THE PHYSIOLOGICAL EFFECTS OF ARTIFICIAL LIGHT AT NIGHT ON THE AUSTRALIAN BLACK FIELD CRICKET

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Who’s jovial excitement and curiosity fostered my love of science
Abstract

The presence of artificial light at night (ALAN) is one of the fastest growing, most pervasive and, until recently, under-appreciated forms of global pollution. Current ALAN levels in urban environments are associated with changes to animal behaviour, dramatic shifts in the timing of life history events, reductions in individual fitness and disrupted physiological processes, including immune function. This thesis explores the physiological effects of ecologically relevant levels of ALAN on a model invertebrate species, the Australian black field cricket, *Teleogryllus commodus*.

In Chapter 1, I reviewed the literature with a particular emphasis on the physiological effects of ALAN, including growth, survival, reproductive success, and immune function. I also speculate as to the potential mechanistic links behind these ALAN induced biological effects.

In Chapter 2, I explored experimentally the effects of ecologically relevant levels of ALAN (1, 10 and 100 lux) on life history and fitness traits of the black field cricket. Under controlled laboratory conditions, I reared crickets from egg to adult in an environment with either no ALAN (0 lux) or one of the above dim-ALAN intensities and assessed the consequences of ALAN for growth, survival and reproductive success. I demonstrated that egg hatch, adult survival and reproductive measures were largely unaffected by the presence of ALAN, however juvenile development time was longer and adults were larger when crickets were exposed to any light at night (1, 10 or 100 lux).

In Chapter 3, I examined the effects of ALAN (1, 10 and 100 lux) on three key measures of adult immune function (haemocyte concentration, lytic activity, and phenoloxidase activity). The presence of any ALAN (1, 10 or 100 lux) had a clear negative effect on the cellular immune response. Specifically, individuals exposed to any ALAN were unable to increase their haemocyte concentration in response to a stressor challenge.

In Chapter 4, I investigated a novel method for the measurement of circulating melatonin.
in small samples of cricket haemolymph using high-performance liquid chromatography tandem mass spectrometry, with methyl tert-butyl ether (MTBE)/ethyl acetate as an extraction agent. The calibration curve for melatonin was linear in the range of 0.25 and 10 pM (R² = 0.999), and the limit of detection was 0.25pM. When applied to a set of pilot data from crickets reared under different ALAN environments (0, 1, 10, and 100 lux), the results were however inconclusive, due to small sample sizes.

In Chapter 5, I discuss the significance of these findings and their ecological implications. My thesis advances our understanding of the biological effects of ALAN for invertebrates, a key taxon contributing to ecological community structure and composition. It is one of the first set of studies to simultaneously investigate multiple traits in the same individuals exposed to lifelong ALAN, and to assess changes in immune function throughout their adult life.

Combined, the results presented demonstrate a disruption to physiological processes, and highlight the potential for ALAN to alter the phenology of communities and reduce the overall fitness of individuals.

**KEYWORDS:** light pollution, invertebrate, development, life history traits, immune function, melatonin, antioxidant, phenology, urbanisation, *Teleogryllus commodus*
Declaration

This is to certify that:

(i) This thesis comprises of my own original work towards the degree of Master of Philosophy, except where indicated in the Preface.

(ii) Due acknowledgement has been made in the text to all other material used.

(iii) This thesis is less than 50,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Joanna Durrant

June 2018
Preface

This thesis contains one study (Chapter 2) published in a peer-reviewed academic journal (Journal of Experimental Zoology Part B: Molecular and Developmental Evolution) and one study (Chapter 3) to be submitted to an academic journal (Journal of Insect Physiology). These studies appear as in the thesis as chapters with minor modifications from their published/prepared format. As such, there is some necessary repetition within the introductions, descriptions of husbandry, and discussions. For all chapters I am the primary author and contributor and all manuscripts are included with the permission of all co-authors. Manuscripts are presented in their published format as appendices. Contributions to each chapter are outlined below.

For Chapter 2, *Artificial light at night prolongs juvenile development time in the black field cricket, Teleogryllus commodus*, I contributed to experimental design, undertook the data collection, data analysis, and manuscript preparation; L. Michael Botha was responsible for data collection and manuscript revision; Mark P. Green participated in experimental design, and manuscript preparation; Therése M. Jones conceived the study, participated in experimental design, data analysis and manuscript preparation.

For Chapter 3, *Dim artificial light at night reduces the cellular immune response of the black field cricket, Teleogryllus commodus*, I contributed to experimental design, undertook the data collection, data analysis, and manuscript preparation; Mark P. Green participated in experimental design, data analysis and manuscript preparation; Therése M. Jones conceived the study, and participated in experimental design, data analysis and manuscript preparation.

For Chapter 4, *The effect of ALAN on melatonin in crickets: A novel HPLC-MS method for the measurement of melatonin in haemolymph*, I contributed to experimental design, method development, undertook the data collection, data analysis, and wrote the chapter content; Thusitha Rupasinghe assisted with the HPLC-MS method development; Mark P. Green participated in experimental design, data analysis and content revision; Therése M. Jones conceived the study, and participated in experimental design, data analysis and
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# Table of Contents

ABSTRACT .................................................................................................................. I
DECLARATION ............................................................................................................. III
PREFACE ......................................................................................................................... IV
ACKNOWLEDGEMENTS .............................................................................................. VI
TABLE OF CONTENTS ............................................................................................... VIII
LIST OF TABLES .......................................................................................................... XI
LIST OF FIGURES ........................................................................................................... XII

## CHAPTER 1: GENERAL INTRODUCTION

ARTIFICIAL LIGHT AT NIGHT (ALAN) ........................................................................ 2
ALAN AND LIFE HISTORY TRAITS ........................................................................... 5
ALAN AND IMMUNE FUNCTION ................................................................................. 7
ALAN AND MELATONIN – A POTENTIAL MECHANISTIC LINK ................................ 8
THE RESEARCH CHALLENGE ................................................................................... 13
THESIS OBJECTIVES AND AIMS ............................................................................. 15

## CHAPTER 2: ARTIFICIAL LIGHT AT NIGHT PROLONGS JUVENILE DEVELOPMENT TIME IN THE BLACK FIELD CRICKET, *TELEOGRYLLUS COMMODUS*

ABSTRACT .................................................................................................................. 18
INTRODUCTION ............................................................................................................. 19
METHODS ......................................................................................................................... 21
Experimental ALAN treatments and rearing conditions .......................................... 21
Juvenile development and survival .......................................................................... 22
Adult morphology and survival ................................................................................ 23
Reproductive output .................................................................................................. 23
Statistical analyses ..................................................................................................... 25
RESULTS ......................................................................................................................... 26
Juvenile development and survival .......................................................................... 26
Adult morphology and survival ................................................................................ 27
Reproductive output .................................................................................................. 28
List of Tables

TABLE 2.1: Generalised mixed models exploring the effect of ALAN treatment (0 lux, 1 lux, 10 lux or 100 lux) on (A) the probability of egg hatch, (B) juvenile survival, (C) juvenile development time, (D) adult size, (E) adult scaled mass index at mating, (F) adult female survival, (G) adult male survival, (H) number of eggs laid per day by treatment females (I) number of eggs laid per day by stock females, and the (J) proportion live sperm. ................................................................. 29

TABLE 3.1: Generalised linear mixed outputs from models exploring the initial (week 0 after final moult) effect of lifelong ALAN exposure (0, 1, 10 and 100 lux) Ln (haemocyte concentration in cells/ml x 10^6) (A, D), change in lytic activity (B, E) and change in Phenoloxidase (PO) activity (C, F) for females and males respectively .. 47

TABLE 3.2: Mixed models exploring the effect of lifelong ALAN treatment (0, 1, 10 and 100 lux) over time (Weeks 0, 2 and 4 after final moult) for (A) haemocyte concentration (cells/ml x 10^6), (B) lytic activity (Δ absorbance) and (C) Phenoloxidase (PO) activity (Δ absorbance). ............................................. 48

TABLE 3.3: Spearman rank correlations (r_s) between haemocyte concentrations, lytic activity and Phenoloxidase (PO) activity for females and males across the three sampling periods (Week 0, Week 2 and Week 4). .............................................. 50

TABLE 4.1: Mixed models exploring the effect of ALAN treatment (0, 1, 10 and 100 lux) on circulating melatonin concentrations in cricket haemolymph: (A) Excluding extreme outliers, and (B) including extreme outliers. .................................................. 68

Table II.1: Mixed models (prior to model reduction) exploring the effect of lifelong ALAN treatment (0, 1, 10 and 100 lux) over time (Weeks 0, 2 and 4 after final moult) for (A) haemocyte concentration (cells/mL x 10^6), (B) lytic activity (Δ absorbance) and (C) Phenoloxidase (PO) activity (Δ absorbance). Family, Individual ID and Assay Plate number were included as random terms in all models................................. 110
List of Figures

FIGURE 1.1: World map depicting the extent of artificial sky brightness at night, shown as a ratio to the natural sky brightness. ................................................................. 3

FIGURE 1.2: Simplified schematic biosynthetic pathway depicting the production of melatonin in the pineal gland of vertebrates. Neural input is required for the rate-limiting step in the vertebrate pathway involving the conversion of serotonin to N-acetyl-serotonin (NAS). Enzymes shown in italics; c-AMP = cyclic AMP; βR, NA = β-adrenergic receptors. ................................................................. 10

FIGURE 1.3: A simplified depiction of the daily rhythm and natural oscillation of melatonin (A) without ALAN, and (B) with the introduction of ALAN. ......................... 11

FIGURE 1.4: The hypothesized key physiological implications of artificial light at night (ALAN). Solid arrows represent proposed causal mechanistic cascades, and dashed arrows represent proposed biological links. Grey boxes are aspects that will be directly addressed in this thesis. ................................................................. 13

FIGURE 2.1: The median proportion of juveniles within each family surviving to complete the final moult for each of the four ALAN treatments (n = 13 families per ALAN treatment). ................................................................. 30

FIGURE 2.2: Median (A) juvenile development time (days), and (B) femur length for crickets reared under each ALAN treatment (of 0 lux, 1 lux, 10 lux and 100 lux) ............ 31

FIGURE 3.1: Variation across ALAN treatments in (A) haemocyte concentration (B) lytic activity, and (C) Phenoloxidase (PO) activity for adult crickets reared from egg through to adults under night-time light levels (ALAN treatment) of 0 lux, 1 lux, 10 lux and 100 lux. ................................................................. 51

FIGURE 3.2: Variation in (A) haemocyte concentration (B) lytic activity, and (C) Phenoloxidase (PO) activity for adult cricket at Week 0, Week 2 and Week 4 after the final moult. ................................................................. 52
FIGURE 3.3: Variation in lytic activity between sampling week and (A) sex and (B) ALAN treatment.

FIGURE 4.1: The composite curve made up of three melatonin standard curves placed at the beginning, middle and end of the sample HPLC-MS run. $R^2 = 0.999$ ($y = 416.2x - 35.6$).

FIGURE 4.2: Circulating melatonin concentration of pooled samples from crickets reared in each ALAN treatment (A) excluding outliers; and (B) including outliers.

FIGURE 5.1: A summary of the potential physiological processes and interactions under artificial light at night (ALAN) conditions, with the biological effects demonstrated in this thesis presented in grey boxes, and bold text indicating significant effects. Solid arrows represent proposed causal mechanistic cascades, and dashed arrows represent proposed biological links.

FIGURE I.1: The spectral output of “day” lights in the incubators used for Chapters 2, 3 and 4.

FIGURE I.2: The spectral output of “night” lights in the incubators used for Chapters 2, 3 and 4.

FIGURE III.1: (A) The sample mass spectrum, and (B) chromatogram for the HPLC-MS melatonin detection method presented in Chapter 4.
CHAPTER 1: General Introduction
Throughout the course of the evolution of life on earth one factor that has remained constant is the transition between day and night. The daily and seasonal changes in the presence of light and dark was a reliable signal until around 140 years ago with the invention of electrical lighting (Hughes 1987). Now, artificial light pervades the night environment, altering this fundamental daily signal that for many species is a biological cue. While organisms have continually adapted to the changing environment of Earth’s atmosphere and landscapes, rarely have the environmental shifts been as rapid as those caused by recent human intervention (Vitousek et al. 1997; Grimm et al. 2008).

**Artificial light at night (ALAN)**

Light can be measured in several ways, but two of the most common units are Lumen and Lux. Lumen (symbol: lm) is a measure of the total amount of visible light emitted from a light source such as light globes (Thimijan & Heins 1983). Lux (symbol: lx), on the other hand, takes into account the area that the light covers, where 1 lx = 1 lm/m\(^2\) (Thimijan & Heins 1983). The further away from the source of light, the lower the light intensity, therefore Lux is a more useful term when measuring the amount of light at a specific geographical location. Natural variation in light intensity across a given day can be enormous. A bright sunny day can reach 100,000 lx, or be as low as 1000 lx on an overcast day. In contrast, natural night-time light levels are around a million times dimmer, varying between 0.0001 (cloudy moonless night) and 0.3 lx (cloudless night with a full moon) (Gaston et al. 2013). Light can also be measured by the colour temperature it produces, known as Kelvin (symbol: K), on a scale from 1,000 to 10,000, with temperatures below about 4000 K appearing reddish, and temperatures above about 7500 K appearing bluish. It is worth noting that both Lux and Kelvin are measures of light that are based on human vision, and therefore do not necessarily capture the relative experiences for other species, many of which are unknown (Gaston et al. 2015b). However, given their use in the design and mitigation of artificial lighting systems by policy makers and practitioners, both Lux and Kelvin are useful measures in ecological studies, due to their applied relevance (Gaston et al. 2012).
Light pollution, the excessive, misdirected, and unwanted consequence of outdoor artificial lighting, is considered one of the fastest growing and most pervasive forms of environmental pollution, increasing globally at a rate of around 6% per year (Holker et al. 2010). In many urban regions and peri-urban environments, the level of artificial light at night (ALAN) can be as high as 15 lx, around 100-fold brighter than the average of a full moon (approximately 0.1 to 0.3 lx) (Garstang 2004; Gaston et al. 2013; Bennie et al. 2015b; Bennie et al. 2016; Kyba et al. 2017). Skyglow is light reflected back down from the brightness of the sky around areas with high levels of light pollution (Kyba et al. 2015). In contrast to natural light from the moon, skyglow increases with increased cloud cover, and unlike direct ALAN, can extend far beyond the source, reaching environmentally protected national parks and marine areas (Davies et al. 2014; Gaston et al. 2015a; Guetté et al. 2018; see Figure 1.1).

Prior to the introduction of ALAN, variation in natural light cycles existed on a daily level with the transition between day and night; on a seasonal level, as determined by relative day length; as well as by lunar cycles, with the natural variation in the amount of light given off by different phases of the moon. Natural light cues are crucial for individuals to maintain the daily and seasonal rhythms of life and, given the evolutionary consistency of this environmental cue, many, if not all, behavioural patterns and physiological

Figure 1.1: World map depicting the extent of artificial sky brightness at night, shown as a ratio to the natural sky brightness (Falchi et al. 2016).
processes are highly influenced by the cycles of light and dark, either directly and indirectly (Bradshaw & Holzapfel 2007). Even the relatively tiny fraction of variation in night-time lunar illumination (between 0.0001 and 0.3 lx) drives behavioural patterns of nocturnal animals (Navara & Nelson 2007). Anthropogenic sources of ALAN, including skyglow (which can be as bright as 1.5 lx), potentially mask such natural variation in light and consequently disrupt the biological processes driven by the environmental cue that this provides (Davies et al. 2013; Falchi et al. 2016).

The ecological consequences of chronic exposure to even relatively low intensity ALAN are reported for many species groups, including plants, invertebrates and vertebrates. Behavioural disruption includes shifts in foraging (Bird et al. 2004; Santos et al. 2010; Becker et al. 2013), daily movement and migration (Moore et al. 2001; Poot et al. 2008; Stone et al. 2009; Riley et al. 2012), and reproductive behaviour (Miller 2006; Kempenaers et al. 2010). Physiological effects include endocrine disruption (Dominoni et al. 2013b; Brüning et al. 2015; Robert et al. 2015; Ouyang et al. 2018), developmental shifts (van Geffen et al. 2014), altered reproductive physiology (Dominoni et al. 2013a), an increased risk of disease and illness (Smolensky et al. 2015; Touitou et al. 2017), and reduced immune function (Bedrosian et al. 2011b).

Increasingly apparent is that species respond differently to the presence of ALAN. For example, the presence of constant illumination affects the timing of fish egg hatch, but the direction of this shift is species specific (Bruning et al. 2011). The level of exposure to ALAN is also not uniform across species and taxa. Nocturnal animals (including 30% of vertebrates and >60% of invertebrate species) that are active during the night when artificial light is present, are likely to experience greater effects of an increase in ALAN (Holker et al. 2010). This is particularly pertinent for crepuscular species whose activity patterns and behaviours are centred around the naturally semi-dark periods of dawn and dusk (Kelber et al. 2005; Haim & Portnov 2013).

The effects of ALAN also extend beyond individuals and can have impacts at an ecosystem level (Gaston et al. 2014). For example, changes to seasonal biology and the timing of life history events, as shown in European songbirds (Da Silva et al. 2015) could have cascading effects and cause disruption of ecosystem phenology; Bennie et al.
(2015a) showed that ALAN can alter the demographics of specialist herbivores through trophic cascade bottom-up effects; and different levels of ALAN tolerance, as shown in bat species (Lacoeuilhe et al. 2014), could ultimately result in biodiversity loss through competitive advantages. Given the negative consequences of ALAN, both at the individual and ecosystem level, as well as its current distribution and potential for spread, ALAN is currently considered to be a potential threat to biodiversity (Holker et al. 2010; Stone et al. 2012; Kyba & Hölker 2013).

**ALAN and life history traits**

Life history traits are the biological processes and behaviours in an organism’s life that have been shaped by natural selection to ensure the optimum survival and ultimately fitness of each individual in their physical and ecological environment (Flatt & Heyland 2011). Key life history measures include size, growth patterns, time to maturity, reproductive investments, the number and sex ratio of offspring, as well as mortality and length of life (Stearns 1992).

Many of the observed biological effects of ALAN, particularly in terms of life history, can be linked to a distorted circadian rhythm caused by an altered perception of day and night, and an associated masking of seasonal variation in day length (Gaston et al. 2014; Gaston et al. 2017). Plants and animals use photoperiod as a primary cue to stimulate physiological processes and inform the timing of various life history stages, such as egg hatch, germination and the onset of reproduction, given that seasonally optimal timing enhances survival (Dawson et al. 2001; Lyons-Sobaski & Berlocher 2009). Changes to key life history traits are usually adaptive, but when in response to an artificially altered environmental stimulus these changes can often have a negative effect on an individual’s overall fitness (Stearns 1976).

