assay Probe information

Table 7.1. GenBank accession numbers and probe size (base pairs) for each gene target used in the QuantiGene® Plex RNA Assay. CE = Capture Extender, LE = Label Extender, BL = Blockers

| ***Bead#*** | ***Accession (GenBank)*** | ***Symbol*** | ***Probeset Region*** | ***Full Size*** | ***CEs*** | ***LEs*** | ***BLs*** | ***Total*** |
|---|---|---|---|---|---|---|---|---|
| 21 | XM_015645462 | AANAT | 180-577 | 1274 | 6 | 10 | 6 | 22 |
| 61 | XM_015630436 | BMAL1 | 674-1080 | 2608 | 6 | 10 | 3 | 19 |
| 36 | XM_015624072 | CLOCK | 2087-2747 | 8226 | 6 | 12 | 11 | 29 |
| 53 | XM_015628865 | CRY1 | 594-978 | 4505 | 6 | 10 | 2 | 18 |
| 46 | XM_015641820 | CSNK1A1 | 381-746 | 2994 | 6 | 10 | 1 | 17 |
| 30 | XM_015627700 | CSNK1E | 743-1125 | 2580 | 6 | 10 | 2 | 18 |
| 33 | XM_015629245 | GATA3 | 595-1010 | 2980 | 6 | 10 | 5 | 21 |
| 12 | XM_015649617 | HMBS | 396-778 | 1509 | 6 | 10 | 1 | 17 |
| 43 | XM_015628060 | IGF1 | 349-792 | 8910 | 6 | 10 | 4 | 20 |
| 14 | XM_015648784 | IL-1 | 1310-1722 | 2485 | 6 | 10 | 4 | 20 |
| 39 | XM_015651893 | IL6 | 310-751 | 9027 | 6 | 10 | 6 | 22 |
| 44 | XM_015618120 | LY86 | 422-829 | 1398 | 6 | 10 | 1 | 17 |
| 22 | XM_015634446 | NFE2L2 | 377-741 | 2961 | 6 | 10 | 0 | 16 |
| 18 | XM_015629571 | NFKBIA | 343-806 | 1779 | 6 | 12 | 4 | 22 |
| 47 | XM_015626243 | NKRF | 617-1008 | 3191 | 6 | 10 | 2 | 18 |
| 13 | XM_015651038 | NR1D1 | 293-675 | 1436 | 6 | 10 | 5 | 21 |
| 55 | XM_015637543 | PER2 | 2713-3069 | 6569 | 6 | 10 | 1 | 17 |
| 51 | XM_015645435 | PRKCA | 1587-2019 | 4135 | 6 | 10 | 4 | 20 |
| 34 | XM_015651012 | RPL19 | 101-513 | 751 | 6 | 10 | 7 | 23 |
| 29 | XM_015619761 | SDHA | 565-1014 | 2307 | 6 | 10 | 4 | 20 |
| 15 | XM_015644481 | TLR4 | 1157-1608 | 6908 | 6 | 10 | 3 | 19 |

Table 7.2 Great tit target gene sequences for probes used in the QuantiGene® Plex RNA Assay

| Gene | Sequence |
|---|---|
| **IGF1** | ACACTGTGTCCTACATCCATTTCTTCTACCTTGGCCTGTGTTTGCTTACC<br>TT<br>AACCAGTTCTGTTGCTGCTGGCCCAGAAACACTCTGTGGTGCTGAGCT<br>GGTTGATGCTCTTCAGTTCGTGTGTGGAGACA<br>GAGGCTTTTACTTCAGTAAGCCCACGGGGTATGGATCCAGCAGTAGAC<br>GTTTACACCACAAGGGAATAGTGGATGAATGC<br>TGCTTCCAGAGTTGTGACCTGAGAAGGCTGGAGATGTATTGTGCTCCA<br>ATAAAGCCGCCTAAATCCGCACGGTCCGTACG<br>TGCCCAGCGCCACACCGACATGCCAAAAGCACAAAAGGAAGTGCATT<br>TGAAGAACACAAGTAGAGGGAACACAGGAAACA<br>GAAACTACAGAATGTAGGAACATGCCATCCACAAGAATGAAGAATGA<br>ATGTGCCATCTGCAGGATACTTTGCT |
| **LY86** | ATATTGCAACCCACACCTTTAACATCAGAGCTGCAATGGTCCTAAGAC<br>ACAGCATCAAG<br>GAACTCTATGTCAAGCTGGACATGATCATAAATGGAAAGACTGTCTTA<br>ACCTACTCAGAGACGCTTTGTGGGCCGGGTCA<br>TGATAAGCTTATTTTCTGTGGAATGAAGAAAGGAGAACATCTCTATTA<br>TGAGGGACCAGTCACACTGGGAATCAAAGAAA<br>TCCCTCAGGGAGATTACACACTTTCAGCAAAGCTGACTAATCAAGATC<br>ACGTCACTGTCGCTTGTGCCGATTTTACTGTG<br>AAAAATTATTTAGACTATTATTAGCAACAAACCAATTTCATGAAAGCT<br>ACAGACTACCAAAACTATTCAAGTCAAGTAGG<br>CTGCTTGCATGCTACAGTGATTCTGAAGA |
| **RPL19** | TGAGTATGCTCCGGCTCCAGAAGAGGCTGGCCTCCAGCGTCCTGCGCT<br>GCGGCAAGAAGA<br>AGGTGTGGCTGGATCCCAACGAGACCAACGAGATCGCCAACGCCAAC<br>TCCCGGCAGCAGATCCGGAAGCTGATCAAGGAT<br>GGGCTGATCATCCGCAAGCCCGTGACCGTGCACTCCCGCGCCCGCTGC<br>AGGAAGAACACGCTGGCCCGGCGGAAGGGCCG<br>GCACATGGGCATCGGTAAGAGGAAGGGCACGGCCAACGCCCGCATGC<br>CCGAGAAGGTCACCTGGATGCGGCGGATGCGGA<br>TCCTGCGGCGGCTGCTCCGGCGCTACCGCGAGTCCAAGAAGATCGACC<br>GCCACATGTGAGCCCTGTGCTCGACCCAGAGC<br>CTGGAGGATCAGGGACAGGGGACAGGGGACAGG |
| **AANAT** | CTGTCCCCTGCACCTGGATGAGATCCGCCACTTTCTGAGCCTGTGCCCG<br>GAGCTGTCCCTC<br>GGCTGGTTTGAGGAAGGGCGGCTGGTGGCATTCATCATCGGCTCCCTC<br>TGGGACCAGGAGAGGCTCAGCCAGGCAGCACT<br>GACCCTGCACAAGCCACAGGGCTCAGCTGTGCACATCCACGTGCTGGC<br>CGTGCACCGCACCGTCCGCCAGCAGGGCAAGG<br>GCTCCATCCTGATGTGGCGCTACCTGCAGCACCTGCGCTGCCTGCCCTT<br>CGCCCGCCGCGCCCTGCTCATGTGCGAGCAC<br>TTCCTCGTGCCCTTCTACCAGAAGTGCGGCTTCGAGGCGCTGGGGCCCT<br>GCGAGGTGACCGTGAGGGACCTGGCCTTCGT<br>GGAGATGCAGCACCCCG |
| **IL1** | TGGCATTCGTCCCTGATTTGGACACACTGGAGAGCGGCAGCTTGAATG<br>AGG<br>AGACACTGTATGGCCCCAACTGTCTCTGCCTGCAGAAGAAGCCCCGAC<br>TGGACTTGGAGGTGACATCACCTGAGGTGGGC<br>ATCCAAGTGACAGTGACAAAAGGACACCTTTCCAGGACCTTCCGCCAG<br>GCTGCCATCCTGGTGGTTGCTGTGACCAAGCT |

| | |
|---|---|
| | CCTAAAGCAGCCATCGCACAAGGACTTTGCTGATAGTGACCTGGGCAG<br>CTTCTTGGATGATATTTTTGAGCCCATCTCCT<br>TCCAGTGCATCAAGGGCAGTTATACCAGGCCACCCGTTTTCCGCTACA<br>CTCGCTCCCAGTCCTTTGACATCCTGGACATT<br>GACCACAAGTGCTTCGTACTGGAGTCACCCACCCAGCTGGTG |
| **IL6** | CCCAACGCAGA<br>CTCCTCGGGGGACGCCGAGCTGGAGGAGGTGGCGAAGCGGCGGGCGG<br>CGCTGCCCGGCAGCCTGGAGCAGGCGCGGCTGC<br>TGCACGCCCGGGCGGCAGAGCTGCGGGACGAGATGTGCGAGAAGTTT<br>ACCGTCTGCCAGAACAGCATGGAAATGCTCCTC<br>CACAACAACCTCAACCTCCCCAAGGTGACGGAGGAAGATGGGTGTCTG<br>CTCGCCGGCTTTAATGAGGATAAATGCTTGAG<br>AAAAATCTCCAGTGGGCTTTATACATTTCAGATATACCTCAAATACAT<br>ACAAGAAACTTTTATTAGTGAAAACCAAAATG<br>TTGAATCGCTATCCCATACTACAGGGCACCTGGCACGTACCCTAAGAG<br>AGATGCTGATCAATCCCAAAGAAGTGATCATC<br>CCCGATGCAGCTACCCAGGAATCCCTCCACA |
| **NFKBIA** | CTGACGGAGGACGGGGACACTTTTCTGCACTTGGCCATTATTCACGAA<br>GAAAAATCCC<br>TGAGCCTGGAGGTGATCCAGCAGGCAGCCGGTGACCGGGCTTTCCTGA<br>ACTTCCAGAACAACCTCAGCCAGACTCCTCTT<br>CACCTGGCAGTGATAACTGATCAGCCTGAAATTGCTGAGCATCTTCTG<br>AAGGCTGGATGTGACCTGGAAATCAGGGACTT<br>CCGAGGAAACACCCCCCTGCACATTGCCTGTCAGCAGGGCTCCCTCAG<br>GAGCGTCAGCGTGCTCACACAGTATTGCCAGC<br>CACACCACCTTCTCACTGTCCTGCAGGCAGCCAACTACAACGGACATA<br>CATGTCTTCATTTAGCATCTATTCAAGGATAC<br>CTGGCTATTGTCGAGTACCTGCTGTCCTTGGGAGCAGATGTAAATGCA<br>CAGGAGCCATGCAATGGCAGAACAGCACTGCA<br>TTTGGC |
| **CSNK1A1** | AGCAGCAGCGGCTCCAAGGC<br>CGAGTTCATTGTCGGAGGCAAATACAAGCTGGTGCGGAAGATCGGGTC<br>GGGCTCCTTCGGGGACATCTATCTGGCGATCA<br>ACATCACCAACGGGGAGGAAGTTGCTGTGAAGTTGGAGTCTCAGAAG<br>GCTCGGCACCCCCAGCTGCTCTATGAAAGCAAA<br>CTGTACAAGATCCTGCAGGGAGGAGTTGGCATCCCACATATACGGTGG<br>TATGGTCAAGAAAAGGACTACAATGTTCTAGT<br>CATGGATCTTCTCGGGCCCAGCCTGGAAGACCTCTTCAACTTCTGTTCT<br>CGCAGGTTTACGATGAAAACAGTACTTATGC<br>TAGCAGACCAGATGATCAGTAGAATT |
| **HMBS** | CAACTTCTCTTCCTCCTGGCTTTACGATTGGCGCTGTCTGCAAGCATTC<br>TGCACTTGCATTATTTTGCAGAAGGGAAAACCCACT<br>TGATGCTGTTGTCTTTCATCCCAAAAACTGTGGAAAAACACTGAGCCT<br>CCTTCCTGAAAAGAGTGTGATTGGAACCAGTT<br>CACTTCGGAGAGCAGCTCAGCTCAAGAAGAAGTTCCCTCATTTAGAAT<br>TCAGGGATATTAGAGGAAACTTAAATACCCGT<br>TTAAAGAAGCTAGATGAGAAAGAAGACTTCAGCGCCATCATCCTAGCT<br>GCTGCTGGGCTGAAGAGAATGGGCTGGGAAAA<br>TCGCATTGGCCAGCTCCTAGGCCCTGAAGATTGTCTGTATGCTGTTGGA<br>CAGGGTGCC |
| **CSNK1E** | CCACCAGCACATCCCTTATCGGGAAAACAAGAATCTGACTGGCACAGC<br>CCGTTATGCCTCTATCAACACCCACCTGGGAA<br>TTGAACAAAGTCGCCGTGATGACCTGGAGAGCCTGGGTTATGTGCTCA<br>TGTATTTCAACCTGGGCTCGCTGCCCTGGCAG<br>GGCCTCAAGGCTGCCACCAAGCGCCAAAAGTATGAGAGGATCAGCGA<br>GAAAAAGATGTCAACGCCCATCGAGGTGCTCTG<br>CAAAGGGTACCCTTCTGAGTTCTCAACATACCTCAACTTCTGCCGTTCA |