Demonstrated changes to growth and development in response to varying levels of ALAN (between 1 and 100 lx) are reported for a range of species (Dominoni et al. 2016). In fish, the presence of ALAN (between 1 and 100 lx) during early embryonic development is linked to variation in egg hatch and swim bladder inflation (Bruning et al. 2011).
Similarly, in the fruit fly, *Drosophila jambulina*, synchronicity of adult eclosion is negatively related to both the presence and intensity of ALAN (5 and 50 lx) (Thakurdas et al. 2009), while in the moth, *Mamestra brassicae*, males reared from the second instar under dim (7 lx) green or white lights at night spent less time in pupal diapause than moths reared with no light at night (van Geffen et al. 2014). Raap et al. (2016a) found that great tit chicks (*Parus major*) experienced lower growth rates after just two nights of exposure to ALAN of 3 lx.

Artificial light at night also affects the timing of reproduction. A study investigating the effects of ALAN on five common forest-breeding birds found that individuals breeding in territories with street lights started egg-laying on average 1.5 days sooner (Kempenaers et al. 2010). In Tammar wallabies (*Macropus eugenii*), individuals exposed to consistent levels of ALAN less than 1 lx gave birth to their offspring one month later than when no ALAN was present (Robert et al. 2015) and blackbirds (*Turdus merula*) exposed to just 0.3 lx ALAN developed their reproductive system up to one month sooner than those under natural lighting conditions (Dominoni et al. 2013a). Such shifts to growth, development and the timing of reproduction have the potential to create trophic mismatches that may lead to resource depletion and reductions in offspring success (Robert et al. 2015; Gaston et al. 2017).

Whether ALAN can directly affect egg and sperm production remains relatively undocumented. In Siberian hamsters (*Phodopus sungorus*), short days are naturally associated with a decrease in sperm production, but when kept under short-day conditions with the presence of ALAN (5 lx), male hamsters did not demonstrate this natural decrease in sperm production. A recent study using *Drosophila melanogaster* also showed that the presence of ALAN (between 1 and 100 lx) reduced the probability of a female commencing oviposition, as well as the number of eggs laid (McLay et al. 2017). Additionally, indirect evidence exists suggesting that clock-gene deficient *Drosophila* (who therefore lack a normal circadian rhythm) produce less sperm and lay fewer eggs (Beaver et al. 2002).

Finally, very few studies have directly assessed differences in survival of individuals living under ALAN conditions, with the exception of a recent study, which found that
Drosophila melanogaster housed under ALAN of 10 and 100 lx had lower survival than those kept in 0 lx (McLay et al. 2017). It has also been shown that constant illumination can accelerates aging in mice (Mus musculus) and rats (Rattus norvegicus) resulting in a shorter lifespan (Anisimov et al. 2004; Vinogradova et al. 2009). A shorter life span and reduced survival could ultimately decrease the chances of successful reproduction and affect species persistence.

**ALAN and immune function**

An area that has been less well studied in relation to the presence of ALAN is its impact on immune function. The immune system is critical for defending an organism against bacterial and viral infections and other pathogens, and is therefore a crucial component of individual fitness (Schmid-Hempel 2005). The immune strategies of vertebrates and invertebrates differ in many ways, although similar pathways exist between the two. Vertebrates possess an innate immune system, as well as a highly adaptive immune system, which has the ability to “remember” previous immune insults and challenges (Flajnik & Kasahara 2010). In contrast, invertebrates have an innate immune system, but lack the highly adaptive immune responses typical of vertebrates (Beck & Habicht 1996) (but see: Moret & Siva-Jothy 2003; Loker et al. 2004). The innate (or non-specific) immune system of invertebrates includes the cellular response, which refers to the immune cells (haemocytes) that attack foreign or infected cells in the body (Beck & Habicht 1996; Murphy & Weaver 2016). It also includes chemicals, enzymes and antimicrobial peptides in body fluids that are upregulated in response to intrusion of foreign bodies (Cooper & Lemmi 1981; Murphy & Weaver 2016).

The physiological effects of exposure to ALAN include an increased risk of disease and illness (Hastings et al. 2003; Fonken et al. 2013; Borniger et al. 2014; Smolensky et al. 2015; Dominoni et al. 2016), in part due to the overwhelmingly negative ALAN effects on immune function, which are evident in a range of species, both vertebrate and invertebrate (Moore & Siopes 2000; Bedrosian et al. 2011b; Aubrecht et al. 2014; Durrant et al. 2015; Raap et al. 2016b). There are several different factors that can modulate innate immunity and an individual’s responsiveness, and many trade-offs exist between immune
function and life history traits (Lochmiller & Deerenberg 2000; Martin et al. 2008). Many of the observed immunological effects of ALAN can, at least in part, be linked to light-induced circadian disruption and its subsequent impact on the immune system (Logan & Sarkar 2012; Scheiermann et al. 2013; Borniger et al. 2017; Markowska et al. 2017). Seasonal variation in the amount of light present over a given 24 hour period can initiate physiological responses to appropriately equip an individual for the environmental conditions, including driving the onset and cessation of reproduction and regulating immune capacity (Nelson et al. 1995).

Given the importance of a functional immune system for individual fitness and survival (Schmid-Hempel 2005), understanding the effect that ALAN can have on these key physiological processes for organisms living in and around highly lit urban environments is of vital importance. The majority of studies to date exploring the effects of ALAN on immune function are vertebrate focused and typically immune function is assessed at a single time point; longitudinal studies exploring the effect of ecological relevant levels of ALAN at an individual level are relatively rare. The relatively simple immune systems of invertebrates, when compared to vertebrates, makes them a good model to explore the effects of ALAN on immune function over the life of an individual.

**ALAN and melatonin – a potential mechanistic link**

There are numerous mechanistic factors and interactions that are likely to play a role in the physiological effects of ALAN, such as the effect that alterations to daily and seasonal light cycles has on circadian rhythms (Dominoni et al. 2016; Touitou et al. 2017; Aulsebrook et al. 2018), clock-genes and metabolic function (Marcheva et al. 2013; Gaston et al. 2017), and a multitude of endocrine hormones including gonadotrophins, corticosterone, thyroid hormones, and melatonin (Ouyang et al. 2018; Russart & Nelson 2018). Alterations to these hormones can have flow on effects due to their complex interaction with other biological compounds and hormones, such as ghrelin and leptin, which are associated with appetite (Kirsz & Zieba 2012; Kirsz et al. 2017). The compound which has gained the most traction as a core mechanistic link between ALAN and reduced fitness is the indoleamine melatonin (Navara & Nelson 2007; Haim & Zubidat 2015;
Melatonin is a biological compound that has been identified in almost all major taxonomic groups, including bacteria, plants and animals (Vivien-Roels & Pevet 1993; Hardeland & Poeggeler 2003; Pandi-Perumal et al. 2006a), and is best known as a key player in the maintenance of circadian systems (Reiter 1993; Claustrat et al. 2005). The formation and key roles of melatonin are thought to be highly conserved across taxa (Poeggeler 1993; Vivien-Roels & Pevet 1993) to the extent that its biosynthesis from tryptophan to serotonin to melatonin is believed to be comparable for both vertebrates and invertebrates (Figure 1.2) (Vivien-Roels & Pevet 1993; Tan et al. 2007). The primary site of circadian production of melatonin is the pineal gland in vertebrates (Tan et al. 2010), and the cerebral ganglion in invertebrates (Binkley 1993). However, melatonin is also synthesised and present in many other tissues and organs including the reproductive organs (Ralph 1981; Tamarkin et al. 1985; Itoh et al. 1994), circulating body fluids (Zachmann et al. 1992; Huether 1993), and the gut (Ralph 1981; Huether 1993; Itoh et al. 1995). It is less clear whether these sites demonstrate circadian production like the pineal gland and cerebral ganglion, although the persistence of nocturnal rises in circulating melatonin following pinealectomy suggest that this is likely the case (Huether 1993).
Melatonin is known as the biological timekeeper because it translates environmental information about the relative length of night and day (and thus both time of day and season) into an internal biological cue (Zawilska 1996; Arendt & Skene 2005; Pandi-Perumal et al. 2006a). It is able to do this because it is typically only synthesised during darkness, and is rapidly metabolised through multiple different pathways (Tan et al. 2007). Light suppresses the synthesis of melatonin during the day, resulting in an oscillating diurnal rhythm with nocturnal peaks and daily troughs in melatonin concentrations (Figure 1.3A) (Zawilska 1996; Arendt & Skene 2005).
The natural synthesis patterns of melatonin and its responsiveness to the stimulus of light and dark means that the presence of ALAN, which is likely to severely reduce the amount of true darkness, has the potential to reduce the overall production of melatonin. Ultimately this will disrupt the biochemical mechanisms that underpin many aspects of an individual’s circadian rhythm (Brainard et al. 1982; Chepesiuk 2009). Figure 1.3B depicts the hypothesised effect of ALAN on daily melatonin levels.

Beyond the fundamental importance of melatonin for the maintenance of a regular circadian rhythm, it is also a powerful antioxidant (for reviews, see Tan et al. 2010; Garcia et al. 2014). As an antioxidant, melatonin aids in “mopping up” free radicals and thus mitigates oxidative stress that can lead to cellular and tissue damage, reduced immune function and an associated increased risk of disease and illness (Reiter et al. 2003; Reiter et al. 2010). Gametes are particularly susceptible to free radical insult and oxidative stress given their relative lack of mechanisms to counter oxidative stress, compared to somatic cells (Agarwal et al. 2006), and accordingly, melatonin has a demonstrated role in the production and preservation of gametes and embryos (Cruz et al. 2014).

Melatonin also functions as a key neuroendocrine signal that interacts with various
receptors and cytoplasmic proteins to regulate many aspects of biological function, including the immune system (Srinivasan et al. 2005; Carrillo-Vico et al. 2013; Esteban et al. 2013; Pohanka 2013; Weil et al. 2015). Several studies have recorded a positive relationship between melatonin and immune function (Drazen & Nelson 2001; Moore & Siopes 2003; Cuesta et al. 2008), particularly in relation to the cellular innate immune response in vertebrates (Calvo et al. 2013). Comparable data on invertebrates are lacking (but see Jones et al. 2015), however given the ubiquitous presence of melatonin in the animal kingdom, as well as its conserved structure and functional versatility, it seems highly likely that it will be similarly important (Poeggeler 1993; Pandi-Perumal et al. 2006a). The loss of melatonin in individuals exposed to ALAN is also implicated in a range of changes to life history traits, including shifts in behaviours associated with circadian rhythm, impaired growth, altered sexual maturation, and altered reproductive output (Reiter et al. 2009; Dominoni et al. 2013c; Brüning et al. 2015; Le Tallec et al. 2016; McLay et al. 2017). Additionally, the known trade-offs between immune function and many life history traits (Lochmiller & Deerenberg 2000; Martin et al. 2008) mean that the impact that ALAN may have on each of these traits may have both direct and indirect consequences. Figure 1.4 depicts a hypothesised schematic of the mechanistic pathway demonstrating the impact of exposure to ALAN for life history and immune traits, and ultimately its effect on overall individual fitness.

Combined, a reduction in overall melatonin production associated with the presence of ALAN, and the subsequent disruption to circadian rhythms and hormonal interactions, is likely to be a major driver underpinning the observed behavioural and physiological effects of ALAN. However, there is currently little evidence directly linking melatonin with the biological effects of ALAN.
The research challenge

Artificial light at night is one of the most rapidly growing forms of environmental pollution (Falchi et al. 2016) and thus understanding how different species are responding to this rapid shift in the nocturnal environment, the underlying mechanisms causing change, and the degree to which the presence of ALAN affects individual fitness traits, is of key concern. Interest in this topic first appeared around 100 years ago (Squires & Hanson 1918; Matzke 1936; McFarlane 1963), however the field has seen an explosion of attention over the past 15 years (Longcore & Rich 2004; Navara & Nelson 2007;
Gaston et al. 2015b). It is now well established that the negative impacts of ALAN on individuals spans across numerous genera and includes behavioural as well as physiological effects (Rich & Longcore 2006; Navara & Nelson 2007; Gaston et al. 2014; Bennie et al. 2016; Dominoni et al. 2016). However, there remain gaps in our current understanding of how ALAN is likely to affect individuals and the ecosystems which they inhabit.

Research on the biological effects of ALAN is typically observational or correlational, and experimental studies have historically used intensities of ALAN well above that experienced in a natural or urban setting (Rich & Longcore 2006; Navara & Nelson 2007; Gaston et al. 2015b). Studies exploring effects on life history traits typically expose individuals to ALAN for a brief period or have only assessed one or two traits. This makes it impossible to determine whether the effects vary over time throughout an individual’s life, or whether biological trade-offs, such as those between reproductive and immune function, are occurring. Additionally, while evidence is building in support of melatonin acting as a key mechanistic link in the observed biological effects of ALAN (Durrant et al. 2015; Jones et al. 2015) direct evidence for melatonin as a causal mechanism is limited. This is likely due, in part, to the difficulty in quantifying the compound, given that levels of circulating and tissue bound melatonin are often extremely low and difficult to measure without harming the individual, making it hard to measure melatonin simultaneously with other behavioural and physiological measures.

Finally, aside from a few notable exceptions, invertebrate studies are largely absent from exploration of the impact of ALAN in ecology (Rich & Longcore 2006; Spoelstra & Visser 2013). This is surprising given the importance of invertebrates in ecosystems, particularly as a food source for higher trophic levels. The majority of invertebrate species are also nocturnal (more than 60%, compared to 30% of vertebrates), making them particularly susceptible to changes in the nocturnal environment (Holker et al. 2010).

Invertebrate species, such as the Australian black field cricket, *Teleogryllus commodus*, are ideal to research the effects of ALAN. Crickets are both crepuscular and nocturnal, with life history and mating strategies evolved to be optimal during darkness (Loher 1962; Loher 1974; Loher 1979). Crickets, like most insect, possess compound eyes and ocelli,
or simple eyes. The photoreception and entrainment of circadian rhythms is thought to occur through the compound eye to the optic lobe, as well as through extra retinal photoreception (Tomioka & Yukizane 1996; Shiga et al. 1999). *T. commodus* is a native inhabitant of heath and grassland landscapes (where ALAN is typically absent) but, critically for this research, it also inhabits urban and agricultural environments (where ALAN is present) (Robinson 2005). Crickets rely on being able to traverse the landscape at night in search of food and mates, so a certain amount of exposure to ALAN, if present, is unavoidable. Crickets also have a relatively fast life cycle and are easily maintained in a laboratory environment, offering a highly tractable system in which to explore a range of life history traits over a full lifetime. Additionally, invertebrates such as these lack a complex adaptive immune system, so their immune responses are relatively easy to assess (Beckage 2008), and preliminary studies have detected circulating melatonin in this species (Durrant et al. 2015). Finally, there are known trade-offs between immune function and many life history traits in this and similar species (Adamo et al. 2001; Dowling & Simmons 2012), meaning that a study incorporating both aspects is likely to be able to shed light on the interactions and relative impacts ALAN can have on each of these contributors to an individual’s overall fitness. All of this makes crickets an ideal candidate to be able to measure the response to ALAN in a range of traits simultaneously, as well as to attempt to elucidate the role of melatonin as one potential mechanistic link.

Laboratory experiments are fundamental to enhance our understanding of the physiological effects of ALAN. In a controlled environment it is possible to manipulate potential confounders and largely eliminate variation due to behavioural change, focusing on the physiological effects and their interactions. Although field studies are crucial to establish how the impacts of ALAN translate into a more malign, variable, and complex setting, laboratory experiments provide a first step, and a base from which to increase our understanding.

**Thesis objectives and aims**

In this thesis, I use a model invertebrate species, the Australian black field cricket, *T. commodus*, to explore the physiological impacts of artificial light at night (ALAN) at a
range of ecologically relevant intensities. There are two key areas of focus; the effects of ALAN on a range of life history traits (egg hatch, juvenile survival and development, adult size, number of eggs produced, sperm viability, and adult survival), and the effects of ALAN on immune function (haemocyte concentration, lytic activity, and phenoloxidase activity), as well as the potential for melatonin to be one key mechanistic factor between ALAN and physiological changes. Few studies have explicitly tested the effect of variation in the intensity of night lighting across many different traits simultaneously using an invertebrate model. I predicted that both immune function and several life history traits would be affected by the presence of ALAN and that there would be correlations between them. I also predicted that circulating melatonin would decrease with increasing levels of ALAN.

The thesis is structured as follows: Chapter 2 investigates experimentally the effect of lifelong exposure to different ecologically relevant intensities of ALAN (0, 1, 10 or 100 lx) on different life stages - egg hatch, juvenile development, adult morphology and condition, survival and the reproductive output of crickets. I also explore whether responses to ALAN are consistent across different family groups. Chapter 3 investigates the effect of lifelong exposure to the same ALAN intensities on three key indicators of immune function at multiple time points throughout adult cricket life. Chapter 4 presents a novel method for measuring exceptionally low concentrations of melatonin in cricket haemolymph, using high performance liquid chromatography and mass spectrometry (HPLC-MS). A pilot data set is presented using pooled haemolymph samples from the same individuals as Chapters 2 and 3. Finally, Chapter 5 synthesises the results of the preceding chapters, and provides a discussion of future research directions. Chapters 2 and 3 are written in the form of manuscripts. Chapter 2 has been accepted for publication by the *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*; Chapter 3 is written in the form of a manuscript, to be submitted to the *Journal of Insect Physiology*.
CHAPTER 2: Artificial light at night prolongs juvenile development time in the black field cricket, *Teleogryllus commodus*

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Abstract

A growing body of evidence exists to support a detrimental effect of the presence of artificial light at night (ALAN) on life-history and fitness traits. However, few studies simultaneously investigate multiple traits and the life stages at which changes manifest. We experimentally manipulated ALAN intensities, within those found in the natural environment, to explore the consequences for growth, survival and reproductive success of the field cricket, *Teleogryllus commodus*. We reared crickets from egg to adult under a daily light-cycle consisting of 12 hr bright daylight (2600 lx) followed by either 12 hr darkness (0 lux) or dim-light environments (1, 10 or 100 lux). We found egg hatch, adult survival and reproductive measures were largely comparable for all treatments. However, juvenile development time (number of days from egg to adult) was on average 10 days (14%) longer and adults were also larger when crickets were exposed to any light at night (1 lux, 10 lux or 100 lux). Our data demonstrate that chronic lifetime exposure to ALAN can modulate the timing of life-history events and may disrupt phenology to a similar extent as other abiotic factors.

**Key words:** light pollution, invertebrate, development, phenology, urbanisation
Introduction

The presence of artificial light at night (ALAN) has dramatically altered the nocturnal environment resulting in night-time light levels in urban areas that are three to 100-fold brighter than that of a full moon (approximately 0.3 lx) (Gaston et al. 2013; Bennie et al. 2015b; Gaston et al. 2015a; Falchi et al. 2016). The ecological consequences of chronic exposure to even relatively low intensity ALAN, if consistently present, includes major disruption to the behavioural and physiological processes of individuals (Longcore & Rich 2004; Rich & Longcore 2006; Navara & Nelson 2007; Bennie et al. 2015b; Gaston et al. 2015b; Bennie et al. 2016). ALAN has become one of the most rapidly growing forms of environmental pollution (Holker et al. 2010). Thus, understanding how species are responding to this rapid shift in the nocturnal environment and the degree to which the presence of ALAN affects individual fitness traits is of key concern.

One of the effects of artificially illuminating the night sky is that it masks natural day-night changes in light intensity. Behaviourally, the presence of artificial night lighting is negatively associated with changes in the movement and migration patterns of a diversity of animals, including birds, fish, turtles, bats and insects (see, Rich & Longcore 2006). Moreover, direct effects on the foraging capacity (Freeman 1981; Bird et al. 2004; Santos et al. 2010; Polak et al. 2011; Rotics et al. 2011; Titulaer et al. 2012), and an increased risk of death, due to disorientation (Witherington 1992; Merkel & Johansen 2011; Rodriguez et al. 2012) are reported for both nocturnal and diurnal vertebrates. The presence of ALAN also has reproductive consequences: in European passerines, urban populations not only commence singing earlier in the day but they also tend to have a more protracted breeding season compared to their rural counterparts (Partecke et al. 2004; Kempenaers et al. 2010; Dominoni et al. 2013c). Most recently, such changes have been linked to potentially adaptive shifts in behaviour (Altermatt & Ebert 2016).

Accumulating evidence highlights the physiological implications of ALAN for key life-history traits (Dominoni et al. 2016). In fish, the presence of constant artificial light during early embryonic development results in species-specific variation in hatch rates and swim bladder inflation (Bruning et al. 2011). Similarly, in Drosophila jambulina, synchronicity
of adult eclosion is negatively related to both the presence and intensity of nocturnal illumination (Thakurdas et al. 2009) and in the cricket *Gryllus bimaculatus* egg hatch is under circadian control (Itoh & Sumi 2000). While in the moth *Mamestra brassicae*, males (but not females) reared from the second instar under dim green or white lights at night spent less time in pupal diapause than moths reared with no light at night (van Geffen et al. 2014). ALAN also potentially masks seasonal variation in light. This is critical for any species whose reproduction is seasonally controlled. For example, a five-year study on the Tammar wallaby (*Macropus eugenii*), a species highly dependent on changes in day length with respect to its reproductive events, determined that individuals living in dimly lit areas delayed the birth of their offspring by one month compared to those living in darker areas (Robert et al. 2015). Collectively, these studies highlight the potential for ALAN to create trophic mismatches that may lead to resource depletion and reductions in offspring growth rates (Robert et al. 2015; Raap et al. 2016a). However, whether ecologically relevant levels of light directly affect egg and sperm production in such species is largely undocumented. Indirect evidence, using male and female *Drosophila*, suggest that clock-gene deficient individuals (who therefore lack a normal circadian rhythm) produce less sperm and lay fewer eggs, respectively (Beaver et al. 2002). Hence, ALAN is likely to be associated with changes in gamete maturation (Dominoni et al. 2013a; Dominoni et al. 2013d). Furthermore, current research on the effect of ALAN on life history traits typically explores individual traits in relative isolation; to our knowledge few studies have explicitly tested the effect of variation in the intensity of night lighting across multiple traits using an invertebrate model.

When considering the effects of ALAN on life history traits, there are potentially several mechanisms by which changes in the duration or intensity of ALAN may result in perturbed behavioural and physiological responses. The presence of ALAN may extend the temporal diurnal or crepuscular photic niche or conversely, may contract (or potentially eliminate) the true nocturnal temporal space. Theoretically, species that are exclusively active in one of these time-periods are likely most obviously affected, but species that occupy both the diurnal and nocturnal environments may also be vulnerable. Such species are less likely to be photophobic; however, the presence of extended periods of light may drive behavioural adaptation and have physiological consequences (Santos et al. 2010). Here, we investigated experimentally the effect of lifelong exposure to
different ALAN intensities (0, 1, 10 or 100 lux) on the growth, survival and reproductive success using an invertebrate model, the Australian black field cricket, *Teleogryllus commodus*. The black field cricket is largely nocturnal with life history and mating strategies evolved to be optimal during darkness (Loher 1962; Loher 1974; Loher 1979). Male courtship song commences prior to sunset but continues long into the night and it is therefore a species that is potentially exposed to ALAN, where it is present (Loher 1962; Sokolove 1975). Naturally, *T. commodus* is a native inhabitant of heath and grassland landscapes (where ALAN is typically absent) but, critically for the current study, it also inhabits urban and agricultural environments (where ALAN is present) (Robinson 2005). Previous research in this species suggests that the presence of constant bright illumination reduces adult immune function (Durrant et al. 2015) and some aspects of mating (Botha, Jones, & Hopkins, 2017) but whether ALAN affects other life history traits is untested. Here, we assessed the effects of variation in the intensity of ALAN on egg hatch, juvenile development, adult morphology, survival and reproductive output in *Teleogryllus commodus*.

**Methods**

Experimental crickets were sourced from a 10th generation laboratory-adapted population of founders captured from a dark location in Victoria, Australia (37.56238 S, 145.31920 E). Stock population crickets (approximately 1000 per generation) were maintained under standard conditions (Durrant et al. 2015) and held in a climate-controlled laboratory under a 12 hr light: 12 hr dark lighting regimen.

**Experimental ALAN treatments and rearing conditions**

To investigate the effect of artificial light at night on the life history of *T. commodus*, we maintained experimental individuals from eggs to adults under comparable simulated daylight conditions (2600 lx, equivalent to a cloudy day, 6800 K) for 12 hours followed by one of four ALAN treatments (0 lux, 1 lux, 10 lux or 100 lux, all at 5900 K) for a further 12 hours. The ALAN treatments were chosen to fall within the range of conditions currently present in and around urban areas, where 100 lux is acknowledged as an extreme but is an intensity present in a heavily urbanised city centre where crickets are present.
(TMJ personal observations). To create the different ALAN treatments, we used retrofitted Westinghouse incubators (model number WRM4300WB-R) set at a constant 28°C and lit by cool-white LED strip lighting on the front panels (World of Thought; Melbourne, Australia). Individual incubator effects were eliminated by alternating the light regimen in each incubator thrice weekly, and swapping the crickets accordingly, ensuring each incubator contributed equally to each regimen, but crickets were always maintained on their designated light regimen. Each cricket was maintained individually throughout the experiment but was rotated within each incubator, so that each experimental individual experienced a range of positions. It should also be noted that while crickets were isolated in an incubator in terms of light regimen, incubators were not sound proofed and thus no cricket was acoustically isolated from one another regardless of their ALAN treatment or incubator.

**Juvenile development and survival**

To explore whether responses to variation in ALAN were consistent across all families, we allocated offspring from known families equally to each of the ALAN treatments. To create known family groups, we paired 60 virgin adult females with virgin adult males from the stock population (24 ± 3 days post the final juvenile moult to the adult stage). After 24 hrs, we removed the male and provided each female with a sand pad for oviposition (standard 60 ml Petri dish base filled with 1 cm deep sand). The sand pad was replaced with a fresh sand pad every two days for a maximum of 10 days (five sand pads per female) or until she had laid at least 100 eggs. Any female that laid less than 100 eggs during this time-period was discarded (n = 47 females); a random sample of 100 eggs from the remaining 13 females were divided equally between the four ALAN treatments (n = 25 eggs per family per treatment), transferred to moistened cotton wool pads (Swisspers, Australia) in transparent round 100 mL containers and then placed in their designated ALAN treatment. Eggs were initially maintained in family groups of comparable age (designated by the date when laid) at densities of between 3 and 25 per container (dependent on the number laid during the given two-day oviposition period). Eggs were checked daily for four weeks or until all nymphs had hatched.

Within 24 hr of hatching, each first instar cricket nymph was transferred to an individual
transparent rearing container (70 x 70 x 40 mm) which contained a folded 10 x 30 mm cardboard shelter, *ad libitum* water and dried cat food (Friskies Senior; Rhodes, Australia). We monitored each nymph daily until death or until after its final juvenile moult to an adult. The time taken for a hatched individual to reach the adult stage of the lifecycle (herein defined as juvenile development time) or the time to death during the juvenile phase of the lifecycle was recorded.

**Adult morphology and survival**

Adult crickets were sexed within a day of completing their final moult and then transferred individually to larger transparent containers (150 x 90 x 50 mm) containing a piece of egg carton for shelter and *ad libitum* water and dried cat food (as above). Adults were similarly checked daily until death or until the completion of the experiment (33 ± 1 days after the final moult) at which point they were euthanised. A small haemolymph sample was taken from each cricket at 3, 17 and 31 ± 1 days after the final moult (following, Durrant et al. 2015), which equated to an additional stressor at the adult phase of the lifecycle (notably imposed on all individuals). Haemolymph samples were assayed for immune function and these data are presented in Chapter 3. All adult crickets were treated in the same manner and all surviving crickets were terminated 33 ± 1 days after the final moult. Body weight (to the nearest mg) was taken prior to mating and, following death, both back leg femurs were removed; taped to a glass slide and then digitally photographed with a Canon EOS 60D (Tokyo, Japan) mounted at x 10 magnification on an Olympus SZX7 stereomicroscope (Tokyo, Japan). Femur length was determined using Image J (Version 1.48V, NIH; Maryland, USA). As femur size is fixed in adult crickets, the average length of the two femurs was used as an index of body size (Mousseau & Roff 1989; Danielson-Francois et al. 2002; Jones et al. 2015). We used the scaled mass index as a measure of body condition, as it provides a better indicator of the relative size of body components than residuals from a standard least square regression of body weight and length (Peig & Green 2009).

**Reproductive output**

To assess the reproductive output of individuals in each ALAN treatment, at 21 ± 3 days after the final moult both male and female crickets were paired to virgin stock population
individuals (reared under a normal 12 hr light: 12 hr dark cycle) of similar age and
provided a 45-minute period to mate (for details on the experimental protocol and results,
see Botha et al. 2017). If this first mating did not result in the successful transfer of a
spermatophore, the stock individual was replaced and the experimental cricket was
provided a second opportunity. If the experimental cricket failed to mate after these two
opportunities, they were considered unmated and either they (for females) or their stock
population partner (for males) were discarded from further analyses of fecundity
(individuals discarded in 0 lux: n = 14/23 females, 5/29 males; 1 lux: n = 9/27 females,
7/37 males; 10 lux: n = 10/29 females, 9/39 males; 100 lux: n = 8/44 females, 4/45 males;
Botha et al 2017).

**Number of eggs laid**

To assess whether variation in ALAN treatment affected egg production, mated
experimental females and stock females mated to experimental males were provided with
a sand pad for oviposition (see above) for a total of seven days. After this period, the sand
pad was removed and the total number of eggs counted as a measure of reproductive
output.

**Sperm quality**

To assess whether variation in ALAN treatment affected sperm quality (measured as
sperm viability), males were provided a second mating opportunity (as above) with a
virgin stock-female two days following their initial mating trial. If this second mating
was successful, the spermatophore was removed from the female (within one minute of
a male attaching it to the female’s genitalia), transferred to an Eppendorf tube (1.5 mL)
containing 80 μl Beadle saline solution (128.3 mM NaCl, 4.7 mM KCl, and 23 mM
CaCl₂) and left for 10 mins. After this time, the spermatophore was removed and the
solution mixed using a pipette to ensure homogeneity of sperm within the solution. This
protocol maximised sperm effusion whilst avoiding sperm damage caused by the
common forceful spermatophore rupturing technique (Gress & Kelly 2011).

To determine sperm viability we used the standard live-dead sperm assay containing
SYBER-14 dye and propidium iodide (Invitrogen Molecular Probes, U.S.A) with a
protocol adapted from Gress and Kelly (2011) and García-González and Simmons (2005). A 5 μl aliquot of the sperm solution was transferred to 10 μl of diluted 1 mM SYBR-14 dye and left for ten minutes on a glass slide in darkness. After 10 minutes, 4 μl of 2.4 mM propidium iodide was added to the sample, and left in darkness for a further 10 minutes. A cover slip was then added and samples were viewed using a fluorescent microscope (Nikon Eclipse Ti-U, Japan) under a blue excitation filter (λ=470) and at x 100 magnification. Viability was assessed electronically using the NIS-Elements (Br 3.0) software. Both live and dead images were taken at four different areas of the slide (where sperm was present) and an average proportion live sperm calculated for each sample, with a minimum of 200 sperm assessed per slide. Samples with less than 200 sperm were discarded from the analysis.

**Statistical analyses**

We used JMP 12.1.0 (SAS Institute, NC, USA) for all analyses except variation in the adult survival and proportion of viable sperm which were assessed using a multi-level approach in MLwiN Version 3.00 (Centre for Multilevel Modelling, University of Bristol) (Rasbash et al. 2000). We used two-level models with ID and family as the first and second levels, respectively and assumed a binomial error distribution with logit link function. The cause of early (first and second instar) juvenile death was not always possible to determine, however the number of individuals whose death was undetermined was comparable across all ALAN treatments (0 lux = 81/269, 1 lux = 72/278, 10 lux = 70/280, 100 lux = 63/287; $\chi^2 = 4.93$, P = 0.18). To ensure that these differences did not affect our results, for juvenile survival, we ran two models: the first included all juveniles, the second we discarded individuals where the death was undetermined. Both models were qualitatively similar and thus only the second model is presented (see, Table 2.1B).

Due to the experimental design, adults were euthanised at 33 days (see above) at which point 80% of females and 83% of males were still alive. Variation in adult survival was therefore assessed using probability models (probability of surviving to the end of the experiment) rather than with a formal survival analysis incorporating censoring. Each model was reduced using hierarchical removal of all terms with a significance of P > 0.1 (except our designated light regimen). Once the minimum adequate model was obtained, excluded terms (P > 0.10) were reintroduced back to confirm the model fit did not
improve with their inclusion. Maximal models included, where biologically appropriate, the fixed factors ALAN treatment, days until hatch, juvenile development time, sex, femur length, mating status, body condition and whether or not they survived to the end of the experiment, as well as appropriate interactions between these terms. Family was included as a random term. Unless otherwise stated, significant interactions were assessed using post-hoc planned contrast tests; data presented are untransformed means ± SE and all tests were two-tailed with a significance level of $P < 0.05$.

**Results**

**Juvenile development and survival**

*Egg hatch*

The number of eggs hatched (out of each family set of 25; $N = 13$ families) was comparable for all ALAN treatments (mean number ± standard error of eggs hatched under 0 lux = 17.5 ± 1.35, 1 lux = 17.5 ± 1.10, 10 lux = 18.1 ± 1.30, and 100 lux = 17.9 ± 1.26; $P = 0.31$; Table 2.1A). The random term *family* explained 79.5% of the observed variation ($P = 0.02$).

*Juvenile survival*

All 13 families produced at least one adult cricket, but there was significant variation between the four ALAN treatments (Table 2.1B; $P = 0.04$). Post-hoc analyses revealed that 100 lux juveniles were more likely to reach the adult stage than 0 lux crickets (Figure 2.1). The random term *family* explained 40.4% of the observed variation ($P = 0.08$).

*Juvenile development time*

For the subset of crickets that completed their final moult, the time from oviposition through to the adult stage of the life cycle (Table 2.1C) varied significantly with ALAN treatment ($P < 0.0001$). Post-hoc analyses revealed that crickets exposed to light at night (1 lux, 10 lux and 100 lux treatments) took 10 days longer to reach the adult phase of the lifecycle compared to 0 lux crickets (Figure 2.2A), with no difference in development
time between any other groups. There was also a significant sex difference: juvenile development time for females was two days shorter (64.55 ± 0.85 days, n = 112) compared to males (66.44 ± 0.63 days, n = 137; P = 0.001). The random term family explained 24.9% of the observed variation (P = 0.07).

**Adult morphology and survival**

**Femur length and body condition**

Adult femur length (Table 2.1D) varied between treatments: crickets exposed to light at night (1 lux, 10 lux and 100 lux treatments) were significantly larger than 0 lux crickets (P < 0.0001; Figure 2.2B). There was a positive relationship between femur length and juvenile development time (β ± SE = 0.03 ± 0.005; P < 0.0001), and females had larger femurs (11.46 ± 0.07 mm, n = 112) compared to males (11.28 ± 0.06 mm, n = 137; P < 0.0001). The random term family explained 11.8% of the observed variation (P = 0.08).

Body condition (measured as the scaled mass index) at the time of mating was comparable across the four ALAN treatments for females but not males (Table 2.1E and 2.1F). Planned comparisons revealed 0 lux treatment males had a higher mass scaled index than 10 and 100 lux males (both P < 0.05) and tended to have a higher index compared to 1 lux males. Female body condition was also weakly negatively related to leg length (P = 0.07). The random term family explained 21.4% (female, P = 0.10) and 33.5% (male, P = 0.04) of the variation respectively.

**Adult female survival**

The probability that a female survived to the end of the experiment (Table 2.1G) was unrelated to her ALAN treatment (P = 0.09). None of the variation in the model was explained by the random term family (P = 1.0).

**Adult male survival**

The probability of a male surviving to the end of the experiment (Table 2.1H) was unrelated to ALAN treatment (P = 0.73). The random term family did not explain significant levels of variation (P = 0.29).
Reproductive output

Egg number

There were no significant difference across the ALAN treatments in the number of eggs laid per day by either treatment females mated to stock males (0 lux = 11.27 ± 4.60, n = 13; 1 lux = 11.78 ± 4.07, n = 15; 10 lux = 19.22 ± 4.98, n = 14; 100 lux = 20.56 ± 3.63, n = 23; P = 0.18; Table 2.1I) or the number of eggs laid by stock females mated to treatment males (0 lux = 17.89 ± 3.43, n = 19; 1 lux = 11.73 ± 2.86, n = 16; 10 lux = 13.74 ± 15.30, n = 19; 100 lux = 12.85 ± 2.75, n = 24; P = 0.35; Table 2.1J). The random term family explained 12.08% (treatment females; P = 0.42) and 12.63% (treatment males; P = 0.49) of the variation respectively.

Sperm viability

The proportion of viable sperm transferred during a male’s second mating attempt was comparable across the four ALAN treatments (median [interquartile range] proportion of viable sperm transferred by 0 lux males = 0.26 [0.15-0.35], n = 15; 1 lux = 0.16 [0.07-0.29], n = 13; 10 lux = 0.15 [0.11-0.33], n = 12; 100 lux = 0.25 [0.16-0.39], n = 11; P = 0.97; Table 2.1K). The random term family explained significant levels of variation; P < 0.001).
Table 2.1: Generalised mixed models exploring the effect of ALAN treatment (0 lux, 1 lux, 10 lux or 100 lux) on (A) the probability of egg hatch, (B) juvenile survival, (C) juvenile development time, (D) adult size, (E) adult scaled mass index at mating, (F) adult female survival, (G) adult male survival, (H) number of eggs laid per day by treatment females (I) number of eggs laid per day by stock females, and the (J) proportion live sperm.