| | |
|---|---|
| | CTGAGGTTTGATGATAAACCTGACTACTCGT<br>ACCTGCGGCAACTCTTCCGCAACCTCTTCCACCGCCAAGGCTTCTCCTA<br>CGACTACGTCTTTGATTGGAACATGCTTAAA<br>TTTGGAGCAGCCCGG |
| **GATA3** | CTGGAACCTCAGCCCGTTCTCCAAGACCTCCATCCATCACAGCTCACC<br>GGGACCCCTCTCCGTCTACCCACCCGCCTCCT<br>CTTCCACTTTATCCGCCGGCCACTCCAGCCCGCACCTTTTCACCTTCCC<br>GCCGACCCCTCCTAAAGATGTGTCCCCGGAT<br>CCGTCCATCTCCACCCCCGGCTCCACCGGCTCCACCCGGCAGGACGAG<br>AAGGAATGCATCAAATACCAGGTGTCCCTGGC<br>CGATACCATGAAGCTGGAGTCCTCTCACTCTCGGAGCAGCATGGCCTC<br>TTTAGGAGGAGCCACCTCCTCCGCTCATCACC<br>CCATCACTACCTACCCTCCGTATGTCCCAGAATATGGCTCTGGACTTTT<br>TCCCCCCAGTAGCCTCTTAGGAGGATCGCCA<br>ACCGGGTTTGGGTGTAAATCGCGACCGAAAGCACGGTCAAGCACAGA<br>AGGCA |
| **NR1D1** | TCCAGGATTCCCGCGCCTATGGTGGAGCCACGCTGGCCCCCCATGAAG<br>ACGGTTCCCCTTCCTCCTCCTCCTCATCCTCC<br>TCCTCCACCTCCTCTTCCTCGTACGGCTCCTCGGTGAATTTCCCCGGGG<br>TTCAGCCGGTCCCCGCGGACGAGCGGCGCCG<br>CAGCTCGCCAAGCAAAGCCGGCAGCACCGTCACGAAGCTCAACGGCA<br>TGGTGCTGCTCTGCAAGGTCTGCGGGGACGTCG<br>CCTCCGGCTTCCACTACGGCGTCCACGCCTGCGAGGGCTGCAAGGGCT<br>TTTTCCGCCGCAGCATCCAGCAGAACATCCAG<br>TACAAGAAGTGCCTCAAGAACGAGAACTGCTCTATCGTCCGCATCAAC<br>CGCAACCGCTGCCAGCAGTGCCGCTTCAAGAA<br>GTGCCTGCTGGTCGGCATGTCCCGTGATGCCGTGCGCT |
| **CRY1** | CTTGAAGAGCTGGGTTTTGACACAGATGGTCTGCCTTCTGCAGTATGG<br>CCAGGGGGAGAAACTGAAGCCCTCACACGATT<br>AGAAAGACACTTAGAGCGAAAGGCTTGGGTGGCAAACTTTGAAAGAC<br>CAAGAATGAATGCAAATTCCCTCCTGGCAAGCC<br>CTACGGGGCTTAGTCCCTACCTCCGCTTTGGCTGTTTGTCCTGTCGGCT<br>CTTTTACTTCAAGTTAACGGATCTGTACAAA<br>AAGGTAAAAAAGAACAGCTCCCCTCCCCTCTCCCTCTATGGCCAGCTG<br>TTATGGCGTGAATTTTTCTACACAGCGGCGAC<br>TAACAATCCACGGTTTGATAAAATGGAGGGGAATCCTATCTGTGTTCA<br>AATTCCATGGGATAAGAATCCTGAGGCTTTGG<br>CCAAATGGGCAGAAGGCA |
| **NFE2L2** | GCTGGAGTTAGACGAAGAGACAGGTGAATTCATTCCTGTGCAGCCAGC<br>TCAGTGCATTCAGTCAAAAGATACTGAGCCAC<br>CCGCTGTTTTCTCACAGACCACAGAGCCTTCAAAACCAGAAGCAGAGG<br>CCTTGTCCTTTGATGACTGCATGCAGCTCTTA<br>GCAGAAGCATTCCCTTTTCTAGATGAGCATGAGGCTTCTTCAGCTGCAT<br>TTCAGTCACTGGCTCCTGCTCAGGTCAATAG<br>CAACCCAGCCTTTGTTTCCTCTGATCAAACTCAGCCACCTGAATCCTCT<br>GATCTAGTACAACCCACTGATGCAGAGAATA<br>TGCAGAACATAGAACAAGTTTGGGAAGAATTACTGTCCCTTCCAGAGT<br>TACAGTGCCTGAACATCGAAAACGATAACCTG<br>GCTGAGGTGAGCACAATCACG |
| **NKRF** | TTTGCAGGCCCAGCAGCAGCAGCC<br>AAGAAGGCTGTGAATAATGTGGACTCTACCACAAGCAGCGCCTTGCAG<br>GTTTCTGTTCCCCCTTCAACTGCCCCATCTGC<br>ATCAGAGACTTTCCCTCCGTCAGCAATAATGCTGAAGCAGAGCTTTAT<br>GGAGAAACTATCCGCAGCAGTCTGGAAAAATC<br>TTACTAATCCAGATGCCAACACTGGGACAGATAAAATTAACTTCACGT<br>ATCTTTTGACACGTTCAATTCAGGCATGCAAG<br>ACAAACCCTGAATATATTTATGTTCCTCTGAAAGAGATTGCCCCTGCTG |

| | |
|---|---|
| | ACCTCCCCAAGAGCAAGAAGCTCTTGACAGA<br>TGGCTTTGCTTGTGAGGTGCGATGTCAGAATGTCTACCTGGCCACCGG<br>CTACGCCGGCAGCAAGAACGGATCCCGGGATC |
| **ARNTL** | TCATTCTCAGGGCAGCAGATGGATTTCTTTTTGTTGTGGGCTGTGACAG<br>AGGAAAGATACTGTTTGTTTCAGAATCTGTC<br>TTCAAGATCCTCAACTACAGTCAGAATGATTTGATTGGTCAAAGTTTAT<br>TTGATTATCTCCATCCTAAAGACATTGCCAA<br>AGTGAAGGAGCAGCTCTCTTCTTCTGACACCGCGCCACGAGAAAGACT<br>CATAGACGCAAAAACCGGACTCCCGGTCAAGA<br>CGGACATCACGCCCGGGCCCTCGCGGCTCTGCTCCGGAGCGCGCCGCT<br>CCTTCTTCTGCAGGATGAAGTGCAACAGGCCC<br>TCTGTCAAAGTAGAAGACAAGGATTTCCCTTCAACCTGCTCAAAGAAG<br>AAAGCAGACCGCAAGAGCTTTTGCACGATCCA<br>CAGCACGGGCTACTTGAAAAGCTGGCCTCCCACCAAGATGGGGCTGGA<br>CGAGGATAACGAGCCAGATAACGAGGGCTGCA |
| **SDHA** | GTGGACAGAGTCTTCAGTTTGGAAAAGGAGGACAGGCCCACAGATGC<br>TGTTGTGTTGCAGACAGGACTGGACATTC<br>ACTCTTACATACTTTGTATGGCAGGTCTCTACGATATGATACAAGCTAC<br>TTTGTTGAATATTTTGCCTTGGACCTACTTA<br>TGGAGAATGGAGAATGCCGTGGAGTTATTGCCCTCTGCATTGAAGATG<br>GAACCATACATCGTTTTAGAGCAAAGAACACG<br>GTCATTGCCACTGGTGGGTATGGCCGCACTTACTTCAGCTGCACATCTG<br>CTCATACCAGTACTGGTGATGGCACGGCCAT<br>GGTCACACGAGCTGGGCTCCCTTGCCAGGACTTAGAATTTGTGCAGTT<br>TCACCCTACAGGTATCTATGGGGCTGGCTGCC<br>TTATCACAGAAGGATGTCGTGGAGAGGGAGGTATTCTGATCAACAGCC<br>AAGGTG |
| **PRKCA** | AACAACCAGGACCT<br>TCTGTGGCACTCCAGACTACATTGCACCAGAGATTATTGCTTACCAGCC<br>CTATGGGAAGTCGGTGGATTGGTGGGCATAT<br>GGTGTGCTGCTCTATGAGATGTTGGCTGGCCAGCCTCCATTTGATGGG<br>GAAGACGAAGATGAGCTGTTCCAGTCCATAAT<br>GGAGCATAATGTTTCCTACCCAAAATCACTGTCCAAAGAAGCTGTCTC<br>CATCTGCAAGGGGCTAATGACTAAACACCCTG<br>CAAAACGCCTTGGCTGTGGCCTGGAAGGTGAAAGAGACATCAGGGAA<br>CACGCTTTCTTCAGGAGAATTGACTGGGAAAAA<br>TTGGAGAACAGAGAGATTCAGCCACCTTTCAAACCTAAAGTGTGCGGC<br>AAAGGGGCTGAAAACTTCGATAAGTTCTTCAC<br>ACGAGGACAGCCGGTGTTG |
| **TLR4** | AGCTGAGAGTGCTCCGTATTACCAAGAACAAAAGACTCAAAAAC<br>TTCAGTCAGAATTTTGAGGGTCTCACTAACCTGGAGGTTATAGATTTGA<br>GTGAGAACAGACTCACCTTCAGCAGTTGCTG<br>CTCCCCTCAGTTTCAAAATTGTCCAAATTTGAAACACTTGAACTTAAGC<br>TTCAATTCTAATATCAGATTGACTGGAGATT<br>TCACTAATGTGAAGAATTTGTTATATTTGGACCTTCAGCACACAACTTT<br>ATTTGGTCCTGGCTCCTACCCTGTCTTTCTG<br>TCCCTTCAGAAACTGATTTACCTTGATATTTCCCATACCAAAACCGAAG<br>TTAAATCCCAGTGCACATTTTGTGGCTTGAA<br>CTCTTTGCAAGTGCTCAAGATGGCAGGAAACTCCTTTGAGGGCAATAA<br>GCTGGCAGGCAACTTCAAAAACCTAAGTCACC<br>TCCACACCTTGGATATTTCAAGCTGCAAATTAGAACATGTGGATCAAA<br>GTACTTTTGATGCCCTCTCTGAGCTAAAGGAG |
| **CLOCK** | AACATGCAAGGTCAAGTTGTTCAGACTAATCAGACTCAAAGTGGGATG<br>AACACAGGCCATATAAATACTCCACA<br>CATGATACAGCAGCAGCAGCCTTTGCAGAGTACTGCAACACAGCATAA<br>TCAACAAAATGTGCTTAGTGGACACAATCAGC<br>AGTCTTCTCTTGCCAGCCAGTCACAGAACACAGTCTCGGCACCTTTGTA |

| | |
|---|---|
| | CAACACTATGGTGATTTCTCAGCCAACAGCA<br>GGAAATGTGGTGCAGGTTCCCTCTAGCTTATCACAGAACAACAACCAG<br>AATGCTGCTGCAGTAACCACTTTTACACAGGA<br>CAGACAAATCAGATTTTCTCAAGGTCAGCAACTTGTAACAAAACTTGT<br>CACGGCCCCAGTAGCATGTGGAGCGGTAATGG<br>TACCAAGTACTATGTTTATGGGACAGGTGGTGACAGCTTATCCCACTTT<br>TGCTGCCCAACAGCAGCAGACACAGACTTTG<br>TCAATAACACAACAACAGCAACAGCAGCAGCAGCAGCAGCAACAACA<br>GCAAAGTCAGCAGGACCATCAGCAGCAGCTCAC<br>CACAGTACAACAGCCAGCTCAGTCACAGCTGACCCAGCACCCTCAGCA<br>GTTCCTACAGACATCCAGGTTACTTCATGGAA<br>ATCAGTCGGCTCAGCTTATTCTCTCTG |
| **PER2** | TTACTACT<br>TACAAGATAAACCAAAAGGACGGCCTGCTGAACGTGGTGGCCGTGGA<br>CAAAGGAATGCCGCTTCTGGAATGGATCAGCCT<br>TGGAAGAAAAGTGGAAAGAACAGGAAATCAAAGCGCATTAAACCACA<br>GGAATCTTCAGACAGCACAACTTCTGGAGCTAA<br>ATTCCCCCATCGGTTCCCCCTGCAAGGTTTAAATGCCACAGCCTGGTCA<br>CCATCAGAGACTTCCCAAGCCAGCTACTCAG<br>CAGTGTCCTTCCCCACTGTCATGCCTGCATATTCCCTTCCTGTTTTTCCA<br>GCAGCAGGGACCGTGCCGCCGGCTCCTGAA<br>ACCTCACTCTCTGGTTTTAATCACTTGCC |

# List of References

Affymetrix, 2015. Application Guide QuantiGene Plex Assay.

Akira, S., Takeda, K., 2004. Toll-like receptor signalling. *Nat. Rev. Immunol*. 4, 499–511. https://doi.org/10.1038/nri1391

Alaasam, V.J., Duncan, R., Casagrande, S., Davies, S., Sidher, A., Seymoure, B., Shen, Y., Zhang, Y., Ouyang, J.Q., 2018. Light at night disrupts nocturnal rest and elevates glucocorticoids at cool color temperatures. *J. Exp. Zool. Part A Ecol. Integr. Physiol*. 329, 465–472. https://doi.org/10.1002/jez.2168

Albalawi, B., 2019. Urbanisation and avian fitness: an investigation of avian malaria prevalence and feather corticosterone level of blue tit *Cyanistes caeruleus* nestlings across two breeding seasons. PhD Thesis [Submitted 1 September 2019], University of Glasgow.

Alvarez, M., Schrey, A.W., Richards, C.L., 2015. Ten years of transcriptomics in wild populations: What have we learned about their ecology and evolution? *Mol. Ecol.* 24, 710–725. https://doi.org/10.1111/mec.13055

Amin, B., Slabbekoorn, H., Schaaf, M., Tudorache, C., 2016. "Early birds" take it easy: Diurnal timing is correlated with overall level in activity of zebrafish larvae. *Behaviour* 153, 1745–1762. https://doi.org/10.1163/1568539X-00003376

Anderson, R.M., May, R.M., 1982. Coevolution of Hosts and Parasites. *Parasitology* 85, 411–426. https://doi.org/10.1017/S0031182000055360

Ando, H., Takamura, T., Matsuzawa-Nagata, N., Shima, K.R., Eto, T., Misu, H., Shiramoto, M., Tsuru, T., Irie, S., Fujimura, A., Kaneko, S., 2009. Clock gene expression in peripheral leucocytes of patients with type 2 diabetes. *Diabetologia* 52, 329–335. https://doi.org/10.1007/s00125-008-1194-6

Ar, A. & Sidis, Y., 2002. Nest microclimate during incubation, in: Avian Incubation: Behaviour, Environment, and Evolution. Oxford University Press, New York, NY, pp. 143–160.

Archer, S.N., Laing, E.E., Möller-Levet, C.S., van der Veen, D.R., Bucca, G., Lazar, A.S., Santhi, N., Slak, A., Kabiljo, R., von Schantz, M., Smith, C.P., Dijk, D., 2014. Mistimed sleep disrupts circadian regulation of the human transcriptome. *Proc. Natl. Acad. Sci*. 111, E682–E691. https://doi.org/10.1073/pnas.1316335111

Ardia, D.R., Pérez, J.H., Clotfelter, E.D., 2010. Experimental cooling during incubation leads to reduced innate immunity and body condition in nestling tree swallows. *Proc. R. Soc. B Biol. Sci.* 277, 1881–1888. https://doi.org/10.1098/rspb.2009.2138

Aschoff, J., 1965. Circadian rhythms in man. *Science* 148:1427-1432.

Aschoff, J., Gerecke, U., Wever, R., 1967. Desynchronization of human circadian rhythms. *Jpn. J. Physiol*. 17, 450–457. https://doi.org/10.2170/jjphysiol.17.450

Asghar, M., Hasselquist, D., Bensch, S., 2011. Are chronic avian haemosporidian infections costly in wild birds? *J. Avian Biol*. 42, 530–537. https://doi.org/10.1111/j.1600-048X.2011.05281.x

Asghar, M., Hasselquist, D., Hansson, B., Zehtindjiev, P., Westerdahl, H., Bensch, S., Asghar, M., Hansson, B., Zehtindjiev, P., Hasselquist, D., 2015. Hidden costs of infection: Chronic malaria accelerates telomere degradation and senescence in wild birds. *Science* (80-. ). 347, 436–438. https://doi.org/10.1126/science.1261121

Aste, N., Cozzi, B., Stankov, B., Panzica, G., 2001. Sexual Differences and Effect of Photoperiod on Melatonin Receptor in Avian Brain. *Microsc. Res. Tech*. 55, 37–47.

Atema, E., Oers, K. van, Verhulst, S., 2013. GAPDH as a Control Gene to Estimate Genome Copy Number in Great Tits, with Cross-Amplification in Blue Tits. *Ardea* 101, 49–54. https://doi.org/10.5253/078.101.0107

Atkinson, C.T., Van Riper, C., 1991. Pathogenicity and epizootiology of avian haematozoa: *Plasmodium*, *Leucocytozoon*, and *Haemoproteus*. *Bird-parasite Interact*. 19–48.

Atkinson, C.T., Woods, K.L., Dusek, R.J., Sileo, L.S., Iko, W.M., 1995. Wildlife disease and conservation in Hawaii: Pathogenicity of avian malaria (*Plasmodium relictum*) in experimentally infected Iiwi (*Vestiaria coccinea*). *Parasitology* 111, S59–S69. https://doi.org/10.1017/S003118200007582X

Avery, M.I., Krebs, J.R., 1984. Temperature and foraging success of Great Tits *Parus major* hunting for spiders. *Ibis*. 126, 33–38. https://doi.org/10.1111/j.1474-919X.1984.tb03661.x

Bailey, M.J., Chong, N.W., Xiong, J., Cassone, V.M., 2002. Chickens' Cry2: molecular analysis of an avian cryptocrhome in retinal and pineal photoreceptors. *FEBS Lett*. 513, 161–174.