<table>
<thead>
<tr>
<th>Model Parameters</th>
<th>$\beta \pm SE$</th>
<th>Statistic</th>
<th>P Value</th>
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<td><strong>(A) Number of eggs hatching</strong></td>
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<td>ALAN treatment</td>
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<td>$F_{3,36} = 1.23$</td>
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<td><strong>(B) Juvenile survival</strong></td>
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<td><strong>(C) Juvenile development time</strong></td>
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<td>$F_{3,233.9} = 33.8$</td>
<td>&lt;0.0001</td>
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<tr>
<td>ALAN treatment</td>
<td></td>
<td>$F_{1,233.9} = 10.8$</td>
<td>0.001</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td>$F_{1,233.9} = 10.8$</td>
<td>0.001</td>
</tr>
<tr>
<td>Days until adult from hatch</td>
<td>$0.03 \pm 0.005$</td>
<td>$F_{1,224.4} = 36.2$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>(D) Adult size (femur length)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALAN treatment</td>
<td></td>
<td>$F_{3,227} = 9.27$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td>$F_{1,227.8} = 14.1$</td>
<td>0.0002</td>
</tr>
<tr>
<td><strong>(E) Adult female scaled mass index at mating</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALAN treatment</td>
<td></td>
<td>$F_{3,98.6} = 0.56$</td>
<td>0.56</td>
</tr>
<tr>
<td>Leg length (mm)</td>
<td>$-0.01 \pm 0.008$</td>
<td>$F_{3,103.6} = 3.46$</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>(F) Adult male scaled mass index at mating</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALAN treatment</td>
<td></td>
<td>$F_{3,116.1} = 3.56$</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>(G) Adult female survival</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALAN treatment</td>
<td></td>
<td>$\chi^2_{3,132} = 49$</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>(H) Adult male survival</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALAN treatment</td>
<td></td>
<td>$\chi^2_{3,109} = 1.32$</td>
<td>0.73</td>
</tr>
<tr>
<td><strong>(I) Number of eggs laid per day (treatment females mated to stock males)</strong></td>
<td></td>
<td>$F_{3,58.08} = 1.71$</td>
<td>0.18</td>
</tr>
<tr>
<td><strong>(J) Number of eggs laid per day (stock females mated to treatment males)</strong></td>
<td></td>
<td>$F_{3,71.72} = 1.11$</td>
<td>0.35</td>
</tr>
<tr>
<td><strong>(K) Proportion live sperm</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALAN treatment</td>
<td></td>
<td>$\chi^2_{3} = 0.24$</td>
<td>0.97</td>
</tr>
</tbody>
</table>
Figure 2.1: The median proportion of juveniles within each family surviving to complete the final moult for each of the four ALAN treatments ($n = 13$ families per ALAN treatment). Box plots denote median and interquartile ranges, whiskers denote the lower 5th and upper 95th percentiles and the x denotes the mean; different letters denote significant ($P < 0.05$) differences between treatments.
Figure 2.2: Median (A) juvenile development time (days) for crickets reared under 0 lux \((n = 50)\), 1 lux \((n = 60)\), 10 lux \((n = 59)\), 100 lux \((n = 80)\); and (B) femur length for crickets reared under 0 lux \((n = 50)\), 1 lux \((n = 62)\), 10 lux \((n = 60)\), 100 lux \((n = 84)\). Box plots denote median and interquartile ranges, whiskers denote the lower 5\(^{th}\) and upper 95\(^{th}\) percentiles and the x denotes the mean; different letters denote significant \((P < 0.05)\) differences between treatments.
**Discussion**

Our experiment yielded three key findings related to the presence of artificial light at night. First, the presence of any ALAN (1, 10 or 100 lux) resulted in a consistent 10 day (equating to approximately 14%) increase in the time taken for crickets to complete the juvenile phase of their development compared to crickets reared in the absence of light at night (0 lux crickets). Second, crickets reared under artificial light at night were significantly larger than crickets under 0 lux, even when accounting for variation in juvenile development time. Third, despite the significant effect of ALAN on juvenile development and resulting adult size, its presence had limited effect on adult survival or reproductive output. Our experiment also revealed substantial variation in adult body size.

Combined our data highlight the potential for artificial light at night to disrupt the physiology and development of organisms and alter the timing of life history events, potentially disrupting phenology in much the same way as other recent abiotic or climatic shifts (Visser et al. 2004; Neil & Wu 2006).

Shifts in the timing of juvenile development linked to the presence of artificial light at night are demonstrated for invertebrates (Thakurdas et al. 2009; van Geffen et al. 2014) and vertebrates (Bruning et al. 2011; de Jong et al. 2015) but the mechanisms underpinning these differences are rarely tested. We suggest that two types of physiological responses may contribute to the observed variation: disruption of the hormonal system around appetite control and growth, as well as disruption of perception of day-length and its implicit seasonality. Initially, exposure to ALAN may interfere with key hormones involved in appetite, satiety and growth rate (such as ghrelin, leptin, insulin, insulin-like growth factor (IGF), growth hormone and melatonin). Ghrelin stimulates appetite and potentially lead to increased weight gain in an environment where food is available *ad libitum* (Peric-Mataruga et al. 2009; Mataruga et al. 2012; Mataruga et al. 2015). The indolamine melatonin, in particular, is a powerful chemical driver of circadian rhythm, whose production is reduced in the presence of light at night (Poeggeler 1993; Reiter et al. 1993; Vivien-Roels & Pevet 1993; Pandi-Perumal et al. 2006b; Tan et al. 2007; Tan et al. 2010). The complex interacting effects between melatonin and ghrelin, (i.e. lower melatonin concentration from a reduced dark phase is unable to suppress ghrelin
expression and elevated appetite, as well as normal metabolism and energy expenditure from food) possibly explain the increased weight gain in crickets reared under ALAN (Kirsz & Zieba 2012; Kirsz et al. 2017). This interaction is supported experimentally in numerous vertebrate species, by removal of the pineal gland resulting in obesity, as well as administration of melatonin reversing weight gain (Kirsz & Zieba 2012; Kirsz et al. 2017). Reduced melatonin concentrations, through decreased dark phase, also explain the slower growth of juvenile crickets exposed to light at night (even the lowest intensity of 1 lx). The actions of melatonin are mediated through its receptor that are widely distributed in the body in many tissues (Slominski et al. 2012). This allows melatonin to regulate growth and development via multiple mechanisms. For example, melatonin is known to induce IGF (Picinato et al. 2008), a potent cellular mitogen required for growth and development, as well as modulating growth hormone secretion (Recabarren et al. 2000). Melatonin is a thyroid antagonist thought to have a role in metamorphosis, as reported in amphibian studies (Wright 2002). However, our findings are in contrast to comparable studies, exploring the effect of dim night lighting on offspring growth that found reduced juvenile to adult development times and reduced body size for ALAN individuals (van Geffen et al. 2014; Raap et al. 2016a). One potential explanation for the observed variation in development is that the 0-lux condition with its 12 hour dark period may have triggered a short day-length response in T. commodus, a species with demonstrated facultative diapause (Hogan 1965; Hogan 1967). While this is yet to be demonstrated in T. commodus, in the closely related T. emma, juveniles developed faster with shorter day-lengths (< 13 hours light) compared to longer day-lengths (14 to 16 hours of light) and adult body weight was positively related to the juvenile period (Kim et al. 2008).

We did not measure melatonin in this study (but see Chapter 4), however chronic exposure to ecologically relevant levels of ALAN can reduce melatonin concentrations (Evans et al. 2007; Brüning et al. 2015) and in T. commodus the presence of constant light negatively affects both melatonin concentrations and immune function (Durrant et al. 2015; Jones et al. 2015). Moreover, the loss of melatonin in species exposed to ALAN is implicated in a range of downstream negative fitness effects including shifts in circadian rhythm, impaired growth and compromised immune function (Reiter et al. 2003; Srinivasan et al. 2005; Navara & Nelson 2007; Cuesta et al. 2008; Reiter et al. 2009;
Calvo et al. 2013; Pohanka 2013; Brüning et al. 2015; Chapter 3). Furthermore, direct links between the presence of ALAN, melatonin and sexual maturation are demonstrated (Asher et al. 2011; Dominoni et al. 2013a; Dominoni et al. 2013d; Robert et al. 2015; Le Tallec et al. 2016) and it is increasingly clear that even dim night lighting (1 lx and below) can affect circulating levels of melatonin (Bruning et al. 2011; Dominoni et al. 2013b; Durrant et al. 2015). The adaptive benefit of a longer development period may be reduced in a natural more malign and likely colder environment, where a protracted juvenile phase of the lifecycle and resulting larger individuals may increase predation risk within the population (Dixon & Baker 1988) thus reducing species fitness.

Given the large developmental difference observed between ALAN and 0 lux crickets, and the likely physiological mechanism underpinning it, the lack of a subsequent effect on reproduction or adult survival is perhaps surprising, although comparable with other studies where it has been explicitly investigated (van Geffen et al. 2014; de Jong et al. 2015). The lack of a detectable difference in the proportion of eggs hatching after their initial allocation to the ALAN treatments (c.f. Bruning et al. 2011) likely arose because we used initially stock population individuals and thus sperm and eggs matured and eggs were subsequently laid under a natural light:dark cycle prior to transferring to the ALAN treatments. Moreover, our protocol artificially selected only clutches with equal to or more than 100 eggs and thus more sensitive females/families were potentially discarded at this initial phase. Moreover, the nature of our subsequent experimental protocol, which promoted extreme selection throughout the entire juvenile phase of the lifecycle (compared with studies that have focussed on either one stage or a partial stage of the life-history, Dominoni et al. 2013a; van Geffen et al. 2014; Robert et al. 2015; Altermatt & Ebert 2016; Raap et al. 2016b) again likely resulted in only the fittest juveniles surviving to the adult phase of the lifecycle. Our experimental design does not permit us to determine the relative importance of ALAN at the early and late juvenile phases of the lifecycle but given that these phases differ in the nature of resource investment – the former typically involves rapid growth and somatic investment, whereas the latter must allocate resources to reproductive development also (Imms 1925) – our data perhaps hint at high susceptibility of the earliest instars.
Conclusion

Our results concur with previous studies that find even extremely low levels of ALAN affect key life-history traits (Bruning et al. 2011; Dominoni et al. 2013c; de Jong et al. 2015). Variation in the timing of development and sexual maturation, due to the presence of ALAN, has broad ecological implications (Visser et al. 2004; Neil & Wu 2006; Holker et al. 2010; Dominoni et al. 2013d), which may lead to a mismatch within populations in terms of the reproductive window (van Geffen et al. 2014; de Jong et al. 2015; Robert et al. 2015). This is particularly important for species with multiple generations within a breeding season, such as *T. commodus*. What is perhaps most challenging is that species clearly respond differently to urbanisation but we have a limited understanding of the capability of most species to adapt to this significant environmental change (Evans et al. 2010; Swaddle et al. 2015). Assessing the amount of natural genetic variation is a first step, although further research is needed to identify if species in their natural habitats are actually adapting to this rapid environmental shift.
CHAPTER 3: Dim artificial light at night reduces the cellular immune response of the black field cricket, *Teleogryllus commodus*

This work was submitted to the *Journal of Insect Physiology* on 02/10/18 as: Durrant J, Green MP, and Jones TM. Dim light at night reduces the cellular immune response of the black field cricket, *Teleogryllus commodus*. 
Abstract

A functioning immune system is crucial for protection against disease and illness, yet increasing evidence suggests that species living in urban areas could be suffering from immune suppression, due to the presence of artificial light at night (ALAN). This study examined the effects of ecologically relevant levels of ALAN on three key measures of immune function (haemocyte concentration, lytic activity, and phenoloxidase activity) using a model invertebrate species, the Australian black field cricket, *Teleogryllus commodus*. We reared crickets under a daily light-cycle consisting of 12 hr bright daylight (2600 lx) followed by either 12 hr darkness (0 lux) or dim environmentally-relevant ALAN (1, 10, 100 lux), and assessed immune function at multiple time points throughout adult life using haemolymph samples (n = 231). We found that the presence of ALAN had a clear negative effect on haemocytes, while the effects on lytic activity and phenoloxidase activity were more complex or largely unaffected by ALAN. Furthermore, the effects of lifelong exposure to ALAN of 1 lux were comparable to those of 10 and 100 lux. Hence the effects of ALAN could be large and widespread, and such reductions in the core immune response of individuals will likely have greater consequences for fitness and survival under more malign conditions, such as those of the natural environment.

Key words: light pollution, urbanisation, immune function, immunomodulation, ecophysiology, circadian rhythm, melatonin, invertebrate.
Introduction

The global presence of artificial light at night (ALAN) is increasing rapidly in terms of its intensity and distribution making it one of the most pervasive recent forms of anthropogenic pollution (Bennie et al. 2015b; Gaston et al. 2015a; Falchi et al. 2016). The ecological consequences of ALAN have been demonstrated across a range of taxa and ecological levels (Rich & Longcore 2006; Gaston et al. 2014; Henneken & Jones 2017; Tierney et al. 2017). A growing body of evidence links the presence of ALAN with physiological effects including altered development (Bruning et al. 2011; van Geffen et al. 2014; Raap et al. 2016a; Durrant et al. 2018; Chapter 2), survival (McLay et al. 2017), aging (Vinogradova et al. 2009), shifts to the timing of reproductive events (Dominoni et al. 2013a; Dominoni et al. 2013d; Robert et al. 2015; Brüning et al. 2016; Botha et al. 2017), and increased risk of disease and illness, including cancer (Hastings et al. 2003; Stevens 2009; Bedrosian et al. 2011a; Fonken et al. 2013; Borniger et al. 2014; Smolensky et al. 2015; Dominoni et al. 2016). Correlated with (and potentially underpinning) these physiological impacts are the largely negative effects of ALAN on immune function demonstrated in birds and mammals, including Japanese quail (Moore & Siopes 2000), rates (Oishi et al. 2006, Siberian hamsters {Aubrecht, 2014 #997; Cisse et al. 2017a), passerine (Russ et al. 2015a), great tits (Raap et al. 2016b; Ouyang et al. 2017).

The immune system is critical for defending an organism against insult from bacterial and viral infections, as well as other diseases or damage, and as such is a crucial component of individual fitness (Schmid-Hempel 2005). The majority of studies to date, including those highlighted above, are vertebrate focused and typically immune function is assayed at a single time point; longitudinal studies exploring the effect of ecologically relevant levels of ALAN at the level of the individual are rare. Invertebrates, and particularly insects, offer an attractive opportunity to address some of these gaps in our current understanding of the effects of ALAN on invertebrate immune function, and in particular, the long-term effects throughout an entire lifetime. Invertebrates lack an adaptive immune system but their innate immune system is highly effective and has cellular recognition and defensive pathways that are analogous with vertebrates (for a
comprehensive coverage of the discipline see, Beckage 2008). The immune system of invertebrates includes immune cells, as well as chemicals, enzymes and antimicrobial peptides in body fluids (Cooper & Lemmi 1981; Beck & Habicht 1996; Murphy & Weaver 2016). These systems interact to mount an effective immune response (Siva-Jothy et al. 2005; Haine et al. 2008).

Three common measures are used to explore variation in insect immunocompetence: (i) The concentration of circulating haemocytes provides a measure of the capacity for an individual to mediate the core cellular defence pathway, including phagocytosis and encapsulation (Ribeiro & Brehélin 2006); (ii) Lysozyme-like (herein referred to as lytic) activity provides an indication of the capacity of antibacterial enzymes principally involved in degrading bacterial cell walls to resist bacterial a challenge (Beckage 2008); and (iii) Phenoloxidase (PO), which becomes activated upon cuticular wounding or infection and is an important precursor instigating repair and encapsulation following infection or parasitism of larger foreign bodies (Kanost & Gorman 2008).

In this study, we explore patterns of immune activity following lifelong exposure to varying levels of ALAN in the nocturnal Australian black field cricket, *Teleogryllus commodus*. The black field cricket is an ideal species to explore ALAN related questions. It is a native inhabitant of grassland areas in Australia, but it is also present in urban environments and thus it is likely directly exposed to the presence of ALAN (Otte & Alexander 1983). Previous research demonstrates that exposure to ecologically relevant levels of ALAN (1 lux, 10 lux and 100 lux) consistently prolongs the juvenile developmental period, results in increased adult size (Durrant et al. 2018; Chapter 2) and affects some aspects of reproduction (Botha et al. 2017). *Teleogryllus commodus* is a model species for invertebrate immune studies (Simmons et al. 2005; Simmons et al. 2010; Bailey et al. 2011; Drayton & Jennions 2011; Dowling & Simmons 2012; Drayton et al. 2012; McNamara et al. 2014a) and exposure to bright illumination, beyond that commonly found in natural environments, during development negatively affects multiple measures of immune function (Durrant et al. 2015; Jones et al. 2015). However, whether exposure to more ecologically relevant ALAN has a comparable effect on immune function is untested.
We investigated the effect of lifelong exposure to four different light levels (0 lux, 1 lux, 10 lux or 100 lux) of ecologically-relevant ALAN on lifetime adult immune function in male and female crickets. We predicted that ALAN would reduce immune function in crickets, as previously demonstrated with constant bright light (Durrant et al. 2015; Jones et al. 2015). Given recent studies highlighting that even dim ALAN (<5 lx) is capable of affecting other physiological processes including circadian rhythm (Evans et al. 2007; Brüning et al. 2015), melatonin release (Dominoni et al. 2013b), sleep (Aulsebrook et al. 2018) the timing of foraging and reproduction (Robert et al. 2015; Russ et al. 2015b), physiological condition (Raap et al. 2016b), survival (McLay et al. 2017), and immune function in vertebrates (Aubrecht et al. 2014), we also predict that these effects on immune function would be seen down to a level of at least 1 lx.

**Materials and methods**

Experimental crickets were sourced from a 10th generation laboratory-adapted population of founders captured in Victoria, Australia (37.56238 S, 145.31920 E). Stock population crickets (approximately 1000 per generation) were maintained under standard conditions, as detailed in (Durrant et al. 2015), and held in a climate-controlled laboratory under a 12hr light: 12hr dark cycle.

**ALAN treatments & rearing conditions**

To investigate the effect of ecologically-relevant levels of ALAN on cricket immune function, we maintained experimental individuals from eggs to adults under comparable simulated daylight conditions (2600 lux; equivalent to a cloudy day) for 12 hours followed by one of four ALAN treatments (0 lux, 1 lux, 10 lux or 100 lux) for a further 12 hours (for further information regarding the rearing and treatment groups see, Botha et al. 2017; Durrant et al. 2018; Chapter 2). To create the different ALAN treatments, we used retrofitted Westinghouse incubators (model number WRM4300WB-R) set at a constant 28°C and lit by cool-white (6800 Kelvin during simulated day and 5900 Kelvin simulated night) LED strip lighting on the front panels (World of Thought; Melbourne, Australia). While this is a laboratory based study, the ALAN treatments were chosen to fall within the range of conditions present in and around urban areas, where 100 lux is
acknowledged as an extreme but is an intensity present in a heavily urbanised city centre where crickets are present (Gaston et al. 2013). We note that sensu Gaston et al (2017), we use lx as our unit of light. While this measure is based on human photopic vision, and thus does not necessarily capture the relative effects of light influencing crickets per se, its use ensures a direct link to illuminance as commonly measured in the environment and employed in the design and mitigation of artificial lighting systems. Individual incubator effects were accounted for and mitigated by alternating the light regimen in each incubator thrice weekly, and swapping the crickets accordingly, ensuring each incubator contributed equally to each regimen, but crickets were always maintained on their designated light regimen. Crickets were also rotated within incubators so that each experimental individual experienced a range of positions in each incubator. It should also be noted that while crickets were isolated in an incubator in terms of light regimen, incubators were not sound proofed and thus no cricket was acoustically isolated from one another regardless of their ALAN treatment or incubator.

Eggs were initially maintained in family groups of comparable age (designated by the date when laid) at densities of between 3 and 25 per container (dependent on the number laid during the given two-day oviposition period). Within 24 hr of hatching, each first instar cricket nymph was transferred to an individual transparent rearing container (70 x 70 x 40 mm) which contained a folded 10 x 30 mm cardboard shelter, ad libitum water and dried cat food (Friskies Senior; Rhodes, Australia), and on the day of the final juvenile moult, adult crickets were weighed (to the nearest mg) and each individual placed in a larger transparent container (150 mm length x 90 mm width x 50 mm height) with a piece of egg carton for shelter and ad libitum food and water. Adults were checked daily until death or until the completion of the experiment (33 ± 1 days after the final juvenile moult) at which point they were killed by freezing. Following death, both femurs were removed from an individual cricket; taped to a glass slide and then digitally photographed with a Canon EOS 60D (Tokyo, Japan) mounted at x 10 magnification on an Olympus SZX7 stereomicroscope (Tokyo, Japan). Mean femur length for each cricket was determined using image J (Version 1.48V, NIH; Maryland, USA).

There was significant variation in adult survival across the ALAN treatments, as presented in Chapter 2, resulting in more 100 lux juveniles surviving to the adult stage.
(Durrant et al. 2018) and, while not critical to the current experiment, to ensure families contributed equitably, we selected a maximum of six individuals from each ALAN treatment and, where possible, ensured that the number of males and females per family per ALAN treatment was comparable (mean ± SE number of females per family per treatment = 2.20 ± 0.07; males = 2.55 ± 0.09).

**Haemolymph extraction for immune measures**

For each individual cricket we assessed immune function at 3 ± 1 days, 17 ± 1 days and 31 ± 1 days after the final juvenile moult, herein referred to as Week 0, Week 2 and Week 4 respectively. These three time-points provide a baseline measure of immune function following juvenile development (Week 0) and also a longitudinal measure of adult immune function (comparisons between Week 0, Week 2 and Week 4).

To collect the haemolymph, a small puncture was made in the left side of the cricket abdomen using a 27G sterile needle (Becton Dickinson and Co.; Melbourne, VIC, Australia). The resulting wound exudes a small haemolymph bubble on the cuticle surface from which we collected a maximum of 6 µL. For haemocyte concentration 1 µL of this haemolymph was collected using a micropipette and immediately transferred to a 0.5 mL Eppendorf tube (Sarstedt; Mawson Lakes, SA, Australia) maintained on ice, containing 20 µL of pre-prepared anticoagulant solution (100 mM NaOH, 150 mM NaCl, 22 mM EDTA, 45 mM citric acid in distilled water). For the lytic and phenol oxidase activity assays, a further 4.5 µL of haemolymph was collected, transferred to a 0.5 mL Eppendorf tube containing 60 µL of phosphate buffer saline (PBS; 11.9 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4), snap frozen in liquid nitrogen and then stored at -80°C until later analysis. We note that crickets have a very efficient coagulation system, as small puncture wounds are common during fights or interactions, so crickets recovered immediately from these procedures and there were no obvious effects on survival.

**Haemocyte concentration**

To determine the concentration of haemocytes, a 10 µL sample of the haemolymph-anticoagulant solution (described above) was placed onto a Neubauer haemocytometer (Blaubrand; Wertheim, Germany) at x 100 magnification on an Olympus BX50
stereomicroscope (Olympus; Tokyo, Japan). The number of haemocytes was counted and expressed as a concentration (Drayton & Jennions 2011).

**Lytic activity**

To assess lytic activity, 10 µl of the haemolymph-PBS solution (described above) was added to a round bottom 96-well plate (Sarstedt; Mawson Lakes, SA, Australia). Each sample was added twice, as technical duplicates. To each well, 80 µL of *Micrococcus lutus* (lysochiticus) bacteria solution (3 mg/mL PBS; Sigma-Aldrich; North Ryde, NSW, Australia) was then added. Lysozymes in the haemolymph gradually degrade the bacteria in the solution causing the turbidity of the solution to decrease. Relative change in absorbance at 490 nm and 30°C was measured over a 120-minute period in a microplate spectrometer (BioTek EL x800 Absorbance Microplate Reader, Millenium Science; Mulgrave, Australia), with higher values indicating greater lytic activity. Samples were stratified across plates and each plate included negative controls of PBS only, as well as the same pooled biological quality control run on every plate. The mean intra- and inter-coefficients of variation for all lytic assays were 13.7% and 12.3% respectively (n = 19 plates).

**Phenoloxidase (PO) activity**

When in circulation in the haemolymph, PO is stored as an inactive precursor, pro-phenoloxidase, thus to measure the total PO present we first cleaved all pro-phenoloxidase with the proteolytic enzyme, α-chymotrypsin (Moreno-Garcia et al. 2013). For each sample, 5 µL of the haemolymph-PBS solution was added to a round-bottom 96-well plate, followed by 7 µL of 1.3 mg/mL bovine pancreas α-chymotrypsin (Sigma-Aldrich; North Ryde, NSW, Australia). After a 20-minute incubation period at room temperature we added 90 µL of L-dihydroxyphenylalanine (L-DOPA; Sigma-Aldrich; North Ryde, NSW, Australia). The relative change in absorbance at 490 nm was measured over a 120-min period in a microplate spectrometer. PO converts L-DOPA to dopachrome causing the solution to darken. The greater change in absorbance indicated greater PO activity. As above, samples were stratified across plates and each plate included technical duplicates of each sample, negative controls of PBS only, as well as the same pooled biological control run on every plate. The mean intra- and inter-coefficients of variation
for all PO assays were 6.1% and 5.7% respectively (n = 19 plates).

**Statistical analyses**
Statistical analyses were performed in JMP 12.1.0 (SAS Institute, NC, USA). We used generalized linear mixed models to explore the effect of ALAN on immune function throughout the adult life of the crickets. Variables were assessed for normality and haemocyte data were subsequently ln transformed prior to analyses of immune function. We first assessed baseline variation across the treatment groups by differences in Week 0 measures. We then performed a second set of analyses exploring the change over time, with week as a categorical variable. ALAN treatment, sex, week and whether they survived to the end of the experimental period (Yes or No) were included as categorical and femur length as a continuous fixed effect in all models; family ID, individual ID and plate number were included as random effects. All biologically appropriate interactions were included. Each model was reduced using hierarchical removal of all terms with a significance of P > 0.1 except the main ALAN treatment. For reference full models prior to model reduction are included in Appendix III. Once the minimum adequate model was obtained, excluded terms (P > 0.1) were reintroduced back to confirm the model fit did not improve with their inclusion. To assess correlations between the three immune measures we used Spearman rank correlation tests using untransformed data throughout. All tests were two-tailed with a significance level of P < 0.05. Unless otherwise stated, significant interactions were assessed using post hoc planned contrast tests, and data presented are means ± SE.

**Results**

**Baseline Week 0 immune measures**
Baseline measures at Week 0 were comparable for all treatments and both sexes (Table 3.1 A-F; P > 0.10 for all analyses). There was also significant variation across families for the majority of immune measures (percentage of family level variation for female haemocytes counts = 18.8%, lytic activity = 5.3%, PO activity = 0.0%; and for male haemocytes = 9.4%, lytic activity = 14.4%, PO = 10.3%).
Longitudinal variation in immune function (Week 0 to Weeks 2 and 4)

Haemocyte concentration

Haemocyte concentrations (cells/mL x 10^6) varied with ALAN treatment (P = 0.0003; Table 3.2A; Figure 3.1A), sampling week (P = 0.04; Table 3.2A; Figure 3.2A) and were greater for females (mean ± SE = 2.83 ± 0.04) compared to males (mean ± SE = 2.65 ± 0.03; P = 0.0002; Table 3.2A). Post hoc analyses revealed crickets in the 1 lux, 10 lux and 100 lux ALAN treatments had lower haemocyte concentrations compared to 0 lux crickets (Table 3.2A) and, on average, haemocyte concentrations increased marginally between the Week 0 and Week 2 samples and then declined at the Week 4 sample (Figure 3.1A). There was also significant variation between families (percentage variation explained by the random family term = 9.1%, P = 0.05; Table 3.2A).

Lytic activity

Patterns of lytic activity were more complex. There was no main effect of ALAN treatment (P = 0.14; Table 3.2B, Figure 3.1B) or Sex (P = 0.56) on lytic activity. However, lytic activity increased across the three sampling periods (P < 0.0001; Figure 3.2B). This latter relationship was driven by significant interactions between ALAN treatment and Week (P = 0.008; Figure 3.3B) and also between Sex and Week (P = 0.001; Figure 3.3B). Post hoc analyses revealed that lytic activity typically increased over the three sampling periods and was thus higher at the Week 4 compared to the Week 0 sampling period for ALAN treatments, but there was variation with respect to the amount of increase at the Week 2 sampling period (Figure 3.3A). Similarly, lytic activity was comparable for males and females at Week 0 and Week 4 but males had higher lytic activity than females in Week 2 (Figure 3.3B). There was considerable individual variation in lytic activity (percentage variation explained by the random term individual = 24.1%, P < 0.0001).

Phenoloxidase (PO) activity

PO activity was comparable across the ALAN treatments (P = 0.76; Table 3.2C, Figure 3.1C) but it increased significantly across the three sampling periods (P < 0.0001; Figure 3.2C). It was significantly greater for females (mean ± SE = 0.90 ± 0.02) compared to
males (mean ± SE = 0.65 ± 0.02; P < 0.0001) and was higher in individuals that survived to the end of the experiment (mean ± PO for individuals that died = 0.56 ± 0.04 (n = 48); survived = 0.80 ± 0.02 (n = 183); P < 0.0001). There was considerable individual variation in PO activity (percentage variation explained by the random term individual = 10.7%, P = 0.03).

**Correlations between immune parameters**

Haemocyte concentration was positively correlated to lytic activity for males and females across all weeks (all P < 0.05) but was unrelated to PO activity (All P > 0.1, Table 3.3). In contrast, lytic activity was correlated with PO activity for Weeks 0 and 4 but unrelated at Week 2.
Table 3.1: Generalised linear mixed outputs from models exploring the initial (week 0 after final moult) effect of lifelong ALAN exposure (0, 1, 10 and 100 lux) Ln (haemocyte concentration in cells/mL x 10⁶) (A, D), change in lytic activity (B, E) and change in Phenoloxidase (PO) activity (C, F) for females and males respectively. Family and Assay Plate Number were included as random terms in all models.

<table>
<thead>
<tr>
<th>Model Parameters</th>
<th>0 lux</th>
<th>1 lux</th>
<th>10 lux</th>
<th>100 lux</th>
<th>Statistic</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(A) Ln (Haemocyte concentration)</td>
<td>2.89 ± 0.14</td>
<td>2.77 ± 0.13</td>
<td>2.78 ± 0.14</td>
<td>2.60 ± 0.12</td>
<td>F₃,₇₇.₀₃ = 1.59</td>
<td>0.20</td>
</tr>
<tr>
<td>(B) Lytic activity (Δ absorbance)</td>
<td>0.27 ± 0.03</td>
<td>0.37 ± 0.04</td>
<td>0.28 ± 0.03</td>
<td>0.31 ± 0.02</td>
<td>F₃,₇₈.₀₅ = 1.66</td>
<td>0.18</td>
</tr>
<tr>
<td>(C) PO activity (Δ absorbance)</td>
<td>0.75 ± 0.10</td>
<td>0.55 ± 0.06</td>
<td>0.44 ± 0.05</td>
<td>0.56 ± 0.08</td>
<td>F₃,₈₁.₅₆ = 2.15</td>
<td>0.10</td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(D) Ln (Haemocyte concentration)</td>
<td>2.69 ± 0.13</td>
<td>2.58 ± 0.09</td>
<td>2.69 ± 0.12</td>
<td>2.61 ± 0.10</td>
<td>F₃,₉₅.₁ = 0.25</td>
<td>0.86</td>
</tr>
<tr>
<td>(E) Lytic activity (Δ absorbance)</td>
<td>0.24 ± 0.03</td>
<td>0.26 ± 0.02</td>
<td>0.31 ± 0.03</td>
<td>0.30 ± 0.02</td>
<td>F₃,₉₅ = 1.80</td>
<td>0.16</td>
</tr>
<tr>
<td>(F) PO activity (Δ absorbance)</td>
<td>0.24 ± 0.03</td>
<td>0.28 ± 0.03</td>
<td>0.30 ± 0.03</td>
<td>0.30 ± 0.03</td>
<td>F₃,₈₉.₆₂ = 0.94</td>
<td>0.42</td>
</tr>
</tbody>
</table>
Table 3.2: Mixed models exploring the effect of lifelong ALAN treatment (0, 1, 10 and 100 lux) over time (Weeks 0, 2 and 4 after final moult) for (A) haemocyte concentration (cells/mL x 10^6), (B) lytic activity (Δ absorbance) and (C) Phenoloxidase (PO) activity (Δ absorbance). Family, Individual ID and Assay Plate number were included as random terms in all models.

<table>
<thead>
<tr>
<th>Model Parameters</th>
<th>0 lux</th>
<th>1 lux</th>
<th>10 lux</th>
<th>100 lux</th>
<th>Statistic</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(A) Haemocyte concentration (cells/mL x 10^6) (Ln transformed)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALAN treatment</td>
<td>2.93 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.66 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.72 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.64 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>F&lt;sub&gt;3,205.3&lt;/sub&gt; = 6.48</td>
<td>0.0003</td>
</tr>
<tr>
<td>Week</td>
<td>F&lt;sub&gt;2,358.5&lt;/sub&gt; = 3.32</td>
<td></td>
<td></td>
<td></td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Sex (F &gt; M)</td>
<td>F&lt;sub&gt;1,205.2&lt;/sub&gt; = 14.4</td>
<td></td>
<td></td>
<td></td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>Week x Sex</td>
<td>F&lt;sub&gt;2,392.4&lt;/sub&gt; = 2.64</td>
<td></td>
<td></td>
<td></td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Family (variation = 9.1%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Individual (variation = 4.5%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>Assay Plate number (variation = 1.6%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td><strong>(B) Lytic activity (Δ absorbance)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALAN treatment</td>
<td>0.38 ± 0.02</td>
<td>0.36 ± 0.01</td>
<td>0.36 ± 0.02</td>
<td>0.38 ± 0.03</td>
<td>F&lt;sub&gt;3,201.3&lt;/sub&gt; = 1.86</td>
<td>0.14</td>
</tr>
<tr>
<td>Week</td>
<td>F&lt;sub&gt;2,342.5&lt;/sub&gt; = 51.7</td>
<td></td>
<td></td>
<td></td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>F&lt;sub&gt;1,200.6&lt;/sub&gt; = 0.56</td>
<td></td>
<td></td>
<td></td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>ALAN treatment x Week</td>
<td>F&lt;sub&gt;6,351.9&lt;/sub&gt; = 2.90</td>
<td></td>
<td></td>
<td></td>
<td>0.008</td>
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<tr>
<td>Week x Sex</td>
<td>F&lt;sub&gt;2,357.3&lt;/sub&gt; = 7.04</td>
<td></td>
<td></td>
<td></td>
<td>0.001</td>
<td></td>
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<tr>
<td>Femur length (mm)</td>
<td>F&lt;sub&gt;1,210.2&lt;/sub&gt; = 3.45</td>
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<td></td>
<td></td>
<td>0.06</td>
<td></td>
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<tr>
<td>Family (variation = 12.1%)</td>
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<td></td>
<td></td>
<td></td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Individual (variation = 24.1%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Assay Plate number (variation = 2.1%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.19</td>
<td></td>
</tr>
</tbody>
</table>
### (C) PO activity (Δ absorbance)

<table>
<thead>
<tr>
<th>Model Parameters</th>
<th>0 lux</th>
<th>1 lux</th>
<th>10 lux</th>
<th>100 lux</th>
<th>Statistic</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALAN treatment</td>
<td>0.83 ± 0.04</td>
<td>0.72 ± 0.03</td>
<td>0.74 ± 0.04</td>
<td>0.78 ± 0.03</td>
<td>$F_{3,217.5} = 0.38$</td>
<td>0.76</td>
</tr>
<tr>
<td>Week</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$F_{2,398.1} = 204.2$</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>Sex (F &gt; M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$F_{1,219} = 83.1$</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>Survived (Y, N)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$F_{1,307.9} = 15.7$</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>Family (variation = 0.4%)</td>
<td>0.36</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Individual (variation = 10.7%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>0.03</strong></td>
</tr>
<tr>
<td>Assay Plate number (variation = 5.0%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.07</td>
</tr>
</tbody>
</table>
Table 3.3: Spearman rank correlations ($r_s$) between haemocyte concentrations, lytic activity and Phenoloxidase (PO) activity for females and males across the three sampling periods (Week 0, Week 2 and Week 4). Significant $r_s$ values shown in bold; * denotes $P < 0.05$, ** $P < 0.01$ and *** $P < 0.0001$.

<table>
<thead>
<tr>
<th></th>
<th>Week 0</th>
<th>Week 2</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lytic activity</td>
<td>PO activity</td>
<td>Lytic activity</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemocyte</td>
<td>0.24 *</td>
<td>0.16</td>
<td>0.32 **</td>
</tr>
<tr>
<td>Lytic activity</td>
<td>-</td>
<td>0.21 *</td>
<td>-</td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemocyte</td>
<td>0.38 ***</td>
<td>0.16</td>
<td>0.36 **</td>
</tr>
<tr>
<td>Lytic activity</td>
<td>-</td>
<td>0.35 **</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 3.1: Variation across ALAN treatments in (A) haemocyte concentration (B) lytic activity, and (C) Phenoloxidase (PO) activity for adult crickets reared from egg through to adults under night-time light levels (ALAN treatment) of 0 lux, 1 lux, 10 lux and 100 lux. Data presented are means ± SE; different letters where present denote significant (P < 0.05) differences between ALAN treatments.
Figure 3.2: Variation in (A) haemocyte concentration (B) lytic activity, and (C) Phenoloxidase (PO) activity for adult cricket at Week 0, Week 2 and Week 4 after the final moult. Data presented are means ± SE; different letters, where present, denote significant (P < 0.05) differences between treatments.
Figure 3.3: Variation in lytic activity between sampling week and (A) ALAN treatment and (B) sex. Data presented are means ± SE; different letters, where present, denote significant (P < 0.05) differences between weeks for Fig 3.3A and males and females for Fig 3.3B.
Our experiment yielded several key findings related to the impact of dim light at night on immune function. First baseline levels of adult immune function were comparable regardless of sex or juvenile rearing treatment. Second, following a wounding challenge (in the Week 0 sampling period), adults reared under the presence of artificial light at night were less able to upregulate their haemocytes and thus had a potentially reduced core cellular defence pathway compared with crickets reared under 0 lux at night. We found a correlation between haemocyte count and lytic activity but neither lytic activity nor PO activity varied in response to the presence of ALAN, although they did increase across the three sampling periods. Finally, there was little concordance across the immune parameters measured with respect to their lifetime response to the presence of ALAN, however our data are in alignment with previous data in *T. commodus* using significantly higher night-time lux levels (Durrant et al. 2015; Jones et al. 2015). Critically, our data suggest that the intensity of nighttime lighting required to generate shifts in immune function (and particularly haemocyte concentrations) in the field cricket are several orders of magnitude lower than previously reported.