Bailey, M.J., Beremand, P.D., Hammer, R., Reidel, E., Thomas, T.L., Cassone, V.M., 2004. Transcriptional Profiling of Circadian Patterns of mRNA Expression in the Chick Retina. *J. Biol. Chem.* 279, 52247–52254. https://doi.org/10.1074/jbc.M405679200

Bailly, J., Scheifler, R., Belvalette, M., Garnier, S., Boissier, E., Clément-Demange, V.A., Géte, M., Leblond, M., Pasteur, B., Piget, Q., Sage, M., Faivre, B., 2016. Negative impact of urban habitat on immunity in the great tit *Parus major*. *Oecologia* 182, 1053–1062. https://doi.org/10.1007/s00442-016-3730-2

Barrett, R.K., Underwood, H., 1991. Retinally perceived light can entrain the pineal melatonin rhythm in Japanese quail. *Brain Res*. 563, 87–93. https://doi.org/10.1016/0006-8993(91)91518-6

Bates, D., Mächler, M., Bolker, B.M., Walker, S.C., 2015. Fitting linear mixed-effects models using lme4. *J. Stat. Softw*. 67. https://doi.org/10.18637/jss.v067.i01

Bednekoff, P.A., Houston, A.I., 1994. Avian daily foraging patterns: Effects of digestive constraints and variability. *Evol. Ecol.* 8, 36–52. https://doi.org/10.1007/BF01237664

Bedrosian, T.A., Fonken, L.K., Walton, J.C., Nelson, R.J., 2011. Chronic exposure to dim light at night suppresses immune responses in Siberian hamsters. *Biol. Lett*. 7, 468–471. https://doi.org/10.1098/rsbl.2010.1108

Bedrosian, T.A., Galan, A., Vaughn, C.A., Weil, Z.M., Nelson, R.J., 2013. Light at Night Alters Daily Patterns of Cortisol and Clock Proteins in Female Siberian Hamsters. *J. Neuroendocrinol*. 25, 590–596. https://doi.org/10.1111/jne.12036

Beier, J.C., 1998. Malaria Parasite Development in Mosquitoes. *Annu. Rev. Entomol.* 43, 519–543. https://doi.org/10.1146/annurev.ento.43.1.519

Bennett, G.F., Peirce, M., Earle, R., 1994. A checklist of the valid avian species of Babesia (Apicomplexa: Piroplasmorida), *Haemoproteus , Leucocytozoon* (Apicomplexa: Haemosporida), and *Hepatozoon* (Apicomplexa: Haemogregarinidae). *Syst. Parasitol.* 29, 61–73. https://doi.org/10.1080/00222930500340534

Bensch, S., Stjernman, M., Hasselquist, D., Orjan, O., Hannson, B., Westerdahl, H., Pinheiro, R.T., 2000. Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. *Proc. R. Soc. B Biol*. Sci. 267, 1583–1589. https://doi.org/10.1098/rspb.2000.1181

Bensch, S., Waldenström, J., Jonzén, N., Westerdahl, H., Hansson, B., Sejberg, D., Hasselquist, D., 2007. Temporal dynamics and diversity of avian malaria parasites in a single host species. *J. Anim. Ecol*. 76, 112–122. https://doi.org/10.1111/j.1365-2656.2006.01176.x

Bentley, G.E., Goldsmith, A.R., Dawson, A., Briggs, C., Pemberton, M., 1998. Decreased Light Intensity Alters the Perception of Day Length by Male European Starlings (*Sturnus vulgaris*). *J. Biol. Rhythms* 13, 148–158. https://doi.org/10.1177/074873098128999998

Bernard, M., Iuvone, P.M., Cassone, V.M., Roseboom, P.H., Coon, S.L., Klein, D.C., 2002. Avian Melatonin Synthesis: Photic and Circadian Regulation of Serotonin N-Acetyltransferase mRNA in the Chicken Pineal Gland and Retina. *J. Neurochem*. 68, 213–224. https://doi.org/10.1046/j.1471-4159.1997.68010213.x

Berntsen, H.H., Bech, C., 2016. Incubation temperature influences survival in a small passerine bird. *J. Avian Biol*. 47, 141–145. https://doi.org/10.1111/jav.00688

Bichet, C., Scheifler, R., Cœurdassier, M., Julliard, R., Sorci, G., Loiseau, C., 2013. Urbanization, Trace Metal Pollution, and Malaria Prevalence in the House Sparrow. *PLoS One* 8, 1–10. https://doi.org/10.1371/journal.pone.0053866

Binkley, S., Kluth, E., Menaker, M., 1971. Pineal function in sparrows: circadian rhythms and body temperature. *Science* 174, 311–4. https://doi.org/10.1126/science.174.4006.311

Bishop, A., Tate, P., Thorpe, M.V., 1938. The duration of *Plasmodium relictum* infection in canaries. *Parasitology* 30, 388–391. https://doi.org/10.1017/S0031182000025944

Both, C., Visser, M.E., Verboven, N., 1999. Density-dependent recruitment rates in great tits: The importance of being heavier. *Proc. R. Soc. B Biol. Sci*. 266, 465–469. https://doi.org/10.1098/rspb.1999.0660

Bradley, C.A., Altizer, S., 2007. Urbanization and the ecology of wildlife diseases. *Trends Ecol. Evol.* 22, 95–102. https://doi.org/10.1016/j.tree.2006.11.001

Brossy, J.J., 1992. Malaria in wild and captive jackass penguins spheniscus demersus along the southern african coast. *Ostrich* 63, 10–12. https://doi.org/10.1080/00306525.1992.9634174

Brown, S.A., Fleury-Olela, F., Nagoshi, E., Hauser, C., Juge, C., Meier, C.A., Chicheportiche, R., Dayer, J.M., Albrecht, U., Schibler, U., 2005. The period length of fibroblast circadian gene expression varies widely among human individuals. *PLoS Biol.* 3. https://doi.org/10.1371/journal.pbio.0030338

Bueno-Enciso, J., Barrientos, R., Sanz, J.J., 2017. Incubation Behaviour of Blue *Cyanistes caeruleus* and Great Tits *Parus major* in a Mediterranean Habitat . *Acta Ornithol*. 52, 21–34. https://doi.org/10.3161/00016454ao2017.52.1.003

Buhr, E.D., Takahashi, J.S., 2013. Molecular Components of the Mammalian Circadian Clock, in: Kramer, A., Merrow, M. (Eds.), Circadian Clocks. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 3–27. https://doi.org/10.1007/978-3-642-25950-0_1

Bulla, M., Valcu, M., Dokter, A.M., Dondua, A.G., Kosztolányi, A., Rutten, A., Helm, B., Sandercock, B.K., Casler, B., Ens, B.J., Spiegel, C.S., Hassell, C.J., Küpper, C., Minton, C., Burgas, D., Lank, D.B., David, C., Loktionov, E.Y., Nol, E., Kwon, E., Smith, F., Gates, H.R., Vitnerová, H., Prüter, H., James, A., Clair, J.J.H.S., Lamarre, J., Rausch, J., Reneerkens, J., Conklin, J.R., Burger, J., Bêty, J., Coleman, J.T., Figuerola, J., Hooijmeijer, J.C.E.W., Alves, J.A., Smith, J.A.M., Koivula, K., Gosbell, K., Exo, K., Niles, L., Koloski, L., Mckinnon, L., Praus, L., Giroux, M., Sládeček, M., Boldenow, M.L., Goldstein, M.I., Šálek, M., Senner, N., Rönkä, N., Lecomte, N., Gilg, O., Vincze, O., Johnson, O.W., Smith, P.A., Woodard, P.F., Pavel, S., Battley, P.F., Bentzen, R., Lanctot, R.B., Porter, R., Saalfeld, S.T., Freeman, S., Brown, S.C., Yezerinac, S.,

Székely, T., Montalvo, T., Piersma, T., Loverti, V., Pakanen, V., Tijsen, W., Kempenaers, B., 2016. Unexpected diversity in socially synchronized rhythms of shorebirds. *Nat. Lett.* 540, 1–22. https://doi.org/http://dx.doi.org/10.1101/084806

Cabannes, E., Khan, G., Aillet, F., Jarrett, R.F., Hay, R.T., 1999. Mutations in the IkBa gene in Hodgkin's disease suggest a tumour suppressor role for IκBα. *Oncogene* 18, 3063–3070. https://doi.org/10.1038/sj.onc.1202893

Calero-Riestra, M., Garcia, J.T., 2016. Sex-dependent differences in avian malaria prevalence and consequences of infections on nestling growth and adult condition in the Tawny pipit, *Anthus campestris. Malar. J.* 15, 178. https://doi.org/10.1186/s12936-016-1220-y

Caorsi, V., Sprau, P., Zollinger, S.A., Brumm, H., 2019. Nocturnal resting behaviour in urban great tits and its relation to anthropogenic disturbance and microclimate. *Behav. Ecol. Sociobiol*. 73, 1–9. https://doi.org/10.1007/s00265-018-2624-1

Capilla-Lasheras, P., Dominoni, D.M., Babayan, S.A., O'Shaughnessy, P.J., Mladenova, M., Woodford, L., Pollock, C.J., Barr, T., Baldini, F., Helm, B., 2017. Elevated immune gene expression is associated with poor reproductive success of urban blue tits. *Front. Ecol. Evol*. 5, 1–13. https://doi.org/10.3389/fevo.2017.00064

Capilla-Lasheras, P., 2018. incR: a new R package to analyse incubation behaviour. *J. Avian Biol.* 49, 1–8. https://doi.org/10.1111/jav.01710

Carlsson, G., 1967. Environmental factors influencing blackfly populations. *Bull. World Health Organ.* 37, 139–150.

Casasole, G., Raap, T., Costantini, D., AbdElgawad, H., Asard, H., Pinxten, R., Eens, M., 2017. Neither artificial light at night, anthropogenic noise nor distance from roads are associated with oxidative status of nestlings in an urban population of songbirds. *Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol.* 210, 14–21. https://doi.org/10.1016/j.cbpa.2017.05.003

Cassone, V.M., Menaker, M., 1984. Is the avian circadian system a neuroendocrine loop? J. *Exp. Zool.* 232, 539–549. https://doi.org/10.1002/jez.1402320321

Cassone, V.M., 2014. Avian circadian organization: A chorus of clocks. *Front. Neuroendocrinol*. 35, 76–88. https://doi.org/10.1016/j.yfrne.2013.10.002

Chabot, C.C., Menaker, M., 1992. Circadian feeding and locomotor rhythms in pigeons and house sparrows. *J. Biol. Rhythms* 7, 287–299. https://doi.org/10.1177/074873049200700403

Chahad-Ehlers, S., Fushita, A.T., Lacorte, G.A., Assis, P.C.P. De, Del Lama, S.N., 2018. Effects of habitat suitability for vectors, environmental factors and host characteristics on the spatial distribution of the diversity and prevalence of haemosporidians in waterbirds from three Brazilian wetlands. *Parasites and Vectors* 11, 1–12. https://doi.org/10.1186/s13071-018-2847-z

Chong, N.W., Chaurasia, S.S., Haque, R., Klein, D.C., Iuvone, P.M., 2003. Temporal-spatial characterization of chicken clock genes: circadian expression in retina, pineal gland, and peripheral tissues. *J. Neurochem*. 85, 851–860. https://doi.org/10.1046/j.1471-4159.2003.01723.x

Christe, P., Glaizot, O., Strepparava, N., Devevey, G., Fumagalli, L., 2012. Twofold cost of reproduction: An increase in parental effort leads to higher malarial parasitaemia and to a decrease in resistance to oxidative stress. *Proc. R. Soc. B Biol. Sci*. 279, 1142–1149. https://doi.org/10.1098/rspb.2011.1546

Clemens, M.J., Elia, A., 1997. The double-stranded RNA-dependent protein kinase PKR: Structure and function. *J. Interf. Cytokine Res*. 17, 503–524. https://doi.org/10.1089/jir.1997.17.503

Conway, C. J., Martin, T.E., 2000. Effects of ambient temperature on avian incubation behavior. *Behav. Ecol.* 11, 178–188. https://doi.org/10.1093/beheco/11.2.178

Coon, C.A.C., Garcia-Longoria, L., Martin, L.B., Magallanes, S., de Lope, F., Marzal, A., 2016. Malaria infection negatively affects feather growth rate in the house sparrow *Passer domesticus*. *J. Avian Biol.* 47, 779–787. https://doi.org/10.1111/jav.00942

Cooper, C.B., Voss, M.A., 2013. Avian Incubation Patterns Reflect Temporal Changes in Developing Clutches. *PLoS One* 8, 1–6. https://doi.org/10.1371/journal.pone.0065521

Cosgrove, C.L., Wood, M.J., Day, K.P., Sheldon, B.C., 2008. Seasonal variation in Plasmodium prevalence in a population of blue tits *Cyanistes caeruleus*. *J. Anim. Ecol.* 77, 540–548. https://doi.org/10.1111/j.1365-2656.2008.01370.x

Craig, D.A., Chance, M.M., 1982. Filter feeding in larvae of *Simuliidae* (Diptera: Culicomorpha): aspects of functional morphology and hydrodynamics. *Can. J. Zool*. 60, 712–724. https://doi.org/10.1139/z82-100

Cravens, Z.M., Boyles, J.G., 2019. Illuminating the physiological implications of artificial light on an insectivorous bat community. *Oecologia* 189, 69–77. https://doi.org/10.1007/s00442-018-4300-6

Crosskey, R., 1990. The Natural History of Blackflies. John Wiley & Sons Ltd, Chichester.

Cuesta, M., Boudreau, P., Dubeau-Laramee, G., Cermakian, N., Boivin, D.B., 2016. Simulated Night Shift Disrupts Circadian Rhythms of Immune Functions in Humans. *J. Immunol.* 196, 2466–75. https://doi.org/10.4049/jimmunol.1502422

Curio, E., Onnebrink, H., 1995. Brood defense and brood size in the great tit (*Parus major*): a test of a model of unshared parental investment. *Behav. Ecol*. 6, 235–241.

Da Silva, A., Valcu, M., Kempenaers, B., 2015. Light pollution alters the phenology of dawn and dusk singing in common european songbirds. *Philos. Trans. R. Soc. B Biol. Sci*. 370. https://doi.org/10.1098/rstb.2014.0126

Dadam, D., Robinson, R.A., Clements, A., Peach, W.J., Bennett, M., Rowcliffe, J.M., Cunningham, A.A., 2019. Avian malaria-mediated population decline of a widespread iconic bird species. *R. Soc. Open Sci.* 6, 182197. https://doi.org/10.1098/rsos.182197

Damiola, F., Le Minh, N., Preitner, N., 2000. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev*. 14, 2950–2961. https://doi.org/10.1101/gad.183500

Dananay, K.L., Benard, M.F., 2018. Artificial light at night decreases metamorphic duration and juvenile growth in a widespread amphibian. *Proc. R. Soc. B Biol. Sci*. 285. https://doi.org/10.1098/rspb.2018.0367

Dawson, A., King, V.M., Bentley, G.E., Ball, G.F., 2001. Photoperiodic Control of Seasonality in Birds. *J. Biol. Rhythms* 16, 365–380. https://doi.org/10.1177/074873001129002079

De Aguilar, J.R., Castillo, F., Moreno, A., Peñafiel, N., Browne, L., Walter, S.T., Karubian, J., Bonaccorso, E., 2018. Patterns of avian haemosporidian infections vary with time, but not habitat, in a fragmented Neotropical landscape. *PLoS One* 13, 1–18. https://doi.org/10.1371/journal.pone.0206493

de Jong, M., Jeninga, L., Ouyang, J.Q., van Oers, K., Spoelstra, K., Visser, M.E., 2016. Dose-dependent responses of avian daily rhythms to artificial light at night. *Physiol. Behav*. 155, 172–179. https://doi.org/10.1016/j.physbeh.2015.12.012

de Jong, M., Lamers, K.P., Eugster, M., Ouyang, J.Q., Da Silva, A., Mateman, A.C., van Grunsven, R.H.A., Visser, M.E., Spoelstra, K., 2018. Effects of experimental light at night on extra-pair paternity in a songbird. *J. Exp. Zool. Part A Ecol. Integr. Physiol*. 329, 441–448. https://doi.org/10.1002/jez.2193

De Lima, L.H.R.G., Dos Santos, K.P., De Lauro Castrucci, A.M., 2011. Clock genes, melanopsins, melatonin, and dopamine key enzymes and their modulation by light and glutamate in chicken embryonic retinal cells. *Chronobiol. Int.* 28, 89–100. https://doi.org/10.3109/07420528.2010.540685

Deeming, D.C., 2002. Importance and evolution of incubation in avian reproduction, in: Avian Incubation: Behaviour, Environment, and Evolution. Oxford University Press, New York, NY, pp. 1–7.

Deviche, P., Davies, S., 2014. Reproductive phenology of urban birds: environmental cues and mechanisms, in: Gil, D., Brumm, H. (Eds.), Avian Urban Ecology: Behavioural and Physiological Adaptations. Oxford University Press, Oxford, pp. 98–115.

Doi, M., Okano, T., Yujnovsky, I., Sassone-Corsi, P., Fukada, Y., 2004. Negative Control of Circadian Clock Regulator E4BP4 by Casein Kinase Iϵ-Mediated Phosphorylation. *Curr. Biol*. 14, 975–980. https://doi.org/10.1016/j.cub.2004.05.043

Dominoni, D.M., Helm, B., Lehmann, M., Dowse, H.B., Partecke, J., 2013. Clocks for the city: circadian differences between forest and city songbirds. *Proc. R. Soc. B Biol. Sci*. 280, 20130593–20130593. https://doi.org/10.1098/rspb.2013.0593

Dominoni, D., Goymann, W., Helm, B., Partecke, J., 2013a. Urban-like night illumination reduces melatonin release in European blackbirds (*Turdus merula*): implications of city life for biological time-keeping of songbirds. *Front. Zool*. 10, 60. https://doi.org/10.1186/1742-9994-10-60

Dominoni, D., Quetting, M., Partecke, J., 2013b. Artificial light at night advances avian reproductive physiology. *Proc. R. Soc. B Biol. Sci*. 280, 20123017–20123017. https://doi.org/10.1098/rspb.2012.3017

Dominoni, D.M., Carmona-Wagner, E.O., Hofmann, M., Kranstauber, B., Partecke, J., 2014. Individual-based measurements of light intensity provide new insights into the effects of artificial light at night on daily rhythms of urban-dwelling songbirds. *J. Anim. Ecol*. 83, 681–692. https://doi.org/10.1111/1365-2656.12150

Dominoni, D.M., 2015. The effects of light pollution on biological rhythms of birds: an integrated, mechanistic perspective. *J. Ornithol*. 156, 409–418. https://doi.org/10.1007/s10336-015-1196-3

Dominoni, D.M., Partecke, J., 2015. Does light pollution alter daylength? A test using light loggers on free-ranging European blackbirds (*Turdus merula*). *Philos. Trans. R. Soc. B Biol. Sci.* 370, 20140118–20140118. https://doi.org/10.1098/rstb.2014.0118

Dominoni, D.M., Borniger, J.C., Nelson, R.J., 2016. Light at night, clocks and health: from humans to wild organisms. *Biol. Lett.* 12, 20160015. https://doi.org/10.1098/rsbl.2016.0015

Dominoni, D.M., de Jong, M., Bellingham, M., O'Shaughnessy, P., van Oers, K., Robinson, J., Smith, B., Visser, M.E., Helm, B., 2018. Dose-response effects of light at night on the reproductive physiology of great tits (*Parus major*): Integrating morphological analyses with candidate gene expression. J. *Exp. Zool. Part A Ecol. Integr. Physiol.* 329, 473–487. https://doi.org/10.1002/jez.2214

Dominoni, D.M., Nelson, R.J., 2018. Artificial light at night as an environmental pollutant: An integrative approach across taxa, biological functions, and scientific disciplines. *J. Exp. Zool. Part A Ecol. Integr. Physiol*. 329, 387–393. https://doi.org/10.1002/jez.2241

Downs, C.T., Brown, M., 2002. Nocturnal Heterothermy and Torpor in the Malachite Sunbird (*Nectarinia Famosa*). *Auk* 119, 251–260. https://doi.org/10.1093/auk/119.1.251

Dunlap, J.C., 1999. Molecular Bases for Circadian Clocks. *Cell* 96, 271–290. https://doi.org/10.1016/S0092-8674(00)80566-8

Dunn, E., 1977. Predation by Weasels (*Mustela nivalis*) on Breeding Tits (*Parus* Spp.) in Relation to the Density of Tits and Rodents. *J. Anim. Ecol.* 46, 633–652.

Dunn, J.C., Cole, E.F., Quinn, J.L., 2011. Personality and parasites: sex-dependent associations between avian malaria infection and multiple behavioural traits. *Behav. Ecol. Sociobiol*. 65, 1459–1471. https://doi.org/10.1007/s00265-011-1156-8

Eck, S., Töpfer, T., Fiebig, J., Heynen, I., Fiedler, W., Nicolai, B., van den Elzen, R., Winkler, R., Woog, F., 2011. Measuring Birds. Minden : Christ Media Natur.

Eide, A., Fallis, A.M., 1972. Experimental Studies of the Life Cycle of *Leucocytozoon simondi* in Ducks in Norway. *J. Protozool.* 19, 414–416. https://doi.org/10.1111/j.1550-7408.1972.tb03494.x

Ellis, V.A., Kunkel, M.R., Ricklefs, R.E., 2014. The ecology of host immune responses to chronic avian haemosporidian infection. *Oecologia* 176, 729–737. https://doi.org/10.1007/s00442-014-3048-x

Evans, K.L., Gaston, K.J., Sharp, S.P., Mcgowan, A., Hatchwell, B.J., Oikos, S., May, N., Gaston, K.J., Sharp, S.P., Mcgowan, A., Simeoni, M., Hatchwell, B., 2009. Effects of Urbanisation on Disease Prevalence and Age Structure in Blackbird *Turdus merula* Populations, *Oikos* 118, 774–782.

Fair, J., Whitaker, S., Pearson, B., 2007. Sources of variation in haematocrit in birds. *Ibis*, 149, 535–552. https://doi.org/10.1111/j.1474-919X.2007.00680.x

Fallis, A.M., Bennett, G.F., 1961. Sporogony of *Leucocytozoon* and *Haemoproteus* in Simuliids and Ceratopogonids and a revised classification of the Haemosporidiida. *Can. J. Zool*. 39, 215-228.

Fargallo, J.A., Laaksonen, T., Pöyri, V., Korpimäki, E., 2002. Inter-sexual differences in the immune response of Eurasian kestrel nestlings under food shortage. *Ecol. Lett*. 5, 95–101. https://doi.org/10.1046/j.1461-0248.2002.00290.x

Ferraguti, M., Martínez-de la Puente, J., Ruiz, S., Soriguer, R.C., Figuerola, J., Roiz, D., 2016. *Culex pipiens* forms and urbanization: effects on blood feeding sources and transmission of avian *Plasmodium. Malar. J*. 15, 1–8. https://doi.org/10.1186/s12936-016-1643-5

Fitzpatrick, S., 1997. The timing of early morning feeding by tits. *Bird Study* 44, 88–96. https://doi.org/10.1080/00063659709461041

Fokidis, H.B., Greiner, E.C., Deviche, P., Fokidis, H.B., Greiner, E.C., Deviche, P., 2008. Interspecific Variation in Avian Blood Parasites and Haematology Associated with Urbanization in a Desert Habitat. *J. Avian Biol*. 39, 300–310. https://doi.org/10.1111/j.2008.0908-8857.04248.x

Fonken, L.K., Nelson, R.J., 2011. Illuminating the deleterious effects of light at night. *F1000 Med. Rep.* 3, 1–7. https://doi.org/10.3410/M3-18

Fonken, L.K., Aubrecht, T.G., Meléndez-Fernández, O.H., Weil, Z.M., Nelson, R.J., 2013. Dim light at night disrupts molecular circadian rhythms and affects metabolism. *J. Biol. Rhythms* 28, 262–71. https://doi.org/10.1177/0748730413493862.

Fonken, L.K., Nelson, R.J., 2014. The effects of light at night on circadian clocks and metabolism. *Endocr. Rev.* 35, 648–670. https://doi.org/10.1210/er.2013-1051

Fontaine C, Dubois G, Duguay Y, et al. The orphan nuclear receptor Rev-Erbalpha is a peroxisome proliferator-activated receptor (PPAR) gamma target gene and promotes PPARgam- ma-induced adipocyte differentiation. *J Biol Chem*. 2003;278: 37672–37680

Fuller, R.A., Warren, P.H., Gaston, K.J., 2007. Daytime noise predicts nocturnal singing in urban robins. *Biol. Lett.* 3, 368–370. https://doi.org/10.1098/rsbl.2007.0134

Gaillard, R.C., Spinedi, E., 1998. Sex- and stress-steroids interactions and the immune system: Evidence for a neuroendocrine-immunological sexual dimorphism. *Domest. Anim. Endocrinol*. 15, 345–352. https://doi.org/10.1016/S0739-7240(98)00028-9

Garnham, P.C.C., 1966. Malaria Parasites and other Haemosporidia. Blackwell Scientific Publications Ltd., Oxford.

Gaspar, L., Brown, S.A., 2015. Measuring circadian clock function in human cells, 1st ed, Methods in Enzymology. Elsevier Inc. https://doi.org/10.1016/bs.mie.2014.10.023

Gaston, S., Menaker, M., 1968. Pineal Function: The Biological Clock in the Sparrow? *Science* 80, 160, 1125–1127.

Gaynor, K.M., Hojnowski, C.E., Carter, N.H., Brashares, J.S., 2018. The influence of human disturbance on wildlife nocturnality. *Science* 80. 360, 1232–1235. https://doi.org/10.1126/science.aar7121

Gekakis, N., Staknis, D., Nguyen, H.B., Davis, F.C., Wilsbacner, L.D., King, D.P., Takahashi, J.S., Weitz, C.J., 1998. Role of the CLOCK protein in the mammalian circadian mechanism. *Science*, 80. 280, 1564–1569. https://doi.org/10.1126/science.280.5369.1564

Gil, D., Honarmand, M., Pascual, J., Pérez-Mena, E., Macías Garcia, C., 2015. Birds living near airports advance their dawn chorus and reduce overlap with aircraft noise. *Behav. Ecol*. 26, 435–443. https://doi.org/10.1093/beheco/aru207

Golombek, D.A., Rosenstein, R.E., 2010. Physiology of circadian entrainment. *Physiol. Rev*. 90, 1063–1102. https://doi.org/10.1152/physrev.00009.2009

Gonçalves, L., Vinhas, M., Pereira, R., Pais De Azevedo, T., Bajanca, F., Palmeirim, I., 2012. Circadian clock genes Bmal1 and Clock during early chick development. *Dev. Dyn*. 241, 1365–1373. https://doi.org/10.1002/dvdy.23821

Gonzalez, G., Sorci, G., Moller, A.P., Ninni, P., Haussey, C., de Lope, F., 1999. Immunocompetence and Condition-Dependent Sexual Advertisement in Male House Sparrows (*Passer domesticus*). *J. Anim. Ecol*. 68, 1225–1234.

Gore, T.C., Noblet, G.P., Noblet, R., 1982. Effects of Pinealectomy and Ocular Enucleation on Diurnal Periodicity of *Leucocytozoon smithi* (Haemosporina) Gametocytes in the Peripheral Blood of Domestic Turkeys. *J. Protozool*. 29, 415–420. https://doi.org/10.1111/j.1550-7408.1982.tb05424.x

Gosler, A.G., 1993. The great tit. Hamlyn, London.

Götmark, F., Andersson, M., 2005. Predation by sparrowhawks decreases with increased breeding density in a songbird, the great tit. *Oecologia* 142, 177–183. https://doi.org/10.1007/s00442-004-1715-z

Graham, J.L., Cook, N.J., Needham, K.B., Hau, M., Greives, T.J., 2017. Early to rise, early to breed: A role for daily rhythms in seasonal reproduction. *Behav. Ecol*. 28, 1266–1271. https://doi.org/10.1093/beheco/arx088

Granthon, C., Williams, D.A., 2017. Avian Malaria, Body Condition, and Blood Parameters In Four Species of Songbirds. *Wilson J. Ornithol*. 129, 492–508. https://doi.org/10.1676/16-060.1

Greives, T.J., Kingma, S.A., Kranstauber, B., Mortega, K., Wikelski, M., van Oers, K., Mateman, A.C., Ferguson, G.A., Beltrami, G., Hau, M., 2015. Costs of sleeping in: Circadian rhythms influence cuckoldry risk in a songbird. *Funct. Ecol*. 29, 1300–1307. https://doi.org/10.1111/1365-2435.12440

Griffiths, R., Double, M.C., Orr, K., Dawson, R.J.G., 1998. A DNA test to sex most birds. *Mol. Ecol.* 7, 1071–1075. https://doi.org/10.1046/j.1365-294x.1998.00389.x

Grunst, M.L., Raap, T., Grunst, A.S., Pinxten, R., Eens, M., 2019. Artificial light at night does not affect telomere shortening in a developing free-living songbird: A field experiment: Artificial light at night and telomere dynamics. *Sci. Total Environ.* 662, 266–275. https://doi.org/10.1016/j.scitotenv.2018.12.469

Gunawardana, T., Ahmed, K.A., Goonewardene, K., Popowich, S., Kurukulasuriya, S., Karunarathna, R., Gupta, A., Lockerbie, B., Foldvari, M., Tikoo, S.K., Willson, P., Gomis, S., 2019. Synthetic CpG-ODN rapidly enriches immune compartments in neonatal chicks to induce protective immunity against bacterial infections. *Sci. Rep*. 9, 1–14. https://doi.org/10.1038/s41598-018-36588-6

Gwinner, E., 2008. Circannual clocks in avian reproduction and migration. *Ibis*, 138, 47–63. https://doi.org/10.1111/j.1474-919x.1996.tb04312.x

Gwinner, E., Hau, M., Heigl, S., 1997. Melatonin: Generation and modulation of avian circadian rhythms. *Brain Res. Bull*. 44, 439–444. https://doi.org/10.1016/S0361-9230(97)00224-4

Gwinner, E., Brandstätter, R., 2001. Complex bird clocks. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 356, 1801–1810. https://doi.org/10.1098/rstb.2001.0959

Gwinner, H., Capilla-Lasheras, P., Cooper, C., Helm, B., Gwinner, H., Helm, B., 2018. ‘ Green incubation ’: avian offspring benefit from aromatic nest herbs through improved parental incubation behaviour. *Proc. R. Soc. B .Biol.* 285, 20180376.