These data concur with a growing number of studies demonstrating that even low levels of ALAN can generate shifts in life history traits of many species, including immune function (Bruning et al. 2011; Dominoni et al. 2013d; Dominoni et al. 2014; van Geffen et al. 2014; Brüning et al. 2015; Dominoni 2015; Van Geffen et al. 2015a; van Geffen et al. 2015b; McLay et al. 2017; van Langevelde et al. 2017; Durrant et al. 2018; Chapter 2). Our longitudinal approach offers some additional insights into immune function in *T. commodus*. First, the fact that all initial responses were comparable regardless of immune assay, ALAN treatment or sex suggests either that ALAN has little impact on juvenile immune function (potentially because conditions were otherwise so benign) or that only the highest quality individuals survive through to the adult phase. The increased up-regulation of haemocytes observed for 0 lux crickets suggests a strong capacity to respond to future challenges which is lacking when crickets are exposed to even relatively low levels of ALAN. While few studies have explored the effects of low levels of ALAN on immune function in invertebrates, similar effects on analogous cellular components of
the immune system in vertebrates are reported (Bedrosian et al. 2011b; Aubrecht et al. 2014; Ikeno et al. 2014).

The effects of ALAN on lytic and PO activity were less pronounced than that of haemocytes. Our data suggest that ALAN may have some effect on the degree of upregulation of the lytic response, as indicated by the differences across the treatments in how rapidly they changed at the Week 2 sample, but these were somewhat more complex than those identified for haemocyte concentration and as identified by our findings, are highly variable between individuals. Such negative impacts on haemocyte concentration and potentially lytic activity are likely to result in significant reductions in the overall fitness of individuals living in urban environments (Ryder & Siva-Jothy 2001; Adamo 2004a; Adamo 2004b; Schmid-Hempel 2005).

Given the above, it may seem surprising that haemocyte concentration and lytic activity were not correlated with adult survival probability in this experiment, as seen in Table 3.2. However, the reason for this becomes clear given the nature of the experimental design. The relatively benign laboratory conditions, *ad libitum* food and water and an absence of any disease or parasite risk, means the greatest immunological threat to the survival of these crickets was the repeated wounding events for the extraction of haemolymph. The component of a cricket immune system that is primarily responsible for responding to such an event is phenoloxidase (Kanost & Gorman 2008), and indeed, a particularly large up-regulation of PO activity was determined at Week 2, following the initial wounding event. Furthermore, individuals who survived until the end of the experiment had significantly higher PO activity than those that did not survive but this was unrelated to the presence of ALAN. Future studies could try to elucidate some of the potential fitness consequences of ALAN by either imposing additional stressors in a laboratory setting or conducting experiments in a semi-natural scenario, where there is lower food availability, increased risk of parasitic load and thus, a greater variety of immune challenges. For nocturnal species, such as *T. commodus*, the consequences of a reduced immune capacity are likely to be far greater under natural conditions and will most likely impact their overall survival and reproductive success (Rolff & Siva-Jothy 2003).
It is likely that further insights could be gained from a multigenerational study that could also assess the transgenerational effects of ALAN. The large amount of family and individual variation demonstrated in this experiment, particularly for lytic and PO activity, suggest that there is likely a genetic component to an individual’s response to ALAN. To date no study has specifically explored heritable variation in species resilience in relation to the presence of light at night but there is mounting evidence suggesting variation in the genetic structure of urban vs rural populations (Evans 2010; Hopkins et al. 2018) which makes this highly likely.

Our data were unable to elucidate the underlying mechanism driving the observed variation in immune function. It is possible that wounding alone imposes a significant immune challenge promoting upregulation of the immune system but we are unable to exclude age-related changes as a possible interacting factor (Adamo et al. 2001; Park et al. 2011; Pinera et al. 2013). Experimental manipulation of the timing of initial wounding would further our understanding in this context. Similarly, the ALAN related changes in haemocyte concentrations are likely to be linked to the light sensitive melatonin pathway. The indolamine melatonin is a key regulator of circadian rhythm and is a powerful antioxidant found in both vertebrates and invertebrates (Poeggeler 1993; Vivien-Roels & Pevet 1993; Tan et al. 2010). Exposure to light at night suppresses melatonin production in vertebrates (Dominoni et al. 2013b; Brüning et al. 2015; Robert et al. 2015) and invertebrates including *Teleogryllus commodus*. Several experiments, including our own in *T. commodus*, report a link between melatonin and the cellular immune response (Demas & Nelson 1998; Drazen & Nelson 2001; Raghavendra et al. 2001; Jones et al. 2015). Moreover, we have previously demonstrated that dietary supplementation with melatonin can mitigate the negative effects on immune function of exposure to even extremely bright light (Jones et al. 2015). Further work is required to assess whether a comparable mechanism operates at lower intensities of ALAN as is observed for other species (Dominoni et al. 2013b; Brüning et al. 2015; Robert et al. 2015), and there is a need to develop highly sensitive methods for the measurement of melatonin in invertebrate model species (see Chapter 4). Given melatonin’s ubiquity across the animal kingdom and its largely comparable modes of action, it seems highly likely that it is acting as a key mechanistic link in the observed effects of ALAN on immune function.
Conclusion

Our study demonstrates that low levels of ALAN have a negative effect on cellular immune response of haemocyte concentration. Furthermore, correlations identified between aspects of the immune system hint towards a causal mechanistic link between ALAN and reductions in biological fitness, with a substantial degree of familial variation potentially being a factor to mask a strong correlation. Perhaps more critically, the effects of lifelong exposure to ALAN of 1 lux are comparable to those of 100 lux. Hence the effects of ALAN could be large and widespread, particularly in more malign conditions such as those of the natural environment. Understanding the physiological impacts of ALAN, as well as why and how they are occurring and to what extent they will impact individual fitness, is crucial to inform the management of this growing environmental concern.
CHAPTER 4: The effect of ALAN on melatonin in crickets: A novel HPLC-MS method for the measurement of melatonin in haemolymph
Abstract

Artificial light at night (ALAN) has negative physiological effects in a range of species. Attempts to explain the associations between ALAN and the observed biological effects have included a focus on the processes of melatonin production and suppression in the presence of ALAN. Uncovering these links presents an analytical challenge given the low concentrations of melatonin and often small obtainable sample volumes. This chapter presents a novel method for the measurement of circulating melatonin in 10 µL samples of cricket haemolymph using high-performance liquid chromatography tandem mass spectrometry. I used methyl tert-butyl ether (MTBE)/ethyl acetate to extract the total free and protein-bound melatonin from samples, and following dry-down and resuspension in acetonitrile (ACN) I injected 5 µL onto a 50 mm × 2.1 mm × 2.7 µm C18 column using an Agilent LC1200 system with a gradient of ammonium acetate solution and ACN at a flow rate of 0.4 mL/min. Separated melatonin was detected by electrospray ionisation-mass spectrometry (ESI-MS) using an Agilent Triple Quad 6460 system. The calibration curve for melatonin was linear in the range of 0.25 to 10 pM ($R^2 = 0.999$), and the limit of detection was 0.25 pM. This method was applied to a set of pilot data collected from crickets reared under different ALAN environments (0, 1, 10, and 100 lux). Due to samples having exceptionally low sample melatonin concentrations, with many below the detection limit, the results were inconclusive. Further work is required to refine the assay in this species and to determine an approach that reduces the loss of samples below the detection limit.

Key words: light pollution, melatonin, HPLC-MS, haemolymph, invertebrate,
Introduction

There are many biological compounds that interact as part of the internal signaling system of organisms. Hormones and neurotransmitters regulate many biological functions and are often responsible for translating external environmental cues into internal biological signals (Zawilska 1996; Jacobs & Wingfield 2000; Dufty et al. 2002). As such these endocrine and neuroendocrine systems are highly susceptible to the large amount of recent human-induced rapid environmental change (Sih et al. 2011), including artificial light at night (ALAN) (Russart & Nelson 2018). Although the precise internal mechanisms underpinning the biological impacts of ALAN remain somewhat undetermined, and are likely the result of multiple interacting systems (Ouyang et al. 2018), one widely proposed biological compound that is likely to play a key mechanistic role is melatonin (Navara & Nelson 2007; Haim & Zubidat 2015; Jones et al. 2015; Touitou et al. 2017).

Melatonin is a ubiquitous biological compound, which is derived from tryptophan (Vivien-Roels & Pevet 1993; Hardeland & Poeppgeler 2003; Hardeland et al. 2006; Pandi-Perumal et al. 2006a; Figure 1.2). It is typically synthesised during darkness and its production is reduced during the day due to the photosensitivity of the metabolic pathway (Tan et al. 2007). This, coupled with its rapid metabolism, results in a typical oscillating diurnal rhythm with nocturnal peaks in melatonin concentrations, and diurnal lows (Reiter 1993; Claustrat et al. 2005). Melatonin is best known as a biological timekeeper because it translates information about the relative length of night and day into an internal biological clock (Zawilska 1996; Arendt & Skene 2005; Pandi-Perumal et al. 2006a). The dependence on darkness for production makes melatonin particularly susceptible to ALAN, the presence of which is likely to greatly reduce the overall production of this compound (Brainard et al. 1982; Chepesiuk 2009).

In addition to its role in the maintenance of circadian rhythm, melatonin is a powerful antioxidant (Tan et al. 2010; Garcia et al. 2014), and its loss is implicated in a range of changes to life history traits, such as impaired growth, altered sexual maturation, and altered reproductive output (Reiter et al. 2009; Dominoni et al. 2013c; Le Tallec et al.
2016; McLay et al. 2017). As a neuroendocrine signal melatonin also either directly or indirectly regulates many aspects of biological function, including the immune system (Srinivasan et al. 2005; Carrillo-Vico et al. 2013; Esteban et al. 2013; Pohanka 2013; Weil et al. 2015).

Correlational evidence linking melatonin with the observed negative effects of ALAN have accumulated over the past decade, but there is little experimental demonstration of a direct relationship between reductions in melatonin and fitness costs in the presence of ALAN, and data from invertebrates is particularly lacking (Gaston et al. 2015b; Jones et al. 2015). This is potentially due to the difficulty in measuring this biological compound, which is produced and found at its highest concentration in the pineal gland of vertebrates (Hardeland et al. 2006; Tan et al. 2010), and the cerebral ganglia of invertebrates (Vivien-Roels & Pevet 1993; Hardeland & Poeggeler 2003). Measurement of melatonin in these organs requires termination of the individual, making it difficult to measure changes over time in correlation with other biological traits, and impossible to assess individual variation over time. To better understand the connections between ALAN, melatonin and other biological traits, we need to be able to assess the compound at multiple time points throughout an individual’s life. Relatively few studies have attempted this, particularly in an invertebrate model.

The development of a relatively non-invasive method to assess the circulating concentrations of melatonin presents an analytical challenge given the low concentration of melatonin in bodily fluids, and that it coexists with numerous compounds with similar structures and/or properties (De Almeida et al. 2011). Historically, the most common methods for measuring melatonin have been radioimmunoassays (RIAs) and enzyme-linked immunosorbent assays (ELISAs) (Chegini et al. 1995; De Almeida et al. 2011). The benefits of these methods are their low cost and relative ease of use, however, there is a high risk of cross-reactions and measurement of non-specific effects, and precise quantification is difficult due to unreliable standard curves (Yalow 1982; Wu et al. 2000; Gan & Patel 2013). Additionally, large sample volumes are required to run samples in duplicate or triplicate. These limitations are particularly important to consider when measuring low concentrations of a biological compound, as is the case with melatonin. A relatively new technique for measuring melatonin that is gaining traction is High
Performance Liquid Chromatography coupled with Mass Spectrometry (HPLC-MS). Compared to other methods it provides a more targeted approach, reducing the risk of non-specific detection. It is also highly sensitive and only requires small sample volumes, making it ideal for detecting low concentrations of melatonin when only small sample volumes can be obtained (Lu et al. 2008; Shackleton 2010), such as is often the case with invertebrate models.

I have previously developed an HPLC-MS method for the detection of circulating melatonin in the haemolymph of crickets with very small (10 µL) sample volumes (Durrant et al. 2015; Jones et al. 2015), however the detection limit of this method (25 pg/mL or 107 pM) needed to be improved to accurately assess melatonin in this species. The aims of this chapter were: 1) To improve the existing HPLC-MS method for measuring melatonin in haemolymph with small sample volumes, and 2) To test the effectiveness of this method in a pilot study, using samples collected under different ALAN environments (0, 1, 10 and 100 lux), as described in the experiment presented in Chapter 3. I predicted that I would be able to develop a more sensitive method to detect circulating melatonin concentration in haemolymph using HPLC-MS, and that when applied to a set of pilot data I would find melatonin concentrations to be lower in samples taken from crickets in high ALAN treatments compared to dark controls.

**Methods**

**ALAN treatments & rearing conditions**

Experimental crickets were reared from eggs in four different ALAN treatments (0, 1, 10 or 100 lux), which were provided by incubators (retrofitted Westinghouse: model number WRM4300WB-R) set at a constant 28°C (as per Chapters 2 and 3). Each incubator provided 12 hours of simulated daylight conditions (2600 lx - equivalent to a cloudy day, at 6800 K) followed by 12 hours of simulated night, with each ALAN treatment (0, 1, 10 and 100 lux, all at 5900 K).
**Haemolymph sample extraction**

Up to two haemolymph samples were taken from individual adult crickets at 17 ± 1 days and 31 ± 1 days after the final juvenile moult. These samples were collected between three to four hours prior to the incubators simulated day commencement, which is assumed to be the time of peak melatonin (Reiter 1991; Reiter 1993). To collect the haemolymph samples a small puncture was made in the left side of the cricket abdomen using a 27 G sterile needle (Becton Dickinson and Co.; Melbourne, VIC, Australia), which results in a small haemolymph bubble on the cuticle surface that can be collected with a pipette. Cricket haemolymph coagulates rapidly following extraction, so the 10 µL of haemolymph collected was immediately transferred to a 0.5 mL Eppendorf tube (Sarstedt; Mawson Lakes, SA, Australia) containing 20 µL of heparin (Hospira; Melbourne, VIC, Australia). It was then snap frozen in liquid nitrogen and stored at -80 °C until analysis.

**Measurement of circulating melatonin concentration using HPLC-MS**

Previously developed methods for the measurement of melatonin in cricket haemolymph (Durrant et al. 2015) were further adapted to increase assay sensitivity. Unless otherwise stated all chemicals and reagents were purchased from Sigma Aldrich (North Ryde, NSW, Australia).

To assess the circulating melatonin concentration in cricket haemolymph samples, it is first necessary to process the samples to extract the total melatonin (free and protein-bound). Methyl tert-butyl ether (MTBE) and ethyl acetate were used as the primary extraction agents, rather than dichloromethane (DCM) (For a description of the DCM method, see Durrant et al. 2015), Ethyl acetate is less toxic than DCM and has recently been used for melatonin extraction in milk samples (Özcan & Bagci 2018); MTBE has similarly been shown to be a very effective extraction solvent for metabolomics analysis (Sitnikov et al. 2016). Liquid phase extraction methods such as these typically result in high recovery rates (Harumi & Matsushima 2000).

Only small volumes of haemolymph (10 µL) were able to be collected from individual crickets, and preliminary trials had determined melatonin concentrations were extremely
Thus, it was necessary to pool individual samples into sets of three individuals per pool prior to melatonin analysis to increase the chance of detection. Individuals contributing to these pools were in the same treatments and the same sex. Prior to pooling, 7 μL of 100 mM ammonium hydroxide was added to each 30 μL individual haemolymph-heparin sample (described above) and incubated at 4°C for 10 minutes. Subsequently, 100 μL of cold (4°C) 1M MTBE/1M ethyl acetate solution (1:1, v/v) was added, before combining the resultant solutions from each of the three designated individuals into one 1.5 mL Eppendorf tube (Eppendorf South Pacific; North Ryde, NSW, Australia). Pooled samples were then vortexed at 1400 rpm and 4°C for five minutes and centrifuged (17500 g) at 4°C for five minutes. The lower MTBE/ethyl acetate phase (~300 μL), with the extracted melatonin, was transferred to a second set of 1.5 mL Eppendorf tubes, and dried under a nitrogen atmosphere (Coregas, Thomastown, VIC, Australia) at 4°C in a CHRIST SpeedDry Vacuum Concentrator RCV 2-33, with a CHRIST Cooling Trap CT 04-50 (Osterode am Harz, Germany) for approximately 30 minutes or until dry. Each original individual sample tube was then rinsed with a further 100 μL of 1M MTBE/1M ethyl acetate solution (1:1, v/v), which was again pooled with the remaining haemolymph-heparin phase in the first 1.5 mL tube set, vortexed, and centrifuged as above. The resultant lower MTBE/ethyl acetate phase (~300 μL), with the second round of extracted melatonin, was added to the solution in the second tube set and dry down continued. This whole step was repeated for a third time. The final dried, pooled samples were re-suspended in 50 μL of 750 mM acetonitrile (ACN) solution, vortexed for 15 minutes at 1400 rpm and 4°C, centrifuged for 5 minutes at 17500 g at room temperature, and the supernatant transferred to a 300 μL High Performance Liquid Chromatography (HPLC) sample vial insert (Agilent Technologies; Mulgrave, VIC, Australia).

Pooled biological quality control samples (PBQCs) were prepared by combining the supernatant of eight pools of three haemolymph samples from both male and female stock crickets (21 ± 4 days after the final juvenile moult) housed under a 12hr light:12 hr dark environment, and then re-aliquoted (30 μL) into HPLC sample vials to generate PBQC samples (two injections per aliquot).

A melatonin standard curve was prepared using 1 mM melatonin stock in pure ACN, and serially diluted with ACN to generate six different concentrations over the range 10 to
0.25 pM (2.3 pg/mL to 0.058 pg/mL). Three sets of this standard curve were prepared and placed at the start, middle and end of the sample run. A composite standard curve, used to compare samples against, was derived from all standard curves (Figure 4.1, n = 3, CV = 13.9%). After every fourth experimental sample, a blank pure 1 M ACN sample was run and after every eighth experimental sample, PBQCs were run (n = 16, CV = 7.6%). The MTBE/ethyl acetate extraction solution also contained deuterated melatonin (melatonin-D4) at a concentration of 21.5 pM (5 pg/mL), to be used as an internal standard for all experimental samples, PBQCs and standards, to quantify and verify relative extraction efficiencies, as well as to identify samples for removal from analyses due to extraction issues.

**Figure 4.1:** The composite curve made up of three melatonin standard curves placed at the beginning, middle and end of the sample HPLC-MS run. $R^2 = 0.999$ ($y = 416.2x - 35.6$). Error bars indicate the standard error about the mean.