Haftorn, S., 1981. Incubation during the Egg-Laying Period in Relation to Clutch-Size and Other Aspects of Reproduction in the Great Tit *Parus major*. *Scand. J. Ornithol*. 12, 169–185.

Hahn, S., Bauer, S., Dimitrov, D., Emmenegger, T., Ivanova, K., Zehtindjiev, P., Buttemer, W.A., 2018. Low intensity blood parasite infections do not reduce the aerobic performance of migratory birds. *Proc. R. Soc. B Biol*. Sci. 285. https://doi.org/10.1098/rspb.2017.2307

Hainsworth, F.R., Moonan, T., Voss, M.A., Sullivan, K.A., Weathers, W.W., 1998. Time and Heat Allocations to Balance Conflicting Demands during Intermittent Incubation by Yellow-Eyed Juncos. *J. Avian Biol.* 29, 113. https://doi.org/10.2307/3677188

Hamilton, W., Zuk, M., 1982. Heritable true fitness and bright birds: a role for parasites? *Science*. 218, 384–387. https://doi.org/10.1126/science.7123238

Harmer, S.L., Hogenesch, J.B., Straume, M., Chang, H.S., Han, B., Zhu, T., Wang, X., Kreps, J.A., Kay, S.A., 2000. Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. Science, 290, 2110–2113. https://doi.org/10.1126/science.290.5499.2110

Hastings, M.H., 2013. Circadian Clocks, Current biology : CB, Handbook of Experimental Pharmacology. Springer Berlin Heidelberg, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-25950-0

Hau, M., Gwinner, E., 1992. Circadian entrainment by feeding cycles in house sparrows, *Passer domesticus. J. Comp. Physiol. A* 170, 403–409. https://doi.org/10.1007/BF00191457

Hau, M., Dominoni, D., Casagrande, S., Buck, C.L., Wagner, G., Hazlerigg, D., Greives, T., Hut, R.A., 2017. Timing as a sexually selected trait: The right mate at the right moment. *Philos. Trans. R. Soc. B Biol. Sci.* 372. https://doi.org/10.1098/rstb.2016.0249

Hayano, J., Sakata, S., Okada, A., Mukai, S., Fujinami, T., 1998. Circadian Rhythms of Atrioventricular Conduction Properties in Chronic Atrial Fibrillation With and Without Heart Failure. *J. Am. Coll. Cardiol*. 31, 158–166. https://doi.org/10.1016/S0735-1097(97)00429-4

Hellgren, O., Waldenström, J., Bensch, S., 2006. a New PCR Assay for Simultaneous Studies of *Leucocytozoon, Plasmodium*, and *Haemoproteus* From Avian Blood. *J. Parasitol*. 90, 797–802. https://doi.org/10.1645/ge-184r1

Helm, B., Visser, M.E., Schwartz, W., Kronfeld-Schor, N., Gerkema, M., Piersma, T., Bloch, G., 2017. Two sides of a coin: Ecological and chronobiological perspectives of timing in the wild. *Philos. Trans. R. Soc. B Biol. Sci*. 372. https://doi.org/10.1098/rstb.2016.0246

Helm, B., Womack, R., 2018. Timing Matters: Allochronic Contributions to Population Divergence, in: Tietze, D.T. (Ed.), Bird Species: How They Arise, Modify and Vanish. Springer International Publishing, Cham, pp. 95–107. https://doi.org/10.1007/978-3-319-91689-7_6

Hepp, G.R., DuRant, S.E., Hopkins, W.A., 2015. Influence of incubation temperature on offspring phenotype and fitness in birds, in: Deeming, D.C., Reynolds, S.J. (Eds.), Nests, Eggs, and Incubation: New Ideas about Avian Reproduction. Oxford University Press, Oxford, pp. 171–178. https://doi.org/10.1086/499988

Hinde, C.A., 2006. Negotiation over offspring care? - A positive response to partner-provisioning rate in great tits. *Behav. Ecol*. 17, 6–12. https://doi.org/10.1093/beheco/ari092

Holzenberger, M., Dupont, J., Ducos, B., Leneuve, P., Géloën, A., Even, P.C., Cervera, P., Le Bouc, Y., 2003. IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. *Nature* 421, 182–187. https://doi.org/10.1038/nature01298

Hõrak, P., Ots, I., Vellau, H., Spottiswoode, C., Møller, A.P., 2001. Carotenoid-based plumage coloration reflects hemoparasite infection and local survival in breeding great tits. *Oecologia* 126, 166–173. https://doi.org/10.1007/s004420000513

Huang, W., Ramsey, K.M., Marcheva, B., Bass, J., 2011. Circadian rhythms, sleep, and metabolism. *J. Clin. Invest*. 121, 2133–2141. https://doi.org/10.1172/JCI46043.rons

Hubálek, Z., 2017. How sunrise and weather affect timing of rooks' (*Corvus frugilegus*) morning departure from the winter communal roost. *Folia Zool*. 66, 227–230. https://doi.org/10.25225/fozo.v66.i4.a3.2017

Ilgunas, M., Bukauskaite, D., Palinauskas, V., Iezhova, T., Fragner, K., Platonova, E., Weissenböck, H., Valkiunas, G., 2019. Patterns of *Plasmodium homocircumflexum* virulence in experimentally infected passerine birds. *Malar. J*. 18, 1–14. https://doi.org/10.1186/s12936-019-2810-2

Isaksson, C., Andersson, S., 2007. Carotenoid diet and nestling provisioning in urban and rural great tits *Parus major. J. Avian Biol*. 38, 564–572. https://doi.org/10.1111/j.2007.0908-8857.04030.x

Isaksson, C., Sturve, J., Almroth, B.C., Andersson, S., 2009. The impact of urban environment on oxidative damage (TBARS) and antioxidant systems in lungs and liver of great tits, *Parus major,*. *Environ. Res.* 109, 46–50. https://doi.org/10.1016/j.envres.2008.10.006

Jacobs, L., Vezzoli, G., Beerda, B., Mench, J.A., 2019. Northern fowl mite infestation affects the nocturnal behavior of laying hens. *Appl. Anim. Behav. Sci.* 216, 33–37. https://doi.org/10.1016/j.applanim.2019.04.007

Jacquin, L., Lenouvel, P., Haussy, C., Ducatez, S., Gasparini, J., 2011. Melanin-based coloration is related to parasite intensity and cellular immune response in an urban free living bird: The feral pigeon *Columba livia. J. Avian Biol*. 42, 11–15. https://doi.org/10.1111/j.1600-048X.2010.05120.x

Jansen, J.K., Boveng, P.L., Bengtson, J.L., 1998. Foraging modes of chinstrap penguins: Contrasts between day and night. *Mar. Ecol. Prog. Ser*. 165, 161–172. https://doi.org/10.3354/meps165161

Jenkins, T., Delhaye, J., Christe, P., 2015. Testing local adaptation in a natural great tit-malaria system: An experimental approach. *PLoS One* 10, 1–20. https://doi.org/10.1371/journal.pone.0141391

Jiménez-Peñuela, J., Ferraguti, M., Martínez-de la Puente, J., Soriguer, R., Figuerola, J., 2019. Urbanization and blood parasite infections affect the body condition of wild birds. *Sci. Total Environ*. 651, 3015–3022. https://doi.org/10.1016/j.scitotenv.2018.10.203

Jin, E., Jia, L., Li, J., Yang, G., Wang, Z., Cao, J., Chen, Y., 2011. Effect of monochromatic light on melatonin secretion and arylalkylamine n-acetyltransferase mrna expression in the retina and pineal gland of broilers. *Anat. Rec*. 294, 1233–1241. https://doi.org/10.1002/ar.21408

Kacelnik, A., Krebs, J.R., 1983. The Dawn Chorus in the Great Tit (*Parus Major*): Proximate and Ultimate Causes. *Behaviour* 83, 287–308. https://doi.org/10.1163/156853983X00200

Karaganis, S.P., Bartell, P.A., Shende, V.R., Moore, A.F., Cassone, V.M., 2009. Modulation of metabolic and clock gene mRNA rhythms by pineal and retinal circadian oscillators. *Gen. Comp. Endocrinol.* 161, 179–192. https://doi.org/10.1016/j.ygcen.2008.12.015

Karatsoreos, I.N., Bhagat, S., Bloss, E.B., Morrison, J.H., McEwen, B.S., 2011. Disruption of circadian clocks has ramifications for metabolism, brain, and behavior. *Proc. Natl. Acad. Sci*. 108, 1657–1662. https://doi.org/10.1073/pnas.1018375108

Keitt, B.S., Tershy, B.R., Croll, D.A., 2004. Nocturnal behavior reduces predation pressure on Black-vented Shearwaters *Puffinus opisthomelas*. *Mar. Ornithol*. 32, 173–178.

Kempenaers, B., Borgström, P., Loës, P., Schlicht, E., Valcu, M., 2010. Artificial night lighting affects dawn song, extra-pair siring success, and lay date in songbirds. *Curr. Biol*. 20, 1735–1739. https://doi.org/10.1016/j.cub.2010.08.028

Kendeigh, S. (1969). Energy Responses of Birds to Their Thermal Environments. *The Wilson Bulletin*, 81(4), 441-449.

Kensler, T.W., Wakabayashi, N., Biswal, S., 2007. Cell Survival Responses to Environmental Stresses Via the Keap1-Nrf2-ARE Pathway. *Annu. Rev. Pharmacol. Toxicol*. 47, 89–116. https://doi.org/10.1146/annurev.pharmtox.46.120604.141046

Kernbach, M.E., Hall, R.J., Burkett-Cadena, N.D., Unnasch, T.R., Martin, L.B., 2018. Dim light at night: physiological effects and ecological consequences for infectious disease. *Integr. Comp. Biol*. 58, 995–1007. https://doi.org/10.1093/icb/icy080

Kilpatrick, A.M., LaPointe, D.A., Atkinson, C.T., Woodworth, B.L., Lease, J.K., Reiter, M.E., Gross, K., 2006. Effects of Chronic Avian Malaria (*Plasmodium Relictum*) Infection on Reproductive Success of Hawaii Amakihi (*Hemignathus Virens*). *Auk* 123, 764–774. https://doi.org/10.1093/auk/123.3.764

Klasing, K.C., 1998. Avian Macrophages: Regulators of Local and Systemic Immune Responses. *Poult. Sci*. 77, 983–989. https://doi.org/10.1093/ps/77.7.983

Klett-Mingo, J.I., Pavón, I., Gil, D., 2016. Great tits, *Parus major*, increase vigilance time and reduce feeding effort during peaks of aircraft noise. *Anim. Behav*. 115, 29–34. https://doi.org/10.1016/j.anbehav.2016.02.021

Knowles, S.C.L., Palinauskas, V., Sheldon, B.C., 2010a. Chronic malaria infections increase family inequalities and reduce parental fitness: Experimental evidence from a wild bird population. *J. Evol. Biol*. 23, 557–569. https://doi.org/10.1111/j.1420-9101.2009.01920.x

Knowles, S.C.L., Palinauskas, V., Sheldon, B.C., 2010b. Chronic malaria infections increase family inequalities and reduce parental fitness: Experimental evidence from a wild bird population. *J. Evol. Biol.* 23, 557–569. https://doi.org/10.1111/j.1420-9101.2009.01920.x

Kocan, R.M., Clark, D.T., 1966. Prepatent Period and Parasitemia in *Leucocytozoon simondi* Infections Resulting from Short Exposures to Sporozoites. *J. Parasitol*. 52, 962–966.

Kohsaka, A., Laposky, A.D., Ramsey, K.M., Estrada, C., Joshu, C., Kobayashi, Y., Turek, F.W., Bass, J., 2007. High-Fat Diet Disrupts Behavioral and Molecular Circadian Rhythms in Mice. *Cell Metab*. 6, 414–421. https://doi.org/10.1016/j.cmet.2007.09.006

Kowalska, E., Moriggi, E., Bauer, C., Dibner, C., Brown, S.A., 2010. The circadian clock starts ticking at a developmentally early stage. *J. Biol. Rhythms* 25, 442–449. https://doi.org/10.1177/0748730410385281

Krama, T., Krams, R., Cīrule, D., Moore, F.R., Rantala, M.J., Krams, I.A., 2015. Intensity of haemosporidian infection of parids positively correlates with proximity to water bodies, but negatively with host survival. *J. Ornithol*. 156, 1075–1084. https://doi.org/10.1007/s10336-015-1206-5

Krams, I.A., Suraka, V., Rantala, M.J., Sepp, T., Mierauskas, P., Vrublevska, J., Krama, T., 2013a. Acute infection of avian malaria impairs concentration of haemoglobin and survival in juvenile altricial birds. *J. Zool*. 291, 34–41. https://doi.org/10.1111/jzo.12043

Kronfeld-Schor, N., Bloch, G., Schwartz, W.J., 2013. Animal clocks: When science meets nature. *Proc. R. Soc. B Biol. Sci.* 280, 3–6. https://doi.org/10.1098/rspb.2013.1354

Kubista, M., Andrade, J.M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B., Strömbom, L., Ståhlberg, A., Zoric, N., 2006. *Mol. Aspects Med*. 27, 95–125. https://doi.org/10.1016/j.mam.2005.12.007

Kumar, V., Singh, B.P., Rani, S., 2004. The Bird Clock: A Complex, Multi-Oscillatory and Highly Diversified System. *Biol. Rhythm Res*. 35, 121–144. https://doi.org/10.1080/09291010412331313287

Kumar, J., Malik, S., Bhardwaj, S.K., Rani, S., 2018. Bright light at night alters the perception of daylength in Indian weaver bird (*Ploceus philippinus*). *J. Exp. Zool. Part A Ecol. Integr. Physiol*. 329, 488–496. https://doi.org/10.1002/jez.2201

Kumar, V., Sharma, A., 2018. Common features of circadian timekeeping in diverse organisms. *Curr. Opin. Physiol.* 5, 58–67. https://doi.org/10.1016/j.cophys.2018.07.004

Kume, K., Zylka, M.J., Sriram, S., Shearman, L.P., Weaver, D.R., Jin, X., Maywood, E.S., Hastings, M.H., Reppert, S.M., 1999. mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. *Cell* 98, 193–205. https://doi.org/10.1016/S0092-8674(00)81014-4

Kwiatkowski, D., Greenwood, B.M., 1989. Why is malaria fever periodic? A hypothesis. *Parasitol. Today* 5, 264–266. https://doi.org/10.1016/0169-4758(89)90261-5

Kwon, J.S., Lee, H.J., Lee, D.H., Lee, Y.J., Mo, I.P., Nahm, S.S., Kim, M.J., Lee, J.B., Park, S.Y., Choi, I.S., Song, C.S., 2008. Immune responses and pathogenesis in immunocompromised chickens in response to infection with the H9N2 low pathogenic avian influenza virus. *Virus Res*. 133, 187–194. https://doi.org/10.1016/j.virusres.2007.12.019

Kyba, C.C.M., Kuester, T., De Miguel, A.S., Baugh, K., Jechow, A., Hölker, F., Bennie, J., Elvidge, C.D., Gaston, K.J., Guanter, L., 2017. Artificially lit surface of Earth at night increasing in radiance and extent. *Sci. Adv.* 3, 1–9. https://doi.org/10.1126/sciadv.1701528

Labrecque, N., Cermakian, N., 2015. Circadian Clocks in the Immune System. *J. Biol. Rhythms* 30, 277–90. https://doi.org/10.1177/0748730415577723

Labyak, S.E., Lee, T.M., Goel, N., 1997. Rhythm chronotypes in a diurnal rodent, *Octodon degus*. *Am. J. Physiol. - Regul. Integr. Comp. Physiol*. 273.