Melatonin samples and standards were resolved by injecting 5 µL aliquots onto a 50 mm × 2.1 mm × 2.7 μm C18 column (Agilent Technologies; Mulgrave, VIC, Australia) using an Agilent LC1200 system with gradient of 1 mM ammonium acetate solution and pure 1 M ACN at a flow rate of 0.4 mL/min. Separated melatonin was detected by electrospray ionisation-mass spectrometry (ESI-MS) using an Agilent Triple Quad 6460 (Agilent Technologies).
Technologies) instrument. Multiple reaction monitoring (MRM) with the transitions m/z 233/174 and m/z 233/159, as quantifier and qualifier MRMs respectively, was used to detect the melatonin, in positive mass spectrometer mode (see appendix IV: Sample mass spectrum and chromatogram). The MS parameters, capillary voltage, fragmentor voltage, and collision energy were 3500V, 130V, and 5V and 20V, respectively. In all cases, the collision gas and sheath gas was nitrogen at a flow rate of 10 L/min and temperatures were maintained at 320°C and 350°C respectively. LCMS data were processed using Agilent MassHunter quantitative software, version 5 (Agilent Technologies), followed by manual checking of the integration peaks to ensure a correct and consistent area was integrated.

**Statistical analyses**

The areas underneath integrated response peaks were converted to melatonin concentrations (pM) using the composite melatonin standard curve, and this was used as the response variable. Statistical analyses were performed in JMP 14.0.0 (SAS Institute, NC, USA) and tests were two-tailed with a significance level of \( P < 0.05 \). Sample sizes were small and data was non-normally distributed, so difference between ALAN treatments were analysed with a non-parametric Kruskal-Wallis Rank Sums test. When processing and verifying data exclusions differences between treatment groups were determined using a Pearson’s Chi-squared contingency analyses.

**Results**

**Method validation**

Assay sensitivity was determined as 0.25 pM melatonin in sample solution (translating to 0.42 pM in cricket haemolymph, given that the final sample volume was 50 µm, which was derived from 30 µm of haemolymph). This was the point at which the concentration could be accurately back calculated from the standard curve formula, and was roughly double the integrated response area of a blank sample, and so was clearly detectable above the noise level. The calibration curve for melatonin was linear in the range of 0.25 to 10 pM (\( R^2 = 0.999 \)), and the method showed good consistency with a PBQC variation co-
efficient of 7.6%, and recovery rates that matched previous methods (>80%).

Data processing and verification

Given the lengthy protocol for sample processing it was necessary to verify the data to check and control for potential processing errors along the way. First, despite pooling individuals, the concentration of melatonin in a large number of samples fell below the assays quantification limit and were thus excluded from further analysis. The number of samples excluded was lowest in the 0 lux treatment, however there was no difference in the number of samples excluded between the four groups (0 lux = 13/25, 1 lux = 18/26, 10 lux = 16/26, and 100 lux = 18/28, P = 0.64). The second step was to check the internal standard levels, so as to eliminate samples where processing errors had occurred. Due to a reasonably large variation in the integrated response area of the internal standard across samples, samples were excluded if they were in the top and bottom 5% of internal standard variation as determined by the median (0 lux = 0/12, 1 lux = 2/8, 10 lux = 0/10, and 100 lux = 3/10, P = 0.07). Finally, some samples had exceptionally large values for melatonin concentration, most likely due to the chance of piercing the gut when extracting haemolymph. These values were determined as outliers using the interquartile range (IQR) method, where any data point falling more than 1.5 times the IQR above the third quartile is deemed an outlier (0 lux = 3/12, 1 lux = 2/6, 10 lux = 3/10, and 100 lux = 3/7, P = 0.88). An analysis was performed both excluding and including these high outliers. The total number of samples excluded did not vary across ALAN treatments when all of the above steps were combined (0 lux = 16/25, 1 lux = 22/26, 10 lux = 19/26, and 100 lux = 24/28, P = 0.20). Consequently, the number of validated samples used in the analysis to determine differences between ALAN treatments were 0 lux (n = 9), 1 lux (n = 4), 10 lux (n = 7), and 100 lux (n = 4).

Circulating melatonin concentration

Although there was a trend for decreasing circulating melatonin concentration with increasing light intensity, melatonin did not vary significantly between ALAN treatments (Table 4.1A, Figure 4.2A). For the sake of transparency, a second analysis was performed including the 11 high outliers. Again, melatonin concentrations were highest in the 0 lux treatment, but there were no statistical differences (P > 0.1; Table 4.1B, Figure 4.2B).
Table 4.1: Mixed models exploring the effect of ALAN treatment (0, 1, 10 and 100 lux) on circulating melatonin concentrations in cricket haemolymph: (A) Excluding extreme outliers; 0 lux \((n = 9)\), 1 lux \((n = 4)\), 10 lux \((n = 7)\), and 100 lux \((n = 4)\) (B) including extreme outliers; 0 lux \((n = 12)\), 1 lux \((n = 6)\), 10 lux \((n = 10)\), and 100 lux \((n = 7)\) including outliers.

<table>
<thead>
<tr>
<th>Model Parameters</th>
<th>Statistic</th>
<th>P Value</th>
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<tr>
<td><strong>(A) Circulating melatonin concentration (pM)</strong></td>
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<tr>
<td>ALAN treatment</td>
<td>( F_{3,21} = 4.98 )</td>
<td>0.17</td>
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<tr>
<td><strong>(B) Circulating melatonin concentration including outliers (pM)</strong></td>
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<tr>
<td>ALAN treatment</td>
<td>( \chi^2_{3,32} = 0.40 )</td>
<td>0.94</td>
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Figure 4.2: (A) Circulating melatonin concentration of pooled samples from crickets reared in 0 lux (n = 9), 1 lux (n = 4), 10 lux (n = 7), and 100 lux (n = 4), excluding outliers; and (B) circulating melatonin concentration of pooled samples from crickets reared in 0 lux (n = 12), 1 lux (n = 6), 10 lux (n = 10), and 100 lux (n = 7) including outliers. Data presented are medians and interquartile range; different letters denote significant (P < 0.05) differences between treatment conditions.
**Discussion**

The HPLC-MS method presented here for the detection of circulating melatonin in cricket haemolymph achieved a high level of sensitivity (0.25 pM or 0.058 pg/mL), several magnitudes lower than the previously reported method in this species (107 pM or 25 pg/ml; Durrant et al. 2015; Jones et al. 2015). No difference was seen in circulating melatonin concentration between ALAN treatments, however, due to low sample numbers, these results are inconclusive.

To date, most commercially available RIA and ELISA kits for measuring plasma melatonin require large sample volumes (200 to 500 µL) that are far greater than is possible from most invertebrate models, and only achieve assay sensitivities as low as 2 to 3 pM (Fourtillan et al. 1994; De Almeida et al. 2011; Carter et al. 2012; Jaffe 2012), which is nearly 10-fold higher than that of the current method. HPLC-MS is increasingly being used for the measurement of melatonin in body fluid, with recent methods reporting assay sensitivities of 603 pM in plasma (Yin et al. 2016), 25 pM in human serum (Wolrab et al. 2016), and as low as 0.14 pM in human saliva (Ishizaki et al. 2017). To my knowledge, very few studies (with the above exception) have achieved sensitivities as low as 0.25 pM, particularly in species other than humans. And few extraction processes require sample volumes low enough so as to be extracutable from invertebrate species.

However, there are a number of limitations with the current method that should be further explored and refined. Despite the low sensitivity of the method, the majority of the samples (61.9%) still fell below the detection limit, making the repeated lifetime measurement of melatonin in this species difficult. Ideally a larger sample volume would be a simple way to increase the efficacy of the method. However, given the impracticalities of this in this species, further refinements to the machine parameters should be made to increase the assay sensitivity still further, including larger injection volumes, changing the gradients of the buffer. Moreover, refinements could be made to the extraction process to reduce the potential for loss of melatonin throughout, and alternative extraction solvents could be tested. It is also worth considering the potential loss of melatonin due to degradation during storage. Samples were stored at -80°C for
several months, with potential melatonin degradation resulting in artificially low concentrations (De Almeida et al. 2011). Future studies should attempt to measure melatonin as soon as possible following extraction.

The large number of samples falling below the detection made it hard to draw meaningful conclusions from the pilot data. Given the visible trend for decreasing melatonin with increasing ALAN, and the fact that several previous studies demonstrate reduced melatonin concentrations following exposure to ALAN levels of less than 1 lx (Dominoni et al. 2013b; Brüning et al. 2015; Robert et al. 2015), it is highly possible that this lack of significance is due to limited sample size, and is not conclusive evidence that no actual biological difference exists. Further work and either a larger sample volume or even more sensitive method, these differences would indeed be biologically significant, and join the growing body of evidence to support ALAN induced reductions in melatonin concentration.

By developing a method to measure circulating melatonin concentrations in small volumes of cricket haemolymph I hoped to be able to assess relative changes in melatonin concentrations at multiple time points in the same individual, without harming the cricket. This would allow the correlation of circulating melatonin concentration with other biological variables within the same individual across the course of their lifespan, and shed light on the potential mechanistic cascade behind the physiological effects of ALAN. Unfortunately, the exceptionally low concentrations of circulating melatonin in cricket haemolymph, combined with the small amount of haemolymph that could be extracted at one time without harming the cricket, meant that I had to pool samples into groups of three, significantly reducing the power of any such correlations. Furthermore, the loss of samples with incredibly low concentrations of melatonin, and the high amount of variability in the remaining samples made such correlations impractical in this current study.

Despite the limitations in the study, with further refinements and validations the method presented could be readily adapted to suit other species and body fluids, particularly for species for which only small sample volumes are obtainable. Furthermore, this method could be used to measure precursors in the melatonin synthesis pathway (see Chapter 1,
Figure 1.2) to better characterise how ALAN affects different parts of the pathway. Tryptophan and serotonin were both detectable using the method developed, and with the inclusion of a relevant standard curve and slight adjustments to the final injection volume, could be easily quantified. Finally, future studies should determine the 24 hr rhythm of circulating melatonin concentrations in this species to determine whether three to four hours before sunrise is actually when peak concentration occurs, both with and without the presence of ALAN.

Conclusion

This chapter investigated a novel technique for the measurement, via HPLC-MS, of circulating melatonin concentration in cricket haemolymph. The method is consistent, targeted, and achieved a sensitivity many magnitudes lower than the previously reported method in this species. Due to low sample numbers, limited conclusions can be drawn about the effect of ALAN on circulating melatonin in crickets. Further work is required to refine the assay in this species and determine an approach that reduces the loss of samples below the detection limit. Despite the limitations in this species, sensitive methods such as this, which require small sample volumes, could be readily adapted to suit other species and body fluid. Such techniques will be valuable in determining the associations and direct effects of melatonin on fitness traits, particularly in association with ALAN.
CHAPTER 5: General discussion
Overview

The aim of this thesis was to explore the physiological effects of artificial light at night (ALAN) using a model invertebrate species, the Australian black field cricket, *Teleogryllus commodus*. The focus was on life history traits, immune function and the potential role of melatonin as a key mechanistic link explaining the observed biological effects of exposure to ALAN. In a series of laboratory experiments, I evaluated the consequences of lifelong exposure to varying intensities of ALAN and assessed specifically its effect on the growth, development and reproductive physiology of crickets, along with three independent measures of adult immune function. Prior to this research relatively little was known about the effects of dim, ecologically relevant levels of ALAN on invertebrate life history and physiology. My thesis therefore significantly broadens our taxonomic understanding of the potential ecological impacts of increasing environmental light pollution.

A diagrammatic summary of the key findings of this thesis is presented in Figure 5.1. In Chapter 2, I assessed the effect of lifelong exposure to different intensities of ALAN (0, 1, 10 or 100 lux) on egg hatch, juvenile development, adult morphology and condition, survival and the reproductive physiology of crickets. Key results included an increase in the time taken for juveniles to reach their final moult under any intensity of ALAN, as well as an increase in size under any intensity of ALAN when compared to crickets reared in the absence of light at night (0 lux). In Chapter 3, I explored the immunological effects of lifelong exposure to ALAN using haemolymph samples taken from adult crickets. I found that two of the three measured indicators of immune function were reduced under all three ALAN intensities (1, 10 or 100 lux). Cricket survival was unaffected by ALAN (Chapter 2), although interesting correlations between survival and each immune indicator may explain this lack of effect (Chapter 3). Finally, in Chapter 4 I present a novel method for the measurement of circulating melatonin in cricket haemolymph using high performance liquid chromatography and mass spectrometry (HPLC-MS) that is highly sensitive and targeted. This method was tested with a small set of pilot data from crickets reared under different ALAN conditions (0, 1, 10 or 100 lux), however due to exceptionally low concentrations and small sample volumes, the results are inconclusive.
Further explanation of these findings along with the probable causal mechanisms are discussed below, outlined in sub-headings that align with the key variables presented in the grey boxes of Figure 5.1. I also discuss the potential ecological implications, future research directions and possible management strategies to reduce the environmental impacts of artificial light at night.

**Figure 5.1:** A summary of the potential physiological processes and interactions under artificial light at night (ALAN) conditions, with the biological effects demonstrated in this thesis presented in grey boxes, and bold text indicating significant effects. Solid arrows represent proposed causal mechanistic cascades, and dashed arrows represent proposed biological links.
Circulating melatonin concentration

The naturally occurring indolamine, melatonin, is often suggested as the putative mechanism underpinning many of the observed physiological effects of ALAN (Navara & Nelson 2007; Jones et al. 2015; Gaston et al. 2017; Russart & Nelson 2018). However, few studies have experimentally linked an ALAN induced reduction in melatonin with the observed biological effects.

By developing a highly targeted and sensitive high-performance liquid chromatography and mass spectrometry (HPC-MS) method to measure circulating melatonin concentrations in cricket haemolymph (Chapter 4), I had hoped to assess relative changes in melatonin concentrations at multiple time points in the same individual, without harming the cricket. My aim was then to correlate circulating melatonin concentration and other biological variables within the same individual across the course of their lifespan. The method developed was successful and I was able to achieve a sensitivity of 0.25 pM, which is far lower than most commercially available kits and previously published methods in this species (De Almeida et al. 2011; Durrant et al. 2015; Jones et al. 2015). Unfortunately, the exceptionally low concentrations of circulating melatonin in cricket haemolymph meant that I had to pool samples into groups of three, reducing the potential to make correlations at the individual level. Furthermore, even with the pooling of samples, the vast majority of samples still fell below the assay detection limit, significantly reducing the number of samples to analyse and rendering the results somewhat inconclusive.

Although there were no significant differences in circulating melatonin concentrations between treatments, there was a slight trend for decreasing melatonin with increasing ALAN intensity. It is possible that with further work and either a larger sample volume or even more sensitive method, these differences would indeed be biologically significant, and join a growing group of studies which have identified a significant reduction in melatonin levels under ALAN condition of less than 1 lux in a range of species (Evans et al. 2007; Dominoni et al. 2013b; Brüning et al. 2015; Robert et al. 2015).
Future research could also use the method which I developed to measure precursors in the melatonin synthesis pathway (see Chapter 1, Figure 1.2) in order to better characterise how ALAN affects different parts of the pathway. Tryptophan and serotonin were both detectable using the method developed, and with the inclusion of a relevant standard curve and slight adjustments to the final injection volume, could be readily quantified.

**Growth and development**

Developmental shifts in response to ALAN have been observed in both vertebrates and invertebrates (Thakurdas et al. 2009; van Geffen et al. 2014; Brüning et al. 2015; de Jong et al. 2015). In *T. commodus*, the presence of any ALAN (1, 10 or 100 lux) resulted in a 10-day (or approximately 14 %) increase in the time taken for crickets to complete the juvenile phase of their development compared to crickets reared in the absence of light at night (0 lux). This also resulted in crickets reared under ALAN moulting into the adult phase approximately 8.5% larger than crickets reared under 0 lux (Chapter 2). The key physiological responses that may have contributed to these shifts include an altered perception of day length and therefore season and a disruption to the hormonal system that controls appetite and growth. Both these mechanisms are associated with melatonin. Melatonin naturally suppresses ghrelin, a hormone that stimulates appetite (Peric-Mataruga et al. 2009; Mataruga et al. 2012; Mataruga et al. 2015), so reduced melatonin production in response to ALAN could lead to increases in circulating ghrelin, increased appetite, and associated weight gain in an environment with *ad libitum* food (Kirsz & Zieba 2012; Fonken et al. 2013; Kirsz et al. 2017). A reduction in melatonin synthesis may also explain the slower growth of juvenile crickets in ALAN treatments. Melatonin directly and indirectly regulates growth and development via multiple mechanisms such as modulating growth hormone secretion (Recabarren et al. 2000), inducing compounds required for cell division and growth (Picinato et al. 2008), and is also thought to have a role in metamorphosis (Wright 2002). Therefore, an ALAN induced reduction in melatonin could hinder these natural processes and delay sexual maturation and development. Future work should further explore these potential relationships.
We note that the direction of growth observed here contrast with comparable studies where individuals reared under some colours of light at night emerged more rapidly and were smaller compared to individuals reared under dark conditions at night (van Geffen et al. 2014; Raap et al. 2016a). This lack of concordance may have arisen because *T. commodus* is a species with a demonstrated seasonally sensitive facultative diapause (Hogan 1965; Hogan 1967). If crickets in the ALAN treatments interpreted their environment as having a longer day than the 0 lux treatment with its 12 hr dark period, then they may have developed slower in response, as has been demonstrated in a closely related species (Kim et al. 2008). Or conversely, it could be that the 12-hour period of dark caused 0 lux crickets to develop faster as a result of interpreting their environment as winter approaching (Tauber & Tauber 1976). This could be explored further in future studies by altering the relative length of day and night under natural and ALAN conditions.

*Gamete quality*

There were a number of life history traits that were unaffected by the presence of ALAN in this study, including both the sperm quality and number of eggs laid by experimental crickets (Chapter 2), which is somewhat surprising given previous studies showing the effects of ALAN on reproductive physiology (Dominoni et al. 2013a; van Geffen et al. 2014; Robert et al. 2015; Raap et al. 2016a; McLay et al. 2017). The lack of effect could potentially be explained by the experimental design. Eggs used for rearing experimental individuals in this experiment were laid under a natural light:dark environment before being placed into their respective ALAN environments to hatch, meaning that maternal investment into the eggs was the same across treatments. Moreover, the nature of the experimental design, and high juvenile mortality rate during the first few moults promoted extreme selection during the juvenile phase of the lifecycle and resulted in only the fittest individuals surviving to the adult phase of the lifecycle, when reproductive investment was assessed. Whereas the majority of previous studies have focussed on either one or a partial stage of life

Despite there being no difference in sperm quality or the number of eggs laid in this study,
it is possible that the epigenetics and antioxidant status of both sperm and eggs are altered, which could in some way affect the next generation if they were allowed to mature (Youngson & Whitelaw 2008; Haim & Zubidat 2015). A multigenerational experiment would better assess changes in gamete quality, and explore the potential epigenetic effects that may emerge over multiple generations. Furthermore, a full factorial design, in which eggs laid under both a natural and an ALAN environment where crossed and reared, would allow the assessment of both multigenerational effects as well as the potential genetic adaptation to ALAN. It would also be interesting to measure whether lifelong exposure to ALAN results in a reduced concentration of melatonin in both sperm and eggs.

**Immune function**

Lifelong exposure to low levels of ALAN had negative effects on the cellular immune response of adult crickets, while the effects on lytic activity and PO activity were more complex or largely unaffected by ALAN (Chapter 4). Crickets reared in the presence of any ALAN (1, 10 or 100 lux) had lower haemocyte concentrations throughout their adult life. The precise mechanism behind these ALAN induced changes to some aspects of immune capacity remains uncertain, although given the known relationships between melatonin and immune function (Guerrero & Reiter 2002; Calvo et al. 2013; Carrillo-Vico et al. 2013; Jones et al. 2015; Weil et al. 2015; Borniger et al. 2017) it seems highly likely that melatonin is acting in some way as a causal link. It is also highly likely that the effects of ALAN on immune function are linked to circadian rhythms and a disruption to the perception of relative day length. Many organisms, including invertebrates, use photoperiod to inform seasonal biological changes (Nelson 2004; Mydlarz et al. 2006). Typically, immune function is suppressed during breeding seasons, in preference for reproductive requirements, and is highest during winter months (Schmid-Hempel 2003; Demas et al. 2011). Therefore, if crickets under this current experimental design perceived their ALAN environment to be an extension of daylight hours, this may have suppressed their ‘short-day response’ and resulted in a lower baseline immune function (Aubrecht et al. 2014; Ikeno et al. 2014).
The consequences of these reductions remain unclear, as lytic and PO activity were less affected by the presence of ALAN. Given that haemocytes are thought to represent the core cellular immune response in invertebrate immunity (Ribeiro & Brehélin 2006) it is likely that such reductions in the upregulation of these cells when faced with a wounding challenge in the presence of ALAN should greatly reduce an individual’s ability to fight disease and infection in a natural environment. It is possible that my ability to detect this was compromised by the high-quality environment crickets were reared under. To better explore how ALAN induced reductions in immune capacity could ultimately affect survival, future studies could rear crickets in a more malign, semi-natural environment where immune capacity is likely to be tested. Moreover, it would be interesting to introduce a specific immune stressor, such as a bacterial challenge that would test the lytic response of crickets potentially at both the adult and juvenile phases of the lifecycle. Such an immune challenge under ALAN conditions could also result in a greater impact of ALAN on reproductive measures, given the known trade-offs between reproduction and immune function in this species (McNamara et al. 2014a; McNamara et al. 2014b). Finally, haemocyte concentration in this experiment was taken as all cells combined. However, as with vertebrates, there are a range of cell types within the haemolymph of invertebrates (Ribeiro & Brehélin 2006). Further work is needed to functionally characterise the different cell types in cricket haemolymph, and their functions, and to assess the relative effects that ALAN may have on each (Cima et al. 2000; Matozzo et al. 2007).

**Survival**

Given the detrimental effects of ALAN on cricket immune function, it may seem surprising that the survival of these crickets was the same across all treatments, regardless of light conditions (Chapter 2). This lack of effect can potentially be explained by two key aspects of the experimental design. First, the design promoted extreme selection throughout the egg hatch and early juvenile phase of the lifecycle, so that those more tolerant of ALAN were likely to survive to the adult phase of the lifecycle. Second, and perhaps more significant, are the experimental conditions during the period of immune assessment, and the specific immune challenges faced by the crickets. As highlighted
above, the relatively benign laboratory conditions, with *ad libitum* food and water and an absence of any disease or parasite risk, means the greatest threat to the survival of these crickets was the repeated wounding events for the extraction of haemolymph. The component of a cricket’s immune system that is primarily responsible for responding to such an event is phenoloxidase (PO) (Kanost & Gorman 2008), which is emphasised in this species by the large up-regulation of PO activity following the initial wounding event (Chapter 4). This means that the component of the immune system which is likely to be the most crucial for the survival of these crickets, PO activity, was in fact the one component that was unaffected by the presence of ALAN. Further evidence that PO activity was the largest determinant of survival is the fact that individuals who survived until the end of the experiment had significantly higher PO activity than those that did not survive, particularly at older ages (Chapter 4). It is therefore not surprising that the probability of adult survival was unaffected by ALAN in this experiment.

**Genetic variability**

There was a strong degree of family level variation in life history traits as well as immune function (Chapters 2 and 3), however, across variables there was no interaction between ALAN treatment and family group. This suggests that although the mechanism promoting variation is underpinned by genetic as well as environmental variation, no specific genetic makeup is more suited to deal with exposure to ALAN than any other. However, as above it is possible that the highly selective juvenile stage of this experimental design, and the resultant selection of only the fittest individuals from each family group masked the potential to see genetically based variability in the physiological responses to ALAN. It is also worth noting that these were a 10th generation laboratory adapted population, and the degree of genetic variability is likely far lower than would be the case if wild caught individuals were used for this experiment. So we cannot rule out whether in a natural setting there would be individuals with a genetic makeup more suited to deal with exposure to ALAN than others.

An important consideration in the response of organisms to ALAN is that species can have plastic responses to selection pressures, or can be capable of rapid adaptation. To
date, no study has specifically explored heritable variation in species’ resilience to the presence of ALAN, but mounting evidence suggesting variation in genetic structure between urban and rural populations (Evans 2010) indicates that this is possible. In alignment with assessing the potential effects of ALAN on gametes, a multi-generational heritability study is needed, in which males and females are crossed from each a natural light and an ALAN environment, in order to quantitate variation attributable to genetics vs the ALAN environment, and to evaluate whether this potential genetic variability could ultimately result in adaptation to this emerging environmental threat. At an ecological level, the capacity of a given species for genetic change across generations needs to be considered when examining their response (Chown et al. 2010). ALAN is likely to have a far greater impact on species with small populations, low fecundity, and long generational times.

Ecological consequences

The findings within this thesis add to a growing body of evidence showing that ALAN has a range of, largely negative, biological implications for wildlife and humans alike (Navara & Nelson 2007; Fonken & Nelson 2011; Dominoni et al. 2016; Gaston et al. 2017; Davies & Smyth 2018). This is concerning given that the prevalence of ALAN is currently increasing at a rate of around 6% per year worldwide (Holker et al. 2010; Falchi et al. 2016), particularly for the 30% of vertebrate species and >60% of invertebrate species which are nocturnal (Holker et al. 2010). Furthermore, the consequences of the effects seen here are likely to be far more severe in a natural setting.

Reduced immune capacity in a natural environment will impact a cricket’s ability to fight disease and infections and will most likely reduce their overall survival and reproductive success (Rolff & Siva-Jothy 2003; Adamo 2004a; Schmid-Hempel 2005). It is also possible that a protracted juvenile phase of the lifecycle and resulting larger individuals would reduce survival, as a result of increased juvenile predation risk within the population (Sibly & Calow 1986; Dixon & Baker 1988). An increase in development time could lead to delayed generational times, which may reduce the number of generations in a season and prolong species adaptation to other biological stressors (Chevin et al. 2010).
And shifts in the timing of life history stages could disrupt phenology and result in wider reaching ecological disruption (Da Silva et al. 2015; Bennie et al. 2017; Gaston et al. 2017).

There are many urban and natural environments around the globe that are currently experiencing levels of ALAN as high as 1 lux, and there is potential for even greater spread (Davies et al. 2014; Gaston et al. 2015a; Guetté et al. 2018). Therefore the effects of 1 lux ALAN observed here demonstrate that in a natural setting ALAN could ultimately reduce species persistence (Stearns 1976; Schmid-Hempel 2005) and disrupt ecological processes through altered phenology, trophic cascades and resulting competitive advantages (Gaston et al. 2014). For these reasons ALAN is considered an emerging anthropogenic threat to biodiversity (Holker et al. 2010; Stone et al. 2012; Kyba & Hölker 2013).

**Future research directions**

The field of ALAN has seen an explosion of interest over the last decade, and the extent of the known biological effects is increasing rapidly. However, there are still several gaps in our understanding (Gaston et al. 2015b; Davies & Smyth 2018). The following are some broad research avenues following on from the questions answered in this thesis.

It is clear that ALAN affects many physiological processes, but it is not clear whether individuals can avoid these consequences by altering their behaviours. A recent study found that birds are protected from the effects of ALAN when inside cavities (Raap et al. 2018), so it is possible that individuals could adjust their behaviour to reduce the effects of ALAN. While many behavioural changes in response to ALAN are reported, these are largely behaviours and movement patterns associated with the natural presence of light, rather than an instinct to avoid it (Witherington & Bjorndal 1991; Baker & Richardson 2006; Kempenaers et al. 2010; Dominoni et al. 2013c). Future studies could assess whether individuals display any specific avoidance behaviours, such as an increased amount of time in shelters. Furthermore, it is unclear what the relationships are between the observed physiological effects of ALAN and the behavioural effects and whether one
might be having a causative effect on the other. This could be explored through experiments that integrate both behavioural and physiological measures into their design.

Similarly, in regards to mitigating the effects of ALAN there is already some evidence that dietary melatonin can act as a recovery agent against the effects of ALAN (Durrant et al. 2015), but further work is needed to assess these effects under ecologically relevant levels of ALAN and with quantities and sources of melatonin that may actually be available to individuals in a natural environment. It should also be determined whether a behavioural preference would indeed be given to melatonin rich food sources for individuals experiencing chronic ALAN. Finally, it is unclear whether it is the dietary melatonin acting as a biological signal that is having a rescue effect, or rather that it is merely the effect of melatonin as a dietary anti-oxidant/protein metabolite source. Therefore, supplementation experiments with both melatonin and an alternative anti-oxidant/protein metabolite source are necessary to determine the relative potential for rescue effects of each.

The experiments in this thesis have demonstrated the potential for ALAN to result in negative physiological impacts under controlled laboratory conditions. However, while laboratory experiments are incredibly useful for determining relationships and causal mechanisms, their extrapolations to the natural environment are limited. The experiments in this thesis should now be extended to a more malign semi-natural environment, where food is less available and natural pathogens are present. Such experiments would also allow a control ALAN treatment to be natural moonlight rather than 0 lx, which is more ecologically relevant. Additionally, it has been shown in this model species that, in many aspects, the effects of 1 lx ALAN are much the same as those of 100 lx and even 4000 lx (Chapter 2; Chapter 3; Durrant et al. 2015). Therefore, future studies should lower the ALAN intensity to determine the threshold of effects. Furthermore, the shifts in the timing of life history stages, as demonstrated in Chapter 2, could disrupt phenology and have wide reaching effects on community structure and ecosystem processes (Visser et al. 2004; Neil & Wu 2006; Bennie et al. 2017; Gaston et al. 2017). This too could be explored further in a semi-natural environment with small scale community structure experiments under different ALAN conditions.
Beyond ecological applicability, we also need a better understanding of the long term effects of this environmental pollutant. We are lacking a well-developed understanding of how biological characteristics and genetics play a role in individual variations in the physiological response to ALAN (Gaston et al. 2015b), or whether the effects of ALAN on individuals persist physically and genetically in the following generation and could even result in speciation. Recent studies in Siberian hamsters have started to provide evidence that parental exposure to dim light at night impairs offspring immune function (Cisse et al. 2017a), and increases depressive-like behaviours (Cisse et al. 2017b). Whether organisms have the potential to adapt to this significant environmental change remains unknown and few studies have begun to think about and investigate this phenomenon (Gaston et al. 2015b; Swaddle et al. 2015; Hopkins et al. 2018). 

Finally, different species experiences light in different ways (Gaston et al. 2012), and increasing evidence shows that the spectral composition of ALAN greatly alters its impacts, particularly for physiology that is linked to melatonin (Santhi et al. 2012; Aubé et al. 2013; Dimovski & Robert 2018). The spectral output varies greatly between different lighting technologies, and evidence highlights the relatively low impacts of amber light when compared to white light (Dimovski & Robert 2018; Grubisic et al. 2018), although this is not always the case (Bennie et al. 2015a). Further research is needed into both understanding the diverse ways in which light is registered by different invertebrate and vertebrate species, and determining lighting strategies that can balance the need for energy efficiency with the potential impact on wildlife and humans (Zielinska-Dabkowska 2018).

**Closing remarks**

My thesis advances our understanding of the biological effects of ALAN for invertebrates, a key taxon contributing to ecological community structure and composition. Combined, the results presented demonstrate a disruption to physiological processes, and highlight the potential for ALAN to alter the phenology of communities and reduce the overall fitness of individuals. Although the extrapolations from a laboratory setting to the natural environment can only be made tentatively, the results of
this research will inform future studies and help to progress our understanding of this growing environmental concern.
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light-at-night accelerates aging and promotes tumorigenesis in young but not in old rats. *Aging* 2:82-92.


APPENDIX I: Spectral output of incubator lights
Figure I.1: The spectral output of “day” lights in the incubators used for Chapters 2, 3 and 4.
Figure I.2: The spectral output of “night” lights in the incubators used for Chapters 2, 3 and 4.
APPENDIX II: Chapter 3 mixed models prior to model reduction
Table II.1: Mixed models (including all variables prior to model reduction) exploring the effect of lifelong ALAN treatment (0, 1, 10 and 100 lux) over time (Weeks 0, 2 and 4 after final moult) for (A) haemocyte concentration (cells/mL x 10<sup>6</sup>), (B) lytic activity (Δ absorbance) and (C) Phenoloxidase (PO) activity (Δ absorbance). Family, Individual ID and Assay Plate number were included as random terms in all models.

<table>
<thead>
<tr>
<th>Model Parameters</th>
<th>0 lux</th>
<th>1 lux</th>
<th>10 lux</th>
<th>100 lux</th>
<th>Statistic</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Haemocyte concentration (cells/mL x 10&lt;sup&gt;6&lt;/sup&gt;) (Ln transformed)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALAN treatment</td>
<td>2.93 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.66 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.72 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.64 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>F&lt;sub&gt;3,195.1&lt;/sub&gt; = 4.82</td>
<td>0.003</td>
</tr>
<tr>
<td>Week</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F&lt;sub&gt;2,358.8&lt;/sub&gt; = 3.53</td>
<td>0.03</td>
</tr>
<tr>
<td>Sex (F &gt; M)</td>
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<td></td>
<td></td>
<td></td>
<td>F&lt;sub&gt;1,195.5&lt;/sub&gt; = 14.52</td>
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<tr>
<td>Survived (Y, N)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F&lt;sub&gt;1,283.4&lt;/sub&gt; = 0.62</td>
<td>0.43</td>
</tr>
<tr>
<td>Femur length (mm)</td>
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<td></td>
<td></td>
<td></td>
<td>F&lt;sub&gt;1,201.8&lt;/sub&gt; = 0.08</td>
<td>0.77</td>
</tr>
<tr>
<td>ALAN treatment x Week</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F&lt;sub&gt;6,392.4&lt;/sub&gt; = 2.64</td>
<td>0.07</td>
</tr>
<tr>
<td>ALAN treatment x Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F&lt;sub&gt;2,365.6&lt;/sub&gt; = 0.41</td>
<td>0.87</td>
</tr>
<tr>
<td>Week x Sex</td>
<td></td>
<td></td>
<td></td>
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<td>F&lt;sub&gt;2,365.8&lt;/sub&gt; = 0.70</td>
<td>0.65</td>
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<td>ALAN treatment x Sex x Week</td>
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<td></td>
<td></td>
<td>F&lt;sub&gt;6,392.4&lt;/sub&gt; = 2.64</td>
<td>0.07</td>
</tr>
<tr>
<td>Family (variation = 8.38%)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
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<tr>
<td>Individual (variation = 4.86%)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.31</td>
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<tr>
<td>Assay Plate number (variation = 0.0%)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.32</td>
</tr>
<tr>
<td>(B) Lytic activity (Δ absorbance)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F&lt;sub&gt;3,196.9&lt;/sub&gt; = 1.37</td>
<td>0.25</td>
</tr>
<tr>
<td>ALAN treatment</td>
<td>0.38 ± 0.02</td>
<td>0.36 ± 0.01</td>
<td>0.36 ± 0.02</td>
<td>0.38 ± 0.03</td>
<td>F&lt;sub&gt;2,327.2&lt;/sub&gt; = 54.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Week</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F&lt;sub&gt;1,197.4&lt;/sub&gt; = 0.46</td>
<td>0.75</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F&lt;sub&gt;1,252&lt;/sub&gt; = 1.42</td>
<td>0.23</td>
</tr>
<tr>
<td>Survived (Y, N)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</table>
## Appendix II

<table>
<thead>
<tr>
<th>Factor</th>
<th>F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Femur length (mm)</td>
<td>$F_{1,206.3} = 3.58$</td>
<td>0.06</td>
</tr>
<tr>
<td>ALAN treatment × Sex</td>
<td>$F_{3,197.4} = 41$</td>
<td>0.74</td>
</tr>
<tr>
<td>ALAN treatment × Week</td>
<td>$F_{6,347.7} = 3.07$</td>
<td>0.006</td>
</tr>
<tr>
<td>Week × Sex</td>
<td>$F_{3,349.4} = 6.55$</td>
<td>0.002</td>
</tr>
<tr>
<td>ALAN treatment × Sex × Week</td>
<td>$F_{6,348.2} = 0.95$</td>
<td>0.46</td>
</tr>
<tr>
<td>Family (variation = 11.2%)</td>
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<td></td>
</tr>
<tr>
<td>Individual (variation = 24.5%)</td>
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<td>&lt; 0.0001</td>
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<tr>
<td>Assay Plate number (variation = 0.01%)</td>
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<td>0.19</td>
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### (C) PO activity (Δ absorbance)

<table>
<thead>
<tr>
<th>Factor</th>
<th>$F$</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALAN treatment</td>
<td>0.83 ± 0.04</td>
<td>0.16</td>
</tr>
<tr>
<td>Week</td>
<td>0.72 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Sex (F &gt; M)</td>
<td>0.74 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Survived (Y, N)</td>
<td>0.78 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Femur length (mm)</td>
<td>$F_{3,212.25} = 1.72$</td>
<td>0.16</td>
</tr>
<tr>
<td>ALAN treatment × Sex</td>
<td>$F_{2,373.6} = 194.3$</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Week × Sex</td>
<td>$F_{1,213.5} = 75.2$</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>ALAN treatment × Week</td>
<td>$F_{3,378.7} = 0.68$</td>
<td>0.67</td>
</tr>
<tr>
<td>Week × Sex × Sex</td>
<td>$F_{3,379.9} = 0.77$</td>
<td>0.46</td>
</tr>
<tr>
<td>Family (variation = 0.29%)</td>
<td></td>
<td>0.36</td>
</tr>
<tr>
<td>Individual (variation = 9.7%)</td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>Assay Plate number (variation = 0.03%)</td>
<td></td>
<td>0.07</td>
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</tbody>
</table>
APPENDIX III: Sample mass spectrum and chromatogram for melatonin method
Figure III.1: (A) The sample mass spectrum, and (B) chromatogram for the HPLC-MS melatonin detection method presented in Chapter 4.
APPENDIX IV: List of conference presentations
Conference presentations