Lack, D., 1964. A Long-Term Study of the Great Tit (*Parus major*). *J. Anim. Ecol*. 33, 159–173.

Laine, V.N., Gossmann, T.I., Schachtschneider, K.M., Garroway, C.J., Madsen, O., Verhoeven, K.J.F., de Jager, V., Megens, H., Warren, W.C., Minx, P., Crooijmans, R.P.M.A., Corcoran, P., Adriaensen, F., Belda, E., Bushuev, A., Cichon, M., Charmantier, A., Dingemanse, N., Doligez, B., Eeva, T., Erikstad, K.E., Fedorov, S., Hau, M., Hille, S., Hinde, C., Kempenaers, B., Kerimov, A., Krist, M., Mand, R., Matthysen, E., Nager, R., Norte, C., Orell, M., Richner, H., Slagsvold, T., Tilgar, V., Tinbergen, J., Torok, J., Tschirren, B., Yuta, T., Sheldon, B.C., Slate, J., Zeng, K., van Oers, K., Visser, M.E., Groenen, M.A.M., 2016. Evolutionary signals of selection on cognition from the great tit genome and methylome. *Nat. Commun.* 7, 10474. https://doi.org/10.1038/ncomms10474

Laine, V.N., Verhagen, I., Mateman, A.C., Pijl, A., Williams, T.D., Gienapp, P., van Oers, K., Visser, M.E., 2019. Exploration of tissue-specific gene expression patterns underlying timing of breeding in contrasting temperature environments in a song bird. *BMC Genomics* 20, 1–16. https://doi.org/10.1186/s12864-019-6043-0

Lambrechts, M.M., Perret, P., 2000. A long photoperiod overrides non-photoperiodic factors in blue tits' timing of reproduction. *Proc. R. Soc. B Biol. Sci.* 267, 585–588. https://doi.org/10.1098/rspb.2000.1041

Lapointe, D.A., Atkinson, C.T., Samuel, M.D., 2012. Ecology and conservation biology of avian malaria. *Ann. N. Y. Acad. Sci*. 1249, 211–226. https://doi.org/10.1111/j.1749-6632.2011.06431.x

Laposky, A.D., Bass, J., Kohsaka, A., Turek, F.W., 2008. Sleep and circadian rhythms : Key components in the regulation of energy metabolism. *FEBS Lett*. 582, 142–151. https://doi.org/10.1016/j.febslet.2007.06.079

Lee, K.A., Martin, L.B., Hasselquist, D., Ricklefs, R.E., Wikelski, M., 2006. Contrasting adaptive immune defenses and blood parasite prevalence in closely related *Passer sparrows. Oecologia* 150, 383–392. https://doi.org/10.1007/s00442-006-0537-6

Lehmann, M., Spoelstra, K., Visser, M.E., Helm, B., 2012. Effects of temperature on circadian clock and chronotype: an experimental study on a passerine bird. *Chronobiol. Int.* 29, 1062–71. https://doi.org/10.3109/07420528.2012.707159

Levy, O., Dayan, T., Porter, W.P., Kronfeld-Schor, N., 2019. Time and ecological resilience: can diurnal animals compensate for climate change by shifting to nocturnal activity? *Ecol. Monogr*. 89, 1–22. https://doi.org/10.1002/ecm.1334

Linnebjerg, J.F., Huffeldt, N.P., Falk, K., Merkel, F.R., Mosbech, A., Frederiksen, M., 2014. Inferring seabird activity budgets from leg-mounted time-depth recorders. *J. Ornithol*. 155, 301–306. https://doi.org/10.1007/s10336-013-1015-7

Lochmiller, R.L., Deerenberg, C., 2000. Trade-offs in evolutionary immunology: Just what is the cost of immunity? *Oikos* 88, 87–98. https://doi.org/10.1034/j.1600-0706.2000.880110.x

Longcore, T., Rich, C., 2004. Ecological light pollution. *Front. Ecol. Environ.* 2, 191–198. https://doi.org/10.1890/1540-9295

Lu, Z., Li, Yan, Takwi, A., Li, B., Zhang, J., Conklin, D.J., Young, K.H., Martin, R., Li, Yong, 2011. miR-301a as an NF-κB activator in pancreatic cancer cells. *EMBO J.* 30, 57–67. https://doi.org/10.1038/emboj.2010.296

Lupu, F., Terwilliger, J.D., Lee, K., Segre, G. V., Efstratiadis, A., 2001. Roles of growth hormone and insulin-like growth factor 1 in mouse postnatal growth. *Dev. Biol.* 229, 141–162. https://doi.org/10.1006/dbio.2000.9975

Marasco, V., Spencer, K.A., 2015. Improvements in our understanding of behaviour during incubation, in: Nests, Eggs, and Incubation. Oxford University Press, pp. 142–151. https://doi.org/10.1093/acprof:oso/9780198718666.003.0012

Markowska, M., Majewski, P.M., Skwarło-Sońta, K., 2017. Avian biological clock – Immune system relationship. *Dev. Comp. Immunol*. 66, 130–138. https://doi.org/10.1016/j.dci.2016.05.017

Martin, L.B., Boruta, M., 2014. The impacts of urbanization on avian disease transmission and emergence, in: Gil, D., Brumm, H. (Eds.), *Avian Urban Ecology*. Oxford University Press, Oxford, pp. 116–127.

Martinez-Bakker, M., Helm, B., 2015. The influence of biological rhythms on host-parasite interactions. *Trends Ecol. Evol.* https://doi.org/10.1016/j.tree.2015.03.012

Marzal, A., De Lope, F., Navarro, C., Møller, A.P., 2005. Malarial parasites decrease reproductive success: An experimental study in a passerine bird. *Oecologia* 142, 541–545. https://doi.org/10.1007/s00442-004-1757-2

Marzal, A., Bensch, S., Reviriego, M., Balbontin, J., De Lope, F., 2008a. Effects of malaria double infection in birds: One plus one is not two. *J. Evol. Biol*. 21, 979–987. https://doi.org/10.1111/j.1420-9101.2008.01545.x

McClung, C.A., 2013. How might circadian rhythms control mood? Let me count the ways... *Biol. Psychiatry.* https://doi.org/10.1016/j.biopsych.2013.02.019

McFadden, E., Jones, M.E., Schoemaker, M.J., Ashworth, A., Swerdlow, A.J., 2014. The relationship between obesity and exposure to light at night: Cross-sectional analyses of over 100,000 women in the breakthrough generations study. *Am. J. Epidemiol*. 180, 245–250. https://doi.org/10.1093/aje/kwu117

McKinney, M., 2002. Urbanization, Biodiversity, and Conservation. *Bioscience* 52.

Medzhitov, R., 2001. Toll-like receptors and innate immunity. *Nat. Rev. Immunol*. 1.

Meitern, R., Andreson, R., Hõrak, P., 2014. Profile of whole blood gene expression following immune stimulation in a wild passerine. *BMC Genomics* 15, 533. https://doi.org/10.1186/1471-2164-15-533

Menaker, M., Zimmerman, N., 1976. Role of the Pineal in the Circadian System of Birds. *J. Am. Zool*. 16, 45–55.

Merino, S., Moreno, J., Sanz, J.J., Arriero, E., 2000. Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). *Proc. R. Soc. B Biol. Sci*. 267, 2507–2510. https://doi.org/10.1098/rspb.2000.1312

Merrow, M., Spoelstra, K., Roenneberg, T., 2005. The circadian cycle: daily rhythms from behaviour to genes. *EMBO Rep*. 6, 930–5. https://doi.org/10.1038/sj.embor.7400541

Mideo, N., Reece, S.E., Smith, A.L., Metcalf, C.J.E., 2013. The Cinderella syndrome: Why do malaria-infected cells burst at midnight? *Trends Parasitol*. https://doi.org/10.1016/j.pt.2012.10.006

Mills, M.G., Gallagher, E.P., 2016. A targeted gene expression platform allows for rapid analysis of chemical-induced antioxidant mRNA expression in zebrafish larvae. *PLoS One* 12, 1–20. https://doi.org/10.1371/journal.pone.0171025

Mishra, I., Knerr, R.M., Stewart, A.A., Payette, W.I., Richter, M.M., Ashley, N.T., 2019. Light at night disrupts diel patterns of cytokine gene expression and endocrine profiles in zebra finch (*Taeniopygia guttata*). *Sci. Rep*. 9, 1–12. https://doi.org/10.1038/s41598-019-51791-9

Möller-levet, C.S., Archer, S.N., Bucca, G., Laing, E.E., Slak, A., Kabiljo, R., Lo, J.C.Y., Santhi, N., Schantz, M. Von, Smith, C.P., Dijk, D., 2013. Effects of insufficient sleep on circadian rhythmicity and expression amplitude of the human blood transcriptome. *PNAS*. https://doi.org/https://doi.org/10.1073/pnas.1217154110

Moore, C.B., Siopes, T.D., 2000. Effects of lighting conditions and melatonin supplementation on the cellular and humoral immune responses in Japanese quail *Coturnix japonica. Gen. Comp. Endocrinol.* 119, 95–104. https://doi.org/10.1006/gcen.2000.7496

Mueller, J.C., Steinmeyer, C., Kempenaers, B., 2012. Individual variation in sleep-wake rhythms in free-living birds. *Chronobiol. Int.* 29, 1216–26. https://doi.org/10.3109/07420528.2012.705404

Mukherjee, S., Karmakar, S., Babu, S.P.S., 2016. TLR2 and TLR4 mediated host immune responses in major infectious diseases: A review. *Brazilian J. Infect. Dis*. https://doi.org/10.1016/j.bjid.2015.10.011

Naef-daenzer, A.L., Naef-daenzer, B., Nager, R.G., 2016. Prey Selection and Foraging Performance of Breeding Great Tits *Parus major* in Relation to Food Availability, *J. Avian. Biol.* , 31, 206–214.

Naguib, M., Diehl, J., van Oers, K., Snijders, L., 2019. Repeatability of signalling traits in the avian dawn chorus. *Front. Zool*. 16, 1–11. https://doi.org/10.1186/s12983-019-0328-7

Naidu, K.S., Morgan, L.W., Bailey, M.J., 2010. Inflammation in the avian spleen: timing is everything. *BMC Mol. Biol*. 11, 104. https://doi.org/10.1186/1471-2199-11-104

Navara, K.J., Nelson, R.J., 2007. The dark side of light at night: Physiological, epidemiological, and ecological consequences. *J. Pineal Res*. 43, 215–224. https://doi.org/10.1111/j.1600-079X.2007.00473.x

Nicolaides, N.C., Charmandari, E., Chrousos, G.P. and Kino, T. 2014. Circadian endocrine rhythms: the hypothalamic–pituitary–adrenal axis and its actions. *Ann. N.Y. Acad. Sci*., 1318: 71-80. doi:10.1111/nyas.12464

Nishikawa, K., Toker, A., Johannes, F.J., Songyang, Z., Cantley, L.C., 1997. Determination of the specific substrate sequence motifs of protein kinase C isozymes. *J. Biol. Chem*. 272, 952–960. https://doi.org/10.1074/jbc.272.2.952

Noordwijk, A.J. Van, McCleery, R.H., Perrins, C.M., 1995. Selection for the Timing of Great Tit Breeding in Relation to Caterpillar Growth and Temperature. *J. Anim. Ecol*. 64, 451. https://doi.org/10.2307/5648

Nord, A., Nilsson, J.Å., 2011. Incubation temperature affects growth and energy metabolism in blue tit nestlings. *Am. Nat.* 178, 639–651. https://doi.org/10.1086/662172

Nordling, D., Andersson, M., Zohari, S., Gustafsson, L., 1998. Reproductive effort reduces specific immune response and parasite resistance. *Proc. R. Soc. B Biol. Sci.* 265, 1291–1298. https://doi.org/10.1098/rspb.1998.0432

Norris, K., 1993. Heritable variation in a plumage indicator of viability in male great tits *Parus major*. *Nature* 362, 537–539. https://doi.org/10.1038/255243a0

Nourbakhsh, M., Hauser, H., 1999. Constitutive silencing of IFN-β promoter is mediated by NRF (NF-κB-repressing factor), a nuclear inhibitor of NF-κB. *EMBO J.* 18, 6415–6425. https://doi.org/10.1093/emboj/18.22.6415

O'Donnell, A.J., Schneider, P., McWatters, H.G., Reece, S.E., 2011. Fitness costs of disrupting circadian rhythms in malaria parasites. *Proc. R. Soc. B Biol. Sci*. 278, 2429–2436. https://doi.org/10.1098/rspb.2010.2457

O'Donnell, A.J., Mideo, N., Reece, S.E., 2013. Disrupting rhythms in *Plasmodium chabaudi:* Costs accrue quickly and independently of how infections are initiated. *Malar. J.* 12, 1–9. https://doi.org/10.1186/1475-2875-12-372

O'Neill, J., Reddy, A., 2011. Circadian clocks in human red blood cells. *Nature* 469, 498–503. https://doi.org/10.1038/nature09702.Circadian

Ocampo-Garcés, A., Mena, W., Hernández, F., Cortés, N., Palacios, A.G., 2006. Circadian chronotypes among wild-captured west Andean octodontids. *Biol. Res.* 39, 209–220. https://doi.org/10.4067/S0716-97602006000200003

Oke, T.R., 1982. The energetic basis of the urban heat island. *Quart. J. R. Met. Soc*. 108, 1–24.

Olias, P., Adam, I., Meyer, A., Scharff, C., Gruber, A.D., 2014. Reference Genes for Quantitative Gene Expression Studies in Multiple Avian Species. *PLoS One* 9, e99678. https://doi.org/10.1371/journal.pone.0099678

Ots, I., Murumägi, A., Hõrak, P., 1998. Haematological health state indices of reproducing Great Tits: Methodology and sources of natural variation. *Funct. Ecol*. 12, 700–707. https://doi.org/10.1046/j.1365-2435.1998.00219.x

Ouyang, J.Q., de Jong, M., Hau, M., Visser, M.E., van Grunsven, R.H.A., Spoelstra, K., 2015. Stressful colours: corticosterone concentrations in a free-living songbird vary with the spectral composition of experimental illumination. *Biol. Lett*. 11, 13–20. https://doi.org/10.1098/rsbl.2015.0517

Ouyang, J.Q., de Jong, M., van Grunsven, R.H.A., Matson, K.D., Haussmann, M.F., Meerlo, P., Visser, M.E., Spoelstra, K., 2017. Restless roosts: Light pollution affects behavior, sleep, and physiology in a free-living songbird. *Glob. Chang. Biol.* 23, 4987–4994. https://doi.org/10.1111/gcb.13756

Ouyang, J.Q., Davies, S., Dominoni, D., 2018. Hormonally mediated effects of artificial light at night on behavior and fitness: Linking endocrine mechanisms with function. *J. Exp. Biol*. 221. https://doi.org/10.1242/jeb.156893

Owen, J.C., 2011. Collecting, processing, and storing avian blood: A review. *J. F. Ornithol.* 82, 339–354. https://doi.org/10.1111/j.1557-9263.2011.00338.x

Pagani, L., Semenova, E.A., Moriggi, E., Revell, V.L., Hack, L.M., Lockley, S.W., Arendt, J., Skene, D.J., Meier, F., Izakovic, J., Wirz-Justice, A., Cajochen, C., Sergeeva, O.J., Cheresiz, S. V., Danilenko, K. V., Eckert, A., Brown, S.A., 2010. The physiological period length of the human circadian clock In Vivo is directly proportional to period in human fibroblasts. *PLoS One* 5. https://doi.org/10.1371/journal.pone.0013376

Palinauskas, V., Valkiunas, G., Bolshakov, C. V., Bensch, S., 2008. *Plasmodium relictum* (lineage P-SGS1): Effects on experimentally infected passerine birds. *Exp. Parasitol*. 120, 372–380. https://doi.org/10.1016/j.exppara.2008.09.001

Palinauskas, V., Žiegyte, R., Ilgunas, M., Iezhova, T.A., Bernotiene, R., Bolshakov, C., Valkiunas, G., 2015. Description of the first cryptic avian malaria parasite, *Plasmodium homocircumflexum* n.

sp., with experimental data on its virulence and development in avian hosts and mosquitoes. *Int. J. Parasito*l. 45, 51–62. https://doi.org/10.1016/j.ijpara.2014.08.012

Partecke, J., 2014. Mechanisms of phenotypic responses following colonization of urban areas, in: Gil, D., Brumm, H. (Eds.), *Avian Urban Ecology*. Oxford University Press, Oxford, pp. 131–142. https://doi.org/10.1093/acprof:osobl/9780199661572.003.0010

Perrins, C.M., 1965. Population Fluctuations and Clutch-Size in the Great Tit , *Parus major. J. Anim. Ecol.* 34, 601–647.

Perrins, C.M., 1991. Tits and their caterpillar food supply. *Ibis*. 133, 49–54. https://doi.org/10.1111/j.1474-919X.1991.tb07668.x

Perrins, C.M., McCleery, R.., 1989. Laying Dates and Clutch Size in the Great Tit. Wilson Bull. 101, 236–253.

Pigeault, R., Vézilier, J., Cornet, S., Zélé, F., Nicot, A., Perret, P., Gandon, S., Rivero, A., 2015. Avian malaria: A new lease of life for an old experimental model to study the evolutionary ecology of Plasmodium. Philos. Trans. R. Soc. B Biol. Sci. 370. https://doi.org/10.1098/rstb.2014.0300

Pigeault, Romain, Caudron, Q., Nicot, A., Rivero, A., Gandon, S., 2018. Timing malaria transmission with mosquito fluctuations. *Evol. Lett*. 2, 378–389. https://doi.org/10.1002/evl3.61

Pigeault, R., Cozzarolo, C.-S., Choquet, R., Strehler, M., Jenkins, T., Delhaye, J., Bovet, L., Wassef, J., Glaizot, O., Christe, P., 2018. Haemosporidian infection and co-infection affect host survival and reproduction in wild populations of great tits. *Int. J. Parasitol*. 48, 1079–1087. https://doi.org/10.1016/j.ijpara.2018.06.007

Pittendrigh, C.S., 1954. On temperature independence in the clock system controlling emergence time in *Drosophila. Proc. Natl. Acad. Sci*. 40, 1018–1029. https://doi.org/10.1073/pnas.40.10.1018

Pittendrigh, C.S., 1981. Circadian Systems: Entrainment. *Biol. Rhythm.* 95–124. https://doi.org/10.1007/978-1-4615-6552-9_7

Poesel, A., Kunc, H.P., Foerster, K., Johnsen, A., Kempenaers, B., 2006. Early birds are sexy: male age, dawn song and extrapair paternity in blue tits, *Cyanistes* (formerly *Parus*) *caeruleus. Anim. Behav.* 72, 531–538. https://doi.org/10.1016/j.anbehav.2005.10.022

Pollock, C.J., Capilla-Lasheras, P., McGill, R.A.R., Helm, B., Dominoni, D.M., 2017. Integrated behavioural and stable isotope data reveal altered diet linked to low breeding success in urban-dwelling blue tits (*Cyanistes caeruleus*). *Sci. Rep.* 7, 1–14. https://doi.org/10.1038/s41598-017-04575-y

Potter, G.D.M., Cade, J.E., Grant, P.J., Hardie, L.J., 2016. Nutrition and the circadian system. *Br. J. Nutr*. 116, 434–442. https://doi.org/10.1017/S0007114516002117

Preuss, F., Tang, Y., Laposky, A.D., Arble, D., Keshavarzian, A., Turek, F.W., 2008. Adverse effects of chronic circadian desynchronization in animals in a "challenging" environment. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 295, R2034–R2040. https://doi.org/10.1152/ajpregu.00118.2008

Prior, K.F., O'Donnell, A.J., Rund, S.S.C., Savill, N.J., van der Veen, D.R., Reece, S.E., 2019. Host circadian rhythms are disrupted during malaria infection in parasite genotype-specific manners. *Sci. Rep.* 9, 1–12. https://doi.org/10.1038/s41598-019-47191-8

Pritchett, D., Reddy, A.B., 2015. Circadian Clocks in the Hematologic System. *J. Biol. Rhythms*, 1–15. https://doi.org/10.1177/0748730415592729

Raap, T., Pinxten, R., Eens, M., 2015. Light pollution disrupts sleep in free-living animals. *Sci. Rep.* 5, 13557. https://doi.org/10.1038/srep13557

Raap, T., Casasole, G., Costantini, D., AbdElgawad, H., Asard, H., Pinxten, R., Eens, M., 2016a. Artificial light at night affects body mass but not oxidative status in free-living nestling songbirds: an experimental study. *Sci. Rep.* 6, 35626. https://doi.org/10.1038/srep35626

Raap, T., Casasole, G., Pinxten, R., Eens, M., 2016b. Early life exposure to artificial light at night affects the physiological condition: An experimental study on the ecophysiology of free-living nestling songbirds. *Environ. Pollut.* 218, 909–914. https://doi.org/10.1016/j.envpol.2016.08.024

Raap, T., Pinxten, R., Eens, M., 2016c. Artificial light at night disrupts sleep in female great tits (Parus major) during the nestling period, and is followed by a sleep rebound. *Environ. Pollut.* 215, 125–134. https://doi.org/10.1016/j.envpol.2016.04.100

Radford, A.N., Mc Cleery, R.H., Woodburn, R.J.W., Morecroft, M.D., 2001. Activity patterns of parent great tits *Parus major* feeding their young during rainfall. *Bird Study* 48, 214–220. https://doi.org/10.1080/00063650109461220

Raspe E, Duez H, Mansen A, et al. Identification of Rev- erbalpha as a physiological repressor of apoC-III gene tran- scription. *J Lipid Res.* 2002;43:2172–2179

Reddy, A.B., 2013. Genome-Wide Analyses of Circadian Systems, in: Kramer, A., Merrow, M. (Eds.), Circadian Clocks. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 379–388. https://doi.org/10.1007/978-3-642-25950-0_16

Reece, S.E., Prior, K.F., Mideo, N., 2017. The Life and Times of Parasites: Rhythms in Strategies for Within-host Survival and Between-host Transmission. *J. Biol. Rhythms* 32, 516–533. https://doi.org/10.1177/0748730417718904

Refinetti, R., 2006. Variability of diurnality in laboratory rodents. *J. Comp. Physiol. A Neuroethol. Sensory, Neural, Behav. Physiol.* 192, 701–714. https://doi.org/10.1007/s00359-006-0093-x

Refinetti, R., Menaker, M., 1992. The circadian rhythm of body temperature. *Physiol. Behav.* 51, 613–637. https://doi.org/10.2741/3634

Reppert, S.M., Weaver, D.R., Cassone, V.M., Godson, C., Kolakowski, L.F., 1995. Melatonin receptors are for the birds: Molecular analysis of two receptor subtypes differentially expressed in chick brain. *Neuron* 15, 1003–1015. https://doi.org/10.1016/0896-6273(95)90090-X

Reppert, S.M., Weaver, D.R., 2002. Coordination of circadian timing in mammals. *Nature* 418, 935–41. https://doi.org/10.1038/nature00965

Ricklefs, R.E., Fallon, S.M., 2002. Diversification and host switching in avian malaria parasites. *Proc. R. Soc. B Biol. Sci.* 269, 885–892. https://doi.org/10.1098/rspb.2001.1940

Ringsby, T.H., Saether, B.-E., Solberg, E.J., 1998. Factors Affecting Juvenile Survival in House Sparrow *Passer domesticus. J. Avian Biol.* 29, 241. https://doi.org/10.2307/3677106

Rivero, A., Gandon, S., 2018. Evolutionary Ecology of Avian Malaria: Past to Present. *Trends Parasitol.* 34, 712–726. https://doi.org/10.1016/j.pt.2018.06.002

Roenneberg, T., Wirz-Justice, A., Merrow, M., 2003. Life between clocks: Daily temporal patterns of human chronotypes. *J. Biol. Rhythms* 18, 80–90. https://doi.org/10.1177/0748730402239679

Roller, N.., Desser, S., 1973. Diurnal periodicity in peripheral parasitemias in ducklings (*Anas boschas*) infected with *Leucocytozoon simondi. Can. J. Zool.* 51.

Romero, L. M. (2004), Physiological stress in ecology: lessons from biomedical research. *Trends in Ecology & Evolution*, 19, 249–255.

Rund, S., O'Donnell, A., Gentile, J., Reece, S., 2016. Daily Rhythms in Mosquitoes and Their Consequences for Malaria Transmission. *Insects* 7, 14. https://doi.org/10.3390/insects7020014

Russ, A., Rüger, A., Klenke, R., 2015. Seize the night: European Blackbirds (*Turdus merula*) extend their foraging activity under artificial illumination. *J. Ornithol*. 156, 123–131. https://doi.org/10.1007/s10336-014-1105-1

Saini, C., Hutton, P., Gao, S., Simpson, R.K., Giraudeau, M., Sepp, T., Webb, E., McGraw, K.J., 2019. Exposure to artificial light at night increases innate immune activity during development in a precocial bird. *Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol*. 233, 84–88. https://doi.org/10.1016/j.cbpa.2019.04.002

Santure, A.W., Gratten, J., Mossman, J.A., Sheldon, B.C., Slate, J., 2011. Characterisation of the transcriptome of a wild great tit *Parus major* population by next generation sequencing. *BMC Genomics* 12, 283. https://doi.org/10.1186/1471-2164-12-283

Saper, C.B., Scammell, T.E., Lu, J., 2005. Hypothalamic regulation of sleep and circadian rhythms. *Nature* 437, 1257–1263. https://doi.org/10.1038/nature04284

Schaper, S. V, Dawson, A., Sharp, P.J., Gienapp, P., Caro, S.P., Visser, M.E., 2012. Increasing Temperature , Not Mean Temperature , Is a Cue for Avian Timing of Reproduction. *Am. Nat*. 179. https://doi.org/10.1086/663675

Scheiermann, C., Kunisaki, Y., Frenette, P.S., 2013. Circadian control of the immune system. *Nat. Rev. Immunol.* 13, 190–198. https://doi.org/10.1038/nri3386

Schoenle, L.A., Schoepf, I., Weinstein, N.M., Moore, I.T., Bonier, F., 2018. Higher plasma corticosterone is associated with reduced costs of infection in red-winged blackbirds. *Gen. Comp. Endocrinol.* 256, 89–98. https://doi.org/10.1016/j.ygcen.2017.07.006

Schneider, P., Rund, S.S.C., Smith, N.L., Prior, K.F., O'Donnell, A.J., Reece, S.E., 2018. Adaptive periodicity in the infectivity of malaria gametocytes to mosquitoes. *Proc. R. Soc. B Biol. Sci*. 285. https://doi.org/10.1098/rspb.2018.1876

Schroeder, A.M., Colwell, C.S., 2013. How to fix a broken clock. *Trends Pharmacol. Sci.* 34, 605–619. https://doi.org/10.1016/j.tips.2013.09.002

Shearman, L.P., Sriram, S., Weaver, D.R., Maywood, E.S., Chaves, I., Zheng, B., Kume, K., Lee, C.C., Van Der Horst, G.T.J., Hastings, M.H., Reppert, S.M., 2000. Interacting molecular loops in the mammalian circadian clock. *Science*, 288, 1013–1019. https://doi.org/10.1126/science.288.5468.1013

Shuboni, D., Yan, L., 2010. Nighttime dim light exposure alters the responses of the circadian system. *Neuroscience* 170, 1172–1178. https://doi.org/10.1016/j.neuroscience.2010.08.009

Skutch, A.F., 1962. The Constancy of Incubation, *The Wilson Ornithological Society*, 74, 115–152.

Slabbekoorn, H., Peet, M., 2003. Ecology: Birds sing at a higher pitch in urban noise. *Nature* 424, 267. https://doi.org/10.1038/424267a

Slagsvold, T., Lifjeld, J.T., 1985. Variation in plumage colour of the Great tit *Parus major* in relation to habitat, season and food. *J. Zool.* 206, 321–328. https://doi.org/10.1111/j.1469-7998.1985.tb05661.x

Sol, D., González-Lagos, C., Moreira, D., Maspons, J., Lapiedra, O., 2014. Urbanisation tolerance and the loss of avian diversity. *Ecol. Lett.* 17, 942–950. https://doi.org/10.1111/ele.12297

Song, P., Rockwell, C.E., Cui, J.Y., Klaassen, C.D., 2015. Individual bile acids have differential effects on bile acid signaling in mice. *Toxicol. Appl. Pharmacol*. 283, 57–64. https://doi.org/10.1016/j.taap.2014.12.005

Spencer, R., 1976. The ringer's manual, British Trust for Ornithology.

Spoelstra, K., Visser, M.E., 2013. The impact of artificial light on avian ecology, in: Gil, D., Brumm, H. (Eds.), Avian Urban Ecology. Oxford University Press, pp. 21–28. https://doi.org/10.1093/acprof:osobl/9780199661572.003.0002

Spoelstra, K., Verhagen, I., Meijer, D., Visser, M.E., 2018. Artificial light at night shifts daily activity patterns but not the internal clock in the great tit (*Parus major*). *Proc. R. Soc. B Biol. Sci*. 285. https://doi.org/10.1098/rspb.2017.2751

Steiger, S.S., Valcu, M., Spoelstra, K., Helm, B., Wikelski, M., Kempenaers, B., 2013. When the sun never sets: diverse activity rhythms under continuous daylight in free-living arctic-breeding birds. *Proc. Biol. Sci.* 280, 20131016. https://doi.org/10.1098/rspb.2013.1016

Steinmeyer, C., Kempenaers, B., Mueller, J.C., 2012. Testing for associations between candidate genes for circadian rhythms and individual variation in sleep behaviour in blue tits. *Genetica* 140, 219–228. https://doi.org/10.1007/s10709-012-9673-6

Stephan F.K., Zucker, I., 1972. Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. *Proc Natl Acad Sci USA* 69:1583–1586

Stokkan, K.A., Yamazaki, S., Tei, H., Sakaki, Y., Menaker, M., 2001. Entrainment of the circadian clock in the liver by feeding. *Science*, 291, 490–493. https://doi.org/10.1126/science.291.5503.490

Stuber, E.F., Grobis, M.M., Abbey-Lee, R., Kempenaers, B., Mueller, J.C., Dingemanse, N.J., 2014. Perceived predation risk affects sleep behaviour in free-living great tits, *Parus major*. *Anim. Behav*. 98, 157–165. https://doi.org/10.1016/j.anbehav.2014.10.010

Stuber, E.F., Dingemanse, N.J., Kempenaers, B., Mueller, J.C., 2015. Sources of intraspecific variation in sleep behaviour of wild great tits. *Anim. Behav*. 106, 201–221. https://doi.org/10.1016/j.anbehav.2015.05.025

Stuber, E.F., Baumgartner, C., Dingemanse, N.J., Kempenaers, B., Mueller, J.C., 2016. Genetic correlates of individual differences in sleep behavior of free-living great tits (*Parus major*). *G3 Genes, Genomes, Genet*. 6, 599–607. https://doi.org/10.1534/g3.115.024216

Stuber, E.F., Dingemanse, N.J., Mueller, J.C., 2017. Temperature affects frequency but not rhythmicity of nocturnal awakenings in free-living great tits, *Parus major*. *Anim. Behav*. 128, 135–141. https://doi.org/10.1016/j.anbehav.2017.03.004

Tan, J., Levine, S.L., Bachman, P.M., Jensen, P.D., Mueller, G.M., Uffman, J.P., Meng, C., Song, Z., Richards, K.B., Beevers, M.H., 2016. No impact of DvSnf7 RNA on honey bee (*Apis mellifera L.*) adults and larvae in dietary feeding tests. *Environ. Toxicol. Chem*. 35, 287–294. https://doi.org/10.1002/etc.3075

Team, Core R., 2018. R: A language and environment for statistical computing. R Found. Stat. Comput.

Thakur, S., Kumar, V., 2015. Avian photoreceptors and their role in the regulation of daily and seasonal physiology. *Gen. Comp. Endocrinol*. 220, 13–22. https://doi.org/10.1016/j.ygcen.2014.06.001

Tinbergen, J.., Boerlijst, M.., 1990. Nestling Weight and Survival in Individual Great Tits (*Parus major). J. Anim. Ecol*. 59, 1113–1127.

Titulaer, M., Spoelstra, K., Lange, C.Y.M.J.G., Visser, M.E., 2012. Activity patterns during food provisioning are affected by artificial light in free living great tits (*Parus major*). *PLoS One* 7, 5–8. https://doi.org/10.1371/journal.pone.0037377

Townroe, S., Callaghan, A., 2014. British container breeding mosquitoes: The impact of urbanisation and climate change on community composition and phenology. *PLoS One* 9. https://doi.org/10.1371/journal.pone.0095325

Tschirren, B., Fitze, P.S., Richner, H., 2003. Sexual dimorphism in susceptibility to parasites and cell-mediated immunity in great tit nestlings. *J. Anim. Ecol*. 72, 839–845. https://doi.org/10.1046/j.1365-2656.2003.00755.x

Turkowska, E., Rai, S., Majewski, P.M., Skwarło-Sońta, K., 2013. Diurnal and seasonal changes in IL-6 and IL-18 gene expression in blood leukocytes of male chickens with experimental peritonitis: The impact of lighting conditions and melatonin. *J. Anim. Feed Sci*. 22, 149–157. https://doi.org/10.22358/jafs/66006/2013

Turkowska, E., Majewski, P.M., Rai, S., Skwarlo-Sonta, K., 2014. Pineal oscillator functioning in the chicken--effect of photoperiod and melatonin. *Chronobiol. Int*. 31, 134–43. https://doi.org/10.3109/07420528.2013.832279

Ueda, H.R., Chen, W., Adachi, A., Wakamatsu, H., Hayashi, S., Takasugi, T., Nagano, M., Nakahama, K.I., Suzuki, Y., Sugano, S., Lino, M., Shigeyoshi, Y., Hashimoto, S., 2002. A transcription factor response element for gene expression during circadian night. *Nature* 418, 534–539. https://doi.org/10.1038/nature00906

Ueda, H.R., Hayashi, S., Chen, W., Sano, M., Machida, M., Shigeyoshi, Y., Iino, M., Hashimoto, S., 2005. System-level identification of transcriptional circuits underlying mammalian circadian clocks. *Nat. Genet*. 37, 187–192. https://doi.org/10.1038/ng1504

Underwood, H., Steele, C.T., Zivkovic, B., 2001. Circadian organization and the role of the pineal in birds. *Microsc. Res. Tech*. https://doi.org/10.1002/jemt.1068

Vallance, T.M., Zeuner, M.T., Williams, H.F., Widera, D., Vaiyapuri, S., 2017. Toll-Like Receptor 4 Signalling and Its Impact on Platelet Function, Thrombosis, and Haemostasis. *Mediators Inflamm*. 2017. https://doi.org/10.1155/2017/9605894

Van't Hof, T.J., Gwinner, E., 1996. Development of post-hatching melatonin rhythm in zebra finches (*Poephila guttata*). *Experientia* 52, 249–252. https://doi.org/10.1007/BF01920717

van Langevelde, F., Ettema, J.A., Donners, M., WallisDeVries, M.F., Groenendijk, D., 2011. Effect of spectral composition of artificial light on the attraction of moths. *Biol. Conserv*. 144, 2274–2281. https://doi.org/10.1016/j.biocon.2011.06.004

Van Noordwijk, A.J., Keizer, L.C.P., Van Balen, J.H., Scharloo, W., 1981. Genetic variation in egg dimensions in natural populations of the Great Tit. *Genetica* 55, 221–232. https://doi.org/10.1007/BF00127206

van Oosterhout, F., Lucassen, E.A., Houben, T., vanderLeest, H.T., Antle, M.C., Meijer, J.H., 2012. Amplitude of the SCN clock enhanced by the behavioral activity rhythm. *PLoS One* 7. https://doi.org/10.1371/journal.pone.0039693

van Riper, C., van Riper, S.G., Goff, M.L., Laird, M., 1986. The Epizootiology and Ecological Significance of Malaria in Hawaiian Land Birds. *Ecol. Monogr*. 56, 327–344. https://doi.org/10.2307/1942550

Videvall, E., Cornwallis, C.K., Palinauskas, V., Valkiunas, G., Hellgren, O., 2015. The avian transcriptome response to malaria infection. *Mol. Biol. Evol*. 32, 1255–1267. https://doi.org/10.1093/molbev/msv016

Visser, M.E., Noordwijk, A.J. Van, Tinbergen, J.M., Lessells, C.M., 1998. Warmer springs lead to mistimed reproduction in great tits (*Parus major*). *Proc. R. Soc. B Biol. Sci*. 265, 1867–1870. https://doi.org/10.1098/rspb.1998.0514

Visser, M.E., Lessells, C.M., 2001. The costs of egg production and incubation in great tits (Parus major). *Proc. R. Soc. B Biol. Sci.* 268, 1271–1277. https://doi.org/10.1098/rspb.2001.1661

Vitaterna, M.H., King, D.P., Chang, A.M., Kernhauser, J.M., Lowrey, P.L., McDonald, J.D., Dove, W.F., Pinto, L.H., Turek, F.W., Takahashi, J.S., 1994. Mutagenesis and mapping of a mouse gene, clock, essential for circadian behavior. *Science*, 264, 719–725. https://doi.org/10.1126/science.8171325

Vitaterna, M.H., Selby, C.P., Todo, T., Niwa, H., Thompson, C., Fruechte, E.M., Hitomi, K., Thresher, R.J., Ishikawa, T., Miyazaki, J., Takahashi, J.S., Sancar, A., 1999., Differential regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2. *Proc Natl Acad Sci USA* 96:12114–12119

Vivanco P, Rol MÁ, Madrid JA. (2010). Temperature cycles trigger noc- turnalism in the diurnal homeotherm *Octodon degus. Chronobiol. Int*. 27:517–534

Wang, G., Harpole, C.E., Trivedi, A.K., Cassone, V.M., 2012. Circadian Regulation of Bird Song, Call, and Locomotor Behavior by Pineal Melatonin in the Zebra Finch. *J. Biol. Rhythms* 27, 145–155. https://doi.org/10.1177/0748730411435965

Wang, G., Harpole, C.E., Paulose, J., Cassone, V.M., 2014. The role of the pineal gland in the photoperiodic control of bird song frequency and repertoire in the house sparrow, *Passer domesticus. Horm. Behav*. 65, 372–379. https://doi.org/10.1016/j.yhbeh.2014.02.008

Wang, Y., Su, M.A., Wan, Y.Y., 2011. An Essential Role of the Transcription Factor GATA-3 for the Function of Regulatory T Cells. *Immunity* 35, 337–348. https://doi.org/10.1016/j.immuni.2011.08.012

Watson, H., Videvall, E., Andersson, M.N., Isaksson, C., 2017. Transcriptome analysis of a wild bird reveals physiological responses to the urban environment. *Sci. Rep.* 7, 44180. https://doi.org/10.1038/srep44180

Webb, D.R., 1987. Thermal Tolerance of Avian Embryos: A Review. *Condor* 89, 874. https://doi.org/10.2307/1368537

Weibel, L., Maccari, S., Reeth, O. Van, 2002. Circadian Clock Functioning Is Linked to Acute Stress Reactivity in Rats. *J. Biol. Rhythms* 17, 438–446. https://doi.org/10.1177/074873002237138

Westwood, M.L., O'Donnell, A.J., de Bekker, C., Lively, C.M., Zuk, M., Reece, S.E., 2019. The evolutionary ecology of circadian rhythms in infection. *Nat. Ecol. Evol.* 3, 552–560. https://doi.org/10.1038/s41559-019-0831-4

Wickham, H., 2014. Package "ggplot2" Title An implementation of the Grammar of Graphics. Bioinformatics.

Williams, R.B., 2005. Avian malaria: Clinical and chemical pathology of Plasmodium gallinaceum in the domesticated fowl *Gallus gallus. Avian Pathol*. 34. https://doi.org/10.1080/03079450400025430

Wolfson, F., 1936. Synchronicity, periodicity, and the length of the asexual cycle of *Plasmodium rouxi* in the canary. *Am. J. Epidemiol*. 23, 340–348. https://doi.org/10.1093/oxfordjournals.aje.a118222

Woodford, L., Bianco, G., Ivanova, Y., Dale, M., Elmer, K., Rae, F., Larcombe, S.D., Helm, B., Ferguson, H.M., Baldini, F., 2018. Vector species-specific association between natural *Wolbachia* infections and avian malaria in black fly populations. *Sci. Rep*. 8, 1–11. https://doi.org/10.1038/s41598-018-22550-z

Wright, K.P., McHill, A.W., Birks, B.R., Griffin, B.R., Rusterholz, T., Chinoy, E.D., 2013. Entrainment of the human circadian clock to the natural light-dark cycle. *Curr. Biol*. 23, 1554–1558. https://doi.org/10.1016/j.cub.2013.06.039

Xue, M., Shi, X., Zhao, Y., Cui, H., Hu, S., Cui, X., Wang, Y., 2013. Effects of reticuloendotheliosis virus infection on cytokine production in SPF chickens. *PLoS One* 8, 1–10. https://doi.org/10.1371/journal.pone.0083918

Yagita, K., Tamanini, F., Van der Horst, G.T.J., Okamura, H., 2001. Molecular mechanisms of the biological clock in cultured fibroblasts. *Science*, 292, 278–281. https://doi.org/10.1126/science.1059542

Yamazaki S, Kerbeshian MC, Hocker CG, Block GD, Menaker M., 1998. Rhythmic properties of the hamster suprachiasmatic nucleus in vivo. *J Neurosci* 18:10709–10723

Yasuo, S., Watanabe, M., Okabayashi, N., Ebihara, S., Yoshimura, T., 2003. Circadian clock genes and photoperiodism: Comprehensive analysis of clock gene expression in the mediobasal hypothalamus, the suprachiasmatic nucleus, and the pineal gland of Japanese quail under various light schedules. *Endocrinology* 144, 3742–3748. https://doi.org/10.1210/en.2003-0435

Yin, L., Wu, N., Curtin, J.C., Qatanani, M., Szwergold, N.R., Reid, R.A., Waitt, G.M., Parks, D.J., Pearce, K.H., Wisely, G.B., Lazar, M.A., 2007. Rev-erb , a Heme Sensor That Coordinates Metabolic and Circadian Pathways. *Science* (80-. ). 318, 1786–1789. https://doi.org/10.1126/science.1150179

Yoo SH, Ko CH, Lowrey PL, Buhr ED, Song EJ, Chang S, Yoo OJ, Yamazaki S, Lee C, Takahashi JS., 2005. A noncanonical E-box enhancer drives mouse Period2 circadian oscillations in vivo. *Proc Natl Acad Sci USA* 102:2608–2613

Yoshimura, T., Makino, E., Kuroiwa, A., Matsuda, Y., Namikawa, T., Ebihara, S., 2000. Molecular analysis of avian circadian clock genes. *Mol. Brain Res*. 78, 207–215. https://doi.org/10.1016/S0169-328X(00)00091-7

Zeileis, A., Hothorn, T., 2002. Diagnostic checking in regression relationships. http://cran.r-project.org/doc/Rnews/. *R News* 2(3), 7—10.

Zeman, M., Gwinner, E., 1993. Ontogeny of the rhythmic melatonin production in a precocial and an altricial bird, the Japanese quail and the European starling. *J. Comp. Physiol*. A 172, 333–338. https://doi.org/10.1007/BF00216615

Zeman, M., Szántóová, K., Herichová, I., 2009. Ontogeny of circadian oscillations in the heart and liver in chicken. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol*. 154, 78–83. https://doi.org/10.1016/j.cbpa.2009.05.005

Zeman, M., Herichová, I., 2011. Circadian melatonin production develops faster in birds than in mammals. *Gen. Comp. Endocrinol*. 172, 23–30. https://doi.org/10.1016/j.ygcen.2010.12.022

Zhang, X., Yang, W., Liang, W., Wang, Y., Zhang, S., 2019. Intensity dependent disruptive effects of light at night on activation of the HPG axis of tree sparrows (*Passer montanus*). *Environ. Pollut*. 249, 904–909. https://doi.org/10.1016/j.envpol.2019.03.008

Zhang, Y., Zhu, X., Feng, Y., Pang, W., Qi, Z., Cui, L., Cao, Y., 2016. TLR4 and TLR9 signals stimulate protective immunity against blood-stage *Plasmodium yoelii* infection in mice. *Exp. Parasitol*. 170, 73–81. https://doi.org/10.1016/j.exppara.2016.09.003

Zhang, K., Wang, R., Shen, C. and Da, L., 2010. Temporal and spatial characteristics of the urban heat island during rapid urbanization in Shanghai, China. *Environmental monitoring and assessment*, 169(1-4), pp.101-112.

Zhao, W., Liu, J., Xu, R., Zhang, C., Pang, Q., Chen, X., Liu, S., Hong, L., Yuan, J., Li, X., Chen, Y., Li, J., Su, X., 2015. The gametocytes of *Leucocytozoon sabrazesi* infect chicken thrombocytes, not other blood cells. *PLoS One* 10, 1–17. https://doi.org/10.1371/journal.pone.0133478